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Mechanisms of Resistance to Anti-Thymidylate Synthase (TS) Chemotherapeutic Agents in Colon Cancer

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Mechanisms of Resistance to Anti-Thymidylate Synthase (TS) Chemotherapeutic Agents in Colon Cancer

Seema Bissoon-Haqqani

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Doctor of Philosophy

University of Ottawa
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DEDICATION

To my loving parents,

Marilyn Coolman Bissoon & Roop Bissoon

ABSTRACT

Thymidylate Synthase (TS) catalyzes the final step in the synthesis of thymidylate, a nucleotide required for DNA synthesis. This enzyme is the target enzyme for several important anti-cancer drugs including 5-fluorouracil (5-FU) and 5-fluorodeoxyuridine (5-FUdR). 5-FU and 5-FUdR are metabolized to FdUMP which, in the presence of a folate cofactor, inhibits TS by forming a tight covalent "ternary complex". TS is usually considered to be a cytoplasmic enzyme, but some reports suggest it may also be present in the nucleus. One recent report has indicated that high levels of nuclear TS were associated with poorer clinical response to 5-FU.

Using a combination of immunocytochemistry, confocal microscopy, immunohistochemistry, western blotting and cell fractionation techniques, evidence is presented that a fraction of cellular TS is indeed located in the nucleus of some colorectal cancer cell lines and tissues. The specificity for TS of the polyclonal antibody used was first confirmed. The effect of TS overexpression on cellular localization was studied in HeLa-55 cells, a 5-FUdR-resistant line containing an amplified TS gene. Nuclear TS was clearly evident in HeLa-55 but not in parental HeLa cells. Fractionation of HeLa-55 growing in 5-FUdR showed that both the ternary complex and free TS were present in the cytoplasm, while only free TS was present in the nucleus. From a panel of 6 colorectal cancer lines and normal fibroblasts, RKO and HCT-116 clearly showed nuclear TS. Strong evidence of nuclear TS in some colorectal cancer tissues is also presented. A putative leucine-rich nuclear export signal sequence (NES) was identified in TS protein. TS constructs lacking this sequence accumulated more nuclear TS, but the sequence did not function as a nuclear export signal when fused to GFP.

A recent report suggests that p53 status can affect the sensitivity of HCT-116 to 5-FUdR. I examined HCT-116 p53 wt and HCT-116 p53 null cells and observed that p53 null cells, in contrast to p53 wt cells, failed to form a ternary complex when exposed to 5-FUdR. p53 wt cells were also more sensitive to killing by 5-FUdR. However, the differences observed were in fact independent of p53 status. 5-FUdR-sensitive cell lines became resistant after several passages in culture. Current evidence suggests that the basis of resistance is related to either drug metabolism or uptake. This thesis has contributed towards a better understanding of TS, a cellular target for an important class of anti-cancer drugs.

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ABBREVIATIONS

2D	two-dimensional
2R	two tandem repeats
3R	three tandem repeats
5-AdCd	5-azacytidine
5-FdUMP	5-fluorodeoxyuridine monophosphate
5-FdUTP	5-fluorodeoxyuridine triphosphate
5-FU	5-fluorouracil
5-FUdR	5-fluorodeoxyuridine
5-FUTP	5-fluorouridine triphosphate
ATCC	American type culture collection
bp	basepair
cDNA	complementary DNA
CFU	colony forming unit
CH_2-THF	5,10-methylenethetrahydrofolic acid
CO_2	carbon dioxide
DHF	dihydrofolic acid
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
dTDP	(deoxy)thymidine diphosphate
dTMP	(deoxy)thymidine monophosphate (thymidylate)
dTTP	(deoxy)thymidine triphosphate
dUMP	deoxyuridine monophosphate
EMSA	electrophoretic mobility shift assay
ENT	equilibrative nucleotide transporter
FACS	fluorescence-activated cell sorting
Fig.	figure
h	hour
H_6	six histidine
H_6-TS	six histidine-tagged TS
HAT	hypoxanthine-aminopterin-thymidine
hENT	human ENT
HF-9	human factor-9
HNPCC	hereditary non-polyposis colorectal cancer
HRP	horseradish peroxidase
hTS	human TS
kb	kilobasepair
kD	kilodalton
kDa	kilodalton
LDH	lactate dehydrogenase
min	minute
MLH	MutL homologues
mRNA	messenger RNA
NES	nuclear export signal
Ni-NTA	Nickel nitrilotriacetic acid
NLS	nuclear localization signal

null	$p53^{-/-}$ cells
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVDF	polyvinylidene fluoride
rhTS	recombinant human TS
RNA	ribonucleic acid
s	second
SDS	sodium dodecyl sulfate
TdR	thymidine (or deoxythymidine)
THF	tetrahydrofolic acid
TK	thymidine kinase
TS	Thymidylate synthase
UTR	untranslated region
WBC	white blood cells
WBC-F	WBC from female
WBC-M	WBC from male
WT	wildtype
WTR	WT cells resistant to 5-FUdR

GENERAL INTRODUCTION

(i) *Thymidylate Synthase (TS)*

Thymidylate synthase (TS) (EC 2.1.1.45) catalyses the conversion of deoxyuridylate (dUMP) to thymidylate (dTMP) by reductive methylation using 5,10-methylenetetrahydrofolate (CH_2-THF) as a cofactor (1)(Fig. 1A). Once synthesized, dTMP is phosphorylated to dTDP and then to the triphosphate, dTTP, which is the direct precursor for DNA synthesis. While dTMP can be formed through the salvage pathway *via* phosphorylation of thymidine (TdR) by thymidine kinase, the TS-catalysed reaction is the sole *de novo* source of dTMP (1)(Fig. 1A). Due to its central role in DNA synthesis, TS is recognized as an attractive target for cancer chemotherapy (2).

In its native form TS exists as a homodimer, the molecular weight of which varies from 60 to 75 kDa depending on the species (3). The DNA sequences of 29 different species of TS (including *E.coli*, yeast, viruses, mouse, rat and human) have been determined, and establishes TS as one of the most phylogenetically conserved proteins known (3). The mRNA of human TS (hTS) is 1.5 kb in length and codes for a protein of 313 amino acids with a monomer molecular weight of 35 kDa (4). The human TS gene has been mapped to chromosome 18p11(5).

(ii) *Anti-TS Chemotherapeutic Agents and Colorectal Cancer*

Human TS has been purified (6) and crystallized (7). As an attractive target for anti-cancer drug design, since the 1950s, many TS analogues of both the substrate, dUMP, and the cofactor, CH_2-THF, have been synthesized and tested as potential anti-cancer therapeutics. Until the late 1980's, 5-fluorouracil, (5-FU) (base) and 5-fluoro-deoxyuridine (5-FUdR) (nucleoside) (Fig. 1B) were the sole TS-targeted drugs approved for clinical application. Later, when the three-dimensional structure of human TS was determined (8), it allowed the design of novel folate analogues such as Tomudex (ZD 1694), BW1843U89, and Thymitaq (AG337) (8).

5-FU is used extensively in the treatment of gastric, colorectal, head and neck and breast cancers (9,10), while 5-FUdR is used in the treatment of renal cancers, hepatomas and metastatic colorectal carcinomas (11-13). Mechanisms of action of the fluoropyrimidines, 5-FUdR and 5-FU, are mutifactorial. Intracellularly, the fluoropyrimidines are metabolized to FdUMP, which forms a stable inhibitory covalently-bound "*ternary complex*" with the target

FIGURE 1. TS-catalyzed *de novo* synthesis of dTMP and the various substrate and substrate-analogues of TS

A. TS-catalyzed synthesis of dTMP, a precursor of DNA synthesis. Thymidine kinase (TK) can also catalyze dTMP *via* salvage pathway (dotted line). ***B.*** Structures of dUMP, dTMP, TdR, 5-FdUMP, 5-FU and 5-FUdR.

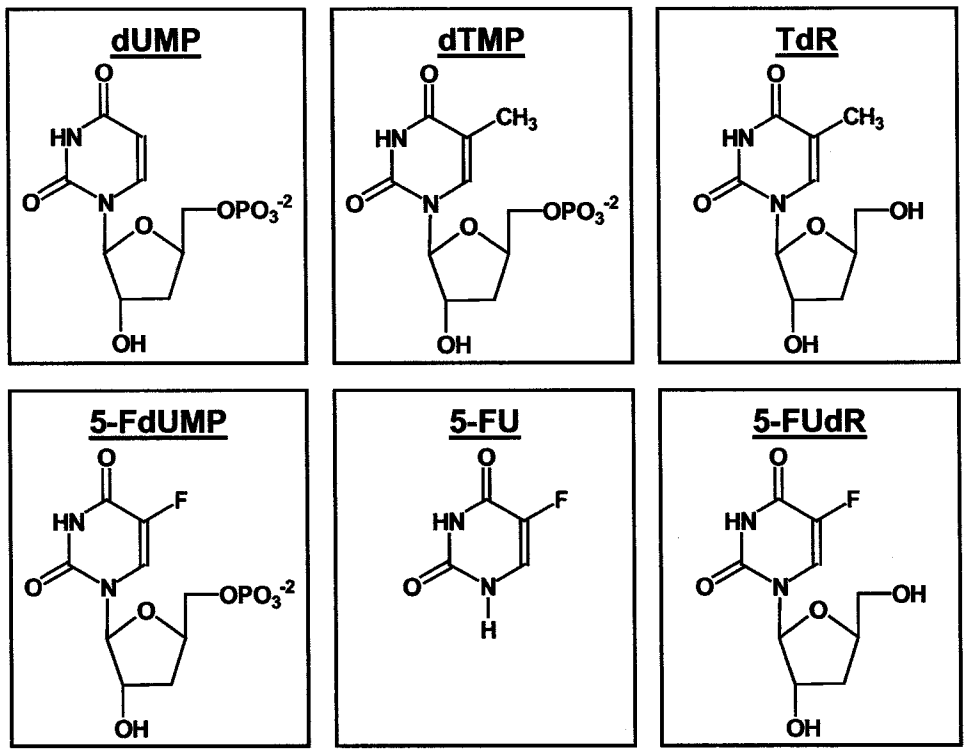
A) CH_2THF DHF

TS

dUMP dTMP → dTDP → dTTP → DNA synthesis

TK ..... TdR

B)



enzyme TS, and the cofactor CH_2-THF (Fig. 2)(14,15). Inactivation of TS results in decreased production of dTMP, cessation of DNA synthesis and ultimately thymineless death (16). Additionally, cytotoxic effects may be mediated by incorporation of the metabolic products FdUTP and FUTP into DNA and RNA, respectively (17) (18)(Fig. 2). The predominant mechanism of fluoropyrimidine action may vary depending on tumor type and metabolic state which include the activities of metabolic enzymes, levels of competing substrates, availability of cofactors, and schedule of drug administration (19).

The inhibition of TS can be enhanced by stabilization of the ternary complex; this can be achieved by providing an excess of folate precursors by administration of leucovorin (LV), also known as folinic acid (10). LV is converted into CH_2-THF, which serves as a cofactor for the binding of FdUMP to TS. 5-FU in combination with LV is now considered to be one of the standard treatments for colorectal cancer (10).

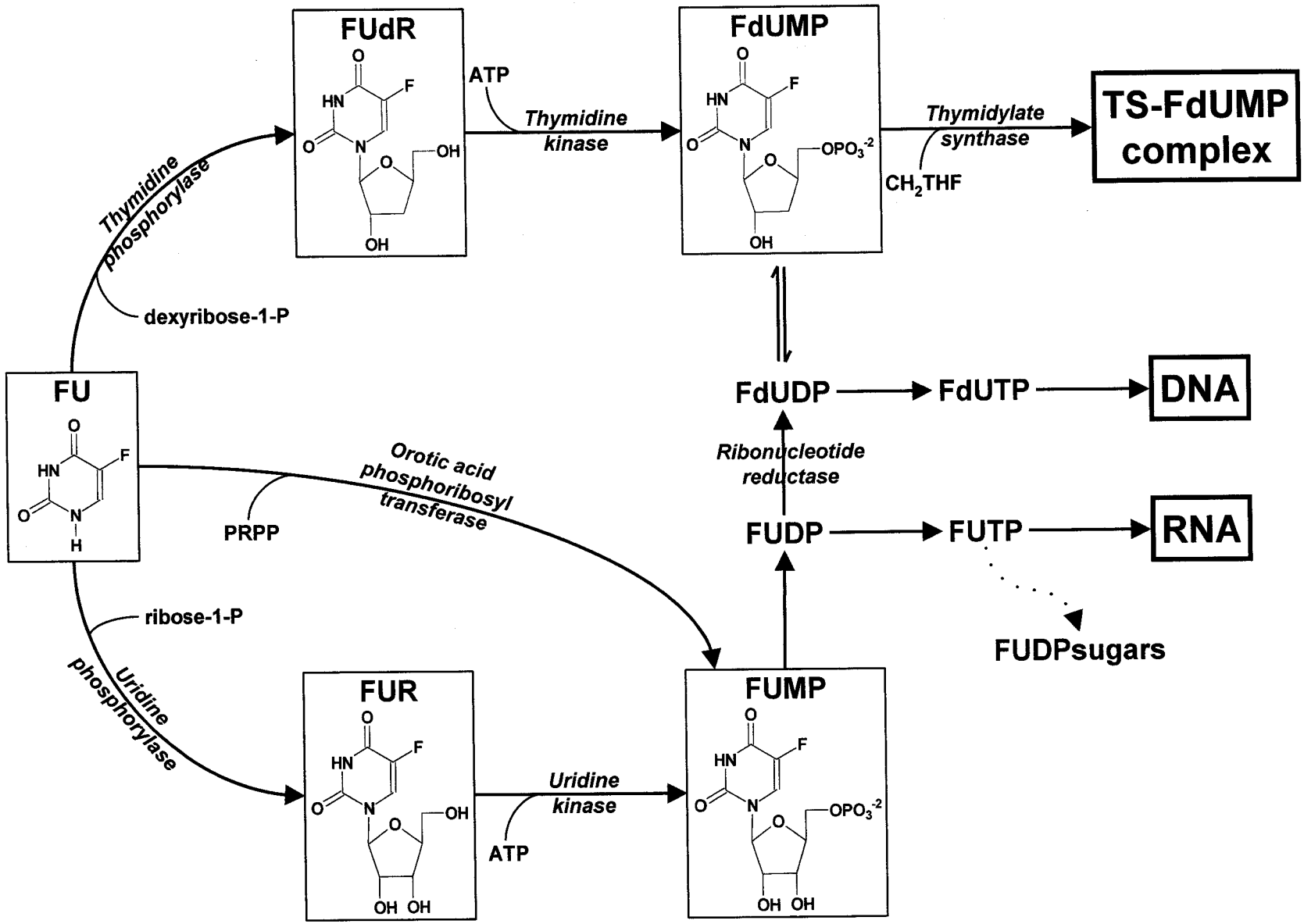
Inhibitors of TS that are analogues of the folate cofactor offer therapeutic advantages over the fluoropyrimidines since antifolates are less susceptible to metabolic degradation than are pyrimidines, and antifolates are not incorporated into DNA or RNA (20). The specific and potent inhibition of TS by Tomudex is due to its rapid cellular uptake *via* the reduced folate carrier and its prolonged retention by intracellular metabolism to polyglutamate forms (21).

Several lines of evidence support the concept of TS being an important chemotherapeutic target. From *in vitro, in vivo* and clinical studies, a strong correlation has been observed between the level of TS enzyme activity and/or TS protein expression and response to fluoropyrimidines (22-24). In addition, there is a strong correlation between the level of TS enzyme inhibition within patient tumor samples following 5-FU therapy and clinical response (25,26).

An estimated 139,900 new cases of cancer and 67,400 deaths from cancer will occur in Canada in 2003 (National Cancer Institute of Canada). Although both the incidence and mortality rates for colorectal cancer have declined steadily since the mid 1980s, overall, colorectal cancer remains the second leading cause of death from cancer (National Cancer Institute of Canada).

FIGURE 2. Metabolism of 5-fluorouracil (FU)

Abbrevations are FUR, 5-fluorouridine; FUMP, 5-fluorouridine-5'-monophosphate; FUDP, 5-fluorouridine-5'-diphosphate; FUTP, 5-fluorouridine-5'-triphosphate; FUdR, 5-fluorodeoxyuridine; FdUMP, 5-fluorodeoxyuridine-5'-monophosphate; FdUDP, 5-fluorodeoxyuridine-5'-diphosphate; FdUTP, 5-fluorodeoxyuridine-5'-triphosphate.


FUdR
FdUMP
ATP
Thymidine kinase
Thymidylate synthase
CH2THF
TS-FdUMP complex
Thymidine phosphorylase
dexyribose-1-P
FU
FdUDP
FdUTP
DNA
Orotic acid phosphoribosyl transferase
PRPP
Ribonucleotide reductase
FUDP
FUTP
RNA
FUDPsugars
ribose-1-P
Uridine phosphorylase
FUR
FUMP
Uridine kinase
ATP

(iii) *Regulation of TS*

Several of the initial studies examining the regulation of TS expression focused on the cell cycle-directed events. Maximal TS enzyme levels occur during periods of active DNA synthesis, and several groups have demonstrated that this increase in TS enzyme activity, as cells enter the S phase of the cell cycle, appears to be regulated at the transcriptional level (27,28). However, other studies have implicated the involvement of post-translational events in regulating the level of TS enzyme activity when cells leave the S-phase of the cell cycle. Studies using an MCF-7 breast cancer line showed that there was almost 40-fold increase in TS enzyme levels during S phase, with no accompanying change in TS mRNA levels (29). In addition, following S phase, there was a dramatic decrease in TS enzyme levels with no associated change in TS mRNA levels (29). These findings suggested that both translational and post-translational events govern the regulation of TS expression during the cell cycle.

When normal or tumor cells are exposed to anti-TS chemotherapeutic agents, a rapid increase in TS enzyme levels is observed *in vitro, in vivo* and in clinical experimental systems (25,30-32). A study using MCF-7 breast cancer cells showed a 10 to 40-fold increase in TS enzyme levels following exposure to the folate analog, Tomudex (29). There was no change in TS mRNA levels and the protein synthesis inhibitor, cycloheximide, inhibited this increase in TS enzyme levels (29). The results of this study suggested that a translational regulatory event was involved. Later work by Chu and co-workers showed that the increases in both TS enzyme activity and TS protein expression, which resulted in the human colon caner line H630 after 5-FU exposure, were not associated with a corresponding change in TS mRNA levels (33,34). Further work by this group showed that the increase in TS protein expression was the direct result of new synthesis of TS protein (33).

Chu and co-workers went on to characterize TS mRNA translation more completely. From all of their studies, this group put forward a model of *translational autoregulation of TS* (35) (Fig. 3A). According to this model, if the TS protein is not interacting with its substrates (either of its physiological substrates dUMP or CH_2-THF, or the 5-FU and 5-FUdR metabolite, FdUMP), it can bind to specific sites on its own mRNA resulting in translational repression (35). In contrast, when TS is bound to substrate, it is no longer able to bind its mRNA and translational inhibition is relieved, resulting in increased translation of

the TS message (35). The RNA binding activity of TS protein is also dependent on its redox state (36). It binds to its mRNA only when it is in a reduced state (36).

Two sites on TS mRNA to which TS protein binds with comparable affinity to the full-length mRNA, have been identified (37). One of the site maps to a 30 nucleotide fragment in the 5'-UTR of the TS mRNA that just includes the start codon (37). The other site has been mapped to a 70 nucleotide region corresponding to nucleotides 480 to 550 within the coding region (38). The RNA binding site in the 5'-UTR is capable of forming a stable hair pin loop structure (37). The model of translational autoregulation of TS provides a rational mechanism for the acute induction of TS that arises in response to exposure to 5-FU, 5-FUdR and to antifolate analogs that specifically target TS (35). In addition, it has been demonstrated by the same research group that TS protein also binds to c-myc and p53 mRNAs and inhibits their translation thereby implicating TS as a translational regulator of cellular gene expression (39,40).

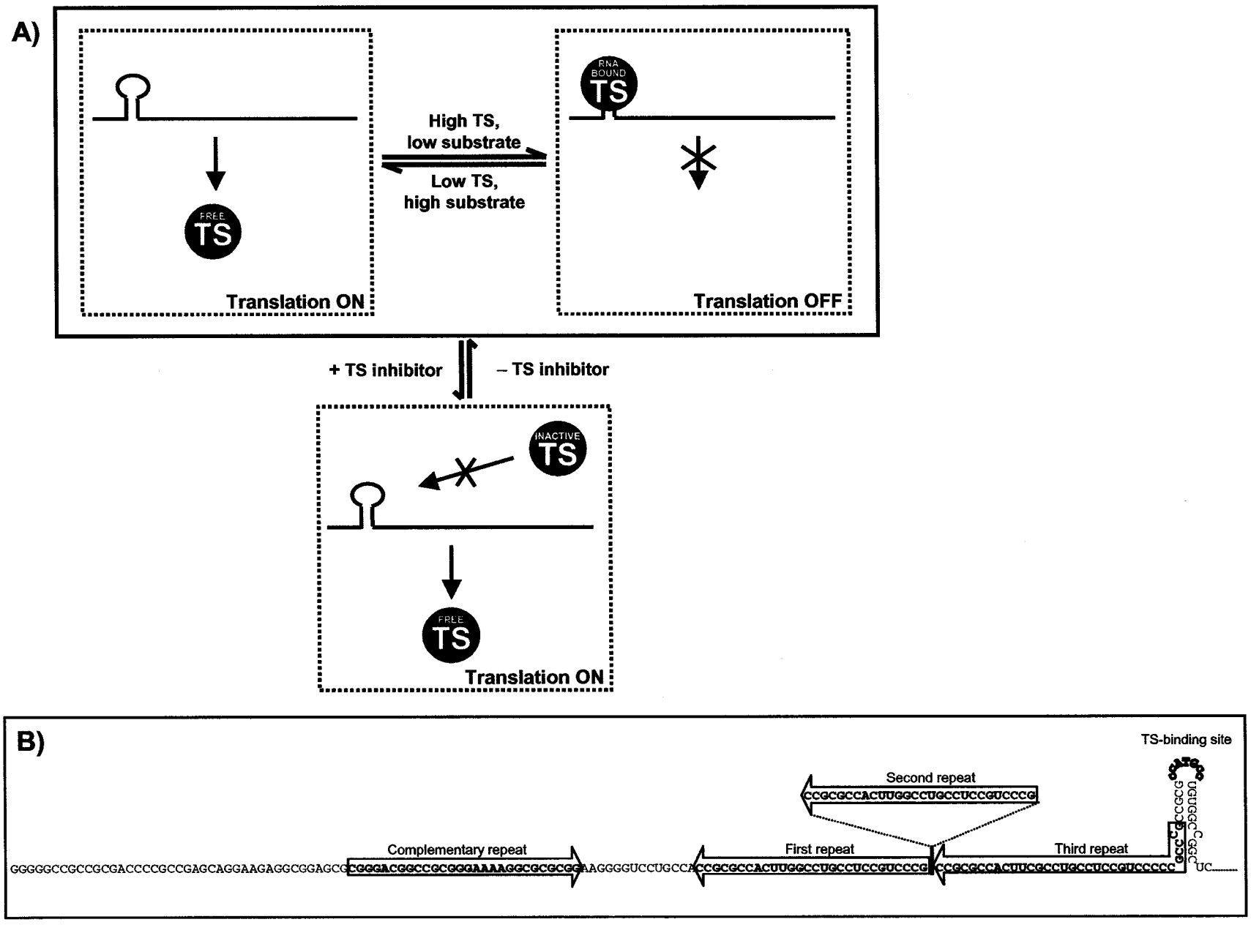
The human TS gene is polymorphic with either triple (3R) or double (2R) tandem repeats of a 28 base-pair sequence downstream of the cap-site in the 5'-terminal regulatory region (41) (Fig. 3B). In *in vitro* studies, the activity of a reporter gene linked to the 5'-terminal fragment of the TS gene with triple tandem repeats was found to be 2.6 times higher than that with double tandem repeats (41). This polymorphic region therefore appeared to be functional and involved in the modulation of TS gene expression. It is important to note that in both TS alleles (3R and 2R), one of the tandem repeats lies just within the TS mRNA binding site in the 5'-UTR (Fig. 3B).

(iv) *Localization of TS*

Attempts to establish the intracellular location of TS have been inconclusive. Most studies have reported TS to be associated with the cytoplasm (22,42-46). In comparison, very few cases have reported it in the nucleus (22,47-50) none of which utilized human cell lines. A recent study characterized the nuclear expression of TS in human tissue *in vivo* and showed that higher TS nuclear expression had a significant association with poorer response to protracted venous infusional 5-FU therapy (50). Thus, this study suggested a physiological relevance for nuclear TS.

FIGURE 3. Mechanism of TS auto regulation at the translational level and the 5'UTR of TS mRNA showing tandem repeats and stem-loop structure

A. TS translation is turned "on" at low TS and/or high substrate levels and is turned "off" by RNA-binding of TS at high TS and/or low substrate level. In the presence of TS inhibitor, the inactivated TS is unable to bind RNA and hence the translation is turned "on". ***B.*** 5'UTR sequence of TS mRNA showing a complementary repeat followed by either three or two tandem polymorphic repeats. The stem-loop secondary structure (in *blue*) is the TS-binding site and contains the start codon (AUG) in the loop. Note that the third repeat partially overlaps with the secondary structure.


A)
RNA BOUND
TS
High TS,
low substrate
Low TS,
high substrate
FREE
TS
Translation ON
Translation OFF
+ TS inhibitor
– TS inhibitor
INACTIVE
TS
FREE
TS
Translation ON
B)
TS-binding site
Second repeat
Complementary repeat
First repeat
Third repeat
GGGGGCCGCCGCGACCCCGCCGAGCAGGAAGAGGCGGAGCG
AAGGGGUCCUGCCA
UC

(v) *Mechanisms of Resistance to Anti-TS Chemotherapeutic Agents*

One of principal obstacles to the clinical efficacy of anti-TS chemotherapeutic agents has been the rapid emergence of cellular resistance. Several mechanisms of resistance to fluoropyrimidines and TS folate cofactor analogues have been reported *in vitro, in vivo* and in the clinical setting and include the following (Table 1): (*i*) increased levels of target enzyme TS resulting from TS gene amplification (51,51-54), increased TS mRNA levels (53,54), TS gene polymorphisms affecting transcription of TS gene (55) and decreased translational regulation of TS (56-59); (*ii*) altered TS enzyme with decreased affinity for FdUMP or CH_2-THF folate cofactor resulting from mutations in the TS gene (60,61); (*iii*) decreased FdUMP synthesis resulting from either increased phosphatase activity resulting in decreased accumulation of FdUMP (62), or decreased activity of 5-FU and 5-FUdR metabolizing enzymes such as thymidine kinase (63-65), orotate phosphoribosyltransferase (OPRT) (66), uridine kinase (UK) (67) and ribonucleotide reductase (RNR) (68); (*iv*) decreased transport of the drug into the cells either through a nucleoside transporter (69-71), a nucleobase transporter (72) or reduced folate carrier (73,74); (*v*) decreased intracellular pools of the reduced folate cofactor resulting from decreased activity of enzymes involved in folate metabolism (66,75-77); (*vi*) decreased incorporation into RNA (66,78) and DNA (67,79) for the fluoropyrimdines; and (*vii*) nuclear localization of TS, since it was recently shown to be associated with poorer response to 5-FU therapy (50).

(vi) *Overview of Thesis*

This thesis focuses on some of the aforementioned mechanisms of resistance to anti-TS chemotherapeutic agents. I have divided my thesis into 4 chapters; each chapter focuses on one particular mechanism of resistance.

- In Chapter 1, I examine factors responsible for the nuclear localization of TS in as much as TS nuclear localization was recently reported to be associated with a poorer response to 5-FU therapy.
- In Chapter 2, I describe the development and characterization of TS overexpressing cell lines keeping in mind that TS overexpression is one of the most common mechanisms of resistance to anti-TS drugs.

- In Chapter 3, I use the lack of TS ternary complex formation as a marker of 5-FUdR resistance and identify a "phenotypic instability" that causes resistance.
- Finally, in Chapter 4, I set out to examine differences in translational regulation between two alleles of the TS gene that could account for differences in TS levels between individuals of different TS genotypes.

TABLE 1. Factors associated with resistance to anti-TS chemotherapeutic agents

	Factors	Probable causes
i	Increased TS levels	Gene amplification Polymorphism Increase TS mRNA levels Reduced translational autoregulation
ii	Reduced TS affinity for drugs	Mutation/modification of TS protein
iii	Reduced 5-FdUMP synthesis	Reduced activity of 5-FU and 5-FUdR metabolizing enzymes (e.g., TK) Increased activity of phosphatase
iv	Reduced uptake of drugs	Reduced activity of nucleoside and nucleobase transporters Reduced folate carrier
v	Reduced folate levels	Reduced activity of enzymes involved in folate metabolism
vi	Reduced RNA and DNA toxicity for fluoropyrimidines	Reduced incorporation of fluoropyrimidine into DNA and RNA
vii	Nuclear localization of TS	?

CHAPTER 1: FACTORS AFFECTING NUCLEAR LOCALIZATION OF THYMIDYLATE SYNTHASE

1.1. INTRODUCTION

Thymidylate Synthase (TS) catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) and is the sole *de novo* source of deoxythymidine monophosphate (dTMP) (80). Once synthesized, dTMP is then metabolized intracellularly to the dTTP triphosphate form, an essential precursor for DNA synthesis. Because of its key role in DNA synthesis, TS represents a key target of cancer chemotherapeutic drugs, including the fluoropyrimidines and various anti-folate analogs (1,3).

The fluoropyrimidines are an important group of antineoplastic agents widely used in the treatment colorectal and other tumors (81). Both 5-fluorouracil (5-FU) and 5-fluorodeoxyuridine (5-FUdR) are converted to FdUMP; in the presence of 5,10-methylenetetrahydrofolate cofactor, FdUMP binds to TS to form a tight-binding covalent ternary complex leading to prolonged inhibition of the enzyme (82).

Attempts to establish the intracellular location of TS have been inconclusive. Most studies have reported TS to be associated with the **cytoplasm** (22,22,42,42-46). In comparison, very few cases have reported it in the **nucleus** (22,47-50) none of which utilized human cell lines. Studies conducted with yeast have shown the enzyme to be localized at the nuclear periphery in association with the nuclear envelope and/or ER membrane (83) and one study utilizing a rat hepatoma cell line reported the enzyme's association with the nucleolus of the cell (49).

Why might TS be present in the nucleus of a cell? The first explanation was proposed in earlier studies which suggested that TS formed a multienzyme complex involved in DNA synthesis in T-even phage infected *E.coli* and in eukaryotes (48). These enzymes including DNA polymerase alpha and various dNTP metabolizing enzymes formed a multienzyme complex, referred to as "replitase", that synthesized and incorporated dNTP directly into replication forks inside the nucleus (83). Multienzyme complex would allow the advantage of metabolite channeling (48). However, debate has revolved around the existence of a multienzyme complex. Countering the replitase model, several investigators have characterized TS and ribonucleotide reductase, constituents of the proposed replitase model, as cytoplasmic (83,84).

The other reason TS might be present in the nucleus comes from more recent studies which show that in addition to its critical catalytic function, TS serves an important role as an

RNA binding protein. TS has been found to bind with high affinity to its own mRNA and directly regulate its own biosynthesis through a translational autoregulatory feed back mechanism (85) (General Introduction, Fig. 3A)(37). The state of ligand occupancy of TS appears to play a key role as a determinant of RNA binding (36,85). When TS is ligand free, maximal RNA binding activity is maintained, thereby resulting in complete translational repression of TS mRNA. In contrast, when TS is bound either to its physiologic substrates, dUMP or 5,10-methylenetetrahydrofolate, or bound to the 5-FU metabolite, FdUMP, or by an antifolate analog such as Tomudex, the RNA binding activity of TS is dramatically decreased. The net effect of reduced RNA binding activity is relief of translational repression leading to increased synthesis of new TS protein. Through its role as an RNA binding protein, it interacts with several other cellular mRNAs including those corresponding to p53 tumor suppressor gene and the myc family of transcription factors (39,40,86). In each of these cases, TS serves as a translational repressor to coordinately regulate the expression and or function of these important genes. It has been speculated that p53 mRNA and TS complex may be localized to the nucleolus (86).

The single report that characterized the nuclear expression of TS in human tissue *in vivo* showed that higher TS nuclear expression had a significant association with poorer response to protracted venous infusional 5-FU therapy (50). This study suggested a physiological relevance for nuclear TS.

When TS localization was examined in mouse L929 cells, it was found to be cytoplasmic (42). The authors compared their results to an earlier study that reported considerable TS activity in the nuclear fraction of CHEF/18 Chinese hamster embryo fibroblasts (48). It was suggested that differences in cell lines and culture conditions could account for the variation in TS localization between the two studies. In past studies examining TS localization in cultured cell lines, only one cell line was investigated in each study (42,49,83,84). Before we can acertain whether cellular differences account for variation in TS localization there is the need for a study in which the same antibody and staining techniques are used to examine and to compare the localization of TS amongst several cell lines. This would control for differences in antibodies and techniques used amongst the past studies.

In carrot cells TS exists as a bifunctional dihyrofolate reductase-thymidylate synthase (DHFR-TS) gene. The intracellular distribution and localization of DHFR-TS of wild type suspension carrot cells was analysed using cytochemical and immunocytochemical techniques (84). In both resting and growing normal cells the activity appeared to be predominantly cytoplasmic. However, metotrexate-resistant cells (metotrexate is an inhibitor of DHFR), overproducing DHFR-TS showed, irrespective of their physiological state, a relevant nuclear or perinuclear immunofluorescence. Results of this study indicated that the overexpression of DHFR-TS in a cell resulted in nuclear localization of the enzyme.

The separation of eukaryotic cells into cytoplasm and nucleus provides an important way to regulate cellular processes through compartmentalization. The exchange of proteins and RNA between cytoplasm and nucleus occurs through the nuclear pore complex (87). Many nuclear proteins are imported by virtue of a nuclear localization sequence (NLS) rich in basic amino acids (87). Nuclear export sequences (NES) (88) promote the export of proteins out of the nucleus. The leucine-rich NES (which consists of four critically spaced leucines or other large hydrophobic residues (89) has been identified in many proteins including protein kinase A inhibitor (PKI) (90), p53 (91), and the RNA binding proteins HIV-1 Rev (89), and HTLV-1 Rex (89). Masking of the NLS or NES through their binding to other factors or through post-translational modifications would allow for controlled shuttling of factors between the cytoplasm and the nucleus (92). Preliminary work in RKO cells revealed that deletion of the first 10 amino acids at the amino-terminus and deletion of 9 amino acids (291-299) at the carboxy terminus resulted in loss of nuclear TS localization (93). These preliminary results suggests that these sequences appear to act as NLS signals (93). These putative NLS of TS do not conform to the consensus of basic rich stretches of amino acids as in established NLSs (94). To date there have been no reports on any nuclear export sequences within the TS gene.

To place confidence in localization studies employing immunohistochemistry, immunocytochemistry and immunofluorescence techniques, the specificity of the antibodies used must be first demonstrated. Since not all studies reporting on the localization of TS employed fully characterized antibodies, it meant that differences in specificities of antibodies could have contributed to the controversy surrounding TS localization. Therefore,

an important first step in any study reporting on the localization of TS should be the demonstration of the specificity of the antibodies used.

From the clinical study that described an association between TS nuclear localization and resistance to 5-FU (50), several important questions ensue. Is nuclear localization causing resistance? Or is it just associated with resistance? If nuclear localization is causing resistance, then I need to understand the *mechanism* of resistance. To understand this mechanism, a more detailed understanding of the *cause* of nuclear localization is needed (ie, the factors involved). On the other hand, if nuclear localization is just associated with resistance, then I need to know the factors that lead to nuclear localization as they may be the same factors that cause the resistance. Either way, it is important to know the factors responsible for TS nuclear localization. In this chapter, I have reported on some of the possible factors that can affect the nuclear localization of human TS. I hypothesized that nuclear localization of TS depends upon several factors including TS overexpression. Cells overexpressing TS will show more prominent nuclear TS than cells with normal TS levels. In addition, some cell-types will show more prominent nuclear TS than others.

1.2. INITIAL OBJECTIVES

My overall initial objective was to examine whether (i) overexpression of TS and (ii) the type of cell, affect the nuclear localization of TS. To test this, the following specific objectives were examined.

- Demonstrate specificity of an anti-TS antibody
- Examine TS localization in HeLa cells overexpressing TS (developed by step-wise FUdR resistance in Chapter 2) using immunohistochemistry
- Examine TS localization in a range of human cancer cell lines (HeLa, HCT-116, HCT-15, HCC-2998, HT-29, SW-620 and RKO) and normal human fibroblast cells using immunohistochemistry
- Use confocal microscopy to demonstrate localization of nuclear TS (if any)
- Separate nuclei of cells showing nuclear TS staining (if any) and detect TS by western blot analysis

1.3. MATERIALS AND METHODS

1.3.1 Antibodies

Sheep anti-hTS antiserum used in these studies was obtained from Rockland Immunochemicals (Gilbertsville, PA; cat. no. 100-601-199). This polyclonal antibody was raised against recombinant $(his)_6$-rhTS (95). Horseradish peroxidase (HRP)-conjugated rabbit anti-sheep secondary antibody (cat. no. 613-4328) and goat anti-lactate dehydrogenase polyclonal antibody (cat. no. 100-1173) were also from Rockland Immunochemicals. CY3-conjugated donkey anti-sheep secondary antibody (cat. no. 716-165-147) and HRP-conjugated donkey anti-goat secondary antibody (cat. no. 705-036-147) were from Jackson ImmunoResearch Laboratories (West Grove, PA). HRP-conjugated anti-rabbit/anti-mouse secondary antibody ("Polymer") was from DAKO (Carpinteria, CA).

The specificity of the anti-hTS antibody was determined using recombinant $(his)_6$-tagged hTS protein (rhTS) as a competitor. This protein was expressed in *E. coli*, purified by affinity chromatography on a nickel column and then twice-purified by SDS-PAGE as previously described (95). After electroelution, the protein was precipitated with ethanol and dissolved as a stock solution at a concentration of 0.5 mg/mL in 0.2% SDS. The detergent was required to maintain the solubility of the denatured TS protein. When used as a competitor for Western blot analysis, immunofluorescence, immunocytochemistry or immunhistochemistry, the stock was diluted such that the final concentration of SDS was $\leq$0.02%. For competition experiments, rhTS was pre-incubated overnight at 4 °C with 100 µL of a 1:500 dilution of sheep anti-TS antiserum. To control for any inhibitory effect of this low concentration of SDS, control experiments were carried out in which the same antiserum was incubated with an equivalent amount of SDS.

Other tests of the specificity of the anti-hTS antibody involved selective depletion of anti-TS antibody from the sheep antiserum using an affinity column with immobilized rhTS. Sheep anti-TS antiserum was diluted 1:100 and passed 20 times through a bed of CN Br-activated Sepharose 4B (Sigma-Aldrich Chemicals, St. Louis, MO) to which rhTS protein was coupled (95). As a control, diluted anti-serum was passed through a similar matrix containing immobilized bovine serum albumin (BSA) (OmniPur ^{R} BSA, Fraction V, EM science- MERCK kGaA, Darmstadt, Germany).

1.3.2 Cell lines

HeLa cells were obtained from ATCC (Rockville, MD). A series of 5-fluorodeoxyuridine (FUdR)-resistant HeLa cells clones were isolated by stepwise selection over several weeks in up to 0.1 μM in this drug. Clone HeLa-55 overexpressed TS protein most strongly and was shown to have a highly amplified TS gene (Chapter 2). The following colon cancer cell lines from the NCI anti-cancer screening panel of 60 cell lines were used: HCT-116; HCT-15, HCC-2998, HT-29 and SW-620; RKO is a colon cancer line originally derived by Brattain *et al.* (96). Normal primary human fibroblasts were from neonatal foreskin. Cells were cultured as monolayers in Dulbecco's modified Eagle's medium (GIBCO-BRL, Burlington, Canada) supplemented with 10% fetal calf serum in 100 mm dishes in 5% CO_2 at 37 °C. RKO cells were maintained in McCoy's 5A media plus 10% fetal calf serum (GIBCO-BRL).

1.3.3 Immunofluorescence

Cells were grown on 22 mm^2 glass cover slips in 10 cm dishes. Log phase cultures were used in all experiments. Coverslips were removed and washed twice with phosphate-buffered saline (PBS). All subsequent steps were carried out at room temperature. Cells were fixed for 10 min with acetone:methanol (1:1). Coverslips were blocked for 30 min with Universal Blocking solution (DAKO). All antibody dilutions were in 'Antibody Diluting buffer' (DAKO). Coverslips were incubated for 1 h with primary anti-TS antibody (1:500 dilution). They were washed 5 times (5 min per wash) with PBS. Cells were then incubated for 30 min with secondary anti-sheep IgG- CY3 antibody (1:1000 dilution) and washed again. The coverslips were mounted onto slides with 'Fluorescent Mounting medium' (DAKO) containing 5 μg/mL Hoechst 33258 (Sigma-Aldrich). Stained cells were examined and photographed with a Zeiss Axiophot fluorescence microscope. Confocal images were obtained using a Zeiss inverted LSM 410 confocal laser scanning miscroscope. Z-series images were obtained through the depth of cells using a step size range of 1-2μm.

1.3.4 Immunocytochemistry

Coverslips containing cells in log phase were removed and washed twice with phosphate-buffered saline (PBS). All subsequent steps were carried out at room temperature.

Cells were fixed for 10 min with acetone:methanol (1:1). Coverslips were blocked for 30 min with Casein Blocking Solution in PBS (2% bovine milk casein (Sigma-Aldrich), 2 mM cyclohexanediamine tetraacetate (Sigma-Aldrich), in PBS). All antibody dilutions were in PBS. Coverslips were incubated for 1 h with primary anti-TS antibody (1:500 dilution). They were washed 5 times (5 min per wash) with PBS. Cells were then incubated for 30 min with secondary rabbit anti-sheep-IgG-HRP antibody (1:1500 dilution) and washed again. Visualization was achieved with diaminobenzidine substrate. The coverslips were mounted onto slides with 'Permount Mounting medium' (Fisher Scientific, Toronto, Canada). Stained cells were examined and photographed with a Zeiss Axiophot microscope.

1.3.5 Immunohistochemistry

Colorectal cancer specimens were obtained from the Ottawa Colorectal Cancer Bank; most were fixed in GenoFix (DNA Genotek Inc, www.DNAgenotek.com). Four μm sections were deparaffinized and hydrated by standard methods. Slides were pre-treated with 3% H_2O_2 in methanol for 10 min and blocked for 30 min at room temperature with Casein Blocking solution in PBS. All antibodies were diluted in PBS. The sections were exposed overnight at 4°C to 1:500 dilution of primary anti-TS antibody. They were washed 5 times (5 min per wash) with PBS and then exposed to 1:200 dilution of rabbit anti-sheep-IgG-HRP antibody for 30 min at room temperature. Sections were washed again and visualization was achieved with diaminobenzidine substrate (DAKO liquid DAB^+ Substrate-Chromogen Solution, K3467, DAKO). The sections were then counterstained with hematoxylin. A negative control (that is lacking primary antibody) was included.

1.3.6 Preparation of whole cell and nuclear extracts

Cultured cells were detached by trypsin treatment, then washed twice and suspended in cold PBS. To prepare whole cell extracts, an equal volume of 2×SDS sample buffer (100 mM MOPS, pH 6.8, 4% SDS, 20% glycerol, 2 % β-mercaptoethanol) was added. The extracts were boiled for 20 min and then sonicated briefly to reduce viscosity. Protein was quantified using fluorescamine (97) (Sigma-Aldrich), using BSA as a standard. For isolation of nuclei, cells were washed twice in PBS and suspended in nuclear extraction buffer (10 mM Tris-HCl, pH7.5, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM spermidine,

2 μg/mL aprotinin and 1 mM PMSF) at ~ 1×10^6 cells/mL. After 20 min incubation at 0°C, the swollen cells were homogenized (50 strokes) with a tight-fitting glass Dounce homogenizer. Samples were centrifuged (800x*g*) at 2-4°C and the pellet was washed twice with nuclear extraction buffer containing 0.1% NP-40. Protein in the nuclear pellet was extracted by dispersion in SDS sample buffer, brief sonication and heating at 100°C for 15 min.

1.3.7 Western blot analysis

Proteins in cell or nuclear extracts in SDS sample buffer were resolved by 12% discontinuous SDS-PAGE and then electrophoretically transferred to a PVDF membrane (Millipore, Nepean, Canada) at 15V for 18 h in transfer buffer (3 mM Na_2CO_3, 10 mM $NaHCO_3$, 10% methanol) (95). The membrane was stained with Ponceau S and then washed twice in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8). All steps were carried out at room temperature. When using anti-TS as the primary antibody, the membrane was blocked for 1 h with freshly prepared Casein Blocker solution (100 mM NaCl, 50 mM sodium acetate, pH 6.0, 1% bovine milk casein, 2 mM cyclohexanediamine tetraacetate). When anti-LDH primary antibody was used, the membrane was blocked with 5% horse serum in TBST. Sheep anti-TS antibody was diluted 1:5000 in Casein Blocking solution and goat anti-LDH primary antibody was diluted 1:1000 in TBST. In all cases, the primary antibody was incubated with the membrane for 1 h. The membrane was washed 5 times in TBST and incubated for 1 h with a secondary antibody as follows: rabbit anti-sheep-IgG-HRP was used at 1:3000 dilution in Casein Blocking solution; DAKO 'Polymer' anti-mouse-IgG-HRP was used at 1:100 dilution in TBST; donkey anti-goat-IgG-HRP was used at 1:25000 dilution in TBST. The membrane was washed 5 times with TBST and HRP was detected using the LumiGLO Chemiluminescent Substrate Kit (Kirkegaard & Perry Lab; Maryland, USA).

1.3.8 Preparation of Plasmid DNA Contructs

GFP-NES: pEGFP-C1 plasmid was obtained from BD Biosciences Clontech (Mississauga, Canada). Two 46-mer oligonucleotides, hTSNESF (AATTCTATTTACCTGAATCACATCGAGCCACTGAAAATTCAGCTTG)

and hTSNESR
(GATCCAAGCTGAATTTTCAGTGGCTCGATGTGATTCAGGTAAATAG), encoding the sense and antisense sequences of a putative TS nuclear export signal (NES) respectively, were synthesized. The oligonucleotides were annealed and the resulting fragment consisted of overhanging sequences compatible to EcoR1 and BamH1 sites. The annealed fragment was ligated to EcoR1/BamH1 digested pEGFP-C1. The resulting plasmid, GFP-NES contained the putative NES of TS fused in frame to the carboxyl end of GFP; its sequence was confirmed by sequencing.

H_6TS^{313} : PQE-hTS (95) encoded human TS with the first 5 amino acids deleted and 16 additional amino acids including a 6 × histidine tag inserted at the N-terminus. This His_6-TS (1 kb) fragment was isolated from PQE-hTS using EcoR1/Xba1 and ligated to EcoR1/Xba1 digested pCR3 (Invitrogen, Burlington, Canada).

H_6TS^{248} : In pcDNA3/HA-hTS29 there was a base pair deletion in the codon for amino acid 248 of human TS (98). This resulted in a frameshift mutation causing amino acids 248-259 to become mutated and ended with a premature stop codon at amino acid 260. This resulted in non expression of amino acids 260-313. An 870bp fragment containing this frameshift mutation was isolated from pcDNA3/HA-hTS29 using Pst1/Xba1. H_6TS-PCR3 was digested with Pst1/Xba1 and the 5167bp fragment was ligated to the 870bp of pcDNA3/HA-hTS29.

H_6TS^{186} : The nucleotide fragment corresponding to amino acids 186-313 in H_6TS-PCR3 was cut out with BglII/Xba1. The remaining construct was blunted-ended with mung bean nuclease and then ligated.

fAeqTS-pCDNA3 was synthesized to contain the full-length TS cDNA. The coding region was obtained from pWHTS-1(6) and the flanking 5'-UTR containing triple GC-rich repeats (41) was synthesized by PCR amplification of genomic DNA. The correctness of the construct was confirmed by sequencing.

1.3.9 Transfection

HeLa and RKO cells were seeded at 1.25×10^5 cells in a 3 cm dish on glass coverslips. The following day, cells were transfected with 2 μg of purified plasmid DNA and 3 μl of Fugene 6 Transfection Reagent (Roche Diagonostics Corporation, Indianapolis,

USA), according to the manufacturer's protocol. Twenty-four hours post-transfection, coverslips were removed and immunofluorescence staining was performed as described above.

1.4. RESULTS

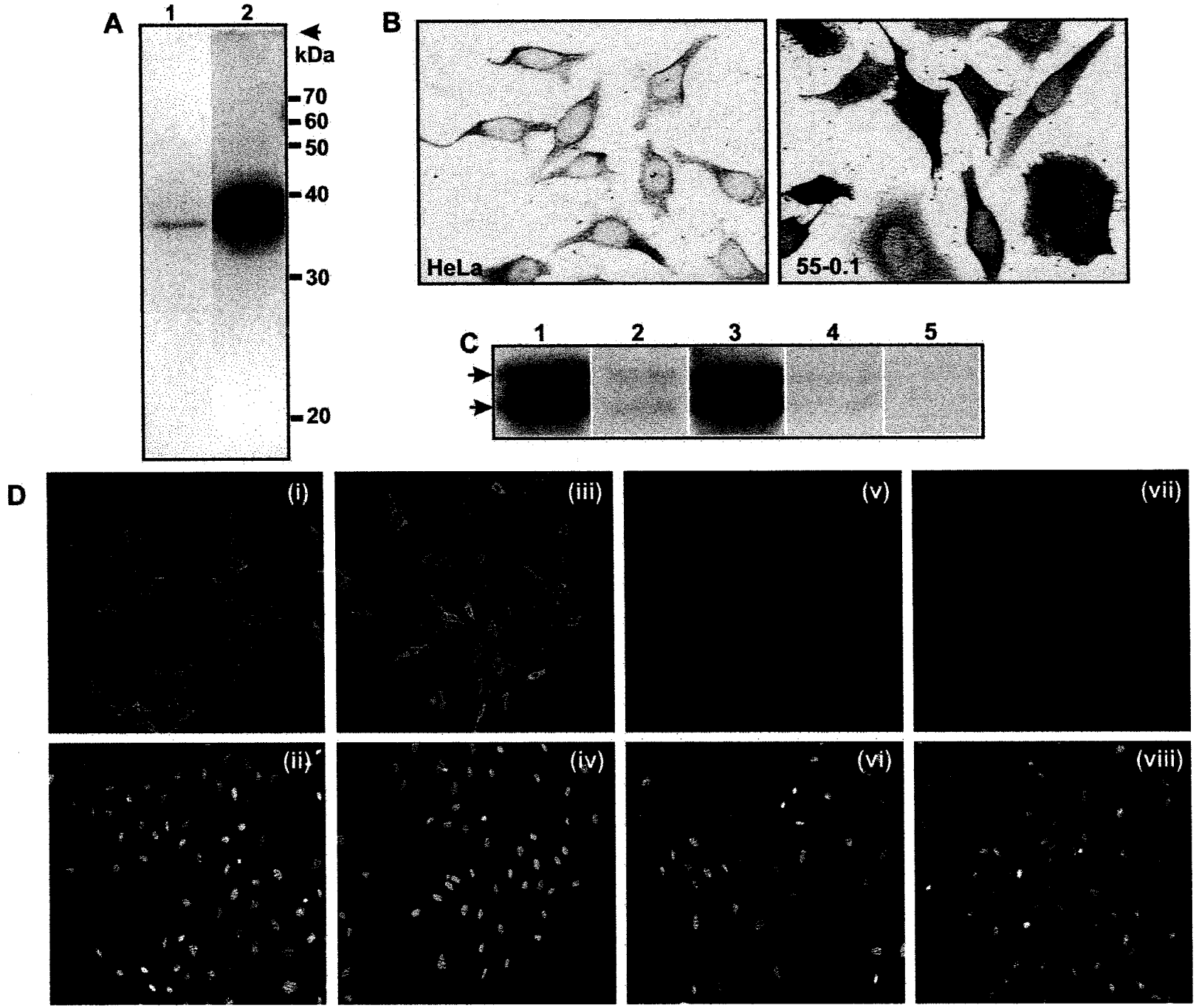
1.4.1. Specificity of anti-hTS antiserum

The interpretation of results employing immunohistochemistry depends critically on the specificity of the antibodies used. Previous immunohistochemical or immunocytochemical studies of TS have employed polyclonal anti-TS antibodies whose specificity was not fully documented. We chose to use multiple techniques to confirm the specificity of the primary anti-hTS sheep polyclonal antibody used in our experiments. Fig. 1.1A (lane 1) shows Western blot analysis of a HeLa cell extract probed with anti-hTS; the only detectable signal was a band of the expected molecular size for TS (35.7 kDa). Fig. 1.1A (lane 2) shows a similar analysis of an extract of a 5-FUdR-resistant HeLa cell line, HeLa-55, growing in 5-FUdR. Two bands are seen in Fig. 1.1A (lane 2) and Fig. 1.1C, which correspond to free TS and to the previously described "ternary complex" containing TS-5-FdUMP-CH_2-THF (22). The high level of TS in HeLa-55 cells was due to amplification of the TS gene (Chapter 2). No other bands were detected in extracts of either cell line, providing one line of evidence supporting claims for the specificity of the primary and secondary antibodies used. Fig. 1.1B shows immunocytochemical analysis of the same preparation of HeLa and HeLa-55 cells as used in panel A. The correspondence in amount of TS in these cell lines by the two methods and the single band seen on the Western blot provide strong evidence that the immunocytochemical signal is indeed due to TS. These results provide one line of evidence that the combination of primary and secondary rabbit anti-sheep antibodies used was highly specific for TS.

Competition with a specific antigen (usually a synthetic peptide) is another approach to establish specificity of antibodies. Since the anti-TS polyclonal antibody was raised against recombinant hTS protein (rhTS) (i.e., not against a synthetic peptide), we prepared highly purified protein and tested this as a competitor. Competition was carried out by either pre-incubating rhTS with the anti-hTS antibody or using anti-hTS antibody passed through an rhTS affinity column. Western blot analysis of HeLa-55 cells probed with either control or treated antibodies is shown in Fig. 1.1C. Control antibody and antibody passed through a

FIGURE 1.1. Proof of specificity of the sheep anti-TS Ab using Western blot and immunohistochemical analyses.

A. Western blot analysis for TS of whole cell extract of HeLa (lane 1) and HeLa-55 (lane 2). 10μg protein was loaded per lane. Arrow-head indicates position of top of the gel. ***B.*** Immunocytochemistry for TS of HeLa and HeLa-55 cells. Same primary and secondary antibodies were used. ***C.*** Competition of TS signal on Western blots of whole cell extract of HeLa-55 cells (10μg/lane). Top arrow indicates TS in a 'ternary complex' and bottom arrow indicates free TS protein. Anti-TS antibody was preincubated with SDS buffer-alone (lane 1) or SDS buffer containing 10μg of soluble rhTS protein (lane 2), or the antibody was preabsorbed using a control 10μg BSA-immobilized column (lane 3) or a 10μg rhTS-immobilized column (lane 4). Lane 5 is a secondary antibody-alone control. ***D.*** Competition of TS signal on immunofluorescence of HeLa-55 cells. Anti-TS antibody was preincubated with standard buffer (i), SDS buffer (iii) or SDS buffer containing 10μg of soluble rhTS protein (v). Secondary antibody-alone control is shown in (vii). The same fields stained with Hoechst are shown in bottom panels (ii), (iv), (vi) and (viii). Other details are in *Materials and Methods*.


A
1
2
kDa
70
60
50
40
30
20
B
HeLa
55-0.1
C
1
2
3
4
5
D
(i)
(iii)
(v)
(vii)
(ii)
(iv)
(vi)
(viii)

control affinity column showed strong TS and TS-ternary complex bands (Fig. 1C lanes 1 and 3). Essentially complete disappearence of the bands was seen with either soluble rhTS or immobilized rhTS as competitor (lanes 2 and 4). Secondary antibody alone showed no signal (lane 5). Immunofluorescence staining of HeLa-55 cells using the same antibodies is shown Fig. 1.1D. An equivalently strong fluorescent signal was seen in these cells when standard buffer (panel i) or 'competition' buffer (panel iii) was used. Pretreatment of the primary antibody with soluble rhTS almost completely eliminated the signal (panel v). Similar disappearence of signal was seen when immobilized rhTS was used as competitor (data not shown). Fluorescent secondary antibody alone showed no signal (panel vii). The same fields stained with Hoechst 33258 to indicate the position of nuclear DNA are shown in the bottom panels. Taken together, this combination of techniques provides strong evidence that the primary and secondary antibodies were highly specific for TS and therefore suitable for the following study.

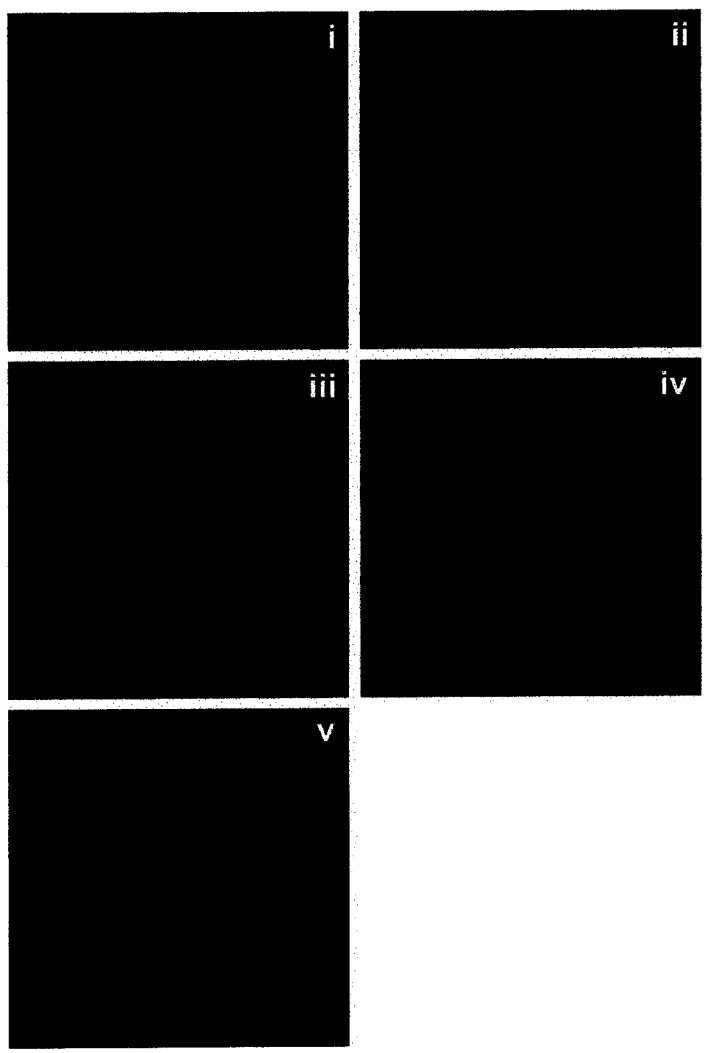
1.4.2. Nuclear localization of TS in overexpressing HeLa cell lines

Cell line HeLa-55, which contains a highly amplified TS gene, strongly overexpressed TS protein (Fig. 1.1). In addition to the strong staining of the cytoplasm of these cells, nuclear staining also appeared to be present (Fig. 1.1B). To confirm this observation, we used immunofluorescence confocal microscopy to study cellular localization. As shown in Fig. 1.2, serial optical sections provide clear evidence for the presence of nuclear staining in these TS-overexpressing cells.

HeLa-55 cells were observed to exhibit heterogeneous intensity of staining (Fig. 1.2). Nuclear staining was detectable in both high- and low-TS expressing HeLa-55 cells, although lower frequency of nuclear staining was seen in cells expressing lower amounts of TS compared to high TS expressors (Fig. 1.2 and data not shown). Variable TS expression was also seen in other 5-FUdR-resistant HeLa clones by immunofluorescence microscopy. In general, it appeared that clones that expressed less TS had a lower frequency of cells with nuclear TS staining (data not shown). Nuclear TS could still be detected in HeLa-55 cells grown in the absence of 5-FUdR for a period of up to 1 week (data not shown).

FIGURE 1.2. Confocal microscopy of immunohistochemically analyzed TS staining in HeLa- 55

2 μm serial sections were taken through the depth of cells from bottom to top, panels (i) to (v). Other details are in *Materials and Methods*.


i
ii
iii
iv
v

1.4.3. Nuclear localization of TS in Other Cell Lines

It has been previously reported that TS can be detected in the nucleus of a rat hepatoma cell line (99) and in human colon cancer specimens (50). To extend these observations, we examined a number of human cell lines for nuclear TS. Cell lines included normal human fibroblasts and 6 colorectal cancer lines (RKO, HCT-116, HCT-15, HT-29, HCC2998 and SW620). Amongst the various cell lines examined, differences in both the intensity and localization of TS protein were seen (Fig. 1.3). Normal fibroblasts and HCT-15 cells showed the lowest total level of TS staining (Fig. 1.3). There were only slight differences in TS staining amongst the other 5 cell lines. Fibroblasts (Fig. 1.3) and parental HeLa cells (Fig. 1.1B) showed no discernible nuclear staining. Both cytoplasmic and nuclear TS staining was observed in the other cell lines but the relative intensity of cytoplasmic TS compared to nuclear TS staining varied. HCT-15 showed a very low level and HCC2998 and SW620 (Fig. 1.3) showed low levels of nuclear TS staining. The strongest evidence of nuclear staining was seen in RKO, HCT-116 and HT-29 cells (Fig. 1.3).

To strengthen these observations, we performed immunofluorescence confocal microscopy on one nuclear TS positive and one negative cell line. As shown in Fig. 1.4, panels i-iii, serial optical sections clearly indicated the presence of nuclear TS staining in cell line RKO, and the absence of nuclear TS in cell line HeLa (Fig. 1.4, panels iv-v). These findings confirmed the conventional microscopy results.

1.4.4. Western Blot Analysis of extracts of isolated nuclei for TS

To provide an independent line of evidence for the presence of TS in nuclei, we isolated nuclei from some cell lines and performed Western blot analysis for TS on nuclear extracts. Nuclei were isolated from RKO and HCT-116, the two colorectal cell lines that showed strongest nuclear TS staining. Total cell extracts from an equivalent number of the same cells were analyzed. HeLa cells, which showed no nuclear TS, was used as a negative control. A strong TS band was detected in total cellular lysates of HeLa, RKO and HCT-116 (Fig. 1.5A, lanes 1-3). A weaker signal was detected in nuclear extracts of RKO and HCT-116 cells (Fig. 1.5A, lanes 5,6), but no indication of TS in the nuclear extract of HeLa cells was detected (Fig. 1.5A, lane 4). It appeared roughly 5-10% of total TS protein was

FIGURE 1.3. Survey of TS localization in variety of cell lines using immunohistochemical analysis.

Most cell lines seem to show nuclear localization; RKO, HCT-116, HT-29; fibroblast do not. There is clumping of some colon cancer lines. The same conditions of conventional immunofluorescence microscopy were used for all cell lines. Other details are in *Materials and Methods*.

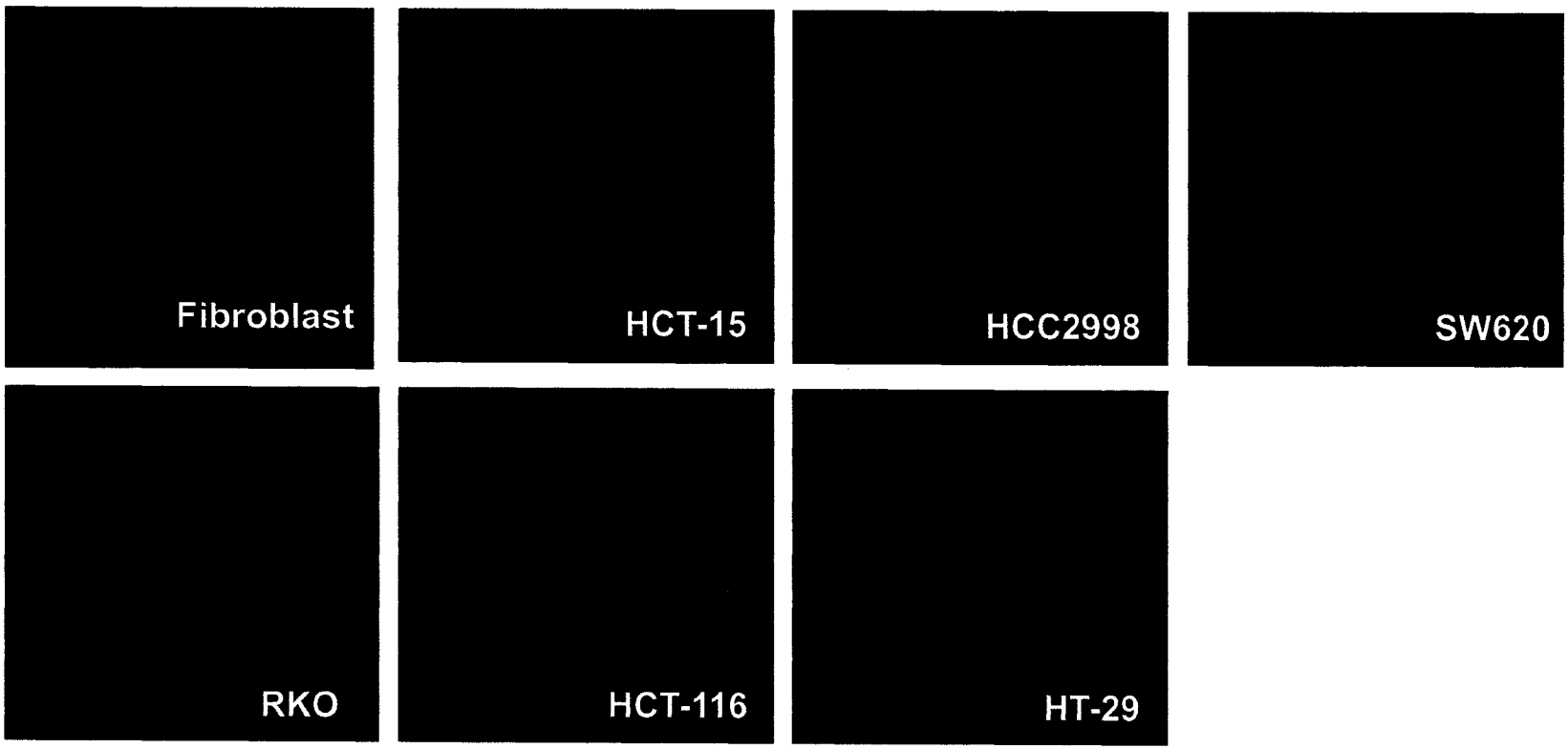

Fibroblast
HCT-15
HCC2998
SW620
RKO
HCT-116
HT-29

FIGURE 1.4. Confocal microscopy of immunohistochemically analyzed TS staining in RKO and HeLa cells.

2 μm serial sections were taken through the depth of cells from bottom to top. RKO cells, panels (i) to (iii); HeLa cells, panels (iv) to (vi). Other details are in *Materials and Methods*.

