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Mechanisms Underlying Thymidylate Synthase Regulation in Human Cells

Brice Le François

Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of philosophy

Department of Biochemistry, Microbiology and Immunology

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Abstract

Thymidylate synthase (TS) catalyzes the last step of the only *de novo* synthesis pathway for deoxythymidine monophosphate (dTMP), a nucleotide required for DNA synthesis and repair. The central role of TS in nucleic acid metabolism has made it an attractive target for antineoplastic drugs. 5-fluorouracil is a prototypic drug targeting TS activity, which blocks the enzyme by forming a stable inhibitory complex with the protein. Depletion of dTMP following TS inhibition leads to repeated cycles of incorporation of uridine into DNA, causing single strand breaks, and ultimately triggering apoptosis. TS is commonly believed to be a S-phase dependent enzyme, an assumption largely based on a score of studies performed in rodent cells. Recent reports do not support this view and suggest that TS is a cell proliferation marker rather than a S-phase enzyme. The mechanisms controlling TS expression in human cells are poorly understood and could potentially provide new therapeutics approaches to decrease TS levels in cancer cells and to sensitize them to anti-TS drugs. We showed that, in human cells synchronized by serum starvation or subjected to serum deprivation, the expression patterns of TS and cyclin E (a well characterized E2F target) were significantly different. Ectopic expression of E2F1 or cyclin E had no effect on TS levels. However, inhibition of CDK4 but not CDK2 was associated with a clear decrease in TS levels in human cells. We also demonstrated that inhibition of MEK1 was associated with a decrease in TS levels, but this event was independent of ERK2 activity. Using reporter assays, we showed that MEK1 controlled TS promoter transcription, and that deletion of the Sp1 site from the essential promoter region greatly reduced the ability of MEK1 to stimulate the TS promoter. Surprisingly, unlike in rodent cells, LSF and GABP failed to

increase TS levels in human cells. Taken together, our data demonstrate that TS levels in human cells are largely independent of S-phase and are primarily controlled by CDK4 and MEK1 activity during G_1. Furthermore, we provide strong evidence that the mechanisms controlling TS expression in human cells significantly differ from those of rodent cells.

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As these 6 years of study come to an end, it is finally time to give credit to the many people that made this work possible. First, I would like to acknowledge my supervisor, Dr. Birnboim, for giving me the opportunity to undertake, continue and eventually finish my Ph.D. research project in his lab under his supervision. I would like to thank him for his time, his numerous advice and ideas that contributed so much to this project. I will remember our weekly Wednesday morning discussions (sometimes quite heated) where I learned despite myself that my supervisor was indeed right most of time. I would also like to thank Dr. Jim Dimitroulakos, Dr. Dennis Bulman, Dr. John Bell and Dr. Paul Albert, my advisory committee members, for their help and valuable suggestions over these 6 long years. I would also like to thank present and past lab mates for helping out over the years and more specifically Catherine Soo, Ivan Gorn, and Denise Proulx. Special thanks to Arsalan and Seema for training me and helping me in the early parts of project. They both taught me most of their lab tricks, which did prove very helpful in the long run.

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Table of Contents

List of Abbreviations

5-FdUMP: 5-fluoro 2'-deoxyuridine monophosphate

5-FU: 5-fluorouracil

APP (amyloid precursor protein)

APP: amyloid precursor protein

ATCC: American Type Culture Collection.

BER: base excision repair pathway

bp: base pair

BSA: bovine serum albumin

CDK: cyclin-dependent kinase

CMV: Cytomegalovirus

DHFR: Dihydrofolate reductase.

DPD: dihydropyrimidine dehydrogenase

dTMP: 2'-deoxythymidine 5'-monophosphate

DTT: dithiothreitol

dUMP: 2'-deoxyuridine 5'-monophosphate

ERK: Extracellular-regulated kinase

ERK: extracellular-regulated kinase

FCS: fetal calf serum

FPGS: folylpoly-γ-glutamate synthetase

GABP: GA binding protein

HDACs: histone deacetylases

HIV: human immunodeficiency virus

IMAGE: Integrated Molecular Analysis of Genomes.

IPG: Immobilized pH gradient.

LSF: late simian virus factor

MAPK: mitogen-activated protein kinase

MAPK: mitogen-activated protein kinase

MDR: multidrug resistance transporter

MEM: Modified Eagle's medium

MOI: Multiplicity of infection.

mTHF: 5,10-methylene tetrahydrofolate

NES: nuclear export signal

NIGMS: National Institute of General Medical Sciences.

PAGE: Polyacrylamide gel electrophoresis.

PBS: phosphate buffer saline

PCR: Polymerase chain reaction

PDPA:1,3-propanediphosphonic acid

PI: propidium iodide

RFC: reduced folate carrier

RNR: ribonucleotide reductase.

SDM: Site-directed mutagenesis.

SDS: sodium dodecyl sulfate

SHT: Serine hydroxymethyl transferase.

SNP: single nucleotide polymorphism

Sp1: specificity protein 1

TFCP2L2: transcription factor CP2 like 2

TK: thymidine kinase

TS: thymidylate synthase

UNG: uracil-DNA glycosylase

USF: upstream stimulating factor

UTR: untranslated region

List of Figures:

List of Tables:

Chapter 1: INTRODUCTION

1.1 Perspective

Cancer is one of the leading causes of death, accounting for 12.5% of total deaths worldwide and 50 % in developed countries. There are currently 20 million individuals affected by cancer, and the numbers keep rising every year as the population ages. It is expected that, by 2020, the number of new cases of cancer diagnosed each year will rise to 15 million (World Health Organisation, 2007).

Over the past 30 years, considerable scientific progress has been made and has provided a better understanding of the disease, highlighting an immense number of mechanisms that lead to the conversion of normal cells to malignant cancer cells. One of the critical aims of cancer treatment is to identify drugs that specifically target and kill cancer cells, with minimum toxicity for normal cells and tissues. This search has yielded a number of drugs that specifically target cellular proteins necessary for cell growth.

One of the earliest anti-cancer drugs that continues to be used in clinical practice since 1958 is 5-fluorouracil (5-FU), which is used as an anti-metabolite in treatment of colon cancer and many other types of cancer. 5-FU targets the cellular enzyme thymidylate synthase (TS), which is involved in nucleotide synthesis and thereby blocks tumour growth by inhibition of DNA synthesis in rapidly proliferating cancer cells. While other compounds inhibiting TS activity have since been developed, their mechanism of action is similar to that of 5-FU, which remains widely used for chemotherapy. Cancer cells can overcome the toxicity of these drugs by a variety of mechanisms, including upregulation of TS levels. Since TS is a very well

established cancer target and drugs targeting this enzyme have been extensively characterized and used clinically, my research focused on ways that cancer cells might develop resistance to anti-TS drugs. In particular, since elevated TS levels are known to decrease the sensitivity of tumour cells to this class of drugs, I focused on improving our understanding of the mechanisms regulating the expression of TS. Such information could aid in the design of new strategies to TS enzyme levels in cancer cells to render them more sensitive to treatment.

1.2 Thymidylate synthase

Thymidylate synthase (TS, EC 2.1.1.45) is the enzyme responsible for the conversion of 2'-deoxyuridine 5'-monophosphate (dUMP) to 2'-deoxythymidine 5'-monophosphate (dTMP) using 5,10-methylene tetrahydrofolate (mTHF) as a cofactor. It represents one of the most highly conserved enzymes across organisms with prokaryotic and eukaryotic homologues of TS having a very high sequence homology (67% homology between human and *Escherichia coli* TS), underscoring the critical function of this protein in cellular metabolism. In particular, a number of key amino acids are conserved across species, including multiple residues critical for the catalytic reaction (Carreras and Santi, 1995). The TS protein in protozoa and plants exists as a bifunctional polypeptide that contains both the dihydrofolate reductase (DHFR) and the thymidylate synthase activities (Ivanetich and Santi, 1990; Lazar et al., 1993). In contrast, eukaryotic cells possess two independent genes coding for TS and DHFR enzymes that function in parallel. The general folding of the TS protein is also quite conserved across species. It is composed of 8 α-helices and 10 β-sheets connected by more or less ordered coiled segments

(Fig.1.1). In human cells, the native protein is found as a homodimer containing two 35 kDa subunits. The interface between the two polypeptide chains is mediated by the interaction of six β-sheets from each subunit and reinforced by specific residues (Prasanna et al., 1999). The two TS monomers have been shown to negatively cooperate (Dev et al., 1994). Binding of the substrate and co-factor to one site of the enzyme triggers conformational changes, decreasing the affinity of the second site for the substrate and co-factor (Anderson et al., 1999). This particular negative cooperativity observed between dimers defines TS as a "half-the-site" enzyme. The N-terminal region of mammalian TS proteins contains approximately 30 additional amino acids that are not found in bacterial homologues. This region is highly disordered and it has been suggested that it is involved in the turn-over of the enzyme (Forsthoefel et al., 2004). Deletion of the first few amino acids of the protein blocks proteosome-dependent degradation of the protein and dramatically increases its half-life (Pena et al., 2006).

1.2. Biological function of Thymidylate Synthase.

1.2.1 TS and nucleic acid metabolism.

Thymidylate synthase catalyzes the reductive methylation of dUMP using tetrahydrofolate (mTHF) as a cofactor to form dTMP and dihydrofolate (Fig.1.2). This catalytic reaction constitutes the last step of the only *de novo* synthesis pathway of dTMP, a nucleotide required for DNA replication and repair. In cells, deoxyribonucleotides are directly synthesized from their ribonucleotide precursors by an enzyme called ribonucleotide reductase (RNR) (Nordlund and Reichard, 2006)

Figure 1.1 Ribbon drawing of a human TS enzyme monomer. Crystal structure of hTS bound to 1,3-propanediphosphonic acid (PDPA), an allosteric inhibitor of the enzyme (from (Lovelace et al., 2007)). β-sheets are represented in blue arrows, α-helices in red cylinder and loops in grey tubes.

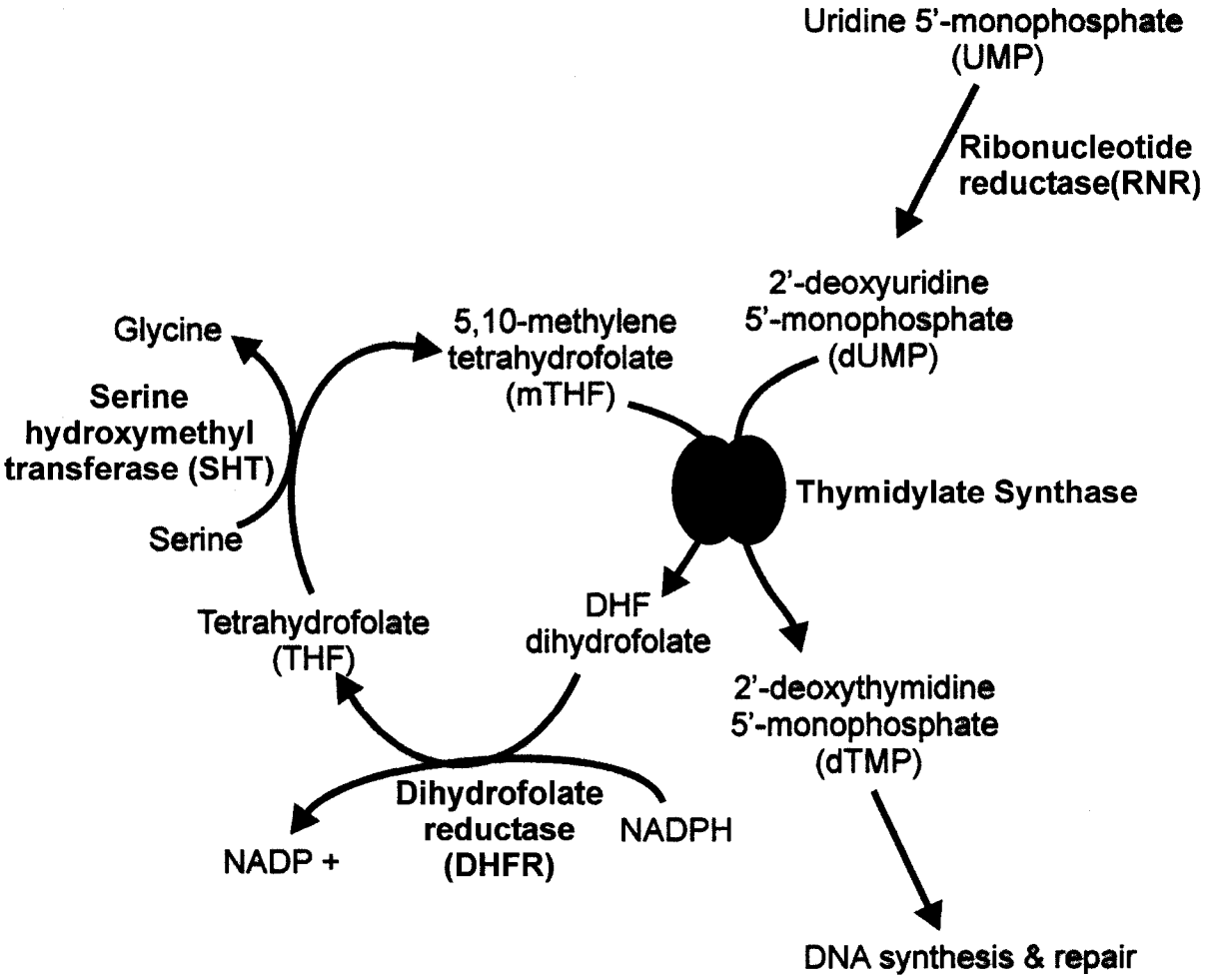
RNR is composed of two subunits called R1 and R2, which function as heterodimers and catalyze the substitution of the 2'-OH group by a hydrogen atom. dTMP is the only deoxyribonucleotide that is not directly synthesized by RNR. Its newly formed precursor, deoxyuridine monophosphate (dUMP), requires one additional catalytic step to form dTMP that is catalyzed by the thymidylate synthase enzyme. In this respect, TS occupies a unique role in nucleic acid metabolism. It is required to maintain both low dUMP concentrations and also sufficient dTMP levels in order for DNA synthesis to proceed normally.

Two other cellular enzymes are required for proper thymidylate synthase function: dihydrofolate reductase (DHFR) and serine hydroxymethyl transferase (SHT). Following synthesis of dTMP by TS, the one-carbon donor dihydrofolate is reduced back to tetrahydrofolate (THF) by DHFR, a process that is dependent on NADPH (Fig.1.2). A second enzyme, SHT, is then required to replenish the 5,10-methylene tetrahydrofolate pools (mTHF) by transferring a methyl group from serine to THF. 5,10-methylene tetrahydrofolate is required as a methyl source for the production of thymidylate by TS. The critical role of DHFR in the synthesis mTHF has also made this enzyme a target for chemotherapeutic drugs that interfere with dTMP synthesis.

1.2.2 Catalytic reaction of thymidylate synthase.

The catalytic reaction of this enzyme has been extensively studied. Crystal structures of the TS enzyme of several bacterial species as well as human were solved between 1987 and 1991 (Hardy et al., 1987; Schiffer et al., 1991), and once again highlighted a highly conserved folding of the TS protein across species.

Figure 1.2 Enzymes and metabolic pathways involved in the *de novo* synthesis pathway of dTMP. RNR synthesizes dUMP; TS then catalyzes the reductive methylation of dUMP using mTHF as a cofactor (methyl donor) to produce dTMP and DHF. mTHF pools are replenished from DHF by two enzymatic reaction catalyzed by DHFR and SHT.


Uridine 5'-monophosphate
(UMP)
Ribonucleotide
reductase(RNR)
2'-deoxyuridine
5'-monophosphate
(dUMP)
Thymidylate Synthase
5,10-methylene
tetrahydrofolate
(mTHF)
Glycine
Serine
hydroxymethyl
transferase (SHT)
Serine
DHF
dihydrofolate
Tetrahydrofolate
(THF)
2'-deoxythymidine
5'-monophosphate
(dTMP)
Dihydrofolate
reductase
(DHFR)
NADPH
NADP +
DNA synthesis & repair

Following these initial steps, crystal structures of the enzyme bound to its substrate, cofactor and various analogues were solved and gave further insight into the catalytic reaction. Binding of dUMP and mTHF to the active site is sequential (Stroud and Finer-Moore, 2003) and this triggers a major conformational change, which isolates the substrate and co-factor from the solvent (Montfort et al., 1990). This structural switch also brings important catalytic residues toward the active site (Berger et al., 2004). Upon substrate binding, the catalytic cysteine 195 in the human enzyme becomes available and forms a covalent bond with the C6 of the uracil ring. Subsequently, the methyl group of mTHF attacks the C5 position of the uracil ring. At that step of the reaction, the enzyme is covalently bound to both its substrate and cofactor in a "ternary complex". A tyrosine residue present in the active site then captures a proton at position C5, triggering the final step of the reaction where dTMP and dihydrofolate are produced and released from the enzyme.