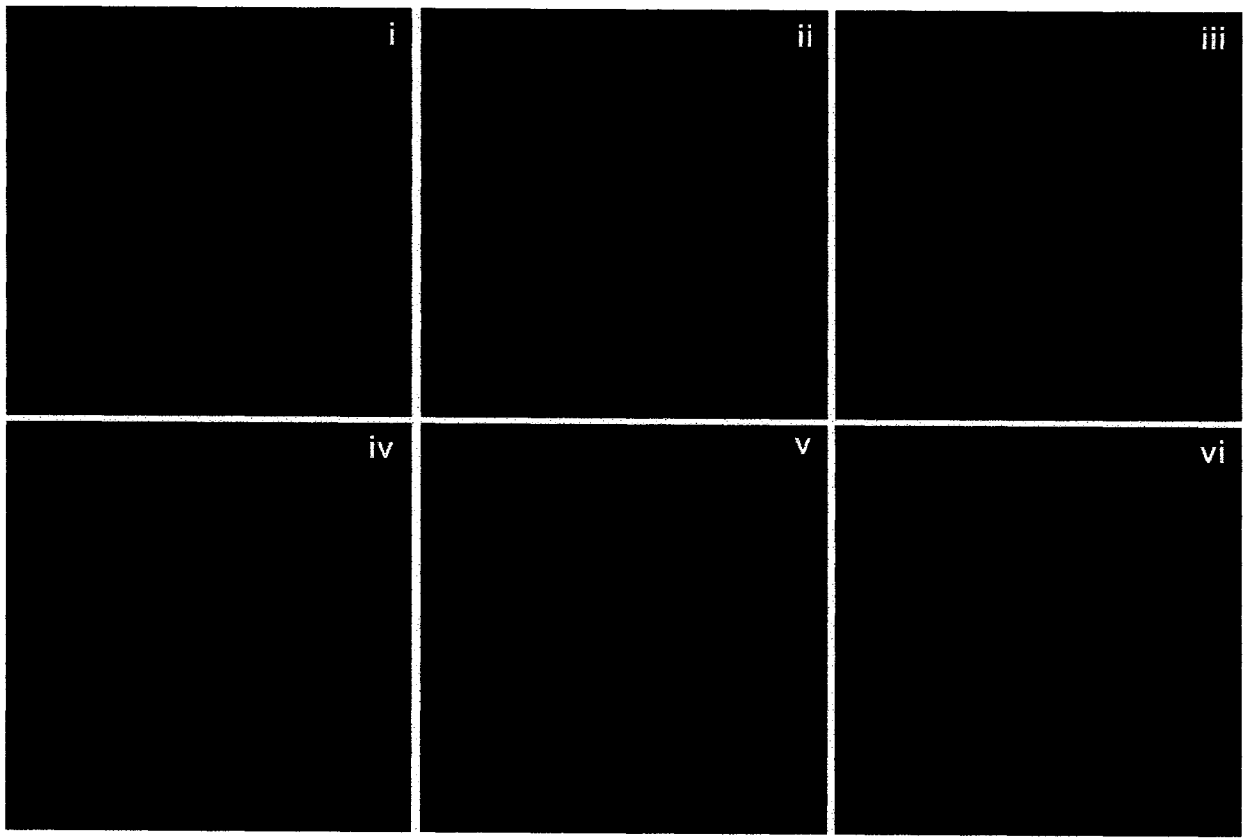
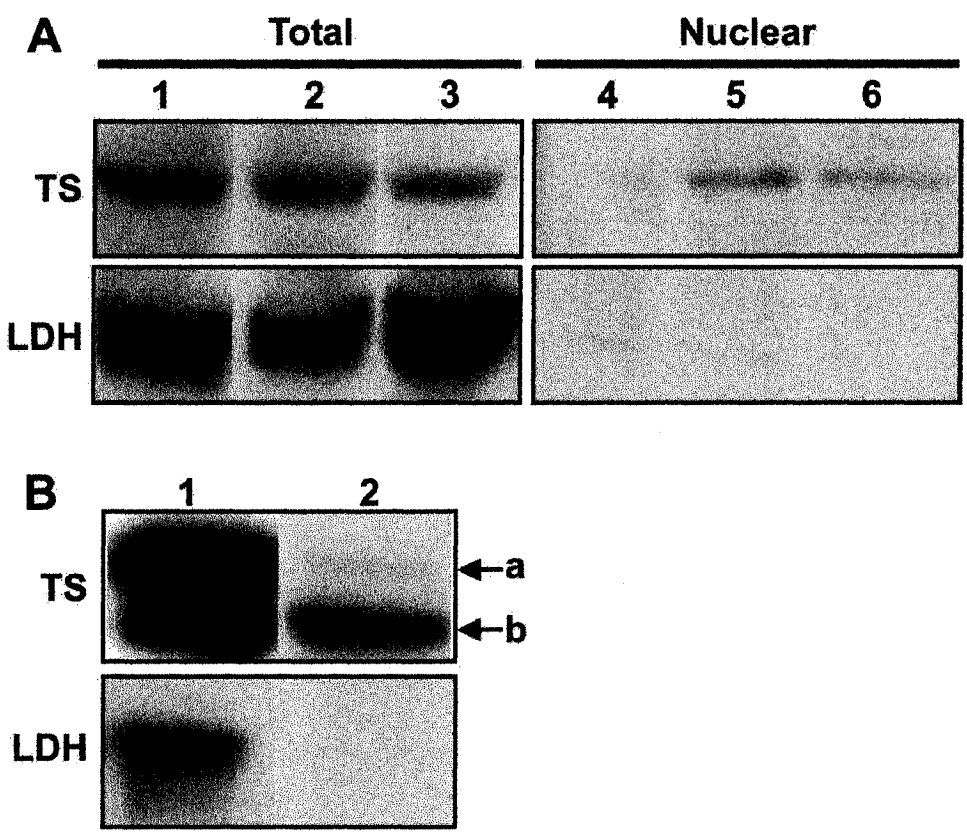

i
ii
iii
iv
v
vi

FIGURE 1.5. Western blot analysis for TS and LDH on the whole-cell or nuclear extracts in selected cell lines

A. Western blot analysis for TS and LDH on whole cell (lanes 1, 2, 3) and nuclear (lanes 4, 5, 6) extracts of HeLa (lanes 1, 4), RKO (lanes 2, 5) and HCT-116 cells (lanes 3, 6). Equivalent number of cells (5×10^5) was loaded in each lane. LDH is a cytoplasmic marker absent in nuclei. ***B***. Western blot analysis for TS and LDH on whole cell (lane 1, 5×10^3 cells) and nuclear (lane 2, 5×10^5 cells) extracts of HeLa-55 cells. Note that one hundred-fold less cells in lane 1 was loaded compared to lane 2 and the lanes in panel A due to a very high level of cytoplasmic TS in HeLa-55. Arrow 'a' indicates TS in a 'ternary complex' and arrow 'b' indicates free TS protein. Other details are in *Materials and Methods*.


A
Total
Nuclear
1
2
3
4
5
6
TS
LDH
B
1
2
TS
a
b
LDH

present in the nucleus of RKO and HCT-116 cells. Lactate dehydrogenase (LDH), a previously established cytoplasmic marker (100), was used to confirm the absence of cytoplasmic contamination in nuclear extracts (Fig. 1.5A).

1.4.5. Nuclear localization of free- and Ternary Complex-bound TS

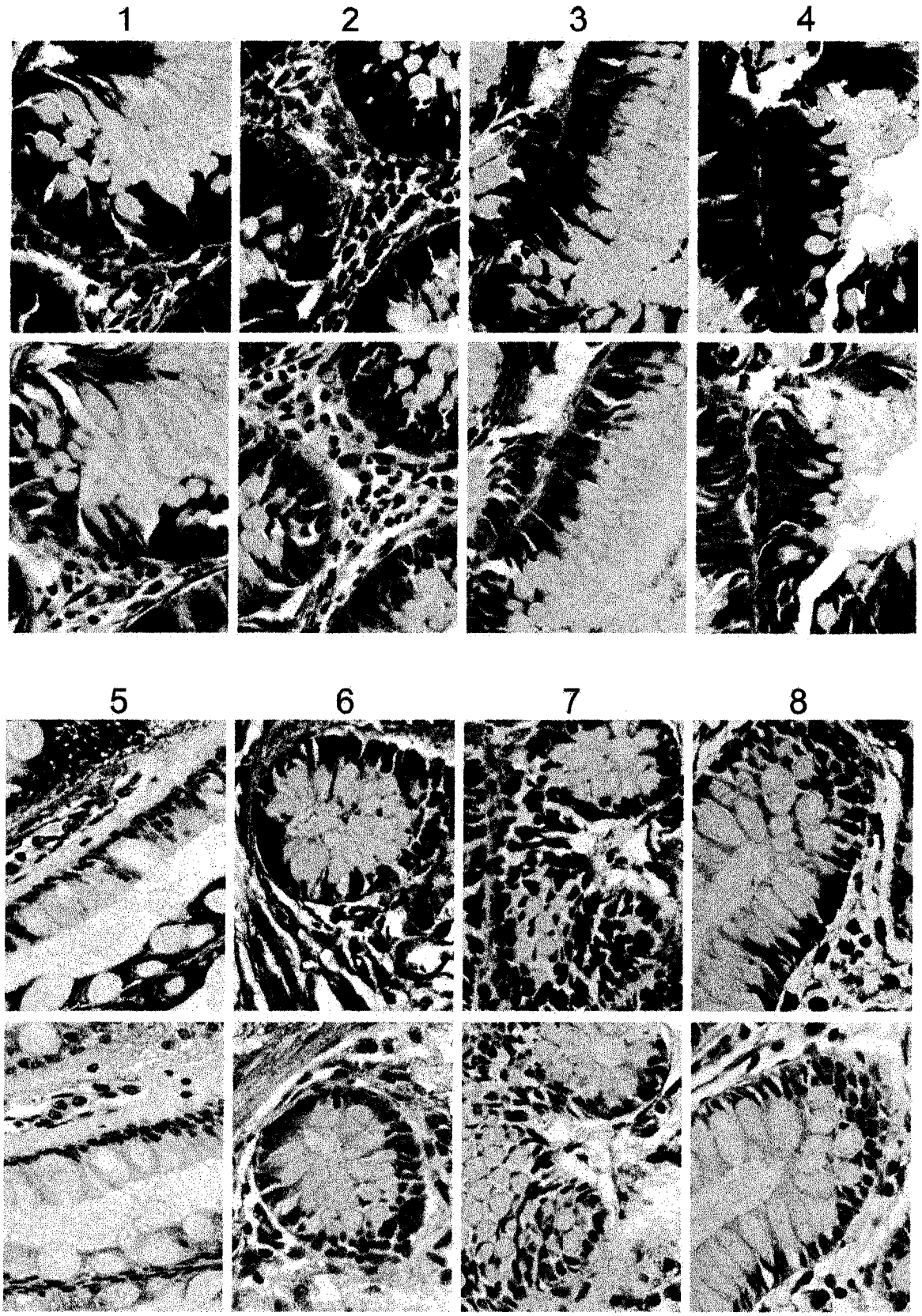
TS-overexpressing HeLa-55 cells grown in the presence of 0.1 μM 5-FUdR were fractionated and a nuclear extract was probed for TS by Western blot analysis. Total cell extract (100-fold less cell equivalents than used for nuclear extract) was similarly analysed. In addition to intense TS staining obtained in the total cell lysate (Fig. 1.5B, lane 1), a strong TS signal was detected in the nuclear extract (Fig. 1.5B, lane 2). From the relative intensities of the two, we estimate that about 0.5% of the total *free* TS was in the nuclear fraction. Interestingly, almost none of the *ternary complex-bound* TS was in the same nuclear fraction. The LDH control confirmed the virtual absence of cytoplasmic contamination in the nuclear extract.

1.4.6. Nuclear TS in Colorectal cancer specimens

Wong et al. (50), in agreement with our own unpublished observations, have identified nuclear staining of colorectal specimens probed with anti-TS antibodies. To confirm that this staining is indeed due to nuclear TS and not due to an unidentified cross-reacting species, we first identified some specimens of colorectal cancer in which nuclear staining could be observed using the same primary and secondary antibodies described above. Panels 1-4 (Fig. 1.6) shows regions selected from 1 case of colorectal cancer and panels 5-8 shows regions selected from 1 normal colonic mucosa. In each case, the lower section shows that the majority (but not all) of the strong TS signal decreased using recombinant hTS protein as a competitor, as in Fig. 1.1D. Noteworthy, both cytoplasmic and nuclear staining was similarly decreased by the competitor, providing strong evidence that nuclear staining was indeed due to the presence of TS protein. Interestingly, some nuclei in normal mucosa also showed the presence of nuclear TS.

FIGURE 1.6. Immunohistochemical analysis for TS in cancerous and normal colorectal tissue sections.

Different regions from a section of a cancer case (panels 1-4) and a normal case (panels 5-8) are shown. Each panel (1-8) shows two serial sections stained with anti-TS antibody containing either no competitor (top section) or 10μg rhTS competitor (bottom section). Pictures were taken at magnification × 400.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8

1.4.7. Putative Nuclear Export Sequence in TS Gene

To explain the presence of TS in the nuclei of some cells, we examined TS for the presence of motifs corresponding to nuclear localization sequences (NLS) and or nuclear export sequences (NES) that might influence its cellular localization. A putative leucine-rich nuclear export sequence (Fig. 1.7A) at position 257-269 was identified near the carboxyl terminus of TS. This putative NES containing a '4-2-1' spacing is conserved in human, mouse, rat and worms and is very similar to the '4-2' spacing found in Drosophilia and yeast and in the bifunctional DHFR-TS of carrot cells (Fig. 1.7A). To test whether the putative NES of TS could function as an NES, we prepared a construct, GFP-NES, in which TS-NES is fused in frame to the carboxyl end of GFP. Using pEGFP-C1 vector as a control, transfected HeLa cells strongly expressed GFP in both nucleus and cytoplasm (Fig. 1.7B). The localization of GFP in HeLa cells transfected with GFP-NES was indistinguishable from cells transfected with pEGFP-C1 control (Fig. 1.7B). Similar results were obtained in RKO cells (data not shown).

To explore the functionality of the putative TS NES further, we generated two His_6-tagged constructs of TS, H_6TS^{248} and H_6TS^{186}, lacking carboxyl terminal regions encompassing the NES and surrounding sequences (Fig. 1.8A). Full-length TS bearing an amino-terminal $histidine_6$ tag (H_6TS^{313}) (Fig. 1.8B), and wild type TS (wt TS) (data not shown) both localized predominantly to the cytoplasm, with only a small amount in the nucleus. However, HeLa cells transfected transiently with H_6TS^{248} and H_6TS^{186} showed an increased amount of nuclear TS (Fig. 1.8B). Cells transfected with H_6TS^{186} contained cytoplasmic punctate speckling and a noticeable decrease in the number of transfected cells expressing this truncated form of TS.

FIGURE 1.7. Putative leucine rich nuclear export signal (NES) of TS.

A. Multiple sequence alignment of some of the known NES (in PKI, HDM2, p53 and REV) with the putative NES in TS of several species (Hs, Homo sapiens; Mm, Mus musculus; Rn, Rattus norvegicus; Ce, Caenorhabditis elegans; Dm, Drosophila melanogaster; Sc, Saccharomyces cerevisiae; Dc, Daucus carota; Ec, Escherichia coli). Arrow indicates human TS sequence, first of the listed TS sequences. Letters in bold highlight amino acids that fit the putative leucine-rich NES motif. ***B***. Fluorescence analysis of HeLa cells transfected with control GFP construct or GFP fused to TS-NES (GFP-NES).

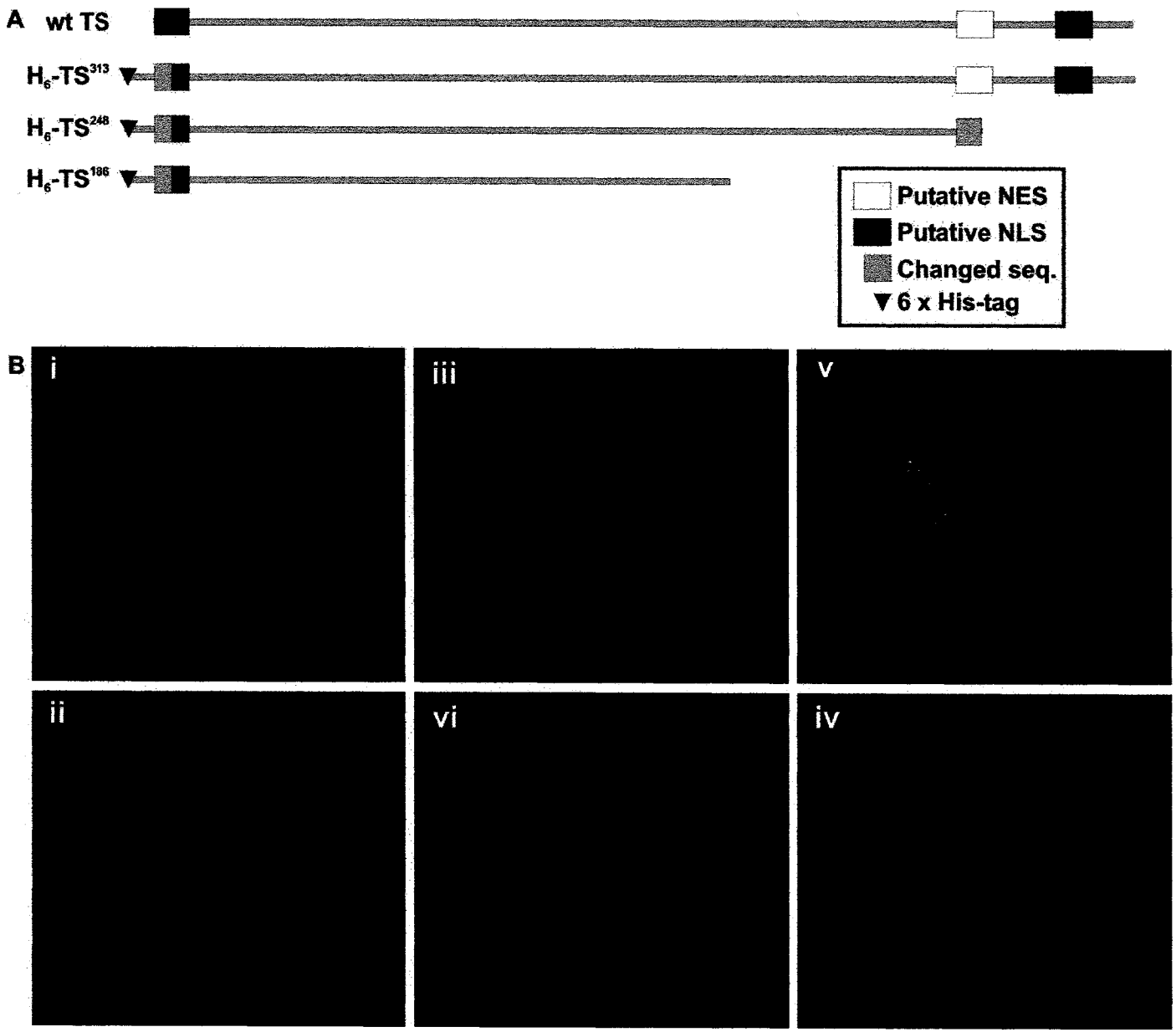
A

PKI^{NES}	36	--NE**L**ALK-**L**AG-**L**D-**I**NKT	50
HDM2^{NES}	194	---S**I**SLS-**F**DES**L**A-**L**CVI	209
Hs-p53^{cNES}	339	---E**M**FRE-**L**NEA**L**E-**L**KD-	352
Mm-p53^{cNES}	335	---E**M**FRE-**L**NEA**L**E-**L**KD-	348
Hs-p53^{nNES}	11	-EPP**L**SQET**F**SD-**L**WK**L**LP-	27
Mm-p53^{nNES}	14	-ELP**L**SQET**F**SG-**L**WK**L**LP-	30
HIV-1 REV^{NES}	75	----**L**PP--**L**ER-**L**T-**L**D--	84
→ Hs-TS	255	--AH**I**YLNH**I**EP-**L**K-**I**QL-	269
Mm-TS	249	--AH**I**YLNH**I**EP-**L**K-**I**QL-	263
Rn-TS	249	--AH**I**YLNH**I**EP-**L**K-**I**QL-	263
Ce-TS	254	--AH**V**YSNH**V**DA-**L**K-**I**QL-	268
Dm-TS	263	--TH**V**YLNH**V**EP-**L**K-EQL-	277
Sc-TS	201	--AH**V**YKDH**I**DA-**L**K-EQI-	215
Dc-DHFR/TS	518	--AH**V**YSNH**L**SD-**L**F-ETS-	532
Ec-DHFR/TS	206	--TH**L**YSNH**M**DQ-TH-**L**QL-	220

B

FIGURE 1.8. Effect of mutation in NES and surrounding sequences in TS protein on nuclear localization.

A. Schematic of various TS constructs. ***B.*** Immunofluorescence analysis of TS in HeLa cells transfected with H_6TS^{313} (i), H_6TS^{248} (iii) or H_6TS^{186} (v). The same fields stained with Hoechst are shown in bottom panels (ii), (iv), and (vi).


A
wt TS
H6-TS313
H6-TS248
H6-TS186
Putative NES
Putative NLS
Changed seq.
6 x His-tag
B
i
iii
v
ii
vi
iv

1.5. DISCUSSION

Confidence in immunohistochemistry results requires that highly specific antibodies be utilized. I therefore thought it necessary to first ascertain the specificity of the primary anti-hTS sheep polyclonal antibody and the secondary rabbit anti-sheep antibody. This was first demonstrated by the detection of only a single band corresponding to human TS in Western blot analysis of HeLa cell extract. I also employed a 5-FUdR-resistant HeLa cell line, HeLa-55, maintained in 5-FUdR, as an example of a cell line expressing increased level of TS. As expected, I obtained intense signal of both the ternary complexed and free TS protein in Western blot analysis of HeLa-55 extract. In addition, immunocytochemical analysis of HeLa and HeLa-55 showed correspondingly low and high levels of TS, respectively, indicating that my combination of antibodies was detecting a specific TS signal. As further proof of the specificity of my primary antibody, I performed competition experiments with highly purified recombinant TS protein. Whether I pre-incubated the primary antibody with my purified TS protein competitor or pre-absorbed the primary antibody onto an affinity column to which my TS competitior had been previously immobilized, I obtained the same results. There was essentially complete disappearance of TS bands when I used these treated primary antibody preparations in Western blot analysis of HeLa-55 cell extract, whereas control treated antibodies detected strong TS bands. Similarly, I obtained almost complete elimination of fluorescence signal when pre-treated or preabsorbed primary antibody was used for immunofluorescence on HeLa-55 cells. I concluded from all of these results that my primary anti-TS antibody was highly specific for TS and that I could be confident in the results of my later experiments utilizing this antibody.

I confirmed nuclear TS expression in colorectal tissue, in both normal, as well as cancer cells. This was important since it substantiates the physiological relevance of nuclear TS. The specificity of the nuclear TS in the colorectal specimens was demonstrated by use of competitor protein with which we obtained over 75% competition. This is comparable to, or even better than the result obtained by Samsonoff and colleagues who reported 50% inhibiton of nuclear TS in the rat hepatoma cell line in their study (99). In addition, since not all nuclei were TS positive, this indicated some specificity for the nuclear TS signal among nuclei within the colorectal specimens. At this point, the reason for nuclear TS in only some cells of normal and cancer tissue remains to be explained.

Since the level of TS protein is often found overexpressed in colorectal cancer (101,102), I was interested to investigate what effect overexpression of the TS protein may have on its localization. To test this, I generated a 5-FUdR resistant HeLa cell line, HeLa-55, that contained a highly amplified TS gene resulting in strong overexpression of the TS protein (Chapter 2). Immunocytochemistry results showed that the parental HeLa cells had only cytoplasmic TS staining. In HeLa-55 cells, TS was found to be strongly expressed in the cytoplasm, but that there also appeared to be some nuclear TS signal. In order to confirm the presence of nuclear TS, I performed immunofluorescence confocal microscopy on HeLa-55 cells. Serial optical sections through these cells confirmed clearly visible nuclear TS. Nuclear TS in HeLa-55 cells could have resulted from significant increase in the relative level of TS protein in these cells or could be attributed to the treatment of the cells with 5-FUdR and folinic acid. However, I confirmed that short term treatment of cells with 5-FUdR and folinic acid was not responsible for cytoplasmic TS translocating to the nucleus in HeLa cells (data not shown). Conversely, after the growth of HeLa-55 cells in the absence of 5-FUdR and folinic acid for 5 days, which is sufficient time to remove most traces of 5-FUdR, nuclear TS could still be detected in these cells (data not presented). These results indicated that in my HeLa cell system, when I manipulated the cell line to overexpress TS protein, some of TS protein translocated from the cytoplasm to the nucleus leading me to conclude that the level of expression of TS affected it's relative cellular localization.

I also detected heterogeneity for TS staining in HeLa-55 and found that stronger nuclear TS staining was detected in stronger TS expressing HeLa-55 cells. This confirmed my previous conclusion that the level of TS in the cell affected its localization and followed the trend that more cellular TS resulted in more nuclear expression. These results are consistent with a previous report using carrot cells in which wildtype cells lacked nuclear DHFR-TS expression but cells manipulated to overexpress DHFR-TS showed it to be present in the nucleus (84).

The majority of past reports have described TS as being cytoplasmic with few reports having mentioned TS as being nuclear. Since these reports used different cell lines and excluded human cell lines, I decided to investigate whether TS localization depended on the cell type examined. To this end, I chose to survey TS localization in a panel of several human cell lines consisting of normal fibroblasts, HeLa and six colorectal cancer lines.

From immunofluorescence results I found that there was not significant differences in the relative level of TS amongst the cell lines examined. TS was localized predominantly to the cytoplasm in two cell lines: normal fibroblasts and HeLa cells. The six colorectal cell lines diplayed some amount of nuclear TS, but there was variation in the relative proportion of nuclear to cytoplasmic TS. I chose the RKO cell line as one most positive for nuclear TS and confirmed nuclear TS in these cells by both confocal microscopy and cellular fraction and Western blot analysis. I used HeLa cells as a negative control throughout these experiments. The collective results of the RKO cell line clearly illustrated that the immunofluorescent signal I detected was specific for TS. My nuclear detection of TS in RKO cells is consistent with earlier reports (93). I therefore demonstrated that within the category of colorectal cancer, although the total cellular levels of TS were not very different among cell lines examined, there were differences in localization of TS. This lead me to conclude that, in addition to TS localization being dependent upon relative level of cellular TS as displayed by HeLa and clone-55 results, other uncharacterized factors also influence the localization of TS within a cell and can be described as cellular differences.

I next questioned whether the TS gene contained any sequences that resembled established motifs of nuclear localization sequence (NLS) and or nuclear export sequences (NES) which may be utilized in the translocation of TS between the nucleus and cytoplasm. Preliminary work in RKO cells has mapped a putative NLS to the first 10 amino acids at the amino-terminus and 9 amino acids (291-299) at the carboxy terminus (93) of the TS gene. The other possibility that TS could be imported into the nucleus with the aid of chaperones (49) has not been ruled out. It is also possible that although TS exists as a dimer in its native state, TS monomer (35kD) might diffuse freely in and out of the nucleus through the nuclear pore complex (60kD pore size) (87). However, this should lead to equivalent amounts of TS in the cytoplasm and nucleus, which is not the case, suggesting that TS would somehow be excluded from or exported from the nucleus. In contrast to NLS reports, there has been no mention to date of a nuclear export sequence in TS. Therefore, I decided to scan the sequence of the TS gene to look for whether it contained any sequence motifs resembling established nuclear export sequences. I found that sequence 257-269 fit the leucine rich putative nuclear export sequence motif which consists of four critically spaced leucines or other large hydrophobic residues. A loose consensus sequence describing leucine rich

NESs of Leu- X_{2-3}-Phe/Ile/Leu/Val/Met-X_{2-3}-Leu-X-Leu/Ile was first proposed where spacings 3-3-1(89,91), 3-2-1 (90), and 2-2-1(89) were acceptable but 2-3-1 was not (89). Interestingly, the putative NES of TS contains a 4-2-1 spacing. This leucine rich NES of TS is conserved in human, mouse, rat and worms and almost conserved with a 4-2 spacing in Drosophilia, yeast and the bifunctional DHFR-TS in carrot cells. A recently described divergence of the NES consensus to a Val-X-X-X-X-X-Leu-X-Val motif with a 5-1 spacing has been described for a subset of HDACs (103). Thus the 4-2 spacing of the almost conserved NES in yeast and the bifunctional DHFR-TS in carrot cells could quite possibly be functional.

I tested whether this putative NES of TS could function independently as an NES by preparing a fusion construct, GFP-NES, in which TS-NES was fused in frame to the carboxyl end of GFP. However, when GFP-NES was expressed, there was no change in the localization of GFP as compared to control GFP vector. I concluded from these results that the putative leucine rich NES of TS cannot function independently as an NES when fused to the carboxyl end of GFP and transfected into HeLa and RKO cells. Several studies have shown that similar leucine rich putative NESs of other proteins fused to the carboxyl end of GFP results in nuclear export of GFP (103). However, one report has described the inability of an intrinsic functional NES to confer nuclear export to GFP and suggested that such NESs might function in a protein context-dependent manner, possibly requiring additional sequences for full activity (103).

To determine whether surrounding sequences were necessary for TS NES to be functional, I generated two His_6-tagged constructs of TS lacking carboxyl terminal regions encompassing the NES and surrounding sequences. Construct H_6TS^{248} had putative NES disrupted partly due to mutated amino acids at positions 257 to 259 and deletion of amino acids 260 to 269. H_6TS^{186} had the full TS NES deleted. I first showed that my His-tagged version of full-length TS, H_6TS^{313}, and wt TS, localized similarly when overexpressed in HeLa cells. Overexpression of TS mutants lacking this putative NES and surrounding sequences resulted in increased accumulation of nuclear TS in addition to some cytoplasmic speckling for H_6TS^{186}. These results suggested that additional sequences between 270 - 313 at carboxy end of TS are necessary for the NES to be functional, although further work is necessary to delineate the minimal carboxy sequences necessary. In cases showing increased

nuclear TS expression, future studies could check for possible mutations or deletions in the delineated sequence responsible for TS nuclear export.

In addition to confocal microscopy, I confirmed nuclear TS in Clone-55 cells by cell fractionation. When Clone-55 cells grown in 5-FUdR were fractionated and the cellular fractions probed for TS levels, I estimated that approximately 0.5% of the free TS protein was present in the nuclear fraction of a population of heterogenous TS-expressing clone-55 cells. More importantly, compared to roughly equivalent amounts of free and ternary complexed TS in the total cellular lysate, there was a far greater predominance of the free TS protein in the nuclear extract of Clone-55 cells. In a similar fashion, when carrot cells, which contain a bifunctional DHFR-TS gene was examined for TS localization, it was found to be cytoplasmic. On overexpression of the DHFR-TS gene by isolation of a methotrexate resistant clone, it was found that DHFR-TS was largely localized to the cytoplasm with some nuclear signal (84). While cytoenzymatic studies pointed to a general cytoplasmic localization of the enzyme, results of immunocytochemical localization showed that in the majority of methotrexate resistant cells, there was also a nuclear localization of the protein (84). The authors suggested that the discrepancy between cytochemical and immunocytochemical results observed with methotrexate resistant cells could be explained by the nuclear accumulation of the inactive form of the enzyme. In this study I show that the ternary complexed form of TS was nearly absent from the nucleus of HeLa-55 cells suggesting that the nuclear TS may not be able to bind the 5-FUdR substrate to form the ternary complex. It is possible that if nuclear TS is incapable of binding substrate, this could explain why nuclear DHFR-TS in the methotrexate resistant carrot cells showed no enzymatic activity.

In contrast to the results in HeLa and carrot cells, when the localization of TS was examined in a rat hepatoma cell line, H35, through autoradiographic assay and immunogold labeling, it was found to be mostly nuclear with some cytoplasmic staining. When these authors selected a 5-FUdR resistant TS overexpressing clone, H35R, they found that from an autoradigraphic assay, most of the overexpressed TS was in the cytoplasm with some in the nucleus. Results of my study indicate that localization of TS is dependent on cellular differences which offers one explanation for the difference in TS localization in the rat hepatoma cells compared to HeLa and carrot cells. Contrary to my results, authors of the rat

hepatoma cell line report also showed that ternary complexed TS was predominantly expressed in the nucleus and that TS was phosphorylated in the 5-FUdR resistant rat hepatoma cell line. Preliminary evidence from our lab studying HeLa TS failed to show that TS was phosphorylated in this cell line (Sanjay Krishnamurthy, unpublished data). To this date there has been no report of phosphorylated TS in any other cell lines and hence we cannot conclude whether in fact phosphorylated TS is present in a normal or abnormal situation. There has been no characterization of phosphorylated TS protein in terms of its catalytic activity, stability and localization. Thus it is very easy to speculate that the differences seen between H35 and HeLa cells could be due to the fact that H35 contains a phosphorylated TS protein. TS nuclear localization dependency on cell type could in fact be due to dependency in part on the phosphorylation status of TS and is something that can be investigated in the future. Therefore, comparison of our results leads to another possible factor that may affect TS nuclear localization, its phosphorylation status, which could in turn be due to differences in the yet to be characterized kinases and signaling pathways that may be directly responsible for the phosphorylation of TS.

I can propose several possibilities to explain my observation of lack of ternary complexed TS protein in the nucleus of Clone-55 cells. If I assume that the work on TS putative NLS is correct, it is possible that FdUMP-bound TS results in the masking of TS NLS so it no longer efficiently enters the nucleus. If, however, TS does not enter the nucleus through an NLS but is instead guided into the nucleus by chaperones, it is possible that FdUMP-bound TS is no longer able to interact with these chaperones as efficiently and therefore not able to enter the nucleus. Conversely, FdUMP-bound TS could result in the exposure of TS NES allowing it to be efficiently exported out of the nucleus. Another possibility is that the predominantly free TS protein detected in the nuclear fraction of Clone 55 as assessed by Western Blot could actually be RNA bound TS. The role of TS in the nucleus may be due to its RNA-binding activity (86) and not to its catalytic activity. If we envision nuclear TS as RNA-bound TS, according to Chu's TS autotranslational regulatory model (General Introduction, Fig. 3A), once FdUMP binds this nuclear TS, the RNA is released and exits the nucleus and can be translated. This would lead to increased expression of genes translationaly regulated by TS, and could constitute another mechanism by which 5-FU treatment could affect target cells. It is also possible that RNA-bound TS exposes TS

NLS so it can efficiently enter the nucleus or that the RNA-bound TS masks TS NES so it is trapped in the nucleus. Alternatively, if I also consider the results of the methotrexate resistant carrot cells where nuclear TS was enzymatically inactive, it is possible that nuclear TS is modified in such a way that it can no longer bind substrate and so is incapable of forming a ternary complex.

5-FU, one of the most commonly used chemotherapeutic agents in the treatment of colorectal cancer, is metabolized in the cell to generate FdUMP. FdUMP binds to TS protein in the presence of the folate co-factor leading to a strong ternary complexed TS protein. This results in prolonged inhibition of the enzyme (82). My model provides one explanation for the recently reported correlation between higher nuclear TS expression and poorer response to 5-FU therapy (50). From my data on the fractionation of Clone 55, the majority of nuclear TS was in the free form. If I believe that this is true for the majority of nuclear TS staining detected in the colorectal cancer cases examined in the above study, then this nuclear TS can be thought of as a store of free TS protein, non ternary complexed, therefore non-inhibited. Nuclear TS represents a store of free TS that might be thought of as providing "resistance" to the inhibition of 5-FU effects possibly due to its inability to bind substrate. This could explain why these higher nuclear TS cases showed poorer response to 5-FU therapy.

1.6. CONCLUSION

In this chapter I have confirmed the nuclear presence of TS in both human cultured cell lines and human colorectal tissue specimens using a highly specific anti-TS antibody. In addition, I have established that TS nuclear localization is dependent upon several factors: (1) level of TS protein in a cell (2) presence of TS NES and surrounding sequences between amino acids 270 - 313 at carboxy end of TS gene and (3) factors that differ between cell types that remain to be characterized.

CHAPTER 2: DEVELOPMENT AND CHARACTERIZATION OF TS OVEREXPRESSING, 5-FUDR RESISTANT HELA CELL LINES

2.1. INTRODUCTION

To examine the effect of TS overexpression on its cellular localization (chapter 1) I planned to develop a TS overexpressing cell line. Previously I had tried to make a stable clone of HeLa cells using a plasmid encoding the full-length cDNA of TS (chapter 4). However, the level of TS over-expression obtained in this way was only 5-7 times greater than the parental level. Several past studies had reported that cell lines exposed *in vitro* to stepwise increasing concentrations of anti-TS inhibitor drugs lead to the development of TS overexpressing cell lines due to TS gene amplification. Therefore I decided to use this method to generate TS over-expressing cell lines.

Amplification is one of the mechanisms by which cells can meet the demand for synthesis of specific gene products in amounts exceeding the transcriptional capacity of a single gene copy (47). Increase of the gene dosage by DNA amplification is one common mechanism for upregulating gene expression. In some species, scheduled amplification can be part of the developmental program, for example, amplification of actin genes during myogenesis in the chicken (47). In mammals, however, amplification is more commonly seen as a form of genomic instability, often in tumor cells (104-107). Gene amplification as a mechanism of drug resistance was first observed in Chinese Hamster Ovary cells and then in murine and human leukemic cells following exposure *in vitro* to increasing concentrations of methotrexate (MTX) (108). The DHFR gene was amplified several hundred fold. Later DHFR gene amplification was observed in clinical specimens taken from patients treated with MTX (109,110).

The TS gene is located on chromosome 18p11 (5). Several past studies have reported on the use of anti-TS inhibitors in the development of TS overexpressing cell lines due to TS gene amplification. Drugs used were 5-FU (54), 5-FUdR (51,111-115) or analogs of the folate cofactor such as ZD9331 (63), CB3717 (116), Tomudex, C2-desamino-C2-methyl-N10-propargyl-5,8-dideazafolic acid and 10-propargyl-5,8-dideazafolate (PDF) (117).

The cytologic features of gene amplification in cultured animal cells are known. Amplified genes occur on small acentromeric chromosomes called double minute chromosomes (DMs) or on chromosomes associated with homogenously staining regions (HSRs) (118). DMs were first described by Spriggs et al, in 1962 as occurrence in a

malignant cell line (119). They lack centromeres and thus have unequal separation at cell division. They tend to cluster at the end of chromosomes and are drawn with them during metaphase. They have a wide variation in size, even in a specific cell line. When cell lines are followed in culture, the DMs tend to be lost, especially in the absence of selection, whereas HSRs are preserved (120). Thus the stability of amplified genes in the absence of continued drug selection reflects their chromosomal location: DMs are unstable, while HSR are stable (118).

There have been two studies reporting on the clinical significance of TS gene amplification. One found TS gene amplification in one patient whose colorectal tumor showed a 4-6 fold increase in TS gene copy number after prolonged treatment with 5-FU in combination with leucovorin, suggesting gene amplification may be important *in vivo* (52). The other study reported high levels of TS expression in association with low-level (2-3 fold) increases in TS gene copy numbers in pulmonary metastatic cancer (121). The authors concluded that high levels of TS enzyme, due in part to TS gene amplification, may be the basis of the lack of response of pulmonary metastases to 5-FU treatment.

In this chapter I present experiments describing the development and characterization of cell lines resistant to 5-FUdR that overexpress TS.

2.2. OBJECTIVES

The main objectives of this chapter are to:

- Isolate 5-FUdR-resistant HeLa cell lines (for use in Chapter 1) using a step-wise selection method
- Determine whether any of the 5-FUdR-resistant lines overexpress TS using Western blot analyses
- Examine the growth characteristics of the TS-overexpressing 5-FUdR-resistant cell lines
- Determine the stability of the TS overexpression in the absence of selective pressure
- Establish a quantitative PCR method for measuring relative TS gene-copy number and utilize it to determine whether the TS-overexpressing 5-FUdR-resistant cell lines have amplified TS gene copies
- If TS gene is amplified, determine whether only one or both polymorphic TS alleles in HeLa cells are amplified

2.3. MATERIALS AND METHODS

2.3.1. Antibodies

Sheep anti-hTS antiserum used in these studies was obtained from Rockland Immunochemicals (Gilbertsville, PA; cat. no. 100-601-199). This polyclonal antibody was raised against recombinant $(his)_6$-rhTS (95)and its specificity has been previously illustrated (Chapter 1). Mouse monoclonal antibody to β-tubulin was a kind gift from Dr. Doug Gray (University of Ottawa, Ont). Horseradish peroxidase (HRP)-conjugated rabbit anti-sheep secondary antibody (cat. no. 613-4328) was also from Rockland Immunochemicals. CY3-conjugated donkey anti-sheep secondary antibody (cat. no. 716-165-147) was from Jackson ImmunoResearch Laboratories (West Grove, PA). HRP-conjugated anti-rabbit/anti-mouse secondary antibody ("Polymer") was from DAKO (Carpinteria, CA).

2.3.2. Establishment of 5-FUdR resistant HeLa cells

HeLa cells were obtained from ATCC (Rockville, MD). 5-FUdR-resistant HeLa clones were isolated by a procedure that is similar to a previous report (112). Briefly, log phase cultures of HeLa cells were exposed to 25 μM folinic acid (Sigma-Aldrich Chemicals, St. Louis, MO) and step-wise increasing concentrations of 5-FUdR (Sigma-Aldrich); they were grown for two weeks in increasing concentrations of 5-FUdR, (0.01 μM followed by 0.03μM and then 0.1μM). Individual clones were isolated and maintained in 25μM folinic acid and 0.1μM 5-FUdR.

2.3.3. Cytotoxicity Studies

$5x10^4$ cells were seeded per well of a 12 well dish and to the media was added varying concentrations of 5-FUdR: 0 μM, 0.001 μM, 0.01μM, 0.1 μM, 0.5 μM and 1.0 μM. After 4 days of growth, cells were trypsinized and counted (Model ZM, Coulter Electronics Ltd, Luton, Beds., England). Counts for 0 μM were set to 100% and all counts expressed as a percentage of 0 μM 5-FUdR count.

2.3.4. Immunofluorescence Staining for Microscopy and FACS Analysis

Cells attached to coverslips were immunofluorescently stained for microscopic examination. Cells were stained in suspension under similar conditions and used for FLOW analysis and FACS. Log phase cultures were used in all experiments. Cells were grown on 22 mm^2 glass cover slips in 10 cm dishes. Coverslips were removed and washed twice with phosphate-buffered saline (PBS), then fixed for 10 min with acetone:methanol (1:1) at room temperature. For FACS analysis, cells growing in 10 cm dishes were detached by trypsin treatment and ~1x 10^6 cells were pelletd at 200 g and washed twice with PBS. The cell pellet was resuspended in 0.5 mL PBS and added drop-wise to 9 volumes of cold 95% EtOH (~80% final) and fixed at -20^{0}C for ≥ 30min; samples were stored at -20^{0}C for up to 1 week. For both cells attached to coverslips and cells in suspension, all subsequent steps were carried out at room temperature. Cells were blocked for 30 min with Universal Blocking solution (DAKO). All antibody dilutions were in 'Antibody Diluting buffer' (DAKO). Cells were incubated for 1 h with primary anti-TS antibody (1:500 dilution). Cells attached to coverslips were washed 5 times (5 min per wash). Cells in suspension were washed twice. Samples were then incubated for 30 min with secondary anti-sheep IgG- CY3 antibody (1:1000 dilution) and washed again. The coverslips were mounted onto slides with 'Fluorescent Mounting medium' (DAKO) containing 5 μg/mL Hoechst 33258 (Sigma-Aldrich). Stained cells were examined and photographed with a Zeiss Axiophot fluorescence microscope. After final washing, samples stained in suspension were resuspended in 1 mL of PBS, or 1 mL of PBS containing 5 ug/mL Hoechst and fluorochrome staining analysis of TS expression was performed using a BD LSR flow cytometer (Becton, Dickinson & Co., San Jose, CA). TS expression was detected by CY3 fluorochrome. CY3 was excited using an argon-ion (blue) laser (488nm) and signal detected on FL2 detector with 575/26 BP filter in place. DNA was monitered by detecting Hoechst staining. Hoechst was excited using an HeCd (ultraviolet) laser (325nm) and its signal detected on FL5 detector which used a 380LP filter. Data was anaylsed using CELLQuest Pro Software (Becton, Dickinson & Co.). Cells were sorted using MoFlo Cell Sorter.

2.3.5. Preparation of total cell protein extracts

Cultured cells were detached by tryspin treatment, then washed twice and suspended in cold PBS. To prepare whole cell extracts, an equal volume of 2×SDS sample buffer (100

mM MOPS, pH 6.8, 4% SDS, 20% glycerol, 2 % β-mercaptoethanol) was added. The extracts were boiled for 20 min and then sonicated briefly to reduce viscosity. Protein was quantified using fluorescamine (97) (Sigma-Aldrich), using BSA as a standard.

2.3.6. Western and dot immunoblot analysis

For dot blot, 10 μg of proteins in cell extracts in SDS sample buffer was blotted onto a PVDF membrane (Millipore, Nepean, Canada) using Bio-Dot ™ apparatus (Bio-Rad) with low vacuum. The membrane was then subjected to immunoblot analysis as described below. For immunoblot, proteins in cell extracts in SDS sample buffer were resolved by 12% discontinuous SDS-PAGE and then electrophoretically transferred to a PVDF membrane at 15V for 18 h in transfer buffer (3 mM Na_2CO_3, 10 mM $NaHCO_3$, 10% methanol) (95). The membrane was stained with Ponceau S and then washed twice in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8). For immunoblot analysis, all steps were carried out at room temperature. The membrane was blocked for 1 h with freshly prepared Casein Blocker solution (100 mM NaCl, 50 mM sodium acetate, pH 6.0, 1% bovine milk casein(Sigma-Aldrich), 2 mM cyclohexanediamine tetraacetate (Sigma-Aldrich)). Sheep anti-TS antibody was diluted 1:5000 in Casein Blocking solution and anti-β tubulin primary antibody was used at 1:10 dilution in TBST. In both cases, the primary antibody was incubated with the membrane for 1 h. The membrane was washed 5 times in TBST and incubated for 1 h with a secondary antibody as follows: rabbit anti-sheep-IgG-HRP was used at 1:3000 dilution in Casein Blocking solution and HRP-conjugated anti-rabbit/anti-mouse secondary antibody DAKO ("Polymer") was used at 1:100 dilution in TBST. The membrane was washed 5 times with TBST and HRP was detected using the LumiGLO Chemiluminescent Substrate Kit (Kirkegaard & Perry Lab; Maryland, USA). Western blot was stripped using Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL) according to maufacturer's protocol.

2.3.7. DNA Isolation from Cultured Cells

An almost confluent 10 cm plate of cells was washed twice with PBS. 1.5 mL of REBS (0.5 M LiCl, 1.0 M Urea, 1.5 mM $CaCl_2$, 0.25% SDS, 20 mM BES, pH6.8) was added and DNA was extracted as previously described (122). DNA was resuspended in CT buffer

(10mM Tris-HCl pH 7.5, 1mM CDTA). For genomic DNA isolation from HeLa-55 sorted cellular fractions, each fraction contained ~ $1x10^5$ cells in 0.6 mL sheath fluid. Cells were centrifuged at 16000 g for 10 min and to the pellet 10 µL of DES solution (0.5 M LiCl, 1M Urea, 2 mM CDTA, 50 mM Tris-HCl pH 9.5, 0.1% SDS) containing Proteinase K (20 µg/mL) was added. Sample was mixed and incubated at 50 °C overnight. Next day 40 µL of distilled water was added, sample was mixed and then sonicated briefly. DNA samples were quantitated using DABA assay as previously reported (123).

2.3.8. Semi Quantitative PCR Analysis

Independent PCR reactions were performed with one set of TS primers and one set of quantitative control, HF9, X-chromosome linked, primers. A 143 bp region within exon 5 of TS gene was amplified using Primer 1(sense): 5' GCCCTCTGCCAGTTCTA and Primer 2 (anti-sense): 5' TTCAGGCCCGTGATGT. A 422 bp region within HF9 was amplified using Primer 3 (sense): 5' CATGACATTGCCCTTCT and Primer 4 (anti-sense): 5' ATCCAGTTGACATACCG. Each 50 µL TS PCR reaction contained 3 mM $MgCl_2$, 1x PCR buffer (supplied with Qiagen Taq DNA polymerase, Qiagen Inc., Mississauga, Canada), 0.2 mM each dNTP, 0.2 mM each primer, 100 ng of total genomic DNA(boiled/sonicated), and 2 units of DNA Polymerase from *Thermus aquaticus* (Qiagen Inc.). The mixture was transferred to a thermal cycler (Gene Amp PCR system 9700, PE Applied Biosystems, Roche Inc., Branchburg, N.J.) for 27 cycles of PCR. Each cycle consisted of 1 min at 97°C, 1 min at 56 °C and 2 min at 72°C. After the reaction, the mixture was incubated at 72°C for 5 min. Each 50 µL HF9 PCR reaction contained all components as in TS PCR reaction except for use of 2 mM $MgCl_2$. The PCR reaction for HF9 consisted of 30 cycles of 1 min at 97°C, 1 min at 53 °C and 2 min at 72°C. After the reaction, the mixture was incubated at 72°C for 5 min. The amplified DNA fragments were analyzed by electrophoresis in 2% agarose gel using equivalent amounts of each PCR reaction. The gel was stained with 2 ug/mL ethidium bromide for 20 min and destained in water for 10 min. DNA bands were visualized and quantitation of band intensities was performed using LabWorks Image Acquisition and Analysis Software (UVP Bioimaging system, UVP Inc, Upland, CA).

2.3.9. *PCR Analysis of Amplified TS Allele in 5-FUdR Resistant TS Over-expressing Clones*

A 380-400 bp region spanning the 5'UTR to within 100bp of exon 1of TS gene was amplified using Primer TS170(sense): 5' GTGGCTCCTGCGTTTCCCCC and Primer JT1(anti-sense): 5' GCCGCAGCGGAGGATGTGTT. The 50 μL PCR reaction contained 2 mM $MgCl_2$, 50 mM KCl,10 mM Tris-HCL (pH 9), 0.01% Triton X-100, 0.2 mM each dNTP, 0.4 mM dITP, 2 % DMSO, 1.2 mg/mL DMSO, 0.2 mM each primer, 100 ng of total genomic DNA (boiled/sonicated), and 3 units of DNA Polymerase from *Thermus aquaticus* (Qiagen Inc.). The mixture was transferred to an Eppendorf Mastercycler Gradient (Gene Amp PCR system 9700, PE Applied Biosystems, Roche Inc., Branchburg, N.J.). The sample was first denatured at 99^0C for 5 min and then subjected to 35 cycles of PCR. Each cycle consisted of 30 sec at 99^0C, 1 min at 63 ^{0}C and 1 min at 72^0C. The reaction was paused after cycle 20 and an additional 2 units of Taq Polymerase was added to each reaction. After 30 PCR cycles, the mixture was incubated at 72^0C for 5 min extension step. The amplified DNA fragments were analyzed by electrophoresis in 6% polyacrylamide gel using equivalent volumes of each PCR reaction. The gel was run at 140 V for 1hr 15min to allow sufficient separation of PCR amplified fragments.

2.4. RESULTS

2.4.1. Establishing and Screening TS Over-Expressing Clones

To obtain a TS-overexpressing cell line, I generated 5-FUdR-resistant HeLa clones by selection in step-wise increasing concentration of 5-FUdR. Over 50 resistant HeLa clones were isolated and screened to determine the level of TS protein by Western dot blot analysis using anti-TS antibody. Most of the clones isolated showed overexpression of TS protein compared to parental HeLa cells (Fig. 2.1). Western blot analysis on 6 clones confirmed that they all overexpressed TS protein compared to parental; clones 55 and 54 overexpressed much more TS than other clones (12 and 70) (Fig. 2.2). Only the free form of the TS protein (~36 kD) was present in the parental cells, whereas both the free TS form and a stable "ternary complex" (~38 kD) of TS with FdUMP and CH_2THF were detected in all resistant clones (Fig. 2.2). This band "shift" corresponding to the stable ternary complex has been previously described (Fig. 2.2) (22). Clones 18, 54, 55 and 66 showed about equal amount of each form of TS protein; clones 12 and 70 showed slightly more of the ternary complex form than free protein. The blot was stripped and re-probed with anti-β tubulin antibody as a loading control (Fig. 2.2). Clone 55 (HeLa-55), which expressed the highest amount of TS protein, was chosen for further experiments.

2.4.2. 5-FUdR resistance, growth and morphology of HeLa-55 clone

Several characteristics of HeLa-55 and parental HeLa were compared. HeLa-55 showed >90% cell survival when continuously grown in 0.1 μM 5-FUdR, compared to parental HeLa which showed ~10% cell survival after only 4 days of growth in the same drug (Fig. 2.3a). Even in 10-fold higher concentration of the drug (1 μM), HeLa-55 showed a high rate of cell survival (>60%) (Fig. 2.3a). Doubling time of HeLa-55 (maintained in 0.1μM 5-FUdR) was 28 hr, which was slightly longer than the doubling time of 20 hr for parental HeLa (maintained in drug-free media). Cellular morphology of HeLa-55 was also observed to be different than parental HeLa. The parental cells showed a fibroblast-like appearance (Fig. 2.3b). HeLa-55, however, consisted of a morphologically heterogeneous population (Fig. 2.3b): most of the cells were larger and appeared flatter than the parental;

FIGURE 2.1. Western Dot-blot analysis for TS in parental HeLa cells and 5-FUdR-resistant clones

Whole cell proteins were extracted from each cell type and 10 μg dot-blotted on a PVDF membrane. The membrane was immunoblotted using anti-TS antibody. Also shown are the clone numbers (to the left) of some of the clones that were further analyzed in Fig. 2.2. Other details are described in *Materials and Methods*.

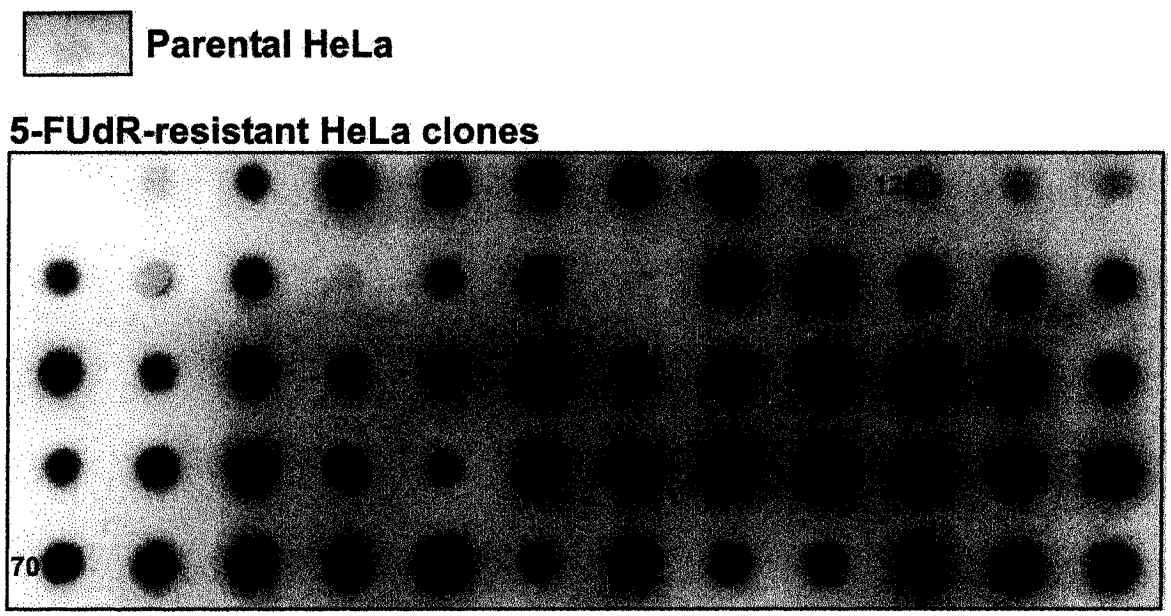

Parental HeLa
5-FUdR-resistant HeLa clones
70

FIGURE 2.2. Western blot analysis for TS in HeLa cells and selected 5-FUdR-resistant clones

Whole cell proteins were extracted from each cell type and analyzed by western blotting using anti-TS antibody. Each lane contains 10 μg of protein. Indicated are the positions for the free TS form (35 kD) and the ternary complex form (~37 kD). Also shown is the overexposed blot indicating the presence of TS in HeLa cells. The blot was striped and re-analyzed for β-tubulin as a loading control (last panel). Other details are in *Materials and Methods*.

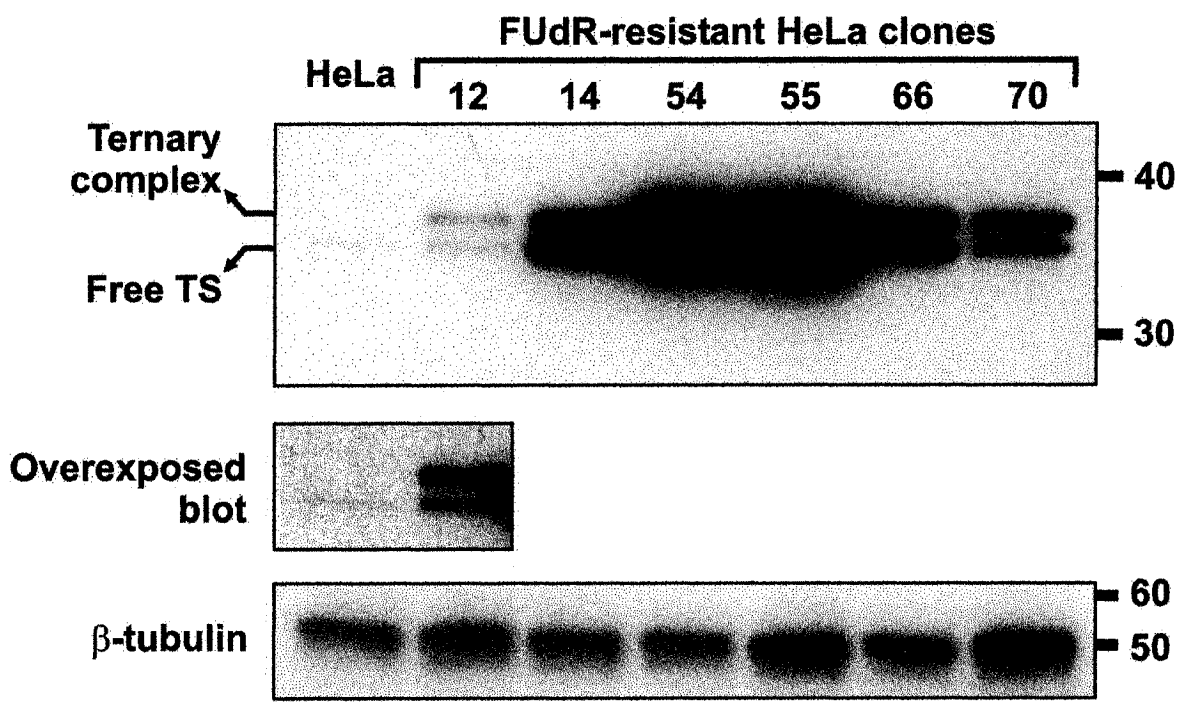
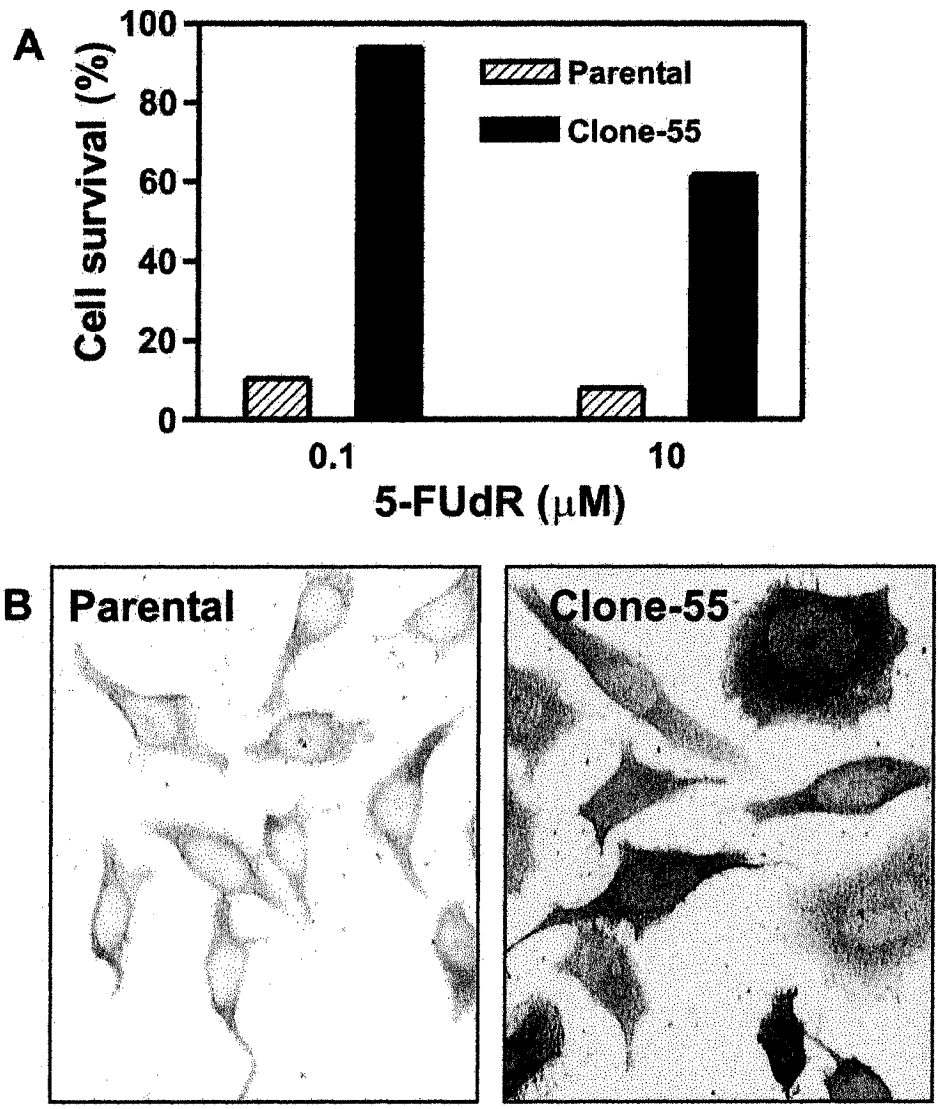

FUdR-resistant HeLa clones
HeLa
12
14
54
55
66
70
Ternary complex
Free TS
40
30
Overexposed blot
β-tubulin
60
50

FIGURE 2.3. Cell growth and morphology of parental HeLa cells and 5-FUdR-resistant clone-55

A. Effect of 0.1 and 10 μM 5-FUdR treatment on cell growth. After treatment, the cells attached to the plates were harvested and counted using Coulter counter. The counts were corrected for the untreated cells (0 μM 5-FUdR). (B) Morphology of parental HeLa and clone-55 cells stained for TS by immunohistochemistry. Other details are in *Materials and Methods*.


A
100
80
60
40
20
0
Cell survival (%)
Parental
Clone-55
0.1
10
5-FUdR (μM)
B
Parental
Clone-55

some were rounder; some were spindle-shaped; others were fibroblast-like as the parental cells.

2.4.3. *TS Gene Amplification in HeLa-55*

To determine whether the observed overexpression of TS protein in HeLa-55 (Fig. 2.1) was due to amplification of the TS gene, a semi-quantitative PCR system was developed. The ability of the system to detect amplification was demonstrated by PCR-amplifying a 422-bp fragment of an X-linked HF-9 gene from white blood cell genomic DNA from a male and a female. The intensity of the HF-9 product was found to be ~2-fold higher in female (XX) than male (XY) (Fig. 2.4), indicating that the PCR method could detect 2-fold difference in gene copy number. To quantify the relative number of TS genes, a 143-bp portion of exon 1 of TS gene was PCR-amplified from genomic DNA isolated from parental HeLa, HeLa-55 and the white blood cells of a male and a female. Equal amounts of DNA were used for each sample. The relative intensities of the TS-specific signal from male and female white blood cell DNA was almost the same as that obtained from parental HeLa cells (Fig. 2.5). This indicated that the relative number of TS gene copies was the same in these three samples. In contrast, HeLa-55 showed a very strong TS signal (Fig. 2.5), demonstrating a significant amplification of the TS gene in this cell line.

The number of TS gene copies in HeLa-55 was more precisely estimated as follows. A series of TS PCR reactions were performed using a mixture of different ratios of HeLa-55 DNA and HeLa DNA (0:100, 1:99, 2:98, 4:98 ng), while maintaining same total amount of DNA (100ng) (Fig. 2.6). With the addition of 2 ng of HeLa-55 DNA to parental-cell DNA (i.e., the 2:98 ratio), the TS-specific signal increased by about 2-fold (Fig. 2.6). I concluded that HeLa-55 cells contain about 50 times more copies of the TS gene than parental HeLa.

2.4.4. *Heterogeneity for TS Staining in HeLa-55*

Parental HeLa cells show low, uniform, cytoplasmic TS staining whereas TS-overexpressing HeLa-55 cells show heterogeneity in both intensity and localization of TS protein (Fig. 2.3b). Most cells in the HeLa-55 population show increased TS staining compared to parental, and the majority of staining is localized to the cytoplasm. However, some cells in the HeLa-55 population also showed nuclear TS staining (Fig. 2.3b and

FIGURE 2.4. Semi-quantitative PCR analysis for X-linked gene Human Factor 9 (HF9) in human DNA from white blood cells of male (WBC-M) or female (WBC-F)

Equal amount of white blood cell DNA (100 ng) was used for both male (WBC-M) and female (WBC-F). Upper panel shows the amplified PCR fragment on an ethidium bromide-stained agarose gel. Lower panel shows the pixel count of the fragments. Other details are in *Materials and Methods*.

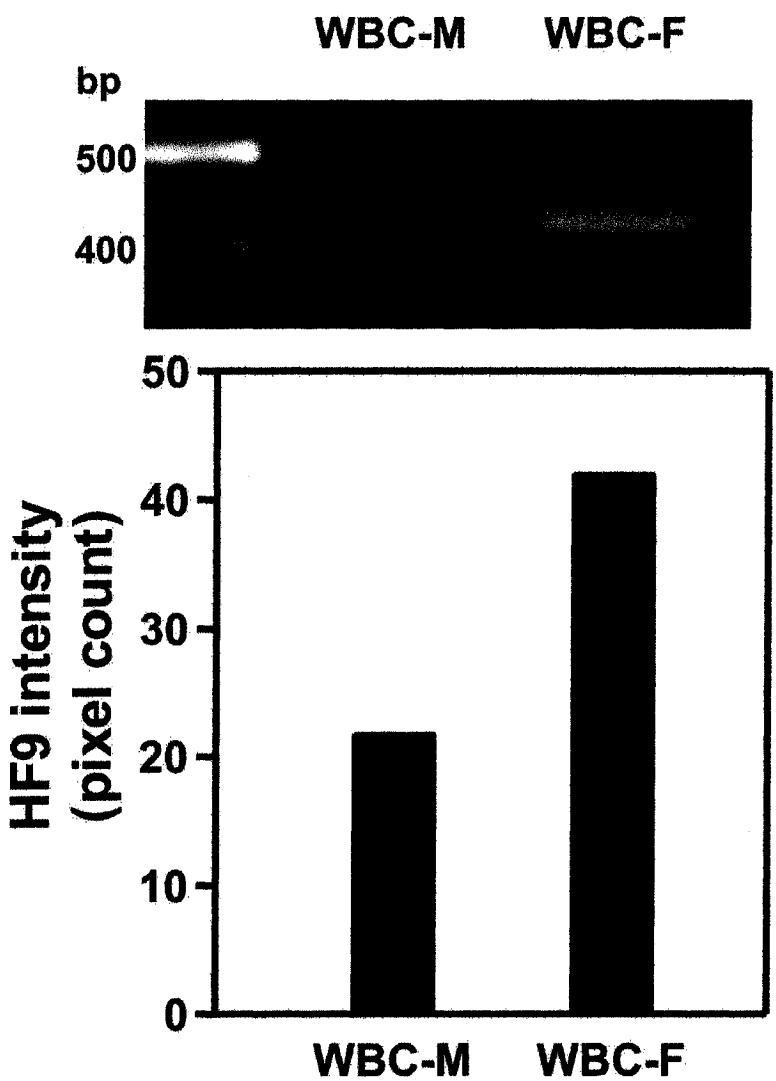

WBC-M
WBC-F
bp
500
400
HF9 intensity
(pixel count)
50
40
30
20
10
0
WBC-M
WBC-F

FIGURE 2.5. Semi-quantitative PCR analysis for TS in DNA from HeLa, HeLa-55 and white blood cells of female (WBC-F) and male (WBC-M)

Equal amount of DNA (100 ng) was used for all reactions. Blank represent PCR analysis in the absence of any DNA. Upper panel shows the amplified PCR fragment on an ethidium bromide-stained agarose gel. Lower panel shows the pixel count of the fragments. Other details are in *Materials and Methods*.