1.2.3 TS activity: a safeguard mechanism for genomic integrity.

1.2.3.1 Uracil in DNA, origin, repair and removal

Under normal conditions, uracil is present in DNA only as a result of the spontaneous chemical deamination of cytosine. This event is potentially highly mutagenic and occurs relatively frequently in cells (Frederico et al., 1990; Shen et al., 1994). During DNA synthesis, DNA polymerases can also misincorporate dUMP into DNA in place of dTMP. The presence of uracil in DNA is promptly detected by a class of enzymes called uracil-DNA glycosylases (UNG), which specifically detect and excise uracil residues from DNA. UNG2 and SMUG1 are the two principal DNA

glycosylases involved in the removal of U/A and U/G genomic mismatches (Akbari et al., 2004). Removal of uracil by DNA glycosylases leaves an apurinic/apyrimidinic site (AP site), which initiates the base excision repair pathway (BER) (Krokan et al., 2000; Lindahl and Barnes, 2000). Following removal of the uracil residue, the AP site is then incised by an AP-endonuclease (APE1/Ref-1), which nicks the 5´ end of the abasic site, creating a single-strand break in the DNA backbone. The final steps of BER can be mediated by one of two distinct sub-pathways, differing in the length of the repair patch produced. Both repair mechanisms are mediated by multiprotein complexes that process the AP site, fill the gap and ligate the nicked DNA to complete the repair process. Typically, a cytosine residue is introduced at the original abasic site. Removal and repair of uracil residues by the base excision repair pathway is a genome safeguard mechanism that prevents GC → AT transition mutations. However, if the original site was A-U pair, then it is converted to a G-C pair, introducing a mutation in the genetic material.

1.2.3.2 Consequences of thymidylate synthase inhibition.

The central role of TS in the production of dTMP has made it an attractive target for cancer treatment. Drugs targeting dTMP synthesis are either analogues of the substrate or of the cofactor and exhibit a high affinity for the enzyme. They bind to the active site of the protein to form stable inhibitory complexes, blocking the catalytic activity of TS and, consequently, the production of dTMP. Apoptotic cell death induced by inhibition of thymidylate synthase is a process that was initially described as *thymineless cell death* (Seno et al., 1985). Thymine/thymidine deprivation in cells treated with anti-TS drugs is associated with nucleotide pool

imbalance and accumulation of DNA strands breaks (Yoshioka et al., 1987). Multiple apoptotic pathways have been reported to be involved in thymineless cell death. Some studies have shown that, in colon carcinoma, signalling through the CD95 pathway is involved (Harwood et al., 2000) with activation of caspase 8. Others have shown that, upon depletion of dTMP, there is release of cytochrome c from mitochondria and activation of caspase 3 involving the mitochondrial pathway (Kottke et al., 1999; Munoz-Pinedo et al., 2001).

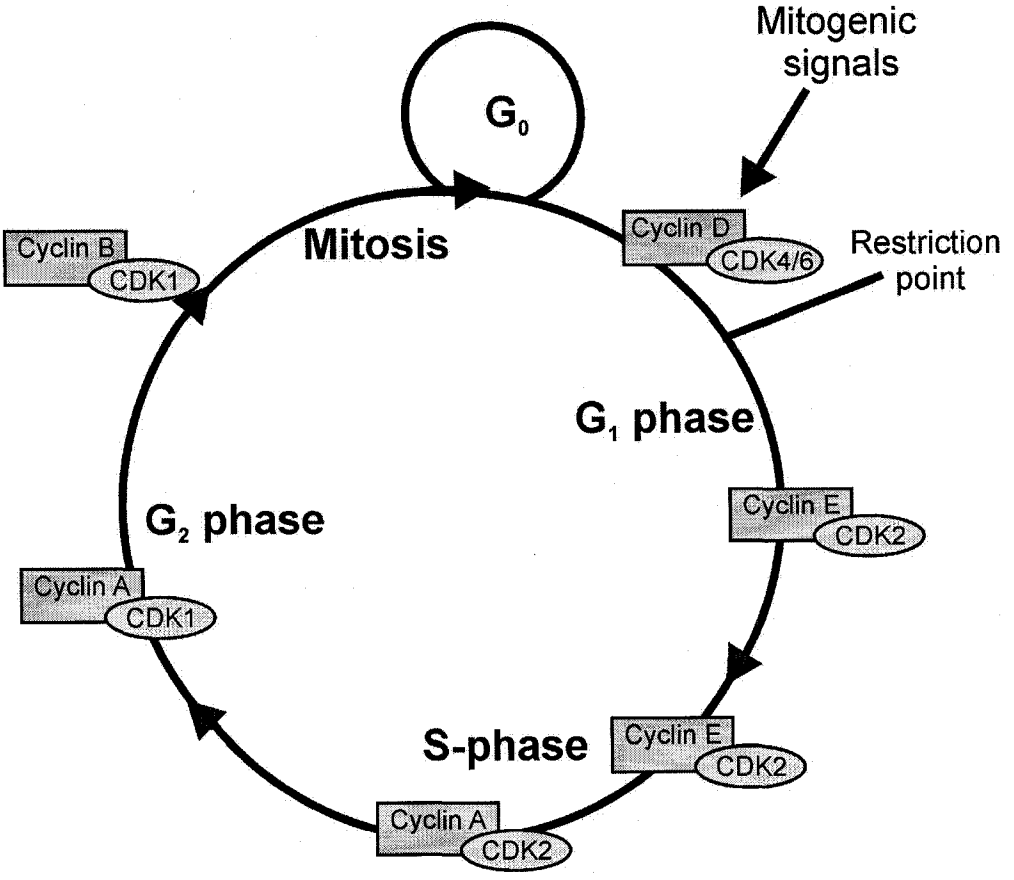
The exact mechanisms underlying cell death following inhibition of TS activity remain to be determined. It is believed that the concomitant decrease in dTMP levels and increase in dUMP levels caused by inhibition of dTMP synthesis fosters massive incorporation of dUMP into DNA by DNA polymerase. Subsequent activation of uracil excision repair pathways leads to repeated futile cycles of dUMP incorporation directly followed by BER-mediated repair. Since the BER repair pathway forms single strand break as repair intermediates, this ultimately results in accumulation of DNA double strand breaks and cell death.

1.3 Cell-cycle dependent regulation of Thymidylate synthase

1.3.1 Overview of the mammalian cell-cycle

The cell cycle is a complex and highly regulated process controlling cell division. The majority of cells in an adult organism are in a resting state (G_0) and do not normally divide. However, upon stimulation with growth factors, some cells can enter the cell-cycle and ultimately commit to undergo division and give rise to two daughter cells. The cell cycle is divided into four major phases: G_1 (Gap 1), S-phase, G_2 (Gap 2) and M (Fig. 1.3). It has long been known that the decision of a

Figure 1.3 The cell-cycle phases and associated cyclins. In the absence of growth factors, cells are in quiescent state (G_0). Upon mitogenic stimulation, cells enter the G_1 phase and cyclin D starts to accumulate. Later during G_1, cyclin D-CDK4/6 complexes phosphorylate pRb, leading to E2F release. Cyclin E is activated and cells enter S-phase. S-phase progression is controlled by cyclin E-CDK2 and cyclin A-CDK2 complexes. When DNA replication is complete, cells enter the G_2 phase controlled by cyclin A-CDK1 activity. Upon activation of cyclin B-CDK1 complexes, cells then enter mitosis and complete the cell-cycle when the two daughter cells are separated. At this time, cells can go back to a resting state (G_0) or continue dividing.


Mitogenic signals
G_0
Cyclin D
CDK4/6
Restriction point
G_1 phase
Cyclin E
CDK2
S-phase
Cyclin E
CDK2
Cyclin A
CDK2
G_2 phase
Cyclin A
CDK1
Mitosis
Cyclin B
CDK1

cell to divide is made during G_1 at a time termed the "restriction point" (Blagosklonny and Pardee, 2002). Traversing this point constitutes an irreversible commitment to division. The G_1 phase of the cell-cycle is the only phase dependent upon the presence of external stimuli (Pardee, 1974). Mitogenic signals lead to the accumulation of cyclin D (Roussel et al., 1995) and, as G_1 progresses, cyclin D-CDK4/6 complexes phosphorylate the retinoblastoma protein (pRb) (Weinberg, 1995). Hyperphosphorylation of pRb ultimately leads to the release of E2F transcription factors that activate a number of genes involved in DNA synthesis and G_1/S transition (DeGregori et al., 1995). Notably, E2Fs activate cyclin E expression through multiple consensus sites found in the cyclin E gene promoter (Geng et al., 1996). Newly synthesized cyclin E proteins associate with cyclin-dependent kinase 2 (CDK2) and further phosphorylate pRb and activate components of the pre-replication machinery to drive S-phase entry (Sauer and Lehner, 1995; Woo and Poon, 2003). Importantly, it has been shown that pRb phosphorylation and cyclin E accumulation are not a prerequisite for passage through the restriction point (Ekholm et al., 2001). This indicates that the restriction point is an early-mid G_1 event preceding pRb phosphorylation and E2F release.

Most of the enzymes necessary for DNA synthesis are upregulated during the G_1/S transition in preparation for DNA replication, which takes place during the S-phase. The S-phase of the cell-cycle is controlled by the activity of cyclin A-CDK2 and cyclin E-CDK2 complexes. Once DNA replication is completed, dividing cells transit through the G_2 phase of the cell cycle and, at that time, cyclin A associates with CDK1. The G_2/M transition is initiated by binding of CDK1 to cyclin B to form an

active complex that drives entry of the cells into mitosis together with cyclin A (Sanchez and Dynlacht, 2005) and leads to completion of cellular division.

1.3.2 Thymidylate synthase and the cell-cycle

Thymidylate synthase is widely assumed to be a cell-cycle dependent enzyme. Since large amounts of dTMP are required during S-phase, one could intuitively assume that TS levels should be upregulated in consequence to provide satisfactory thymidylate levels for DNA replication. In support of this supposition, a number of other enzymes involved in nucleotide synthesis have been shown to be cell-cycle dependent. Thymidine kinase (TK), which is involved the thymidine salvage pathway, has been shown to be a S-phase dependent enzyme and its expression is regulated by E2F transcription factors. Interestingly, RNR is also cell cycle-dependent. RNR-R2 is the rate-limiting subunit for the enzymatic activity of RNR; its level is also cell-cycle dependent (Chabes and Thelander, 2000). Since RNR produces dUMP in a cell-cycle dependent fashion, it would seem reasonable to assume that TS activity should be cell-cycle dependent as well.

Supporting evidence that TS is a S-phase dependent enzyme comes from a number of earlier studies examining TS expression levels in synchronized cells. In mouse fibroblasts synchronized by serum starvation, TS activity increased 17-fold following addition of serum and entry of cells into the S-phase (Navalgund et al., 1980). In the same cells synchronized by mitotic selection, TS mRNA levels were cell-cycle dependent and underwent a 5 to 10-fold increase as cells progressed from G_1 to S-phase (Nagarajan and Johnson, 1989). In human fibroblasts synchronized by serum-starvation, TS mRNA levels were also reported to be associated with the

cell-cycle and increased 6 h following stimulation to reach levels 14 times higher than the baseline. However, nuclear run-on assays showed that the increase in mRNA levels was not associated with increased transcription of the TS gene, suggesting the existence of post-transcriptional mechanisms (Ayusawa et al., 1986). A similar discrepancy between TS gene transcription and mRNA accumulation was also observed in mouse cells (Jenh et al., 1985). Further studies of the TS promoter were able to determine that the sequences required for S-phase dependent expression were located in the essential promoter region as well as in the first intron of the gene (Ash et al., 1995; Ash et al., 1993).

In contrast, other studies of TS gene expression led to a very different conclusion. In mouse cells synchronized in S-phase by aphidicolin treatment, TS mRNA levels appeared to remain constant throughout the different cell-cycle phases following drug removal (Matherly et al., 1989). Moreover, a more recent report using three different asynchronously growing human tumour cell lines showed that TS expression level was not tightly associated with the various cell-cycle phases. Rather, TS expression was indeed associated with cell *proliferation* rather than the percentage of cells in S-phase, and TS levels were low only in confluent cell populations (Pestalozzi et al., 1995). These studies contradicted the dogma that considers TS as a cell-cycle dependent enzyme and presented an alternative point of view on regulatory mechanisms controlling TS expression (Dolnick, 2000).

1.3.3 Cell-cycle events regulating TS gene expression

Members of the E2F transcription factor family play an essential role in cell cycle progression, gene regulation and apoptosis. They have been shown to be

directly involved in the regulation of genes required for the G_1/S transition as well as several genes related to DNA synthesis, including TS (DeGregori et al., 1995). The TS gene promoter contains two E2Fs consensus binding sites which are located in the inverted repeat. However, point mutations in neither of these two sites significantly affect TS gene expression (Dong et al., 2000; Lee and Johnson, 2000). The involvement of E2F in the regulation of TS expression has been confirmed by microarray analyses in rodent cells, which have shown that TS mRNA levels are upregulated following ectopic expression of various E2F family members (Ishida et al., 2001; Kalma et al., 2001; Polager et al., 2002). Although studies of human tumour samples have examined the correlation between E2F levels and TS levels, no consistent picture of regulation has emerged. Some studies have shown a strong correlation between expression levels of TS and E2F while others have shown no significant relationship (Belvedere et al., 2004; Kasahara et al., 2000; Sowers et al., 2003b). An interesting difference between human and murine cells has been described following cytomegalovirus (CMV) infection. CMV infection induced E2F-dependent TS induction in murine cells (Gribaudo et al., 2000) but not in human cells (Gribaudo et al., 2002).

1.4 The TS gene promoter structure and transcriptional regulation

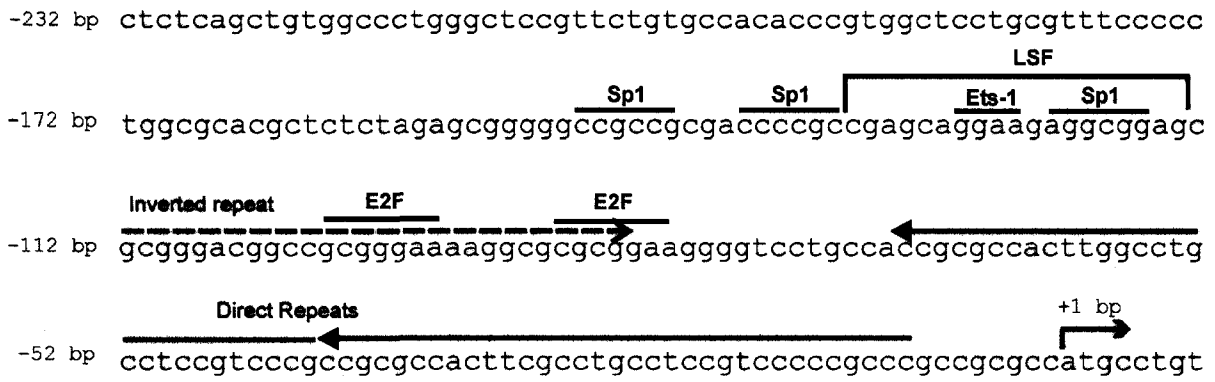
1.4.1 Structure of the TS promoter

To better understand the mechanisms controlling TS expression, various groups have studied the structure of the TS promoter. The genomic region containing the human TS gene promoter was cloned in 1989 (Takeishi et al., 1989) and it demonstrated that the region located at the 5' end of the gene (i.e. 3' end of

the promoter) contained a series of repeats that are also found on the mRNA. This confirmed earlier studies of the TS mRNA that had showed that the TS message contains a series of repeats in the 5' untranslated region (UTR) which were able to form stem-loop structures (Takeishi et al., 1985). In addition to these tandem repeats, the TS genomic fragment also contains an inverted repeat of the same sequence, found just upstream of the tandem repeats. However, the inverted repeat is not found in the TS mRNA suggesting that it is not transcribed. Promoter deletion studies were performed in order to identify the regions of the promoter and the transcription factors required for TS expression. Mapping of the TS gene promoter showed that it extended about 500 bp upstream of the translational start site (Kaneda et al., 1990), and that the sequences necessary for proper expression was located in the region between nucleotides -242 to -148 relative to the first codon (Horie et al., 1993). That region was later found to contain a CACCC box and a Sp1 binding site similar to the one found in the mouse TS promoter (Horie and Takeishi, 1997).

Further analysis of the regulatory region of the TS gene highlighted, once again, highly conserved sequences across species (Lee and Johnson, 2000). The human TS essential promoter region is GC-rich and lacks a conventional TATA box, like many other house-keeping genes. The essential promoter is located between nucleotides -141 to -161, upstream of the translational start site; it contains putative binding sites for Ets and Sp1, as well as a LSF consensus sequence (Fig. 1.4). Point mutations of any of these transcription factor binding sites leads to a significant reduction in the rate of TS transcription, confirming the critical role of this region

Figure 1.4 Structure and sequence of the human TS gene promoter. The region corresponding to nucleotides -232 to +8 bp and including the essential promoter region and the tandem repeats is shown. Consensus binding site for LSF, Ets-1, Sp1, E2F and USF transcription factor are represented by blue lines. The repeat sequences are indicated by the arrows (direct repeats) or the dashed arrow (inverted repeat). The structure of the promoter shown here is the one corresponding to the genotype containing two tandem repeats (2R). Adapted from Dong et al. (2000).