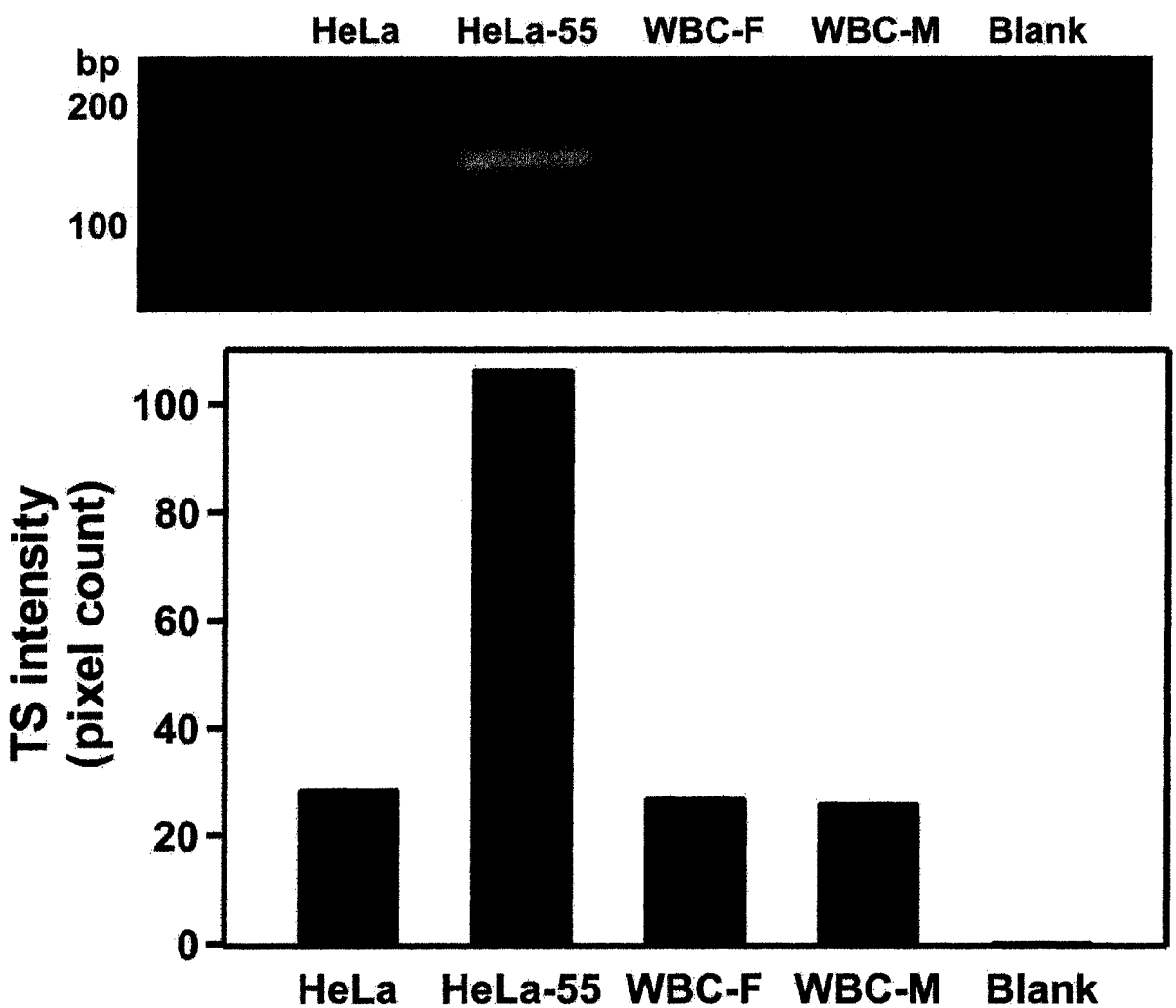
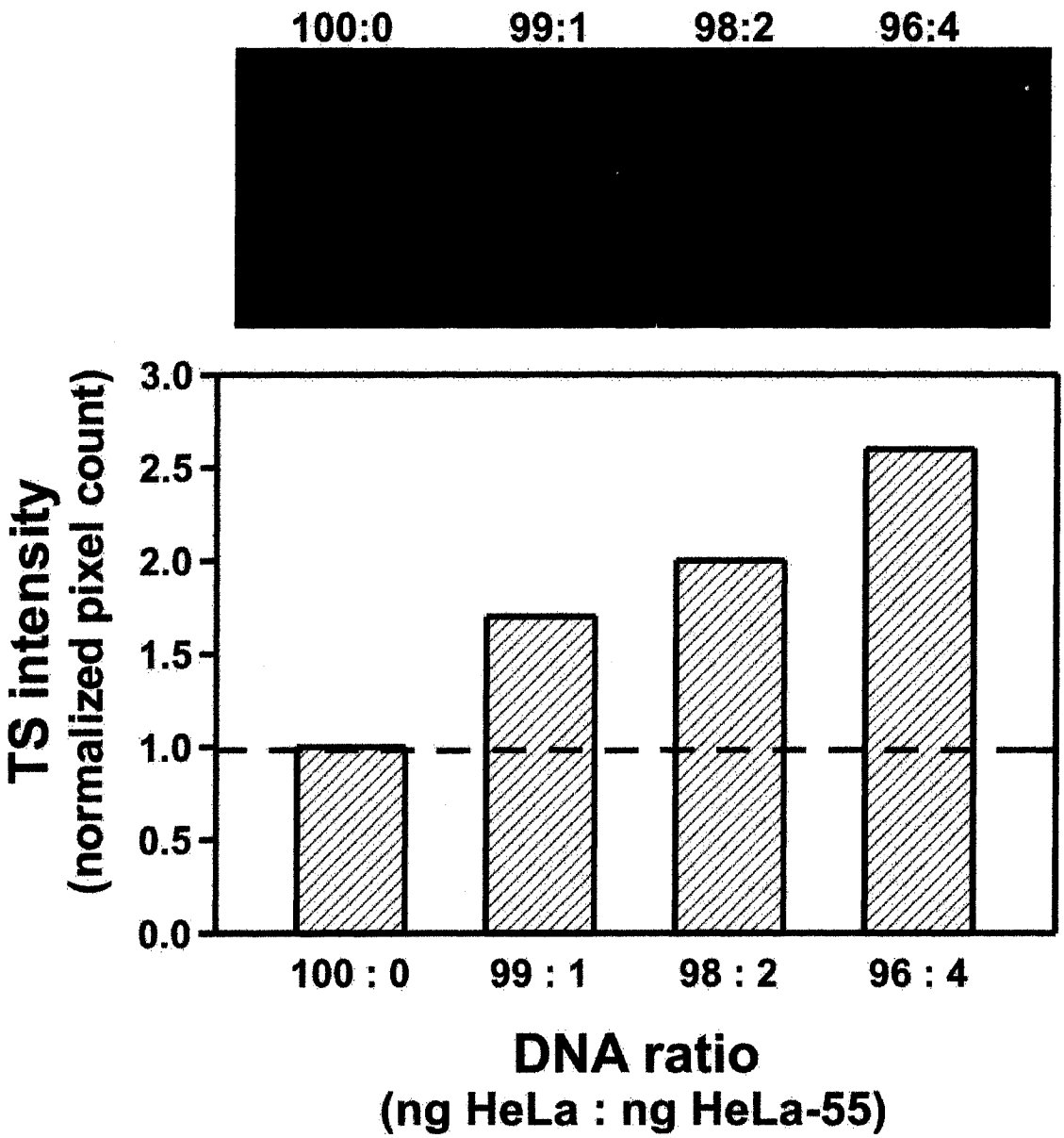

HeLa
HeLa-55
WBC-F
WBC-M
Blank
bp
200
100
TS intensity
(pixel count)
100
80
60
40
20
0
HeLa
HeLa-55
WBC-F
WBC-M
Blank

FIGURE 2.6. Semi-quantitative PCR analysis for TS in a mixture containing different ratios of DNA from HeLa and HeLa-55 cells

Genomic DNA isolated from HeLa and HeLa-55 were mixed at ratios 100:0, 99:1, 98:2 or 96:4 ng:ng and analyzed by PCR. Upper panel shows the amplified PCR fragment on an ethidium bromide-stained agarose gel. Lower panel shows the pixel count of the fragments relative to the 100:0 fragment. Other details are in *Materials and Methods*.


100:0
99:1
98:2
96:4
TS intensity
(normalized pixel count)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
100 : 0
99 : 1
98 : 2
96 : 4
DNA ratio
(ng HeLa : ng HeLa-55)

Chapter 1). To further confirm the heterogeneity of TS intensity in HeLa-55, FLOW analysis was carried out. Both HeLa-55 and parental HeLa cells were labeled with anti-TS primary antibody and subsequently with Cy3-conjugated secondary antibody and then subjected to FLOW analysis. While TS levels in parental HeLa cells showed a narrow peak distribution, the TS levels in HeLa-55 followed a broader distribution (Fig. 2.7). This confirmed heterogeneity of TS expression in HeLa-55 cells.

Since TS protein overexpression in HeLa-55 was associated with TS gene amplification (Fig. 2.5), I next studied whether *heterogeneity* of TS protein expression in HeLa-55 was dependent upon a possible heterogeneity of TS gene amplification. I again fluorescently-labeled HeLa-55 cells for TS expression and sorted the heterogeneous HeLa-55 cell-population using FACS analysis into four fractions (F1-F4) based on increasing level of TS expression (Fig. 2.8A). DNA was extracted from ~$1x10^5$ cells of each sorted fraction, and the relative TS gene level in each fraction was quantified by PCR analysis (Fig. 2.8B). Fractions 1, 2, 3 and 4 showed 4.5, 15.6, 17.6 and 67.5- fold increase in TS gene expression compared to parental HeLa, respectively. As predicted, the sub-populations of HeLa-55 cells with highest TS expression contained the greatest number of TS gene copies. I conclude that the heterogeneity of TS protein expression in HeLa-55 is closely related to heterogeneity of TS gene amplification.

2.4.5. Stability of TS Gene Amplification in HeLa-55

To examine the stability of the amplified TS gene in HeLa-55, the cells were grown in the absence of 5-FUdR for 0-3 weeks and subjected to TS immunofluorescence labeling and FLOW analysis. A progressive reduction in the level of TS staining towards parental HeLa levels was observed, with each successive additional week of maintenance in 5-FUdR-free media (Fig. 2.9). Thus, TS amplification is lost in the absence of selective pressure, indicating that the amplified TS gene was highly unstable.

2.4.6. Amplification of TS alleles in TS-overexpressing 5-FUdR-resistant HeLa clones

The 5′- terminal region of the TS gene is polymorphic for a number of GC-rich tandem repeats (41). PCR analysis on this region indicated that parental HeLa cells contained one TS allele with 3 tandem repeats (3R allele) and another TS allele with 2

FIGURE 2.7. TS levels analyzed by flow cytometry in HeLa and HeLa-55 cells

Cells were first labeled with sheep anti-hTS antibody and then either not labeled (empty) or fluorescently labeled with Cy3-conjugated anti-sheep antibody (filled). Other details are in *Materials and Methods*.

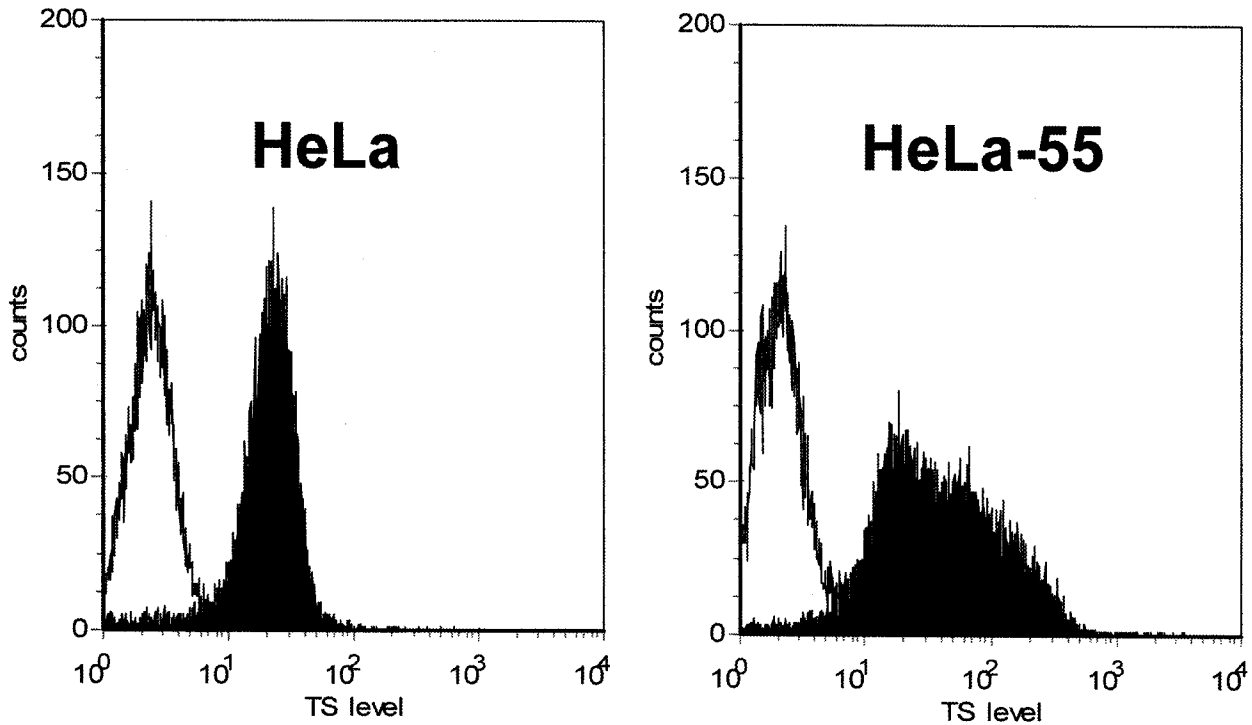

HeLa
HeLa-55
counts
TS level
200
150
100
50
0
10^0
10^1
10^2
10^3
10^4

FIGURE 2.8. Fluorescent-activated cell sorting (FACS) of anti-TS-labeled HeLa-55 cells and TS gene analysis of the sorted cells

A. FACS analysis. Cells were first labeled with sheep anti-hTS antibody and then with Cy3-conjugated anti-sheep antibody. The cell population was separated into four fractions (F1 to F4) with increasing TS intensity. Cell sorting was confirmed by fluorescent microscopy (upper part of the panel). The percentage of cells in each population is shown on the arrows. ***B.*** Semi-quantitative PCR analysis for TS in genomic DNA isolated from HeLa, HeLa-55 and fractions F1 to F4 from panel A. Equal amount of DNA (100 ng) was used for all reactions. Shown is the pixel count of the amplified PCR fragments relative to HeLa. Other details are in *Materials and Methods*.

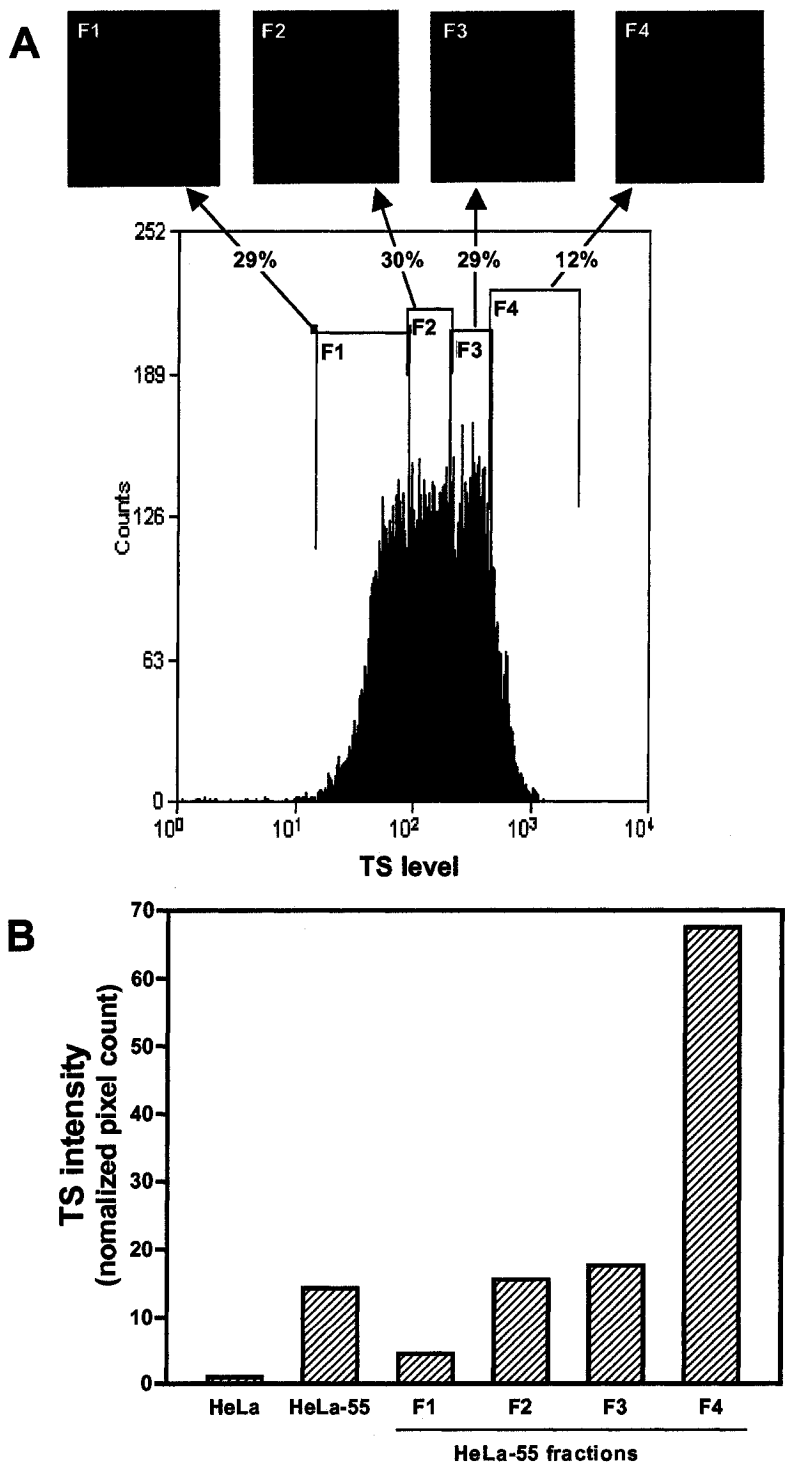
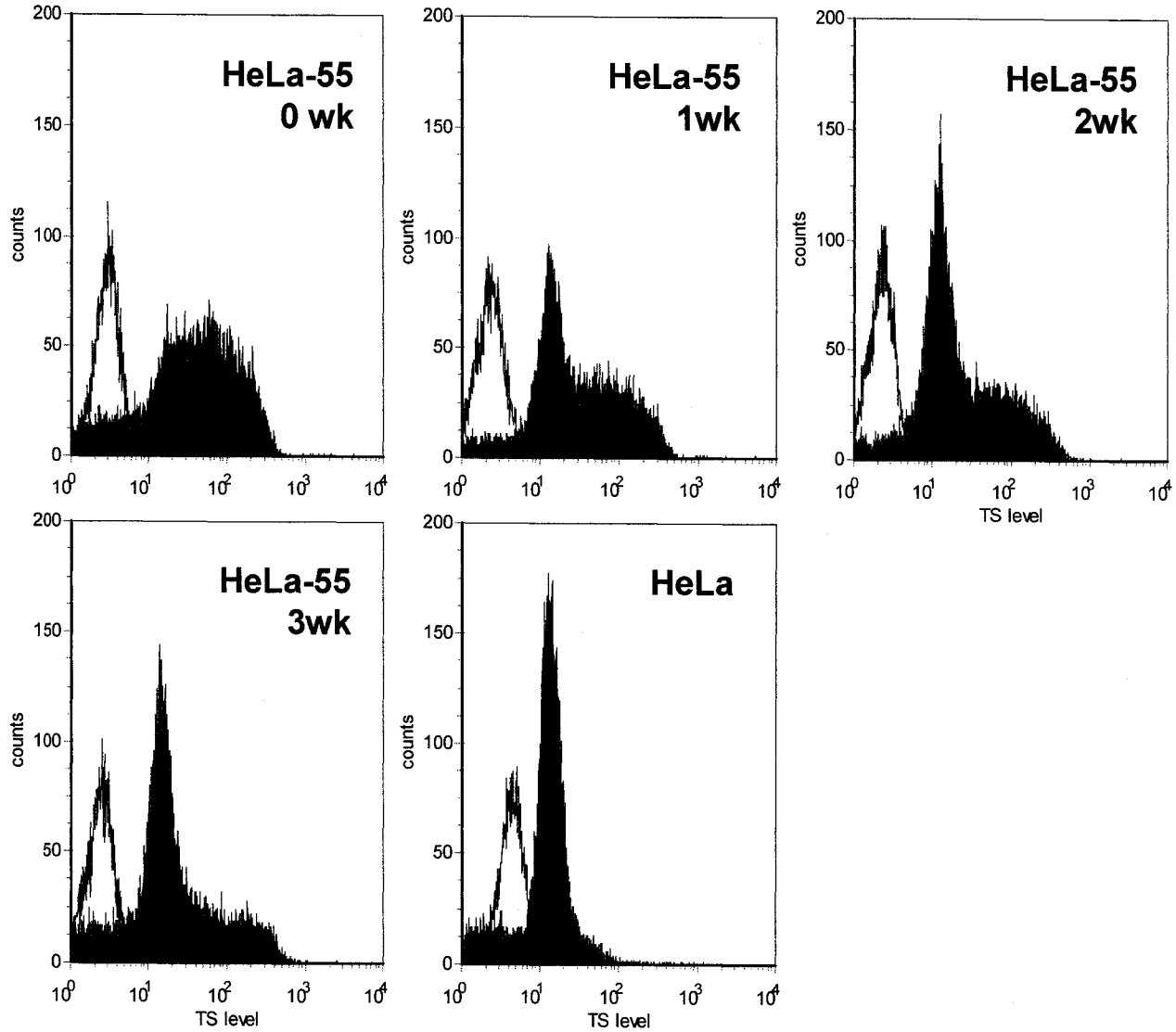

A
F1
F2
F3
F4
29%
30%
29%
12%
252
189
126
63
0
Counts
10^0
10^1
10^2
10^3
10^4
TS level
B
TS intensity
(nomalized pixel count)
70
60
50
40
30
20
10
0
HeLa
HeLa-55
F1
F2
F3
F4
HeLa-55 fractions

FIGURE 2.9. Stability of TS overexpression as analyzed by flow cytometry in HeLa-55 cells at 0, 1, 2 and 3 weeks in the absence of 5-FUdR

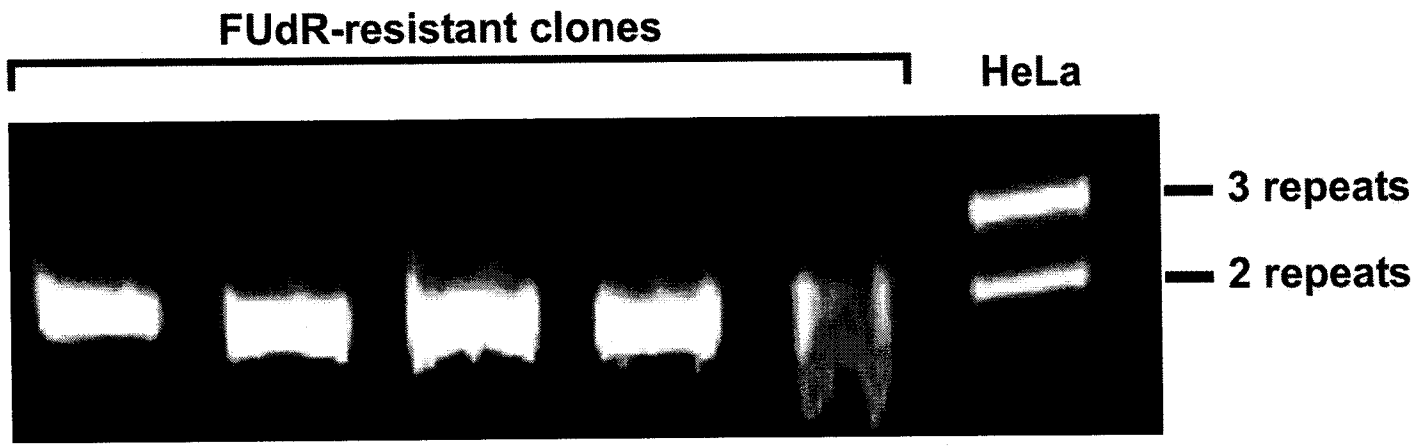
Cells were first labeled with sheep anti-hTS antibody and then either not labeled (empty) or fluorescently labeled with Cy3-conjugated anti-sheep antibody (filled). Also shown is the analysis of parental HeLa cells. Note the gradual loss of TS overexpression in HeLa-55 from 0 to 3 weeks. Other details are in *Materials and Methods*.


HeLa-55
0 wk
HeLa-55
1wk
HeLa-55
2wk
HeLa-55
3wk
HeLa
counts
TS level
0
50
100
150
200
10^0
10^1
10^2
10^3
10^4

tandem repeats (2R allele). My series of 5-FUdR resistant HeLa clones that overexpressed TS protein were examined to determine whether there was preferential amplification of the 3R or the 2R allele. When PCR analysis was conducted on these TS-overexpressing, 5-FUdR-resistant HeLa clones, a striking observation was made: 20 out of 20 (100%) of the clones showed amplification of the 2R allele of TS (Fig. 2.10).

FIGURE 2.10. PCR analysis for polymorphic repeats in the genomic DNA of 5-FUdR-resistant clones and HeLa cells

Shown are the positions for the amplified fragments containing 3 or 2 repeats. Note that *all* the 5-FUdR-resistant clones show amplification of the allele containing 2 repeats, although only five are shown here. Other details are in *Materials and Methods*.


FUdR-resistant clones
HeLa
3 repeats
2 repeats

2.5. DISCUSSION

In eukaryotes, amplification is postulated to arise by mechanisms based on replication origins, either used to form duplications by over-replication or to allow the persistence of episomes seen as double minutes (DMs) (124). DMs are acentric circular episomes that contain a replication origin and therefore persists (124). Once circular episomes are formed, their amplification might occur by several possible routes including unregulated segregation into daughter cells at cell division and crossing-over between two circular episomes. These circles might be able to intergrate into the chromosome either homogenously or ectopically (124). Another mode of amplification in eukaryotes is initiated by a double strand break (DSB), often occurring at a fragile site (124). These DSBs provoke a breakage-fusion-bridge (B-F-B) cycle in which chromatid ends join to each other before anaphase separation. Segregation of the chromatids to opposite poles at mitosis leads to a break. This break then leads to another fusion and subsequent break in succeeding generations (124). Cultured cells exposed to inhibitors of nucleotide biosynthesis, such as 5-FUdR develop intracellular nucleotide pool imbalances (3). Since the regulation of intracellular nucleotide pools plays an important role in controlling genomic stability, exposing cultured cells to 5-FUdR may therefore induce chromosome fragility possibly leading to a B-F-B mechanism of gene amplification (124,125). It must also be noted that initial amplification reactions are typically followed by periods of genomic instability during which a variety of secondary amplification reactions often involving different mechanisms, occur (126).

Past reports utilizing stepwise selection in increasing concentration of 5-FUdR for generating TS over-expressing cell lines, have found that in most cases TS gene amplification was the leading cause of 5-FUdR resistance (51,112-115). In order to generate a TS overexpressing HeLa cell line, I decided to use this fairly simple method. Folinic acid (leucovorin) is a reduced folate, which increases intracellular folate pools as it acts as a precursor of CH_2-THF (the cofactor used by TS). It is included in the selection media so that this cofactor does not become limiting during selection for TS enzyme over-production (51). From several selection plates, over 50 individual clones were isolated and screened by Western dot blot analysis. Most, but not all, overexpressed TS protein to a large extent. Although all clones were maintained in selection media (i.e. 0.1 μM 5-FUdR, 25 μM folinic

acid), not all of the TS protein was in the ternary complex, indicating that there existed some balance between free and ternary complexed TS in the cells.

Clone 55 (HeLa-55) was chosen for further study because it grew most aggressively compared to other clones and was one which overexpressed TS protein to the largest extent. It was expected that HeLa-55 would contain an amplified TS gene. I determined that HeLa-55 amplification of the TS gene was > 50-fold compared to parental HeLa levels through semi-quantitative PCR analysis. Later, our lab obtained access to a Real Time PCR machine and I confirmed by real time PCR that HeLa-55 amplification of the TS gene was 100-fold compared to HeLa levels. Although HeLa-55 grew fastest of the isolated clones, it should be noted that, in selection media, its doubling time was still 33% longer than parental HeLa cells grown in standard media. This indicated that the drug was still partially inhibitory to the cells. Although HeLa-55 was selected by stepwise increasing 5-FUdR concentration and eventually maintained in 0.1μM 5-FUdR, it was capable of growth in 1 μM 5-FUdR. I expected that the mechanism leading to resistance in these cells continued to occur with the further increase in drug concentration.

I observed heterogeneity in both morphology and intensity of TS staining of HeLa-55 while maintained in 5-FUdR. This heterogeneity was confirmed to be due to heterogeneity in TS gene copy number among HeLa-55 cells. From FACS analysis of HeLa-55, I clearly showed that there was correlation with relative TS staining and number of TS gene copies in the various fractions of HeLa-55 cells sorted according to relative TS staining level. It has been reported that there exists heterogeneity with regard to size of the amplicon not only between cell lines carrying amplified DNA but even within cells of the same cell line (126). This may be due to variable trimming of the original amplicon upon propagation of cells under selection pressure (126). On the other hand, it is possible that the substantial homology present in mutimegabase amplicons makes them prone to recombination (127). Thus it has been proposed that high frequency unequal exchanges could occur within such structures to generate the copy number heterogeneity detected during the amplification process (127).

When grown in the absence of selection pressure, HeLa-55 cells quickly lost the amplicon containing the TS gene. Due to the instability of the amplified TS gene in HeLa-55, I anticipate that a substantial percentage of the amplified TS gene resides in unstable

double minute chromosomes (DMs). Since DMs do not contain centromeres, they have the potential for unequal segregation into daughter cells at the time of mitosis (128). In cells containing DMs, it has been shown that individual cells with lower amplified gene copy number grew more rapidly over a number of cell doublings when grown in the absence of selection pressure (128). In addition, early experiments estimated that HeLa-55 unsorted cells contained amplified TS at least 50-fold, whereas later experiments for FACS analysis on HeLa-55 estimated that unsorted HeLa-55 cells contained a 20-40 fold increase in TS level. The two analyses were conducted ~ six months apart and their differences can be explained by the fact that the TS gene amplification in HeLa-55 is unstable and continuously changing.

When gene amplification takes place, usually only one of the two alleles of the gene is amplified (61). This appeared to be the case with our TS over-expressing HeLa clones. The TS gene expression is polymorphic in the 5′ regulatory region of the gene (41). The polymorphism consists mainly of either 3 repeats (3R allele) or 2 repeats (2R allele) of a 28-bp GC-rich sequence, with the 3R allele producing greater TS expression (55). Since parental HeLa cells are heterozygous for this polymorphism, I was able to study whether there was any bias in the selection of which allele would be amplified. Surprisingly, I found that in all 20 TS over-expressing clones, including HeLa-55, amplification of only the 2R allele occurred. The explanation of this is not clear. The simple explanation of "sib-selection" can be ruled out as the clones were derived from several different culture plates. It is possible that in HeLa cells there is something special about that region of chromosome 18 containing the 2R allele that allows it to be more sensitive to amplification. Alternatively, it could be that the 2R allele itself somehow confers sensitivity to gene amplification compared to the 3R allele. It would be of great interest to repeat this type of experiment with other cell lines and with other polymorphic TS markers in homozygous (3R/3R) or (2R/2R) and heterozygous (3R/2R) cell lines.

2.6. CONCLUSION

In this chapter I developed TS overexpressing HeLa cell lines by stepwise selection in 5-FUdR. One clone, HeLa-55 was chosen and characterized in further detail. As expected, HeLa 55 contained an amplified TS gene and heterogeneity for TS expression in

HeLa-55 cells was due to heterogeneity in TS gene amplification. Surprisingly, in all TS-overexpressing 5-FUdR-resistant HeLa clones, the 2R allele of TS was preferentially amplified over the 3R allele.

CHAPTER 3: PARTIAL ELUCIDATION OF THE MECHANISM OF 5-FUDR AND 5-FU RESISTANCE IN A COLORECTAL CANCER CELL LINE

3.1. INTRODUCTION

Due to its importance in supplying a deoxynucleotide essential for DNA synthesis, TS is used as a target for chemotherapeutic agents such as the fluoropyrimidine based analogs, 5-FU and 5-FUdR (1,3). Mechanisms of action of 5-FUdR and 5-FU are mutifactorial. Cytotoxic effects may be mediated by incorporation of the metabolic products FdUTP and FUTP into DNA and RNA, respectively (17) (General Introduction, Fig. 2)(18). Additionally, intracellularly, the fluoropyrimidines are metabolized to FdUMP. FdUMP forms a stable covalent "ternary complex" with the target enzyme TS, and the cofactor 5,10-methylenetetrahydrofolate (CH_2-THF) (14,15). Formation of TS ternary complex prevents binding of the natural substrate dUMP to TS, leading to prolonged inhibition of the enzyme (14). Inactivation of TS results in decreased production of TMP, cessation of DNA synthesis and ultimately thymineless death (16). The predominant mechanism of fluoropyrimidine action may vary depending on tumor type and the activities of metabolic enzymes, levels of competing substrates, availability of cofactors, and schedule of drug administration (19).

The formation of the TS ternary complex can be used as a marker of the metabolism of 5-FU and 5-FUdR to 5-FdUMP. Any cellular mechanism that leads to decreased formation of TS ternary complex would likely cause resistance to 5-FU and 5-FUdR. The formation of the TS ternary complex can be affected by factors involved in the availability of the substrates FdUMP and CH_2THF to the TS protein, or the interaction of the substrates with the TS protein. Decreased availability of the substrates FdUMP and CH_2THF could result from the following mechanisms: (1) decreased transport of 5-FU and 5-FUdR into cells (69-72), (2) altered folypolyglutamylation (75-77), (3) decreased activity of thymidine kinase (63) (64,65)and for 5-FU, additional enzymes involved in its anabolism such as orotate phosphoribosyltransferase (OPRT), uridine kinase (UK) and ribonucleotide reductase (RNR) (66-68), and (4) increased phosphatase activity resulting in decreased accumulation and retention of FdUMP (62). Decreased interaction of the substrates with the TS protein could result from altered TS protein such that the affinities of the enzyme for FdUMP or CH_2THF are decreased (60,61).

In addition to the above mentioned mechanisms, it is possible that other differences between cell lines could affect their response to 5-FU and 5-FUdR. Two recent reports have

suggested that the p53 status of a cell line can have a profound effect on its response to 5-FU. One utilized an HCT-116 p53 null cell line and the other utilized a MCF-7 p53 wt cell line transformed with E6. Both groups reported that the cell lines were resistant to 5-FU (129,130). They interpreted their results as showing that expression of wild-type p53 greatly potentiates 5-FU cytotoxicity.

Genetic instability can be described as the accumulation of DNA damage and mutations, where mutations can be chromosomal abnormalities or nucleotide sequence abnormalities (131). Genetic instability may be either progressive (persistent) instability or episodic (transient) instability (131). Progressive instability is transmitted from generation to generation as in tumor cells with hereditary nonpolyposis colorectal cancer (HNPCC) (132). Episodic instability is sporadic genetic damage and accounts for a large proportion of adaptive mutations occurring in cells (132). Epigenetics (outside "conventional" genetics) describes stable alterations in gene expression that arise during development and cell proliferation (133). Epigenetic processes are essential for development and differentiation, but they can also arise randomly. Molecular mechanisms that appear to mediate epigenetic phenomena include changes in DNA methylation and some histone modifications (133). Numerous studies of genetic instability in cell lines have been reported. However, there have been no previous reports of genetic instability in the context of resistance to 5-FU and 5-FUdR.

In this chapter I present evidence that suggests that decreased transport of 5-FUdR and 5-FU into the colorectal cancer cell line, HCT-116, is responsible for resistance to these fluoropyrimidine base analogs. In addition, my results suggest that this mechanism of resistance is unstable and independent of p53.

3.2. OBJECTIVE

The objective of work described in this chapter was to explain the novel observation that a colon cancer cell line rapidly became resistant to the anti-TS drug 5-fluorodeoxyuridine (5-FUdR).

3.3. MATERIALS AND METHODS

3.3.1. Antibodies

Rabbit anti-hTS antiserum used in these studies was prepared previously in our lab (95). This polyclonal antibody was raised against recombinant $(his)_6$-rhTS and its specificity has been previously illustrated. HRP-conjugated anti-rabbit/anti-mouse secondary antibody ("Polymer") was from DAKO (Carpinteria, CA). Anti-p53 (Ab-6) and anti-p21 antibodies were from Oncogene (Boston, MA), and anti-actin antibody (clone AC-47) was from Sigma-Aldrich (St Louis, MO). Mouse monoclonal antibody to β-tubulin was a kind gift from Dr. Doug Gray (University of Ottawa, Ont).

3.3.2. Cell lines

HeLa cells were obtained from ATCC (Rockville, MD). The following colon cancer cell lines from the NCI anti-cancer screening panel of 60 cell lines were used: HCT-116, HCT-15, HCC-2998, HT-29 and SW-620; RKO is a colon cancer line originally derived by Brattain *et al.*(96). HCT-116 p53 wt and HCT-116 p53 null were originally from Vogelstein's lab (134) and HCT-116 +Chr3 was originally from Koi's Lab(135). Normal primary human fibroblasts were from neonatal foreskin. Cells were cultured as monolayers in Dulbecco's modified Eagle's medium (GIBCO-BRL, Burlington, Canada) supplemented with 10% fetal calf serum in 100 mm dishes in 5% CO_2 at 37 °C. RKO and ATCC HCT-116 cells were maintained in McCoy's 5A media plus 10% fetal calf serum (GIBCO-BRL).

3.3.3. Cytotoxicity Studies

$1x10^3$cells were seeded in 1 mL of standard media per well of a 12 well dish. Next day fresh media with or without drug was added. Where indicated, 5-FUdR (Sigma-Aldrich, St. Louis, MO) and 5-FU (Pharmacia & Upjohn Inc., Bridgewater, NJ) were used at concentrations of 0 M, 1×10^{-9} M, 1×10^{-8} M, 1×10^{-7} M, 1×10^{-6} M and 1×10^{-5} M. Thymidine (Sigma-Aldrich) was used at 2×10^{-5}M. Media was changed until cells were counted by releasing attached cells with trypsin and counting in a Coulter counter (Model ZM, Coulter Electronics Ltd, Luton, Beds., England). Cell counts for 0 M were set to 100% and all

counts were expressed as a percentage of the 0 M count. For 5-FUdR and 5-FU, cells were counted 8 days after addition of drug.

For dose response to 5-Aza-2-deoxycytidine (5-AdCd), $1x10^4$cells were seeded in 1 mL of standard media per well of a 12 well dish. 5-AdCd (Sigma-Aldrich) was used at same concentrations as 5-FUdR. At 24 and 48 hour time points, attached cells were counted. For pre-treatment of cells with 5-AdCd, 5×10^4 cells were plated in 5 mL of standard media in a 5 mL dish. Next day the media was changed to media with 5-AdCd. 24 hr after, the media with drug was removed and drug free media was added. Cells were allowed to recover for 3 days before they were trysinized, counted and used for plating for ^{3}H-thymidine incorporation assay and colony forming unit assay.

3.3.4. Colony forming unit (CFU) assay

300 cells were plated in 5 mL of standard media in 5 mL dishes. 10 days after, the media was removed by gently pouring off. Colonies were fixed and stained with methylene blue stain in methanol (0.6% (w/v)) for > 30 min. The stain was carefully poured off and colonies were rinsed by dipping the plates in water. Plates were air dried and colonies > 1mm in diameter were counted.

3.3.5. HAT selection

Cells were plated for colony forming assay. 24 hr after plating, media was removed and replaced with HAT selection medium [hypoxanthine (Sigma-Aldrich) (1×10^{-4} M), aminopterin (Sigma-Aldrich) (4×10^{-7} M) and thymidine (1×10^{-7} or 1×10^{-5} M)]. A control plate in which standard medium was added, was included in each experiment. 10 days after HAT selection, the medium was removed and colonies were fixed as described in colony forming unit assay. The number of colonies in treated plates was expressed as a percentage of control plate.

3.3.6. Treatment of Cells to measure Formation of Ternary Complex

Cells were plated at 5×10^4 cells per well of a 12 well plate in standard medium. Next day the medium of the cells in log phase were removed and replaced with medium containing 5-FUdR in the absence or presence of folinic acid (2.5×10^{-5}M, Sigma-Aldrich

Chemicals, St. Louis, MO) or 5-FU. 5-FUdR and 5-5-FU were used at concentrations of 0 M, 1×10^{-9} M, 1×10^{-8} M, 1×10^{-7} M, 1×10^{-6} M and 1×10^{-5} M. 24 hr later, the medium was removed and cells were treated for preparation of total cell protein extracts as described below.

3.3.7. Preparation of total cell protein extracts

Cultured cells were detached by light trypsin treatment, then washed twice to remove trypsin and suspended in cold PBS. To prepare whole cell extracts, an equal volume of 2×SDS sample buffer (100 mM MOPS, pH 6.8, 4% SDS, 20% glycerol, 2 % β-mercaptoethanol) was added. The extracts were boiled for 20 min and then sonicated briefly to reduce viscosity. Protein was quantified using fluorescamine (97) (Sigma-Aldrich), using BSA as a standard.

3.3.8. Western blot

Proteins in cell extracts in SDS sample buffer were resolved by 12% discontinuous SDS-PAGE and then electrophoretically transferred to a PVDF membrane (Millipore, Nepean, Canada) at 15V for 18 h in transfer buffer (3 mM Na_2CO_3, 10 mM $NaHCO_3$, 10% methanol) (95). The membrane was stained with Ponceau S and then washed twice in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8). All steps were carried out at room temperature. When using anti-TS as the primary antibody, the membrane was blocked with 1% bovine serum albumin (BSA) (OmniPur ^{R} BSA, Fraction V, EM science- MERCK kGaA, Darmstadt, Germany) in TBST. When using anti-β tubulin as the primary antibody, the membrane was blocked with freshly prepared Casein Blocker solution (100 mM NaCl, 50 mM sodium acetate, pH 6.0, 1% bovine milk casein, 2 mM cyclohexanediamine tetraacetate (Sigma-Aldrich)). When using anti-p53, anti-p21, and anti-actin primary antibodies, the membrane was blocked with 5% non fat milk (Sigma-Aldrich) in TBST. In all cases the membrane was blocked for 1 h. Rabbit anti-TS antibody was used at 1:1000 dilution in 0.5% BSA in TBST, anti-β tubulin primary antibody was used at 1:10 dilution in TBST, anti-p53 primary antibody was used at 1:1000 dilution in 0.5% milk in TBST, anti-p21 primary antibody was used at 1:250 dilution in 0.5% milk in TBST and anti-actin primary antibody was used at 1:15000 dilution in 0.5% milk in TBST. In all cases, the

primary antibody was incubated with the membrane for 1 hr. The membrane was washed 5 times in TBST and incubated for 1 h with the secondary antibody: DAKO 'Polymer' anti-mouse-IgG-HRP used at 1:250 dilution in TBST. The membrane was washed 5 times with TBST and HRP was detected using the LumiGLO Chemiluminescent Substrate Kit (Kirkegaard & Perry Lab; Maryland, USA). Western blot was stripped using Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL) according to the maufacturer's protocol.

3.3.9. 2D-gel analysis Of TS

Cells were sonicated in 20 mM Tris-base and diluted to 1 mg/ml in urea lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.2% Bio-analyte 3-10 ampholytes, 0.001% bromophenol blue). 300 μg of cellular protein was used to rehydrate a 17-cm IPG strip pH 3-10 NL (ReadyStrip, Bio-Rad, Mississauga, Canada) at room temperature for 16 h. For the first dimension, the strip was isoelectrically focused at a voltage of 10,000 V (current 50 μA/strip) until 50,000 volt-hours was reached. Prior to the second dimension, the strip was equilibrated in reducing buffer (6 M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol, 2% DTT) for 15 min and then in alkylating buffer (6 M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide) for an additional 15 min. For the second dimension, the strip was resolved on a 10% preparative SDS-PAGE at a constant voltage of 48 mA for 5 h. The gel was then analysed for western blot analysis to detect TS as described above.

3.3.10. TK in vitro assay

Cells growing in log phase were trypsinized, washed with cold PBS and resuspended in extraction buffer (50mM MOPS pH 7.4, 1 mM EDTA pH 7.4, aprotinin (10 μg/mL) and PMSF (2 mM). Cells in suspension were sonicated briefly, centrifuged at 10 000g at 4 °C for 20 min and the supernatant was used for TK *in vitro* assay.

For preparation of anion exchange resin, 1 gram of anion exchange resin Spectra/Gel Strong base anion chloride form (Spectrum, LA, Ca) was washed with 5 mL water, centrifuged at 500 g and washing was repeated twice. The resin was then washed 3× with 0.1N NaOH and 2× with 0.01 N NaOH and then with binding buffer (10 mM Tris-HCl

pH8.0) and kept stored in 10 mL of binding buffer at 4 °C for up to a week. Each 50 μL TK *in vitro* reaction mixture consisted of 1× assay buffer (10mM ATP, 5mM $MgCl_2$, 5mM MOPS, 100μM EDTA, pH 7.4, thymidine (0.5μCi ^{3}H-thymidine (Amersham Life Sc, Buckinghamshire, England, stock 25Ci/mmol) and cellular extract containing TS enzyme which was the last component added to start the reaction. The reaction mix was incubated at 37°C and stopped by the addition of excess cold thymidine (1mM) and heating at 95°C for 3 min. 0.5 mL of binding buffer (10mM Tris-HCl pH8.0) and 500μL of previously equilibrated anion exchange resin in binding buffer were added and the tube was rotated for 1 hr to allow resin to bind ^{3}H-TMP product. The reaction tube was then centrifuged at 3500 g for 15 secs and the supernatant was removed. The resin was washed 3 × by triturating with 1 mL of binding buffer and a final 0.5 mL wash was collected for counting. The resin was then eluted with 0.5 mL of 0.2 M NaCl and the eluate was counted. For counting, 1mL of Liquid Scintillation counting Fluid Scintran Cocktail X (VWR International, Mississauga, Canada) was added and tubes counted in a Packard model 1600 TR liquid scintillation counter.

3.3.11. Incorporation of ^{3}H- Thymidine (^{3}H-TdR) into DNA

7.5×10^3 cells were plated per well of a 96-well plate. 48 hr later, 0.5 μCi ^{3}H-TdR was added to each well. Cells were incubated at 37°C for 18 hr. The medium was removed and attached cells were washed with PBS (3×200μL). Cells were lysed with 0.1% SDS and the total cell lysate was counted.

3.3.12. Plating Cells As A Mixture:

To test whether 5-FUdR resistance could occur in *trans*, WT and WTR cells were plated at ratios of 90:10, 75:25, and 50:50 (WT:WTR) such that the total number of cells was 7.5×10^3 cells in 200μL. Cells were used for the assay measuring incorporation of ^{3}H-TdR into DNA.

Conditioned Medium : The medium from WT cells was removed and placed with WTR medium for 48 hr before addition of 0.5 μCi of ^{3}H-TdR. The assay measuring incorporation of ^{3}H-TdR into DNA was performed.

Pre-treatment: 7.5×10^3 WT or WTR cells were plated per well in a 96 well plate. 24hr later, the medium of the WT cells was removed and replaced with medium from WTR cells.

24 hr later, this medium was removed and replaced with fresh medium containing 0.5 μCi of ^{3}H-TdR. The assay measuring incorporation of ^{3}H-TdR into DNA was performed. WTR cells were subjected to same treatment as a control.

3.3.13. Uptake of ^{3}H-TdR into Cells

2×10^4 cells in 200μL were plated per well in a 96-well plate. 48 hr later, the medium was changed to 1% serum in DMEM and 0.5 μCi of ^{3}H-TdR (0.1μM)was added. The plate was incubated at 37°C to allow cellular uptake of ^{3}H-TdR. Uptake was stopped by addition of excess cold thymidine (20μM). The medium was removed and attached cells were washed 3× with PBS. Cells were trypsinized and transferred to a Microcon-PCR centrifugal filter device (Millipore; Bedford, MA) and centrifuged at 500 g for 5 min. The cells retained on the filter in ~ 20μL were washed 5× with PBS. The final wash was collected for counting. 0.2% SDS was added to the filter and incubated at RT for 20 min to solubilize the cells. The filter was then inverted and centrifuged into a fresh tube and the cell extract was collected and counted. Cell incorporation of ^{3}H-TdR was defined as counts in the cell extract minus counts in the "final wash".

3.3.14. Infection of p53 null Cells with Adenovirus Expressing wt p53

Cells were plated at the density of $3x10^5$ cells/mL in 35mm wells and incubated overnight. The medium was removed and cells were infected with an adenovirus encoding for wild-type p53 (136) at a MOI of 40 diluted in 250 μL PBS, and incubated for 1 h at 25°C. 2 mL of fresh medium was then added and the cells were incubated at 37°C for another 6 h to ensure high infection efficiency before replating them at a density of $5x10^4$ cells/mL per well of a 12-well plate. After overnight incubation (to ensure sufficient time for cells to express high p53 levels), 5-FUdR was added and cells were exposed to the inhibitor for 24h before extracting total protein as described above.

3.3.15. RNA extraction & cDNA synthesis

Exponentially growing cell monolayers were washed twice with chilled PBS. 1.5 mL of REBS (0.5M LiCl, 1.0M Urea, 1.5mM $CaCl_2$, 0.25% SDS, 20mM BES, pH6.8) was added and RNA was extracted as previously described (122). RNA was resuspended in

sterile water and total RNA concentration was determined by A_{260} reading. 5μg of total RNA was annealed with 1μg of oligo-dT (Invitrogen, Burlington, Canada) and cDNA was synthesized using the reagents and protocols provided with M-MLV Reverse Transcriptase (Invitrogen).

3.3.16. LightCycler Relative mRNA Quantification.

PCR reactions were performed on a cDNA library from each HCT 116 line, targeting a 204 bp fragment of TK cDNA, a 231 bp of ENT1 cDNA, a 366bp fragment of ENT2 cDNA and an internal reference 718 bp fragment of G6PD mRNA. The primers sequences were as follow: TK (sense), 5′-CCTGCCAACTGAGGGACC-3'; TK (antisense), 5′-GCAGACCAGTGGGTAGGAG-3'; ENT1 (sense), 5′-TGTCTGCGTCCGTGTC-3'; ENT1 (antisense), 5′-CACCTGGTTTCTGTCAAAT-3'; ENT2 (sense), 5′-TGCCTGCGGTTCCTGTTC-3′; ENT2 (antisense), 5′-TGAGCCAAGCTCGCCATT-3′; G6PD (sense), 5′-ATCTACCGCATCGACCAC-3′; G6PD (antisense): 5′-CAGGGAGCTTCACGTTCT-3′. Each 20 μL PCR reaction contained 1X buffer (Invitrogen), 0.2 mM dNTPs, 4 mM $MgCl_2$, 4 pmoles of each primer (10 pmoles for G6PD), 0.5 mg/mL BSA, 2 units of Taq polymerase (Invitrogen) and 1/40000 SYBR green I (Molecular probes Inc., Eugene, OR) and 2 μL of cDNA dilution. Target and reference sequences were amplified and quantified in a LightCycler (Roche, Mississauga, Canada) using the following programs: TK denaturation (98°C for 30 sec), amplification of 50 cycles (98°C for 0 sec, 56°C for 8 sec, 72°C for 9 sec with fluorescence acquisition); ENT1 denaturation (98°C for 30 sec), amplification of 50 cycles (98°C for 0 sec, 52°C for 8 sec, 72°C for 10 sec with fluorescence acquisition); ENT2 denaturation (98°C for 30 sec), amplification of 50 cycles (98°C for 0 sec, 58°C for 8 sec, 72°C for 15 sec with fluorescence acquisition); G6PD denaturation (98°C for 30 sec), amplification of 50 cycles (98°C for 0 sec, 62°C for 10 sec, 72°C for 29 sec with fluorescence acquisition). Each run included a standard curve obtained by 6 serial dilutions of the sensitive cDNA library used as a standard, a no template control and the two samples being quantified. PCR specificity was confirmed by melting curve analysis (63°C-99 °C, 0.1°C/sec). The relative values of the target genes were extrapolated from the standard curve and then normalized to the internal reference (G6PD).

3.4. RESULTS

3.4.1. Screening Cell Lines for Differences in TS Ternary Complex Formation

The formation of the "ternary complex" of 5-FdUMP, TS and folate cofactor can be used as a biochemical surrogate of the successful conversion of 5-FUdR or 5-FU to 5-FdUMP. This complex is easily detected using western blot and anti-TS antibody. I screened several cell lines including normal fibroblasts and 8 cancer cells lines: HeLa and 7 colorectal cell lines: RKO, SW-620, HT-29, HCT-15, HCC2998, HCT-116 p53WT, and one recently described as resistant to 5-FU, HCT-116 p53 null, for the presence of TS ternary complex. All cell lines were subjected to the same treatment of 24 h exposure to 10^{-7}M 5-FUdR. Surprisingly, the p53 null cell line formed very little TS ternary complex compared to all others tested (Fig. 3.1). In addition, there was less of an increase in TS protein level in this p53 null line upon 5-FUdR treatment as compared to other cell lines. As expected, in samples not exposed to 5-FUdR, only the free TS protein was detected. The lower panel shows the loading control for β-tubulin detection (Fig. 3.1).

3.4.2. Response of HCT-116p53 wt and null Cell Lines to 5-FUdR

From my initial screen of cell lines to identify differences in ternary complex formation I found that the only cell line that showed obvious differences was HCT-116p53 null cell line. Since the result of this cell line differed from its p53 wt counterpart I decided to focus on this pair of cell lines, HCT-116 p53 wt and p53 null for the rest of my study and my aim was to try to understand the mechanism responsible for this difference in ternary complex formation between the two lines.

My next step was to test whether this difference in TS ternary complex formation between the two lines had an effect on their response to 5-FUdR. When I compared their response to 5-FUdR I found, as expected the p53 wt cell line was sensitive to 5-FUdR as compared to the p53null cell line (Fig. 3.2). I tested whether the sensitivity of the p53 wt cell line could be rescued with the addition of thymidine in the media and found that the inclusion of 2×10^{-5}M thymidine was sufficient to completely rescue the p53 wt cell line from the cytotoxicity of 5-FUdR. Since the p53 null cell line was resistant to cytotoxicity of

FIGURE 3.1. Detection of TS-ternary complex formation in various cell lines after 5-FUdR treatment.

Cells were either not treated (–) or treated (+) with 0.1 μM 5-FUdR for 24 h and analyzed by western blotting using anti-TS antibody. Each lane contains 10 μg of protein. Indicated are the positions for the free TS form (35 kD) and the ternary complex form (~37 kD). The blot was striped and re-analyzed for β-tubulin as a loading control (lower panel). Other details are in *Materials and Methods*.

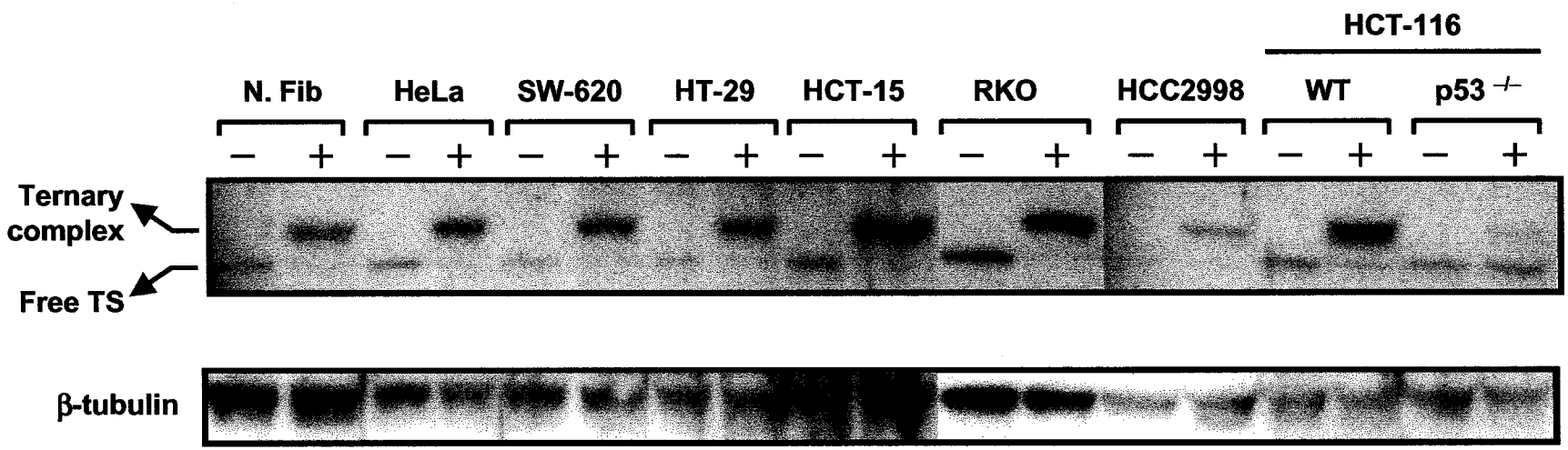
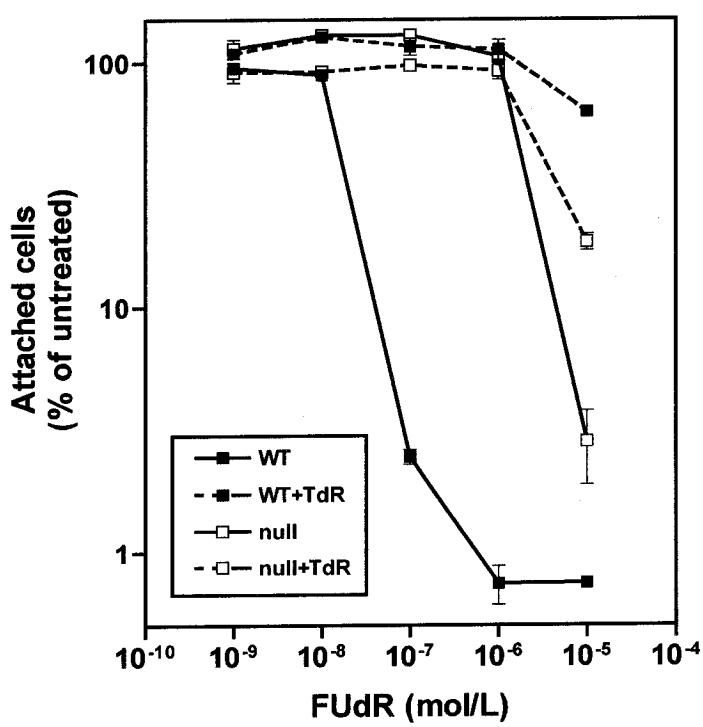

HCT-116
N. Fib
HeLa
SW-620
HT-29
HCT-15
RKO
HCC2998
WT
p53 -/-
– + – + – + – + – + – + – + – + – + – +
Ternary complex
Free TS
β-tubulin

FIGURE 3.2. Dose-dependent effect of 5-FUdR on growth of HCT-116 wild-type (WT) and p53 null cells in the absence or presence of TdR

Cells were treated with various concentrations of 5-FUdR in the absence or presence of 20 μM TdR for 8 d. The cells attached to the plates were harvested and counted using Coulter counter. The counts were corrected for the untreated cells (0 M FUdR). Error bars represent mean ± SD of 3 replicates. Other details are in *Materials and Methods*.


Attached cells
(% of untreated)
100
10
1
WT
WT+TdR
null
null+TdR
10-10
10-9
10-8
10-7
10-6
10-5
10-4
FUdR (mol/L)

5-FUdR and formation of TS ternary complex, my results suggested that uptake or metabolism of 5-FUdR was different in these cells.

3.4.3. *Effect of 5-FUdR Concentration on Ternary Complex Formation*

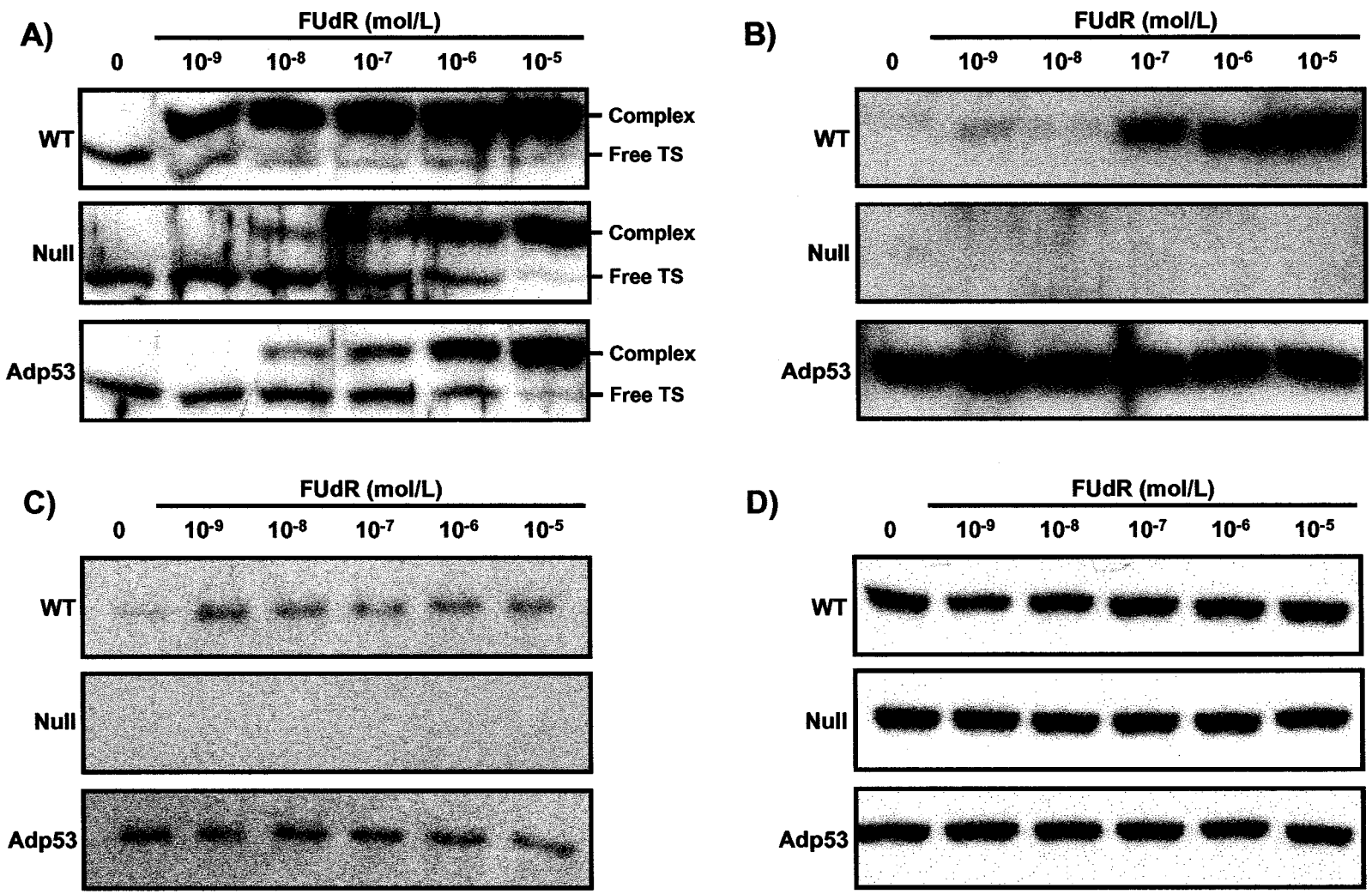
To explore the reason for the difference in TS ternary complex formation in the HCT-116 p53 wt and p53 null lines, I tested various concentrations of 5-FUdR to determine whether this correlated with the differences in 5-FUdR sensitivity to killing between the two lines. I exposed the cells to the same range of doses of 5-FUdR as used in the cytotoxicity experiments (Fig. 3.2) and found that with the lowest dose tested, 10^{-9}M, the majority of TS protein was in the ternary complex form in the p53wt cell line whereas there was no detectable TS ternary complex in the p53 null line (Fig. 3.3A panels wt, null). In the p53 wt cell line, with increasing 5-FUdR concentrations, there was very little increase in the amount of TS in the ternary complex. In comparison, for the p53 null cell line, as the 5-FUdR concentration was increased, the amount of free TS protein gradually decreased and the amount of ternary complex TS gradually increased (Fig. 3.3A panel null). At the highest dose of 5-FUdR tested, 10^{-5}M, most of the TS protein was in a ternary complex form with a trace amount of free TS protein. Therefore, at 5-FUdR doses between 10^{-9} to 10^{-6} M, the two cell lines showed marked differences in TS ternary complex formation, with the p53 null cell line illustrating a reduced ability to form the TS ternary complex. Only at a very high dose, 10^{-5} M 5-FUdR, was the ternary complex formation eventually comparable between the two lines. These results were consistent with a problem in 5-FUdR uptake or metabolism in the p53 null cell line. I chose to use the 5-FUdR dose of 10^{-7} M for subsequent experiments when illustrating the difference in TS ternary complex formation between the two lines. At each 5-FUdR dose, the p53 wt cell line always showed a greater level of TS protein expression as compared to the p53null line (Fig. 3.3A panels wt, null).

3.4.4. *Effect of Folinic acid on Ternary Complex Formation*

To determine whether the amount of folates in the media was limiting and responsible for the difference in ternary complex formation between the two cell lines, I examined the effect of adding folinic acid to the medium of the cells. I compared the ternary complex formation of the two lines in the absence and presence of 2.5×10^{-5}M folinic acid.

FIGURE 3.3. Dose-dependent effect of 5-FUdR on the levels of TS (A), p53 (B), p21 (C) and actin (D) in HCT-116 wild-type (WT), $p53^{-/-}$ (null) and p53-transfected $p53^{-/-}$ (Adp53) cells

Cells were treated with various concentrations of 5-FUdR for 24 h and analyzed by western blotting using anti-TS antibody. Each lane contains 10 μg of protein. Indicated are the positions for the free TS form (35 kD) and the ternary complex form (~37 kD). Other details are in *Materials and Methods*.


A)
FUdR (mol/L)
0
10-9
10-8
10-7
10-6
10-5
WT
Null
Adp53
Complex
Free TS
B)
FUdR (mol/L)
0
10-9
10-8
10-7
10-6
10-5
WT
Null
Adp53
C)
FUdR (mol/L)
0
10-9
10-8
10-7
10-6
10-5
WT
Null
Adp53
D)
FUdR (mol/L)
0
10-9
10-8
10-7
10-6
10-5
WT
Null
Adp53

The only difference observed in the presence of folinic acid was a slight mobility shift (unexplained) in the ternary complex form of TS (Fig. 3.4). The relative levels of TS in the free and ternary complex forms remained the same for both lines and thus the inclusion of folinic acid had no effect on the difference in ternary complex formation between the two cell lines.

3.4.5. Effect of p53 Expression on Ternary Complex Formation

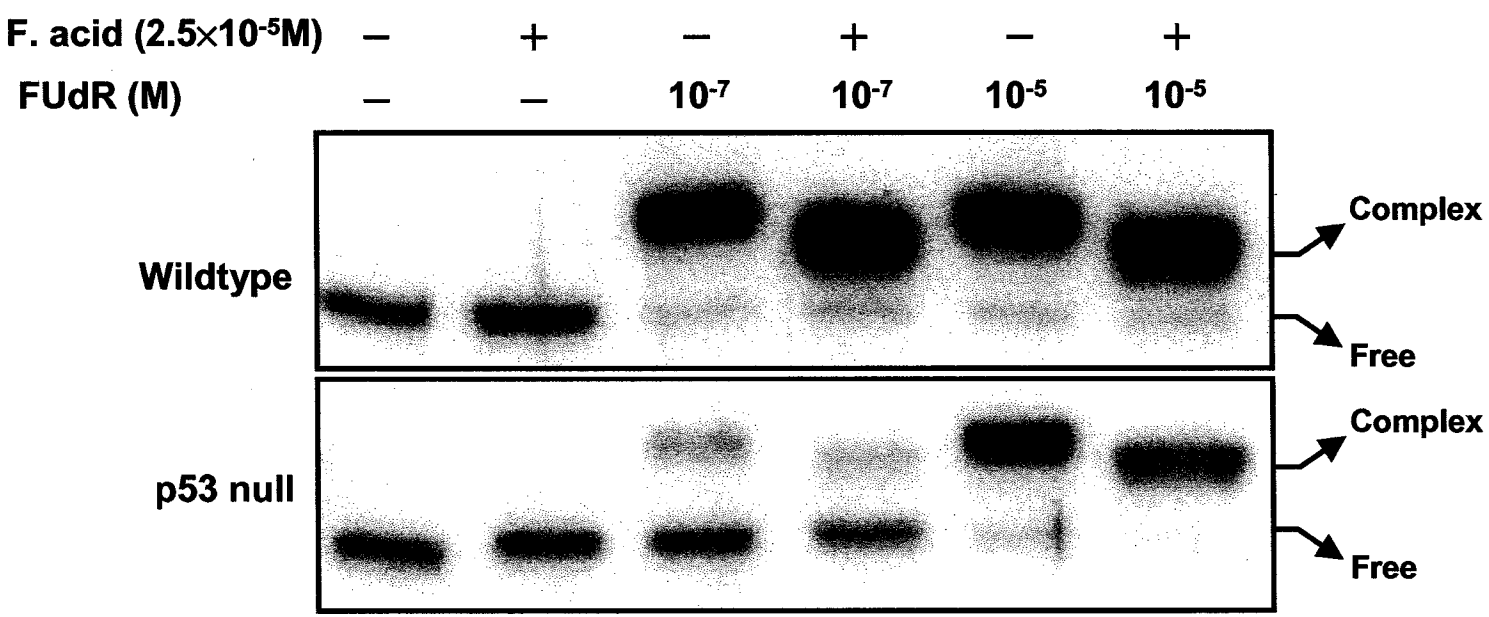
To determine whether the difference in TS ternary complex formation between HCT-116 p53wt and p53 null cell lines was due to the difference in their p53 status, we used p53 null lines (prepared by Brice Le Francois in our lab) infected with an adenovirus expressing wildtype p53. We confirmed that the infected cells were expressing p53 by detecting high levels of p53 in infected cells (Fig. 3.3B panel Adp53) compared to low basal levels in wt cells (Fig. 3.3B panel wt, lane 1). The levels of p53 increased in wt cells as a function of 5-FUdR concentration due to DNA damage (Fig. 3.3B panel wt, lanes 4-6). p53 null cells showed no p53 protein as expected nor any increase with 5-FUdR (Fig. 3.3B panel null). To confirm that this infected p53 was indeed functional we measured levels of p21, a known target of p53 regulation. We found comparable levels of p21 in p53wt (Fig. 3.3C panel wt) and p53null infected cells (Fig. 3.3C panel Adp53). The exposure chosen shows no detectable p21 levels in the p53 null cell line (Fig. 3.3C panel null). However, longer exposures confirmed low basal levels of p21in p53 null cells (data not shown). The next step was to determine whether restoring wild-type p53 to these cells could restore their ability to form the TS ternary complex at levels similar to the wt cell line. p53null infected cells (Fig. 3.3A panel Adp53) were indistinguishable from p53 null cells; both failed to form TS ternary complex as efficiently as wt cells at 5-FUdR doses of 10^{-9}M to 10^{-6}M. The last set of panels (Fig. 3.3D) shows levels of actin as a loading control. This group of experiments provides strong evidence that p53 status of HCT-116 cells is not the sole difference between the two cell lines affecting their ability to form ternary complex.