-232 bp ctctcagctgtggccctgggctccgttctgtgccacacccgtggctcctgcgtttccccc
LSF
Sp1
Sp1
Ets-1
Sp1
-172 bp tggcgcacgctctctagagcgggggccgccgcgaccccgccgagcaggaagaggcggagc
Inverted repeat
E2F
E2F
-112 bp gcgggacggccgcgggaaaaggcgcgcggaaggggtcctgccaccgcgccacttggcctg
Direct Repeats
+1 bp
-52 bp cctccgtcccgccgcgccacttcgcctgcctccgtcccccgcccgccgcgccatgcctgt

(Dong et al., 2000). The inverted repeat found just downstream of the essential promoter contains two putative E2F binding sites and constitutes the major site of transcription initiation. However, mutation of these two E2F sites had little effect on promoter activity. 5' promoter deletion analyses showed that multiple negative elements were found in the distal promoter sequences, as well as in the region just upstream of the essential promoter. This proximal sequence also contains another putative Sp1 binding site.

1.4.2 Transcription factors involved in the regulation of the TS gene

1.4.2.1 Late simian virus factor (LSF)

LSF was first identified in HeLa cells through its ability to transactivate the major late simian virus 40 promoter (Kim et al., 1987). LSF is a 66 kDa transcription factor, related to Drosophila grainyhead transcription factor, and binds DNA as a tetramer (Murata et al., 1998). Its consensus site is composed of two tandem repeats separated by about 4 nucleotides, restricting the binding of LSF tetramer to only one side of the DNA helix. Tissue specific studies in adult mice showed that LSF expression was ubiquitous, suggesting a global role for this transcription factor (Swendeman et al., 1994). LSF is implicated in the regulation of a number of genes including the mouse α-globin gene (Lim et al., 1992) as well as the human HIV long terminal repeat (Romerio et al., 1997). Like many other transcription factors, LSF is regulated by post-translational modifications. Phosphorylation of LSF by mitogen-activated protein kinase (MAPK) was shown to affect its DNA binding activity, increasing or decreasing the affinity of the protein for DNA (Volker et al., 1997;

Ylisastigui et al., 2005). Moreover, LSF can act both as a transcriptional activator and as a repressor, depending upon the promoter context.

In agreement with data obtained by promoter deletion and promoter mutation studies, a study performed in synchronized mouse cells confirmed the role of LSF as a positive regulator of TS expression (Powell et al., 2000). Transfection of a dominant-negative form of LSF into NIH 3T3 cells impaired accumulation of TS as cells reached S-phase and triggered massive apoptosis. Addition of exogenous thymidine rescued the cells, proving that cell death was due to insufficient dTMP levels as a result of low TS activity. A subsequent study showed that overexpression of Fe65, a ligand of APP (amyloid precursor protein), blocked cell-cycle progression and LSF-dependent upregulation of TS (Bruni et al., 2002), confirming the important role of the LSF transcription factor in the regulation of the TS gene expression.

1.4.2.2 Sp1 and Ets-1

Consensus sites for both Ets-1 and Sp1 are found in the TS essential promoter region, suggesting an important role for these transcription factors in the regulation of TS. Sp1 (Specificity protein 1) is a member of the Sp-family and represents a ubiquitous zinc-finger transcription factor involved in the regulation of a number of genes through binding to GC-boxes (Li et al., 2004). Sp1 can act as an activator or repressor of transcription, and its activity is mainly regulated by interactions with co-repressors and co-activators and post-translational modifications that include phosphorylation, acetylation, sumoylation and glycosylation.

The Ets family of transcription factors contains over 30 members, all characterized by a highly conserved helix-turn-helix DNA binding domain (Graves

and Petersen, 1998). They bind to a GGA(A/T) boxes and regulate the activity of a large number of cellular genes. Activity of a number of Ets proteins has been shown to be regulated by post-transcriptional events as well as by interaction with various other proteins that can modulate their activity (Seth and Watson, 2005).

Point mutations of the Sp1 or the Ets-1 sites in the mouse and human TS promoter led to a 3-fold decrease in the rate of TS transcriptional rate. A study performed in 2002 specifically addressed the role of Ets transcription factors in the regulation of TS gene expression. The authors showed that the transcription factor GABP (GA binding protein) was the only Ets family member able to transactivate the mouse TS promoter significantly (Rudge and Johnson, 2002). Furthermore, it was also shown that GABP acted with Sp1 to increase TS promoter activity synergistically.

1.4.2.3 Other promoter elements affecting TS expression

The region of the promoter that contains the tandem repeats has also been extensively studied with respect to its role in TS transcription. Analysis of this region showed that it constituted a polymorphic region in humans. This region was initially shown to contain three 28 bp repeats (3R); however a new allele, containing only two repeats (2R) is found at a fairly high frequency in a number of human populations. Reporter assays comparing the two human TS promoter alleles showed that the expression level of the 3R TS promoter was higher than its counterpart containing only two repeats (2R) (Horie et al., 1995). It was later shown that the first repeat of the 2R allele and the first two repeats of the 3R allele contain functional upstream stimulating factor (USF) binding sites (Mandola et al., 2003). Interestingly,

the USF site found in the second repeat of the 3R allele was shown to contain a single nucleotide polymorphism (SNP) within the USF consensus sequence that could affect USF binding affinity to its consensus site.

1.5 Alternative functions of the TS protein

1.5.1 TS as RNA binding protein

In addition to its well-characterized role in dTMP synthesis, TS has also been reported to be an mRNA binding protein (Liu et al., 2002). It was postulated that the repeats found in 2R/3R alleles described above in the 5'-UTR of the human TS mRNA were able to form stem-loop structures that could affect the translational rate of the TS transcipt (Takeishi et al., 1985). Analysis of TS transcripts where these repeats have been deleted confirmed that this region affected TS translational rate. The presence of these repeats was shown to reduce TS translation (Kaneda et al., 1987). A different line of evidence confirmed that TS expression is regulated by post-transcriptional mechanisms. Following acute exposure of cells to 5-FU (which binds and inhibits TS), a rapid increase in TS protein levels was observed (Washtien, 1984). However, the mechanisms responsible for this increase remained unclear. It was postulated that the observed increase was due to an increase in the TS mRNA translation or an increase in the stability of the TS protein.

One research group has provided evidence that TS protein binds its own mRNA to negatively regulate its translation (Chu et al., 1991). Interestingly, the presence of the substrate, the folate co-factor or the analogue 5-FU abolished binding of the protein to its mRNA, suggesting that translation of the TS mRNA by the TS protein is only repressed in the absence of the enzyme substrates. This

mechanism would constitute a negative autoregulatory feedback loop, where an excess of free inactive enzyme would decrease synthesis of new TS protein. Two binding sites on the TS mRNA were identified through their ability to bind the TS protein. One was located at the 5' end of the transcript overlapping the translational start, while the other one was found within the coding sequence of the protein (Chu et al., 1993b; Lin et al., 2000). Other studies by the same group identified a number of cellular mRNA that can form complexes with the TS protein. IFN-induced 15kDa mRNA as well as p53 mRNA were identified as *in vivo* targets bound by the TS protein (Chu et al., 1996). The ability of TS to regulate p53 expression was studied in more detail and showed that, in an inducible TS expression model, induction of high TS protein led to an almost complete suppression of p53 protein expression without concomitant change in p53 mRNA levels (Chu et al., 1999). Taken together this data suggests that besides its well established metabolic role, TS might be an RNA binding protein that can control the translation of various cellular mRNA including its own. It is worth noting that these findings have never been confirmed by others, including our lab.

1.5.2 TS as an oncogene

Recently, one report described TS as an oncogene. Overexpression of a catalytically active form of the TS protein was able to transform mammalian cells, as demonstrated by growth in soft agar and tumour formation in nude mice (Rahman et al., 2004). The authors suggested that the neoplastic phenotype observed was dependent on increased DNA synthesis initiated by high TS activity. For the first time, this study implied that high TS levels could drive the proliferative potential of

cells. It is interesting to note that RNR activity, which is responsible for dUMP, had also been shown to be involved with neoplastic transformation of cells (Fan et al., 1998). Earlier studies had already provided indications of the potential role of TS in the control of cellular proliferation. For example, comparison of cell cycle regulation of NCI H630 and its paired cell line overexpressing TS (15 to 20-fold) had highlighted striking differences between the two. Confluent cells overexpressing TS showed no evidence of a G_0 population whereas over 25% of the matched wild type cells were quiescent (McGinn et al., 2000). These results suggested that higher TS levels prevented cells from entering a resting state and conferred them a higher proliferative capacity.

1.6 TS as a molecular target and prognostic marker for chemotherapy

1.6.1 Anti-TS drugs

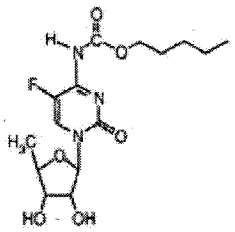
The central role of TS in nucleic acid metabolism and DNA synthesis has made it a very attractive target for cancer treatment. Inhibition of TS leads to cancer specific toxicity presumably because of the high proliferative rate of cancer cells compared to most normal cells in the body. 5-FU was the first anti-TS drug to be used clinically for cancer treatment, as early as 1958 (Bertino, 1997). 5-FU is administered intravenously and is metabolized to 5-FdUMP (5-fluoro-2'-deoxyuridine monophosphate) by cellular enzymes including thymidine phosphorylase, thymidine kinase, and orotate phosphoribosyltransferase (Ichikawa, 2006). 5-FdUMP is an analogue of the substrate, bearing a fluoride atom on carbon 5 of the uracil moiety. During catalysis by TS, the active site cysteine attacks carbon 6 of 5-FdUMP to form a covalent bond. However, the presence of the fluoride atom at that position

prevents further catalysis and attack by the co-factor, leaving the enzyme bound to 5-FdUMP in a stable inhibitory ternary complex. Despite its introduction as a treatment of colorectal cancer over 4 decades ago, 5-FU in combination with leucovorin remains one of the primary treatments used nowadays for the treatment of gastrointestinal, head and neck and breast cancer. Currently, it is often used in combination therapy, along with other compounds such as oxaliplatin and irinotecan, to increase treatment efficacy (Patiyil and Alberts, 2006).

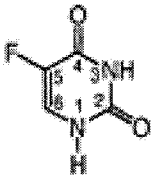
A number of other compounds targeting the TS enzyme have been designed and developed clinically in recent years (Papamichael, 1999). They are either analogues of the substrate or the folate co-factor. Ftorafur and capecitabine are precursors of 5-FU and FdUMP and represent a new generation of antimetabolites that can be administered orally (Fig.1.5). Activation of these molecules involve a multi-enzymatic process that ultimately leads to the formation of 5-FU, which represents the active compound. Even though they exhibit less toxicity than 5-FU, due to activation more specifically within tumours, their mechanism of action is not restricted to TS inhibition since they also affect RNA metabolism (Longley et al., 2003). Raltitraxed, nolatrexed and pemetrexed are folate analogues and are considered to be much more specific TS inhibitors than 5-FU and its precursors because they target TS activity directly (Fig. 1.5). Raltitrexed and pemetrexed were recently approved for the treatment of advanced colorectal cancer and non-small cell lung cancer, respectively.

Figure 1.5 Structure of various inhibitors targeting the TS enzyme. Chemical structures of 5-fluorouracil and its precursors which are metabolized to form the active compound FdUMP. FdUMP inhibits TS by forming a stable ternary complex with the protein. Chemical structures of folate analogues that target the TS enzyme to form inhibitory complexes

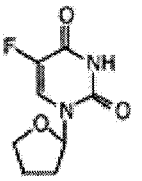
FdUMP precursors



Capecitabine

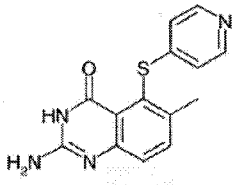


5-FU

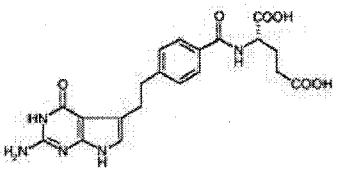


Ftorafur

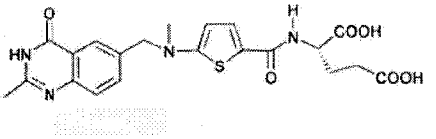
Folate co-factor analogues



Nolatrexed



Pemetrexed



Raltitrexed

1.6.2 Resistance to drugs targeting dTMP synthesis

One of the best documented ways for tumour cells to overcome the toxicity of chemotherapeutics drugs targeting thymidylate synthesis is through upregulation of their primary target: TS enzyme levels. However, the mechanisms responsible for this increase are not completely understood. It has been demonstrated that short exposure of cells to ant-TS drugs lead to a rapid accumulation of the TS protein with no significant changes in mRNA levels, suggesting the presence of post-transcriptional events (Keyomarsi et al., 1993; Chu et al., 1993a). The observed increase has been suggested to be caused by a stabilization of the enzyme when bound to its inhibitors (Berger et al., 2004). Others believe that, upon binding to its inhibitors, the TS protein is unable to inhibit translation of its own mRNA leading to synthesis of new TS protein (Liu et al., 2002). Interestingly, TS levels can be high in some tumours prior to chemotherapy (Haqqani et al., 1999), suggesting the existence of alternative mechanisms leading to TS overexpression such as gene amplification (Wang et al., 2004). Structural studies of the TS enzyme also showed that point mutations of certain amino acid residues, involved in the substrate and co-factor binding, can also affect sensitivity of cells to anti-TS drugs (Kawate et al., 2002; Tong et al., 1998). These mutations decrease the affinity of the enzyme for the antimetabolites; however this mechanism of drug resistance does not appear to occur with a high frequency in tumours.

Other enzymes can also affect the response of cells to drugs targeting TS. For example, 80% of 5-FU is actually degraded in the liver by the enzyme dihydropyrimidine dehydrogenase (DPD). DPD catalyzes the first step in the catabolic pathway of 5-FU and can greatly affect the tumour response to 5-FU and

its prodrugs (Kubota, 2003). In the case of antifolate compounds, several cellular enzymes involved in folate metabolism have been implicated in the acquisition of resistance: (i) decrease uptake of the drugs by the reduced folate carrier (RFC); (ii) increased cellular efflux through multidrug resistance transporter (MDR); (iii) decrease cellular retention due to defects in polyglutamylation by folylpoly-γ-glutamate synthetase (FPGS) (Assaraf, 2007). Upregulation of the level/activity of any of these enzymes can strongly decrease the efficacy of antifolates targeting TS.

1.6.3 TS as a prognostic marker

Recently, efforts have been made to try to identify biomarkers that can help oncologists predict the best clinical approach to treat the disease. Since TS represents a common target for chemotherapy for number of cancers, it is commonly considered to be a prognostic marker for tumour responsiveness to drugs targeting TS or for clinical outcome in general. It has been shown that TS levels can be high in some tumours compared to normal tissues (Haqqani et al., 1999; Mizutani et al., 2003). Clinical correlation studies also demonstrated that high TS expression levels are associated with poorer response to 5-FU in gastrointestinal cancer (Johnston et al., 1995; van Triest et al., 1999; Okumura et al., 2007). Furthermore, high TS levels seem to correlate with overall poorer clinical outcome in non small cell lung carcinoma and cervical cancer, independent of the treatment used for chemotherapy (Shintani et al., 2003; Suzuki et al., 1999). These findings strongly suggest that TS levels, in addition to being a primary determinant of response to drugs targeting thymidylate synthesis, might independently predict poorer clinical outcomes.

1.7 Rationale and Objectives

1.8.1 Rationale

Thymidylate synthase is a critical cellular enzyme involved in the *de novo* synthesis of dTMP and has been widely used as a target for chemotherapy for almost 50 years. A number of compounds directly targeting this enzyme have been developed and used clinically over the years to treat a number of cancers, including gastrointestinal tumours. Despite having been known and used as a target for cancer treatment many years, the mechanisms controlling the expression of the TS gene remain largely unknown. TS expression has been proposed to be controlled by various mechanisms, including transcription, translation and protein stability/activity. A number of studies performed in rodent models established TS as a S-phase dependent enzyme. However, some later studies performed in human cells do not support this dogmatic point of view. Identification and understanding of the factors that control TS transcription in human cells could provide interesting insights into the regulation of this important gene. Furthermore, it might also provide new therapeutic options to decrease expression of TS and increase sensitivity of tumour cells to drugs targeting TS and increase the efficacy of a number of drugs commonly used in the clinic. Moreover, since high TS levels have been recently shown to have oncogenic properties, decreasing this enzyme levels could also decrease the tumorigenic potential of malignant cells.

We hypothesize that the mechanisms controlling TS expression in human cells are different than in rodent cells and are primarily dependent on proliferation signals rather than cell-cycle progression.

1.8.2 Objectives

- Study the cell cycle dependent regulation of thymidylate synthase in human cells and test the role of E2Fs transcription factor in the regulation of human TS transcription.
- Explore the role of proliferative/growth signaling pathways on TS expression in human cells.
- Assess the role of the transcription factor LSF and GABP in the transcriptional regulation of the human TS gene.