3.4.6. Sensitivity to 5-FUdR of HCT-116 wt Cells is an Unstable Phenotype

During the course of my experiments, I observed that some cultures of HCT-116 p53 wt cells displayed 5-FUdR resistance comparable to my p53 null cells (data not shown).

FIGURE 3.4. Effect of folinic acid and 5-FUdR treatment on TS levels in HCT-116 wild-type and $p53^{-/-}$ (null) cells

Cells were not treated or treated with folinic acid and/or 5-FUdR for 24 h and analyzed by western blotting using anti-TS antibody. Each lane contains 10 μg of protein. Indicated are the positions for the free TS form (35 kD) and the ternary complex form (~37 kD). Other details are in *Materials and Methods*.


F. acid (2.5×10-5M) – + – + – +
FUdR (M) – – 10-7 10-7 10-5 10-5
Wildtype
Complex
Free
p53 null
Complex
Free

I explored this systematically using a single stock of cells. These were established in culture and monitored for 5-FUdR sensitivity over 10-14 passages in culture (~6 weeks). At this point they demonstrated resistance to 5-FUdR comparable to p53 null cells. When the ternary complex forming ability of these cultures on exposure to 5-FUdR was tested, I found that passage 13 cells were unable to form TS ternary complex at 10^{-7} M 5-FUdR, as compared to passage 4 cells (Fig. 3.5). Since 5-FUdR resistance appeared to be independent of p53 and was an unstable phenotypic property of a single cell line, I decided to focus on trying to understand the mechanism underlying the conversion of early passage p53 wt 5-FUdR-*sensitive* cells, which I will refer to as WT cultures, to late passage p53 wt 5-FUdR-*resistant* cells, which I will refer to as WTR cultures.

3.4.7. *Two-Dimension Gel Analysis of TS protein in HCT-116 WT and HCT-116 WTR Cells*

It was previously reported that the HCT-116 cell line expressed a variant (more basic) form of TS protein in addition to the normal form. This variant form was characterized and shown to have reduced binding affinity for TS substrates (61). I examined WT and WTR cells to determine if they still contained both structural forms of the TS protein and whether any gross rearrangment of the TS protein had taken place in the conversion of WT cells to WTR phenotype. I performed two-dimensional gel analysis on extracts of WT and WTR cells and detected TS through western blot analysis. I found that there were no obvious differences in the TS protein present in the two cell cultures (Fig. 3.6). I expected to see two spots representing the normal and more basic forms of the TS protein, however, 3 spots were detected for TS in both cultures. The nature of the third spot is unknown.

3.4.8. *Ability to Take up ^{3}H- TdR and Incorporate it Into DNA in WT and WTR cells*

^{3}H-TdR uptake and incorporation into DNA over a period ranging from 4 to 24 h is commonly used as a measure of DNA synthesis. Some of the steps preceeding incorporation of TdR into DNA are similar to those when 5-FUdR is added to cells, *viz*, transport into the cell and phosphorylation by thymidine kinase to the nucleotide (TMP). I next examined WT and WTR cells for differences in their ability to take up and incorporate TdR into DNA. Since the two cultures did not have detectable differences in growth rate (data not shown),

FIGURE 3.5. Effect of high passage number on TS-ternary complex formation in HCT-116 wildtype and p53 null cells after 5-FUdR treatment

Cells were either not treated (0) or treated (10^{-7} or 10^{-5} mol/L) with 5-FUdR for 24 h and analyzed by western blotting using anti-TS antibody. Each lane contains 10 μg of protein. Indicated are the positions for the free TS form (35 kD) and the ternary complex form (~37 kD). Other details are in *Materials and Methods*.

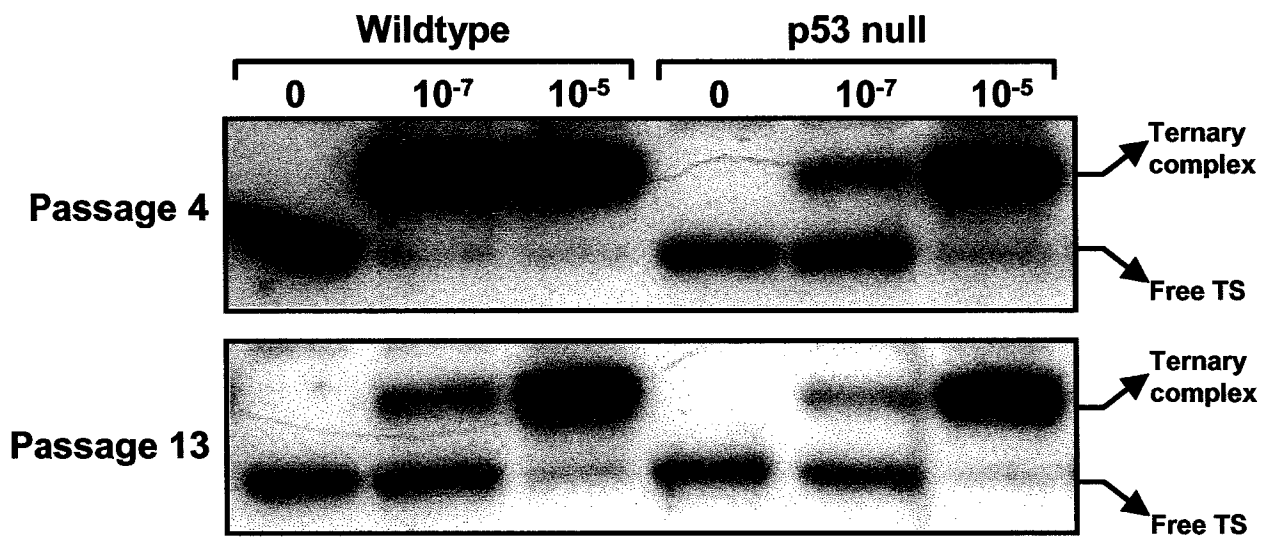
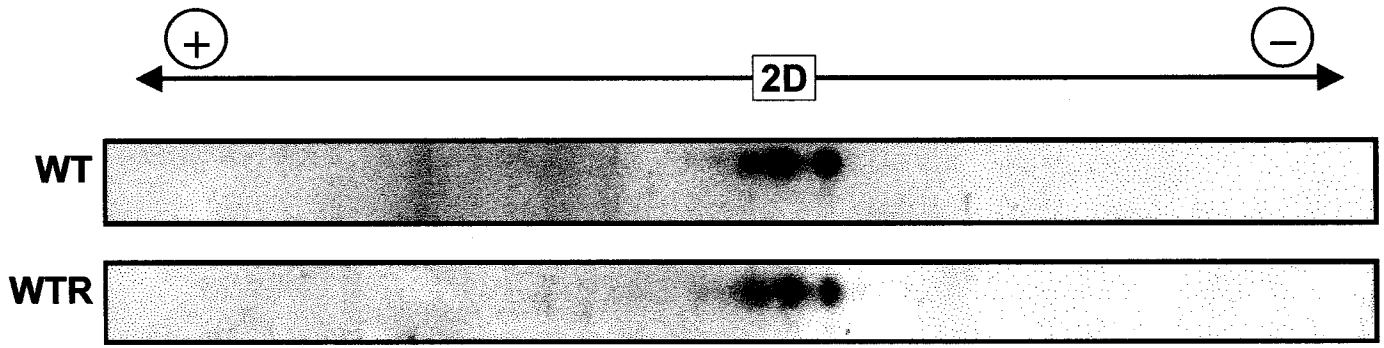

Wildtype
p53 null
0
10-7
10-5
0
10-7
10-5
Passage 4
Ternary complex
Free TS
Passage 13
Ternary complex
Free TS

FIGURE 3.6. 2D gel electrophoresis followed by western blot analysis for TS in WT and WTR cells

For 2D gel analysis, whole cell extract (300 μg protein) was analyzed by isoelectric focusing on pH 3-10 IPG strips followed by 10% SDS-PAGE. The gel was then analysed for western blot analysis using anti-TS antibody. Anode (+) and cathode (-) represent the pH 3 and pH 10 side of the gel, respectively. Other details are in *Materials and Methods*.


+
2D
−
WT
WTR

I anticipated that any differences between the two cultures produced in this assay would be due to differences in their ability to uptake and or metabolise the ^{3}H-TdR. Following exposure to ^{3}H-TdR for 18 hr, I observed a significantly greater amount of ^{3}H-TdR uptake and incorporation into DNA for WT cells than WTR cells (Fig. 3.7). These results utilizing ^{3}H-TdR paralleled resistance to formation of ternary complex by 5-FUdR and suggested that there were differences in either uptake or metabolism (TK or phosphatase activity) of the nucleoside, between the two cell cultures.

3.4.9. HAT Selection of WT and WTR cells

Another method to explore differences between WT and WTR cultures was to compare their viability in **h**ypoxanthine-**a**minopterin-**t**hymidine (HAT) selection media. Aminopterin inhibits DHFR, leading to folate depletion (Fig. 3.17) . This leads to depletion of purine and dTMP levels required for DNA synthesis and cell proliferation. Hypoxanthine and thymidine in HAT replenish the purine and dTMP levels, respectively, *via* HPRT and TK salvage pathways. I performed this assay using two concentrations of thymidine in the HAT media, 10^{-7}M and 10^{-5}M thymidine. I chose these doses based on earlier observations that indicated that, at 10^{-5}M 5-FUdR, both cultures had most of its TS in the ternary complex, but at 10^{-7}M 5-FUdR, the WT culture had most of its TS in the ternary complex while the WTR culture had most of its TS in the free form (Fig. 3.3A). When the two cultures were exposed to HA selection medium (no thymidine), I observed no colony forming units (CFUs) for WTR cultures and a few tiny colonies for WT culture (Fig. 3.8). These results indicated that the amount of thymidine present in the fetal serum in standard medium was insufficient to rescue the cells from the cytotoxicity of aminopterin. When the cultures were exposed to HAT selection media containing 10^{-5}M thymidine, both cultures were completely rescued from aminopterin toxicity; I observed no difference in the number of CFUs between the two cultures and this number was comparable to number of CFUs in control plates (Fig. 3.8). When 10^{-7}M thymidine was used in the HAT selection media, I observed a statistically significant difference ($p< 0.05$) in the number of CFUs between the two cultures as there was 80% of CFUs compared to control plates for the WT culture, and 50% of CFUs compared to control plates for the WTR culture (Fig. 3.8). This assay therefore provided evidence that

FIGURE 3.7. Incorporation of [^{3}H]-TdR into DNA in HCT-116 WT and WTR cells

Cells were exposed to [^{3}H]-TdR for 18 h and analyzed as described in *Materials and Methods*. Error bars represent mean ± SD of 3 replicates.

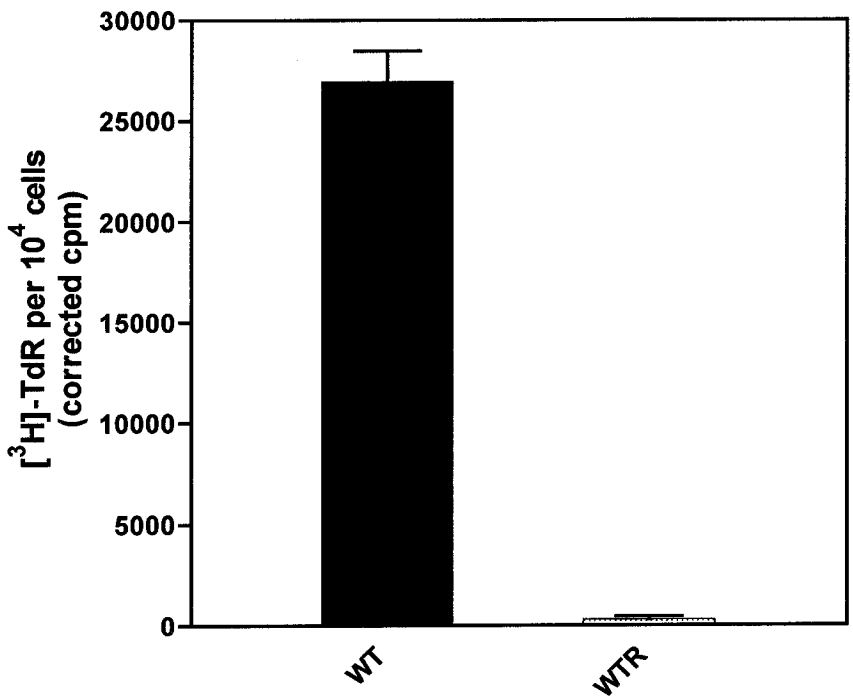
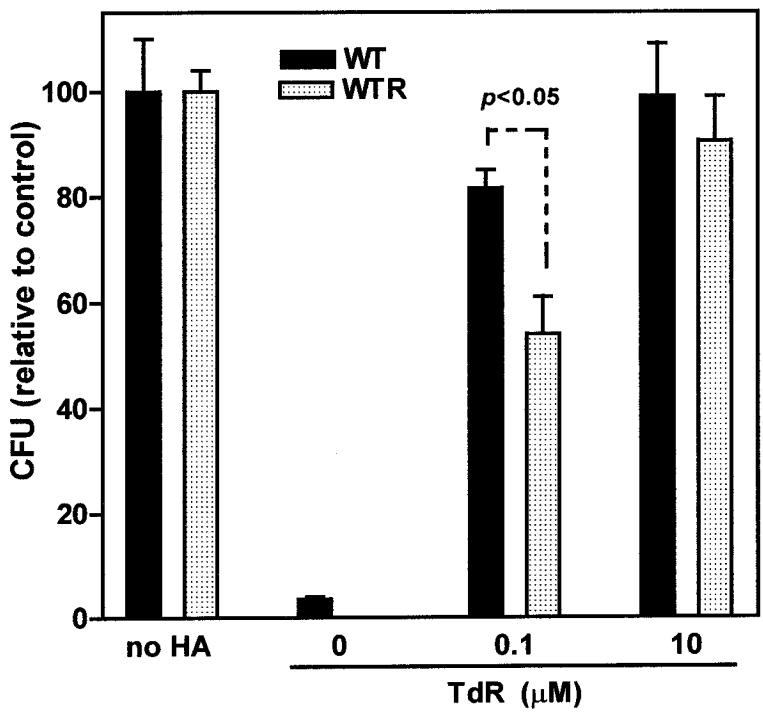

30000
25000
20000
15000
10000
5000
0
[3H]-TdR per 10^4 cells
(corrected cpm)
WT
WTR

FIGURE 3.8. Effect of HAT-treatment on colony-formation by HCT-116 WT and WTR cells

Cells were either not treated (no HA) or treated with HA containing 0, 0.1 or 10 μM of TdR. After 10 d, colonies were counted. Reported are colony forming units (CFU) relative to the control (no HA). Error bars represent mean ± SD of 3 replicates. Other details are in *Materials and Methods*.


WT
WTR
p<0.05
CFU (relative to control)
100
80
60
40
20
0
no HA
0
0.1
10
TdR (μM)

thymidine was either not efficiently taken up or phosphorylated by TK to its active TMP metabolite form, in the WTR culture.

3.4.10. Thymidine Kinase (TK)

To explore whether a deficiency in TK activity was responsible for the observed phenotype, I compared TK levels in the WT and WTR cells. TK enzymatic activity was measured using an *in vitro* biochemical assay. Based upon a single experiment, there appeared to be no difference in TK activity in extracts of both cell types (Fig. 3.9A). Quantitative RT-PCR analysis of TK mRNA also showed no significant differences between the two cultures (Fig. 3.9B).

3.4.11. Cellular Uptake of ^{3}H-Thymidine (^{3}H-TdR)

Given the lack of evidence that differences in TK levels between WT and WTR cells might explain the phenotypic differences observed, I next compared cellular uptake of thymidine between the two cultures. When growing cells are exposed to ^{3}H-TdR, the nucleoside is first taken up into the cell *via* a nucleoside transporter, then it is phosphorylated by thymidine kinase, (trapping ^{3}H-thymidine monophosphate (TMP) inside the cell), then it is further phosphorylated to TTP which can then be incorporated into DNA. Very short labeling times will favour cell uptake and phosphorylation over incorporation into DNA. To distinguish uptake and phosphorylation from incorporation into DNA, I exposed cells to ^{3}H-TdR for short periods of time, 10 and 20 min. Results of Fig. 3.10 show that, at these very early time periods, the amount of ^{3}H-TdR associated with WT cells was much higher than with WTR cells. At the 10 min time point, WT cells contained approximately 3 times as much radioactivity (statistically significant, $p< 0.05$) as WTR cells and at 20 min time point, WT cells contained approximately 20 times as much radioactivity as WTR cells (Fig. 3.10). The results of this and the TK experiment provide strong evidence that the phenotypic differences between WT and WTR cells are due to some defect in uptake of the nucleoside into WTR cells.

FIGURE 3.9. Level of thymidine kinase (TK) enzymatic activity (A) and mRNA (B) in HCT-116 WT and WTR cells

(A) Each TK *in vitro* assay mixture consisted of 1× assay buffer and cellular extract containing TS enzyme. The reaction mix was incubated at 37°C and stopped by the addition of excess cold thymidine and heating at 95°C for 3 min. Binding buffer and anion exchange resin in binding buffer were added and the tube was rotated for 1 hr. The reaction tube was then centrifuged and the supernatant was removed. The resin was washed and then eluted with 0.2 M NaCl.

(B) PCR reactions were performed on a cDNA library from each HCT 116 line, targeting a 204 bp fragment of TK cDNA, a 231 bp of ENT1 cDNA, a 366bp fragment of ENT2 cDNA and an internal reference 718 bp fragment of G6PD mRNA. Each run included a standard curve obtained by 6 serial dilutions of the sensitive cDNA library used as a standard, a no template control and the two samples being quantified. PCR specificity was confirmed by melting curve analysis. The relative values of the target genes were extrapolated from the standard curve and then normalized to the internal reference (G6PD).

See *Materials and Methods* for further details. Error bars represent mean ± SD of 3 replicates.

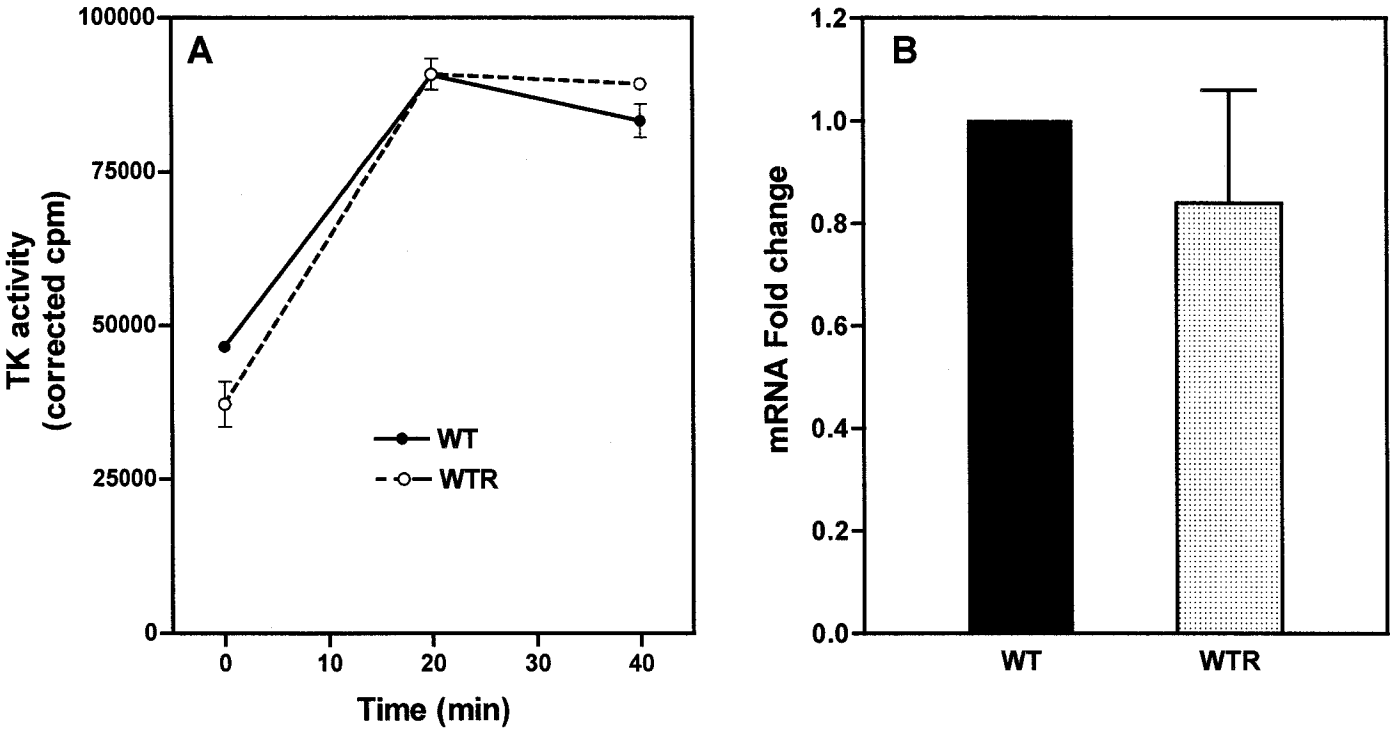
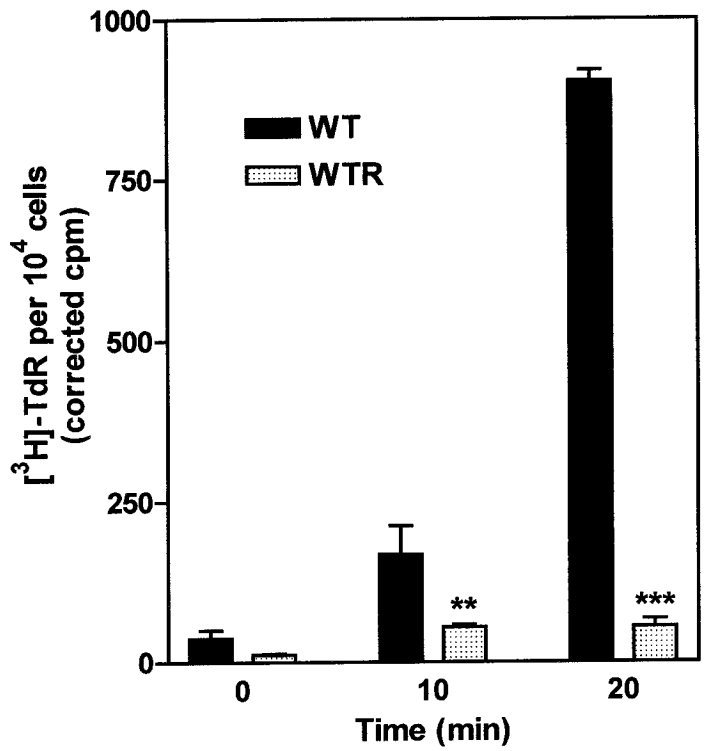

A
100000
75000
50000
25000
0
TK activity
(corrected cpm)
0
10
20
30
40
Time (min)
WT
WTR
B
1.2
1.0
0.8
0.6
0.4
0.2
0.0
mRNA Fold change
WT
WTR

FIGURE 3.10. Uptake of [^{3}H]-TdR in HCT-116 WT and WTR cells

Cells were exposed to [^{3}H]-TdR for 0, 10 or 20 min and analyzed as described in *Materials and Methods*. Error bars represent mean ± SD of 3 replicates. Asterisks (**p=0.015, ***p<0.0001) represent unpaired 2-tailed t tests (WT vs. WTR).


1000
750
500
250
0
WT
WTR
**
***
0
10
20
Time (min)
[3H]-TdR per 10^4 cells
(corrected cpm)

3.4.12. *Comparison of 5-FUdR and 5-FU*

5-FU (the nucleobase) is used more widely than 5-FUdR (the nucleoside) in clinical medicine. Although they may share a common transporter to enter a cell, their metabolism to reach 5-FdUMP is very different (see Fig. 3.17). Earlier workers have also previously tested 5-FU on HCT-116 cells (129). My results showed that p53 WT cells were sensitive to 5-FUdR while p53 null cells were resistant. In these experiments, I also utilized nullR cells which are p53 null cells of later passage number. When I compared the 5-FUdR response of WTR and nullR (p53 null, late passage) cultures, I found that they both behaved as WTR cells, i.e., resistant to 5-FUdR (Fig. 3.11A). When I tested 5-FU at the same doses, I found that p53 null cells were relatively resistant to 5-FU at doses between 10^{-7} to 10^{-6}M compared to the p53 wt cell line (Fig. 3.11B). These results support the trend cited in the earlier study (129), although results of that study reported a far greater extent of 5-FU resistance for the p53 null cell line. Furthermore, nullR (p53 null, later passage) cultures were even more resistant than null cultures at 5-FU doses between 10^{-7} to 10^{-6}M. WTR cultures were found to be more resistant to 5-FU compared to WT cultures. When examined for their ability to form TS ternary complex after exposure to 5-FU or 5-FUdR, WTR, null and nullR cultures all showed similar results that were different from WT cells (Fig. 3.12). At 10^{-7}M, most of the TS protein was in the ternary complex in the WT culture while most of the TS protein was in the free form in the WTR, null and nullR cultures (Fig. 3.12). At 10^{-6}M 5-FU (and higher doses) and at 10^{-5}M 5-FUdR, the amount of TS in ternary complex was similar for all cell lines. It is interesting to note that, at 10^{-6} M 5-FU, there were significant differences among lines in their response to 5-FU (Fig. 3.11B) but little difference in the relative amount of TS in ternary complex.

3.4.13. *Comparison of ATCC HCT-116 Cell Line with WT and WTR cultures*

Since the results of my HCT-116 p53wt cell line showed a significantly reduced sensitivity to 5-FU than previously reported for this cell line (129), I compared my stock of HCT-116 (WT) cell line with the same cell line from ATCC. I tested ATCC HCT-116 (early passage) cell line and found that its response to 5-FU was similar to my WT culture (Fig. 3.11B). When I tested the response of ATCC HCT-116 cells to 5-FUdR, I found that it

behaved exactly the same as my WTR culture, displaying relative resistance to 5-FUdR compared to my WT culture (Fig. 3.11A). In assays measuring cellular uptake of

FIGURE 3.11. Dose-dependent effect of 5-FUdR (A) and 5-FU (B) on growth of HCT-116 WT, WTR, null, nullR and ATCC cells

Cells were treated with various concentrations of 5-FUdR (***A***) or 5-FU (***B***) for 8 d. The cells attached to the plates were harvested and counted using Coulter counter. The counts were corrected for the untreated cells (0 M FUdR). Error bars represent mean ± SD of 3 replicates. Other details are in *Materials and Methods*. Asterisks *** represent $p<0.001$ and * represents $p<0.05$ of one-way ANOVA.

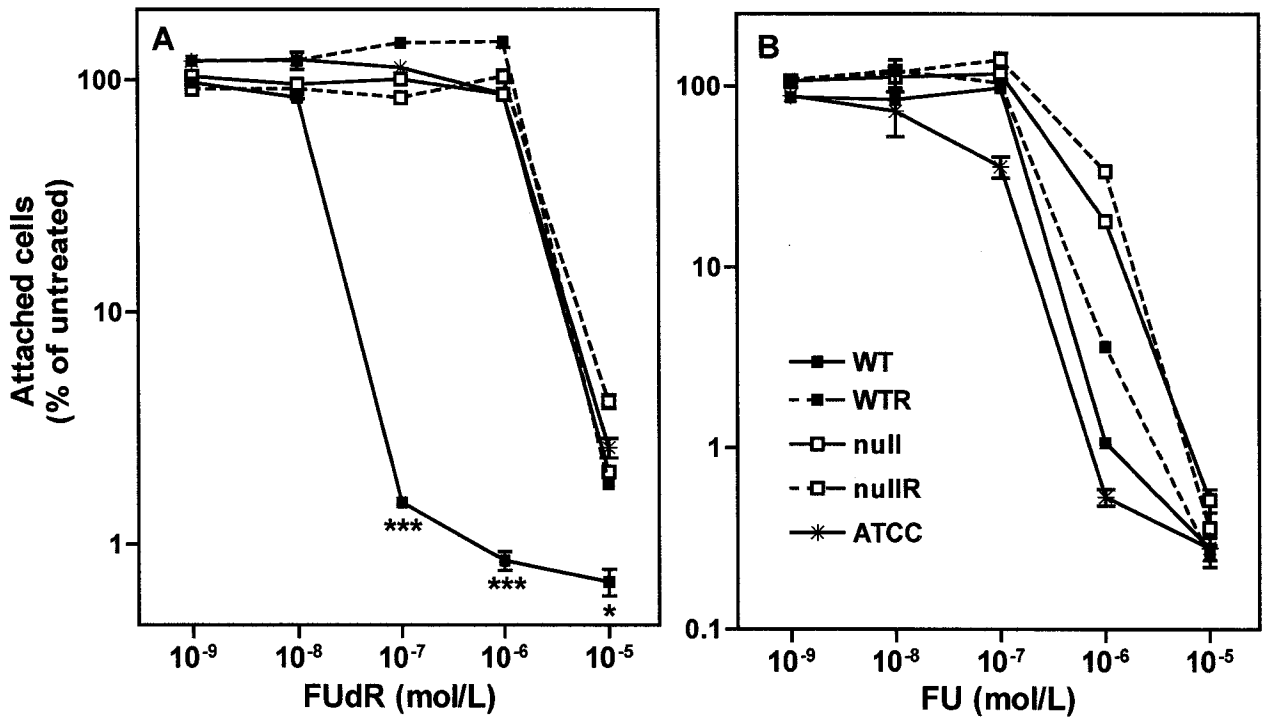
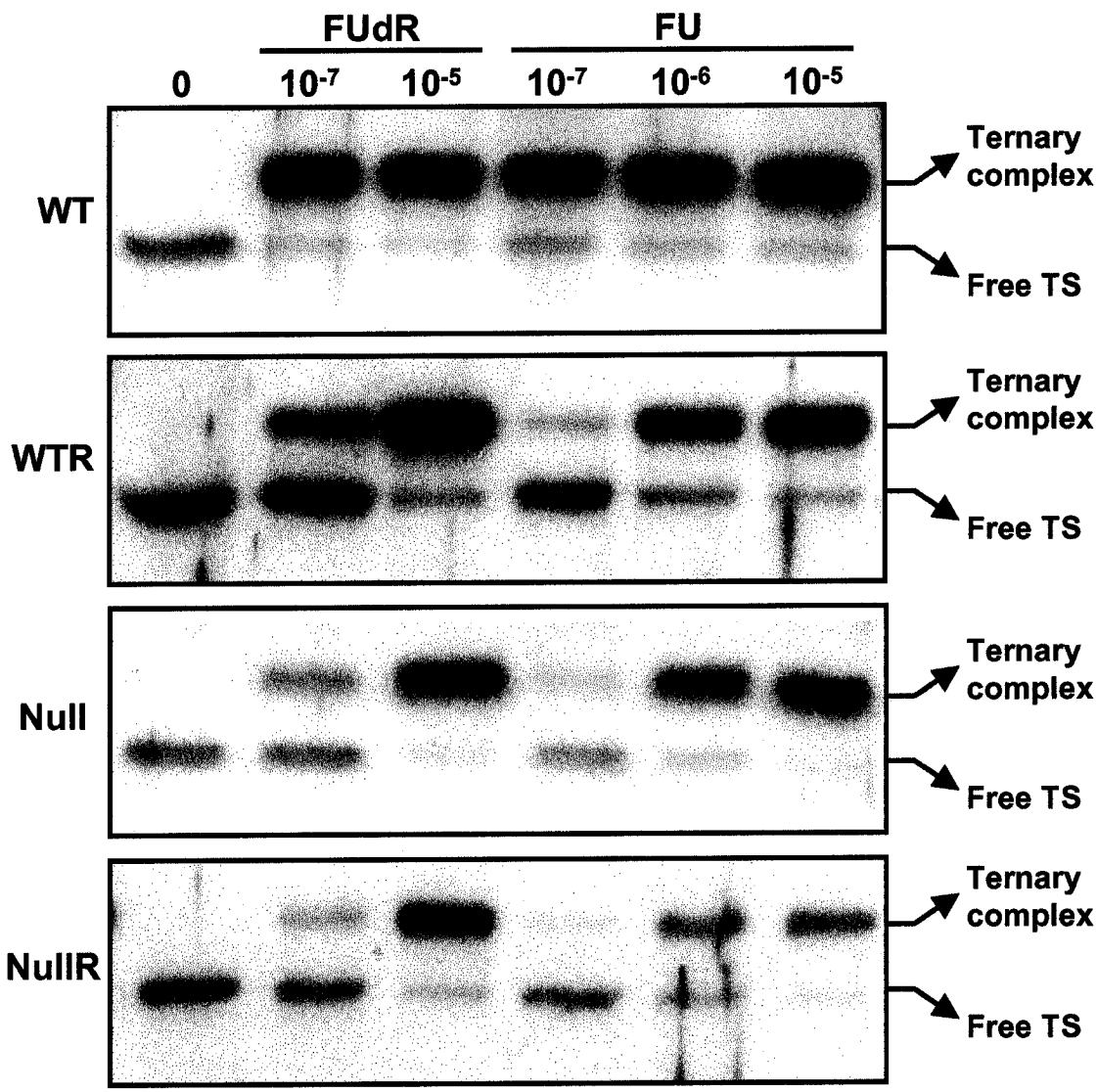

A
B
Attached cells
(% of untreated)
100
10
1
0.1
***
***
*
WT
WTR
null
nullR
ATCC
10-9
10-8
10-7
10-6
10-5
FUdR (mol/L)
FU (mol/L)

FIGURE 3.12. Effect of 5-FUdR and 5-FU on TS-ternary complex formation in HCT-116 WT, WTR, null and nullR cells

Cells were either not treated (0) or treated (10^{-7} or 10^{-5} mol/L) with 5-FUdR or 5-FU for 24 h and analyzed by western blotting using anti-TS antibody. Each lane contains 10 μg of protein. Indicated are the positions for the free TS form (35 kD) and the ternary complex form (~37 kD). Other details are in *Materials and Methods*.


FUdR
FU
0
10-7
10-5
10-7
10-6
10-5
WT
WTR
Null
NullR
Ternary complex
Free TS

^{3}H-thymidine and incorporation into DNA, I found that there was less uptake and incorporation of the radioisotope in the ATCC HCT-116 cells than my WT culture (data not shown). Thus, ATCC HCT-116 cells behave like WTR cells in nucleoside assays and like WT cells in nucleobase assays.

3.4.14. Effect of hMLH-1 on Conversion of Culture from WT to WTR Phenotype

Results thus far strongly suggest a phenotypic instability in HCT-116 cells with respect to nucleoside or nucleobase uptake or metabolism. HCT-116 is an HNPCC cell line due to a defect in the mismatch repair gene, hMLH-1 (located on chromosome 3). I investigated whether this gene defect had any effect on the conversion of WT cells to the WTR phenotype. To test this, I used another HCT-116 derived cell line, HCT-116+Chr3, which was previously characterized and shown to contain a functional transfected copy of chromosome 3 (135). When I tested an early culture of HCT-116+Chr3, I found that it showed comparable results to WT cells (Fig. 3.13) in terms of its ability to take up ^{3}H-TdR and incorporate it into DNA. However, after maintaining HCT-116+Chr3 for several passages, I found that late passage cultures were no longer able to uptake ^{3}H-TdR as efficiently as earlier passages cells and eventually they too developed a WTR (Fig. 3.13) phenotype. These results suggest that a defect in genes on chromosome 3 responsible for the HNPCC phenotype of HCT-116 cell line was not involved in the instability governing the conversion of WT cultures to the WTR phenotype.

3.4.15. Effect of 5-Aza-2-deoxycytidine (5-AdCd)

Methylation of genes is a known mechanism for changing gene expression. To explore whether such events were responsible for the observed phenotypic instability, I tested whether I could revert this phenotype by treatment of WTR cultures with 5-AdCd. My first step was to determine the sensitivity of the cells to 5-AdCd to allow choice of a suitable dose and length of exposure for pre-treatment of the cells. The experimental results indicated that about 80-90% of cells remained attached for both cultures after 24h treatment with doses of 10^{-9} to 10^{-5}M 5AdCd (Fig. 3.14A) and 60-85% after 48h treatment. I decided to use a 24 h pre-treatment period. When I tested the ability of 5-AdCd pre-treated WTR cells to take up ^{3}H-TdR and incorporate it into DNA, I found no difference between

FIGURE 3.13. Incorporation of [^{3}H]-TdR into DNA in early and late passages of chromosome 3-transfected HCT-116 cells as compared to WT and WTR cultures

Cells were exposed to [^{3}H]-TdR for 18 h and analyzed as described in *Materials and Methods*. Error bars represent mean ± SD of 3 replicates.

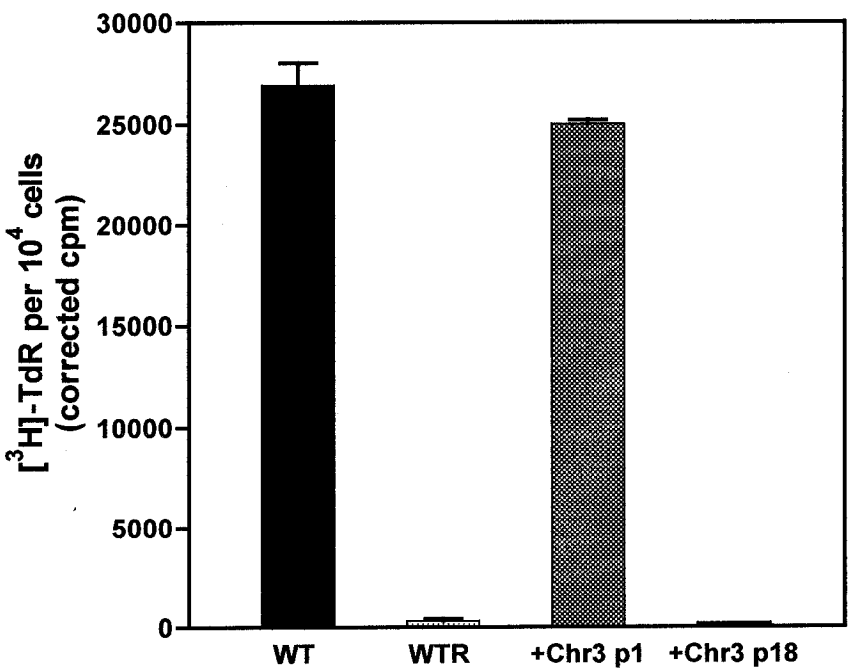
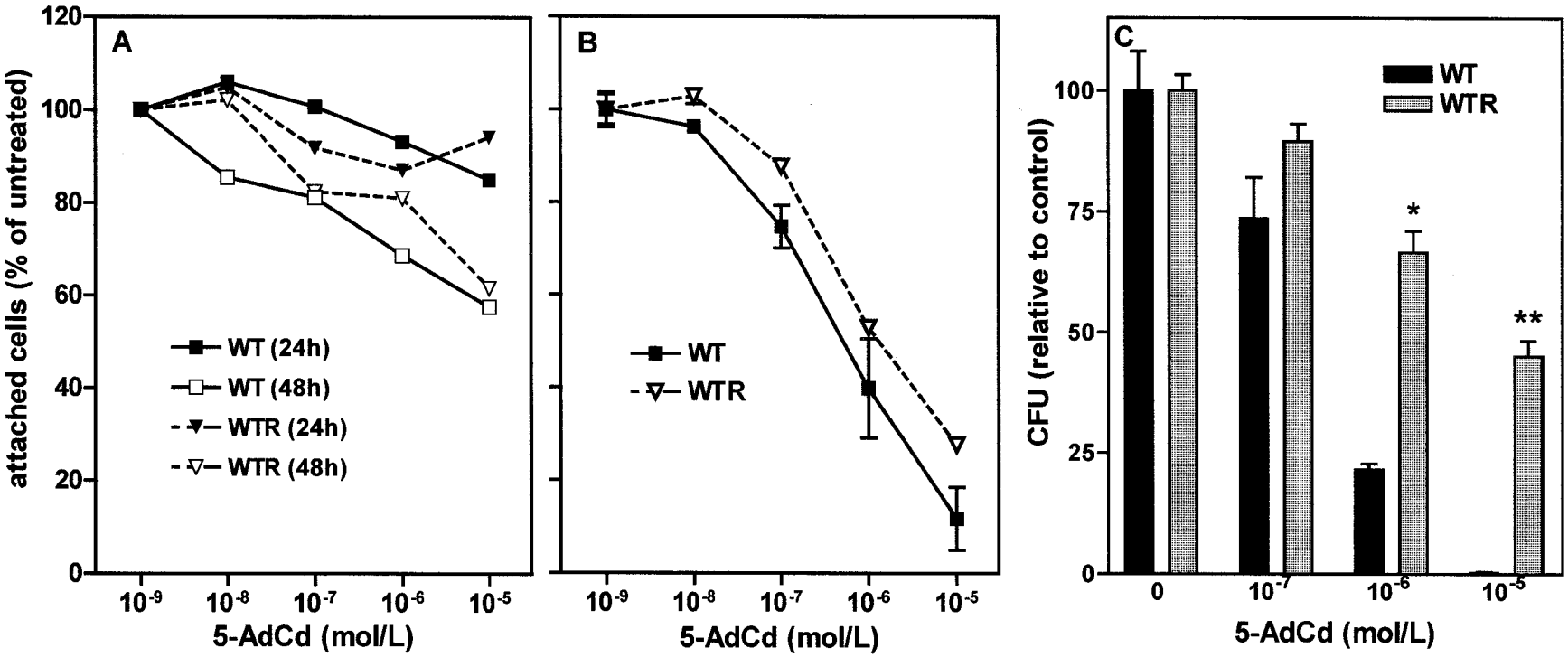

30000
25000
20000
15000
10000
5000
0
[3H]-TdR per 10^4 cells
(corrected cpm)
WT
WTR
+Chr3 p1
+Chr3 p18

FIGURE 3.14. Effect of 5-AdCd-treatment on growth of HCT-116 WT and WTR cells

A. Effect of 24 or 48 h treatment with 5-AdCd. ***B.*** Effect of 24 h 5-AdCd-treatment followed by 3 d recovery. ***C.*** Colony-forming ability of cells in B. In A and B, cells attached to plates were counted using Coulter counter and the counts were corrected for the untreated cells (0 M 5-AdCd). In C, colony forming units (CFU) are reported relative to the untreated cells. Error bars represent mean ± SD of 2 replicates. Asterisks (*p=0.01, **p=0.008) represent unpaired 2-tailed t tests (WT vs. WTR). Other details are in *Materials and Methods*.


A
B
C
attached cells (% of untreated)
120
100
80
60
40
20
0
WT (24h)
WT (48h)
WTR (24h)
WTR (48h)
WT
WTR
10-9
10-8
10-7
10-6
10-5
5-AdCd (mol/L)
CFU (relative to control)
100
75
50
25
0
*
**
WT
WTR
0
10-7
10-6
10-5
5-AdCd (mol/L)

pre-treated and untreated cells (data not shown). Thus, there is no indication that 5-AdCd pre-treatment of WTR cultures reverts their resistant phenotype. This provides no evidence to support the hypothesis that methylation was involved in the observed phenotypic instability.

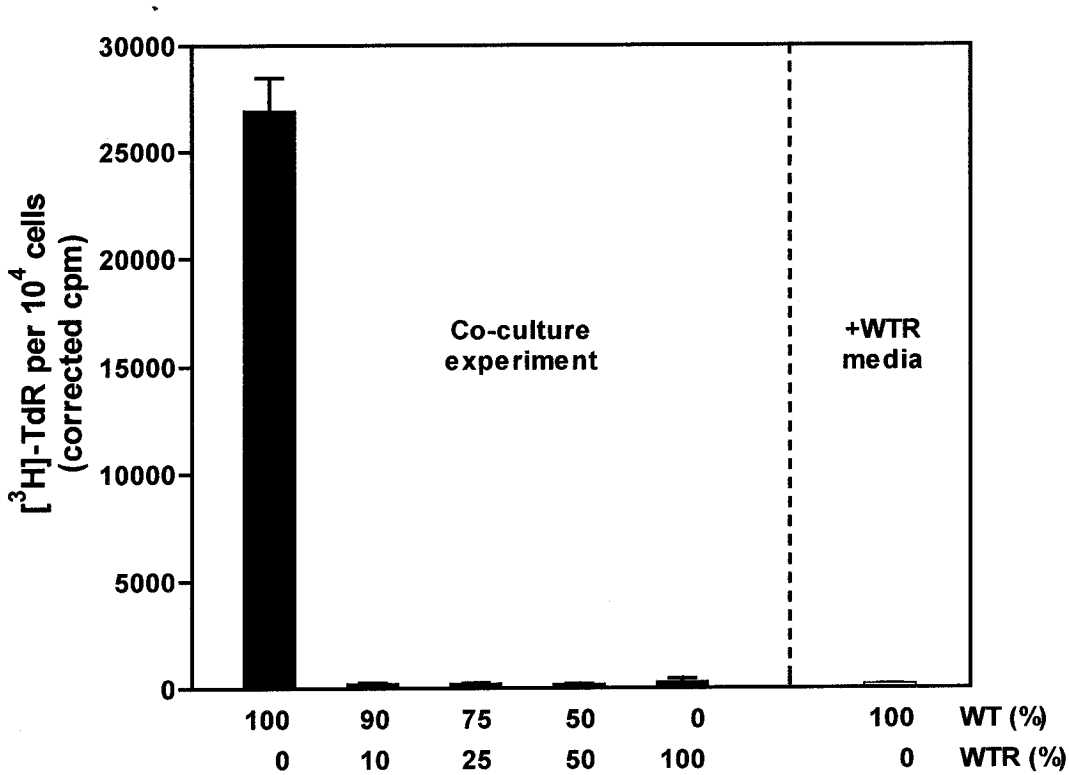
Since 5-AdCd is a nucleoside and my results suggested that nucleoside uptake was different between WT and WTR cultures I decided to examine in more detail the response of the two cell lines to this drug. The number of cells remaining after 3 days following a 24h 5-AdCd pre-treatment, was lower for the WT cells than WTR cells (Fig. 3. 14B). However, this difference was slight, so I switched to a more reliable assay for testing cytotoxicity of drugs, *viz.*, the colony forming unit assay. I observed a statistically significant difference in the number of CFUs between the two cultures at doses of 10^{-7}, 10^{-6} and 10^{-5}M 5-AdCd (Fig. 3.14C). I obtained 75, 22 and 0.5 % respectively of CFUs (compared to control plates) for WT cells and 90, 67 and 45% of CFUs (compared to control plates) for WTR cells. Results from this assay clearly showed that the WTR culture was cross resistant to 5-AdCd and suggested that a common pathway was affected.

3.4.16. Ability of WTR Cells to In Trans Induce Phenotypic Instability in WT Cells

I next wanted to investigate whether this conversion of WT to WTR phenotype might be due to a stable factor released from WTR cells that could alter the phenotype of WT cells. I plated WT and WTR cells in different ratios and tested their ability to take up ^{3}H-TdR and incorporate it into DNA. I found that, at all ratios tested, the mixed cultures showed a WTR phenotype (Fig. 3.15). Thus when WTR cells were used at as low as 10% of the total number of plated cells, they were capable of converting the remaining 90% of WT cells to a WTR phenotype. To further investigate whether this "activity" from the WTR cells was released and stable enough to promote phenotypic conversion, I incubated WT cells with conditioned medium of WTR cells and found that this was sufficient to convert them to a WTR phenotype (Fig. 3.15). In a further experiment, I exposed WT cells to the conditioned medium of WTR cells for 24h and then replaced it with fresh medium; these pre-exposed cells showed similar low levels of ^{3}H-TdR uptake and incorporation into DNA as WTR control cells (data not shown). Thus, it appears that a stable factor is generated by WTR

FIGURE 3.15. Effects of co-culturing WT and WTR cells and exposure of WT cells to conditioned media of WTR cells on incorporation of [^{3}H]-TdR into DNA

For co-culturing experiment, WT and WTR cells were mixed at the indicated ratios and exposed to [^{3}H]-TdR for 18 h and analyzed as described in *Materials and Methods*. For supplementation experiment, WT cells were cultured in the media of WTR cells for 48 h and exposed to [^{3}H]-TdR for 18 h analysis. Error bars represent mean ± SD of 3 replicates.


30000
25000
20000
15000
10000
5000
0
[3H]-TdR per 10^4 cells (corrected cpm)
Co-culture experiment
+WTR media
100 90 75 50 0 100 WT (%)
0 10 25 50 100 0 WTR (%)

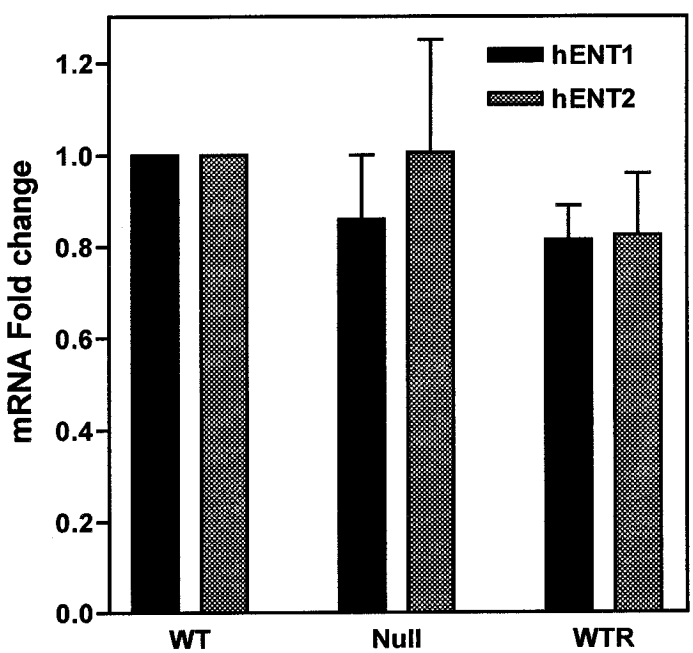
cells and released into the culture medium that is capable of causing phenotypic change in WT cells.

3.4.17. Levels of h-ENT1 and h-ENT2 mRNA

Four human nucleoside transporters, h-ENT1, h-ENT2, h-ENT3 and h-ENT4 have been recently identified (137). However, only the first two, h-ENT1 and h-ENT2 have been recently characterized (138). I prepared RNA from WT and WTR cells and assessed levels of mRNA for the two characterized transporters by quantitative RT-PCR. No significant differences in their mRNA levels were seen (Fig. 3.16).

FIGURE 3.16. Levels of h-ENT1 and h-ENT2 in HCT-116 WT, null and WTR cells

See *Materials and Methods* for details. Error bars represent mean ± SD of 3 replicates.


hENT1
hENT2
mRNA Fold change
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT
Null
WTR

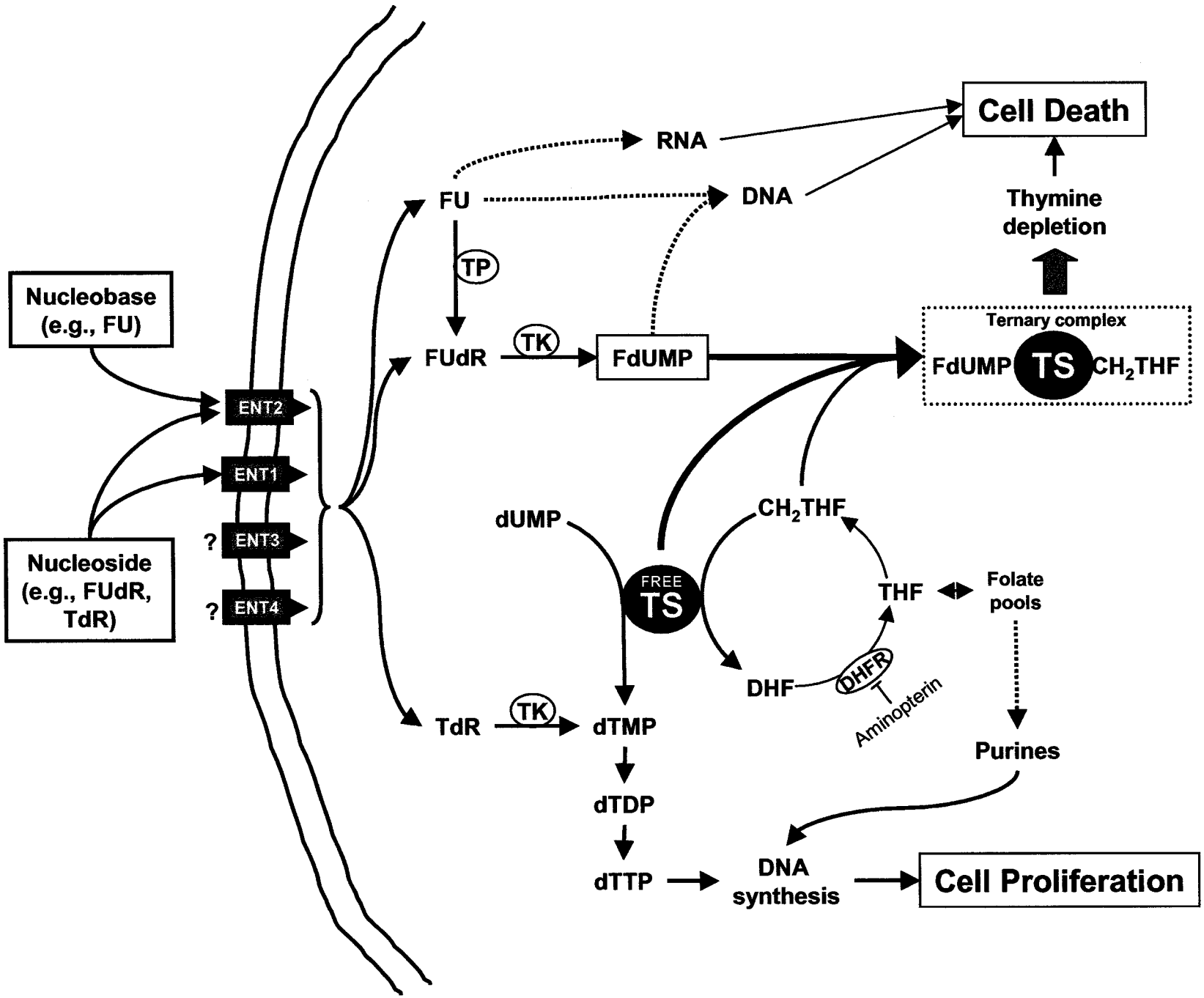
3.5. DISCUSSION

Two forms of TS protein are observed by western blot analysis in cancer cells after treatment with 5-FU or 5-FUdR chemotherapeutic drugs (Fig. 3.17). One form is the free TS protein that may be capable of interacting with its natural substrates (CH_2THF, dUMP) and producing dTMP for DNA synthesis and cell proliferation. The other form is a TS protein covalently bound to CH_2THF and FdUMP (a key metabolite of 5-FU and 5-FUdR) forming a stable ternary complex (14,15). Continuous formation of this inactivated TS protein complex leads to depletion of the free TS protein and hence leads to depletion of thymine-containing nucleotides and eventually cell death (16). Thus, the presence of this complex in cancer cells is a good indication of the extent of TS-specific cytotoxicity caused by 5-FU or 5-FUdR drugs. However, many cancer cells are/develop resistance to these drugs, and one type of resistance is the reduced ability to form the ternary complex after treatment with the drugs. In the present study, while examining a set of cancer cells for ternary complex formation, I found the HCT-116 $p53^{-/-}$ cells showed a "5-FUdR-resistant phenotype", i.e., a lack of ternary complex formation after 5-FUdR treatment and relative 5-FUdR-resistance compared to wildtype (WT) cells. Results presented in this chapter of my thesis suggest a possible *mechanism* for the lack of this complex formation in these cells.

I first examined whether the absence of p53 in HCT-116 $p53^{-/-}$ cells was responsible for the reduced ternary complex formation and 5-FUdR resistance. Recent studies have purported to show that lack or disruption of p53 can affect the sensitivity of cells to 5-FU. Results from two different studies, one utilizing HCT-116 $p53^{-/-}$ cells and the other utilizing MCF-7 cells transformed with E6, both found the cell lines to be resistant to 5-FU (129,130). My initial results are consistent with these studies and show that HCT-116 $p53^{-/-}$ cells demonstrate a 5-FUdR-resistant phenotype. However, my serendipitous finding of a reduced formation of ternary complex suggested that alteration in TS protein or nucleoside metabolism was responsible, not a p53-dependent response to drug-induced DNA damage. The lack of involvement of p53 was demonstrated by re-introduction of a biologically active p53 into HCT-116 $p53^{-/-}$ cells to a level similar to WT cells. This failed to alter the resistant phenotype. Furthermore, I observed that even HCT-116 WT cells were unstable, eventually showed a 5-FUdR-resistant phenotype (WTR), after a few weeks in culture in the absence of

FIGURE 3.17. Schematic showing the factors involved in ternary complex formation in a cell

See *Discussion* for more details.


Cell Death
RNA
DNA
FU
Thymine depletion
TP
Nucleobase (e.g., FU)
FUdR
TK
FdUMP
Ternary complex
FdUMP
TS
CH_2THF
ENT2
ENT1
? ENT3
? ENT4
dUMP
FREE TS
CH_2THF
THF
Folate pools
Nucleoside (e.g., FUdR, TdR)
DHF
DHFR
Aminopterin
TdR
TK
dTMP
Purines
dTDP
dTTP
DNA synthesis
Cell Proliferation

any drug. Thus, the lack of ternary complex formation that I observed in HCT-116 $p53^{-/-}$ cells appeared to be independent of p53 status, and likely dependent upon other factor(s).

Several cellular factors were investigated that could be responsible for decreasing ternary complex formation after 5-FUdR or 5-FU treatment in WTR cells. 1) Reduced affinity of TS for FdUMP could theoretically reduce ternary complex formation. To the best of my knowledge this has been shown in only two reports (60), including the report by Berger and coworkers(61). Berger and coworkers showed the presence of a variant TS enzyme in addition to the wildtype TS in HCT-116 cells (61). Compared to wildtype TS, the variant protein is more basic (higher isoelectric point) and has reduced affinities for both FdUMP and CH_2THF. I therefore used two-dimensional gel electrophoresis to examine whether there were any differences in the two forms of TS protein in WT and WTR HCT-116 cells. The results showed *no difference* in either the levels or the isoforms of wildtype and variant TS. Thus the lack of ternary complex formation in WTR cells is not likely due to differences in the forms of TS protein.

2) A reduction in the level of cellular folate is capable of reducing ternary complex formation since lower folate levels can lead to lower amounts of CH_2THF, one component of the ternary complex. To rule out this explanation, I co-administered folinic acid and 5-FUdR in HCT-116 $p53^{-/-}$ cells to examine the effect on the ternary complex formation. Folinic acid is 5-CHO-THF, which increases the intracellular folates including CH_2THF levels (139). Addition of folinic acid did *not* seem to increase the ternary complex formation in the HCT-116 $p53^{-/-}$ cells. This suggested that low levels of folate are not responsible for the lack of ternary complex formation in HCT-116 $p53^{-/-}$ cells.

3) A reduction in the *formation* of FdUMP is also capable of reducing the ternary complex formation in WTR cells. Two main factors that can reduce intracellular FdUMP are low thymidine kinase (TK) levels and reduced uptake of the 5-FUdR or 5-FU. TK is a non-essential enzyme that catalyzes the salvage biosynthesis of dTMP by phosphorylating TdR (thymidine) (Fig. 3.17). Since TdR and 5-FUdR are nucleosides of very similar structure, TK can also catalyze the phosphorylation of 5-FUdR to form the TS-reactive FdUMP. (Note: when 5-FU is given to the cells, it is first converted to 5-FUdR by thymidine phosphorylase and then to FdUMP by TK (Fig. 3.17). Examination of the TK level by an enzymatic assay and by RT-PCR showed *no change* in WTR cells compared to WT cells.

This suggested that the lack of ternary complex formation in WTR cells is not likely due to low TK levels.

4) I examined the possibility that impaired *uptake* of 5-FUdR is responsible for the lack of ternary complex formation in WTR cells. The first indication of impaired nucleoside uptake came from the experiment that demonstrated a dramatic reduction in the 18 h-uptake of ^{3}H-TdR in WTR cells. Since most nucleosides (including 5-FUdR and TdR) are believed to be taken up *via* a mechanism involving the same transporters, impaired uptake of TdR implies the same will be true for 5-FUdR. 18 h growth of cells in ^{3}H-TdR results in its incorporation into DNA. I used short-term uptake (10-20 min) of ^{3}H-TdR as a measure of cellular uptake. Again, WTR cells showed impaired uptake of TdR. Thus, these results suggest that WTR cells have impaired uptake of nucleosides including 5-FUdR, and this is most likely responsible for the reduced ternary complex formation and increased 5-FUdR resistance.

A clonogenic method involving hypoxanthine-aminopterin-TdR (HAT) treatment was also used to demonstrate impaired TdR uptake into WTR cells. Aminopterin inhibits DHFR, leading to folate depletion. This leads to depletion of purine and dTMP levels required for DNA synthesis and cell proliferation. Hypoxanthine and TdR in HAT replenish the purine and dTMP levels, respectively, *via* HPRT and TK salvage pathways. Treatment with HAT containing various levels of TdR showed that WTR cells require higher concentration of TdR than WT cells to be rescued. The difference between WT and WTR cells observed using HAT treatment was not as dramatic as the difference seen using the TdR uptake experiment (above). This is probably due to the fact that, although TdR uptake is impaired, it is not completely inhibited in WTR cells and the cells are still able to take up TdR at slow rates and supply sufficient dTMP to maintain cell needs.

Impaired uptake of nucleoside is likely due to defect in transport, but this is difficult to prove directly. A method of measuring incorporation of radiolabelled thymidine for short periods of time (10-20 min) was developed. Further uptake was quenched by dilution of ^{3}H-TdR with a 200-fold excess of unlabelled TdR. Lowering of the background to very low levels was possible using centrifugation and extensive washing of cells on a small filter. With this technique, ^{3}H-TdR taken up by cells after as little as 10 min could be detected. At this short time interval, the majority of cell-associated radioactivity is likely to be ^{3}H-TdR or

^{3}H-dTMP. Differences between WT and WTR cell lines are therefore likely to be due to defects in transport of ^{3}H-TdR into the cell or possibly conversion to ^{3}H-dTMP.

To further explore the impaired uptake of nucleoside in WTR cells, I examined the levels of nucleoside and nucleobase transporters, ENT-1 and ENT-2. ENT-1 transports only nucleosides (e.g., 5-FUdR, TdR) while ENT-2 can transport both nucleosides and nucleobases (e.g., 5-FU) (138) (Fig. 3.17). Since reduction in ternary complex formation in WTR cells was seen for both 5-FU and 5-FUdR, I expected that ENT-2 would be affected. However, RT-PCR analysis demonstrated *no difference* in ENT-1 or ENT-2 levels between the WT and WTR cells. This did not rule out the possibility that the activity of these transporters is somehow affected in WTR cells. Furthermore, the involvement of recently described, although not well characterized, transporters ENT-3 and ENT-4 in the impaired uptake in WTR needs to be explored, especially ENT-3, as it has only just recently believed to be a transporter of both nucleosides and nucleobases (137).

Although nucleobase 5-FU showed lower ternary complex formation in WTR than WT cells, it showed only a small difference in the cytotoxicity of the two cell lines. This is different than the effect by 5-FUdR, which showed a lower ternary complex formation *and* significantly lower cytotoxicity in WTR than WT cells. Once 5-FU is inside a cell, it is converted to 5-FUdR by thymidine phosphorylase (TP) and/or incorporated into RNA and DNA (Fig. 3.17) by various metabolic enzymes (19). Both 5-FU-to-5-FUdR conversion and RNA/DNA incorporation pathways can lead to 5-FU-mediated cytotoxicity. Since the effect of 5-FU on ternary complex formation was similar to 5-FUdR, the 5-FU-to-5-FUdR conversion pathway is not likely responsible for the difference in 5-FU-dependent cytotoxicity in WT and WTR cells. Surprisingly, p53 null and nullR cells showed a slight resistance in 5-FU-mediated cytotoxicity compared to WT and WTR cells. A similar but much more pronounced resistance has been seen by other groups in p53 null cells (129,130).

To address the question of the mechanism of instability of WT cells leading to WTR cells demonstrating a 5-FUdR-resistant phenotype, I examined the role of genetic instability as a possible mechanism. Ferguson and Cheng have shown that human colon cancer cells are phenotypically unstable with respect to sensitivities to chemotherapeutic drugs etoposide and vincristine (140). However, the same effect towards 5-FU or 5-FUdR has not previously been shown. Colon cancer HCT-116 cells have been previously shown to demonstrate

hereditary non-polyposis colorectal cancer (HNPCC) phenotype due to mismatch repair deficiency caused by the lack of hMLH-1 gene (135). Since defects of this DNA repair gene can lead to further genetic instability in cells, I first examined whether reintroduction of the missing gene would inhibit the WT to WTR conversion. The hMLH-1 gene is located on chromosome 3p21 and reintroduction of chromosome 3 in the cells (HCT-116+Chr3) has been shown to correct the mismatch repair deficiency and HNPCC phenotype (135). However, I found that HCT-116+Chr3 cells with corrected HNPCC phenotype were still phenotypically unstable and converted to WTR cells with a 5-FUdR-resistant phenotype. I also examined the role of DNA methylation in the conversion of WT to WTR. 5-aza-2-deoxycytidine (5-AdCd) is widely used nucleoside that reverts DNA methylation in cells (141,142). I was unable to "revert" WTR cells back to WT cells using 5-AdCd, suggesting this is not the mechanism of the observed instability.

I also addressed other *in vitro* factors that could possibly induce the unstable phenotype in WT cells. 1) Mycoplasma contamination is believed to decrease TdR uptake by cells (143). However, I found no evidence of mycoplasma contamination in any of my cell cultures using a commercial detection kit. 2) HCT-116 cells have been cultured in DMEM (61) and McCoy's (129) media by different groups. I examined whether culturing my WT cells in McCoy's media instead of DMEM media would prevent the unstable phenotype. Although my HCT-116 cells grew readily in McCoy's medium, they still demonstrated the unstable phenotype. On the other hand, HCT-116 cells obtained from ATCC grew only in McCoy's medium but did not survive the switch to DMEM medium. These ATCC HCT-116 cells showed a 5-FUdR-resistant phenotype from early passage.

I further examined whether factors released into the medium are responsible for the unstable phenotype of WT cells. Co-culturing WT and WTR cells caused all the cells to demonstrate the 5-FUdR-resistant phenotype. Exposing WT cells to conditioned media of WTR cells also converted the former cells to the WTR phenotype. I attempted crude fractionation of the medium from WTR cells by ethanol precipitation of protein and other large molecules. However, neither the alcohol soluble nor the reconstituted precipitated fraction was able to convert WT cells to the WTR phenotype. This indicated that the putative factor(s) responsible for the unstable phenotype in the culture media of WTR cells did not survive ethanol precipitation. Further work will be necessary to identify and

characterize the factors responsible for the unstable phenotype leading to 5-FUdR resistance in WTR cells.

3.6. CONCLUSION

In this chapter, I have used the lack of TS-FdUMP-CH_2THF ternary complex formation to identify a 5-FUdR-resistant mechanism in HCT-116 cells. The mechanism of resistance seems to be impaired uptake of the 5-FUdR drug, likely due to impaired nucleoside uptake. HCT-116 cells were found to be phenotypically unstable as they spontaneously converted to 5-FUdR-resistant phenotype. My results also show that some unknown factor(s) in the culture media of WTR cells is (are) capable of inducing 5-FUdR-resistant phenotype in WT cells.