Chapter 2: MATERIALS & METHODS

2.1 Cell culture and cell synchronization

Human colon cancer cells HCT116, breast cancer cells MCF-7 and prostate cancer cells PC3 were all obtained from ATCC. Normal fibroblast GM38 cells were obtained from NIGMS. HCT116 were maintained in McCoy's 5A medium (Wisent) supplemented with 10% FCS (fetal calf serum) (Wisent) at 37°C in a 5% CO_2/95% air atmosphere. MCF-7, PC3 were maintained in DMEM (HyClone) supplemented with 10% FCS at 37°C in a 5% CO_2/95% air atmosphere. GM38 cells were maintained in DMEM (HyClone) supplemented 15% FCS respectively at 37°C in a 5% CO_2/95% air atmosphere. Cells were regularly tested for Mycoplasma contamination by staining with Hoechst 33258.

For synchronization experiments, HCT116 cells were plated at low density (1 $\times 10^5$ cells/mL) in a 10 cm plate in regular culture medium and incubated overnight to allow the cells to attach. The following day, fresh culture medium supplemented with 0.5% fetal calf serum was added to the plates and cells were incubated in these conditions for 7 days. At the end of the starvation period, cells were stimulated to re-enter the cell cycle by addition of fresh medium supplemented with 20% fetal calf serum.

2.2 Flow cytometry

For flow cytometry analysis, cells were harvested by trypsinization, washed once with cold PBS (phosphate buffer saline) and fixed by suspending the cell pellet in cold 70% ethanol for 1 hr or longer. Cells were then washed with cold PBS and

resuspended and incubated in PBS with propidium iodide (1 µg/mL) containing 40 µg/mL ribonuclease A for 1 hr on ice. Stained cells were then analyzed using a BD LSR flow cytometer (BD bioscience), and separated as a function of side scatter and forward scatter on a logarithmic scale. Cells were gated appropriately in order to remove apoptotic cells and isolate the single cell population. A total of 10,000 gated events were recorded for each individual sample. Cell cycle distribution was then determined using the Modfit® program (Verity Software Inc): cell populations were plotted on a graph FL2 area as a function of FL2 width, in order to gate the single cell population for cell cycle distribution analysis. The peaks corresponding to G_1 and G_2/M populations were adjusted manually, and the ratio of the fluorescence intensity of G_1 and G_2/M fractions was kept between 1.8 and 2.1.

2.3 Drug treatments and siRNA

2.3.1 Inhibitors treatment

CDK2 inhibitor III and CDK4 inhibitor have been previously described (Bhattacharjee et al., 2001; Zhu et al., 2003). Roscovitine, CDK2 inhibitor III, CDK4 inhibitor and UO126 were all purchased from Calbiochem and dissolved in DMSO. Prior to drug treatment, MCF-7, PC3 and HCT116 were plated at a density of $2x10^5$ cells/mL incubated overnight to allow cells to attach. Prior to addition to the cells, stock solutions of inhbitors were diluted in serum free medium and added to the cells at the indicated concentration. Cells were then incubated for 24 h in presence of the drugs before protein and RNA extraction, and flow cytometry analysis. DMSO diluted in culture medium under the same conditions was used as a control in every inhibitor experiment.

2.3.2 siRNA and knock-down experiments

Small interfering RNA (siRNA) targeting MEK1, MEK2, LSF, ERK1 and ERK2 were all purchased from Dharmacon. The control siRNA (siCONTROL non-targeting siRNA #1) was also purchased from Dharmacon and used as a control in knock-down experiments. The siRNAs targeting MAP kinase kinase MEK1 and MEK2 have already been described (Ussar and Voss, 2004). siRNA targeting LSF and ERK2 were designed using Dharmacon siDESIGN centre™. The mRNA sequences targeted by these various siRNAs are as follow: MEK1: 5'-AAGCAACTCATGGTTCATGCTTT; MEK2, 5'-AAGAAGGAGAGCCTCACAGCA; LSF, 5'-AATTACCATGCTATCTATCTA; ERK2, 5-'CCAAAGCTCTGGACTTATT. For siRNA experiments, cells were plated at low density ($1.25x10^5$ cells/mL) in 6-well plates and incubated overnight to let the cells attach. Cell monolayers were then washed 2 times with PBS and 0.8 mL of serum-free Opti-MEM (Invitrogen) was added to the wells. siRNA duplexes were diluted in serum-free Opti-MEM to a final volume of 185 µL and then incubated for 30 min at room temperature in presence of 15 µL of Opti-MEM- oligofectamine (Invitrogen) mix (13 to 2 ratio) prior to addition to the cell monolayers. Following addition of siRNA duplexes to the cells, monolayers were incubated in serum-free media containing the siRNA for 6 h at 37°C before addition of normal culture medium supplemented with 20% FCS. Cells were then incubated for an additional 24 to 72 h before protein and mRNA extraction for further analysis.

2.4 Adenoviruses, infections, plasmids and transfection

2.4.1 Adenoviruses and infections

Adenoviruses encoding cyclin E, Dp1 and E2F1 were supplied by Dr J.R. Nevins and have been previously described (Ekholm et al., 2001; Schwarz et al., 1995). CA35 adenovirus encoding β-galactosidase was provided by Dr. C. Addison. Adenoviruses encoding human p16INK4A and human p27Kip1 were a gift from Dr F. Graham and have been previously described (Schreiber et al., 1999). Adenoviruses were propagated in 293 cells and purified by CsCl banding as previously described (Ng and Graham, 2002). Virus stock titre was determined by plaque assays in soft agar. Cells to be infected were plated a density of $2x10^5$ cells/mL and incubated overnight to allow the cells to attach. Cells were then washed 2 times with PBS and infected at the indicated MOI for 1 h in PBS++ at 37°C. Fresh medium supplemented with 10% FCS was then added to the plates and cells were incubated for another 24 h before protein and mRNA extraction.

2.4.2 Plasmids

Plasmids encoding wild-type MEK1 (pCMV5-MEK1 WT) and the constitutively active form of MEK1 (pCMV5-MEK1-R4F) were a kind gift from Dr N. Ahn and have previously been described (Xu et al., 2001). Plasmids encoding the MEK-ERK2 fusion proteins (myc CMV5- ERK2-MEK1 and myc CMV5- ERK2-MEK1 LA) were a kind gift gift from Dr M. Cobb and have been previously described (Robinson et al., 1998). The expression plasmids for GABP-α and GABP-β1 (pSRSPA-GABP α & pSRSPA-GABP β1) were a kind gift of Dr U. Rapp and have previously been described (Flory et al., 1996).

The pcDNA3.1-LSF- myc-His plasmid was engineered by cloning the human LSF cDNA in pcDNA3.1 myc-His vector. LSF cDNA was obtained by RT-PCR,

using 3 µg of total RNA from HCT116 cells and 1 µg of oligo-dT. cDNA synthesis was carried out using Superscript II (Invitrogen) according to the manufacturer instructions. PCR amplification of LSF cDNA was then carried out using Vent DNA polymerase (New England Biolabs) and the following primers (LSF-S: 5'-TGGTTTGCGTTACTCCTG; LSF-AS: 5'-GCCGCACTCCT**C**CTTCAG). The forward primer included a BamHI site on its 5' end whereas the reverse primer included a XhoI site on its 5' end. The reverse primer also included a point mutation to remove the LSF stop codon (shown in bold in the primer sequence). PCR product were digested with Bam HI and Xho I and cloned into pcDNA 3.1 myc-His in frame with the myc and his tags on the 3' end of the LSF cDNA. The construct was sequenced to confirm the absence of any spurious mutations.

The IMAGE (Integrated Molecular Analysis of Genomes) clone containing TFCP2L2 (transcription factor CP2 like 2) cDNA was purchased from ATCC (IMAGE clone 6971725). TFCP2L2 cDNA was cut out of pDNR-dual by digestion with Sal I and Xba I and subcloned in the Xho I / Xba I sites of pCDNA 3. The pcDNA3-TFCP2L2 was sequenced to confirm the absence of mutations.

2.4.3 Transfections

HCT116 cells were plated at a density of $3x10^5$ cells/mL in 6 well plates and incubated overnight prior to allow the cells to attach. The following day, 2 mL of fresh medium was added to the cells. 1 to 3 µg of plasmid DNA was used to per transfection. Prior to addition to the cells, plasmid DNA was diluted in 250 µL of serum-free medium, and then incubated in presence of oligofectamine 2000 (Invitrogen) diluted in 250 µL of serum-free medium. Typically, 3 µL of

oligofectamine were used per µg of plasmid DNA. Plasmid DNA was incubated in the presence of Oligofectamine at room temperature for 20-30 min prior to addition of the transfection reaction to the cells. Following transfection, cells were incubated for 24 to 48 h before RNA and/or protein extraction.

2.5 RNA extraction, RT-PCR and real-time PCR

2.5.1 RNA extraction and cDNA synthesis

RNA extractions were carried out as previously described (Birnboim, 1988). Briefly, cell monolayers were chilled on ice for 5 min, washed twice with cold PBS and lysed with RES-1 (1 M Urea, 0.5 M LiCl, 20 mM sodium citrate, pH 6.8, 5 mM CDTA, 1% SDS). Cell lysates were sonicated and incubated for 30 min at 55°C in the presence of Proteinase K (100 µg/mL). Nucleic acids were precipitated by addition of 2 vol. of cold ethanol. The pellet was redisolved in RES-1 and incubated another time with proteinase K (100 µg/mL). Samples were purified by phenol-chloroform extraction and RNA precipitation was carried out by addition of 20 mM acetic acid and 1 volume of LiCl/EtOH and incubation overnight at 0°C. To wash out the excess phenol, RNA pellets were resuspended in CCS (1 mM sodium citrate pH 6.8, 0.1% sodium dodecyl sulfate (SDS), 1 mM CDTA) and precipitated by addition of 150mM sodium acetate and 2 volumes of EtOH. The final RNA pellet was resuspended in RNAse free water, and RNA concentration was determined by reading the absorbance at 260 nm. cDNA synthesis was carried out using 3 µg total RNA, 1 µg oligo-dT primer (Invitrogen) and 200 units of M-MLV reverse transcriptase (Invitrogen), following the manufacturer's instructions.

2.5.2 Real time PCR

Quantification of human TS, Cyclin E, E2F1, β-2-microglobulin and ribosomal protein L32 (RPL32) mRNAs was carried out using the following primer pairs: TS (forward 5'-CCCTGACGACAGAAGAA; reverse, 5'-TAGTTGGATGCGGATTG); Cyclin E (forward 5'-CAAGTACACCAGCCACCTC; reverse 5'-GTACAACGGAGCCCAGAA); RPL32 (forward 5'-GCCCTCAGACCCCTTGTG; reverse 5'-AGATGCCAGATGGCAGTTT); E2F1 (forward 5'-GCCACTCGGCTGACGG; reverse 5'-GGCTGATCCCACCTACGGT); β-2-microglobulin (forward 5'-CGCTACTCTCTCTTTCTGGC; reverse 5'-AACTTCAATGTCGGATGGAT).

Real-time PCR reactions were performed on a Roche Lightcycler in a total volume of 20 µL. The PCR buffer contained 2 to 4 mM $MgCl_2$, 2 to 8 pmoles of each primer, 2 units of Taq polymerase (Invitrogen), 1/40,000 dilution of Sybr green I (Molecular Probes), and 2 µL of an appropriate dilution of the cDNA template. Amplification conditions were as follows. For TS, 98°C, 20 sec (1 cycle); 98°C, 3 sec, 51°C, 8 sec, 72°C,17 sec (40 cycles). For Cyclin E , 98°C, 20 sec (1 cycle); 98°C, 1 sec, 60°C, 6 sec, 72°C, 24 sec (40 cycles). For RPL32, 98°C, 20 sec (1 cycle); 98°C, 1 sec, 61°C, 6 sec, 72°C, 19 sec (40 cycles). For E2F1, 99°C, 20 sec (1 cycle); 99°C, 3 sec, 58°C, 8 sec, 72°C, 20 sec (40 cycles). For β-2-microglobulin, 95°C, 30 sec (1 cycle); 95°C, 1 sec, 52°C, 10 sec, 72°C, 6 sec (40 cycles). Relative quantification of unknown samples was achieved by building a standard curve from a 2-fold serial dilution series of one of the samples designated as a reference. Values obtained for individual mRNAs were then normalized to data obtained for RPL32 or β-2-microglobulin, used as references in these experiments.

2.6 Protein extraction, immunoblotting and densitometry

2.6.1 Protein extraction, quantification and SDS PAGE

For western blot analysis, total protein extracts of HCT116, MCF-7, PC3 and GM38 cells were prepared by trypsinization of cell monolayers. Briefly, cells were washed two times with PBS, trypsinized and resuspended in cell culture medium. Cell suspensions were then centrifuged at low speed for 5 min at 4°C and then washed with 1 mL of cold PBS. Cell pellets were then lysed in SDS sample buffer (125 mM MOPS, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.001% bromophenol blue).and the extracts were incubated at 100°C for 10 min prior to loading on SDS-PAGE gels.

Protein quantification was achieved using the fluorescamine assay (Udenfriend et al., 1972) (Sigma). Known concentrations of bovine serum albumin (BSA) was used as a reference to build a standard to determine the protein concentration of unknown samples. 20 to 40 µg of total protein extract was resolved on a 12% polyacrylamide gel (19:1 acrylamide: bisacrylamide) for 1 h at 180 V in SDS running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS). Following electrophoresis gels were equilibrated in transfer buffer (10 mM $NaHCO_3$, 3 mM $NaCO_3$ and 15% Methanol) for 15 min and transferred onto a Immobilon PVDF membrane (Millipore), as previously described (Bissoon-Haqqani et al., 2006).

2.6.2 Western blot and densitometry,

Membranes were blocked for 1 h in TBS-T (10mM Tris-HCl pH 8, 150 mM NaCl, 0.1% Tween 20) supplemented with 1% BSA or 5% milk. Membranes were then incubated with the appropriate dilution of primary antibodies in blocking solution

from 1 h to overnight depending on the strength of the signal. Antibodies specific to cyclin E, cylin D1, E2F1, MEK1/2, ERK1/2 were purchased from Santa Cruz Biotechnologies, phosphor ERK1/2 and p27Kip1 from Cell signalling, LSF from BD Transduction laboratories, β-actin and p16INK4A from Sigma and rabbit anti-thymidylate synthase (Haqqani et al., 1999) from Rockland Immunochemicals. Following incubation with the primary antibody, blots were then washed 5 times with TBS-T and incubated for 1 h with Envision labelled polymer mouse rabbit-HRP (Dako Cytomation) secondary antibody, diluted 1:250 in TBS-T. Membranes were once again washed 5 times in fresh TBS-T before addition of 1 mL of chemiluminescent reagent (KPL) and incubated for 1 min at room temperature to increase the chemical reaction. Membranes were then transferred in a cassette and exposed on X-omat film (Kodak) for increasing amounts of time.

For densitometry analysis, blots were scanned using a GS 800 densitometer (Bio Rad). Individual bands were quantified manually using the Quantity One software. Relative protein quantities were normalized to 0 hr and β-Actin levels, which were used as a reference to correct for loading.

2.7 Two dimension SDS PAGE and mass spectrometry

Following inhibitor treatment or transfection, cells were collected by trypsinization and resuspension in culture medium, centrifuged and washed in ice-cold sucrose buffer (10 mM BES pH 7.4, 0.3 M sucrose) to remove excess salts. After a second centrifugation, cell pellets were lysed in IEF buffer (7 M urea, 2 M thiourea, 4% CHAPS, 130 mM dithiothreitol and 0.0001% bromophenol blue) and homogenized by vortexing extracts for 1 h. Proteins were then precipitated by

addition of 10 volumes of acetone followed by a centrifugation for 5 min at 2500 rpm. After semi-drying, the protein extracts were resuspended in IEF buffer plus 0.4% 3-10 ampholytes (Bio-rad), and protein concentration was determined by a Bradford assay (Bio-rad) using BSA as a standard. IPGs strips (7cm, 3-10 pH range, Bio-Rad) were rehydrated overnight with 50 to 100 µg of total protein in a total volume of 125 µL. Isoelectric focusing for 7cm IPG strips was carried on protein IEF cell (Bio-Rad) under the following conditions: 250 Volts for 20 min, 4,000V for 2 h for desalting and 10,000 Volt hours at 4,000 V for focusing. Strips were then equilibrated for 15 min in reducing buffer (375 mM Tris-HCl pH 8.8, 6 M urea, 2% SDS, 65 mM DTT, 20% glycerol) and 15 min in alkylating buffer (375 mM Tris-HCl pH 8.8, 6 M urea, 2% SDS, 217 mM iodoacetamide, 20% glycerol). The strips were then dipped in SDS running buffer before mounting on top of a 12% SDS PAGE gel using a 1% agarose overlay solution in SDS running buffer. The second dimension and the transfer were performed as previously described in section 2.5.1.