CHAPTER 4: COMPARISON OF TRANSLATIONAL AUTOREGULATION OF TWO ALLELES OF HUMAN TS

4.1. INTRODUCTION

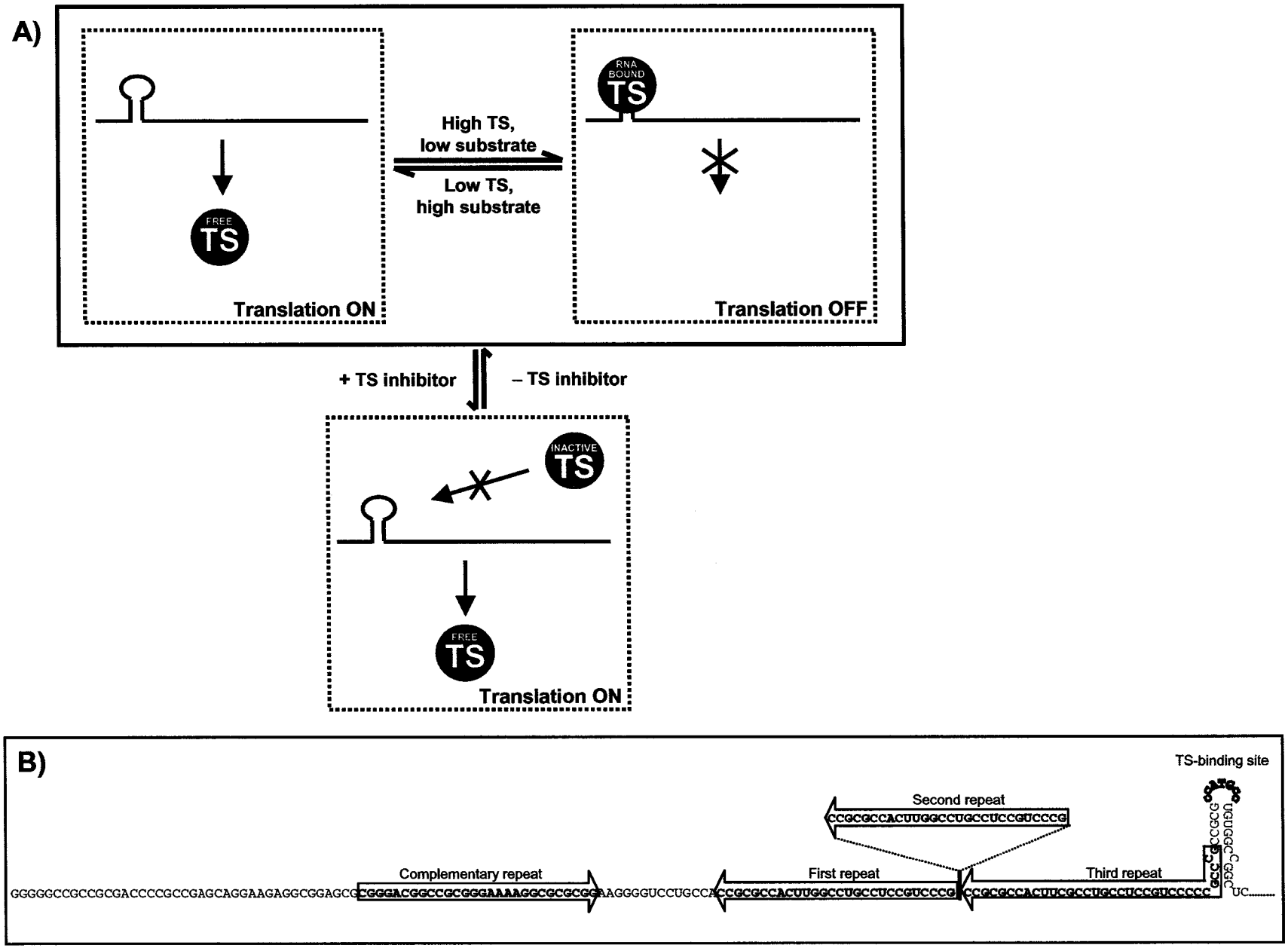
The possibility of translational control of TS was first put forward by Belfort and coworkers upon cloning and analysis of the structural features of the *E.coli* TS thyA gene (144). Subsequently, in characterizing human TS cDNA, Takeshi and coworkers also pointed out the potential of translational control, due to the presence of three tandem 28-34-nucleotide repeat sequences in the 5'-UTR of the TS cDNA (4). Secondary structure analysis of TS cDNA suggested the potential of three interconvertible secondary structures, each with a stable stem-loop structure in the 5'-UTR (4). Kanada and coworkers demonstrated that deletion of any one of these tandem repeat sequences resulted in signification alteration in TS mRNA translational efficiency *in vivo* (145). Their study suggested that these sequences could mediate the regulation of translation of TS mRNA, possibly through its interaction with some cellular factor or protein.

Several studies have reported rapid increases in TS enzyme levels in various *in vitro, in vivo* and clinical experimental model systems after short-term exposure to the fluoropyrimidines, 5-FU and 5-FUdR (25,30-32). One study, using MCF-7 breast cancer cells, showed a 10 to 40-fold increase in TS enzyme levels following exposure to the folate analog, Tomudex (29). There was no change in TS mRNA levels and the protein synthesis inhibitor, cycloheximide, inhibited this increase in TS protein (29). These authors also suggested that a translational regulatory event was involved. Chu and co-workers showed that the increase in both TS enzyme activity and TS protein expression, which occurred in the human colon cancer line H630 following exposure to 5-FU, were not associated with a corresponding change in TS mRNA levels (33,34). Further work by the same group showed that the increase in TS protein expression was the direct result of new synthesis of TS protein (33).

Chu and co-workers went on to characterize TS mRNA translation more completely. Using a rabbit reticulocyte lysate *in vitro* translation system, they demonstrated that the addition of exogenous pure recombinant human TS protein repressed the translation of human TS mRNA in a specific dose-dependent manner (85). They used an RNA electrophorectic gel mobility shift assay system to illustrate a specific direct interaction between human recombinant TS protein and its corresponding TS mRNA (37). This group put forward a model of translational autoregulation of TS (35) (Fig. 4.1). According to this

FIGURE 4.1. Mechanism of TS auto regulation at the translational level (A) and the 5'UTR of TS mRNA showing tandem repeats and stem-loop structure (B)

A. TS translation is turned "on" at low TS and/or high substrate levels and is turned "off" by RNA-binding of TS at high TS and/or low substrate level. In the presence of TS inhibitor, the inactivated TS is unable to bind RNA and hence the translation is turned "on". ***B.*** 5'UTR sequence of TS mRNA showing a complementary repeat followed by either three or two tandem polymorphic repeat. The stem-loop secondary structure (in blue) is the TS-binding site and contains the start codon in the loop. Note that the third repeat partially overlaps with the secondary structure.


A)
FREE
TS
Translation ON
High TS,
low substrate
Low TS,
high substrate
RNA
BOUND
TS
Translation OFF
+ TS inhibitor
– TS inhibitor
INACTIVE
TS
FREE
TS
Translation ON
B)
TS-binding site
Second repeat
Complementary repeat
First repeat
Third repeat
GGGGGCCGCCGCGACCCCGCCGAGCAGGAAGAGGCGGAGCG
AGGGGUCCUGCCA
UC

model, if TS protein is not bound to substrate (either of its physiolocial substrates, dUMP or CH_2-THF, or to the 5-FU and 5-FUdR metabolite, FdUMP), it binds to specific sites on its own mRNA, resulting in translational repression (35). Conversely, when TS is bound to substrate, it is no longer able to bind its mRNA and translational inhibition is relieved, resulting in an increase in translation of the TS message (35). The RNA binding activity of TS protein is also dependent on its redox state (36). It binds to its mRNA only when it is in a reduced state. Upon oxidation, it no longer binds its mRNA (36).

Two sites on TS mRNA to which TS protein binds with comparable affinity to the full-length mRNA, have been reported (37). One of the site maps to a 30 nucleotide fragment in the 5'-UTR of the TS mRNA that just includes the start codon (37). The other site has been mapped to a 70 nucleotide region corresponding to nucleotides 480 to 550 within the coding region (38). Zuker RNA fold-analysis of the RNA binding site in the 5'-UTR indicates that it is capable of forming a stable stem-loop structure (37). It has been shown that the GCCAUG hexanucleotide sequence in this loop structure is the critical determinant of the protein-RNA interaction (37). Preliminary secondary structure analysis predicts the downstream binding site in the coding region to form a complex pseudoknot structure, one significantly different from the stem-loop structure observed for the mRNA binding site in the 5'-UTR (37).

The model of translational autoregulation of TS provides a rational mechanism for the acute induction of TS that arises in response to exposure to 5-FU, 5-FUdR and to antifolate analogs that specifically target TS (35). In addition, it has been demonstrated by the same research group that TS protein also binds to c-myc and p53 mRNAs and inhibit the translation of these messages (39,40).

The human TS gene is polymorphic with either triple (3R) or double (2R) tandem repeats of a 28 base-pair sequence downstream of the cap-site in the 5'UTR (41) (Fig.4.1B). In *in vitro* studies, the activity of a reporter gene linked to the 5'-terminal fragment of the TS gene with 3R was found to be 2.6 times higher than that with 2R (41). Thus this polymorphic region appeared to be functional and involved in the modulation of TS gene expression. It is important to note that in both TS alleles (3R and 2R), one of the tandem repeats lies just within the binding site for TS protein in the 5'-UTR.

In this chapter, due to the proximity and partial overlap of the polymorphic repeats to the TS-mRNA binding site in the 5'-UTR of the TS mRNA, I hypothesize that the translational efficiency of the 3R and 2R alleles of the TS mRNAs will be different in the presence of a functionally active TS protein. Since most of the work on the model of translational autoregulation of TS came from one lab, I also wanted to confirm that this model was true. My additional aim was to investigate possible mutations in the TS protein or RNA binding sites that might alter translational regulation of TS leading to its overexpression; however, this aim could not be addressed due to the technical problems experienced in this chapter.

4.2. OBJECTIVES ADDRESSED

The overall objective is to confirm that translational autoregulation is different in the 3R and 2R alleles of TS. Some of the specific objectives are as follows:

- Develop plasmid constructs for generating full-length 3R and 2R TS mRNA
- Generate 3R and 2R mRNA *in vitro* and determine if they are capable of producing TS protein by *in vitro* translation
- Compare the rate of translation of TS protein between the 3R and 2R allele mRNAs
- Determine if *in vitro* translated TS is enzymatically active
- Express and purify a recombinant TS protein in *E. coli* and determine if it is enzymatically active
- If unable to express enzymatically active TS in *E. coli*, express recombinant TS in HeLa cells by stable transfection and demonstrate its enzymatic activity
- Examine the effect of adding a functionally active TS protein-alone or in the presence of FdUMP or dUMP to *in vitro* translation of TS protein from 3R and 2R allele mRNAs
- Demonstrate the RNA-binding ability of the functionally active TS (purified or in crude extracts) by an electromobility shift assay

4.3. MATERIALS AND METHODS

4.3.1. Generation of pTS10 construct

Plasmid pWHTS-1 (6) containing human TS protein coding-region was a kind gift from Dr. D. Santi. A 1.7 kb fragment encoding the coding region was excised from pWHTS-1 by HindIII/Sma1 digestion and ligated to HindIII/EcoRV digested pBluescript SK $^{+/-}$ plasmid (Stratagene; Cedar Creek, TX). The resulting plasmid, pTS.10, allowed the sequencing of the TS insert. It was sequenced in forward (T7 primer) and reverse orientation (T3 primer), confirming that this 1.7 kb fragment contained the human TS coding region of the gene, flanked by a partial 5-UTR consisting of 1 GC rich repeat lacking the first 5 bp and another full GC rich repeat, i.e., a 2R allele of TS lacking the first 5 bp.

Having confirmed the sequence of the 1.7kb fragment in pWHTS-1 as human TS, I proceeded to use pWHTS-1 in preparing constructs encoding full-length 3R and 2R alleles of TS.

4.3.2. Generation of f2RTS-pCDNA3-5 (p2R-TS)

A 380 bp region spanning the 5'UTR to within 100bp of exon 1of TS gene was amplified using Primer TS170(sense): 5' GTGGCTCCTGCGTTTCCCCC and Primer TS 72(anti-sense): 5' ACAGCCATAAGCCGTACGTC. Genomic DNA extracted from white blood cells of an individual previously determined (in our lab) to be homozygous for the 2R allele was used. The 50 μL PCR reaction contained 1× Buffer (supplied with Taq Polymerase, Qiagen Inc), 1× Q solution (supplied with Taq Polymerase, Qiagen Inc.), 3 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 mM each primer, 100 ng of total genomic DNA (boiled/sonicated), and 2 units of DNA Polymerase from *Thermus aquaticus* (Qiagen Inc.). The sample was first denatured at 94^{0}C for 1 min and then subjected to 35 cycles of PCR. Each cycle consisted of 1 min at 94^{0}C, 1 min at 65 ^{0}C and 2 min at 72^{0}C. After 35 PCR cycles, the mixture was incubated at 72^{0}C for 5 min extension step. The amplified DNA fragments were analyzed by electrophoresis in 6% polyacrylamide gel using equivalent volumes of each PCR reaction. The gel was run at 140 V for 1hr 15min to allow sufficient separation of PCR amplified fragments.

The fresh PCR reaction product, which consisted of PCR products with an extra A-overhang added at the 3'end due to Taq polymerase, was subcloned directly into the pCR2.1 vector with TA overhangs according to manufacture's protocol (Invitrogen; Burlington,

Canada). Several clones were isolated and the orientation of the insert was verified by restriction enzyme-digestion analysis. One clone of proper orientation, 2R7-pCR2.1, was digested with HindIII / SphI and the 400bp fragment containing the PCR amplified 5' region of 2R allele of TS was gel purified. pWHTS-1 was digested with HindIII / SphI and the 4.2 kb fragment was gel purified and ligated to the gel purified 400 bp fragment of 2R7-pCR2.1. The resulting plasmid, f2RTS-10 encoded the full-length 2R allele of TS in pWHTS-1 (PUC-9) backbone. The plasmid f2RTS-10 was digested with SmaI / HindIII so as to generate a 1.7 kb fragment containing the full-length 2R allele of TS which was gel purified and ligated to Hind III/ EcoRV pCDNA3 vector. The resulting plasmid was sequenced and found to contain the full-length 2R allele of TS but there was one silent mutation at bp 42. Since this was not within the RNA binding site and the codon was not changed, I decided that this construct was suitable for my studies and was used as the construct encoding the full-length 2R allele of TS in pCDNA3 mammalian expression vector under control of a T7 promoter.

4.3.3. Generation of f3RTS-pCDNA3-5 (p3R-TS)

The construct encoding the full-length 3R allele of TS was prepared similarly. The only difference was that I started with PCR amplification of the 400 bp region spanning the 5'UTR to within 100bp of exon 1 of TS gene from genomic DNA from an individual previously determined to be homozygous for the 3R allele; all subsequent steps were the same as was performed for the full-length 2R allele construct, f2RTS-pCDNA3-5. However, when the isolated clone, f3RTS-pCDNA3-6 was sequenced, it was found to contain 2 point-mutations of G-A base pair changes, one at position -67 and other at -19. Since these were within the third repeat and at the start of the second repeat, within the RNA binding site, respectively, I could not use this construct as a full-length 3R allele of TS. I went back to the clones encoding the amplified PCR 5'-UTR fragment in pCR2.1 TA vector and had the inserts of several clones sequenced. I found that all 5 of the clones sequenced contained 1 or 2 point-mutations, either in the RNA binding site or a mutation that resulted in a codon change (not the same in all clones) and so none of them could be used for constructing the full-length 3R allele of TS. It is probable that the errors were introduced at the PCR stage by Taq polymerase, which lacks proof-reading function. In order to improve fidelity, I attempted to PCR amplify this region using a higher fidelity polymerase, Vent Polymerase

(New England Biolabs, Inc.; Beverly, MA). I also tried to vary the existing PCR conditions used with Taq Polymerase, including annealing temperatures, concentration of $MgCl_2$, annealing times, use of Qiagen Q solution. However, I was not successful in using Vent polymerase for the priming of the 5'PCR product of TS 3R allele. This was likely because of the high GC content of this region, which can make priming very difficult. I was finally successful by returning to Taq polymerase and managing to reduce its rate of error or nucleotide misincorporation. I decreased the number of PCR reaction cycles from 35 to 30 and used 2 mM $MgCl_2$ with an equimolar amount of dNTP to $MgCl_2$, conditions which have been reported to reduce the error rate of Taq Polymerase (146). All other parameters were kept constant. The resulting PCR product was subcloned directly into TA overhang vector, pCR2.1. When clones containing the insert in the proper orientation were sent for sequencing, 2 of the 3 selected clones were free of errors. One error-free clone, 3R26-pCR2.1 was chosen for use in constructing f3ReqTS-pCDNA3-6 where all steps were the same as conducted for generation of f2RTS-pCDNA3-5, which utilized 2R7-pCR2.1. The correct orientation of f3ReqTS-pCDNA3-6 was confirmed by restriction enzyme analysis and also by sequence analysis and found to encode the full-length 3R allele of TS in pCDNA3 mammalian expression vector under control of a T7 promoter.

4.3.4. In vitro Transcription

Contructs f2RTS-pCDNA3-5 and f3Req-TS-pCDNA3-6 were linearized with Not1. A control construct, CMV-pGL3 was a kind gift from Dr. R. Cowling. It encoded a luciferase gene under the control of a T7 promoter *(R.T. Cowling's Ph.D. thesis)*; it was linearized with Hpa1. All constructs were linearized just beyond the stop codon. Linearized plasmids were phenol-chloroform extracted, ethanol precipitated and dissolved in CT buffer. They were quantitated by a DABA assay and stored at -20 °C. For *in vitro* transcription, the T7 Cap Scribe kit (Roche, Mississauga, Canada) was used according to manufacturer's instructions. Briefly, each *in vitro* transcription reaction consisted of 500 ng of linearized DNA, 20 U RNasin inhibitor (Promega; Madison, WI), 1x T7 Cap Scribe reaction buffer (containing nucleoside triphosphates) and 2U of T7 RNA Polymerase in a 20 µl reaction The reaction mix was incubated at 37 °C for 1h. Production of *in vitro* transcribed RNAs

was confirmed by electrophoresis on agarose gel. *In vitro* transcribed reactions were used directly in *in vitro* translation reactions.

4.3.5. Quantitation of in vitro transcribed RNAs

In vitro transcribed RNAs were quantitated using 2 µl of each *in vitro* transcribed RNA reaction. 48 µl of cold mix of 0.2 N HCl and 0.3 N NaCl was added and the sample was incubated on ice for 1 h to precipitate the DNA and RNA. The sample was centrifuged at 13000 g for 5 min and the pellet was washed with 50 µl of cold 1N HCl to remove ribonucleotide triphosphates. The pellet was then dissolved in 60 µl of 0.2 N NaOH and incubated at 37° C overnight to hydrolyse the RNA. The sample was then cooled to 0°C and 40 µl of 1N HCl was added to neutralize the reaction, which was incubated at 0°C for 4 h or longer to precipitate DNA. The sample was centrifuged and the supernatant containing the hydrolysed RNA was transferred to a fresh tube and its absorbance at wavelength of 260 nm was measured using a cuvette with a 100 µl capacity. This absorbance value was used to calculate the concentration of RNA. This direct biochemical method of RNA quantitation needed to be developed since there is no readily available reliable method for quantifying RNA in an *in vitro* transcribed reaction mix containing DNA template and excess ribonucleotide triphosphates, all of which contribute to absorbance at 260 nm. The reliability of this quantitation method was demonstrated using commercial bacterial RNA as a reference and a negative control consisting of a zero-time NaOH treatment.

4.3.6. In vitro Translation

In vitro translation reactions were performed using a rabbit reticulocyte *in vitro* translation system (Promega; Madison, WI) according to manufacturer's instructions. Each *in vitro* translation reaction consisted of 175 ng of *in vitro* transcribed RNA. The RNA was used directly from *in vitro* transcribed reaction and was heated at 65°C for 3 min and cooled immediately on ice to remove any RNA secondary structures just prior to use for *in vitro* translation. Each reaction also contained 70% (v/v) rabbit reticulocyte lysate, 30 µM amino acid mixture, 10 U RNasin inhibitor (Promega) and 0.4 µM (5 µCi) S^{35}methionine (Amersham Life Sc; Buckinghamshire, England) for hot reactions or 30 µM cold methionine

for cold reactions. The reaction mixtures were incubated at 30°C. The reaction was stopped by incubating on ice. To establish a time course of *in vitro* translation, several reaction mixtures were incubated at 30 °C for 0 to 60 min and one reaction tube was removed every 10 min. Once conditions were determined for a linear time course, these were used for all experiments, unless otherwise stated. The *in vitro* translated products were electrophoresed on a 12% SDS-PAGE gel at 200V for 45 min. The gels were Coomassie Blue stained and destained, then dried and exposed to phosphorscreen for 24 h. The phosphorscreen was scanned.

4.3.7. Western Blot analysis of in vitro translated TS reactions

After the *in vitro* translated reactions were electrophoresed through a 12% SDS-PAGE gel, the protein on the gel was transferred to a PVDF membrane (Millipore, Nepean, Canada) at 15V for 18 h in transfer buffer (3 mM Na_2CO_3, 10 mM $NaHCO_3$, 10% methanol) (95). The membrane was Ponceau S stained and then washed with TBST. For immunoblot analysis, all steps were carried out at room temperature.

The membrane was blocked with 1% BSA in TBST for 1 h and then incubated with primary rabbit anti-TS antibody hTS 8.3 (previously prepared in our laboratory (95) at 1/1000 dilution in 0.5% BSA in TBST. Washing was carried out 5×5 min in TBST and then the blot was incubated in HRP-conjugated anti-rabbit/anti-mouse secondary antibody ("Polymer") (DAKO; Carpinteria, CA) for 1 h. The membrane was washed 5 times with TBST and HRP was detected using the LumiGLO Chemiluminescent Substrate Kit (Kirkegaard & Perry Lab; Maryland, USA).

4.3.8. Effect of synthetic TS protein on in vitro translation of TS

Fresh *in vitro* translation reactions of 3R and 2R alleles to prepare synthetic TS proteins were first carried out using "cold" methionine (unlabeled). A control (no RNA) cold reaction was also set up. An aliquot of the "cold" reaction (17% v/v) was added to a fresh *in vitro* translation "hot" reaction (i.e., containing [^{35}S]-methionine). An aliquot of cold 3R allele reaction was added to hot 3R and a hot 2R allele reaction and similarly an aliquot of cold 2R allele reaction was added to hot 3R and a 2R allele reaction. As control reactions, an aliquot of *no RNA* "cold" reaction was added to hot 3R and a hot 2R allele reaction. The

reaction mixtures were incubated at 30°C for 15 min and treated similarly as described above for SDS-PAGE analysis and densitometry scanning.

4.3.9. Substrate binding ability of Synthetic TS Protein

To examine the ability of the *in vitro* translated TS protein to bind substrate, an *in vitro* translation of 2R allele was performed in presence of dUMP or FdUMP each at 30μM. Substrate was included at the start of the reaction and the reaction was allowed to take place for 1 hr to allow sufficient time for *in vitro* translated protein to be synthesized and bind to the substrate.

4.3.10. Catalytic Activity of Synthetic TS Protein

The determination of TS catalytic activity of *in vitro* synthesized TS was performed using TS activity assay as described below. An aliquot of freshly prepared *in vitro* translation reaction of the 2R allele was tested.

4.3.11. Time-course of Induction and Expression of H_6-TS

E.coli bacterial cells containing PQE31-TS7 and M15[pREP4] plasmids were prepared as described previously (95). These cells were grown at 37°C in 2×YT media with kanamycin (25 μg/mL) and ampicillin (100 μg /mL) until OD at 600 nm was 0.6-0.8. For induction of expression of H_6-TS, IPTG was then added (2 mM). An uninduced control sample was included to which no IPTG was added. Every 30 min beginning with a 0 time point and up to 180 min post induction, an aliquot of bacterial culture was removed. It was centrifuged at 3500 g for 10 min at 4°C. The supernantant was removed and the cell pellet was washed with cold water, after which the cell pellets were stored at -80°C. The pellets were later thawed on ice, resuspended in Sonication buffer (50 mM sodium phosphate, pH 7.8, 300 mM NaCl) containing protease inhibitors (PMSF (1 mM) and aprotinin (2 μg/mL)) and lysozyme (1 mg/mL). The resuspended pellets were incubated on ice for 40 min, sonicated briefly (Sonicator) and centrifuged at 10000 g for 10 min at 4°C. The supernatant was removed and transferred to a fresh tube. The pellet was resuspended in sonication buffer including protease inhibitors and constituted the pellet fraction. An aliquot of the supernatant was subsequently centrifuged in an Ultracentrifuge at 45 000 rpm for 30 min at

4°C. After ultracentrifugation, although there was no noticeable pellet, the upper two-thirds of the volume in the tube was transferred to a fresh tube and designated as the ultracentrifuged supernatant. The amount of protein in each of the supernatants, ultracentrifuged supernatants and pellet fractions was quantified using the fluorescamine assay. Equal amounts of protein were analysed on a 12% SDS-PAGE gel and either Coomasie Blue stained, destained and dried, or transferred to a PVDF membrane and subjected to anti-TS western blot analysis as described above.

4.3.12. Large Scale Production of H_6-TS

Once the time course of induction for expression of H_6-TS protein had been established, a larger quantity of H_6-TS protein was prepared as above with a few changes. Using 100 mL of bacterial culture following 2 hr induction, the culture was centrifuged as above, the pellet was washed with water and then kept stored at -80°C. When needed, the pellet was thawed, resuspended in 20 mL sonication buffer with protease inhibitors and lysozyme and incubated on ice for 40 min. The sample was then homogenized (3× 10 sec pulses) and the lysis of the cells was confirmed by microscopic examination of an aliquot of the sample. After homogenization, dithiorethiol (DTT) (10 mM) was added and the sample centrifuged at 10000 g for 20 min at 4°C. The supernatant was transferred to a fresh tube and this crude lysate constituted the crude soluble H_6-TS protein.

4.3.13. Affinity Purification of H_6-TS using a Ni-NTA column

All purification steps were conducted at 4°C and all buffers used on the columns contained protease inhibitors and DTT (1 mM). For the preparation of the Ni-NTA column, 1 mL of Ni-NTA resin (stored in 50% EtOH, Qiagen) was centrifuged at 500 g, 20 sec and resuspended in 500 µL Sonication buffer. The washing step was repeated 2× and resin kept stored in Sonication buffer for equilibrium overnight. Next day the 1 mL of resin was loaded into a 3 mL syringe to construct a column with 0.5 cm column height. The crude lysate was loaded 1× through an Ni-NTA column. The column was then washed with (~30 mL) sonication buffer until the A_{280} was < 0.01. The column was then washed with wash buffer (~30 mL) (50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 10% glycerol) until the A_{280} was < 0.01. To get rid of stronger affinity impurities, the column was washed with 15 mL of

wash buffer containing 25 mM imidazole. H_6-TS protein was eluted in 1mL fractions of 250 mM imidazole in wash buffer.

4.3.14. Buffer Exchange of Ni-NTA-purified H_6-TS using a PD-10 column

A PD-10 column (Pharmacia,) prepacked with Sephadex G-25M was used for rapid buffer exchange, according to manufacturer's instructions. The fractions of H_6-TS eluted off Ni-NTA column in 250 mM imidazole in wash buffer were loaded onto a PD-10 column. Elution buffer was M buffer (10 mM MOPS buffer, pH 7.4, 10% glycerol). 0.5 mL fractions were collected while loading and eluting from the column.

Fractions were stored at 0°C. Fluorescamine assay was used to quantitate the amount of protein in fractions collected. An aliquot of various fractions collected throughout the purification steps was subjected to SDS-PAGE analysis and Coomassie blue Staining. I estimated that 100 mL of bacterial culture produced about 1 to 1.5 mg of purified H_6-TS protein.

4.3.15. Catalytic Activity of H_6-TS

TS isotopic activity assay was modified from a previous report (147). For preparation of anion exchange resin , 1 gram of anion exchange resin Spectra/Gel Strong base anion Chloride form (Spectrum, LA, Ca) was washed with 5 mL water, centrifuged at 500 g and washing repeated 2×. The resin was then washed 3× with 0.1N NaOH and 2× with 0.01 N NaOH and kept stored in 10 mL of 0.01 N NaOH at 4 °C for up to a week. Each 100 µL TS activity reaction mixture consisted of 10 mM MOPS buffer, pH 7.4, formaldehyde (0.644 nM), tetrahydrofolate (THF) (Sigma-Aldrich, St. Louis, MO) (0.1 mM), dithioreithol (DTT) (Sigma-Aldrich) (10 mM), NaCl (20 mM) and dUMP (Sigma-Aldrich) (46 µM (699:1 molar ration of cold to hot) containing 0.1 µCi ^{3}H-dUMP (Amersham Life Sc, Little Chalfont, Buckinghamshire, Eng, stock 15.5 Ci/mmol). THF, DTT and formaldehyde reagents were freshly prepared. Cellular extract containing TS enzyme was added to start the reaction and the reaction mix was incubated at room temperature for 1 h. The reaction was stopped by addition of 900µL of cold 2 mM NaOH. 100 µL of previously equilibrated anion exchange resin in 0.01 N NaOH was added and tube rotated for 1 h to allow the resin to bind any unreacted ^{3}H-dUMP substrate. The reaction

tube was then centrifuged at 3500 g for 15 secs and 150 μL of supernatant containing ^3H_2O was transferred to a fresh tube to which 1.5 mL of Liquid Scintillation counting Fluid Scintran Cocktail X (VWR International, Mississauga, Canada) was added and tubes counted in a Packard model 1600 TR liquid scintillation counter.

The preparation of CH_2THF cofactor was prepared *in vitro* by the inclusion of THF and formaldehyde in the reaction mixture. Formaldehyde was freshly prepared by dissolving paraformaldehyde in MOPS buffer and boiling it for 10 min. THF is a very unstable reactant. THF powder was kept stored at -80°C. A stock solution of THF was freshly prepared each time for use by resuspending the powder in distilled water, dissolving it by neutralizing with alkali (equimolar NaOH) followed by the addition of 10 mM DTT to maintain the THF in a reduced state. To ensure the fresh THF solution was not oxidized, the spectral peak shift at 290-292 nm of THF incubated in the presence of formaldehyde (148) was monitored.

4.3.16. Generation of Stable TS Overexpressing HeLa Cell Lines

HeLa cells were plated at 3×10^5 cells in a 1 mL dish and 24 h after they were transfected with f3ReqTS-pCDNA3-6 using Fugene 6 (according to manufacturer's instructions). 48 h post-transfection, cells were trypsinized and plated at low density in a series of 10 cm dishes in media containing G418 (500 μg/mL). Selection media was changed every 3 days and two weeks after plating, individual clones from several plates were isolated, expanded and maintained in G418 selection.

4.3.17. Preparation of Total Cell Protein Extracts for SDS-PAGE

Cultured cells were detached by trypsin treatment, then washed twice and suspended in cold PBS. To prepare whole cell extracts, an equal volume of 2×SDS sample buffer (100 mM MOPS, pH 6.8, 4% SDS, 20% glycerol, 2 % β-mercaptoethanol) was added. The extracts were boiled for 20 min and then sonicated briefly to reduce viscosity. Protein was quantified using fluorescamine (97) (Sigma-Aldrich), using BSA as a standard. For immunoblotting, proteins in cell extracts in SDS sample buffer were resolved by 12% discontinuous SDS-PAGE and then electrophoretically transferred to a PVDF membrane at 15V for 18 h in transfer buffer. The membrane was stained with Ponceau S and then

washed in TBST. Immunoblot analysis for TS detection levels was performed as described above.

4.3.18. Mammalian Cell Extracts for TS Activity and RNA Binding Assays

The media was removed from a 10 cm dish of almost confluent cells. Attached cells were washed with PBS and detached by addition of 5 mL PBS with 2 mM CDTA and rocked at room temperature for 10min. Cells were dislodged from the plate with a pipette, transferred to a 15 mL tube and centrifuged at 500 g for 5 min at 4°C. The supernatant was removed and cells were resuspended in 0.5 mL PBS and transferred to a 1.5 mL centrifuge tube and centrifuged at 13000 g for 10 sec. The supernatant was removed and cells were resuspended in 0.2 mL 25% cold PBS in H_2O. 0.2 mL of 2×Extraction buffer (1% triton-X, 40 mM MOPS, pH7.2) was added, sample was vortexed and incubated for 30 min on ice and then sonicated briefly to shear DNA.

4.3.19. Synthesis of radiolabelled RNA Probe for RNA-binding activity

For the synthesis of radiolabelled probe, the 20 µL reaction consisted of 1×transcription buffer (New England Biolabs; Pickering, Canada), 3 ribonucleotide mix (A,G,U) (0.5 mM), CTP (12 µM), α-^{32}P-CTP (800 Ci/mmol or 20 µCi /µL) at 50 µCi (0.8 mM) (Amersham), linearized DNA template (0.6 µg), RNasin inhibitor (Promega) (20 U) and T7 RNA Polymerase (20 U). The reaction mix was incubated at 37°C for 1 h. For the synthesis of full-length 2R allele of TS RNA, linearized f2RTS-pcDNA3-5 was used and for synthesis of radiolabelled control luciferase RNA, linearized CMV-pGL3 was used. RNA labeled probe was purified using a Microcon-PCR centrifugal filter (Millipore, Bedford, MA) according to manufacturer's instructions.

4.3.20. RNA Binding Assay

Recombinant H_6-TS was pre-treated with 25 mM DTT for 15 min at room temperature prior to use. RNA-protein binding experiments were performed similar to the previously described method (36). Briefly, each 20 µL RNA binding assay reaction consisted of full-length RNA probe (1ng, 26 fmol, 3×10^5 cpm), 1× binding buffer (10 mM HEPES pH 7.6, 40 mM KCL, 3 mM MgCL2, 5% glycerol and RNasin inhibitor (10 U).

Various amounts of purified TS protein were used (100 ng, 2.9 fmol- 780ng, 2262 fmol). The reaction mix was incubated at 37°C for 15 min following which RNase T1 (3 units, Boehringer Mannheim,) was added. After 10 min, heparin sulfate (5 mg/mL, Sigma) was added and samples were incubated for 10 min at 37°C. A 4% non-denaturing (i.e., lacking SDS or Urea) polyacrylamide gel (acrylamide/methylenebisacrylamide ratio 60:1) was pre-electrophoresed for 30 min at 100V. Half of the reaction was resolved by electrophoresis for 40 min at 200V. The gel was then dried and exposed to phosphorscreen overnight.
For crosslinking experiments, the final reaction mix was incubated with 5% neutral buffered formalin (VWR; Dorset, England) for 1 h at room temperature and half of this reaction was resolved on 12% SDS-PAGE gel at 200V for 1hr 30 min. The gel was dried and exposed to phosphoscreen overnight.

4.3.21. Effect of Recombinant TS protein on in vitro Translation

These experiments were conducted with the 2R allele since this was the first construct to be obtained. For these experiments, H_6-TS protein purified from PD-10 column in M Buffer was used. It was pretreated with fresh DTT (25 mM) for 15 min at room temperature just prior to use to ensure it was in a reduced state (36). Various amounts of H_6-TS was included in *in vitro* translation reactions at the start of the reaction. A 15 min time point (determined to be in the linear range) was used. To control for any effect of M buffer or DTT on the *in vitro* translation process, control reactions were carried out in the presence of M buffer, and M buffer containing DTT. Although this preparation of H_6-TS was only 80% pure, I decided that this was sufficient for preliminary experiments. *In vitro* translation reactions containing 390 ng of H_6-TS were also conducted in the presence of substrate dUMP (30 μM) and reactions were allowed for 1 h.

4.3.22. Preparation of Control and TS Over-Expressing Crude Bacterial Extracts

Control *E.coli* cells transformed with M15pREP[4] plasmid were grown under the same conditions as *E.coli* cells transformed with both M15pREP[4] and pQE-31-TS7 plasmids (as in the above section). Crude extracts of both bacterial cultures were prepared as described for preparation of crude H_6-TS (as in the above section).

4.4. RESULTS

4.4.1. Construction of plasmids capable of generating 3R and 2R TS RNAs

The entire cDNA of human TS for 3R and 2R allele was required to generate the respective full-length mRNAs (including the 5'-autoregulatory loop and the repeats in the 5'UTR) to test the TS autoregulation hypothesis. A cDNA clone was obtained from Dr. Santi. However, after sequencing it was found to be incomplete since it lacked the full-length 5'UTR. I therefore only utilized the coding-region from this clone and needed to generate the two alleles of the 5'UTR (3R and 2R). PCR analysis using Taq polymerase was used to amplify the complete 5'UTR from genomic DNA of white blood cells isolated from individuals that were homozygous for either 3R or 2R allele. Since the 5'UTR of TS is very GC-rich and Taq polymerase is error-prone, most of the originally amplified 5'UTR clones were found to contain errors. I eventually was able to modify the procedure to reduce the error rate of the Taq polymerase. As a result, I was able to generate error-free 5'UTR clones. Finally, full length TS cDNAs for 3R and 2R alleles were constructed and placed under the CMV promoter to generate p3R-TS and p2R-TS constructs. *In vitro* transcription of these constructs generated 3R and 2R RNAs (Fig. 4.2A). Both constructs produced similar levels of RNA. As control, full-length luciferase RNA was transcribed from CMV-pGL3 plasmid *(R.T. Cowling, Ph.D. thesis).*

4.4.2. Ability of 3R and 2R RNAs to generate TS protein by in vitro translation

The TS and luciferase RNAs were then examined for their ability to produce the respective proteins. *In vitro* translation of the 3R and 2R RNAs using rabbit reticulocyte lysate showed a 35 kD protein corresponding to the expected molecular mass of TS (Fig. 4.2B). The control luciferase RNA also produced an expected molecular mass protein product (~60 kD). To confirm that the 35 kD protein is in fact TS, western blot analysis was carried out on these *in vitro* translated products using an anti-TS polyclonal antibody. As shown in Fig. 4.3, the 35 kD protein products in the *in vitro* reactions of 3R and 2R RNAs were indeed TS. An unknown protein of higher mobility (~37 kD) also cross-reacted with the TS antibody. This protein was also present in the "No RNA" control (Fig. 4.3) suggesting that the anti-TS polyclonal antibody is cross-reacting with a 37 kD rabbit

FIGURE 4.2. *In vitro* transcription and translation of TS from 3R and 2R allele mRNA

A. *In vitro* transcription of mRNA from either no DNA or plasmids p3R-TS, p2R-TS or CMV-pGL3. The p3R-TS, p2R-TS and CMV-pGL3 encode for TS 3R-allele, TS 2R-allele and luciferase, respectively. Shown is the ethidium bromide-stained agarose gel indicating the positions of the plasmid DNA (band *a*) and the transcribed mRNA (band *b*). ***B.*** *In vitro* translation of protein from either no mRNA, positive control mRNA or the mRNAs transcribed in panel A. 175 ng of mRNA was translated using rabbit reticulate lysate and the [^{35}S]-labelled translated proteins were analyzed by electrophoresis and autoradiography. The positive control mRNA is a lucifierase mRNA supplied with the kit. Arrows indicate the bands for luciferase (~60 kD) and TS (~35 kD) proteins. Other details are in *Materials and Methods*.

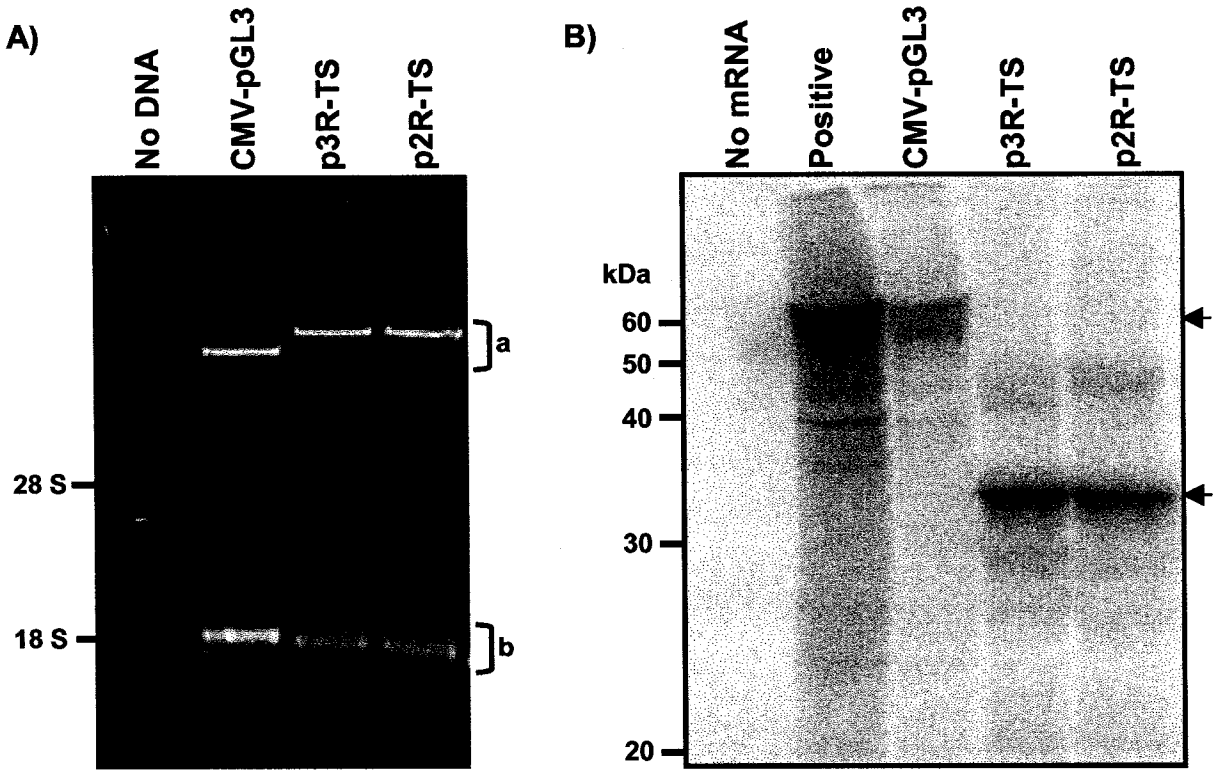
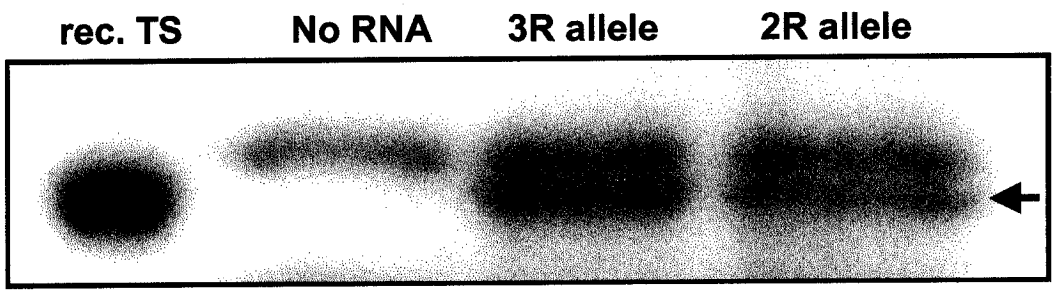

A)
No DNA
CMV-pGL3
p3R-TS
p2R-TS
28 S
18 S
a
b
B)
No mRNA
Positive
CMV-pGL3
p3R-TS
p2R-TS
kDa
60
50
40
30
20

FIGURE 4.3. Western blot analysis of *in vitro* translated TS protein from 3R and 2R allele mRNA

Rabbit reticulate lysate was used to translate either no mRNA or TS mRNA (3R or 2R) and analyzed by Western blot analysis using anti-TS antibody. Recombinant (rec.) TS is used as a size marker. *In vitro* translated TS is the lower band as indicated by the arrow. Other details are in *Materials and Methods*.


rec. TS
No RNA
3R allele
2R allele

reticulocyte lysate protein. Taken together, these results demonstrate that both 3R and 2R alleles of TS RNA are capable of producing human TS protein *in vitro*.

4.4.3. Linear range of in vitro translation reaction of TS

In order to reliably quantify any TS autoregulational difference between the 3R and 2R RNA, the linear range of the time course of *in vitro* translation reaction was determined. Luciferase was used as an internal control to normalize the TS signal. The *in vitro* translation reactions of both 3R and 2R RNAs appeared to be linear between 5 and 20 min (Fig. 4.4). After 20 min, the reactions reached a plateau. No apparent difference between the reactions of 3R and 2R RNA was observed. The 15 min reaction was used for future experiments to detect any TS autoregulational differences between the 3R and 2R RNA.

4.4.4. Ability of in vitro translated TS protein to inhibit its own translation

I next examined the ability of *in vitro* translated TS protein to inhibit the translation reaction of 3R and 2R RNA. Non-radiolabeled TS was generated from *in vitro* translation of either 3R or 2R RNA and used as an inhibitor. [^{35}S]-labeled TS protein was translated from 3R or 2R RNA in the absence or presence of the non-radiolabeled TS protein. Translation reactions containing non-radiolabeled TS produced significantly less [^{35}S]-labelled TS protein compared to reactions containing no non-radiolabeled TS (Fig. 4.5A). This suggested that *in vitro* synthesized TS protein is capable of inhibiting translation. Similar inhibition of translation was seen by either TS synthesized from 3R or 2R. Translation of 2R RNA appeared to be more effectively inhibited than the translation of 3R RNA ($p = 0.006$).

Binding of TS to its RNA can be prevented in the presence of substrate dUMP or inhibitor FdUMP (85). Since the ability of the *in vitro* synthesized TS to inhibit translation (Fig. 4.5) is believed to be due to binding to its RNA, I examined whether the presence of dUMP or FdUMP would have an effect on the translation reaction. A significant increase in the *in vitro* translation of TS RNA was seen in the presence of either dUMP or FdUMP (Fig. 4.6). In addition, a TS ternary complex representing TS-FdUMP-CH_2THF was seen in the presence of FdUMP. Thus, these results indicate that *in vitro* synthesized TS was capable of interacting with its substrate and forming a ternary complex both of which can relieve its inhibitory effect on translation.

FIGURE 4.4. Time-dependent *in vitro* translation of 3R and 2R mRNA of TS

3R or 2R allele TS mRNA was translated in the presence of luciferase mRNA as an internal control for the indicated periods of time using rabbit reticulate lysate. [^{35}S]-labelled translated proteins were analyzed by electrophoresis and autoradiography, and the band intensities corresponding to the TS and luciferase proteins were quantified. Shown are the intensity pixel ratios between TS and luciferase. Bars represent mean ± SD. Other details are in *Materials and Methods*.

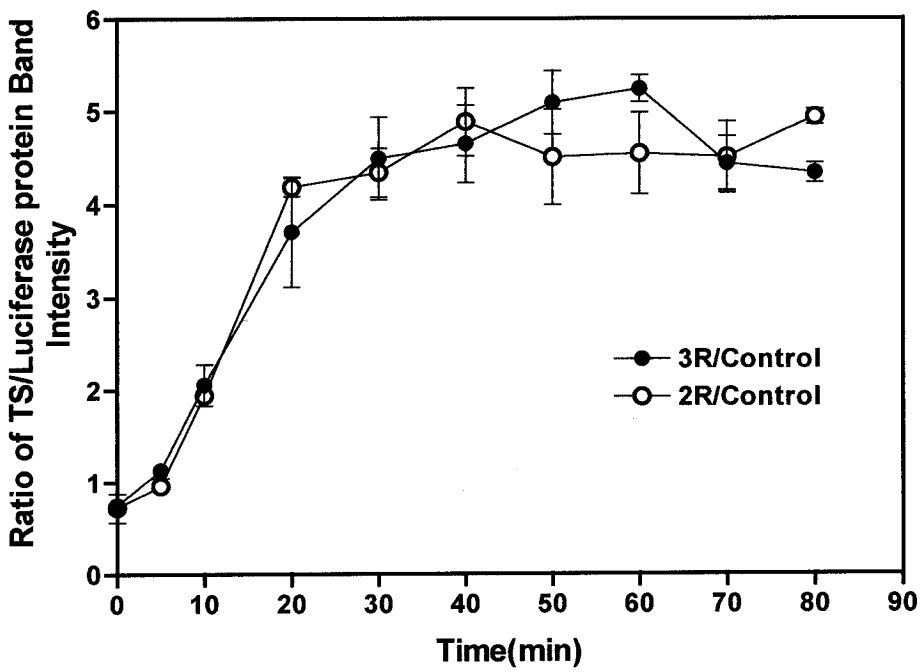

Ratio of TS/Luciferase protein Band Intensity
Time(min)
3R/Control
2R/Control
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90

FIGURE 4.5. Effect of adding "cold" TS protein to *in vitro* translation reaction of 3R and 2R mRNAs

A. [^{35}S]-labeled TS was *in vitro* translated from either no RNA or mRNA of 3R or 2R in the absence or presence of non-radiolabeled "cold" *in vitro* translated TS protein. The "cold" protein was translated from either 3R or 2R mRNA. Translation was carried out for 15 min. Each bar represents mean ± standard error two independent autoradiographs. Asterisks (**, $p<0.01$; ***, $p<0.001$) on the "cold" TS-treated bars indicate significant difference (two-tailed *t* tests) compared to corresponding untreated bars. ***B.*** Western blot analysis of non-radiolabeled translated TS. Recombinant (rec.) TS is used as a size marker. *In vitro* translated TS is the lower band as indicated by the arrow. Other details are in *Materials and Methods*.

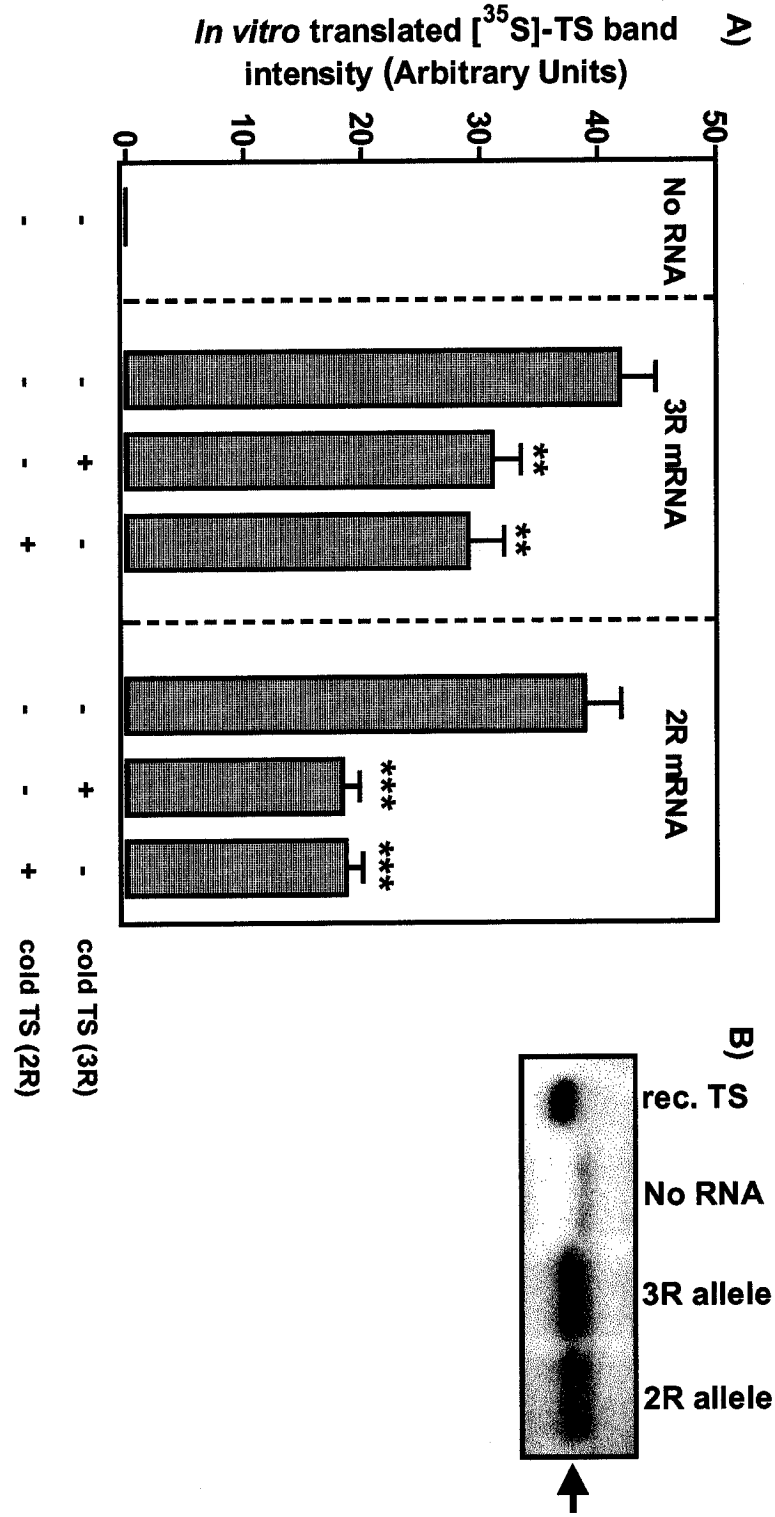
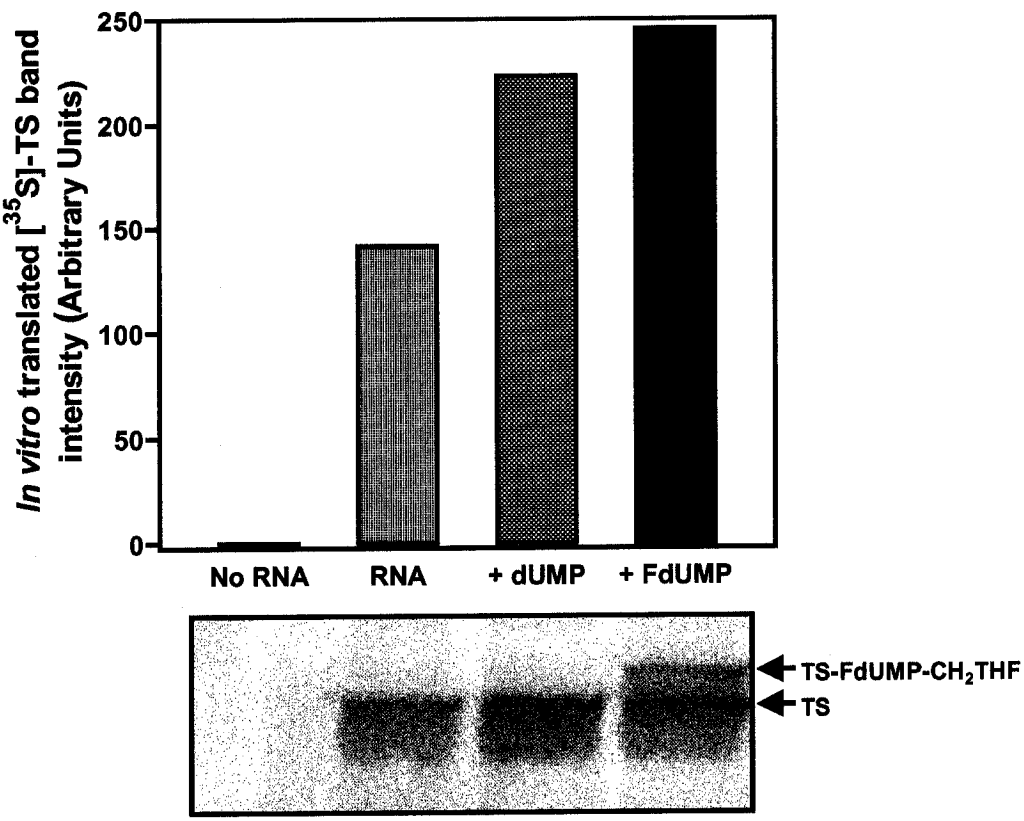

A)
In vitro translated [^{35}S]-TS band intensity (Arbitrary Units)
0
10
20
30
40
50
No RNA
3R mRNA
2R mRNA
**
**
***
***
cold TS (3R)
cold TS (2R)
B)
rec. TS
No RNA
3R allele
2R allele

FIGURE 4.6. Effect of adding dUMP or FdUMP to *in vitro* translation of TS protein

In vitro translation of TS was carried out using rabbit reticulate lysate in the absence or presence of 30 μM of dUMP or FdUMP. The [^{35}S]-labelled translated proteins were analyzed by electrophoresis and autoradiography (lower panel), and the band intensities corresponding to the TS protein was quantified (upper panel). Bottom panel also shows the 2 forms of TS bands: free (TS) or ternary complexed (TS-FdUMP-CH_2THF). The mRNA used for translation was transcribed from p2R-TS construct. Other details are in *Materials and Methods*.


In vitro translated [35S]-TS band intensity (Arbitrary Units)
250
200
150
100
50
0
No RNA
RNA
+ dUMP
+ FdUMP
TS-FdUMP-CH2THF
TS

4.4.5. *Enzymatic activity of in vitro translated TS*

To further characterize the *in vitro* synthesized TS, I examined its enzymatic activity. The activity was measured in rabbit reticulocyte lysate containing either no RNA or *in vitro* translated TS from 2R RNA. (Note: this assay was carried out after optimization, see Fig. 4.9). Surprisingly, a high level of TS activity was endogenously present in the rabbit reticulocyte lysate and this activity was higher than the activity present in an equivalent amount of protein from HeLa cells (Fig. 4.7). No significant difference in the TS activity between *in vitro* translation reaction containing "No RNA" and TS RNA was seen (Fig. 4.7). Thus, due to the high level of the endogenous TS activity present in the rabbit reticulocyte lysate, I was unable to conclude if the *in vitro* synthesized TS was active.

4.4.6. *Induction and expression of recombinant H_6-TS in E. coli*

Our lab has previously expressed a recombinant TS with a six histidine (H_6) affinity tag at the N-terminus (Fig. 4.8A) in *E. coli* and purified it on Ni-NTA column under denaturing conditions (95). However, a soluble TS is required for RNA-binding and autoregulation. I therefore examined various induction times with IPTG to identify the one that will give the most soluble H_6-TS protein. Analysis of the soluble and insoluble protein fractions by Coomassie blue staining showed a continual increase in a 36 kD protein expressed in both fractions with increased periods of IPTG induction (Fig. 4.8B). To more clearly examine the 36 kD band and confirm that it was H_6-TS, I analyzed the soluble and insoluble fractions by western blot analysis (Fig. 4.8D). The western blotting results were consistent with the Coomassie blue staining of the 36 kD protein, suggesting that it was in fact H_6-TS. The results from Fig. 4.8 B and D show that while the levels of insoluble H_6-TS seem to "plateau" off at around 150 min of IPTG induction, the levels of *soluble* H_6-TS still increased at 180 min. Thus, 180 min of IPTG induction appears to contain a higher ratio of soluble-to-insoluble H_6-TS than the 150 min induction and was used for further experiments.

4.4.7. *Enzymatic activity of recombinant H_6-TS in E. coli extracts*

I next set out to examine the various IPTG induced fractions (from Fig. 4.8 B) to identify the one with the highest TS activity as measure of correct confirmation of the protein. Firstly, a TS activity assay was established and optimized using one of the

FIGURE 4.7. Enzymatic activity of *in vitro* translated TS protein

TS activity was measured in rabbit reticulate lysate translating either no mRNA or TS mRNA (2R). Positive is a control extract containing recombinant TS protein. One unit of activity represents production of 1 μmol of product ([^{3}H]-water) per min. Bars represent mean ± SD. Other details are in *Materials and Methods*.

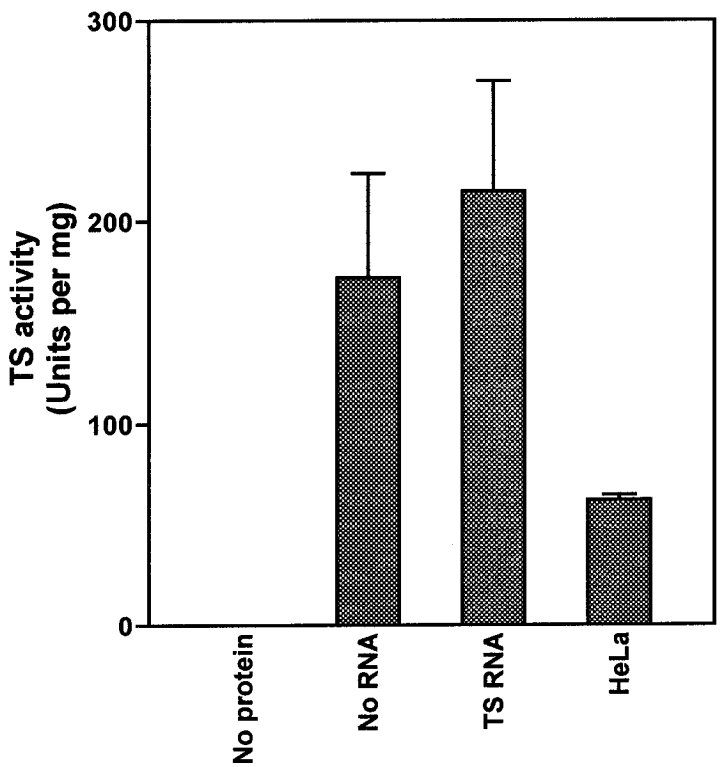
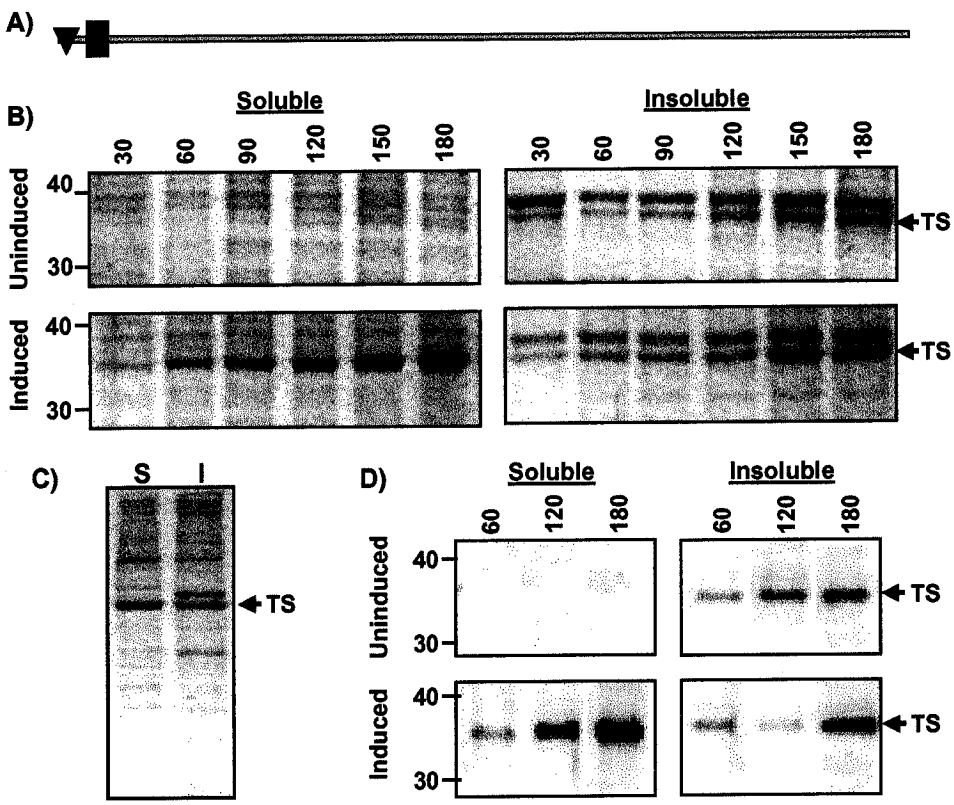

300
200
100
0
TS activity
(Units per mg)
No protein
No RNA
TS RNA
HeLa

FIGURE 4.8. Induction and expression of recombinant H_6-TS in *E. coli*

A. Recombinant human TS containing a 6-histidine tag (▼, H_6) and 10 additional amino acids (▮). ***B.*** Coomassie blue-based detection of a 36 kD protein (corresponding to H_6-TS) in the soluble and insoluble fractions of *E. coli* either not induced or IPTG-induced for 30, 60, 90, 120, 150 or 180 min. ***C.*** Coomassie blue-stained gel showing all the proteins (including H_6-TS) in the soluble (S) and insoluble (I) fractions after IPTG-induction for 150 min. (D) Western blot analysis for TS in the soluble and insoluble fractions from *E. coli* either not induced or IPTG-induced for 60, 120 or 180 min. Other details are in *Materials and Methods*.


A)
B)
Soluble
Insoluble
30
60
90
120
150
180
Uninduced
Induced
40
30
TS
C)
S
I
TS
D)
Soluble
Insoluble
60
120
180
Uninduced
Induced
40
30
TS

IPTG-induced crude *E. coli* extract containing H_6-TS protein. As seen in Fig. 4.9, both a time-dependent and protein-dependent quasi-linear relationship with TS activity was obtained to identify the quasi-linear range of the assay. I then used the optimal conditions to analyze the soluble and insoluble fractions of both IPTG-induced and uninduced *E. coli* extracts for TS activity. With an increase in IPTG induction period, a gradual increase in TS activity was seen (Fig. 4.10A and B), which was consistent with the results of Fig. 4.8. Although the non-IPTG induced cells also showed TS activity, representing *E. coli* TS and "leaky" H_6-TS production, the level of TS activity in the IPTG-induced cells was about 10-fold higher (Fig. 4.10A). Comparison of soluble and insoluble fractions showed that H_6-TS in the soluble fraction is much more active than in the insoluble fraction in IPTG-induced cells (Fig. 4.10B). To confirm the solubility of H_6-TS, the soluble fraction was ultracentrifuged. No significant loss of TS activity was seen after ultracentrifugation suggesting the H_6-TS is in fact soluble (Fig. 4.10B). One limitation was that soluble H_6-TS was best if freshly prepared; a considerable amount of activity was lost after only 3 d of storage at 0 °C (Fig. 4.10C).

4.4.8. Affinity purification of H_6-TS from crude E. coli extracts

Using conditions that gave the most soluble and enzymatically active H_6-TS, IPTG-induced *E. coli* extracts were prepared and used for H_6-TS purification. The H_6-TS in the extracts was affinity purified on a Ni-NTA column. As shown in Fig. 4.11A, while the 36 kD H_6-TS band was present in the crude *E. coli* extracts, it was absent in the flow through from the column. This suggested that majority of the H_6-TS protein efficiently bound to the column. The band was also absent in the 25 mM imidazole washes and could be eluted in a soluble form in 250 mM imidazole (Fig. 4.11A). The imidazole was removed by passing the Ni-NTA-purified H_6-TS proteins onto a PD-10 buffer exchange column (Fig. 4.11B).

4.4.9. Enzymatic activity of purified H_6-TS as a measure of its native confirmation

Enzymatic activity of purified H_6-TS was determined to see if it survived purification on Ni-NTA and PD-10 columns. As Fig. 4.12A shows, H_6-TS purified on Ni-NTA column and passed through and PD-10 buffer exchange column still had TS activity. The activity in Ni-NTA-purified fraction was enriched by 26-fold compared to activity in the crude *E. coli*

FIGURE 4.9. Optimization of TS enzymatic activity assay

Relationship between TS activity versus both time (panel ***A***, 6 μg protein) and amount of protein (panel ***B***, 60 min) in IPTG-induced soluble crude *E. coli* fraction. Other details are in *Materials and Methods*.

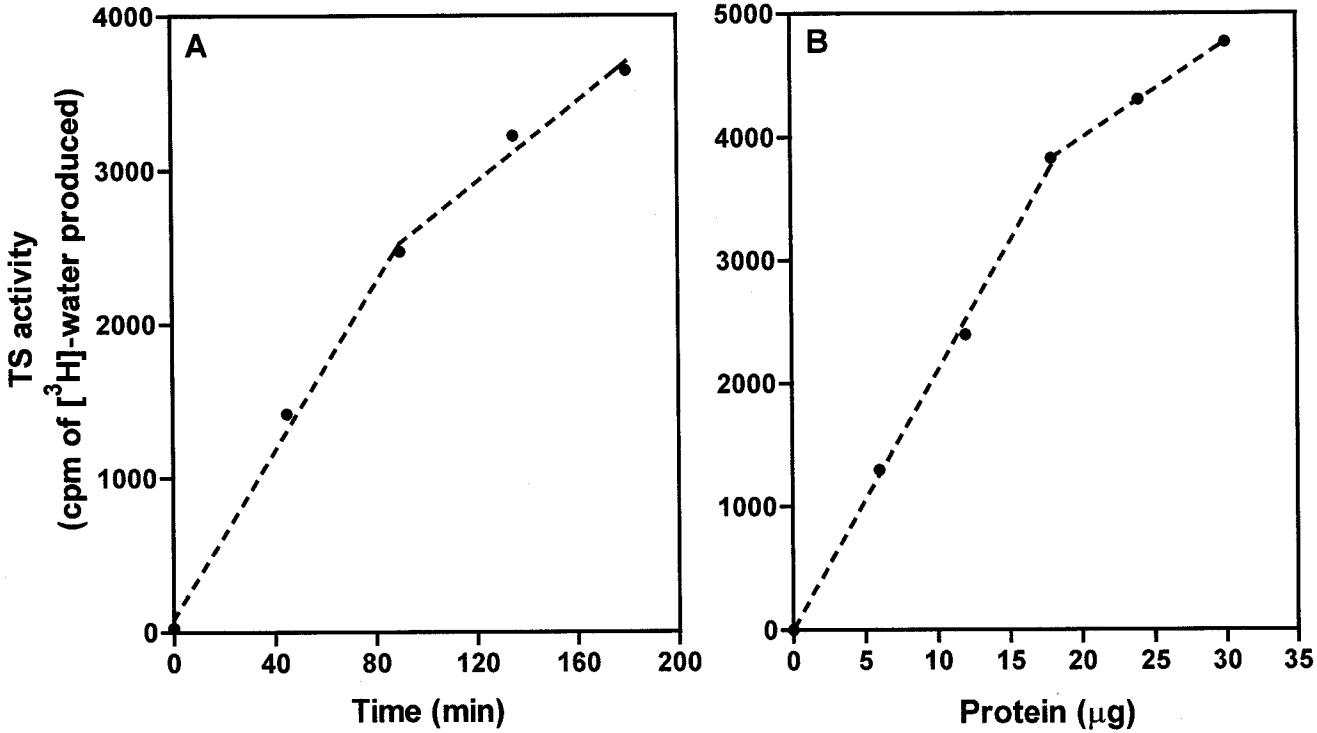

A
B
TS activity
(cpm of [^{3}H]-water produced)
Time (min)
Protein (μg)
0
1000
2000
3000
4000
5000
0
40
80
120
160
200
0
5
10
15
20
25
30
35

FIGURE 4.10. TS activity in crude extracts of *E. coli* expressing recombinant H_6-TS

A. Activity in ultracentrifuged fractions of various IPTG-induced or uninduced extracts. ***B.*** Activity in soluble, soluble-ultracentrifuged (UC) and insoluble fractions of various IPTG-induced soluble extracts. ***C.*** Effect of 0 °C storage on TS activity in ultracentrifuged fractions of various IPTG-induced extracts. Equal amounts of protein were used for each extract (6 μg). Bars represent mean ± SD. Other details are in *Materials and Methods*.