For mass spectrometry analysis, 1 mg of total protein extract was run and focused on a 17 cm pH 3-10 IPG strip (Bio-rad). Isoelectric focusing was carried under the following conditions: 250 Volts for 20 min, 10,000V for 2.5 h for desalting and 40,000 Volt hours at 10,000 V for focusing. Following incubation with reducing and alkylating buffers, the strips were mounted on a Bio-rad protean II xi 12% SDS gel. Following electrophoresis, the gels were either stained with coomassie blue or transferred to a PVDF membrane. Prior to mass spectroscopy analysis, gels were stained with colloidal coomassie solution (0.08% Coomassie brilliant blue G250, 1.6% ortho-phosphoric acid, 8% ammonium sulphate and 20% methanol) for several hours in a closed plastic dish to avoid contamination with

spurious proteins. The gel was then washed several times with distilled water. To identify the regions on the gel containing putative LSF peptides, the coomassie stained gel and the membrane probed for LSF were compared and aligned. The two regions containing proteins reacting with the LSF antibody were cut out of the gel and sent for mass spectrometry analysis. Identification of the peptides present in the various spots was kindly performed by Dr. Arsalan Haqqani (NRC) using a nano liquid chromatography MS/MS system (ESI-Q-TOF instrument from Waters).

2.8 Promoter constructs, site directed mutagenesis and luciferase assay

2.8.1 Promoter constructs.

The human TS reporter construct (pGL2B-TS) was engineered by cloning the human TS promoter upstream of the luciferase cDNA into the pGL2B vector (Promega). The full length TS promoter fragment corresponding to the region from -472 bp to +10 bp relative to the AUG start codon (in the 2R genotype containing only 2 tandem repeats) was amplified by PCR from HCT116 genomic DNA with following primers (TSR+ 5'-AGAAAAGCTGCATTATACCACTTGC; TSR- 5'-CCACAGGCGTGGCGCG). The TSR+ and TSR- primers included a Xho I and an Hind III site respectively for cloning into the multiple cloning site of pGL2B. The various promoter deletions constructs were engineered by PCR amplification of specific regions of the promoter, using pGL2B-TS plasmid as a template (see Table 2.1). The primers used for amplification of the various promoter fragments are shown in Table 1. The forward primers always included a Xho I site at their 5' end whereas the reverse primers always included an Hind III site at their 5' end to permit cloning in the Xho I / Hind III site of the pGL2B vector.

Table 2.1: Sequence of the various primers used to engineer promoter deletion constructs. The primer pairs used to amplify various regions of the human promoter are shown here. The sequences of the forward and reverse primer as well as the corresponding amplified region (relative to the first codon) are indicated. The forward primers always included a Xho I site on their 5' end whereas the reverse primers always included a Hind III site.

Deletion construct	Forward primer	Reverse primer
+132 bp/+10bp	5'-CGAGCAGGAAGAGGCGGAGC	5'-CCACAGGCGTGGCGCG
-472 bp/ -123 bp	5'-AGAAAAGCTGCATTATACCACTTGC	5'-GCTCCGCCTCTTCCTGCT
-472 bp/ -73 bp	5'-AGAAAAGCTGCATTATACCACTTGC	5'-CAGGACCCCTTCCGCGC
-472 bp/ -42 bp	5'-AGAAAAGCTGCATTATACCACTTGC	5'-CGGGACGGAGGCAGGC

Promoter deletions constructs where the LSF, Ets-1 and Sp1 consensus sites have been deleted were engineered by restriction digest of the parental pGL2B-TS plasmid using the XbaI and SacII restriction enzymes. Digestion of the parental pGL2B-TS plasmid with XbaI and SacII enzymes removed a 57 nt region of the TS promoter containing the essential promoter. Double-stranded oligonucleotides containing deletions of several essential promoter sites were synthesized and cloned back into the XbaI-SacII digested pGL2B-TS plasmid. Every promoter construct was sequenced before use in luciferase assays in order to ensure the absence of any spurious mutations introduced by the PCR amplification or cloning strategy.

2.8.2 Luciferase Assays

HCT116 cells were plated at a density of $3x10^5$ cells/mL and incubated overnight prior to the transfection. The following day, cells were transfected with 1 µg of the pGL2B construct containing the various promoter fragments, along with 1 µg of the plasmid encoding the protein of interest and 1 µg of pSV-β-galactosidase plasmid (Promega), used as a control for transient transfection efficiency. Transfections were carried out using the same conditions as described in 2.4.3. Following transfection, cells were incubated for 36 h and then washed 2 times with cold PBS and lysed using 1X reporter lysis buffer (Promega). Extracts were then submitted to two freeze-thaw cycles and then centrifuged at 12,000 rpm for 2 min to remove cellular debris. β-galactosidase activity was determined using the β-galactosidase assay system (Promega) and was used to correct for transient transfection efficiency. Luciferase activity was measured following addition of 100

µL of luciferase assay reagent (Promega) to 100 µL of extract using a Lumat LB9507 luminometer (EG & G Berthold).

2.9 Site-directed mutagenesis (SDM)

For SDM experiments, a pair of complementary primers containing the desired mutation was designed for each site to be mutated. The pGL2B-TS wild-type plasmid was used as a template in PCR reaction using the following mutated primers set (mutations are shown in bold in the primer sequence): LSF mutation (LSF Mut S: 5'-GCCGCGACCCCGC**TC**AGCAGGAAGAGG; LSF Mut AS: 5'-CCTCTTCCTGCT**GA**GCCGGGTCGCGGC), Ets-1 mutation (Ets-1 Mut S: 5'-CGACCCCGCCGAGCAGG**TT**GAGGCGGAGCG; Ets-1 Mut AS: 5'-CGCTCCGCCTC**AA**CCTGCTCGGCGGGGTCG), Sp1 mutation (Sp1 Mut S: 5'-CGCCGAGCAGGAAGA**AT**CGGAGCGCGGGAC; Sp1 Mut AS: 5'-GTCCCGCGTTCCG**AT**TCTTCCTGCTCGGCG). PCR reactions were carried out using 2 units of Vent DNA polymerase, 125 ng of each primer and 20 to 50 ng of pGL2B-TS template in a total volume of 50 µL. The PCR cycle conditions were as follow: 99°C for 2 min (1 cycle); 99°C for 30 sec, 55°C for 1 min, 72°C for 14 min (18 cycles); 72°C for 5 min, 4°C hold (1 cycle). Following PCR amplification, non-mutated parental plasmid DNA was digested by direct addition of 10 units of Dpn I to the PCR reaction and incubation at 37°C for 2 h. *E. coli* DH5α cells were then transformed with 10 µL of restriction product and plated on agar plates supplemented with 50 µg/mL ampicillin (Sigma). The presence of the mutation was determined using restriction digest where possible and always confirmed by sequencing.

Chapter 3: RESULTS

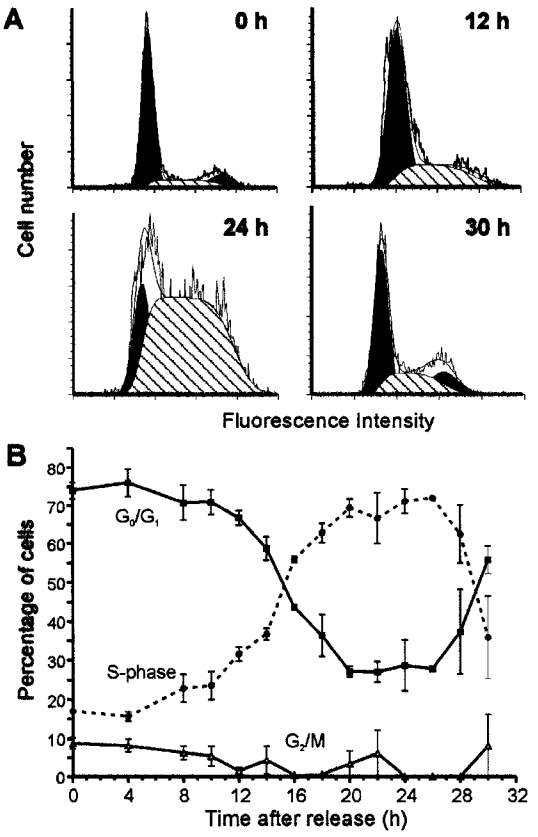
3.1 Cell-cycle dependent regulation of human thymidylate synthase

We first sought to study the cell cycle dependence of thymidylate synthase (TS) in human cells as well as the role of E2F transcription factors and G_1/S protein in the regulation of TS expression. A number of previous studies have used serum-starvation to study the cell-cycle dependent regulation of TS, despite the possibility that serum-starvation may create artifacts. We used this same approach to synchronize human cells rather than using drug treatment in order to be able to compare our data to previously performed experiments. The human colon cancer cell line HCT116 was chosen as a model cell line because it expresses the pRb pocket protein that is essential to inhibit E2F activity in non-cycling cells, and its cell cycle machinery seems to be largely intact. Preliminary experiments were performed to test the serum concentration as well as the time required to achieve synchronization of the cells.

3.1.1 Synchronization of HCT116 cells by serum-starvation and re-feeding

Synchronization of HCT116 cells was achieved by maintaining cells for 7 days in 0.5% fetal calf serum. Under these conditions, more than 80% of the cells were in the G_0/G_1 phase of the cell cycle (Fig. 3.1A) after the seven day starvation period. To stimulate cells and allow them to re-enter the cell cycle in a synchronized fashion, fresh media supplemented with 20% FCS was added to the cells. After a latency period of 8 to 10h, cells resumed growth and started to enter S-phase about 8 h after release. By 18 h post-stimulation, the majority of cells were in S-phase

Figure 3.1 Cell-cycle distribution of synchronized HCT116 following release from serum-starvation. (**A**) Histogram analysis of cells stained with propidium iodide at various time points following release. Data were acquired on a BD LSR Flowcytometer and analyzed using Modfit®. The proportion of cells in G_1 is represented by a red peak on the left and cells in G_2/M on the right in each panel; cells in S-phase are represented by the hatched area. (**B**) Time-dependent changes in cell cycle distribution following re-addition of serum. Percentage of cells in each of the cell-cycle phases was determined by Modfit analysis. Data shown are the mean and SEM of three independent experiments.

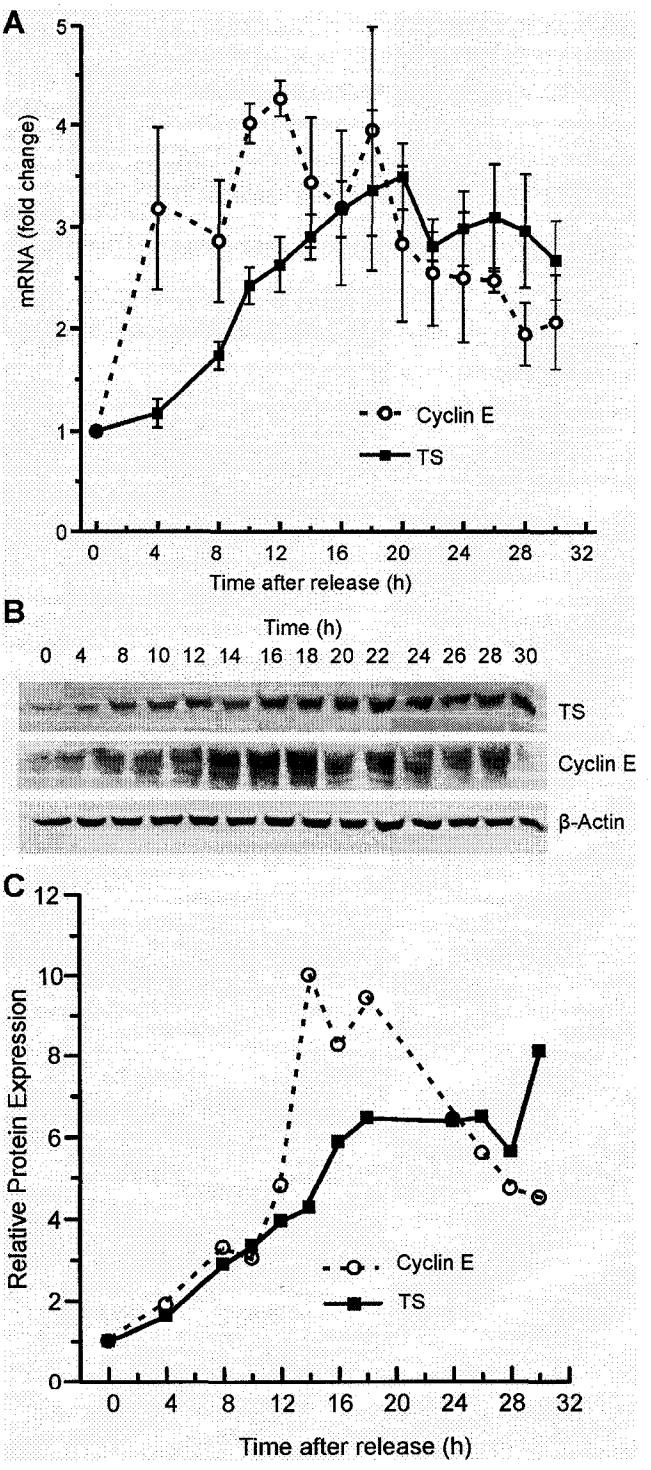

A
0 h
12 h
24 h
30 h
Cell number
Fluorescence Intensity
B
Percentage of cells
G_0/G_1
S-phase
G_2/M
Time after release (h)
0
4
8
12
16
20
24
28
32
10
20
30
40
50
60
70
80

(80%) and were actively replicating their DNA. Cell cycle progression continued steadily and, by 30 h, most cells had completed a full cell cycle and had entered a second G_0/G_1 (Fig. 3.1A, 3.1B). The transit of the cells through the G_2/M phase was not clearly observed in our experiments, probably because G_2/M is a very short phase of the cell cycle. Based on flow cytometry data and the measurement of cell cycle distribution, we concluded that these conditions achieved an adequate level of cell synchrony. As shown in Fig. 3.1B, the majority of cells entered S-phase between 10 and 16 h post-stimulation, indicating the time frame corresponding to the G_1-S transition. The proportion of cells in S-phase remained high until about 24 h, at which time cells entered G_2/M. The completion of one full cycle was observed 28 h post-release and was concomitant with an increase in the G_1 population.

3.1.2 Kinetics of expression of cyclin E and thymidylate synthase in synchronized HCT116 cells

To test the cell cycle dependency of TS and the putative role of E2F transcription factors in its regulation, we compared the relative expression patterns of human TS and cyclin E mRNA and protein levels in HCT116 cells after release from serum-starvation. Cyclin E is a well characterized cell-cycle protein and is a direct transcriptional target of E2F activity. The cyclin E promoter contains a number of E2F consensus sites (Geng et al., 1996) and is known to be expressed in late G_1, at the time of E2F release (Halaban, 1999). In our experimental model, cyclin E mRNA rose sharply by 4 h post-stimulation and reached a maximum (4-fold above baseline) by 10-12 h (Fig. 3.2A). In contrast, changes in TS mRNA followed different kinetics; upregulation of TS mRNA occurred more slowly than cyclin E,

Figure 3.2 Time course of expression of TS and cyclin E in synchronized HCT116 cells. (**A**) Time dependent changes in cyclin E and TS mRNA. Cyclin E and TS mRNA levels were quantified by real-time PCR and normalized to levels of RPL32 mRNA. Data from three independent experiments are shown. Error bars represent the SEM. (**B**) Western blot analysis of cyclin E and TS. (**C**) Relative expression of cyclin E and TS protein levels as determined by densitometry of blots shown in (B). Expression levels were normalized to 0 time and to β-actin levels.


A
mRNA (fold change)
Time after release (h)
Cyclin E
TS
B
Time (h)
0 4 8 10 12 14 16 18 20 22 24 26 28 30
TS
Cyclin E
β-Actin
C
Relative Protein Expression
Time after release (h)
Cyclin E
TS

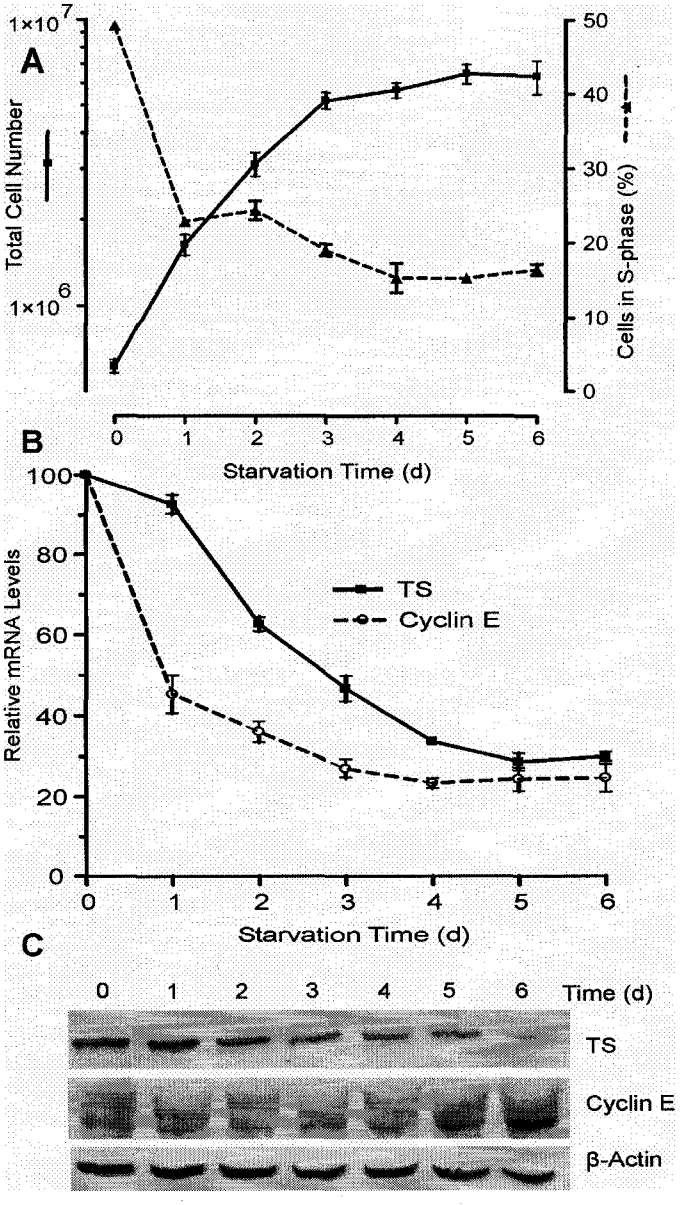
reaching its maximum only after 16-20 h. Cyclin E mRNA levels started to drop after 12 h whereas TS mRNA levels remained relatively constant.

Cyclin E and TS protein levels, as detected by western blot analysis, also followed different kinetics (Fig. 3.2B, 3.2C). The level of both proteins increased in parallel, shortly after re-feeding. Cyclin E increased continuously to a maximum between 14-18 h, decreasing thereafter. In contrast, TS increased gradually to a maximum at 16-18 h, remaining relatively constant thereafter. Thus, in this human colorectal cancer cell line synchronized by serum starvation and re-feeding, prominent upregulation of cyclin E and TS was seen in G_1, reaching a maximum in early S-phase. However, expression of the two genes differed significantly later in the cell cycle: cyclin E mRNA and protein decreased whereas TS did not. Furthermore, transcriptional activation of cyclin E, which is a prototypic E2F-regulated gene, clearly preceded the accumulation of TS mRNA, suggesting that TS expression is not closely dependent on E2F activity.

3.1.3 Kinetics of expression of cyclin E and thymidylate synthase in HCT116 cells during serum deprivation.

To complement our study of HCT116 cells released from synchronization by re-addition of serum, we also studied the same cells as they exited the cell cycle under conditions of serum deprivation. We followed TS and cyclin E levels periodically during the 6-day course of serum deprivation. During serum deprivation, cells continued to grow rapidly for the first 3 days; thereafter the growth rate decreased appreciably (Fig. 3.3A). The percentage of cells in S-phase, as measured by flow cytometry, decreased sharply after 24 h in low serum conditions and continued to decrease at a slower rate until day 4. Thereafter, the fraction of cells in

Figure 3.3 Time dependent changes in growth rate and expression of TS and cyclin E in HCT116 cells during starvation. (**A**) Cell number and percentage of cells in S-phase as a function of time. Viable cell number was determined by trypan blue exclusion. The percentage of cells in S-phase was determined by flow cytometry as in Fig. 1. The mean and SEM of three independent experiments is shown. (**B**) Time-dependent decrease in TS and cyclin E mRNA in starved HCT116 cells. TS and cyclin E mRNA levels were quantified by real-time PCR and normalized to levels of RPL32 mRNA. The mean and SEM of three independent experiments is shown. (**C**) Western blot analysis showing changes in TS and cyclin E during the course of starvation.


A
1×10^7
1×10^6
Total Cell Number
Cells in S-phase (%)
50
40
30
20
10
0
0 1 2 3 4 5 6
Starvation Time (d)
B
100
80
60
40
20
0
Relative mRNA Levels
TS
Cyclin E
0 1 2 3 4 5 6
Starvation Time (d)
C
0 1 2 3 4 5 6 Time (d)
TS
Cyclin E
β-Actin

S-phase remained at a plateau value of about 15% until day 6 (Fig. 3.3A). The expression of cyclin E and TS was measured during this same period. For TS, both mRNA and protein levels decreased about 5-fold almost linearly over the 6 day period (Fig. 3.3B, 3.3C). Cyclin E mRNA levels decreased more rapidly than TS mRNA, most evident at days 1 and 2, followed by a more gradual decrease. Interestingly, cyclin E mRNA was closely associated with the proportion of cells in S-phase, and decreased quite sharply in the first 24 h. Surprisingly, and in contrast with the results obtained in synchronized cells, changes in cyclin E protein levels did not correlate very well with the changes observed at the mRNA level. An increase in cyclin E protein levels was seen after 5 days. Since cyclin E mRNA levels were decreased by a factor of 4 to 5-fold at the same time point, we postulate that this discrepancy was due to impaired degradation of cyclin E under low serum conditions.

Taken together, these results demonstrate that time-dependent changes in TS and cyclin E levels differed significantly in synchronized cells as well as in cells exiting the cell cycle under low serum conditions. Therefore, these differences provide further evidence of differences in the regulatory mechanisms of these two genes.

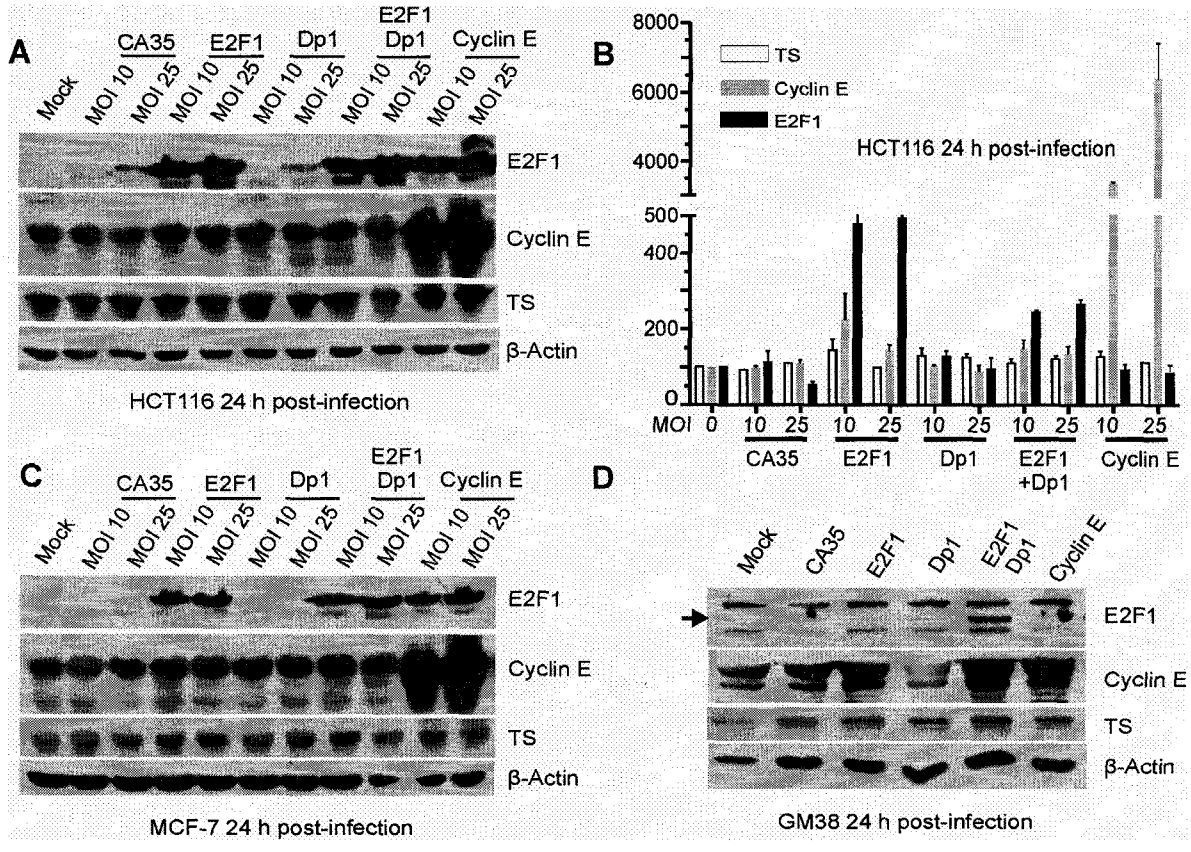
3.1.4 Ectopic expression of cyclin E and E2F1 does not induce TS expression in HCT116, MCF-7 or GM38 cells

Previous reports using mouse and rat embryonic fibroblasts showed that ectopic expression of E2F1 and E2F2 leads to an increase in TS transcripts (DeGregori et al., 1995; Ishida et al., 2001). To further characterize the relationship

between key effectors of G_1/S transition and the regulation of human TS transcription, we infected HCT116 and MCF-7 cells with recombinant adenoviruses expressing human E2F1, Dp1 and cyclin E. Western blot analysis revealed that, 24 h post-infection, both cyclin E and E2F1 were overexpressed in both cell lines (Fig. 3.4A, 3.4C). However, no significant changes were observed in TS levels following infection with any of the viruses. Real-time PCR analysis also showed a dose-dependent increase in E2F1 and cyclin E mRNA expression in infected HCT116 cells (Fig. 3.4B). E2F1 mRNA did not accumulate as much as cyclin E mRNA, likely because of the pro-apoptotic activity of E2F1. As expected, ectopic expression of E2F1 alone or E2F1 together with Dp1 (its binding partner and transcriptional co-activator), upregulated endogenous cyclin E mRNA by a factor of 2-3 fold, but little change was observed in endogenous TS mRNA (Fig. 3.4B).

The increase in cyclin E protein levels observed in HCT116 and MCF-7 cell lines infected with E2F1 and E2F1+ Dp1 was less than expected (Fig. 3.4A and 3.4C), possibly because these are rapidly proliferating cancer cell lines exhibiting a very short doubling time (less than 20 h). We therefore also infected a normal human fibroblast line, GM38, with the same adenoviruses. These cells, which have a longer doubling time (over 30 h), responded to ectopic expression of E2F1 and E2F1+Dp1 with a marked increase in cyclin E levels (Fig. 3.4D), characteristic of an increase in E2F-dependent transcription. However, despite the observed higher E2F1 activity, there was no significant increase in TS protein levels in this cell line either. In agreement with the results obtained with synchronized HCT116, these experiments provide direct evidence that neither E2F nor cyclin E affect TS expression in human cells. Our data is therefore in sharp contrast with data

Figure 3.4 Effect of adenovirus-mediated overexpression of E2F1, Dp1 and cyclin E on TS expression. (**A**) Western blot analysis of HCT116 cells infected for 24 h at MOIs of 10 and 25. (**B**) Relative mRNA expression levels of TS, cyclin E and E2F1 24 h after infection with adenoviruses encoding E2F1, Dp1 and Cyclin E. mRNA levels were normalized to β-2-microglobulin mRNA levels. The mean and SEM of three independent experiments is shown. (**C**) Western blot analysis of MCF-7 cells infected for 24 h at MOIs of 10 and 25. (**D**) Western blot analysis of GM38 cells infected for 24 h at a MOI of 25. Where shown, infection with CA35 adenovirus was used as a control.

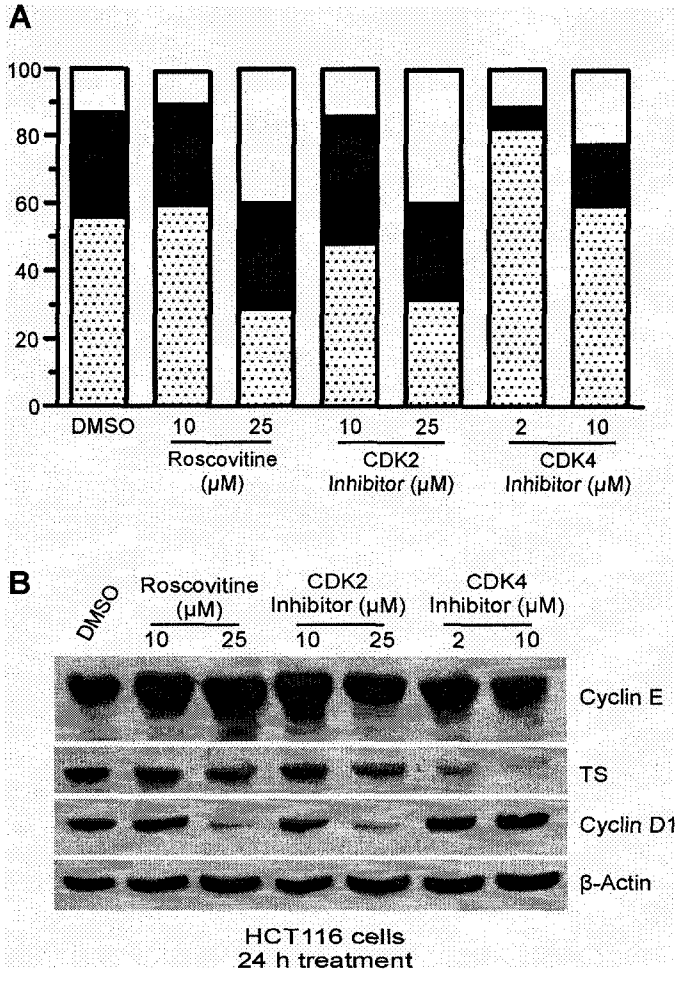

A
CA35
E2F1
Dp1
E2F1 Dp1
Cyclin E
Mock
MOI 10
MOI 25
E2F1
Cyclin E
TS
β-Actin
HCT116 24 h post-infection
B
TS
Cyclin E
E2F1
HCT116 24 h post-infection
8000
6000
4000
500
400
200
0
MOI 0 10 25 10 25 10 25 10 25 10 25
CA35
E2F1
Dp1
E2F1 +Dp1
Cyclin E
C
CA35
E2F1
Dp1
E2F1 Dp1
Cyclin E
Mock
MOI 10
MOI 25
E2F1
Cyclin E
TS
β-Actin
MCF-7 24 h post-infection
D
Mock
CA35
E2F1
Dp1
E2F1 Dp1
Cyclin E
E2F1
Cyclin E
TS
β-Actin
GM38 24 h post-infection

obtained from rodent models where E2F upregulate TS levels. This suggests that the regulatory mechanisms in rodent and human are more divergent than previously understood.

3.1.5 Effect of small molecule inhibitors of CDK4 and CDK2 on cell cycle progression and TS levels

Since we have shown that TS is upregulated during the G_1 phase in synchronized human cells independently of E2F activity, we sought to determine factors that affect TS during the G_1 phase. G_1 phase progression is controlled by two different types of cyclin-dependent kinases (CDKs), CDK4 and CDK2. To assess the role of these two CDKs in the regulation of the TS gene, we performed a series of experiments using small molecule inhibitors of CDK4 and CDK2. Roscovitine is a potent, broad-specificity inhibitor of cyclin-dependent kinases (CDKs), and has been shown to block cell-cycle progression both in G_1 and G_2 (Meijer et al., 1997). The effects of roscovitine and other more specific inhibitors of CDK2 and CDK4 on TS expression were tested on HCT116, MCF-7 and PC3 cells. Following treatment of randomly growing cells for 24 h with the various agents, expression levels of TS and cell cycle proteins were determined by western blot analysis and cell cycle distribution was measured by flow cytometry. As expected, treatment of HCT116 cells with roscovitine increased the G_2/M fraction and decreased the G_1 fraction (Fig. 3.5A). Treatment with CDK2 inhibitor yielded very similar results, with an increase in the number of cells in G_2/M. In contrast, treatment with the CDK4 inhibitor led to an accumulation of cells in G_0/G_1. Inhibition of CDK2 by roscovitine or CDK2 inhibitor was associated with a marked decrease in

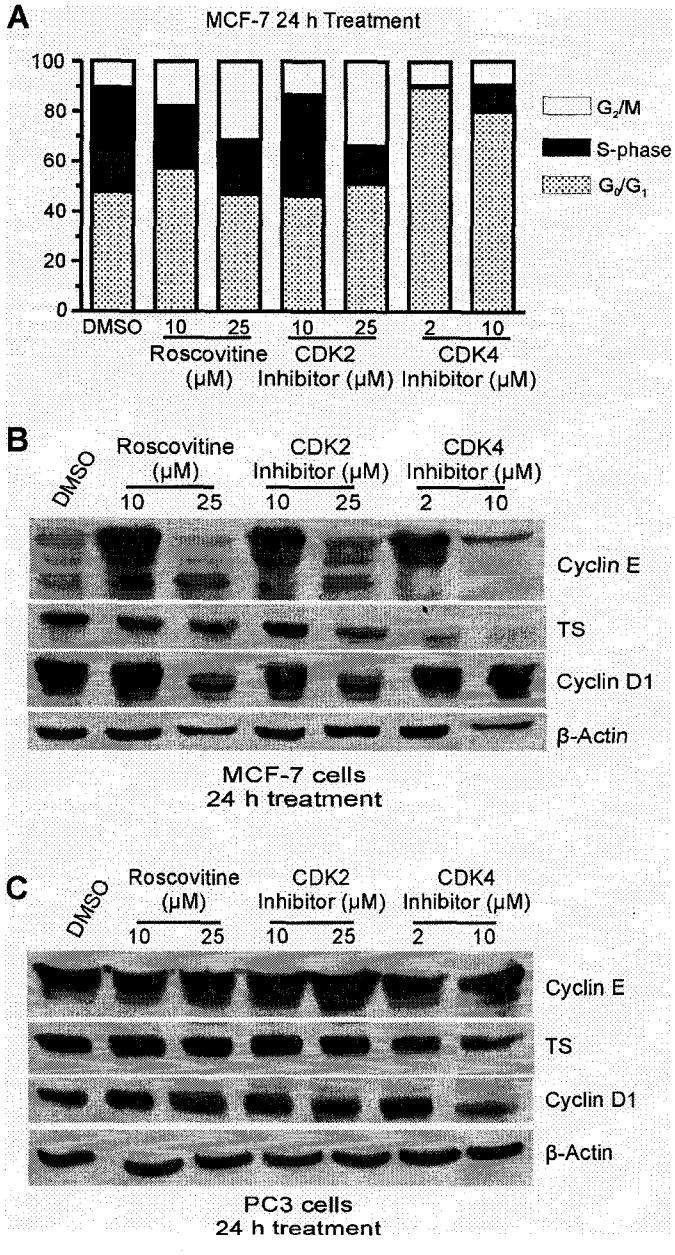
Figure 3.5 Effect of small molecule inhibitors of CDKs on HCT116 cells. (**A**) Cell-cycle distribution in HCT116 cells following 24 h treatment with the indicated inhibitor. Cell-cycle distribution was determined by flow cytometry as in Fig. 3.1. Percentage of cells in G_0/G_1 is represented by the speckled bar, S-phase by the black bar and G_2/M by the white. Data shown are the average of two independent experiments. (**B**) Western blot analysis of TS, cyclin E and cyclin D1 following 24 h treatment with solvent (DMSO), roscovitine, CDK2 inhibitor or CDK4 inhibitor of HCT116


A
100
80
60
40
20
0
DMSO
10
25
Roscovitine (μM)
10
25
CDK2 Inhibitor (μM)
2
10
CDK4 Inhibitor (μM)
B
DMSO
Roscovitine (μM)
10
25
CDK2 Inhibitor (μM)
10
25
CDK4 Inhibitor (μM)
2
10
Cyclin E
TS
Cyclin D1
β-Actin
HCT116 cells
24 h treatment

cyclin D1 protein levels in HCT116 (Fig. 3.5B), but there was no change in TS protein levels. A very different effect was seen following inhibition of CDK4, as treatment of HCT116 cells with the CDK4 inhibitor (even at the lowest concentration) led to a clear decrease in TS protein levels. Under the same conditions, no change in cyclin E or cyclin D levels was detected.