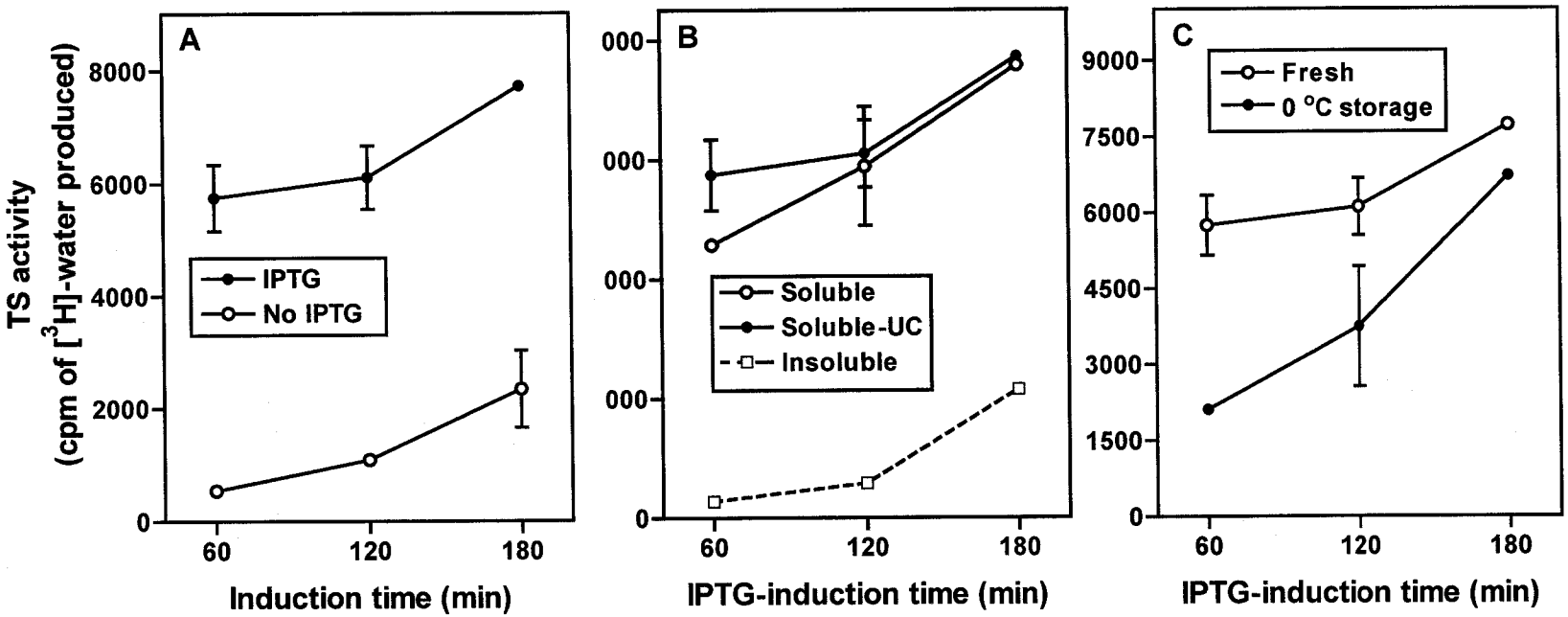
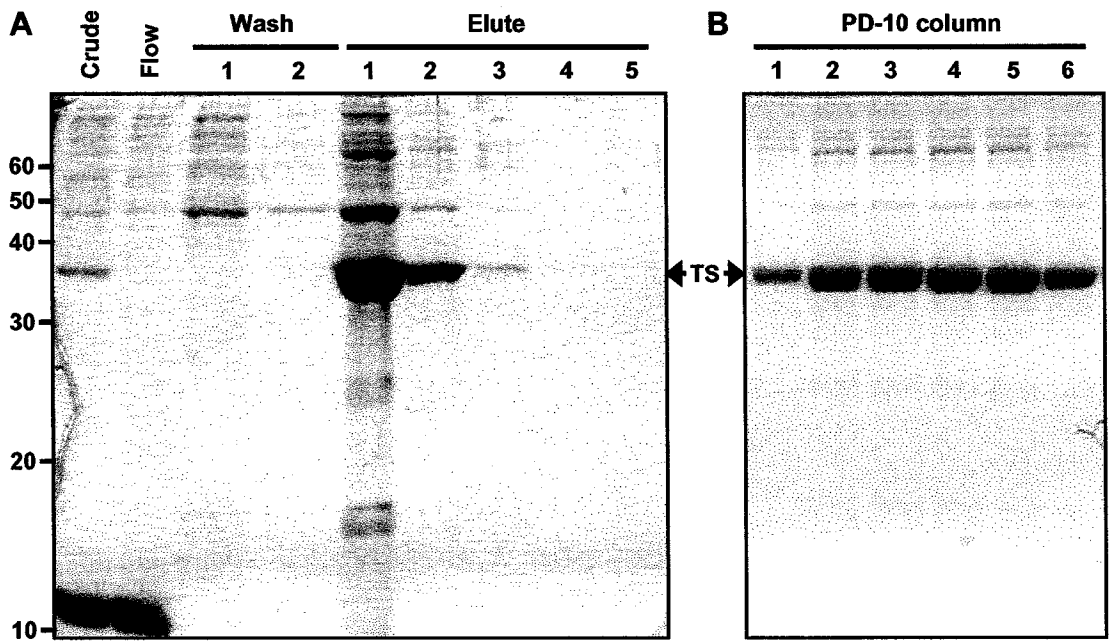

A
TS activity
(cpm of [3H]-water produced)
8000
6000
4000
2000
0
IPTG
No IPTG
60
120
180
Induction time (min)
B
000
000
000
000
0
Soluble
Soluble-UC
Insoluble
60
120
180
IPTG-induction time (min)
C
9000
7500
6000
4500
3000
1500
0
Fresh
0 °C storage
60
120
180
IPTG-induction time (min)

FIGURE 4.11. Purification of recombinant H_6-TS from crude *E. coli* extracts

A. Coomassie blue-stained gel showing Ni-NTA column-based affinity purification of H_6-TS from crude soluble extracts of IPTG-induced *E. coli*. Lanes shown are of crude extract, flow through, column washes in 25 mM imidazole and eluted fractions in 250 mM imidazole. ***B.*** Coomassie blue-stained gel showing PD-10 column-based buffer exchange of the Ni-NTA-purified H_6-TS. Shown are the 5 fractions eluted in buffer 10 mM MOPS, 10% glycerol, pH 7.4. Other details are in *Materials and Methods*.


A
Crude
Flow
Wash
Elute
1
2
1
2
3
4
5
60
50
40
30
20
10
TS
B
PD-10 column
1
2
3
4
5
6

fraction. In addition, the PD-10 buffer exchange column did not significantly affect the activity of the Ni-NTA-purified H_6-TS. Thus, purified H_6-TS still had enzymatic activity.

I next set out to determine the percentage of enzymatically active protein in the Ni-NTA-purified H_6-TS extract. As controls, I used HeLa cells and HeLa *clone 22* overexpressing TS (details of which are described below in Fig. 4.14). Western blot analysis was used to estimate the percentage of active TS protein in an extract with the assumptions that (i) HeLa cells and clone 22 contain 100% active TS and (ii) the western blot signal is linear. Comparison of the crude *E. coli* and clone 22 extracts by western blot analysis showed about 1 : 1 ratio of TS intensity for the two extracts, while a 1 : 1.5 ratio is seen for TS activity (Fig. 4.12B). From this I estimated that ~67% of H_6-TS is active in the crude *E. coli* extract. Similarly, comparison of the crude *E. coli* and Ni-NTA-purified H_6-TS extracts by western blot analysis shows about 1 : 5 ratio of TS intensity for the two extracts, while a 1 : 2.6 ratio is seen for TS activity (Fig. 4.12B). From this I estimated that H_6-TS in crude *E. coli* extract lost ~50% of its activity after Ni-NTA purification. Thus, since 67% of H_6-TS was estimated to be active in crude *E. coli* extract and 50% of it lost the activity after purification, I estimated that only about 33% of Ni-NTA-purified H_6-TS protein is active.

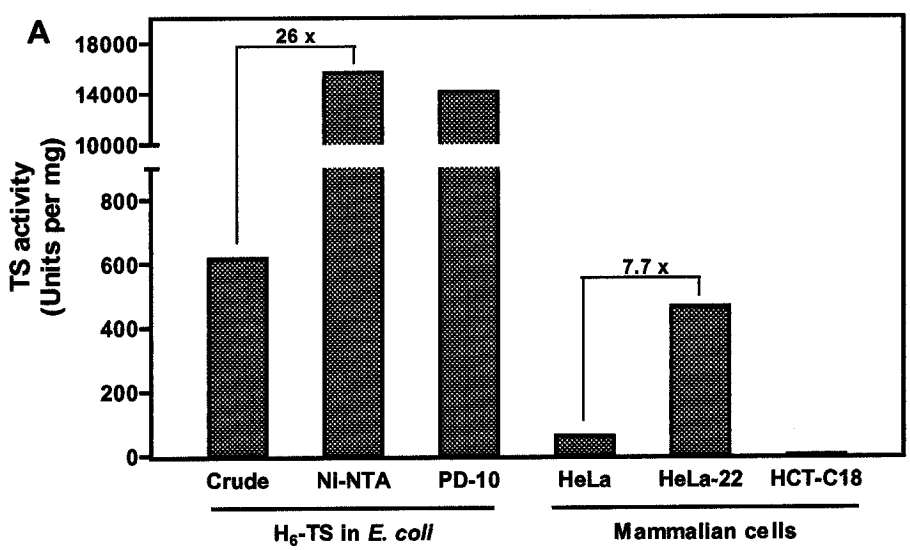
4.4.10. Ability of pure recombinant H_6-TS to inhibit in vitro translation of TS RNA

To examine the autoregulating capability of pure recombinant H_6-TS, *in vitro* translation of TS RNA was carried out in the presence of various amounts of H_6-TS protein. The H_6-TS protein was pre-treated with DTT to keep it in reduced state so it can bind RNA (36). Addition of increasing amount of H_6-TS protein showed a significant dose-dependent inhibition in the *in vitro* translation of TS RNA while buffer-alone or buffer containing DTT had no significant effect (Fig. 4.13A). This suggested that purified H_6-TS was capable of inhibiting translation.

I next *indirectly* tested whether this inhibition was due to RNA-binding activity of H_6-TS. Since RNA-binding activity of TS can be prevented in the presence of its substrates, I examined whether dUMP can prevent the H_6-TS-dependent translational inhibition seen in Fig. 4.13A. Addition of dUMP significantly enhanced the translation of TS (Fig. 4.13B), which was similar to that seen before (see Fig. 4.6). In the experiment of Fig. 4.13B, 0.4 μg H_6-TS failed to inhibit translation to the same extent seen in Fig. 4.13A, likely due to the loss

FIGURE 4.12. Enzymatic activity and western blot analysis for TS in crude and purified H_6-TS extracts and mammalian cell extracts

A. Specific activity of TS in crude, Ni-NTA and PD-10 purified extracts from IPTG-induced *E. coli* and in HeLa, HeLa stably overexpressing transfected TS (HeLa-22) and HCT-C18 cell extracts. One unit of activity represents production of 1 μmol of product ([^{3}H]-water) per min. HCT-C18 is a negative control cell-line. (B) Western blot analysis for TS in crude (5 μg protein) and Ni-NTA purified (0.5 μg prtoein) extracts from IPTG-induced *E. coli* and in HeLa (10 μg protein) and HeLa stably overexpressing transfected TS (HeLa-22) (10 μg protein). TS activity loaded per lane (relative to crude) is indicated on each lane. Values for both panels ***A*** and ***B*** were in the linear range of the standard curve. Other details are in *Materials and Methods*.


A
18000
14000
10000
800
600
400
200
0
TS activity
(Units per mg)
26 x
7.7 x
Crude
NI-NTA
PD-10
HeLa
HeLa-22
HCT-C18
H6-TS in E. coli
Mammalian cells

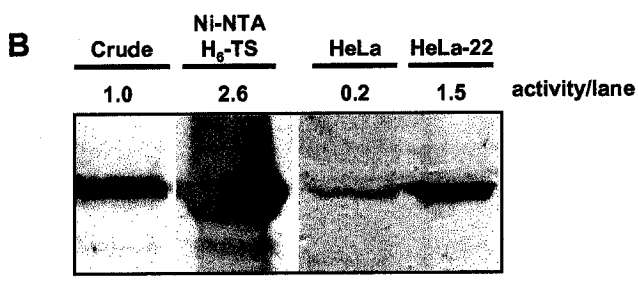
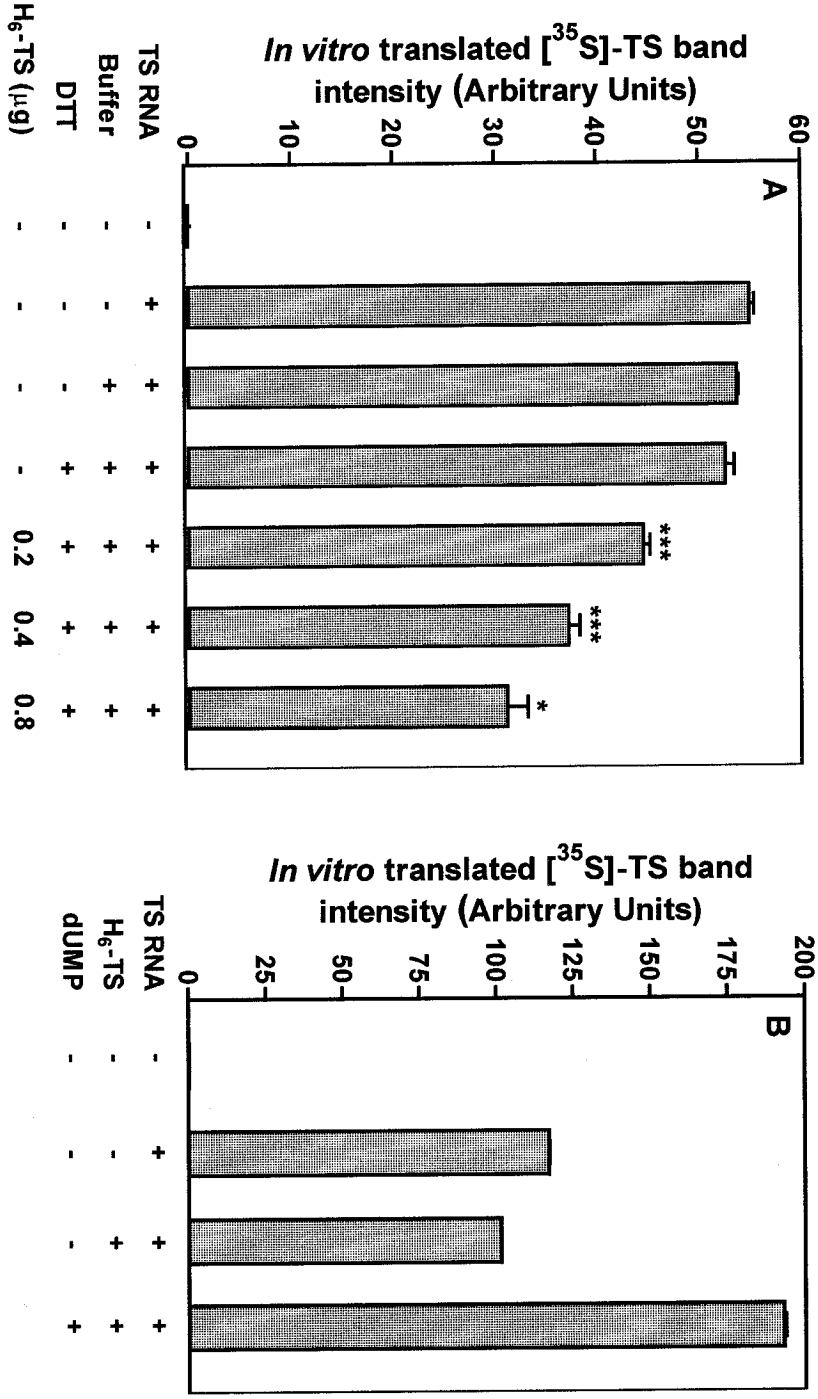

B
Crude
Ni-NTA
H6-TS
HeLa
HeLa-22
1.0
2.6
0.2
1.5
activity/lane

FIGURE 4.13. Effect of adding recombinant H_6-TS-alone or in the presence of dUMP to *in vitro* translation of TS protein

A) Effect of adding various amounts of H_6-TS (0.2-0.8 μg in buffer containing DTT) to the *in vitro* translation of [^{35}S]-labelled protein from TS mRNA. The effect of buffer and DTT is also shown. Translation was carried out for 15 min. Bars represent mean ± SD. Asterisks (*, $p<0.05$ and ***, $p<0.001$) on the H_6-TS-treated bars indicate significant difference (two-tailed *t* tests) compared to DTT buffer-treated bar. (B) Effect of adding H_6-TS (0.4 μg) in the presence of dUMP (30 μM) to the *in vitro* translation of [^{35}S]-labelled protein from TS mRNA. The mRNA used in both panels was transcribed from p2R-TS construct. Other details are in *Materials and Methods*.


A
In vitro translated [^{35}S]-TS band intensity (Arbitrary Units)
0
10
20
30
40
50
60
TS RNA
Buffer
DTT
H_6-TS (μg)
-
+
+
+
+
+
+
-
-
-
-
0.2
0.4
0.8
***
***
*
B
In vitro translated [^{35}S]-TS band intensity (Arbitrary Units)
0
25
50
75
100
125
150
175
200
TS RNA
H_6-TS
dUMP
-
+
+
+

of functionality after 3 d storage at 0 °C (see Fig. 4.10C). Some of the enhanced translation seen on dUMP addition was likely due to relief of inhibition by binding of *in vitro* translated TS, not the added recombinant H_6-TS, to the mRNA.

4.4.11. Development of TS-overexpressing HeLa cell lines

Since the Ni-NTA-purified H_6-TS expressed in *E. coli* was only partially active and gave inconsistent results, I also overexpressed TS in mammalian cells. HeLa cells were used and stably transfected with p3R-TS construct. Stable G418-resistant clones were isolated and examined for TS levels by western blot analysis. As shown in Fig. 4.14, several clones showed high TS levels compared to parental HeLa cells. Clone 22 was among the highest producing clones and used for further analysis. Examination of the TS enzymatic activity showed that this clone had about 7.7-fold higher activity than the parental HeLa cells (see Fig. 4.12).

4.4.12. RNA-binding ability of TS from various sources

To directly test whether H_6-TS could bind to TS RNA, an electromobility shift assay was utilized. *In vitro*-transcribed TS RNA was radiolabeled and either untreated or treated with RNase in the absence or presence of different amounts of H_6-TS protein and analyzed by gel electrophoresis and autoradiography. As shown in Fig. 4.15, addition of increasing amount of H_6-TS protein showed an apparent increase in an RNase-protected product. In addition, less amount of RNase-digested TS RNA was seen when 0.8 μg H_6-TS was added. Although the quality of the electromobility shift assay was rather poor, an H_6-TS-dependent trend was seen, likely due to H_6-TS binding to the TS RNA.

A significant amount of time was spent trying to optimize conditions for the electromobility shift assay to work. Several different conditions were tried including (1) cross-linking of RNA-protein complexes; (2) use of crude *E. coli* IPTG-induced extracts since it was found to contain 50% more TS activity than purified H_6-TS (Fig. 4.16); (3) use of HeLa clone 22 overexpressing TS since it is assumed to contain 100% active TS (Fig. 4.16); (4) use of control luciferase RNA in an attempt to show specificity (Fig. 4.16); (5) use of a 23-nucleotide synthetic RNA oligomer spanning the 5' mRNA-binding region for

FIGURE 4.14. HeLa cells stably overexpressing transfected TS

Western blot analysis for TS (***A***) and Coomassie blue-staining (***B***) of HeLa cells, HCT-C18 and seventeen G418-resistant stably transfected HeLa clones. The transfection was done with a TS-expressing construct containing a neomycin-resistant marker gene (p3R-TS). Other details are in *Materials and Methods*.

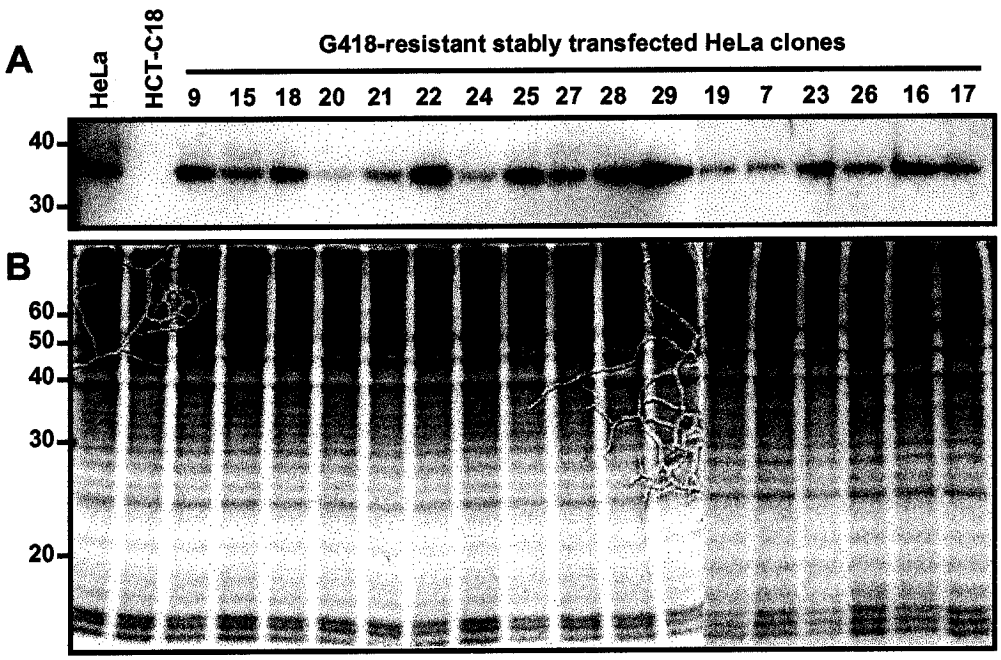

A
HeLa
HCT-C18
G418-resistant stably transfected HeLa clones
9 15 18 20 21 22 24 25 27 28 29 19 7 23 26 16 17
40
30
B
60
50
40
30
20

FIGURE 4.15. mRNA-binding ability of Ni-NTA-purified H_6-TS as analyzed by electromobility shift assay

In vitro-transcribed TS mRNA was [^{32}P]-labelled and either untreated or treated with RNase (1 unit) in the absence or presence of different amounts of H_6-TS (0.2, 0.4 or 0.8 μg). These were electrophoresed and analyzed by autoradiography. The RNA used was transcribed from p2R-TS construct. Other details are in *Materials and Methods*. Shown on the right are three possible scenarios of the signal observed. 1) naked mRNA, 2) RNase-digested mRNA and 3) TS-bound mRNA (mobility shift).

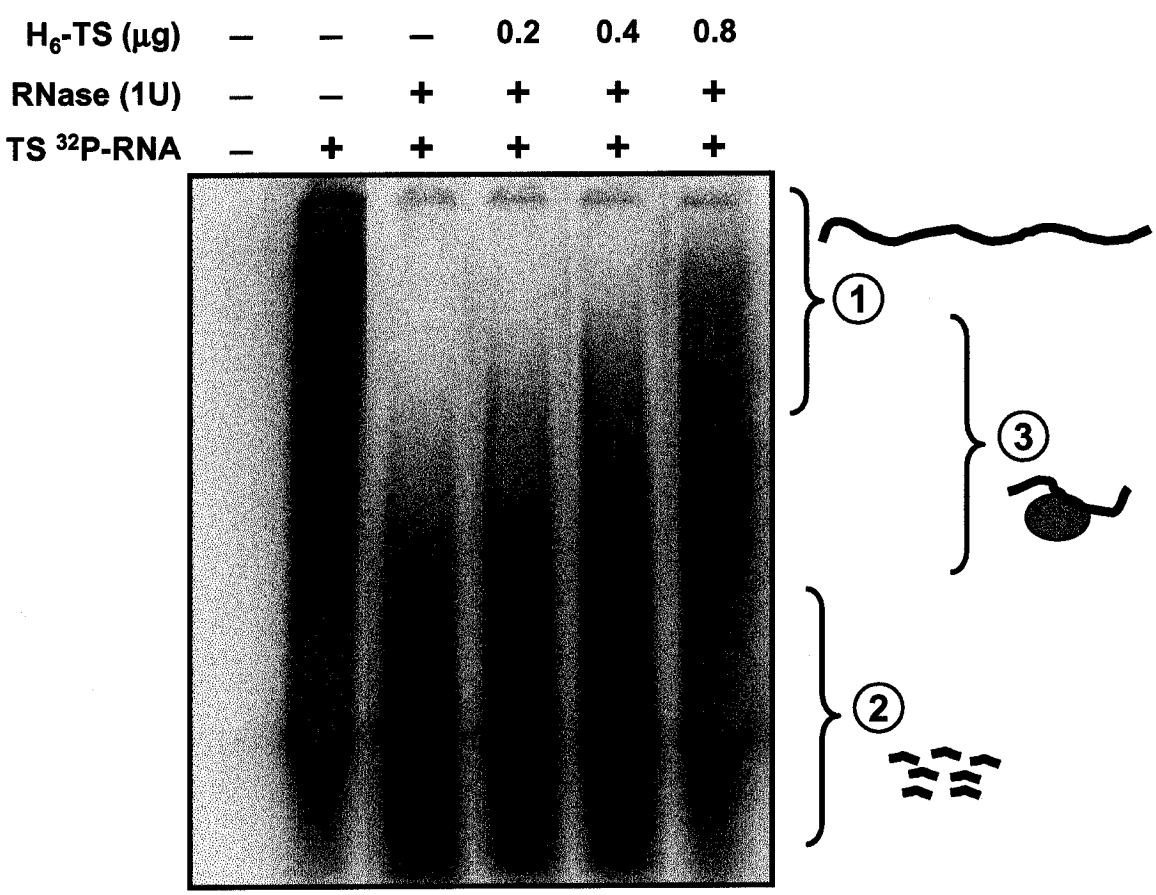
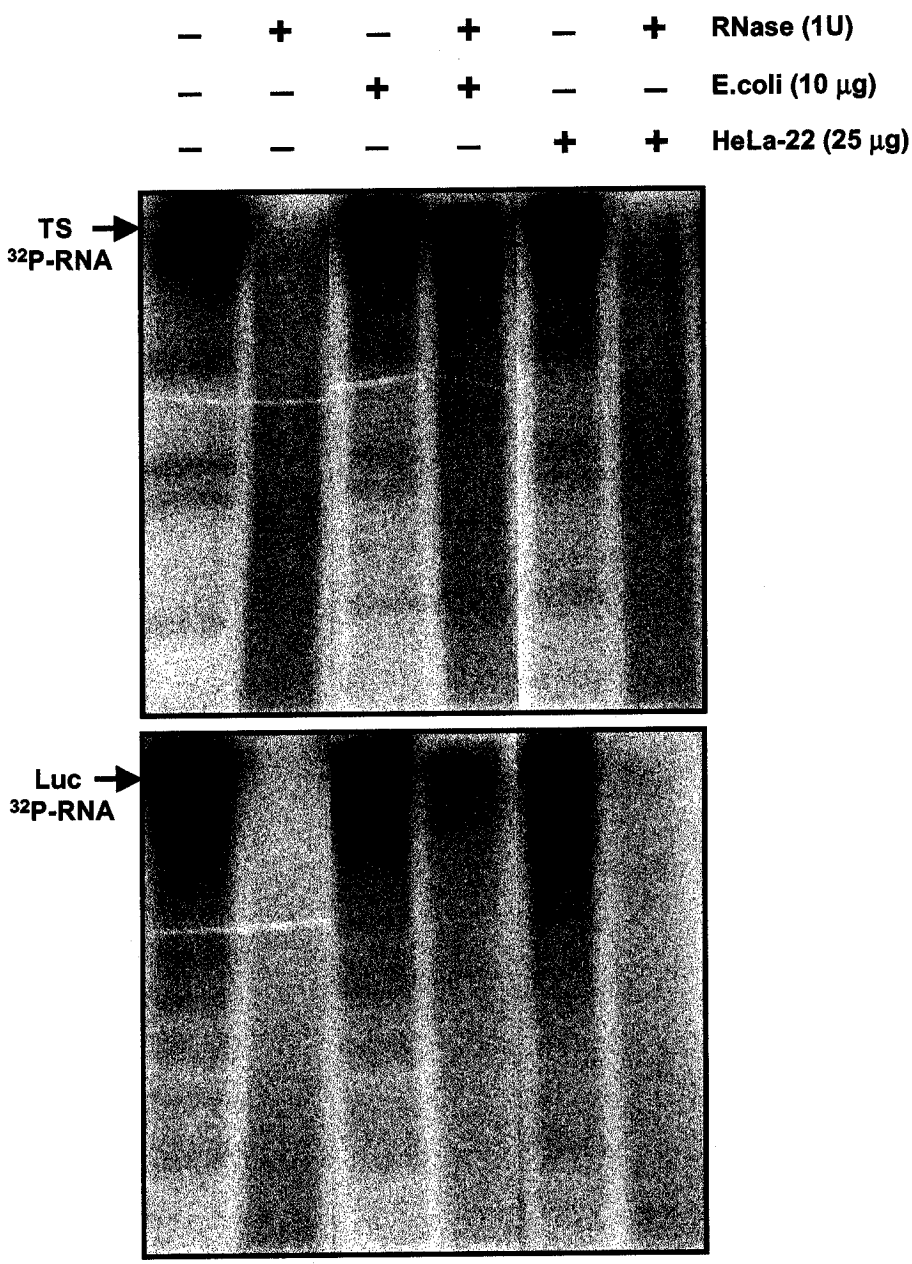

H6-TS (μg) – – – 0.2 0.4 0.8
RNase (1U) – – + + + +
TS 32P-RNA – + + + + +
1
3
2

FIGURE 4.16. mRNA-binding ability of proteins in cell-extracts containing overexpressed TS as analyzed by electromobility shift assay

In vitro-transcribed TS mRNA (top panel) or Luciferase mRNA (bottom panel) was [^{32}P]-labelled and either not incubated or incubated with either crude soluble extract of IPTG-induced *E. coli* or with extract of HeLa-22. These were cross-linked and either not treated or treated with RNase (1 unit). The samples were electrophoresed and analyzed by autoradiography. The mRNA used for TS was transcribed from p2R-TS construct. Other details are in *Materials and Methods*.


– + – + – + RNase (1U)
– – + + – – E.coli (10 μg)
– – – – + + HeLa-22 (25 μg)
TS
32P-RNA
Luc
32P-RNA

TS (149) (as oppose to full-length mRNA). However, none of the conditions tried produced convincing evidence of a well-resolved electromobility shift representing TS-RNA complex.

4.5. DISCUSSION

TS is able to regulate its own expression at the translational level (35) (Fig. 4.1A). The mechanism of this auto-regulation involves binding of TS to its own mRNA and inhibition of protein synthesis. One of the binding site of TS in mRNA is present in the sequences between position -19 and +17 that includes a part of the 5'UTR, start codon and a part of protein-coding region. This site is predicted to form a stem-loop structure and TS is believed to bind to the loop (37) stabilizing the structure and inhibiting translation. The 5'UTR of the mRNA of TS also has unique tandemly repeated sequences and is polymorphic in the number of the repeats (41). The most commonly observed are either three (3R) or two (2R) tandem-repeats proceeded by a complementary reverse repeat (Fig. 4.1B). The last of the repeats in either 3R or 2R mRNA (position -8 and -41) overlaps with the putative stem-loop structure. Horie *et al* have previously shown that expression of a reporter gene is 2.6-fold higher when linked to 5'UTR of 3R than 2R mRNA in HeLa cells (41). Due to the proximity and partial overlap of the repeats to TS-binding site in the 5'UTR of TS mRNA, I proposed that the difference in 3R and 2R expression seen by Horie *et al* was due to differences in translational auto-regulation by TS in the two alleles. In this chapter, I set out examine this difference. At the time this study was started (1998), there was no evidence that TS translational autoregulation contributed to this difference.

As a starting point, two constructs were developed: one capable of expressing 3R mRNA and the other 2R mRNA of TS. It was crucial that the mRNA contain error-free repeats, stem-loop structure and TS protein-coding region in order to examine TS autoregulation. However, since the 5'UTR is very rich in GC content, a higher than usual number of errors was incorporated by Taq polymerase in the PCR reaction. This was especially the case for developing the construct for 3R allele. A considerable amount of time was spent to correct this technical problem. For this reason, I was unable to completely characterize TS auto-regulation in the 3R mRNA and most of the results were carried out on the 2R mRNA (Fig. 4.13, 4.15, 4.16). I was, however, eventually able to generate error-free 3R and 2R constructs by modifying the PCR conditions. The constructs were able to generate 3R and 2R mRNA of TS, and *in vitro* translation of both these mRNAs were able to produce human TS protein.

While determining the linear range of the *in vitro* translation reaction, I observed that the reaction reach a "plateau" after 20 min. This could be due to substrate depletion, inactivation of translational machinery and/or auto-regulation by the translated TS. The third scenario was *not* expected to be true since Chu and co-workers (85) had found that their *in vitro* translated TS was inactive and unable to inhibit its translation. However, further characterization of the *in vitro* synthesized TS in my experiment showed that it was in fact partially functional. My *in vitro* translated TS was found to be capable of inhibiting its own translation. This *indirectly* implicated that this TS has RNA-binding activity.

Further confirmation of the functionality of *in vitro* translated TS came from its treatment with substrate and substrate analogues. Previous studies showed that TS bound to its mRNA could be released from the mRNA in the presence of its substrate (e.g., dUMP) or inhibitor (e.g., 5-FdUMP) (85), augmenting TS translation. I showed that the presence of either dUMP or 5-FdUMP was able to enhance the level of *in vitro* translation, suggesting that my *in vitro* synthesized TS was capable of interacting with its substrates. In fact, this was confirmed by the formation of a ternary complex representing TS-FdUMP-CH_2THF by the [^{35}S]-labeled translated TS. Thus, interaction with dUMP and 5-FdUMP and enhancement of its translation is consistent with the RNA-binding ability of the *in vitro* translated TS.

I was unable to conclude if the *in vitro* translated TS was enzymatically active due to a high level of endogenous TS activity present in the rabbit reticulocyte lysate. It is possible that TS protein can interact with its substrate but be incapable of full enzymatic activity (i.e. unable to make product). However, it is unknown if enzymatic activity is required for TS to bind its mRNA. The high level of endogenous TS activity could be due to rabbit TS protein present in the reticulocyte lysate. In fact, our polyclonal anti-human TS antibody did cross-react with a 37 kDa band on western blots (Fig. 4.3). The molecular mass of rabbit TS is unknown since it has not been cloned. If an active rabbit TS is present in the lysates then this does complicate interpretation of some of the results.

A pure, soluble and enzymatically active human TS protein was required to more reliably understand the mechanism of TS auto-regulation and RNA-binding. This TS would allow me to examine the effect of TS-alone and not other factors in the reticulocyte lysate (e.g. endogenous TS). In addition, this TS was useful in Chapter 1 for antibody competition

studies. Our lab previously expressed recombinant H_6-TS in *E. coli* and purified it under denaturing conditions (95), which rendered it insoluble in non-denaturing buffer. Insoluble protein may non-specifically inhibit *in vitro* translation reactions by aggregating with the translational machinery. I therefore changed the expression and induction conditions and found the best one for expressing *soluble* H_6-TS. About two-thirds of human H_6-TS in *E. coli* was believed to be active. However, after affinity purification on Ni-NTA column, about half of the active protein became inactive. That is, only one-third of *purified* H_6-TS was believed to be active. One possibility is that H_6-TS requires other cellular factor(s) to be in the active state. Alternatively, H_6-TS may become inactivated by the Ni-NTA column. Our lab previously found that H_6-TS became aggregated/misfolded on the Ni-NTA column (unpublished data) when it was purified under denaturing conditions (95). However, the H_6-TS that I used in the present study was still soluble after purification.

Although pure H_6-TS was only partially active, it was able to inhibit translation of TS mRNA in a dose-dependent manner. This *indirectly* implied the RNA-binding ability of this H_6-TS. In addition, direct examination of the RNA-binding activity by electromobility shift assay showed a dose-dependent increase in an apparent H_6-TS-mRNA band, although the quality of the assay was poor. However, consistent ability of pure H_6-TS to inhibit translation and bind mRNA was not observed. I believe that one reason for this was the loss of functionality of H_6-TS after long-term storage. This is because, I have later observed that only when freshly purified H_6-TS was used (from freshly grown *E. coli* cells), I was able to get somewhat convincing results. Due to inconsistencies seen with the pure H_6-TS, I was unable to further examine the effect of H_6-TS addition on translation of 3R and 2R mRNA.

Since purified H_6-TS was only partially active and gave inconsistent results, I also examined the effect of adding *E. coli* and HeLa cells overexpressing TS on RNA-binding. HeLa cells were utilized since possible unknown mammalian cellular factor(s) may be required for proper folding and maintenance of the human TS in an active state[1]. However, since I was unable to get the electromobility shift assay working convincingly, I could not demonstrate the RNA-binding by the TS-overexpressing extracts. Any band-shift seen was likely due to other cellular proteins binding to the RNA.

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[1] Overexpression of TS in HeLa cells was originally intended for use in Chapter 1 to see the effect on TS localization. The 5-FUdR-resistant TS-overexpressing HeLa clone-55 from Chapter 1,2 was developed later and, therefore, not used here.

Although I experienced several technical problems in this study, some important questions were still answered regarding TS auto-regulation and polymorphism. I was able to show that the *in vitro* synthesized TS could inhibit its own translation, which was prevented in the presence of either dUMP or 5-FdUMP. This was not observed by Chu's group (85). In addition, my results provided a tentative indication that translation of 3R mRNA was *less* effectively inhibited than the translation of 2R mRNA (Fig. 4.5A, $p = 0.006$). This is consistent with the observation by Horie *et al* who showed that luciferase activity in HeLa cells transfected with a construct expressing 3R-luciferase mRNA was higher than from a construct expressing 2R-luciferase mRNA (41). A similar finding was recently reported by Danenberg's group except that they also showed that the level of mRNA in the two transfections was unchanged (59), suggesting a post-transcriptional mechanism. My result, although preliminary, is more direct since it shows that it is the *translation* of 3R and 2R mRNA that is differentially inhibited by TS protein.

4.6. CONCLUSION

Despite several technical problems, I was able to partially confirm the work of others that the *in vitro* synthesized TS could inhibit its own translation. In addition, my preliminary results indicated that translation of 3R mRNA was *less* effectively inhibited than the translation of 2R mRNA. This is consistent with observations by others workers (41,59) except that my results more directly show that it is the *translation* of 3R and 2R mRNA that is differentially inhibited by TS protein. My additional aim to investigate possible mutations in the TS protein or RNA binding sites that might alter translational regulation of TS leading to its overexpression could not be addressed due to the technical problems experienced in this chapter.

GENERAL DISCUSSION AND CLINICAL RELEVANCE

Cellular *resistance* to anti-TS chemotherapeutics drugs is a major hindrance in clinical treatment of colorectal and other types of cancers. Understanding the various mechanisms of resistance is thus of obvious clinical importance since it would allow us to more rapidly identify the patients showing resistance and design alternative and more specific targets for their treatment. In this thesis, I have examined various mechanisms of resistance to anti-TS drugs and factors associated with the resistance. Several questions I set out to address were answered and several unexpected and novel findings were also observed. In all, I have identified several important factors associated with different forms of drug resistance in this thesis that may prove to be useful in identifying drug resistance in clinic and/or designing alternative and more specific targets for treatment of the resistance.

Nuclear localization of TS was recently shown to be associated with poorer 5-FU drug response in colorectal cancer patients (50). However, it is unknown if nuclear localization *itself* is causing 5-FU resistance or is a *result* of an unknown factor(s) that is causing the resistance. No factor(s) has been previously described that cause TS to localize to nucleus. I have found several factors that can induce or are associated with nuclear localization of TS (Chapter 1). One of the major factors is TS overexpression, which is widely observed in many types of human tumors and has often been associated with disease state, poor survival and drug resistance. Other factors associated with nuclear localization are (i) differences in cell lines due to unknown cellular factor(s) even though the cells were from cancers of colonic origin and (ii) mutations in putative NES and surrounding sequences in TS protein. Further work needs to done to associate these factors with drug resistance. In addition, cancer tissues need to be examined for the presence of NES mutation in TS protein in order to have a more clinical association with my study. Thus, the factors associated with TS nuclear localization that are identified in my thesis and the unknown ones that still need to be identified might prove to be of significant importance in understanding the mechanism of poorer 5-FU response seen in cancer patients.

Several findings that I have reported in my thesis were consistent with previous studies. (i) Step-wise selection in 5-FUdR was able to produce HeLa clones overexpressing TS protein. I showed that the TS overxpression in these cells was in fact due to amplification of TS gene (Chapter 2), as has been previously described (51,112,114,115). I further extended this study and showed that the heterogeneity in TS protein levels was due to

heterogeneity in the number of TS gene copies. (ii) Previous studies have demonstrated that expression from 3R and 2R alleles is different and regulation at the post-transcriptional level is responsible for the difference (41,59). Although they postulated that translational regulation was responsible, my preliminary results show that the translation of 3R and 2R is differently affected after addition of *in vitro* synthesized TS protein (Chapter 3).

I observed several unexpected and novel findings in the thesis. (i) Preferential gene amplification of TS 2R allele over 3R allele in *all* my 5-FUdR-resistant TS-overexpressing HeLa clones was a surprising finding (Chapter 2). One possible explanation is that sequences surrounding the 2R allele in chromosome 18 were more sensitive to amplification than the sequences surrounding the 3R allele. Alternatively, the lack of one tandem repeat in 2R compared to 3R somehow confers sensitivity to amplification. It would be of interest to demonstrate this in cell lines of other origin and genotype.

(ii) Lack of ternary TS-complex in the nuclei of 5-FUdR-resistant HeLa cells compared to the presence of the complex in large amounts in the cytoplasm was also a surprising finding. I have proposed several possible explanations for this observation (see Chapter 1). Further work needs to be done to examine these possibilities as they will broaden our understanding of the mechanisms of nuclear localization of TS and 5-FUdR resistance.

(iii) Another unexpected finding was the identification of a putative NES in human TS protein (Chapter 2). More work, however, needs to be done to validate the signal. In a number of multifunctional RNA- or DNA-binding proteins (e.g. p53, Smad1, HIV Rev) that contain NES, phosphorylation can affect their cellular localization (91,150,151). Since TS is a also a multifunctional RNA-binding protein that has been shown to exist in a phosphorylated form in one report (99), identification of the putative NES in TS in my thesis warrants the investigation of the role of TS phosphorylation in its cellular localization.

(iv) One of the most novel and clinically significant findings in my thesis was the identification of the *phenotypic instability* in colon cancer HCT-116 cells (Chapter 3). This instability appeared to be spontaneous and led to 5-FUdR-resistance. After careful examination of the possible factors involved in this instability, I concluded that a dramatic reduction in the uptake of the 5-FUdR drug by the cells was the main reason for the resistance. My preliminary results suggest that soluble factor(s) made by HCT-116 cells and

released into the media are likely responsible for the dramatic and rapid conversion of the 5-FUdR-sensitive phenotype to a resistant one. Thus, the *identification* of this instability-inducing factor(s) would prove to be of extreme clinical importance since it might act as a soluble marker of a type of 5-FUdR-resistance. In addition, designing drugs that target this factor(s) might also prove to be useful for co-administration with fluoropyrimidine drugs in the fight against drug resistance in the clinic.

In conclusion, results from my thesis have revealed several aspects about TS protein and its regulation and identified important factors associated with different forms of resistance to anti-TS drugs. These findings are of clinical significance since they contribute to our understanding of various mechanisms of resistance to anti-TS drugs. Further, extension of this work may prove to be useful in identifying anti-TS drug resistance in clinic and/or designing alternative and more specific targets for treatment of resistance.

REFERENCES

1. Danenberg P.V. 1977. Thymidylate synthetase - a target enzyme in cancer chemotherapy. *Biochim. Biophys. Acta* **473**: 73-92.

2. Van der Wilt C.L., Peters G.J. 1994. New targets for pyrimidine antimetabolites in the treatment of solid tumours. 1: Thymidylate synthase. *Pharm. World Sci.* **16**: 84-103.

3. Hardy L.W., Finer-Moore J.S., Montfort W.R., Jones M.O., Santi D.V., Stroud R.M. 1987. Atomic structure of thymidylate synthase: target for rational drug design. *Science* **235**: 448-455.

4. Takeishi K., Kaneda S., Ayusawa D., Shimizu K., Gotoh O., Seno T. 1985. Nucleotide sequence of a functional cDNA for human thymidylate synthase. *Nucleic. Acids. Res.* **13**: 2035-2043.

5. Hori T., Takahashi E., Ayusawa D., Takeishi K., Kaneda S., Seno T. 1990. Regional assignment of the human thymidylate synthase (TS) gene to chromosome band 18p11.32 by nonisotopic in situ hybridization. *Hum. Genet.* **85**: 576-580.

6. Davisson V.J., Sirawaraporn W., Santi D.V. 1989. Expression of human thymidylate synthase in Escherichia coli. *J. Biol. Chem.* **264**: 9145-9148.

7. Schiffer C.A., Davisson V.J., Santi D.V., Stroud R.M. 1991. Crystallization of human thymidylate synthase. *J. Mol. Biol.* **219**: 161-163.

8. Schiffer C.A., Clifton I.J., Davisson V.J., Santi D.V., Stroud R.M. 1995. Crystal structure of human thymidylate synthase: a structural mechanism for guiding substrates into the active site. *Biochemistry* **34**: 16279-16287.

9. Murakami Y., Kazuno H., Emura T., Tsujimoto H., Suzuki N., Fukushima M. 2000. Different mechanisms of acquired resistance to fluorinated pyrimidines in human colorectal cancer cells. *Int. J. Oncol.* **17**: 277-283.

10. Peters G.J., Van der Wilt C.L. 1995. Thymidylate synthase as a target in cancer chemotherapy. *Biochem. Soc. Trans.* **23**: 884-888.

11. Hrushesky W.J., von Roemeling R., Lanning R.M., Rabatin J.T. 1990. Circadian-shaped infusions of floxuridine for progressive metastatic renal cell carcinoma. *J. Clin. Oncol.* **8**: 1504-1513.

12. Chang A.E., Schneider P.D., Sugarbaker P.H., Simpson C., Culnane M., Steinberg S.M. 1987. A prospective randomized trial of regional versus systemic continuous 5-fluorodeoxyuridine chemotherapy in the treatment of colorectal liver metastases. *Ann. Surg.* **206**: 685-693.

13. Hohn D.C., Stagg R.J., Friedman M.A., Hannigan J.F., Jr., Rayner A., Ignoffo R.J., Acord P., Lewis B.J. 1989. A randomized trial of continuous intravenous versus hepatic intraarterial floxuridine in patients with colorectal cancer metastatic to the liver: the Northern California Oncology Group trial. *J. Clin. Oncol.* **7**: 1646-1654.

14. Santi D.V., McHenry C.S., Sommer H. 1974. Mechanism of interaction of thymidylate synthetase with 5- fluorodeoxyuridylate. *Biochemistry* **13**: 471-481.

15. Danenberg P.V., Lockshin A. 1981. Fluorinated pyrimidines as tight-binding inhibitors of thymidylate synthetase. *Pharmacol. Ther.* **13**: 69-90.

16. Seno T., Ayusawa D., Shimizu K., Koyama H., Takeishi K., Hori T. 1985. Thymineless death and genetic events in mammalian cells. *Basic Life Sci.* **31**: 241-263.

17. Parker W.B., Cheng Y.C. 1990. Metabolism and mechanism of action of 5-fluorouracil. *Pharmacol. Ther.* **48**: 381-395.

18. Heidelberger C., Danenberg P.V., Moran R.G. 1983. Fluorinated pyrimidines and their nucleosides. *Adv. Enzymol. Relat Areas Mol. Biol.* **54**: 58-119.

19. Pinedo H.M., Peters G.F. 1988. Fluorouracil: biochemistry and pharmacology. *J. Clin. Oncol.* **6**: 1653-1664.

20. Harrap K.R., Jackman A.L., Newell D.R., Taylor G.A., Hughes L.R., Calvert A.H. 1989. Thymidylate synthase: a target for anticancer drug design. *Adv. Enzyme Regul.* **29**: 161-179.

21. Jackman A.L., Taylor G.A., Gibson W., Kimbell R., Brown M., Calvert A.H., Judson I.R., Hughes L.R. 1991. ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth in vitro and in vivo: a new agent for clinical study. *Cancer Res.* **51**: 5579-5586.

22. Johnston P.G., Liang C.-M., Henry S., Chabner B.A., Allegra C.J. 1991. Production and characterization of monoclonal antibodies that localize human thymidylate synthase in the cytoplasm of human cells and tissue. *Cancer Res.* **51**: 6668-6676.

23. Leichman C.G., Lenz H.J., Leichman L., Danenberg K., Baranda J., Groshen S., Boswell W., Metzger R., Tan M., Danenberg P.V. 1997. Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted-infusion fluorouracil and weekly leucovorin. *J. Clin. Oncol.* **15**: 3223-3229.

24. Lenz H.J., Hayashi K., Salonga D., Danenberg K.D., Danenberg P.V., Metzger R., Banerjee D., Bertino J.R., Groshen S., Leichman L.P., Leichman C.G. 1998. p53 point mutations and thymidylate synthase messenger RNA levels in disseminated colorectal cancer: an analysis of response and survival. *Clin. Cancer Res.* **4**: 1243-1250.

25. Swain S.M., Lippman M.E., Egan E.F., Drake J.C., Steinberg S.M., Allegra C.J. 1989. Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. *J. Clin. Oncol.* **7**: 890-899.

26. Peters G.J., Van der Wilt C.L., van Groeningen C.J., Smid K., Meijer S., Pinedo H.M. 1994. Thymidylate synthase inhibition after administration of fluorouracil with or without leucovorin in colon cancer patients: implications for treatment with fluorouracil. *J. Clin. Oncol.* **12**: 2035-2042.

27. Navalgund L.G., Rossana C., Muench A.J., Johnson L.F. 1980. Cell cycle regulation of thymidylate synthetase gene expression in cultured mouse fibroblasts. *J. Biol. Chem.* **255**: 7386-7390.

28. Ayusawa D., Shimizu K., Koyama H., Kaneda S., Takeishi K., Seno T. 1986. Cell-cycle-directed regulation of thymidylate synthase messenger RNA in human diploid fibroblasts stimulated to proliferate. *J. Mol. Biol.* **190**: 559-567.

29. Keyomarsi K., Samet J., Molnar G., Pardee A.B. 1993. The thymidylate synthase inhibitor, ICI D1694, overcomes translational detainment of the enzyme. *J. Biol. Chem.* **268**: 15142-15149.

30. Washtien W.L. 1984. Increased levels of thymidylate synthetase in cells exposed to 5- fluorouracil. *Mol. Pharmacol.* **25**: 171-177.

31. Keyomarsi K., Moran R.G. 1988. Mechanism of the cytotoxic synergism of fluoropyrimidines and folinic acid in mouse leukemic cells. *J. Biol. Chem.* **263**: 14402-14409.

32. Van der Wilt C.L., Pinedo H.M., Smid K., Peters G.J. 1992. Elevation of thymidylate synthase following 5-fluorouracil treatment is prevented by the addition of leucovorin in murine colon tumors. *Cancer Res.* **52**: 4922-4928.

33. Chu E., Zinn S., Boarman D., Allegra C.J. 1990. Interaction of gamma interferon and 5-fluorouracil in the H630 human colon carcinoma cell line. *Cancer Res.* **50**: 5834-5840.

34. Chu E., Koeller D.M., Johnston P.G., Zinn S., Allegra C.J. 1993. Regulation of thymidylate synthase in human colon cancer cells treated with 5-fluorouracil and interferon-gamma. *Mol. Pharmacol.* **43**: 527-533.

35. Chu E., Allegra C.J. 1996. The role of thymidylate synthase as an RNA binding protein. *BioEssays* **18**: 191-198.

36. Chu E., Voeller D.M., Morrison P.F., Jones K.L., Takechi T., Maley G.F., Maley, Allegra C.J. 1994. The effect of reducing reagents on binding of thymidylate synthase protein to thymidylate synthase messenger RNA. *J. Biol. Chem.* **269**: 20289-20293.

37. Chu E., Voeller D., Koeller D.M., Drake J.C., Takimoto C.H., Maley G.F., Maley F., Allegra C.J. 1993. Identification of an RNA binding site for human thymidylate synthase. *Proc. Natl. Acad. Sci. USA* **90**: 517-521.

38. Lin X., Parsels L.A., Voeller D.M., Allegra C.J., Maley G.F., Maley F., Chu E. 2000. Characterization of a cis-acting regulatory element in the protein coding region of thymidylate synthase mRNA. *Nucleic Acids Res.* **28**: 1381-1389.

39. Chu E., Takechi T., Jones K.L., Voeller D.M., Copur S.M., Maley G.F., Maley F., Segal S., Allegra C.J. 1995. Thymidylate synthase binds to c-myc RNA in human colon cancer cells and in vitro. *Mol. Cell Biol.* **15**: 179-185.

40. Ju J., Pedersen-Lane J., Maley F., Chu E. 1999. Regulation of p53 expression by thymidylate synthase. *Proc. Natl. Acad. Sci. USA* **96**: 3769-3774.

41. Horie N., Aiba H., Oguro K., Hojo H., Takeishi K. 1995. Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate synthase. *Cell Struct. Funct.* **20**: 191-197.

42. Kucera R., Paulus H. 1986. Localization of the deoxyribonucleotide biosynthetic enzymes ribonucleotide reductase and thymidylate synthase in mouse L cells 9. *Exp. Cell Res.* **167**: 417-428.

43. Johnston P.G., Lenz H.-Z., Leichman C.G., Danenberg K.D., Allegra C.J., Danenberg P.V., Leichman L., Lenz H.J. 1995. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res.* **55**: 1407-1412.

44. Suzuki M., Okabe H., Tsukagoshi S., Kawai T., Fukushima M., Sato I. 1998. Immunohistochemical investigation of thymidylate synthase in cervical cancer. *Oncology* **55**: 564-568.

45. Yamachika T., Nakanishi H., Inada K., Tsukamoto T., Kato T., Fukushima M., Inoue M., Tatematsu M. 1998. A new prognostic factor for colorectal carcinoma, thymidylate synthase, and its therapeutic significance. *Cancer* **82**: 70-77.

46. Paradiso A., Simone G., Petroni S., Leone B., Vallejo C., Lacava J., Romero A., Machiavelli M., De Lena M., Allegra C.J., Johnston P.G. 2000. Thymidylate synthase and p53 primary tumour expression as predictive factors for advanced colorectal cancer patients. *Br. J. Cancer* **82**: 560-567.

47. Brown S.S., Neal G.E., Williams D.C. 1965. Subcellular distribution of some folic acid-linked enzymes in rat liver. *Biochem. J.* **97**: 34C-36C.

48. Prem veer R.G., Pardee A.B. 1980. Multienzyme complex for metabolic channeling in mammalian DNA replication
3. *Proc. Natl. Acad. Sci. U. S. A* **77**: 3312-3316.

49. Reston J., Samsonoff W., McJee M., O'Connor B., Galivan J., Maley G., Maley F. 1996. Nuclear location of thymidine synthase (TS) and its state of phosphorylation (Meeting abstract). *Proc. Annu. Meet. Am. Assoc. Cancer Res.* **37**: A3362.

50. Wong N.A., Brett L., Stewart M., Leitch A., Longley D.B., Dunlop M.G., Johnston P.G., Lessells A.M., Jodrell D.I. 2001. Nuclear thymidylate synthase expression, p53 expression and 5FU response in colorectal carcinoma. *Br. J. Cancer* **85**: 1937-1943.

51. Berger S.H., Jenh C.H., Johnson L.F., Berger F.G. 1985. Thymidylate synthase overproduction and gene amplification in fluorodeoxyuridine-resistant human cells. *Mol. Pharmacol.* **28**: 461-467.

52. Clark J.L., Berger S.H., Mittelman A., Berger F.G. 1987. Thymidylate synthase gene amplification in a colon tumor resistant to fluoropyrimidine chemotherapy. *Cancer Treat. Rep.* **71**: 261-265.

53. Johnston P.G., Drake J.C., Trepel J., Allegra C.J. 1992. Immunological quantitation of thymidylate synthase using the monoclonal antibody TS 106 in 5-fluorouracil-sensitive and - resistant human cancer cell lines. *Cancer Res.* **52**: 4306-4312.

54. Copur S., Aiba K., Drake J.C., Allegra C.J., Chu E. 1995. Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil. *Biochem. Pharmacol.* **49**: 1419-1426.

55. Mandola M.V., Stoehlmacher J., Muller-Weeks S., Cesarone G., Yu M.C., Lenz H.J., Ladner R.D. 2003. A novel single nucleotide polymorphism within the 5' tandem repeat polymorphism of the thymidylate synthase gene abolishes USF-1 binding and alters transcriptional activity. *Cancer Res.* **63**: 2898-2904.

56. Iacopetta B., Grieu F., Joseph D., Elsaleh H. 2001. A polymorphism in the enhancer region of the thymidylate synthase promoter influences the survival of colorectal cancer patients treated with 5-fluorouracil. *Br. J. Cancer* **85**: 827-830.

57. Marsh S., McKay J.A., Cassidy J., McLeod H.L. 2001. Polymorphism in the thymidylate synthase promoter enhancer region in colorectal cancer. *Int. J. Oncol.* **19**: 383-386.

58. Kawakami K., Omura K., Kanehira E., Watanabe Y. 1999. Polymorphic tandem repeats in the thymidylate synthase gene is associated with its protein expression in human gastrointestinal cancers. *Anticancer Res.* **19**: 3249-3252.

59. Kawakami K., Salonga D., Park J.M., Danenberg K.D., Uetake H., Brabender J., Omura K., Watanabe G., Danenberg P.V. 2001. Different lengths of a polymorphic repeat sequence in the thymidylate synthase gene affect translational efficiency but not its gene expression. *Clin. Cancer Res.* **7**: 4096-4101.

60. Bapat A.R., Zarow C., Danenberg P.V. 1983. Human leukemic cells resistant to 5-fluoro-2'-deoxyuridine contain a thymidylate synthetase with lower affinity for nucleotides. *J. Biol. Chem.* **258**: 4130-4136.

61. Berger S.H., Barbour K.W., Berger F.G. 1988. A naturally occurring variation in thymidylate synthase structure is associated with a reduced response to 5-fluoro-2'-deoxyuridine in a human colon tumor cell line. *Mol. Pharmacol.* **34**: 480-484.

62. Fernandes D.J., Cranford S.K. 1985. Resistance of CCRF-CEM cloned sublines to 5-fluorodeoxyuridine associated with enhanced phosphatase activities. *Biochem. Pharmacol.* **34**: 125-132.

63. Zhang Z.G., Malmberg M., Yin M.B., Slocum H.K., Rustum Y.M. 1993. Isolation and characterization of a human ileocecal carcinoma cell line (HCT-8) subclone resistant to fluorodeoxyuridine. *Biochem. Pharmacol.* **45**: 1157-1164.

64. Jastreboff M.M., Zielinska Z.M. 1983. Development and characteristics of a subline of Ehrlich ascites carcinoma cells persistently resistant to 5-fluoro-2'-deoxyuridine. *Acta Biochim. Pol.* **30**: 185-191.

65. Houghton P.J., Houghton J.A., Germain G., Torrance P.M. 1987. Development and characterization of a human colon adenocarcinoma xenograft deficient in thymidine salvage. *Cancer Res.* **47**: 2117-2122.

66. Aschele C., Sobrero A., Faderan M.A., Bertino J.R. 1992. Novel mechanism(s) of resistance to 5-fluorouracil in human colon cancer (HCT-8) sublines following exposure to two different clinically relevant dose schedules. *Cancer Res.* **52**: 1855-1864.

67. Chu E., Lai G.M., Zinn S., Allegra C.J. 1990. Resistance of a human ovarian cancer line to 5-fluorouracil associated with decreased levels of 5-fluorouracil in DNA. *Mol. Pharmacol.* **38**: 410-417.

68. Inaba M., Mitsuhashi J., Sawada H., Miike N., Naoe Y., Daimon A., Koizumi K., Tsujimoto H., Fukushima M. 1996. Reduced activity of anabolizing enzymes in 5-fluorouracil-resistant human stomach cancer cells. *Jpn. J. Cancer Res.* **87**: 212-220.

69. Fry D.W., Jackson R.C. 1986. Membrane transport alterations as a mechanism of resistance to anticancer agents. *Cancer Surv.* **5**: 47-79.

70. Sobrero A., Aschele C., Guglielmi A., Nobile M.T., Rosso R. 1990. Resistance to 5-fluorouracil and 5-fluoro-2'-deoxyuridine mechanisms and clinical implications. *J. Chemother.* **2 Suppl 1**: 12-16.

71. Miyachi H., Jiao L., Sowers L.C., Scanlon K.J. 1992. Collateral sensitivity to azidothymidine in methotrexate resistant human leukemia cells. *In Vivo* **6**: 17-21.

72. Tezuka M., Sugiyama H., Tamemasa O., Inaba M. 1982. Biochemical characteristics of a 5-fluorouracil-resistant subline of P388 leukemia. *Gann* **73**: 70-76.

73. Drake J.C., Allegra C.J., Moran R.G., Johnston P.G. 1996. Resistance to tomudex (ZD1694): multifactorial in human breast and colon carcinoma cell lines. *Biochem. Pharmacol.* **51**: 1349-1355.

74. Kobayashi H., Takemura Y., Miyachi H. 1998. Molecular characterization of human acute leukemia cell line resistant to ZD9331, a non-polyglutamatable thymidylate synthase inhibitor. *Cancer Chemother. Pharmacol.* **42**: 105-110.

75. Zhang Z.G., Harstrick A., Rustum Y.M. 1992. Mechanisms of resistance to fluoropyrimidines. *Semin. Oncol.* **19**: 4-9.

76. Romanini A., Lin J.T., Niedzwiecki D., Bunni M., Priest D.G., Bertino J.R. 1991. Role of folylpolyglutamates in biochemical modulation of fluoropyrimidines by leucovorin. *Cancer Res.* **51**: 789-793.

77. Yin M.B., Zakrzewski S.F., Hakala M.T. 1983. Relationship of cellular folate cofactor pools to the activity of 5-fluorouracil. *Mol. Pharmacol.* **23**: 190-197.

78. Glazer R.I., Peale A.L. 1979. The effect of 5-fluorouracil on the synthesis of nuclear RNA in L1210 cells in vitro. *Mol. Pharmacol.* **16**: 270-277.

79. Major P.P., Egan E., Herrick D., Kufe D.W. 1982. 5-Fluorouracil incorporation in DNA of human breast carcinoma cells. *Cancer Res.* **42**: 3005-3009.

80. Carreras C.W., Santi D.V. 1995. The catalytic mechanism and structure of thymidylate synthase. *Annu. Rev. Biochem.* **64**: 721-762.

81. Rodriguez-Bigas M.A., Petrelli N.J. 1993. Biochemical modulation of fluoropyrimidines and other drugs. *In*: Colorectal cancer. *Editors*: H.J. Wanebo. Mosby, St. Loius, pp. 509-522.

82. Drake J.C., Allegra C.J., Johnston P.G. 1993. Immunological quantitation of thymidylate synthase-FdUMP-5,10-methylenetetrahydrofolate ternary complex with the monoclonal antibody TS 106. *Anticancer Drugs* **4**: 431-435.

83. Poon P.P., Storms R.K. 1994. Thymidylate synthase is localized to the nuclear periphery in the yeast Saccharomyces cerevisiae. *J. Biol. Chem.* **269**: 8341-8347.

84. Cella R., Balestrazzi A., Carbonera D., Nano R., Rezzani R. 1991. Cellular localization of dihydrofolate reductase-thymidylate synthase in carrot cells 3. *Eur. J Basic Appl. Histochem.* **35**: 61-71.

85. Chu E., Koeller D.M., Casey J.L., Drake J.C., Chabner B.A., Elwood P.C., Zinn S., Allegra C.J. 1991. Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase. *Proc. Natl. Acad. Sci. USA* **88**: 8977-8981.

86. Chu E., Copur S.M., Ju J., Chen T.M., Khleif S., Voeller D.M., Mizunuma N., Patel M., Maley G.F., Maley F., Allegra C.J. 1999. Thymidylate synthase protein and p53 mRNA form an in vivo ribonucleoprotein complex. *Mol. Cell Biol.* **19**: 1582-1594.

87. Nakielny S., Dreyfuss G. 1999. Transport of proteins and RNAs in and out of the nucleus 1. *Cell* **99**: 677-690.

88. Michael W.M. 2000. Nucleocytoplasmic shuttling signals: two for the price of one. *Trends Cell Biol.* **10**: 46-50.

89. Bogerd H.P., Fridell R.A., Benson R.E., Hua J., Cullen B.R. 1996. Protein sequence requirements for function of the human T-cell leukemia virus type 1 Rex nuclear export signal delineated by a novel in vivo randomization-selection assay. *Mol. Cell Biol.* **16**: 4207-4214.

90. Lindsay M.E., Holaska J.M., Welch K., Paschal B.M., Macara I.G. 2001. Ran-binding protein 3 is a cofactor for Crm1-mediated nuclear protein export. *J. Cell Biol.* **153**: 1391-1402.

91. Zhang Y., Xiong Y. 2001. A p53 amino-terminal nuclear export signal inhibited by DNA damage-induced phosphorylation. *Science* **292**: 1910-1915.

92. Harbers M., Nomura T., Ohno S., Ishii S. 2001. Intracellular localization of the Ret finger protein depends on a functional nuclear export signal and protein kinase C activation. *J. Biol. Chem.* **276**: 48596-48607.

93. Liu J., Schmitz J.C., Lin X., Tai N., Yan W., Farrell M., Bailly M., Chen T., Chu E. 2002. Thymidylate synthase as a translational regulator of cellular gene expression 7. *Biochim. Biophys Acta* **1587**: 174-182.

94. Jans D.A., Xiao C.Y., Lam M.H. 2000. Nuclear targeting signal recognition: a key control point in nuclear transport? *BioEssays* **22**: 532-544.

95. Haqqani A.S., Cowling R.T., Maroun J.A., Birnboim H.C. 1999. Characterization of a polyclonal antibody to human thymidylate synthase suitable for the study of colorectal cancer specimens. *J. Histochem. Cytochem.* **47**: 1563-1574.

96. Brattain M.G., Levine A.E., Chakrabarty S., Yeoman L.C., Willson J.K., Long B. 1984. Heterogeneity of human colon carcinoma. *Cancer Metastasis Rev.* **3**: 177-191.

97. Udenfriend S., Stein S., Bohlen P., Dairman W., Leimgruber W., Weigele M. 1972. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science* **178**: 871-872.

98. Cowling R.T., Birnboim H.C. 1998. Preliminary characterization of the protein encoded by human testis-enhanced gene transcript (TEGT). *Mol. Memb. Biol.* **15**: 177-187.

99. Samsonoff W.A., Reston J., McKee M., O'Connor B., Galivan J., Maley G., Maley F. 1997. Intracellular location of thymidylate synthase and its state of phosphorylation. *J. Biol. Chem.* **272**: 13281-13285.

100. Zaman K., Ryu H., Hall D., O'Donovan K., Lin K.I., Miller M.P., Marquis J.C., Baraban J.M., Semenza G.L., Ratan R.R. 1999. Protection from oxidative stress-induced apoptosis in cortical neuronal cultures by iron chelators is associated with enhanced DNA binding of hypoxia-inducible factor-1 and ATF-1/CREB and increased expression of glycolytic enzymes, p21(waf1/cip1), and erythropoietin. *J. Neurosci.* **19**: 9821-9830.

101. Ardalan B., Dang Z. 1996. Thymidylate synthase gene expression in normal and malignant colorectal tissues: relation to in vivo response and survival. *Proc. Annu. Meet. Am. Assoc. Cancer Res.* **37**: A1376.