The changes in cell-cycle distribution in MCF-7 cells treated with the same inhibitors were very similar to results obtained with HCT116 cells. Inhibition of CDK2 by roscovitine or CDK2 inhibitor led to an accumulation of cells in G_2/M whereas inhibition of CDK4 blocked cells in G_1 (Fig.3.6A). Treatment of MCF-7 cells with roscovitine or CDK2 inhibitor was associated with a moderate decrease cyclin D1 (Fig. 3.6B), but no change in TS levels were observed. In contrast, treatment with the CDK4 inhibitor decreased cyclin E levels as well as TS levels. Treatment of PC3 cells with the same inhibitors had a very similar effect on cell-cycle progression as described for HCT116 and MCF-7 (data not shown). Similarly, inhibition of CDK2 had no effect on TS protein levels. However, once again, inhibition of CDK4 was associated with a decrease in TS expression (Fig.3.6C). In PC3 cells, neither cyclin E nor cyclin D1 levels were affected by any of the inhibitors. Taken together our data demonstrate that TS expression in human cells does not correlate with cyclin D or cyclin E expression levels. Our novel observation is that specific inhibition of CDK4 activity blocked cell-cycle progression in G_1 and led to a consistent decrease in TS expression in all the tested cell lines without affecting other cell-cycle dependent proteins in a consistent manner. These results strongly suggest that CDK4 activity is involved the regulation of TS expression, but that TS expression does not depend on the activity of cyclin E-CDK2 complexes.

Figure 3.6 Effect of small molecule inhibitors of CDKs on MCF-7 and PC3 cells. (**A**) Cell-cycle distribution in HCT116 cells following 24 h treatment with the indicated inhibitor. Cell-cycle distribution was determined by flow cytometry as in Fig. 3.1. Percentage of cells in G_0/G_1 is represented by the speckled bar, S-phase by the black bar and G_2/M by the white. Data shown are the average of two independent experiments. Western blot analysis of TS, cyclin E and cyclin D1 following 24 h treatment with solvent (DMSO), roscovitine, CDK2 inhibitor or CDK4 inhibitor MCF-7 (**B**) and PC3 (**C**) cells.

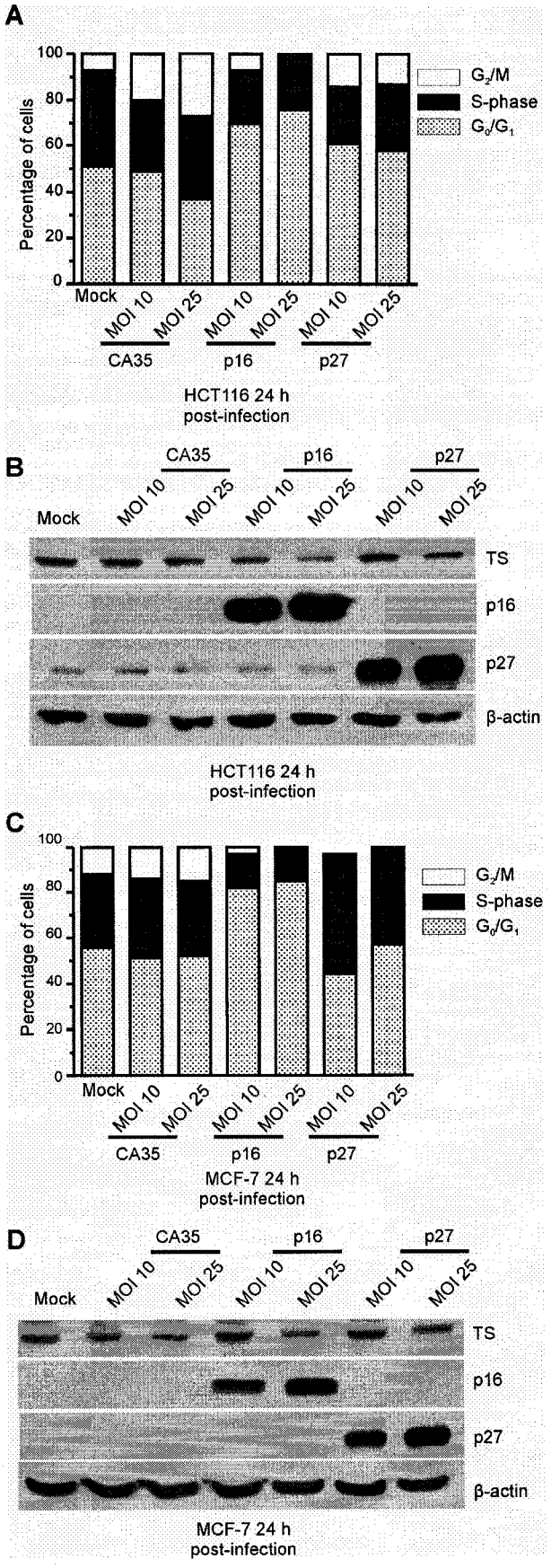

A
MCF-7 24 h Treatment
100
80
60
40
20
0
G2/M
S-phase
G0/G1
DMSO
10
25
10
25
2
10
Roscovitine (μM)
CDK2 Inhibitor (μM)
CDK4 Inhibitor (μM)
B
DMSO
Roscovitine (μM)
CDK2 Inhibitor (μM)
CDK4 Inhibitor (μM)
10
25
10
25
2
10
Cyclin E
TS
Cyclin D1
β-Actin
MCF-7 cells
24 h treatment
C
DMSO
Roscovitine (μM)
CDK2 Inhibitor (μM)
CDK4 Inhibitor (μM)
10
25
10
25
2
10
Cyclin E
TS
Cyclin D1
β-Actin
PC3 cells
24 h treatment

3.1.6 Effect of p16INK4A and p27KIP1 expression on thymidylate synthase levels in human cells

To provide an independent line of evidence about the role of CDK4 in the regulation of TS, we infected HCT116 and MCF-7 cells with adenoviruses encoding human p16INK4A and p27KIP1. p16INK4A is known to bind to CDK4 (and to CDK6) and inhibit their activities, blocking cells in G_1. p27KIP1 is an inhibitor of CDK2 that can also associate and inhibit cyclin D-CDK4/6 complexes. Since both p16INK4A and p27KIP1 are inhibitors of G_1 CDKs, infection of HCT116 with adenoviruses encoding p16INK4A and p27KIP1 led to an arrest of cells in G_0/G_1 (Fig. 3.7A). Upon ectopic expression of p16, 80% of the cells accumulated in G1. However, the extent of the G_1 block was less following expression of p27, with only 60% of the cells arrested in G_1. Western blot analysis of extracts from infected HCT116 showed a strong induction of p16 and p27 levels (Fig.3.7B). Consistent with the data from chemical inhibition of CDK4, the increase in p16 levels was associated with a dose-dependent decrease in endogenous TS protein levels. On the other hand, overexpression of p27 was associated with a less pronounced decrease in TS levels compared to p16INK4A. Overexpression of p16INK4A in MCF-7 cells led to an accumulation of cells in G_0/G_1 while overexpression of p27KIP1 triggered an increase in the S-phase fraction (Fig.3.7C) rather than a G_1 block. In this cell line, high p16 and p27 levels were both associated with a similar decrease in TS protein (Fig.3.7D).

Given the known ability of p16INK4A and p27KIP1 to block CDK4 activity, our data clearly demonstrate for the first time that CDK4 is required for the expression of

Figure 3.7 Effect of adenovirus-mediated overexpression of p16INK4A and p27KIP1 on TS expression and cell-cycle progression in MCF-7 and HCT116. (**A**) Cell-cycle distribution 24 h after infection of HCT116 cells with adenoviruses encoding Lac Z, p16 and p27 at MOIs of 10 or 25 . Cell-cycle distribution was determined by flow cytometry as in Fig. 3.1. Data shown are the average of two independent experiments. (**B**) Western blot analysis of HCT116 cells 24 h after infection with CA35, p16INK4A or p27KIP1 adenoviruses at MOIs of 10 or 25. (**C**) Cell-cycle distribution 24 h after infection of HCT116 cells with adenoviruses encoding Lac Z, p16 and p27 at MOIs of 10 or 25. Cell-cycle distribution was determined by flow cytometry as in Fig. 3.1. Data shown are the average of two independent experiments (**D**) Western blot analysis of MCF-7 cells 24 h after infection with CA35, p16INK4A or p27KIP1 adenoviruses at MOIs of 10 or 25.


A
Percentage of cells
100
80
60
40
20
0
G2/M
S-phase
G0/G1
Mock
MOI 10
MOI 25
MOI 10
MOI 25
MOI 10
MOI 25
CA35
p16
p27
HCT116 24 h
post-infection
B
CA35
p16
p27
Mock
MOI 10
MOI 25
MOI 10
MOI 25
MOI 10
MOI 25
TS
p16
p27
β-actin
HCT116 24 h
post-infection
C
Percentage of cells
100
80
60
40
20
0
G2/M
S-phase
G0/G1
Mock
MOI 10
MOI 25
MOI 10
MOI 25
MOI 10
MOI 25
CA35
p16
p27
MCF-7 24 h
post-infection
D
CA35
p16
p27
Mock
MOI 10
MOI 25
MOI 10
MOI 25
MOI 10
MOI 25
TS
p16
p27
β-actin
MCF-7 24 h
post-infection

TS during G_1. Specific inhibition of this cell cycle dependent kinase is associated with a decrease in TS expression. Since CDK4 activity is vital for a cell to progress through early G1, it suggests that TS upregulation in synchronized cells takes place in the same time frame (early mid G_1), when CDK4 is active.

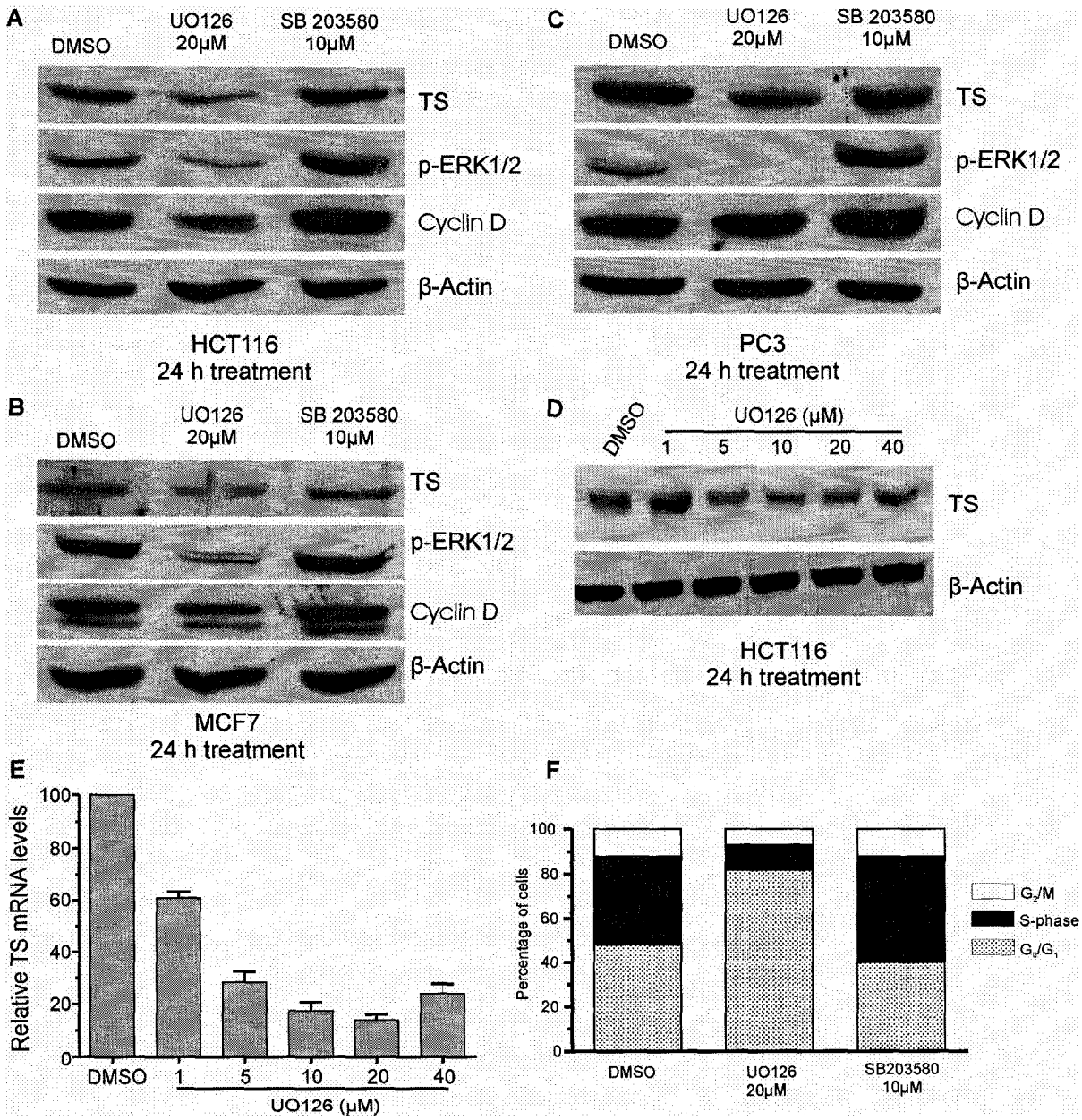
3.2 Regulation of human TS transcription is dependent on growth signaling pathways involving the MEK1 kinase

It is well established that the early part of G1 phase is dependent on the presence of growth factors (Blagosklonny and Pardee, 2002). The MAP kinase pathway is a prototypic signaling cascade that regulates cellular proliferation and survival and is well know to control the early part of G_1 (Massague, 2004). Activation of cell surface receptors following binding of growth factor molecules triggers sequential activation of Ras, Raf, MEK 1/2 and ERK 1 and 2 protein kinases, which activate a number of nuclear transcription factors that initiate a transcriptional cellular response to the stimuli. The MAP kinase pathway is known to be critical for G_1 progression and it has been shown to be in part responsible for the accumulation of cyclin D upon stimulation of cells (Lavoie et al., 1996; Torii et al., 2006). Having established that TS expression is not dependent on S-phase but rather on events taking place early during the G_1 phase, we performed experiments to determine if TS could be regulated by growth factor signaling. To test our hypothesis, we inhibited the MAP kinase pathway by treatment of cells with UO126, a small molecule inhibitor of MEK1 and 2, and tested its effect on TS expression.