102. Findlay M.P., Cunningham D., Morgan G., Clinton S., Hardcastle A., Aherne G.W. 1997. Lack of correlation between thymidylate synthase levels in primary colorectal tumours and subsequent response to chemotherapy. *Br. J. Cancer* **75**: 903-909.

103. McKinsey T.A., Zhang C.L., Olson E.N. 2001. Identification of a signal-responsive nuclear export sequence in class II histone deacetylases. *Mol. Cell Biol.* **21**: 6312-6321.

104. Akiyama K., Kanda N., Yamada M., Tadokoro K., Matsunaga T., Nishi Y. 1994. Megabase-scale analysis of the origin of N-myc amplicons in human neuroblastomas. *Nucleic Acids Res.* **22**: 187-193.

105. al-Kasspooles M., Moore J.H., Orringer M.B., Beer D.G. 1993. Amplification and over-expression of the EGFR and erbB-2 genes in human esophageal adenocarcinomas. *Int. J. Cancer* **54**: 213-219.

106. Alitalo K., Schwab M. 1986. Oncogene amplification in tumor cells. *Adv. Cancer Res.* **47**: 235-281.

107. Ding W., Wang Z., Niu F., Zhang W. 1996. Expression of p53, p21 and amplification of c-myc and c-erbB-2 genes in human esophageal squamous cell carcinoma. *Proc. Annu. Meet. Am. Assoc. Cancer Res.* **37**: A3947.

108. Schimke R.T. 1984. Gene amplification in cultured animal cells. *Cell* **37**: 705-713.

109. Carman M.D., Schornagel J.H., Rivest R.S., Srimatkandada S., Portlock C.S., Duffy T., Bertino J.R. 1984. Resistance to methotrexate due to gene amplification in a patient with acute leukemia. *J. Clin. Oncol.* **2**: 16-20.

110. Göker E., Waltham M., Kheradpour A., Trippett T., Mazumdar M., Elisseyeff Y., Schnieders B., Steinherz P., Tan C., Berman E., Bertino J.R. 1995. Amplification of the dihydrofolate reductase gene is a mechanism of acquired resistance to methotrexate in patients with acute lymphoblastic leukemia and is correlated with p53 gene mutations. *Blood* **86**: 677-684.

111. Ferguson P.J., DeMoor J.M., Vincent M.D., Koropatnick J. 2001. Antisense-induced down-regulation of thymidylate synthase and enhanced cytotoxicity of 5-FUdR in 5-FUdR-resistant HeLa cells. *Br J Pharmacol.* **134**: 1437-1446.

112. Rhee M.S., Balinska M., Bunni M., Priest D.G., Maley G.F., Maley F., Galivan J. 1990. Role of substrate depletion in the inhibition of thymidylate biosynthesis by the dihydrofolate reductase inhibitor trimetrexate in cultured hepatoma cells 4. *Cancer Res.* **50**: 3979-3984.

113. Nagarajan M., Johnson L.F. 1989. Regulation of thymidylate synthase gene expression in mouse fibroblasts synchronized by mitotic selection. *Exp. Cell Res.* **181**: 289-297.

114. Jenh C.H., Geyer P.K., Baskin F., Johnson L.F. 1985. Thymidylate synthase gene amplification in fluorodeoxyuridine- resistant mouse cell lines. *Mol. Pharmacol.* **28**: 80-85.

115. Baskin F., Carlin S.C., Kraus P., Friedkin M., Rosenberg R.N. 1975. Experimental chemotherapy of neuroblastoma. II. Increased thymidylate synthetase activity in a 5-

fluorodeoxyuridine-resistant variant of mouse neuroblastoma 28. *Mol. Pharmacol.* **11**: 105-117.

116. Imam A.M., Crossley P.H., Jackman A.L., Little P.F. 1987. Analysis of thymidylate synthase gene amplification and of mRNA levels in the cell cycle. *J. Biol. Chem.* **262**: 7368-7373.

117. Danenberg K.D., Danenberg P.V. 1989. Activity of thymidylate synthetase and its inhibition by 5- fluorouracil in highly enzyme-overproducing cells resistant to 10-propargyl-5,8-dideazafolate. *Mol. Pharmacol.* **36**: 219-223.

118. Coderre J.A., Beverley S.M., Schimke R.T., Santi D.V. 1983. Overproduction of a bifunctional thymidylate synthetase-dihydrofolate reductase and DNA amplification in methotrexate-resistant Leishmania tropica. *Proc. Natl. Acad. Sci. U. S. A* **80**: 2132-2136.

119. Spriggs A.I., Boddington M.M., Clarke C.M. 1962. *Br Med J* **2**: 1431-1435.

120. Benner S.E., Wahl G.M., Von Hoff D.D. 1991. Double minute chromosomes and homogeneously staining regions in tumors taken directly from patients versus in human tumor cell lines. *Anticancer Drugs* **2**: 11-25.

121. Gorlick R., Metzger R., Danenberg K.D., Salonga D., Miles J.S., Longo G.S., Fu J., Banerjee D., Klimstra D., Jhanwar S., Danenberg P.V., Kemeny N., Bertino J.R. 1998. Higher levels of thymidylate synthase gene expression are observed in pulmonary as compared with hepatic metastases of colorectal adenocarcinoma. *J. Clin. Oncol.* **16**: 1465-1469.

122. Birnboim H.C. 1993. Extraction of high molecular weight RNA and DNA from cultured mammalian cells. *Meth. Enzymol.* **216**: 154-160.

123. Vytasek R. 1982. A sensitive fluorometric assay for the determination of DNA. *Anal. Biochem.* **120**: 243-248.

124. Hastings P.J., Rosenberg S.M. 2002. In pursuit of a molecular mechanism for adaptive gene amplification. *DNA Repair (Amst)* **1**: 111-123.

125. Kuo M.T., Sen S., Hittelman W.N., Hsu T.C. 1998. Chromosomal fragile sites and DNA amplification in drug-resistant cells. *Biochem. Pharmacol.* **56**: 7-13.

126. Wintersberger E. 1994. DNA amplification: new insights into its mechanism. *Chromosoma* **103**: 73-81.

127. Windle B.E., Wahl G.M. 1992. Molecular dissection of mammalian gene amplification: new mechanistic insights revealed by analyses of very early events. *Mutat. Res.* **276**: 199-224.

128. Schimke R.T., Brown P.C., Kaufman R.J. 1982. Gene amplification and drug resistance in mammalian cells. *Natl. Cancer Inst. Monogr* **60**: 79-86.

129. Bunz F., Hwang P.M., Torrance C., Waldman T., Zhang Y., Dillehay L., Williams J., Lengauer C., Kinzler K.W., Vogelstein B. 1999. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J. Clin. Invest* **104**: 263-269.

130. Longley D.B., Boyer J., Allen W.L., Latif T., Ferguson P.R., Maxwell P.J., McDermott U., Lynch M., Harkin D.P., Johnston P.G. 2002. The role of thymidylate synthase induction in modulating p53-regulated gene expression in response to 5-fluorouracil and antifolates. *Cancer Res.* **62**: 2644-2649.

131. Coleman W.B., Tsongalis G.J. 2002. Role of genomic instability in the development of human cancer. *In*: The molecular basis of human cancer. *Editors*: W.B. Coleman, G.J. Tsongalis. Humana Press, New Jersey, pp. 115-142.

132. Rosenberg S.M., Thulin C., Harris R.S. 1998. Transient and heritable mutators in adaptive evolution in the lab and in nature. *Genetics* **148**: 1559-1566.

133. Jaenisch R., Bird A. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* **33 Suppl**: 245-254.

134. Bunz F., Dutriaux A., Lengauer C., Waldman T., Zhou S., Brown J.P., Sedivy J.M., Kinzler K.W., Vogelstein B. 1998. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* **282**: 1497-1501.

135. Hawn M.T., Umar A., Carethers J.M., Marra G., Kunkel T.A., Boland C.R., Koi M. 1995. Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint. *Cancer Res.* **55**: 3721-3725.

136. Fortin A., Cregan S.P., MacLaurin J.G., Kushwaha N., Hickman E.S., Thompson C.S., Hakim A., Albert P.R., Cecconi F., Helin K., Park D.S., Slack R.S. 2001. APAF1 is a key transcriptional target for p53 in the regulation of neuronal cell death. *J Cell Biol.* **155**: 207-216.

137. Baldwin S.A., Beal P.R., Yao S.Y., King A.E., Cass C.E., Young J.D. 2003. The equilibrative nucleoside transporter family, SLC29. *Pflugers Arch.*, in press.

138. Yao S.Y., Ng A.M., Vickers M.F., Sundaram M., Cass C.E., Baldwin S.A., Young J.D. 2002. Functional and molecular characterization of nucleobase transport by recombinant human and rat equilibrative nucleoside transporters 1 and 2. Chimeric constructs reveal a role for the ENT2 helix 5-6 region in nucleobase translocation. *J. Biol. Chem.* **277**: 24938-24948.

139. Van der Wilt C.L., Pinedo H.M., Smid K., Cloos J., Noordhuis P., Peters G.J. 1992. Effect of folinic acid on fluorouracil activity and expression of thymidylate synthase. *Semin. Oncol.* **19**: 16-25.

140. Ferguson P.J., Cheng Y.C. 1989. Phenotypic instability of drug sensitivity in a human colon carcinoma cell line. *Cancer Res.* **49**: 1148-1153.

141. Kim W.J., Vo Q.N., Shrivastav M., Lataxes T.A., Brown K.D. 2002. Aberrant methylation of the ATM promoter correlates with increased radiosensitivity in a human colorectal tumor cell line. *Oncogene* **21**: 3864-3871.

142. Liteplo R.G., Alvarez E., Frost P., Kerbel R.S. 1985. Induction of thymidine kinase activity in a spontaneously enzyme- deficient murine tumor cell line by exposure in

vivo to the DNA- hypomethylating agent 5-aza-2'-deoxycytidine: implications for mechanisms of tumor progression. *Cancer Res.* **45**: 5294-5298.

143. Sinigaglia F., Talmadge K.W. 1985. Inhibition of [3H]thymidine incorporation by Mycoplasma arginini-infected cells due to enzymatic cleavage of the nucleoside. *Eur. J Immunol.* **15**: 692-696.

144. Belfort M., Maley G., Pedersen-Lane J., Maley F. 1983. Primary structure of the Escherichia coli thyA gene and its thymidylate synthase product. *Proc. Natl. Acad. Sci. USA* **80**: 4914-4918.

145. Kaneda S., Takeishi K., Ayusawa D., Shimizu K., Seno T., Altman S. 1987. Role in translation of a triple tandemly repeated sequence in the 5'- untranslated region of human thymidylate synthase mRNA. *Nucleic Acids Res.* **15**: 1259-1270.

146. Eckert K.A., Kunkel T.A. 1990. High fidelity DNA synthesis by the Thermus aquaticus DNA polymerase. *Nucleic Acids Res.* **18**: 3739-3744.

147. Calvert A.H., Jones T.R., Dady P.J., Grzelakowska-Sztabert B., Paine R.M., Taylor G.A., Harrap K.R. 1980. Quinazoline antifolates with dual biochemical loci of action. Biochemical and biological studies directed towards overcoming methotrexate resistance. *Eur. J. Cancer* **16**: 713-722.

148. Blakley R.L. 1960. Spectrophotometric studies on the combinations of formaldehyde with tetrahydropteroylglutamic acid and other hydropteridines. *Biochem. J.* **74**: 71-82.

149. Voeller D.M., Zajac-Kaye M., Fisher R.J., Allegra C.J. 2002. The identification of thymidylate synthase peptide domains located in the interface region that bind thymidylate synthase mRNA
1. *Biochem Biophys Res. Commun.* **297**: 24-31.

150. Xiao Z., Watson N., Rodriguez C., Lodish H.F. 2001. Nucleocytoplasmic shuttling of Smad1 conferred by its nuclear localization and nuclear export signals. *J Biol. Chem.* **276**: 39404-39410.

151. D'Agostino D.M., Ferro T., Zotti L., Meggio F., Pinna L.A., Chieco-Bianchi L., Ciminale V. 2000. Identification of a domain in human immunodeficiency virus type 1 rev that is required for functional activity and modulates association with subnuclear compartments containing splicing factor SC35. *J Virol.* **74**: 11899-11910.

APPENDIX: MANUSCRIPTS FOR SUBMISSION

were resistant to 5-FU (40,41). They interpreted their results as showing that expression of wild-type p53 greatly potentiates 5-FU cytotoxicity.

Genetic instability can be described as the accumulation of DNA damage and mutations, where mutations can be chromosomal abnormalities or nucleotide sequence abnormalities (42). Genetic instability may be either progressive (persistent) instability or episodic (transient) instability (42). Progressive instability is transmitted from generation to generation as in tumor cells with hereditary nonpolyposis colorectal cancer (HNPCC) (43). Episodic instability is sporadic genetic damage and accounts for a large proportion of adaptive mutations occurring in cells (43). Epigenetics (outside "conventional" genetics) describes stable alterations in gene expression that arise during development and cell proliferation (44). Epigenetic processes are essential for development and differentiation, but they can also arise randomly. Molecular mechanisms that appear to mediate epigenetic phenomena include changes in DNA methylation and some histone modifications (44). Numerous studies of genetic instability in cell lines have been reported. However, there have been no previous reports of genetic instability in the context of resistance to 5-FU and 5-FUdR.

In this paper we report that decreased transport of 5-FUdR and 5-FU into the colorectal cancer cell line, HCT-116, leads to its resistance to these fluropyrimidine base analogs. In addition, our results suggest that this mechanism of resistance is unstable and independent of p53.

MATERIALS AND METHODS

Antibodies

Rabbit anti-hTS antiserum used in these studies was prepared previously in our lab (45). This polyclonal antibody was raised against recombinant $(his)_6$-rhTS and its specificity has been previously illustrated. HRP-conjugated anti-rabbit/anti-mouse secondary antibody ("Polymer") was from DAKO (Carpinteria, CA). Anti-p53 (Ab-6) and anti-p21 antibodies were from Oncogene (Boston, MA), and anti-actin antibody (clone AC-47) was from Sigma-Aldrich (St Louis, MO). Mouse monoclonal antibody to β-tubulin was a kind gift from Dr. Doug Gray (University of Ottawa, Ont).

Cell lines

HeLa cells were obtained from ATCC (Rockville, MD). The following colon cancer cell lines from the NCI anti-cancer screening panel of 60 cell lines were used: HCT-116, HCT-15, HCC-2998, HT-29 and SW-620; RKO is a colon cancer line originally derived by Brattain *et al.*(46). HCT-116 p53 wt and HCT-116 p53 null were obtained from (47) and HCT-116 +Chr3 was obtained from (48). Normal primary human fibroblasts were from neonatal foreskin. Cells were cultured as monolayers in Dulbecco's modified Eagle's medium (GIBCO-BRL, Burlington, Canada) supplemented with 10% fetal calf serum in 100 mm dishes in 5% CO_2 at 37 °C. RKO and ATCC HCT-116 cells were maintained in McCoy's 5A media plus 10% fetal calf serum (GIBCO-BRL).

Cytotoxicity Studies

$1x10^3$cells were seeded in 1 mL of standard media per well of a 12 well dish. Next day the media was replaced with fresh media with or without drug was added. Where indicated, 5-FUdR (Sigma-Aldrich, St. Louis, MO) and 5-FU (Pharmacia & Upjohn Inc., Bridgewater, NJ) were used at concentrations of 0 M, 1×10^{-9} M, 1×10^{-8} M, 1×10^{-7} M, 1×10^{-6} M and 1×10^{-5} M. Thymidine (Sigma-Aldrich) was used at 2×10^{-5}M. Media was changed until cells were counted by releasing attached cells with trypsin and counting in a Coulter counter (Model ZM, Coulter Electronics Ltd, Luton, Beds., England). Cell counts for 0 M were set to 100% and all counts were expressed as a percentage of the 0 M count. For 5-FUdR and 5-FU, cells were counted 8 days after addition of drug.

For dose response to 5-AdCd, $1x10^4$cells were seeded in 1 mL of standard media per well of a 12 well dish. 5-AdCd (Sigma-Aldrich) was used at same concentrations as 5-FUdR. At 24 and 48 hour time points, attached cells were counted. For pre-treatment of cells with 5-AdCd, 5×10^4 cells were plated in 5mL of standard media in a 5mL dish. Next day the media was changed to media with 5-AdCd. 24 hr after, the media with drug was removed and drug free media was added. Cells were allowed to recover for 3 days before they were trysinized, counted and used for plating for ^{3}H-thymidine incorporation assay and colony forming unit assay.

Colony forming unit (CFU) assay

300 cells were plated in 5 mL of standard media in 5 mL dishes. 10 days after, the media was removed by gently pouring off. Colonies were fixed and stained with methylene blue stain in methanol (0.6% (w/v)) for > 30 min. The stain was carefully poured off and colonies were rinsed by dipping the plates in water. Plates were air dried and colonies > 1mm in diameter were counted.

HAT selection

Cells were plated for colony forming assay. 24 hr after plating, media was removed and replaced with HAT selection medium [hypoxanthine (Sigma-Aldrich) (1×10^{-4} M), aminopterin (Sigma-Aldrich) (4×10^{-7} M) and thymidine (1×10^{-7} or 1×10^{-5} M)]. A control plate in which standard medium was added, was included in each experiment. 10 days after HAT selection, the medium was removed and colonies were fixed as described in colony forming unit assay. The number of colonies in treated plates was expressed as a percentage of control plate.

Treatment of Cells to measure Formation of Ternary Complex

Cells were plated at 5×10^4 cells per well of a 12 well plate in standard medium. Next day the medium of the cells in log phase were removed and replaced with medium containing 5-FUdR in the absence or presence of folinic acid (2.5×10^{-5}M, Sigma-Aldrich Chemicals, St. Louis, MO) or 5-FU. 5-FUdR was used at concentrations of 0 M, 1×10^{-9} M, 1×10^{-8} M, 1×10^{-7} M, 1×10^{-6} M and 1×10^{-5} M. 5-FU was used at concentrations of 0 M, 1×10^{-11} M, 1×10^{-10} M, 1×10^{-9} M, 1×10^{-8} M and 1×10^{-7} M. 24 hr later, the medium was removed and cells were treated for preparation of total cell protein extracts as described below.

Preparation of total cell protein extracts

Cultured cells were detached by light trypsin treatment, then washed twice to remove trypsin and suspended in cold PBS. To prepare whole cell extracts, an equal volume of 2×SDS sample buffer (100 mM MOPS, pH 6.8, 4% SDS, 20% glycerol, 2 % β-mercaptoethanol) was added. The extracts were boiled for 20 min and then sonicated briefly to reduce viscosity. Protein was quantified using fluorescamine (49) (Sigma-Aldrich), using BSA as a standard.

Western blot

Proteins in cell extracts in SDS sample buffer were resolved by 12% discontinuous SDS-PAGE and then electrophoretically transferred to a PVDF membrane (Millipore, Nepean, Canada) at 15V for 18 h in transfer buffer (3 mM Na_2CO_3, 10 mM $NaHCO_3$, 10% methanol) (45). The membrane was stained with Ponceau S and then washed twice in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8). All steps were carried out at room temperature. When using anti-TS as the primary antibody, the membrane was blocked with 1% bovine serum albumin (BSA) (OmniPur ^{R} BSA, Fraction V, EM science-MERCK kGaA, Darmstadt, Germany) in TBST. When using anti-β tubulin as the primary antibody, the membrane was blocked with freshly prepared Casein Blocker solution (100 mM NaCl, 50 mM sodium acetate, pH 6.0, 1% bovine milk casein, 2 mM cyclohexanediamine tetraacetate (Sigma-Aldrich)). When using anti-p53, anti-p21, and anti-actin primary antibodies, the membrane was blocked with 5% non fat milk (Sigma-Aldrich) in TBST. In all cases the membrane was blocked for 1 h. Rabbit anti-TS antibody was used at 1:1000 dilution in 0.5% BSA in TBST, anti-β tubulin primary antibody was used at 1:10 dilution in TBST, anti-p53 primary antibody was used at 1:1000 dilution in 0.5% milk in TBST, anti-p21 primary antibody was used at 1:250 dilution in 0.5% milk in TBST and anti-actin primary antibody was used at 1:15000 dilution in 0.5% milk in TBST. In all cases, the primary antibody was incubated with the membrane for 1 hr. The membrane was washed 5 times in TBST and incubated for 1 h with the secondary antibody: DAKO 'Polymer' anti-mouse-IgG-HRP used at 1:250 dilution in TBST. The membrane was washed 5 times with TBST and HRP was detected using the LumiGLO Chemiluminescent Substrate Kit (Kirkegaard & Perry Lab; Maryland, USA). Western blot was stripped using Restore^{TM} Western Blot Stripping Buffer (Pierce, Rockford, IL) according to the maufacturer's protocol.

TK *in vitro* assay

Cells growing in log phase were trypsinized, washed with cold PBS and resuspended in extraction buffer (50mM MOPS pH 7.4, 1 mM EDTA pH 7.4, aprotinin (10 μg/mL) and PMSF (2 mM). Cells in suspension were sonicated briefly, centrifuged at 10 000g at 4 °C for 20 min and the supernatant was used for TK *in vitro* assay.

For preparation of anion exchange resin, 1 gram of anion exchange resin Spectra/Gel Strong base anion chloride form (Spectrum, LA, Ca) was washed with 5 mL water, centrifuged at 500 g and washing was repeated twice. The resin was then washed 3× with 0.1N NaOH and 2× with 0.01 N NaOH and then with binding buffer (10 mM Tris-HCl pH8.0) and kept stored in 10mL of binding buffer at 4 °C for up to a week. Each 50 μL TK *in vitro* reaction mixture consisted of 1× assay buffer (10mM ATP, 5mM $MgCl_2$, 5mM MOPS, 100μM EDTA, pH 7.4, thymidine (0.5μCi ^{3}H-thymidine (Amersham Life Sc, Buckinghamshire, England, stock 25Ci/mmol) and cellular extract containing TS enzyme which was the last component added to start the reaction. The reaction mix was incubated at 37°C and stopped by the addition of excess cold thymidine (1mM) and heating at 95°C for 3 min. 0.5 mL of binding buffer (10mM Tris-HCl pH8.0) and 500μL of previously equilibrated anion exchange resin in binding buffer were added and the tube was rotated for 1 hr to allow resin to bind ^{3}H-TMP product. The reaction tube was then centrifuged at 3500 g for 15 secs and the supernatant was removed. The resin was washed 3 × by triturating with 1 mL of binding buffer and a final 0.5 mL wash was collected for counting. The resin was then eluted with 0.5 mL of 0.2 M NaCl and the eluate was counted. For counting, 1mL of Liquid Scintillation counting Fluid Scintran Cocktail X (VWR International, Mississauga, Canada) was added and tubes counted in a Packard model 1600 TR liquid scintillation counter.

Incorporation of ^{3}H-TdR into DNA

7.5×10^3 cells were plated per well of a 96-well plate. 48 hr later, 0.5 μCi ^{3}H-thymidine was added to each well. Cells were incubated at 37°C for 18 hr. The medium was removed and attached cells were washed with PBS (3×200μL). Cells were lysed with 0.1% SDS and the total cell lysate was counted.

Uptake of ^{3}H-TdR into Cells

2×10^4 cells in 200μL were plated per well in a 96-well plate. 48 hr later, the medium was changed to 1% serum in DMEM and 0.5 μCi of ^{3}H-TdR (0.1μM)was added. The plate was incubated at 37°C to allow cellular uptake of ^{3}H-TdR. Uptake was stopped by addition of excess cold thymidine (20μM). The medium was removed and attached cells were washed 3× with PBS. Cells were trypsinized and transferred to a Microcon-PCR centrifugal filter device (Millipore; Bedford, MA) and centrifuged at 500 g for 5 min. The cells retained on the filter in ~ 20μL were washed 5× with PBS. The final wash was collected for counting. 0.2% SDS was added to the filter and incubated at RT for 20 min to solubilize the cells. The filter was then inverted and centrifuged into a fresh tube and the cell extract was collected and counted. Cell incorporation of ^{3}H-TdR was defined as counts in the cell extract minus counts in the "final wash".

Infection of p53 null Cells with Adenovirus Expressing wt p53

Cells were plated at the density of $3\text{x}10^5$ cells/mL in 35mm wells and incubated overnight. The medium was removed and cells were infected with an adenovirus encoding for wild-type p53 (50) at a MOI of 40 diluted in 250 μL PBS, and incubated for 1 hr at 25°C. 2 mL of fresh medium was then added and the cells were incubated at 37°C for another 6 hr to ensure high infection efficiency before replating them at a density of $5\text{x}10^4$ cells/mL per well of a 12-well plate. After overnight incubation (to ensure sufficient time for cells to express high p53 levels), 5-FUdR was added and cells were exposed to the inhibitor for 24h before extracting total protein as described above.

RNA extraction & cDNA synthesis

Exponentially growing cell monolayers were washed twice with chilled PBS. 1.5 mL of REBS (0.5M LiCl, 1.0M Urea, 1.5mM $CaCl_2$, 0.25% SDS, 20mM BES, pH6.8) was added and RNA was extracted as previously described (51). RNA was resuspended in sterile water and total RNA concentration was determined by A_{260} reading. 5μg of total RNA was annealed with 1μg of oligo-dT (Invitrogen, Burlington, Canada) and cDNA was synthesized using the reagents and protocols provided with M-MLV Reverse Transcriptase (Invitrogen).

LightCycler Relative mRNA Quantification.

PCR reactions were performed on a cDNA library from each HCT 116 line, targeting a 204 bp fragment of TK cDNA, a 231 bp of ENT1 cDNA, a 366bp fragment of ENT2 cDNA and an internal reference 718 bp fragment of G6PD mRNA. The primers sequences were as follow: TK (sense), 5′-CCTGCCAACTGAGGGACC-3'; TK (antisense), 5′-GCAGACCAGTGGGTAGGAG-3'; ENT1 (sense), 5′-TGTCTGCGTCCGTGTC-3'; ENT1 (antisense), 5′-CACCTGGTTTCTGTCAAAT-3'; ENT2 (sense), 5′-TGCCTGCGGTTCCTGTTC-3′; ENT2 (antisense), 5′-TGAGCCAAGCTCGCCATT-3′; G6PD (sense), 5′-ATCTACCGCATCGACCAC-3′; G6PD (antisense): 5′-CAGGGAGCTTCACGTTCT-3′. Each 20 μL PCR reaction contained 1X buffer (Invitrogen), 0.2 mM dNTPs, 4 mM $MgCl_2$, 4 pmoles of each primer (10 pmoles for G6PD), 0.5 mg/mL BSA, 2 units of Taq polymerase (Invitrogen) and 1/40000 SYBR green I (Molecular probes Inc., Eugene, OR) and 2 μL of cDNA dilution. Target and reference sequences were amplified and quantified in a LightCycler (Roche, Mississauga, Canada) using the following programs: TK denaturation (98°C for 30 sec), amplification of 50 cycles (98°C for 0 sec, 56°C for 8 sec, 72°C for 9 sec with fluorescence acquisition); ENT1 denaturation (98°C for 30 sec), amplification of 50 cycles (98°C for 0 sec, 52°C for 8 sec, 72°C for 10 sec with fluorescence acquisition); ENT2 denaturation (98°C for 30 sec), amplification of 50 cycles (98°C for 0 sec, 58°C for 8 sec, 72°C for 15 sec with fluorescence acquisition); G6PD denaturation (98°C for 30 sec), amplification of 50 cycles (98°C for 0 sec, 62°C for 10 sec, 72°C for 29 sec with fluorescence acquisition). Each run included a standard curve obtained by 6 serial dilutions of the sensitive cDNA library used as a standard, a no template control and the two samples being quantified. PCR specificity was confirmed by melting curve analysis (63°C-99 °C, 0.1°C/sec). The relative values of the target genes were extrapolated from the standard curve and then normalized to the internal reference (G6PD).

RESULTS

Response of HCT-116p53 wt and null Cell Lines to 5-FUdR

Some cell lines are reported to be dramatically resistant to 5-FU. One such cell line comes from work conducted by Bunz and colleagues where they disrupted the p53 gene in the HCT 116 cell line to generate an HCT-116 p53 null cell line (40). Authors of this report found that the p53 null cell line was remarkably resistant to 5-FU as compared to its p53 wt counterpart (40). Our aim was to try to understand the mechanism responsible for this difference in resistance to drugs between the two lines. We tested their response to 5-FUdR and found that p53 wt and null cell lines were different in response, the p53 wt cell line was sensitive to 5-FUdR as compared to the p53null cell line (Fig. 1). We tested whether the sensitivity of the p53 wt cell line could be rescued with the addition of thymidine in the media and found that the inclusion of 2×10^{-5}M thymidine was sufficient to completely rescue the p53 wt cell line from the cytotoxicity of 5-FUdR. Since the p53 null cell line was resistant to cytotoxicity of 5-FUdR and formation of TS ternary complex, our results suggested that uptake or metabolism of 5-FUdR was different in these cells.

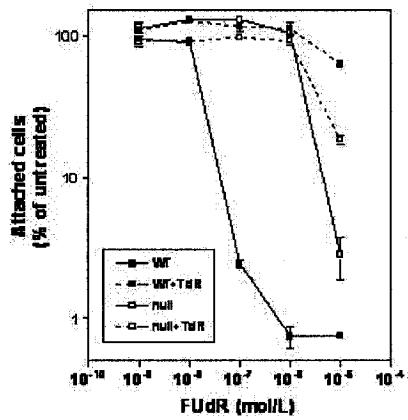

FIGURE 1. Dose-dependent effect of 5-FUdR on growth of HCT-116 wild-type (WT) and p53 null cells in the absence or presence of TdR
Cells were treated with various concentrations of 5-FUdR in the absence or presence of 20 □M TdR for 8 d. The cells attached to the plates were harvested and counted using Coulter counter. The counts were corrected for the untreated cells (0 M FUdR). Error bars represent mean ± SD of 3 replicates. Other details are in Materials and Methods.

Screening Cell Lines for Differences in TS Ternary Complex Formation

The formation of the "ternary complex" of 5-FdUMP, TS and folate cofactor can be used as a biochemical surrogate of the successful conversion of 5-FUdR or 5-FU to 5-FdUMP. This complex is easily detected using western blot and anti-TS antibody. We screened HCT-116 p53 wt and p53 null and several other cell lines: HeLa and 5 colorectal cell lines: RKO, SW-620, HT-29, HCT-15, and HCC2998 for the presence of TS ternary complex. All cell lines were subjected to the same treatment of 24 hr exposure to 10^{-7}M 5-FUdR. Surprisingly, the p53 null cell line formed very little TS ternary complex compared to all others tested (Fig. 2). In addition, there was less of an increase in TS protein level in this p53 null line upon 5-FUdR treatment as compared to other cell lines. As expected, in samples not exposed to 5-FUdR, only the free TS protein was detected. The lower panel shows the loading control for β-tubulin detection (Fig. 2).

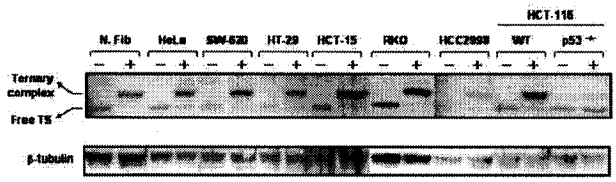

FIGURE 2. Detection of TS-ternary complex formation in various cell lines after 5-FUdR treatment.
Cells were either not treated (-) or treated (+) with 0.1 μM 5-FUdR for 24 h and analyzed by western blotting using anti-TS antibody. Each lane contains 10 μg of protein. Indicated are the positions for the free TS form (35 kD) and the ternary complex form (~37 kD). The blot was striped and re-analyzed for β-tubulin as a loading control (lower panel). Other details are in Materials and Methods.

Effect of 5-FUdR Concentration on Ternary Complex Formation

To explore the reason for the difference in TS ternary complex formation in the HCT-116 p53 wt and p53 null lines, we tested various concentrations of 5-FUdR to determine whether this correlated with the differences in 5-FUdR sensitivity to killing between the two lines. We exposed the cells to the same range of doses of 5-FUdR as used in the cytotoxicity experiments (Fig. 1) and found that with the lowest dose tested, 10^{-9}M, the majority of TS protein was in the ternary complex form in the p53wt cell line whereas there was no detectable TS ternary complex in the p53 null line (Fig. 3A panels wt, null). In the p53 wt cell line, with increasing 5-FUdR concentrations, there was very little increase in the amount of TS in the ternary complex. In comparison, for the p53 null cell line, as the 5-FUdR concentration was increased, the amount of free TS protein gradually decreased and the amount of ternary complex TS gradually increased (Fig. 3A panel null). At the highest dose of 5-FUdR tested, 10^{-5}M, most of the TS protein was in a ternary complex form with a trace amount of free TS protein. Therefore, at 5-FUdR doses between 10^{-9} to 10^{-6} M, the two cell lines showed marked differences in TS ternary complex formation, with the p53 null cell line illustrating a reduced ability to form the TS ternary complex. Only at a very high dose, 10^{-5} M 5-FUdR, was the ternary complex formation eventually comparable between the two lines. These results were consistent with a problem in 5-FUdR uptake or metabolism in the p53 null cell line. We chose to use the 5-FUdR dose of 10^{-7} M for subsequent experiments when illustrating the difference in TS ternary complex formation between the two lines. At each 5-FUdR dose, the p53 wt cell line always showed a greater level of TS protein expression as compared to the p53null line.

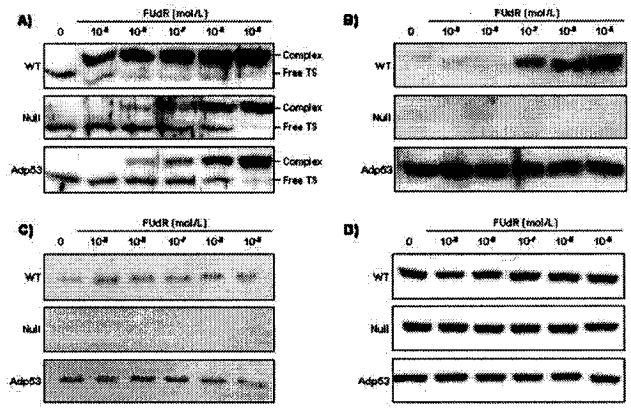

FIGURE 3. Dose-dependent effect of 5-FUdR on the levels of TS (A), p53 (B), p21 (C) and actin (D) in HCT-116 wild-type (WT), p53-/- (null) and p53-transfected p53-/- (Adp53) cells
Cells were treated with various concentrations of 5-FUdR for 24 h and analyzed by western blotting using anti-TS antibody. Each lane contains 10 μg of protein. Indicated are the positions for the free TS form (35 kD) and the ternary complex form (~37 kD). Other details are in Materials and Methods.

Effect of p53 Expression on Ternary Complex Formation

To determine whether the difference in TS ternary complex formation between HCT-116 p53wt and p53 null cell lines was due to the difference in their p53 status, we used p53 null lines infected with an adenovirus expressing wildtype p53. We confirmed that the infected cells were expressing p53 by detecting high levels of p53 in infected cells (Fig. 3B panel Adp53) compared to low basal levels in wt cells (Fig. 3B panel wt, lane 1). The levels of p53 increased in wt cells as a function of 5-FUdR concentration due to DNA damage (Fig. 3B panel wt, lanes 4-6). p53 null cells showed no p53 protein as expected nor any increase with 5-FUdR (Fig. 3B panel null). To confirm that this infected p53 was indeed functional we measured levels of p21, a known target of p53 regulation. We found comparable levels of p21 in p53wt (Fig. 3C panel wt) and p53null infected cells (Fig. 3C panel Adp53). The exposure chosen shows no detectable p21 levels in the p53 null cell line (Fig. 3C panel null). However, longer exposures confirmed low basal levels of p21in p53 null cells (data not shown). The next step was to determine whether restoring wild-type p53 to these cells could restore their ability to form the TS ternary complex at levels similar to the wt cell line. p53null infected cells (Fig. 3A panel Adp53) were indistinguishable from p53 null cells; both failed to form TS ternary complex as efficiently as wt cells at 5-FUdR doses of 10^{-9}M to 10^{-6}M. The last set of panels (Fig. 3D) shows levels of actin as a loading control. This group of experiments provides strong evidence that p53 status of HCT-116 cells is not the sole difference between the two cell lines affecting their ability to form ternary complex.

Sensitivity to 5-FUdR of HCT-116 wt Cells is an Unstable Phenotype

During the course of our experiments, we observed that some cultures of HCT-116 p53 wt cells displayed 5-FUdR resistance comparable to my p53 null cells (data not shown). We

explored this systematically using a single stock of cells. These were established in culture and monitored for 5-FUdR sensitivity over 10-14 passages in culture (~6 weeks). At this point they demonstrated resistance to 5-FUdR comparable to p53 null cells. When the ternary complex forming ability of these cultures on exposure to 5-FUdR was tested, we found that passage 13 cells were unable to form TS ternary complex at 10^{-7} M 5-FUdR, as compared to passage 4 cells (Fig. 4). Since 5-FUdR resistance appeared to be independent of p53 and was an unstable phenotypic property of a single cell line, we decided to focus on trying to understand the mechanism underlying the conversion of early passage p53 wt 5-FUdR-*sensitive* cells, which we will refer to as WT cultures, to late passage p53 wt 5-FUdR-*resistant* cells, which we will refer to as WTR cultures.

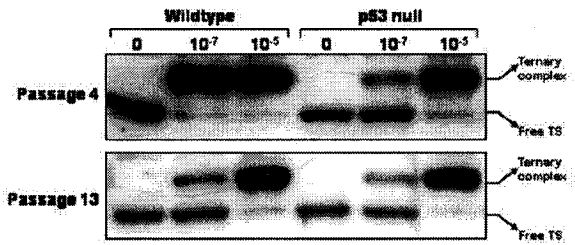

FIGURE 4. Effect of high passage number on TS-ternary complex formation in HCT-116 wildtype and p53 null cells after 5-FUdR treatment
Cells were either not treated (0) or treated (10^{-7} or 10^{-5} mol/L) with 5-FUdR for 24 h and analyzed by western blotting using anti-TS antibody. Each lane contains 10 □g of protein. Indicated are the positions for the free TS form (35 kD) and the ternary complex form (~37 kD). Other details are in *Materials and Methods*.

Differences Between Cell Lines in their Ability to Take up ^{3}H-Thymidine (^{3}H TdR) and Incorporate it Into DNA

^{3}H-TdR uptake and incorporation into DNA over a period ranging from 4 to 24 hr is commonly used as a measure of DNA synthesis. Some of the steps preceeding incorporation of TdR into DNA are similar to those when 5-FUdR is added to cells, *viz*, transport into the cell and phosphorylation by thymidine kinase to the nucleotide (TMP). We next examined WT and WTR cells for differences in their ability to take up and incorporate TdR into DNA. Since the two cultures did not have detectable differences in growth rate (data not shown), we anticipated that any differences between the two cultures produced in this assay would be due to differences in their ability to uptake and or metabolise the ^{3}H-TdR. Following exposure to ^{3}H-TdR for 18 hr, we observed a significantly greater amount of ^{3}H-TdR uptake and incorporation into DNA for WT cells than WTR cells (Fig. 5). These results utilizing ^{3}H-TdR paralleled resistance to formation of ternary complex by 5-FUdR and suggested that there were differences in either uptake or metabolism (TK or phosphatase activity) of the nucleoside, between the two cell cultures.

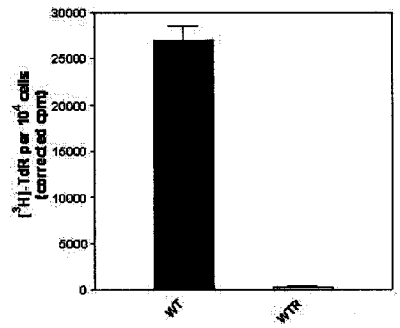

FIGURE 5. Incorporation of [^{3}H]-TdR into DNA in HCT-116 WT and WTR cells
Cells were exposed to [^{3}H]-TdR for 18 h and analyzed as described in *Materials and Methods*. Error bars represent mean ± SD of 3 replicates.

HAT Selection of WT and WTR cells

Another method to explore differences between WT and WTR cultures was to compare their viability in **h**ypoxanthine-**a**minopterin-**t**hymidine (HAT) selection media. Aminopterin inhibits DHFR, leading to folate depletion. This leads to depletion of purine and dTMP levels required for DNA synthesis and cell proliferation. Hypoxanthine and thymidine in HAT replenish the purine and dTMP levels, respectively, via HPRT and TK salvage pathways. We performed this assay using two concentrations of thymidine in the HAT media, 10^{-7}M and 10^{-5}M thymidine. We chose these doses based on earlier observations that indicated that, at 10^{-5}M 5-FUdR, both cultures had most of its TS in the ternary complex, but at 10^{-7}M 5-FUdR, the WT culture had most of its TS in the ternary complex while the WTR culture had most of its TS in the free form (Fig. 3A). When the two cultures were exposed to HA selection medium (no thymidine), we observed no colony forming units (CFUs) for WTR cultures and a few tiny colonies for WT culture (Fig. 6).

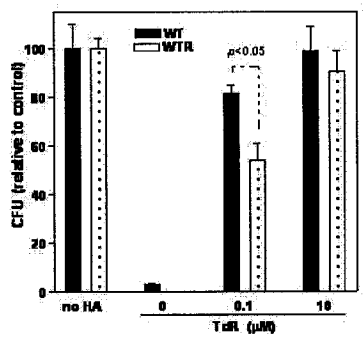

FIGURE 6. Effect of HAT-treatment on colony-formation by HCT-116 WT and WTR cells
Cells were either not treated (no HA) or treated with HA containing 0, 0.1 or 10 μM of TdR. After 10 d, colonies were counted. Reported are colony forming units (CFU) relative to the control (no HA). Error bars represent mean ± SD of 3 replicates. Other details are in *Materials and Methods*.

These results indicated that the amount of thymidine present in the fetal serum in standard medium was insufficient to rescue the cells from the cytotoxicity of aminopterin. When the cultures were exposed to HAT selection media containing 10^{-5}M

thymidine, both cultures were completely rescued from aminopterin toxicity; we observed no difference in the number of CFUs between the two cultures and this number was comparable to number of CFUs in control plates (Fig. 6). When 10^{-7}M thymidine was used in the HAT selection media, we observed a statistically significant difference ($p< 0.05$) in the number of CFUs between the two cultures as there was 80% of CFUs compared to control plates for the WT culture, and 50% of CFUs compared to control plates for the WTR culture (Fig. 6). This assay therefore provided evidence that thymidine was either not efficiently taken up or phosphorylated by TK to its active TMP metabolite form, in the WTR culture.

Thymidine Kinase (TK)

To explore whether a deficiency in TK activity was responsible for the observed phenotype, we compared TK levels in the WT and WTR cells. TK enzymatic activity was measured using an *in vitro* biochemical assay. Based upon a single experiment, there appeared to be no difference in TK activity in extracts of both cell types (Fig. 7A). Quantitative RT-PCR analysis of TK mRNA also showed no significant differences between the two cultures (Fig. 7B).

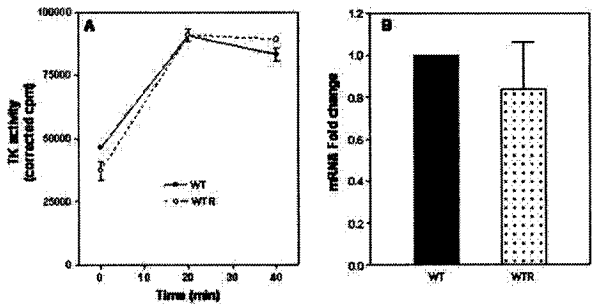

FIGURE 7. Level of thymidine kinase (TK) enzymatic activity (A) and mRNA (B) in HCT-116 WT and WTR cells
See *Materials and Methods* for details. Error bars represent mean ± SD of 3 replicates.

Cellular Uptake of ^{3}H-Thymidine (^{3}H-TDR)

Given the lack of evidence that differences in TK levels between WT and WTR cells might explain the phenotypic differences observed, we next compared cellular uptake of thymidine between the two cultures. When growing cells are exposed to ^{3}H-TdR, the nucleoside is first taken up into the cell via a nucleoside transported, then it is phosphorylated by thymidine kinase, (trapping ^{3}H-thymidine monophosphate (TMP) inside the cell), then it is further phosphorylated to TTP which can then be incorporated into DNA. Very short labeling times will favour cell uptake and phosphorylation over incorporation into DNA. To distinguish uptake and phosphorylation from incorporation into DNA, we exposed cells to ^{3}H-TdR for short periods of time, 10 and 20 min. Results of Fig. 8 show that, at these very early time periods, the amount of ^{3}H-TdR associated with WT cells was much higher than with WTR cells. At the 10 min time point, WT cells contained approximately 3 times as much radioactivity (statistically significant, $p< 0.05$) as WTR cells and at 20 min time point, WT cells contained approximately 20 times as much radioactivity as WTR cells (Fig. 8). The results of this and the TK experiment provide strong evidence that the phenotypic differences between WT and WTR cells are due to some defect in uptake of the nucleoside into WTR cells.

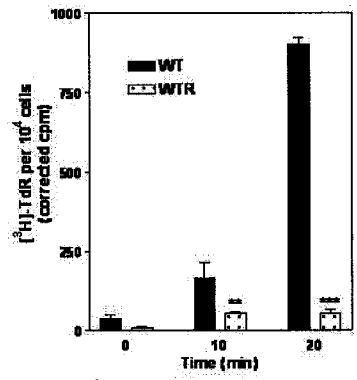

FIGURE 8. Uptake of [^{3}H]-TdR in HCT-116 WT and WTR cells
Cells were exposed to [^{3}H]-TdR for 0, 10 or 20 min and analyzed as described in *Materials and Methods*. Error bars represent mean ± SD of 3 replicates. Asterisks (**p=0.015, ***p<0.0001) represent unpaired 2-tailed t tests (WT vs. WTR).

Comparison of 5-FUdR and 5-FU

5-FU (the nucleobase) is used more widely than 5-FUdR (the nucleoside) in clinical medicine. Although they may share a common transporter to enter a cell, their metabolism to reach 5-FdUMP is very different (Fig. 14). Earlier workers have also previously tested 5-FU on HCT-116 cells (40). Our results showed that p53 WT cells were sensitive to 5-FUdR while p53 null cells were resistant. When we compared the 5-FUdR response of WTR and nullR (p53 null, late passage) cultures, we found that they both behaved as WTR cells, i.e., resistant to 5-FUdR (Fig. 9A). When we tested 5-FU at the same doses, we found that p53 null cells were relatively resistant to 5-FU at doses between 10^{-7} to 10^{-6}M compared to the p53 wt cell line (Fig. 9B). These results support the trend cited in the earlier study, although results of that study reported a far greater extent of 5-FU resistance for the p53 null cell line. Furthermore, NullR (p53 null, later passage) cultures were even more resistant than null cultures at 5-FU doses between 10^{-7} to 10^{-6}M. WTR cultures were found to be more resistant to 5-FU compared to WT cultures. When examined for their ability to form TS ternary complex after exposure to 5-FU or 5-FUdR, WTR, null and nullR cultures all showed similar results that were different from WT cells (Fig. 10). At 10^{-7}M, most of the TS protein was in the ternary complex in the WT culture while most of the TS protein was in the free form in the WTR, null and nullR cultures (Fig. 10). At 10^{-6}M 5-FU (and higher doses) and at 10^{-5}M 5-FUdR, the amount of TS in ternary complex was similar for all cell lines. It is interesting to note that, at 10^{-6} M 5-FU, there were significant differences among lines in their response to 5-FU (Fig. 9B) but little difference in the relative amount of TS in ternary complex.

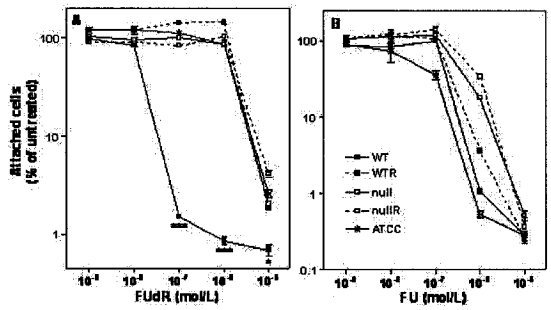

FIGURE 9. Dose-dependent effect of 5-FUdR (A) and 5-FU (B) on growth of HCT-116 WT, WTR, null, nullR and ATCC cells
Cells were treated with various concentrations of 5-FUdR (*A*) or 5-FU (*B*) for 8 d. The cells attached to the plates were harvested and counted using Coulter counter. The counts were corrected for the untreated cells (0 M FUdR). Error bars represent mean ± SD of 3 replicates. Other details are in *Materials and Methods*. Asterisks *** represent $p<0.001$ and * represents $p<0.05$ of one-way ANOVA.

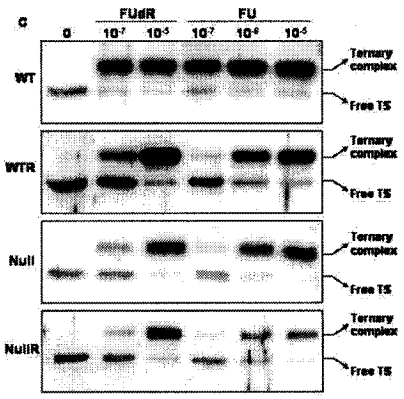

FIGURE 10. Effect of 5-FUdR and 5-FU on TS-ternary complex formation in HCT-116 WT, WTR, null and nullR cells
Cells were either not treated (0) or treated (10^{-7} or 10^{-5} mol/L) with 5-FUdR or 5-FU for 24 h and analyzed by western blotting using anti-TS antibody. Each lane contains 10 μg of protein. Indicated are the positions for the free TS form (35 kD) and the ternary complex form (~37 kD). Other details are in *Materials and Methods*.

Effect of hMLH-1 on Conversion of Culture from WT to WTR Phenotype

Results thus far strongly suggest a phenotypic instability in HCT-116 cells with respect to nucleoside or nucleobase uptake or metabolism. HCT-116 is an HNPCC cell line due to a defect in the mismatch repair gene, hMLH-1 (located on chromosome 3). We investigated whether this gene defect had any effect on the conversion of WT cells to the WTR phenotype. To test this, we used another HCT-116 derived cell line, HCT-116+Chr3, which was previously characterized and shown to contain a functional transfected copy of chromosome 3 (48). When we tested an early culture of HCT-116+Chr3, we found that it showed comparable results to WT cells (Fig. 11) in terms of its ability to take up ^{3}H-TdR and incorporate it into DNA. However, after maintaining HCT-116+Chr3 for several passages, we found that late passage cultures were no longer able to uptake ^{3}H-TdR as efficiently as earlier passages cells and eventually they too developed a WTR (Fig. 11) phenotype. These results suggest that a defect in genes on chromosome 3 responsible for the HNPCC phenotype of HCT-116 cell line were not involved in the instability governing the conversion of WT cultures to the WTR phenotype.

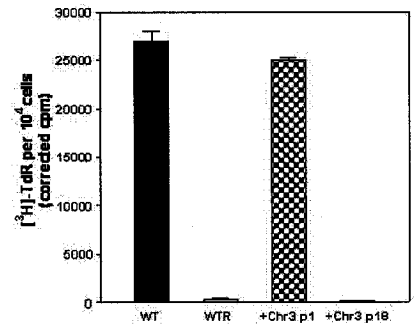

FIGURE 11. Incorporation of [^{3}H]-TdR into DNA in early and late passages of chromosome 3-transfected HCT-116 cells as compared to WT and WTR cultures
Cells were exposed to [^{3}H]-TdR for 18 h and analyzed as described in *Materials and Methods*. Error bars represent mean ± SD of 3 replicates.

Effect of 5-Aza-2-deoxycytidine

Methylation of genes is a known mechanism for changing gene expression. To explore whether such events were responsible for the observed phenotypic instability, we tested whether we could revert this phenotype by treatment of WTR cultures with 5-AdCd. Our first step was to determine the sensitivity of the cells to 5-AdCd to allow choice of a suitable dose and length of exposure for pre-treatment of the cells. The experimental results indicated that about 80-90% of cells remained attached for both cultures after 24hr treatment with doses of 10^{-9} to 10^{-5}M 5AdCd (Fig. 12A) and 60-85% after 48hr treatment. We decided to use a 24 hr pre-treatment period. When we tested the ability of 5-AdCd pre-treated WTR cells to take up ^{3}H-thymidine and incorporate it into DNA, we found no difference between pre-treated and untreated cells (data not shown). Thus, there is no indication that 5-AdCd pre-treatment of WTR cultures reverts their resistant phenotype. This provides no evidence to support the hypothesis that methylation was involved in the observed phenotypic instability.

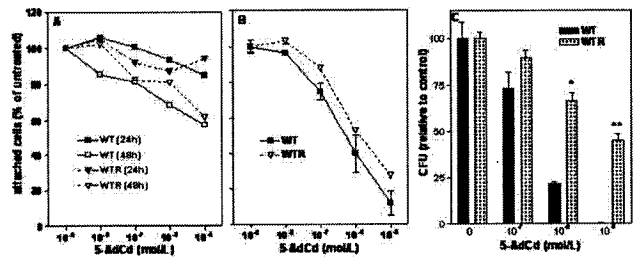

FIGURE 12. Effect of 5-AdCd-treatment on growth of HCT-116 WT and WTR cells
A. Effect of 24 or 48 h treatment with 5-AdCd. *B*. Effect of 24 h 5-AdCd-treatment followed by 3 d recovery. *C*. Colony-forming ability of cells in B. In A and B, cells attached to plates were counted using Coulter counter and the counts were corrected for the untreated cells (0 M 5-AdCd). In C, colony forming units

(CFU) are reported relative to the untreated cells. Error bars represent mean ± SD of 2 replicates. Asterisks (*p=0.01, **p=0.008) represent unpaired 2-tailed t tests (WT vs. WTR). Other details are in *Materials and Methods*.

Since 5-AdCd is a nucleoside and my results suggested that nucleoside uptake was different between WT and WTR cultures we decided to examine in more detail the response of the two cell lines to this drug. The number of cells remaining after 3 days following a 24hr 5-AdCd pre-treatment, was lower for the WT cells than WTR cells (Fig. 12B). However, this difference was slight, so we switched to a more reliable assay for testing cytotoxicity of drugs, *viz.*, the colony forming unit assay. We observed a statistically significant difference in the number of CFUs between the two cultures at doses of 10^{-7}, 10^{-6} and 10^{-5}M 5-AdCd (Fig. 12C). We obtained 75, 22 and 0.5 % respectively of CFUs (compared to control plates) for WT cells and 90, 67 and 45% of CFUs (compared to control plates) for WTR cells. Results from this assay clearly showed that the WTR culture was cross resistant to 5-AdCd and suggested that a common pathway was affected.

Levels of h-ENT1 and h-ENT2 mRNA

Four human nucleoside transporters, h-ENT1, h-ENT2, h-ENT3 and h-ENT4 have been recently identified(52). However, only the first two, h-ENT1 and h-ENT2 have been recently characterized(53). We prepared RNA from WT and WTR cells and assessed levels of mRNA for the two characterized transporters by quantitative RT-PCR. No significant differences in their mRNA levels were seen (Fig. 13).

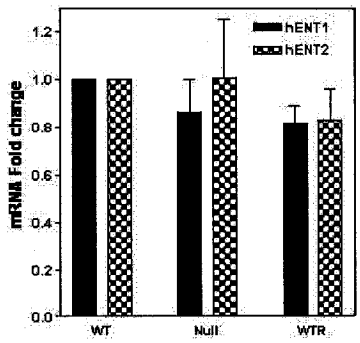

FIGURE 13. Levels of h-ENT1 and h-ENT2 in HCT-116 WT, null and WTR cells
See *Materials and Methods* for details. Error bars represent mean ± SD of 3 replicates.

DISCUSSION

Two forms of TS protein are observed by western blot analysis in cancer cells after treatment with 5-FU or 5-FUdR chemotherapeutic drugs (Fig. 14). One form is the free TS protein that may be capable of interacting with its natural substrates (CH_2THF, dUMP) and producing dTMP for DNA synthesis and cell proliferation. The other form is a TS protein covalently bound to CH_2THF and FdUMP (a key metabolite of 5-FU and 5-FUdR) forming a stable ternary complex (6) (7). Continuous formation of this inactivated TS protein complex leads to depletion of the free TS protein and hence leads to depletion of thymine-containing nucleotides and eventually cell death (8). Thus, the presence of this complex in cancer cells is a good indication of the extent of TS-specific cytotoxicity caused by 5-FU or 5-FUdR drugs. However, many cancer cells are/develop resistance to these drugs, and one type of resistance is the reduced ability to form the ternary complex after treatment with the drugs. In the present study, while examining a set of cancer cells for ternary complex formation, I found the HCT-116 $p53^{-/-}$ cells showed a "5-FUdR-resistant phenotype", i.e., a lack of ternary complex formation after 5-FUdR treatment and relative 5-FUdR-resistance compared to wildtype (WT) cells. Results reported in this paper suggest a possible *mechanism* for the lack of this complex formation in these cells.

We first examined whether the absence of p53 in HCT-116 $p53^{-/-}$ cells was responsible for the reduced ternary complex formation and 5-FUdR resistance. Recent studies have purported to show that lack or disruption of p53 can affect the sensitivity of cells to 5-FU. Results from two different studies, one utilizing HCT-116 $p53^{-/-}$ cells and the other utilizing MCF-7 cells transformed with E6, both found the cell lines to be resistant to 5-FU (40,41). Our initial results are consistent with these studies and show that HCT-116 $p53^{-/-}$ cells demonstrate a 5-FUdR-resistant phenotype. However, our serendipitous finding of a reduced formation of ternary complex suggested that alteration in TS protein or nucleoside metabolism was responsible, not a p53-dependent response to drug-induced DNA damage. The lack of involvement of p53 was demonstrated by re-introduction of a biologically active p53 into HCT-116 $p53^{-/-}$ cells to a level similar to WT cells. This failed to alter the resistant phenotype. Furthermore, we observed that even HCT-116 WT cells were unstable, eventually showed a 5-FUdR-resistant phenotype (WTR), after a few weeks in culture in the absence of any drug. Thus, the lack of ternary complex formation that I observed in HCT-116 $p53^{-/-}$ cells appeared to be independent of p53 status, and likely dependent upon other factor(s).

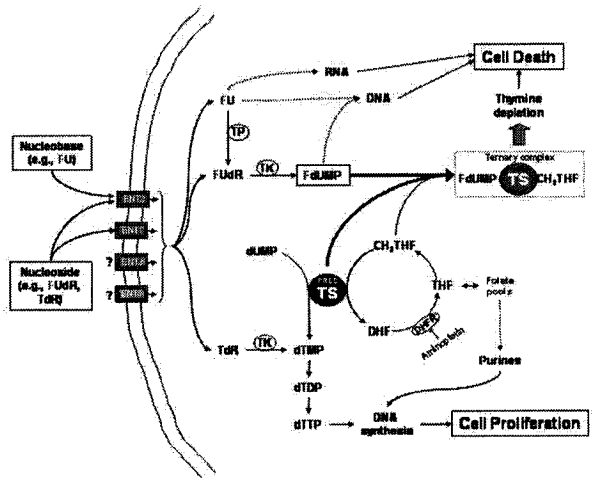

FIGURE 14. Schematic showing the factors involved in ternary complex formation in a cell
See *Discussion* for more details.

Several cellular factors were investigated that could be responsible for decreasing ternary complex formation after 5-FUdR or 5-FU treatment in WTR cells. 1) Reduced affinity of TS for FdUMP could theoretically reduce ternary complex formation. To the best of our knowledge there has been shown in only two reports (13) (14). Berger and coworkers showed the

presence of a variant TS enzyme in addition to the wildtype TS in HCT-116 cells (14). Compared to wildtype TS, the variant protein is more basic (higher isoelectric point) and has reduced affinities for both FdUMP and CH_2THF. We used two dimensional gel electrophoresis to examine whether there were any differences in the two forms of TS protein in WT and WTR HCT-116 cells. The results showed *no difference* in either the levels or the isoforms of wildtype and variant TS (data not shown). Thus the lack of ternary complex formation in WTR cells was not likely due to differences in the forms of TS protein.

2) A reduction in the level of cellular folate is capable of reducing ternary complex formation since lower folate levels can lead to lower amounts of CH_2THF, one component of the ternary complex. To rule out this explanation, we co-administered folinic acid and 5-FUdR in HCT-116 $p53^{-/-}$ cells to examine the effect on the ternary complex formation. Folinic acid is 5-CHO-THF, which increases the intracellular folates including CH_2THF levels (54). Addition of folinic acid did *not* seem to increase the ternary complex formation in the HCT-116 $p53^{-/-}$ cells (data not shown). This suggested that low levels of folate are not responsible for the lack of ternary complex formation in HCT-116 $p53^{-/-}$ cells.

3) A reduction in the *formation* of FdUMP is also capable of reducing the ternary complex formation in WTR cells. Two main factors that can reduce intracellular FdUMP are low thymidine kinase (TK) levels and reduced uptake of the 5-FUdR or 5-FU. TK is a non-essential enzyme that catalyzes the salvage biosynthesis of dTMP by phosphorylating TdR (thymidine). Since TdR and 5-FUdR are nucleosides of very similar structure, TK can also catalyze the phosphorylation of 5-FUdR to form the TS-reactive FdUMP. (Note: when 5-FU is given to the cells, it is first converted to 5-FUdR by thymidine phosphorylase and then to FdUMP by TK (Fig. 14)). Examination of the TK level by an enzymatic assay and by RT-PCR showed *no change* in WTR cells compared to WT cells. This suggested that the lack of ternary complex formation in WTR cells is not likely due to low TK levels.

4) We examined the possibility that impaired *uptake* of 5-FUdR is responsible for the lack of ternary complex formation in WTR cells. The first indication of impaired nucleoside uptake came from the experiment that demonstrated a dramatic reduction in the 18 h-uptake of ^{3}H-TdR in WTR cells. Since most nucleosides (including 5-FUdR and TdR) are believed to be taken up *via* a mechanism involving the same transporters, impaired uptake of TdR implies the same will be true for 5-FUdR. 18 h growth of cells in ^{3}H-TdR results in its incorporation into DNA. We used short-term uptake (10-20 min) of ^{3}H-TdR as a measure of cellular uptake. Again, WTR cells showed impaired uptake of TdR. Thus, these results suggest that WTR cells have impaired uptake of nucleosides including 5-FUdR, and this is most likely responsible for the reduced ternary complex formation and increased 5-FUdR resistance.

A clonogenic method involving hypoxanthine-aminopterin-TdR (HAT) treatment was also used to demonstrate impaired TdR uptake into WTR cells. Aminopterin inhibits DHFR, leading to folate depletion. This leads to depletion of purine and dTMP levels required for DNA synthesis and cell proliferation. Hypoxanthine and TdR in HAT replenish the purine and dTMP levels, respectively, via HPRT and TK salvage pathways. Treatment with HAT containing various levels of TdR showed that WTR cells require higher concentration of TdR than WT cells to be rescued. The difference between WT and WTR cells observed using HAT treatment was not as dramatic as the difference seen using the TdR uptake experiment (above). This is probably due to the fact that, although TdR uptake is impaired, it is not completely inhibited in WTR cells and the cells are still able to take up TdR at slow rates and supply sufficient dTMP to maintain cell needs.

Impaired uptake of nucleoside is likely due to defect in transport, but this is difficult to prove directly. A method of measuring incorporation of radiolabelled thymidine for short periods of time (10-20 min) was developed. Further uptake was quenched by dilution of ^{3}H-TdR with a 200-fold excess of unlabelled TdR. Lowering of the background to very low levels was possible using centrifugation and extensive washing of cells on a small filter. With this technique, ^{3}H-TdR taken up by cells after as little as 10 min could be detected. At this short time interval, the majority of cell-associated radioactivity is likely to be ^{3}H-TdR or ^{3}H-dTMP. Differences between WT and WTR cell lines are therefore likely to be due to defects in transport of ^{3}H-TdR into the cell or possibly conversion to ^{3}H-dTMP.

To further explore the impaired uptake of nucleoside in WTR cells, we examined the levels of nucleoside and nucleobase transporters, ENT-1 and ENT-2. ENT-1 transports only nucleosides (e.g., 5-FUdR, TdR) while ENT-2 can transport both nucleosides and nucleobases (e.g., 5-FU) (53) (Fig. 14). Since reduction in ternary complex formation in WTR cells was seen for both 5-FU and 5-FUdR, we expected that ENT-2 would be affected. However, RT-PCR analysis demonstrated *no difference* in ENT-1 or ENT-2 levels between the WT and WTR cells. This did not rule out the possibility that the activity of these transporters is somehow affected in WTR cells. Furthermore, the involvement of recently described, although not well characterized, transporters ENT-3 and ENT-4 in the impaired uptake in WTR needs to be explored, especially ENT-3, as it is just recently believed to be a transporter of both nucleosides and nucleobases (52).

Although nucleobase 5-FU showed lower ternary complex formation in WTR than WT cells, it showed only a small difference in the cytotoxicity of the two cell lines. This is different than the effect by 5-FUdR, which showed a lower ternary complex formation *and* significantly lower cytotoxicity in WTR than WT cells. Once 5-FU is inside a cell, it is converted to 5-FUdR by thymidine phosphorylase (TP) and/or incorporated into RNA and DNA (Fig. 17) by various metabolic enzymes (9). Both 5-FU-to-5-FUdR conversion and RNA/DNA incorporation pathways can lead to 5-FU-mediated cytotoxicity. Since the effect of 5-FU on ternary complex formation was similar to 5-FUdR, the 5-FU-to-5-FUdR conversion pathway is not likely responsible for the difference in 5-FU-dependent cytotoxicity in WT and WTR cells. Surprisingly, p53 null and nullR cells showed a slight resistance in 5-FU-mediated cytotoxicity compared to WT and WTR cells. A similar but much more pronounced resistance has been seen by other groups in p53 null cells (40,41).

To address the question of the mechanism of instability of WT cells leading to WTR cells demonstrating a 5-FUdR-resistant phenotype, we examined the role of genetic instability as a possible mechanism. Ferguson and Cheng have shown that human colon cancer cells are phenotypically unstable with respect to sensitivities to chemotherapeutic drugs etoposide and vincristine (55). However, the same effect towards 5-FU or 5-

FUdR has not previously been shown. Colon cancer HCT-116 cells have been previously shown to demonstrate hereditary non-polyposis colorectal cancer (HNPCC) phenotype due to mismatch repair deficiency caused by the lack of hMLH-1 gene (48). Since defects of this DNA repair gene can lead to further genetic instability in cells, we first examined whether reintroduction of the missing gene would inhibit the WT to WTR conversion. The hMLH-1 gene is located on chromosome 3p21 and reintroduction of chromosome 3 in the cells (HCT-116+Chr3) has been shown to correct the mismatch repair deficiency and HNPCC phenotype (48). However, we found that HCT-116+Chr3 cells with corrected HNPCC phenotype were still phenotypically unstable and converted to WTR cells with a 5-FUdR-resistant phenotype. We also examined the role of DNA methylation in the conversion of WT to WTR. 5-aza-2-deoxycytidine (5-AdCd) is widely used nucleoside that reverts DNA methylation in cells (56,57). We were unable to "revert" WTR cells back to WT cells using 5-AdCd, suggesting this is not the mechanism of the observed instability.

Mycoplasma contamination is believed to decrease TdR uptake by cells (58). However, we found no evidence of mycoplasma contamination in any of our cell cultures using a commercial detection kit. We further examined whether factors released into the medium are responsible for the unstable phenotype of WT cells. Co-culturing WT and WTR cells caused all the cells to demonstrate the 5-FUdR-resistant phenotype. Supplementing culture media from WTR to WT cells also converted the latter cells to the WTR phenotype. We attempted crude fractionation of the medium from WTR cells by ethanol precipitation of protein and other large molecules. However, neither the alcohol soluble nor the reconstituted precipitated fraction was able to convert WT cells to the WTR phenotype. This indicated that the putative factor(s) responsible for the unstable phenotype in the culture media of WTR cells did not survive ethanol precipitation. Further work will be necessary to identify and characterize the factors responsible for the unstable phenotype leading to 5-FUdR resistance in WTR cells.
In this study we have used the lack of TS-FdUMP-CH_2THF ternary complex formation to identify a 5-FUdR-resistant mechanism in HCT-116 cells. The mechanism of resistance seems to be impaired uptake of the 5-FUdR drug, likely due to impaired nucleoside uptake. HCT-116 cells were found to be phenotypically unstable as they spontaneously converted to 5-FUdR-resistant phenotype. Our results also show that some unknown factor(s) in the culture media of WTR cells is (are) capable of inducing 5-FUdR-resistant phenotype in WT cells.

REFERENCES

1. Carreras C.W., Santi D.V. 1995. The catalytic mechanism and structure of thymidylate synthase. *Annu. Rev. Biochem.* **64**: 721-762.
2. Danenberg P.V. 1977. Thymidylate synthetase - a target enzyme in cancer chemotherapy. *Biochim. Biophys. Acta* **473**: 73-92.
3. Hardy L.W., Finer-Moore J.S., Montfort W.R., Jones M.O., Santi D.V., Stroud R.M. 1987. Atomic structure of thymidylate synthase: target for rational drug design. *Science* **235**: 448-455.
4. Parker W.B., Cheng Y.C. 1990. Metabolism and mechanism of action of 5-fluorouracil. *Pharmacol. Ther.* **48**: 381-395.
5. Heidelberger C., Danenberg P.V., Moran R.G. 1983. Fluorinated pyrimidines and their nucleosides. *Adv. Enzymol. Relat Areas Mol. Biol.* **54**: 58-119.
6. Santi D.V., McHenry C.S., Sommer H. 1974. Mechanism of interaction of thymidylate synthetase with 5- fluorodeoxyuridylate. *Biochemistry* **13**: 471-481.
7. Danenberg P.V., Lockshin A. 1981. Fluorinated pyrimidines as tight-binding inhibitors of thymidylate synthetase. *Pharmacol. Ther.* **13**: 69-90.
8. Seno T., Ayusawa D., Shimizu K., Koyama H., Takeishi K., Hori T. 1985. Thymineless death and genetic events in mammalian cells. *Basic Life Sci.* **31**: 241-263.
9. Pinedo H.M., Peters G.F. 1988. Fluorouracil: biochemistry and pharmacology. *J. Clin. Oncol.* **6**: 1653-1664.
10. Berger S.H., Jenh C.H., Johnson L.F., Berger F.G. 1985. Thymidylate synthase overproduction and gene amplification in fluorodeoxyuridine-resistant human cells. *Mol. Pharmacol.* **28**: 461-467.
11. Zhang Z.G., Malmberg M., Yin M.B., Slocum H.K., Rustum Y.M. 1993. Isolation and characterization of a human ileocecal carcinoma cell line (HCT-8) subclone resistant to fluorodeoxyuridine. *Biochem. Pharmacol.* **45**: 1157-1164.
12. Rhee M.S., Balinska M., Bunni M., Priest D.G., Maley G.F., Maley F., Galivan J. 1990. Role of substrate depletion in the inhibition of thymidylate biosynthesis by the dihydrofolate reductase inhibitor trimetrexate in cultured hepatoma cells
4. *Cancer Res.* **50**: 3979-3984.
13. Bapat A.R., Zarow C., Danenberg P.V. 1983. Human leukemic cells resistant to 5-fluoro-2'-deoxyuridine contain a thymidylate synthetase with lower affinity for nucleotides. *J. Biol. Chem.* **258**: 4130-4136.
14. Berger S.H., Barbour K.W., Berger F.G. 1988. A naturally occurring variation in thymidylate synthase structure is associated with a reduced response to 5-fluoro-2'- deoxyuridine in a human colon tumor cell line. *Mol. Pharmacol.* **34**: 480-484.
15. Fry D.W., Jackson R.C. 1986. Membrane transport alterations as a mechanism of resistance to anticancer agents. *Cancer Surv.* **5**: 47-79.
16. Sobrero A., Aschele C., Guglielmi A., Nobile M.T., Rosso R. 1990. Resistance to 5-fluorouracil and 5-fluoro-2'-deoxyuridine mechanisms and clinical implications. *J. Chemother.* **2 Suppl 1**: 12-16.
17. Miyachi H., Jiao L., Sowers L.C., Scanlon K.J. 1992. Collateral sensitivity to azidothymidine in methotrexate resistant human leukemia cells. *In Vivo* **6**: 17-21.
18. Fernandes D.J., Cranford S.K. 1985. Resistance of CCRF-CEM cloned sublines to 5-fluorodeoxyuridine associated with enhanced phosphatase activities. *Biochem. Pharmacol.* **34**: 125-132.
19. Zhang Z.G., Harstrick A., Rustum Y.M. 1992. Mechanisms of resistance to fluoropyrimidines. *Semin. Oncol.* **19**: 4-9.
20. Romanini A., Lin J.T., Niedzwiecki D., Bunni M., Priest D.G., Bertino J.R. 1991. Role of folylpolyglutamates in biochemical modulation of fluoropyrimidines by leucovorin. *Cancer Res.* **51**: 789-793.
21. Jastreboff M.M., Zielinska Z.M. 1983. Development and characteristics of a subline of Ehrlich ascites carcinoma cells persistently resistant to 5-fluoro-2'-deoxyuridine. *Acta Biochim. Pol.* **30**: 185-191.
22. Houghton P.J., Houghton J.A., Germain G., Torrance P.M. 1987. Development and characterization of a human colon adenocarcinoma xenograft deficient in thymidine salvage. *Cancer Res.* **47**: 2117-2122.
23. Clark J.L., Berger S.H., Mittelman A., Berger F.G. 1987. Thymidylate synthase gene amplification in a colon tumor resistant to fluoropyrimidine chemotherapy. *Cancer Treat. Rep.* **71**: 261-265.
24. Johnston P.G., Drake J.C., Trepel J., Allegra C.J. 1992. Immunological quantitation of thymidylate synthase using the monoclonal antibody TS 106 in 5-fluorouracil-sensitive and - resistant human cancer cell lines. *Cancer Res.* **52**: 4306-4312.
25. Aschele C., Sobrero A., Faderan M.A., Bertino J.R. 1992. Novel mechanism(s) of resistance to 5-fluorouracil in human colon cancer (HCT-8) sublines following exposure to two different clinically relevant dose schedules. *Cancer Res.* **52**: 1855-1864.
26. Copur S., Aiba K., Drake J.C., Allegra C.J., Chu E. 1995. Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil. *Biochem. Pharmacol.* **49**: 1419-1426.
27. Glazer R.I., Peale A.L. 1979. The effect of 5-fluorouracil on the synthesis of nuclear RNA in L1210 cells in vitro. *Mol. Pharmacol.* **16**: 270-277.
28. Major P.P., Egan E., Herrick D., Kufe D.W. 1982. 5-Fluorouracil incorporation in DNA of human breast carcinoma cells. *Cancer Res.* **42**: 3005-3009.
29. Chu E., Lai G.M., Zinn S., Allegra C.J. 1990. Resistance of a human ovarian cancer line to 5-fluorouracil associated with decreased levels of 5-fluorouracil in DNA. *Mol. Pharmacol.* **38**: 410-417.
30. Yin M.B., Zakrzewski S.F., Hakala M.T. 1983. Relationship of cellular folate cofactor pools to the activity of 5-fluorouracil. *Mol. Pharmacol.* **23**: 190-197.

31. Inaba M., Mitsuhashi J., Sawada H., Miike N., Naoe Y., Daimon A., Koizumi K., Tsujimoto H., Fukushima M. 1996. Reduced activity of anabolizing enzymes in 5-fluorouracil-resistant human stomach cancer cells. *Jpn. J. Cancer Res.* **87**: 212-220.
32. Sobrero A.F., Moir R.D., Bertino J.R., Handschumacher R.E. 1985. Defective facilitated diffusion of nucleosides, a primary mechanism of resistance to 5-fluoro-2'-deoxyuridine in the HCT-8 human carcinoma line. *Cancer Res.* **45**: 3155-3160.
33. Plagemann P.G., Woffendin C. 1987. Residual nitrobenzylthioinosine-resistant nucleoside transport in a transport mutant (AE1) of S49 murine T-lymphoma cells. *Mol. Cell Biol.* **7**: 160-166.
34. Aronow B., Ullman B. 1985. Thymidine incorporation in nucleoside transport-deficient lymphoma cells. *J. Biol. Chem.* **260**: 16274-16278.
35. Agarwal R.P., Han T., Fernandez M. 2001. Reduced cellular transport and activation of fluoropyrimidine nucleosides and resistance in human lymphocytic cell lines selected for arabinosylcytosine resistance. *Biochem. Pharmacol.* **61**: 39-47.
36. Lynch T.P., Cass C.E., Paterson A.R. 1977. Defective transport of thymidine by cultured cells resistant to 5-bromodeoxyuridine. *J. Supramol. Struct.* **6**: 363-374.
37. Dumontet C., Bauchu E.C., Fabianowska K., Lepoivre M., Wyczechowska D., Bodin F., Rolland M.O. 1999. Common resistance mechanisms to nucleoside analogues in variants of the human erythroleukemic line K562. *Adv. Exp. Med. Biol.* **457**: 571-577.
38. Tezuka M., Sugiyama H., Tamemasa O., Inaba M. 1982. Biochemical characteristics of a 5-fluorouracil-resistant subline of P388 leukemia. *Gann* **73**: 70-76.
39. Lowe S.W., Ruley H.E., Jacks T., Housman D.E. 1993. p53-Dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **74**: 957-967.
40. Bunz F., Hwang P.M., Torrance C., Waldman T., Zhang Y., Dillehay L., Williams J., Lengauer C., Kinzler K.W., Vogelstein B. 1999. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J. Clin. Invest* **104**: 263-269.
41. Longley D.B., Boyer J., Allen W.L., Latif T., Ferguson P.R., Maxwell P.J., McDermott U., Lynch M., Harkin D.P., Johnston P.G. 2002. The role of thymidylate synthase induction in modulating p53-regulated gene expression in response to 5-fluorouracil and antifolates. *Cancer Res.* **62**: 2644-2649.
42. Coleman W.B., Tsongalis G.J. 2002. Role of genomic instability in the development of human cancer. *In*: The molecular basis of human cancer. *Editors*: W.B. Coleman, G.J. Tsongalis. Humana Press, New Jersey, pp. 115-142.
43. Rosenberg S.M., Thulin C., Harris R.S. 1998. Transient and heritable mutators in adaptive evolution in the lab and in nature. *Genetics* **148**: 1559-1566.
44. Jaenisch R., Bird A. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* **33 Suppl**: 245-254.
45. Haqqani A.S., Cowling R.T., Maroun J.A., Birnboim H.C. 1999. Characterization of a polyclonal antibody to human thymidylate synthase suitable for the study of colorectal cancer specimens. *J. Histochem. Cytochem.* **47**: 1563-1574.
46. Brattain M.G., Levine A.E., Chakrabarty S., Yeoman L.C., Willson J.K., Long B. 1984. Heterogeneity of human colon carcinoma. *Cancer Metastasis Rev.* **3**: 177-191.
47. Bunz F., Dutriaux A., Lengauer C., Waldman T., Zhou S., Brown J.P., Sedivy J.M., Kinzler K.W., Vogelstein B. 1998. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* **282**: 1497-1501.
48. Hawn M.T., Umar A., Carethers J.M., Marra G., Kunkel T.A., Boland C.R., Koi M. 1995. Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint. *Cancer Res.* **55**: 3721-3725.
49. Udenfriend S., Stein S., Bohlen P., Dairman W., Leimgruber W., Weigele M. 1972. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science* **178**: 871-872.
50. Fortin A., Cregan S.P., MacLaurin J.G., Kushwaha N., Hickman E.S., Thompson C.S., Hakim A., Albert P.R., Cecconi F., Helin K., Park D.S., Slack R.S. 2001. APAF1 is a key transcriptional target for p53 in the regulation of neuronal cell death. *J Cell Biol.* **155**: 207-216.
51. Birnboim H.C. 1993. Extraction of high molecular weight RNA and DNA from cultured mammalian cells. *Meth. Enzymol.* **216**: 154-160.
52. Baldwin S.A., Beal P.R., Yao S.Y., King A.E., Cass C.E., Young J.D. 2003. The equilibrative nucleoside transporter family, SLC29. *Pflugers Arch.*, in press.
53. Yao S.Y., Ng A.M., Vickers M.F., Sundaram M., Cass C.E., Baldwin S.A., Young J.D. 2002. Functional and molecular characterization of nucleobase transport by recombinant human and rat equilibrative nucleoside transporters 1 and 2. Chimeric constructs reveal a role for the ENT2 helix 5-6 region in nucleobase translocation. *J. Biol. Chem.* **277**: 24938-24948.
54. Van der Wilt C.L., Pinedo H.M., Smid K., Cloos J., Noordhuis P., Peters G.J. 1992. Effect of folinic acid on fluorouracil activity and expression of thymidylate synthase. *Semin. Oncol.* **19**: 16-25.
55. Ferguson P.J., Cheng Y.C. 1989. Phenotypic instability of drug sensitivity in a human colon carcinoma cell line. *Cancer Res.* **49**: 1148-1153.
56. Kim W.J., Vo Q.N., Shrivastav M., Lataxes T.A., Brown K.D. 2002. Aberrant methylation of the ATM promoter correlates with increased radiosensitivity in a human colorectal tumor cell line. *Oncogene* **21**: 3864-3871.
57. Liteplo R.G., Alvarez E., Frost P., Kerbel R.S. 1985. Induction of thymidine kinase activity in a spontaneously enzyme- deficient murine tumor cell line by exposure in vivo to the DNA- hypomethylating agent 5-aza-2'-deoxycytidine: implications for mechanisms of tumor progression. *Cancer Res.* **45**: 5294-5298.
58. Sinigaglia F., Talmadge K.W. 1985. Inhibition of [3H]thymidine incorporation by Mycoplasma arginini-infected cells due to enzymatic cleavage of the nucleoside. *Eur. J Immunol.* **15**: 692-696.

antibody (cat. no. 100-1173) were also from Rockland Immunochemicals. CY3-conjugated donkey anti-sheep secondary antibody (cat. no. 716-165-147) and HRP-conjugated donkey anti-goat secondary antibody (cat. no. 705-036-147) were from Jackson ImmunoResearch Laboratories (West Grove, PA). HRP-conjugated anti-rabbit/anti-mouse secondary antibody ("Polymer") was from DAKO (Carpinteria, CA).

The specificity of the anti-hTS antibody was determined using recombinant $(his)_6$-tagged hTS protein (rhTS) as a competitor. This protein was expressed in *E. coli*, purified by affinity chromatography on a nickel column and then twice-purified by SDS-PAGE as previously described (16). After electroelution, the protein was precipitated with ethanol and dissolved as a stock solution at a concentration of 0.5 mg/mL in 0.2% SDS. The detergent was required to maintain the solubility of the denatured TS protein. When used as a competitor for Western blot analysis, immunofluorescence, immunocytochemistry or immunhistochemistry, the stock was diluted such that the final concentration of SDS was $\leq$0.02%. For competition experiments, rhTS was pre-incubated overnight at 4 °C with 100 µL of a 1:500 dilution of sheep anti-TS antiserum. To control for any inhibitory effect of this low concentration of SDS, control experiments were carried out in which the same antiserum was incubated with an equivalent amount of SDS.

Other tests of the specificity of the anti-hTS antibody involved selective depletion of anti-TS antibody from the sheep antiserum using an affinity column with immobilized rhTS. Sheep anti-TS antiserum was diluted 1:100 and passed 20 times through a bed of CN Br-activated Sepharose 4B (Sigma-Aldrich Chemicals, St. Louis, MO) to which rhTS protein was coupled (16). As a control, diluted anti-serum was passed through a similar matrix containing immobilized bovine serum albumin (BSA) (OmniPur ^{R} BSA, Fraction V, EM science- MERCK kGaA, Darmstadt, Germany).

Cell lines

HeLa cells were obtained from ATCC (Rockville, MD). A series of 5-fluorodeoxyuridine (FUdR)-resistant HeLa cells clones were isolated by stepwise selection over several weeks in up to 0.1 µM in this drug. Clone HeLa-55 overexpressed TS protein most strongly and was shown to have a highly amplified TS gene (Chapter 2). The following colon cancer cell lines from the NCI anti-cancer screening panel of 60 cell lines were used: HCT-116; HCT-15, HCC-2998, HT-29 and SW-620; RKO is a colon cancer line originally derived by Brattain *et al.*(17). Normal primary human fibroblasts were from neonatal foreskin. Cells were cultured as monolayers in Dulbecco's modified Eagle's medium (GIBCO-BRL, Burlington, Canada) supplemented with 10% fetal calf serum in 100 mm dishes in 5% CO_2 at 37 °C. RKO cells were maintained in McCoy's 5A media plus 10% fetal calf serum (GIBCO-BRL).

Immunofluorescence

Cells were grown on 22 mm^2 glass cover slips in 10 cm dishes. Log phase cultures were used in all experiments. Coverslips were removed and washed twice with phosphate-buffered saline (PBS). All subsequent steps were carried out at room temperature. Cells were fixed for 10 min with acetone:methanol (1:1). Coverslips were blocked for 30 min with Universal Blocking solution (DAKO). All antibody dilutions were in 'Antibody Diluting buffer' (DAKO). Coverslips were incubated for 1 h with primary anti-TS antibody (1:500 dilution). They were washed 5 times (5 min per wash) with PBS. Cells were then incubated for 30 min with secondary anti-sheep IgG- CY3 antibody (1:1000 dilution) and washed again. The coverslips were mounted onto slides with 'Fluorescent Mounting medium' (DAKO) containing 5 µg/mL Hoechst 33258 (Sigma-Aldrich). Stained cells were examined and photographed with a Zeiss Axiophot fluorescence microscope. Confocal images were obtained using a Zeiss inverted LSM 410 confocal laser scanning miscroscope. Z-series images were obtained through the depth of cells using a step size range of 1-2µm.

Immunocytochemistry

Coverslips containing cells in log phase were removed and washed twice with phosphate-buffered saline (PBS). All subsequent steps were carried out at room temperature. Cells were fixed for 10 min with acetone:methanol (1:1). Coverslips were blocked for 30 min with Casein Blocking Solution in PBS (2% bovine milk casein (Sigma-Aldrich), 2 mM cyclohexanediamine tetraacetate (Sigma-Aldrich), in PBS). All antibody dilutions were in PBS. Coverslips were incubated for 1 h with primary anti-TS antibody (1:500 dilution). They were washed 5 times (5 min per wash) with PBS. Cells were then incubated for 30 min with secondary rabbit anti-sheep-IgG-HRP antibody (1:1500 dilution) and washed again. Visualization was achieved with diaminobenzidine substrate. The coverslips were mounted onto slides with 'Permount Mounting medium' (Fisher Scientific, Toronto, Canada). Stained cells were examined and photographed with a Zeiss Axiophot microscope.

Immunohistochemistry

Colorectal cancer specimens were obtained from the Ottawa Colorectal Cancer Bank; most were fixed in GenoFix (DNA Genotek Inc, www.DNAgenotek.com). Four µm sections were deparaffinized and hydrated by standard methods. Slides were pre-treated with 3% H_2O_2 in methanol for 10 min and blocked for 30 min at room temperature with Casein Blocking solution in PBS. All antibodies were diluted in PBS. The sections were exposed overnight at 4°C to 1:500 dilution of primary anti-TS antibody. They were washed 5 times (5 min per wash) with PBS and then exposed to 1:200 dilution of rabbit anti-sheep-IgG-HRP antibody for 30 min at room temperature. Sections were washed again and visualization was achieved with diaminobenzidine substrate (DAKO liquid DAB^+ Substrate-Chromogen Solution, K3467, DAKO). The sections were then counterstained with hematoxylin. A negative control (that is lacking primary antibody) was included.

Preparation of whole cell and nuclear extracts

Cultured cells were detached by tryspin treatment, then washed twice and suspended in cold PBS. To prepare whole cell

extracts, an equal volume of 2×SDS sample buffer (100 mM MOPS, pH 6.8, 4% SDS, 20% glycerol, 2 mM β-mercaptoethanol) was added. The extracts were boiled for 20 min and then sonicated briefly to reduce viscosity. Protein was quantified using fluorescamine (18) (Sigma-Aldrich), using BSA as a standard. For isolation of nuclei, cells were washed twice in PBS and suspended in nuclear extraction buffer (10 mM Tris-HCl, pH7.5, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM spermidine, 2 μg/mL aprotinin and 1 mM PMSF) at ~ 1×10^6 cells/mL. After 20 min incubation at 0°C, the swollen cells were homogenized (50 strokes) with a tight-fitting glass Dounce homogenizer. Samples were centrifuged (800*g*) at 2-4°C and the pellet was washed twice with nuclear extraction buffer containing 0.1% NP-40. Protein in the nuclear pellet was extracted by dispersion in SDS sample buffer, brief sonication and heating at 100°C for 15 min.

Western blot analysis

Proteins in cell or nuclear extracts in SDS sample buffer were resolved by 12% discontinuous SDS-PAGE and then electrophoretically transferred to a PVDF membrane (Millipore, Nepean, Canada) at 15V for 18 h in transfer buffer (3 mM Na_2CO_3, 10 mM $NaHCO_3$, 10% methanol) (16). The membrane was stained with Ponceau S and then washed twice in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8). All steps were carried out at room temperature. When using anti-TS as the primary antibody, the membrane was blocked for 1 h with freshly prepared Casein Blocker solution (100 mM NaCl, 50 mM sodium acetate, pH 6.0, 1% bovine milk casein, 2 mM cyclohexanediamine tetraacetate. When anti-LDH primary antibody was used, the membrane was blocked with 5% horse serum in TBST. Sheep anti-TS antibody was diluted 1:5000 in Casein Blocking solution and goat anti-LDH primary antibody was diluted 1:1000 in TBST. In all cases, the primary antibody was incubated with the membrane for 1 h. The membrane was washed 5 times in TBST and incubated for 1 h with a secondary antibody as follows: rabbit anti-sheep-IgG-HRP was used at 1:3000 dilution in Casein Blocking solution; DAKO 'Polymer' anti-mouse-IgG-HRP was used at 1:100 dilution in TBST; donkey anti-goat-IgG-HRP was used at 1:25000 dilution in TBST. The membrane was washed 5 times with TBST and HRP was detected using the LumiGLO Chemiluminescent Substrate Kit (Kirkegaard & Perry Lab; Maryland, USA).

Preparation of Plasmid DNA Contructs

GFP-NES: pEGFP-C1 plasmid was obtained from BD Biosciences Clontech (Mississauga, Canada). Two 46-mer oligonucleotides, hTSNESF (AATTCTATTTACCTGAATCACATCGAGCCACTGAAAATTCAGCTTG) and hTSNESR (GATCCAAGCTGAATTTTCAGTGGCTCGATGTGATTCAGGTAAATAG), encoding the sense and antisense sequences of a putative TS nuclear export signal (NES) respectively, were synthesized. The oligonucleotides were annealed and the resulting fragment consisted of overhanging sequences compatible to EcoR1 and BamH1 sites. The annealed fragment was ligated to EcoR1/BamH1 digested pEGFP-C1. The resulting plasmid, GFP-NES contained the putative NES of TS fused in frame to the carboxyl end of GFP; its sequence was confirmed by sequencing.

H_6TS^{313} : PQE-hTS (16)encoded human TS with the first 5 amino acids deleted and 16 additional amino acids including a 6 × histidine tag inserted at the N-terminus. This His_6-TS (1 kb) fragment was isolated from PQE-hTS using EcoR1/Xba1 and ligated to EcoR1/Xba1 digested pCR3 (Invitrogen, Burlington, Canada).

H_6TS^{248} : In pcDNA3/HA-hTS29 there was a base pair deletion in the codon for amino acid 248 of human TS(19). This resulted in a frameshift mutation causing amino acids 248-259 to become mutated and ended with a premature stop codon at amino acid 260. This resulted in non expression of amino acids 260-313. An 870bp fragment containing this frameshift mutation was isolated from pcDNA3/HA-hTS29 using Pst1/Xba1. H_6TS-PCR3 was digested with Pst1/Xba1 and the 5167bp fragment was ligated to the 870bp of pcDNA3/HA-hTS29.

H_6TS^{186} : The nucleotide fragment corresponding to amino acids 186-313 in H_6TS-PCR3 was cut out with BglII/Xba1. The remaining construct was blunted-ended with mung bean nuclease and then ligated.

fAeqTS-pCDNA3 was synthesized to contain the full-length TS cDNA. The coding region was obtained from pWHTS-1(20) and the flanking 5'-UTR containing triple GC-rich repeats (21) was synthesized by PCR amplification of genomic DNA. The correctness of the construct was confirmed by sequencing.

Transfection

HeLa and RKO cells were seeded at 1.25×10^5 cells in a 3 cm dish on glass coverslips. The following day, cells were transfected with 2 μg of purified plasmid DNA and 3 μl of Fugene 6 Transfection Reagent (Roche Diagonostics Corporation, Indianapolis, USA), according to the manufacturer's protocol. Twenty-four hours post-transfection, coverslips were removed and immunofluorescence staining was performed as described above.

RESULTS

Specificity of anti-hTS antiserum

The interpretation of results employing immunohistochemistry depends critically on the specificity of the antibodies used. Previous immunohistochemical or immunocytochemical studies of TS have employed polyclonal anti-TS antibodies whose specificity was not fully documented. We chose to use multiple techniques to confirm the specificity of the primary anti-hTS sheep polyclonal antibody used in our experiments. Fig 1A (lane 1) shows Western blot analysis of a HeLa cell extract probed with anti-hTS; the only detectable signal was a band of the expected molecular size for TS (35.7 kDa). Fig. 1A (lane 2) shows a similar analysis of an extract of a 5-FUdR-resistant HeLa cell line, HeLa-55, growing in 5-FUdR. Two bands are seen in Fig. 1A (lane 2) and Fig. 1C, which correspond to free TS and to the previously described "ternary complex" containing TS-5-FdUMP-CH_2-THF(7). The high level of TS in HeLa-55 cells was due to amplification of the TS gene

(Chapter 2). No other bands were detected in extracts of either cell line, providing one line of evidence supporting claims for the specificity of the primary and secondary antibodies used. Fig. 1B shows immunocytochemical analysis of the same preparation of HeLa and HeLa-55 cells as used in panel A. The correspondence in amount of TS in these cell lines by the two methods and the single band seen on the Western blot provide strong evidence that the immunocytochemical signal is indeed due to TS. These results provide one line of evidence that the combination of primary and secondary rabbit anti-sheep antibodies used was highly specific for TS.

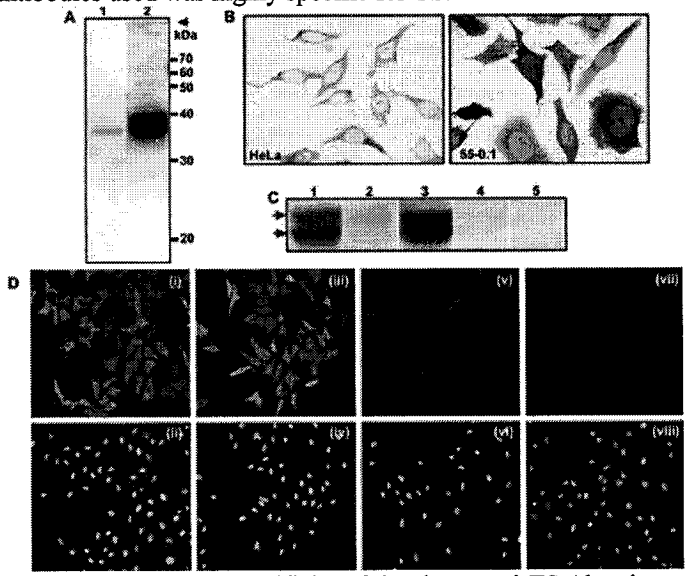

FIGURE 1. Proof of specificity of the sheep anti-TS Ab using Western blot and immunohistochemical analyses.
A. Western blot analysis for TS of whole cell extract of HeLa (lane 1) and HeLa-55 (lane 2). 10μg protein was loaded per lane. Arrow-head indicates position of top of the gel. *B.* Immunocytochemistry for TS of HeLa and HeLa-55 cells. Same primary and secondary antibodies were used. *C.* Competition of TS signal on Western blots of whole cell extract of HeLa-55 cells (10μg/lane). Top arrow indicates TS in a 'ternary complex' and bottom arrow indicates free TS protein. Anti-TS antibody was preincubated with SDS buffer-alone (lane 1) or SDS buffer containing 10μg of soluble rhTS protein (lane 2), or the antibody was preabsorbed using a control 10μg BSA-immobilized column (lane 3) or a 10μg rhTS-immobilized column (lane 4). Lane 5 is a secondary antibody-alone control. *D.* Competition of TS signal on immunofluorescence of HeLa-55 cells. Anti-TS antibody was preincubated with standard buffer (i), SDS buffer (iii) or SDS buffer containing 10μg of soluble rhTS protein (v). Secondary antibody-alone control is shown in (vii). The same fields stained with Hoechst are shown in bottom panels (ii), (iv), (vi) and (viii). Other details are in *Materials and Methods*.

Competition with a specific antigen (usually a synthetic peptide) is another approach to establish specificity of antibodies. Since the anti-TS polyclonal antibody was raised against recombinant hTS protein (rhTS) (i.e., not against a synthetic peptide), we prepared highly purified protein and tested this as a competitor. Competition was carried out by either pre-incubating rhTS with the anti-hTS antibody or using anti-hTS antibody passed through an rhTS affinity column. Western blot analysis of HeLa-55 cells probed with either control or treated antibodies is shown in Fig. 1C. Control antibody and antibody passed through a control affinity column showed strong TS and TS-ternary complex bands (Fig. 1C lanes 1 and 3). Essentially complete disappearence of the bands was seen with either soluble rhTS or immobilized rhTS as competitor (lanes 2 and 4). Secondary antibody alone showed no signal (lane 5). Immunofluorescence staining of HeLa-55 cells using the same antibodies is shown Fig. 1D. An equivalently strong fluorescent signal was seen in these cells when standard buffer (panel i) or 'competition' buffer (panel iii) was used. Pretreatment of the primary antibody with soluble rhTS almost completely eliminated the signal (panel v). Similar disappearence of signal was seen when immobilized rhTS was used as competitor (data not shown). Fluorescent secondary antibody alone showed no signal (panel vii). The same fields stained with Hoechst 33258 to indicate the position of nuclear DNA are shown in the bottom panels. Taken together, this combination of techniques provides strong evidence that the primary and secondary antibodies were highly specific for TS and therefore suitable for the following study.

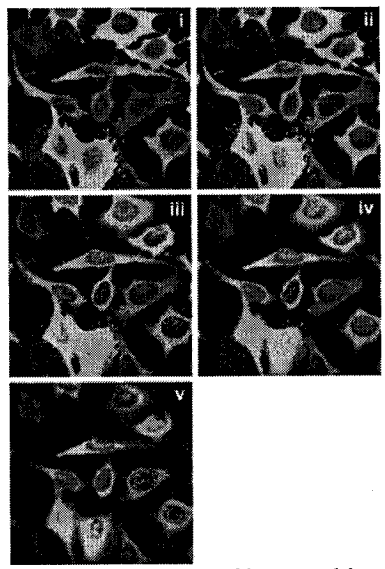

FIGURE 2. Confocal microscopy of immunohistochemically analyzed TS staining in HeLa- 55
2 μm serial sections were taken through the depth of cells from bottom to top, panels (i) to (v). Other details are in *Materials and Methods*.

Nuclear localization of TS in overexpressing HeLa cell lines

Cell line HeLa-55, which contains a highly amplified TS gene, strongly overexpressed TS protein (Fig. 1). In addition to the strong staining of the cytoplasm of these cells, nuclear staining also appeared to be present (Fig. 1B). To confirm this observation, we used immunofluorescence confocal microscopy to study cellular localization. As shown in Fig. 2, serial optical sections provide clear evidence for the presence of nuclear staining in these TS-overexpressing cells.

HeLa-55 cells were observed to exhibit heterogeneous intensity of staining (Fig. 2). Nuclear staining was detectable in both high- and low-TS expressing HeLa-55 cells, although lower frequency of nuclear staining was seen in cells expressing lower amounts of TS compared to high TS expressors (Fig. 2 and data not shown). Variable TS expression was also seen in other 5-FUdR-resistant HeLa clones by immunofluorescence microscopy. In general, it appeared that clones that expressed

less TS had a lower frequency of cells with nuclear TS staining (data not shown). Nuclear TS could still be detected in HeLa-55 cells grown in the absence of 5-FUdR for a period of up to 1 week (data not shown).

Nuclear localization of TS in Other Cell Lines

It has been previously reported that TS can be detected in the nucleus of a rat hepatoma cell line (22) and in human colon cancer specimens (15). To extend these observations, we examined a number of human cell lines for nuclear TS. Cell lines included normal human fibroblasts and 6 colorectal cancer lines (RKO, HCT-116, HCT-15, HT-29, HCC2998 and SW620). Amongst the various cell lines examined, differences in both the intensity and localization of TS protein were seen (Fig. 3). Normal fibroblasts and HCT-15 cells showed the lowest total level of TS staining (Fig. 3). There were only slight differences in TS staining amongst the other 5 cell lines. Fibroblasts (Fig. 3) and parental HeLa cells (Fig. 1B) showed no discernible nuclear staining. Both cytoplasmic and nuclear TS staining was observed in the other cell lines but the relative intensity of cytoplasmic TS compared to nuclear TS staining varied. HCT-15 showed a very low level and HCC2998 and SW620 (Fig. 3) showed low levels of nuclear TS staining. The strongest evidence of nuclear staining was seen in RKO, HCT-116 and HT-29 cells (Fig. 3).

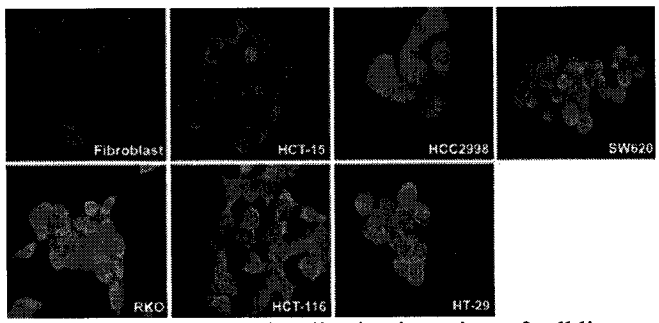

FIGURE 3. Survey of TS localization in variety of cell lines using immunohistochemical analysis.
Most cell lines seem to show nuclear localization; RKO, HCT-116, HT-29; fibroblast do not. There is clumping of some colon cancer lines. The same conditions of conventional immunofluorescence miscroscopy were used for all cell lines. Other details are in *Materials and Methods*.

To strengthen these observations, we performed immunofluorescence confocal microscopy on one nuclear TS positive and one negative cell line. As shown in Fig. 4, panels i-iii, serial optical sections clearly indicated the presence of nuclear TS staining in cell line RKO, and the absence of nuclear TS in cell line HeLa (Fig. 4, panels iv-v). These findings confirmed the conventional microscopy results.

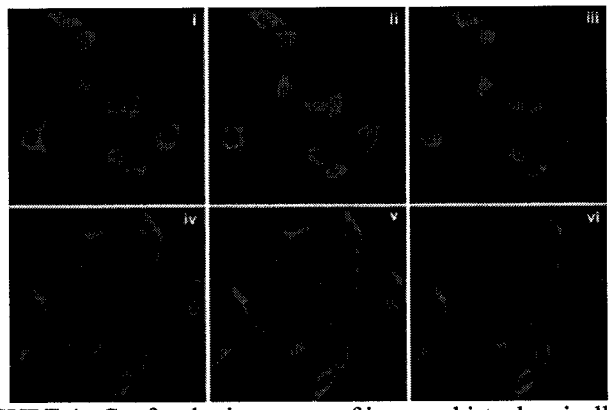

FIGURE 4. Confocal microscopy of immunohistochemically analyzed TS staining in RKO and HeLa cells.
2 μm serial sections were taken through the depth of cells from bottom to top. RKO cells, panels (i) to (iii); HeLa cells, panels (iv) to (vi). Other details are in *Materials and Methods*.

Western Blot Analysis of extracts of isolated nuclei for TS

To provide an independent line of evidence for the presence of TS in nuclei, we isolated nuclei from some cell lines and performed Western blot analysis for TS on nuclear extracts. Nuclei were isolated from RKO and HCT-116, the two colorectal cell lines that showed strongest nuclear TS staining. Total cell extracts from an equivalent number of the same cells were analyzed. HeLa cells, which showed no nuclear TS, was used as a negative control. A strong TS band was detected in total cellular lysates of HeLa, RKO and HCT-116 (Fig. 5A, lanes 1-3). A weaker signal was detected in nuclear extracts of RKO and HCT-116 cells (Fig. 5A, lanes 5,6), but no indication of TS in the nuclear extract of HeLa cells was detected (Fig. 5A, lane 4). It appeared roughly 5-10% of total TS protein was present in the nucleus of RKO and HCT-116 cells. Lactate dehydrogenase, a previously established cytoplasmic marker (23), was used to confirm the absence of cytoplasmic contamination in nuclear extracts (Fig. 5A).

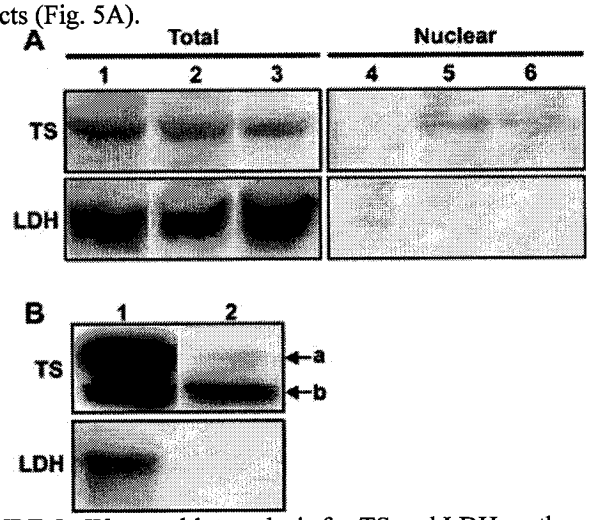

FIGURE 5. Western blot analysis for TS and LDH on the whole-cell or nuclear extracts in selected cell lines
A. Western blot analysis for TS and LDH on whole cell (lanes 1, 2, 3) and nuclear (lanes 4, 5, 6) extracts of HeLa (lanes 1, 4), RKO (lanes 2, 5) and HCT-116 cells (lanes 3, 6). Equivalent number of cells (5×10^5) was loaded in each lane. LDH is a cytoplasmic marker absent in nuclei. *B*. Western blot analysis

for TS and LDH on whole cell (lane 1, 5×10^3 cells) and nuclear (lane 2, 5×10^5 cells) extracts of HeLa-55 cells. Note that one hundred-fold less cells in lane 1 was loaded compared to lane 2 and the lanes in panel A due to a very high level of cytoplasmic TS in HeLa-55. Arrow 'a' indicates TS in a 'ternary complex' and arrow 'b' indicates free TS protein. Other details are in *Materials and Methods.*

Nuclear localization of free- and Ternary Complex-bound TS

TS-overexpressing HeLa-55 cells grown in the presence of 0.1 μM 5-FUdR were fractionated and a nuclear extract was probed for TS by Western blot analysis. Total cell extract (100-fold less cell equivalents than used for nuclear extract) was similarly analysed. In addition to intense TS staining obtained in the total cell lysate (Fig. 5B, lane 1), a strong TS signal was detected in the nuclear extract (Fig. 5B, lane 2). From the relative intensities of the two, we estimate that about 0.5% of the total *free* TS was in the nuclear fraction. Interestingly, almost none of the *ternary complex-bound* TS was in the same nuclear fraction. The LDH control confirmed the virtual absence of cytoplasmic contamination in the nuclear extract.

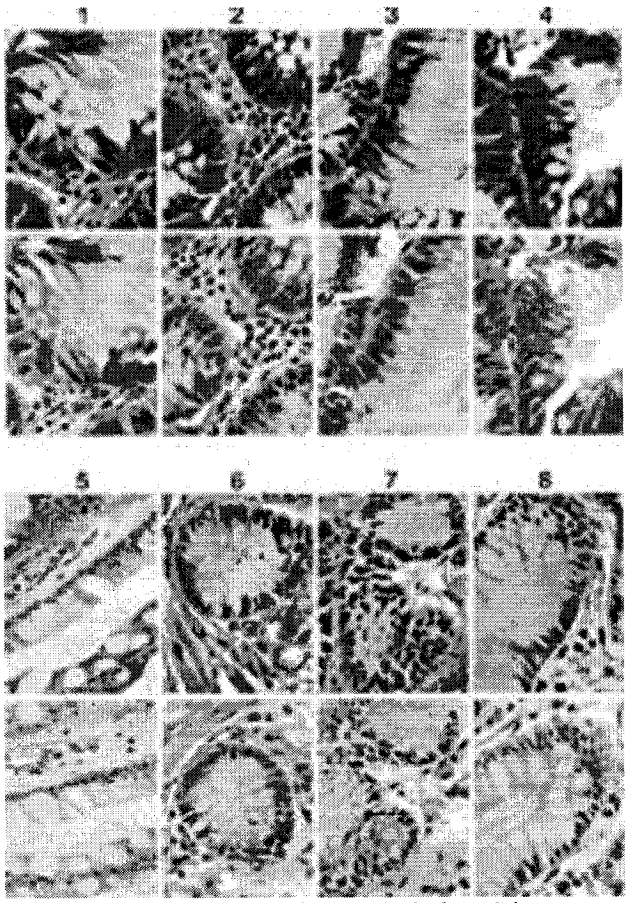

FIGURE 6. Immunohistochemical analysis for TS in cancerous and normal colorectal tissue sections.
Different regions from a section of a cancer case (panels 1-4) and a normal case (panels 5-8) are shown. Each panel (1-8) shows two serial sections stained with anti-TS antibody containing either no competitor (top section) or 10μg rhTS competitor (bottom section). Pictures were taken at magnification × 400.

Nuclear TS in Colorectal cancer specimens

Wong et al. (15), in agreement with our own unpublished observations, have identified nuclear staining of colorectal specimens probed with anti-TS antibodies. To confirm that this staining is indeed due to nuclear TS and not due to an unidentified cross-reacting species, we first identified some specimens of colorectal cancer in which nuclear staining could be observed using the same primary and secondary antibodies described above. Panels 1-4 (Fig. 6) shows regions selected from 1 case of colorectal cancer and panels 5-8 shows regions selected from 1 normal colonic mucosa. In each case, the lower section shows that the majority (but not all) of the strong TS signal decreased using recombinant hTS protein as a competitor, as in Fig. 1D. Noteworthy, both cytoplasmic and nuclear staining was similarly decreased by the competitor, providing strong evidence that nuclear staining was indeed due to the presence of TS protein. Interestingly, some nuclei in normal mucosa also showed the presence of nuclear TS.

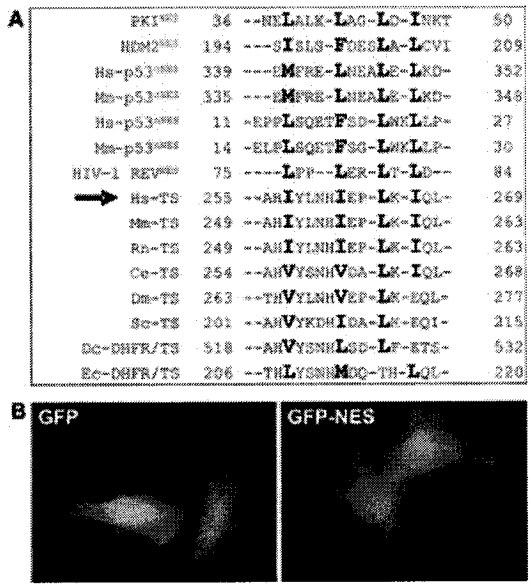

FIGURE 7. Putative leucine rich nuclear export signal (NES) of TS.
A. Multiple sequence alignment of some of the known NES (in PKI, HDM2, p53 and REV) with the putative NES in TS of several species (Hs, Homo sapiens; Mm, Mus musculus; Rn, Rattus norvegicus; Ce, Caenorhabditis elegans; Dm, Drosophila melanogaster; Sc, Saccharomyces cerevisiae; Dc, Daucus carota; Ec, Escherichia coli). Arrow indicates human TS sequence, first of the listed TS sequences. Letters in bold highlight amino acids that fit the putative leucine-rich NES motif. *B.* Fluorescence analysis of HeLa cells transfected with control GFP construct or GFP fused to TS-NES (GFP-NES).

Putative Nuclear Export Sequence in TS Gene

To explain the presence of TS in the nuclei of some cells, we examined TS for the presence of motifs corresponding to nuclear localization sequences (NLS) and or nuclear export sequences (NES) that might influence its cellular localization. A putative leucine-rich nuclear export sequence (Fig. 7A) at position 257-269 was identified near the carboxyl terminus of

TS. This putative NES containing a '4-2-1' spacing is conserved in human, mouse, rat and worms and is very similar to the '4-2' spacing found in Drosophilia and yeast and in the bifunctional DHFR-TS of carrot cells (Fig. 7A). To test whether the putative NES of TS could function as an NES, we prepared a construct, GFP-NES, in which TS-NES is fused in frame to the carboxyl end of GFP. Using pEGFP-C1 vector as a control, transfected HeLa cells strongly expressed GFP in both nucleus and cytoplasm (Fig. 7B). The localization of GFP in HeLa cells transfected with GFP-NES was indistinguishable from cells transfected with pEGFP-C1 control (Fig. 7B). Similar results were obtained in RKO cells (data not shown).

To explore the functionality of the putative TS NES further, we generated two His_6-tagged constructs of TS, H_6TS^{248} and H_6TS^{186}, lacking carboxyl terminal regions encompassing the NES and surrounding sequences (Fig. 8A). Full-length TS bearing an amino-terminal histidine$_6$ tag (H_6TS^{313}) (Fig. 8B), and wild type TS (wt TS) (data not shown) both localized predominantly to the cytoplasm, with only a small amount in the nucleus. However, HeLa cells transfected transiently with H_6TS^{248} and H_6TS^{186} showed an increased amount of nuclear TS (Fig. 8B). Cells transfected with H_6TS^{186} contained cytoplasmic punctate speckling and a noticeable decrease in the number of transfected cells expressing this truncated form of TS.

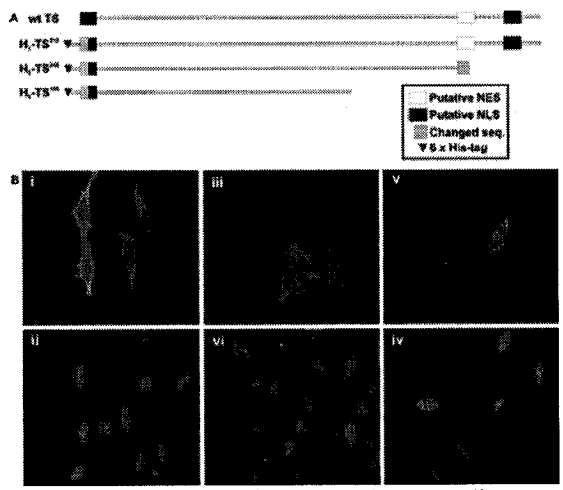

FIGURE 8. Effect of mutation in NES and surrounding sequences in TS protein on nuclear localization.
A. Schematic of various TS constructs. *B.* Immunofluorescence analysis of TS in HeLa cells transfected with H_6TS^{313} (i), H_6TS^{248} (iii) or H_6TS^{186} (v). The same fields stained with Hoechst are shown in bottom panels (ii), (iv), and (vi).

DISCUSSION

Confidence in immunohistochemistry results requires that highly specific antibodies be utilized. We therefore thought it necessary to first ascertain the specificity of the primary anti-hTS sheep polyclonal antibody and the secondary rabbit anti-sheep antibody. This was first demonstrated by the detection of only a single band corresponding to human TS in Western blot analysis of HeLa cell extract. We also employed an 5-FUdR-resistant HeLa cell line, HeLa-55, maintained in 5-FUdR, as an example of a cell line expressing increased level of TS. As expected, we obtained intense signal of both the ternary complexed and free TS protein in Western blot analysis of HeLa-55 extract. In addition, immunocytochemical analysis of HeLa and HeLa 55 showed correspondingly low and high levels of TS, respectively, indicating that our combination of antibodies was detecting a specific TS signal. As further proof of the specificity of our primary antibody, we performed competition experiments with highly purified recombinant TS protein. Whether we pre-incubated the primary antibody with our purified TS protein competitor or pre-absorbed the primary antibody onto an affinity column to which our TS competitior had been previously immobilized, we obtained the same results. There was essentially complete disappearance of TS bands when we used these treated primary antibody preparations in Western blot analysis of HeLa-55 cell extract, whereas control treated antibodies detected strong TS bands. Similarly, we obtained almost complete elimination of fluorescence signal when pre-treated or preabsorbed primary antibody was used for immunofluorescence on HeLa-55 cells. We concluded from all of these results that our primary and secondary antibodies were highly specific for TS and that we could be confident in the results of our later experiments utilizing these antibodies.

We confirmed nuclear TS expression in colorectal tissue, in both normal, as well as cancer cells. This was important since it substantiates the physiological relevance of nuclear TS. The specificity of the nuclear TS in the colorectal specimens was demonstrated by use of competitor protein with which we obtained over 75% competition. This is comparable to, or even better than the result obtained by Samsonoff and colleagues who reported 50% inhibiton of nuclear TS in the rat hepatoma cell line in their study. In addition, since not all nuclei were TS positive, this indicated some specificity for the nuclear TS signal among nuclei within the colorectal specimens. At this point, the reason for nuclear TS in only some cells of normal and cancer tissue remains to be explained.

Since the level of TS protein is often found overexpressed in colorectal cancer (24,25), we were interested to investigate what effect overexpression of the TS protein may have on its localization. To test this, we generated a 5-FUdR resistant HeLa cell line, HeLa-55, that contained a highly amplified TS gene resulting in strong overexpression of the TS protein (Chapter 2). Immunocytochemistry results showed that the parental HeLa cells had only cytoplasmic TS staining. In HeLa-55 cells, TS was found to be strongly expressed in the cytoplasm, but that there also appeared to be some nuclear TS signal. In order to confirm the presence of nuclear TS, we performed immunofluorescence confocal microscopy on HeLa-55 cells. Serial optical sections through these cells confirmed clearly visible nuclear TS. Nuclear TS in HeLa-55 cells could have resulted from significant increase in the relative level of TS protein in these cells or could be attributed to the treatment of the cells with 5-FUdR and folinic acid. However, we confirmed that short term treatment of cells with 5-FUdR and folinic acid was not responsible for cytoplasmic TS translocating to the nucleus in HeLa cells (data not shown). Conversely, after the growth of HeLa-55 cells in the absence of 5-FUdR and folinic acid for 5 days, which is sufficient time to remove most traces of 5-FUdR, nuclear TS could still be detected in these cells (data not presented). These results indicated that in our HeLa cell system, when we manipulated the cell line to overexpress TS protein, some of TS protein translocated from the cytoplasm to the nucleus leading us to conclude that the level of expression of TS affected it's relative cellular localization.

We also detected heterogeneity for TS staining in HeLa-55 and found that stronger nuclear TS staining was detected in stronger TS expressing HeLa-55 cells. This confirmed our previous conclusion that the level of TS in the cell affected its localization and followed the trend that more cellular TS resulted in more nuclear expression. These results are consistent with a previous report using carrot cells in which wildtype cells lacked nuclear DHFR-TS expression but cells manipulated to overexpress DHFR-TS showed it to be present in the nucleus (26).

The majority of past reports have described TS as being cytoplasmic with few reports having mentioned TS as being nuclear. Since these reports used different cell lines and excluded human cell lines, we decided to investigate whether TS localization depended on the cell type examined. To this end, we chose to survey TS localization in a panel of several human cell lines consisting of normal fibroblasts, HeLa and six colorectal cancer lines. From immunofluorescence results we found that there was not significant differences in the relative level of TS amongst the cell lines examined. TS was localized predominantly to the cytoplasm in two cell lines: normal fibroblasts and HeLa cells. The six colorectal cell lines diplayed some amount of nuclear TS, but there was variation in the relative proportion of nuclear to cytoplasmic TS. We chose the RKO cell line as one most positive for nuclear TS and confirmed nuclear TS in these cells by both confocal microscopy and cellular fraction and Western blot analysis. We used HeLa cells as a negative control throughout these experiments. The collective results of the RKO cell line clearly illustrated that the immunofluorescent signal we detected was specific for TS. Our nuclear detection of TS in RKO cells is consistent with earlier reports (27). We therefore demonstrated that within the category of colorectal cancer, although the total cellular levels of TS were not very different among cell lines examined, there were differences in localization of TS. This lead us to conclude that, in addition to TS localization being dependent upon relative level of cellular TS as displayed by HeLa and clone-55 results, other factors also influence the localization of TS within a cell and can be described as cellular differences.

We next questioned whether the TS gene contained any sequences that resembled established motifs of nuclear localization sequence (NLS) and or nuclear export sequences (NES) which may be utilized in the translocation of TS between the nucleus and cytoplasm. Preliminary work in RKO cells has mapped a putative NLS to the first 10 amino acids at the amino-terminus and 9 amino acids (291-299) at the carboxy terminus (27) of the TS gene. The other possibility that TS could be imported into the nucleus with the aid of chaperones (14) has not been ruled out. It is also possible that although TS exists as a dimer in its native state, TS monomer (35kD) might diffuse freely in and out of the nucleus through the nuclear pore complex (60kD pore size) (28). However, this should lead to equivalent amounts of TS in the cytoplasm and nucleus, which is not the case, suggesting that TS would somehow be excluded from or exported from the nucleus. In contrast to NLS reports, there has been no mention to date of a nuclear export sequence in TS. Therefore, we decided to scan the sequence of the TS gene to look for whether it contained any sequence motifs resembling established nuclear export sequences. We found that sequence 257-269 fit the leucine rich putative nuclear export sequence motif which consists of four critically spaced leucines or other large hydrophobic residues. A loose consensus sequence describing leucine rich NESs of Leu- X_{2-3}-Phe/Ile/Leu/Val/Met-X_{2-3}-Leu-X-Leu/Ile was first proposed where spacings 3-3-1(29) (30), 3-2-1 (31), and 2-2-1(29) were acceptable but 2-3-1 was not (29). Interestingly, the putative NES of TS contains a 4-2-1 spacing. This leucine rich NES of TS is conserved in human, mouse, rat and worms and almost conserved with a 4-2 spacing in Drosophilia, yeast and the bifunctional DHFR-TS in carrot cells. A recently described divergence of the NES consensus to a Val-X-X-X-X-X-Leu-X-Val motif with a 5-1 spacing has been described for a subset of HDACs (32). Thus the 4-2 spacing of the almost conserved NES in yeast and the bifunctional DHFR-TS in carrot cells could quite possibly be functional.

We tested whether this putative NES of TS could function independently as an NES by preparing a fusion construct, GFP-NES, in which TS-NES was fused in frame to the carboxyl end of GFP. However, when GFP-NES was expressed, there was no change in the localization of GFP as compared to control GFP vector. We concluded from these results that the putative leucine rich NES of TS cannot function independently as an NES when fused to the carboxyl end of GFP and transfected into HeLa and RKO cells. Several studies have shown that similar leucine rich putative NESs of other proteins fused to the carboxyl end of GFP results in nuclear export of GFP (32). However, one report has described the inability of an intrinsic functional NES to confer nuclear export to GFP and suggested that such NESs might function in a protein context-dependent manner, possibly requiring additional sequences for full activity (32).

To determine whether surrounding sequences were necessary for TS NES to be functional, we generated two His_6-tagged constructs of TS lacking carboxyl terminal regions encompassing the NES and surrounding sequences. Construct H_6TS^{248} had putative NES disrupted partly due to mutated amino acids at positions 257 to 259 and deletion of amino acids 260 to 269. H_6TS^{186} had the full TS NES deleted. We first showed that our His-tagged version of full-length TS, H_6TS^{313}, and wt TS, localized similarly when overexpressed in HeLa cells. Overexpression of TS mutants lacking this putative NES and surrounding sequences resulted in increased accumulation of nuclear TS in addition to some cytoplasmic speckling for H_6TS^{186}. These results suggested that additional sequences between 270 - 313 at carboxy end of TS are necessary for the NES to be functional, however, further work is necessary to delineate the minimal carboxy sequences necessary. In cases showing increased nuclear TS expression, future studies could check for possible mutations or deletions in the delineated sequence responsible for TS nuclear export.

In addition to confocal microscopy, we confirmed nuclear TS in Clone-55 cells by cell fractionation. When Clone-55 cells grown in 5-FUdR were fractionated and the cellular fractions probed for TS levels, we estimated that approximately 0.5% of the free TS protein was present in the nuclear fraction of a population of heterogenous TS-expressing clone-55 cells. More importantly, compared to roughly equivalent amounts of free and ternary complexed TS in the total cellular lysate, there was a far greater predominance of the free TS protein in the nuclear extract of Clone-55 cells. In a similar fashion, when carrot cells, which contain a bifunctional DHFR-TS gene was examined for TS localization, it was found to be cytoplasmic. On overexpression of the DHFR-TS gene by isolation of a

methotrexate resistant clone, it was found that DHFR-TS was largely localized to the cytoplasm with some nuclear signal (26). While cytoenzymatic studies pointed to a general cytoplasmic localization of the enzyme, results of immunocytochemical localization showed that in the majority of methotrexate resistant cells, there was also a nuclear localization of the protein(26). The authors suggested that the discrepancy between cytochemical and immunocytochemical results observed with methotrexate resistant cells could be explained by the nuclear accumulation of the inactive form of the enzyme. In this study we show that the ternary complexed form of TS was nearly absent from the nucleus of HeLa-55 cells suggesting that the nuclear TS may not be able to bind the 5-FUdR substrate to form the ternary complex. It is possible that if nuclear TS is incapable of binding substrate, this could explain why nuclear DHFR-TS in the methotrexate resistant carrot cells showed no enzymatic activity.

In contrast to the results in HeLa and carrot cells, when the localization of TS was examined in a rat hepatoma cell line, H35, through autoradiographic assay and immunogold labeling, it was found to be mostly nuclear with some cytoplasmic staining. When these authors selected a 5-FUdR resistant TS overexpressing clone, H35R, they found that from an autoradigraphic assay, most of the overexpressed TS was in the cytoplasm with some in the nucleus. Results of our study indicate that localization of TS is dependent on cell type differences which offers one explanation for the difference in TS localization in the rat hepatoma cells compared to HeLa and carrot cells. Contrary to our results, authors of the rat hepatoma cell line report also showed that ternary complexed TS was predominantly expressed in the nucleus and that TS was phosphorylated in the 5-FUdR resistant rat hepatoma cell line. Preliminary evidence from our lab studying HeLa TS failed to show that TS was phosphorylated in this cell line (Sanjay Krishnamurthy, unpublished data). To this date there has been no report of phosphorylated TS in any other cell lines and hence we cannot conclude whether in fact phosphorylated TS is present in a normal or abnormal situation. There has been no characterization of phosphorylated TS protein in terms of its catalytic activity, stability and localization. Thus it is very easy to speculate that the differences seen between H35 and HeLa cells could be due to the fact that H35 contains a phosphorylated TS protein. TS nuclear localization dependency on cell type could in fact be due to dependency in part on the phosphorylation status of TS and is something that can be investigated in the future. Therefore, comparison of our results leads to another possible factor that may affect TS nuclear localization, its phosphorylation status, which could in turn be due to differences in the yet to be characterized kinases and signaling pathways that may be directly responsible for the phosphorylation of TS.

We can propose several possibilities to explain our observation of lack of ternary complexed TS protein in the nucleus of Clone-55 cells. If we assume that the work on TS putative NLS is correct, it is possible that FdUMP-bound TS results in the masking of TS NLS so it no longer efficiently enters the nucleus. If, however, TS does not enter the nucleus through an NLS but is instead guided into the nucleus by chaperones, it is possible that FdUMP-bound TS is no longer able to interact with these chaperones as efficiently and therefore not able to enter the nucleus. Conversely, FdUMP-bound TS could result in the exposure of TS NES allowing it to be efficiently exported out of the nucleus. Another possibility is that the predominantly free TS protein detected in the nuclear fraction of Clone 55 as assessed by Western Blot could actually be RNA bound TS. The role of TS in the nucleus may be due to its RNA-binding activity (33) and not to its catalytic activity. If we envision nuclear TS as RNA-bound TS, according to Chu's TS autotranslational regulatory model, once FdUMP binds this nuclear TS, the RNA is released and exits the nucleus and can be translated. This would lead to increased expression of genes translationaly regulated by TS, and could constitute another mechanism by which 5-FU treatment could affect target cells. It is also possible that RNA-bound TS exposes TS NLS so it can efficiently enter the nucleus or that the RNA-bound TS masks TS NES so it is trapped in the nucleus. Alternatively, if I also consider the results of the methotrexate resistant carrot cells where nuclear TS was enzymatically inactive, it is possible that nuclear TS is modified in such a way that it can no longer bind substrate and so is incapable of forming a ternary complex.

5-FU, one of the most commonly used chemotherapeutic agents in the treatment of colorectal cancer, is metabolized in the cell to generate FdUMP. FdUMP binds to TS protein in the presence of the folate co-factor leading to a strong ternary complexed TS protein. This results in prolonged inhibition of the enzyme (5). Our model provides one explanation for the recently reported correlation between higher nuclear TS expression and poorer response to 5-FU therapy (15). From our data on the fractionation of Clone 55, the majority of nuclear TS was in the free form. If we believe that this is true for the majority of nuclear TS staining detected in the colorectal cancer cases examinedmin the above study, then this nuclear TS can be thought of as a store of free TS protein, non ternary complexed, therefore non-inhibited. Nuclear TS represents a store of free TS that might be thought of as providing "resistance" to the inhibition of 5-FU effects possibly due to its inability to bind substrate. This could explain why these higher nuclear TS cases showed poorer response to 5-FU therapy.

In conclusion, in this paper we have confirmed the nuclear presence of TS in both human cultured cell lines and human colorectal tissue specimens using a highly specific anti-TS antibody. In addition, we have established that TS nuclear localization is dependent upon several factors: (1) level of TS protein in a cell (2) presence of TS NES and surrounding sequences between amino acids 270 - 313 at carboxy end of TS gene and (3) factors that differ between cell types that remain to be characterized.

REFERENCES

1. Carreras C.W., Santi D.V. 1995. The catalytic mechanism and structure of thymidylate synthase. *Annu. Rev. Biochem.* **64**: 721-762.
2. Danenberg P.V. 1977. Thymidylate synthetase - a target enzyme in cancer chemotherapy. *Biochim. Biophys. Acta* **473**: 73-92.
3. Hardy L.W., Finer-Moore J.S., Montfort W.R., Jones M.O., Santi D.V., Stroud R.M. 1987. Atomic structure of thymidylate synthase: target for rational drug design. *Science* **235**: 448-455.
4. Rodriguez-Bigas M.A., Petrelli N.J. 1993. Biochemical modulation of fluoropyrimidines and other drugs. *In*: Colorectal cancer. *Editors*: H.J. Wanebo. Mosby, St. Loius, pp. 509-522.
5. Drake J.C., Allegra C.J., Johnston P.G. 1993. Immunological quantitation of thymidylate synthase-FdUMP-5,10-methylenetetrahydrofolate ternary complex with the monoclonal antibody TS 106. *Anticancer Drugs* **4**: 431-435.

6. Kucera R., Paulus H. 1986. Localization of the deoxyribonucleotide biosynthetic enzymes ribonucleotide reductase and thymidylate synthase in mouse L cells
9. *Exp. Cell Res.* **167**: 417-428.
7. Johnston P.G., Liang C.-M., Henry S., Chabner B.A., Allegra C.J. 1991. Production and characterization of monoclonal antibodies that localize human thymidylate synthase in the cytoplasm of human cells and tissue. *Cancer Res.* **51**: 6668-6676.
8. Johnston P.G., Lenz H.-Z., Leichman C.G., Danenberg K.D., Allegra C.J., Danenberg P.V., Leichman L., Lenz H.J. 1995. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res.* **55**: 1407-1412.
9. Suzuki M., Okabe H., Tsukagoshi S., Kawai T., Fukushima M., Sato I. 1998. Immunohistochemical investigation of thymidylate synthase in cervical cancer. *Oncology* **55**: 564-568.
10. Yamachika T., Nakanishi H., Inada K., Tsukamoto T., Kato T., Fukushima M., Inoue M., Tatematsu M. 1998. A new prognostic factor for colorectal carcinoma, thymidylate synthase, and its therapeutic significance. *Cancer* **82**: 70-77.
11. Paradiso A., Simone G., Petroni S., Leone B., Vallejo C., Lacava J., Romero A., Machiavelli M., De Lena M., Allegra C.J., Johnston P.G. 2000. Thymidylate synthase and p53 primary tumour expression as predictive factors for advanced colorectal cancer patients. *Br. J. Cancer* **82**: 560-567.
12. Brown S.S., Neal G.E., Williams D.C. 1965. Subcellular distribution of some folic acid-linked enzymes in rat liver. *Biochem. J.* **97**: 34C-36C.
13. Prem veer R.G., Pardee A.B. 1980. Multienzyme complex for metabolic channeling in mammalian DNA replication
3. *Proc. Natl. Acad. Sci. U. S. A* **77**: 3312-3316.
14. Reston J., Samsonoff W., McJee M., O'Connor B., Galivan J., Maley G., Maley F. 1996. Nuclear location of thymidine synthase (TS) and its state of phosphorylation (Meeting abstract). *Proc. Annu. Meet. Am. Assoc. Cancer Res.* **37**: A3362.
15. Wong N.A., Brett L., Stewart M., Leitch A., Longley D.B., Dunlop M.G., Johnston P.G., Lessells A.M., Jodrell D.I. 2001. Nuclear thymidylate synthase expression, p53 expression and 5FU response in colorectal carcinoma. *Br. J. Cancer* **85**: 1937-1943.
16. Haqqani A.S., Cowling R.T., Maroun J.A., Birnboim H.C. 1999. Characterization of a polyclonal antibody to human thymidylate synthase suitable for the study of colorectal cancer specimens. *J. Histochem. Cytochem.* **47**: 1563-1574.
17. Brattain M.G., Levine A.E., Chakrabarty S., Yeoman L.C., Willson J.K., Long B. 1984. Heterogeneity of human colon carcinoma. *Cancer Metastasis Rev.* **3**: 177-191.
18. Udenfriend S., Stein S., Bohlen P., Dairman W., Leimgruber W., Weigele M. 1972. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science* **178**: 871-872.
19. Cowling R.T., Birnboim H.C. 1998. Preliminary characterization of the protein encoded by human testis-enhanced gene transcript (TEGT). *Mol. Memb. Biol.* **15**: 177-187.
20. Davisson V.J., Sirawaraporn W., Santi D.V. 1989. Expression of human thymidylate synthase in Escherichia coli. *J. Biol. Chem.* **264**: 9145-9148.
21. Horie N., Aiba H., Oguro K., Hojo H., Takeishi K. 1995. Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate synthase. *Cell Struct. Funct.* **20**: 191-197.
22. Samsonoff W.A., Reston J., McKee M., O'Connor B., Galivan J., Maley G., Maley F. 1997. Intracellular location of thymidylate synthase and its state of phosphorylation. *J. Biol. Chem.* **272**: 13281-13285.
23. Zaman K., Ryu H., Hall D., O'Donovan K., Lin K.I., Miller M.P., Marquis J.C., Baraban J.M., Semenza G.L., Ratan R.R. 1999. Protection from oxidative stress-induced apoptosis in cortical neuronal cultures by iron chelators is associated with enhanced DNA binding of hypoxia-inducible factor-1 and ATF-1/CREB and increased expression of glycolytic enzymes, p21(waf1/cip1), and erythropoietin. *J. Neurosci.* **19**: 9821-9830.
24. Ardalan B., Dang Z. 1996. Thymidylate synthase gene expression in normal and malignant colorectal tissues: relation to in vivo response and survival. *Proc. Annu. Meet. Am. Assoc. Cancer Res.* **37**: A1376.
25. Findlay M.P., Cunningham D., Morgan G., Clinton S., Hardcastle A., Aherne G.W. 1997. Lack of correlation between thymidylate synthase levels in primary colorectal tumours and subsequent response to chemotherapy. *Br. J. Cancer* **75**: 903-909.
26. Cella R., Balestrazzi A., Carbonera D., Nano R., Rezzani R. 1991. Cellular localization of dihydrofolate reductase-thymidylate synthase in carrot cells
3. *Eur. J Basic Appl. Histochem.* **35**: 61-71.
27. Liu J., Schmitz J.C., Lin X., Tai N., Yan W., Farrell M., Bailly M., Chen T., Chu E. 2002. Thymidylate synthase as a translational regulator of cellular gene expression
7. *Biochim. Biophys Acta* **1587**: 174-182.
28. Nakielny S., Dreyfuss G. 1999. Transport of proteins and RNAs in and out of the nucleus
1. *Cell* **99**: 677-690.
29. Bogerd H.P., Fridell R.A., Benson R.E., Hua J., Cullen B.R. 1996. Protein sequence requirements for function of the human T-cell leukemia virus type 1 Rex nuclear export signal delineated by a novel in vivo randomization-selection assay. *Mol. Cell Biol.* **16**: 4207-4214.
30. Zhang Y., Xiong Y. 2001. A p53 amino-terminal nuclear export signal inhibited by DNA damage-induced phosphorylation. *Science* **292**: 1910-1915.
31. Lindsay M.E., Holaska J.M., Welch K., Paschal B.M., Macara I.G. 2001. Ran-binding protein 3 is a cofactor for Crm1-mediated nuclear protein export. *J. Cell Biol.* **153**: 1391-1402.
32. McKinsey T.A., Zhang C.L., Olson E.N. 2001. Identification of a signal-responsive nuclear export sequence in class II histone deacetylases. *Mol. Cell Biol.* **21**: 6312-6321.
33. Chu E., Copur S.M., Ju J., Chen T.M., Khleif S., Voeller D.M., Mizunuma N., Patel M., Maley G.F., Maley F., Allegra C.J. 1999. Thymidylate synthase protein and p53 mRNA form an in vivo ribonucleoprotein complex. *Mol. Cell Biol.* **19**: 1582-1594.