3.2.1 Inhibition of MEK1/2 by UO126 is associated with a decrease in TS levels.

HCT116, MCF-7 and PC3 cells were treated for 24 h with UO126, a pharmacological inhibitor of MEK1&2, and SB203580, a pharmacological inhibitor of p38. As expected, treatment with 20 μM UO126 for 24 h significantly decreased phosphorylation of ERK1&2 in all three cell lines (Fig. 3.8A, 3.8B, 3.8C). Treatment

Figure 3.8 Inhibition of MEK 1/2 by UO126 decreases TS protein and mRNA levels in human cell lines. Western blot analysis of cellular extracts treated for 24 h with DMSO alone, 20μM UO126 or 10μM SB203580 **(A)** HCT116, **(B)** MCF-7, **(C)** PC3 cells. **(D)** Western blot analysis of HCT116 extracts following 24 h treatment with increasing concentrations of UO126. **(E)** Real time PCR quantification of TS mRNA levels following treatment with various concentrations of UO126. TS mRNA levels were normalized to β-2-microglobulin levels which were used as a reference. Mean and SEM from three independent experiment shown here. **(F)** Cell cycle distribution following 24 h treatment of HCT116 cells with DMSO, 20μM UO126 or 10μM SB203580. Cells were stained with PI (propidium iodide) and analyzed by flowcytometry. Percentage of cells in each of the cell-cycle phases was determined by Modfit analysis. Data shown here are the mean and SEM of three independent experiments.


A
DMSO
UO126 20μM
SB 203580 10μM
TS
p-ERK1/2
Cyclin D
β-Actin
HCT116
24 h treatment
C
DMSO
UO126 20μM
SB 203580 10μM
TS
p-ERK1/2
Cyclin D
β-Actin
PC3
24 h treatment
B
DMSO
UO126 20μM
SB 203580 10μM
TS
p-ERK1/2
Cyclin D
β-Actin
MCF7
24 h treatment
D
DMSO
UO126 (μM)
1 5 10 20 40
TS
β-Actin
HCT116
24 h treatment
E
Relative TS mRNA levels
100
80
60
40
20
0
DMSO 1 5 10 20 40
UO126 (μM)
F
Percentage of cells
100
80
60
40
20
0
DMSO
UO126 20μM
SB203580 10μM
G2/M
S-phase
G0/G1

of these three cell lines with UO126 was also associated with a clear decrease in TS protein levels. Treatment with SB203580 or DMSO, on the other hand, had no significant effect on ERK phosphorylation status or on TS levels. As previously reported, inhibition of MEK 1/2 produced a decrease in cyclin D levels in HCT116 and MCF-7 cells treated with UO126. Cyclin D levels were unchanged in PC3 cells following treatment with UO126. Flow cytometry analysis showed that, under the same conditions, UO126 blocked cell cycle progression and lead to the accumulation of HCT116 cells in G_0/G_1 of (Fig. 3.8F); the same effect was observed for MCF-7 cells (data not shown). To address the specificity of UO126, we also performed a dose response treatment of HCT116 cells to see if lower concentrations of the inhibitor would still affect phosphorylation of ERK 1/2 and TS expression. UO126 concentrations as low as 5 μM were able to decrease the expression of TS protein as efficiently as higher concentrations of the drug (Fig 3.8D).

Having established that chemical inhibition of the MAP kinase pathway led to a decrease in TS protein levels, we next sought to determine if the observed decrease in TS protein levels was associated with a decrease in TS mRNA. We performed quantitative PCR analysis of the TS mRNA following treatment with increasing concentrations of UO126. Inhibition of the MAP kinase pathway was associated with a decrease in TS mRNA levels up to a factor of 5-fold (Fig.3.8E). Our data demonstrated that pharmacological inhibition of MEK 1/2 by UO126 was associated with decreased TS levels expression. To the best of our knowledge, this is the first evidence that TS expression is regulated by the MAP kinase pathway.

3.2.2 MEK1 kinase regulates human TS transcription

Since MEK activity seems to correlate with TS mRNA and protein levels, it seemed probable that the upstream MAP kinase pathway controlled TS expression at the transcriptional level. To test the effect of MEK activity more directly on TS transcriptional rate, the full length TS promoter corresponding to nucleotides – 472 bp to + 10 bp (relative to the start codon) was cloned into the pGL2 basic luciferase reporter construct. HCT116 cells were transfected with this TS reporter construct for 24 h and then treated for 24 h with the indicated concentrations of UO126. Our results showed that inhibition of MEK 1&2 with UO126 was associated with a decrease in TS transcriptional activity (Fig. 3.9A). To confirm these results, we also performed co-transfection experiments of the full length TS reporter construct along with either wild-type MEK1 or MEK1 R4F, a constitutively active form of the kinase. Co-transfection of the TS reporter construct with MEK1 R4F lead to 8 to 10-fold increase in the rate of TS transcription compared to wild-type MEK1 (Fig.3.9B). These results demonstrate that MEK 1/2 kinases are involved in the transcriptional regulation of the TS gene.

3.2.3 siRNA knock-down of MEK1 but not MEK2 decrease TS levels, independent of ERK 2.

To further study the relationship between MEK 1/2 kinases and TS, we also performed siRNA experiments to specifically knock-down MEK1 or MEK2 mRNAs. HCT116 and MCF-7 cells were transfected with increasing concentrations of a siRNA targeting MEK1 mRNA. 48 h post-transfection, MEK1 levels were decreased significantly in both cell lines (Fig. 3.10A, 3.10B) and the decrease in MEK1 level was clearly associated with lower TS levels. In contrast, specific knock-

Figure 3.9 MEK1 regulates the transcription of the human TS gene.
(A) Relative luciferase activity of the full length TS promoter following 24 h treatment with increasing concentrations of UO126. Cells were co-transfected with pGL2B-TS and pSV-β-gal and incubated for 24 h before addition of the drug. Data shown here is the mean and SEM of three independent experiments. Luciferase activity was normalized to β-galactosidase activity used as a transient transfection efficiency control. **(B)** Relative luciferase activity of the full length TS promoter in HCT116 cells 48 h post-transfection with wild-type MEK1 or MEK1 R4F. Data shown here is the mean and SEM of three independent experiments. Luciferase activity was normalized to β-galactosidase activity used as a transient transfection efficiency control.

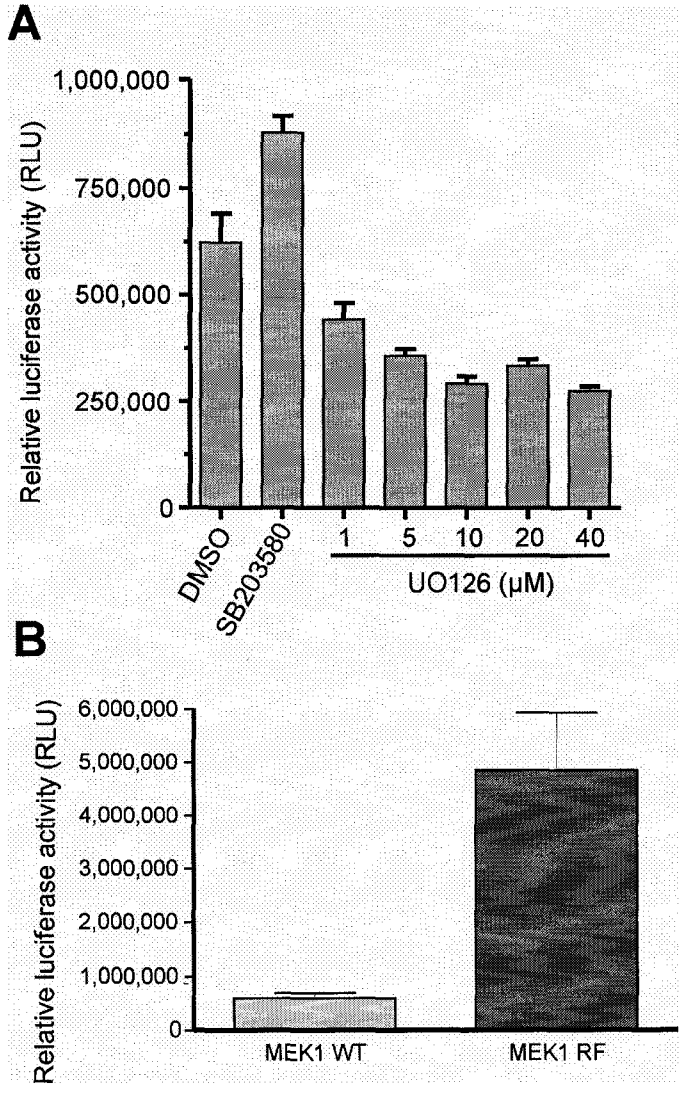
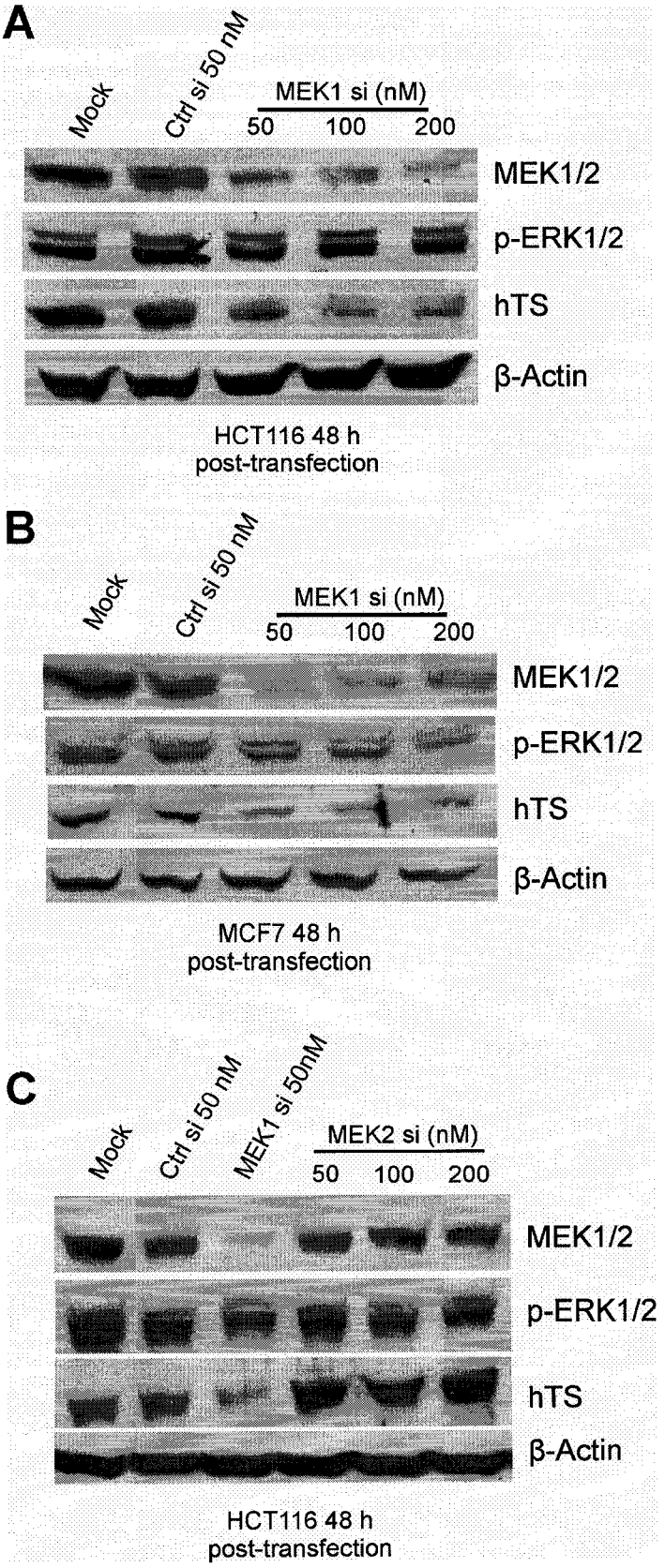

A
1,000,000
750,000
500,000
250,000
0
Relative luciferase activity (RLU)
DMSO
SB203580
1
5
10
20
40
UO126 (μM)
B
6,000,000
5,000,000
4,000,000
3,000,000
2,000,000
1,000,000
0
Relative luciferase activity (RLU)
MEK1 WT
MEK1 RF

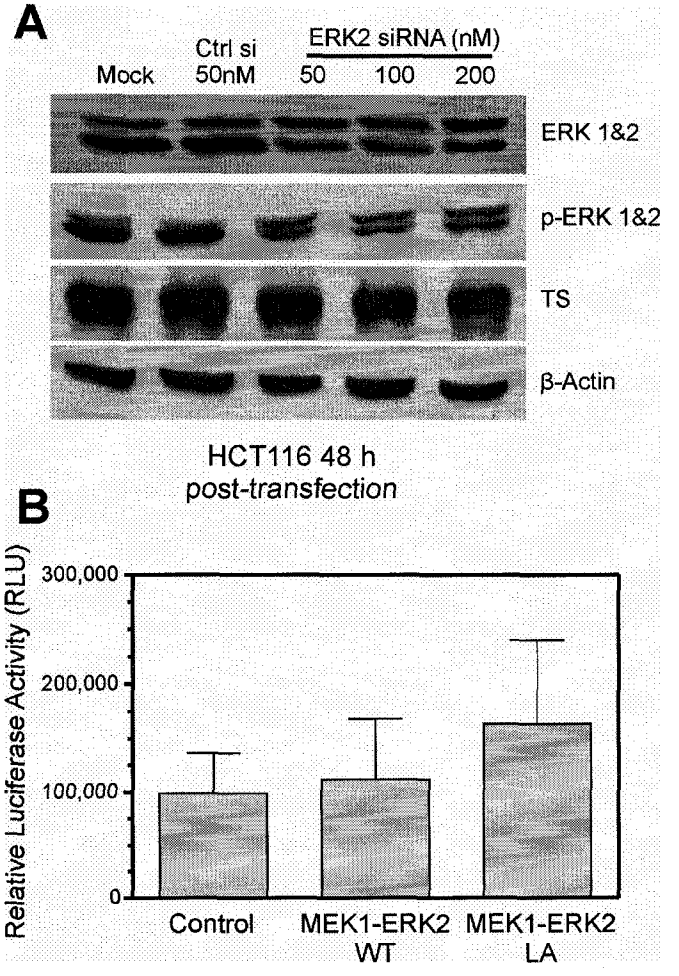
Figure 3.10 Knock-down of MEK1 but not MEK2 decrease TS levels in human cells. (A) Western blot analysis of HCT116 cells transfected for 48 h with increasing concentrations of a MEK1 specific siRNA. **(B)** Western blot analysis of MCF-7 cells transfected for 48 h with increasing concentrations of a MEK1 specific siRNA. **(C)** Western blot analysis of HCT116 cells transfected for 48 h with 50 nM of a MEK1 specific siRNA or increasing concentrations of a MEK2 specific siRNA.


A
Mock
Ctrl si 50 nM
MEK1 si (nM)
50
100
200
MEK1/2
p-ERK1/2
hTS
β-Actin
HCT116 48 h
post-transfection
B
Mock
Ctrl si 50 nM
MEK1 si (nM)
50
100
200
MEK1/2
p-ERK1/2
hTS
β-Actin
MCF7 48 h
post-transfection
C
Mock
Ctrl si 50 nM
MEK1 si 50nM
MEK2 si (nM)
50
100
200
MEK1/2
p-ERK1/2
hTS
β-Actin
HCT116 48 h
post-transfection

down of MEK2 mRNA in HCT116 cells for 48 h did not decrease TS levels but rather seemed to slightly increase TS expression (Fig. 3.10C). Surprisingly, despite the fact that MEK1 levels were decreased by a factor of 4 to 5-fold following siRNA treatment, phosphorylation of ERK 1&2, its downstream targets, were not affected. On the other hand, lower MEK1 levels led to an important decrease in TS expression.

Since ERK 1 and 2 are usually considered to be the downstream targets of MEK 1 and 2, we also tested the role of ERK 1/2 in the regulation of TS. Since ERK 2 is the main isoform expressed in HCT116 cells, we designed a siRNA targeting ERK2 mRNA. 48 h post-transfection in HCT116 cells, we could observe a three to four fold decrease in total ERK 2 as well as phosphorylated ERK 2 (Fig. 3.11A). However, despite the observed decrease in both ERK 2 expression and activity, there was no significant change in TS protein levels. These results therefore suggest that the MEK1-dependent increase of TS expression was independent of ERK 2 activity. To confirm this observation, we performed luciferase assays following co-transfection of the TS luciferase reporter plasmid together with plasmids encoding constitutively active forms of ERK 2 kinase. In this plasmid, the ERK2 protein is fused to its upstream activator, MEK1, and is constantly activated even in the absence of extracellular signals (Robinson et al., 1998). However, this fusion protein can only localize to the cytoplasm because it contains a strong MEK1 nuclear export signal (NES). The MEK1-ERK2 LA fusion protein lacks this NES and can remain in the nucleus where ERK activity is essential to trigger transcription of target genes. Co-transfection of the TS reporter plasmid with the nuclear MEK1-ERK2

Figure 3.11 TS expression is independent of ERK 2 activity.
(A) Western blot analysis of HCT116 cells transfected for 48 h with increasing concentrations of an ERK 2 specific siRNA. **(B)** Relative luciferase activity of the full length TS promoter in HCT116 cells 48 h post-transfection with the cytoplasmic MEK1-ERK2 or the nuclear MEK1-ERK2 LA fusion proteins. Data shown here is the mean and SEM of three independent experiments. Luciferase activity was normalized to β-galactosidase activity used as a transient transfection efficiency control.

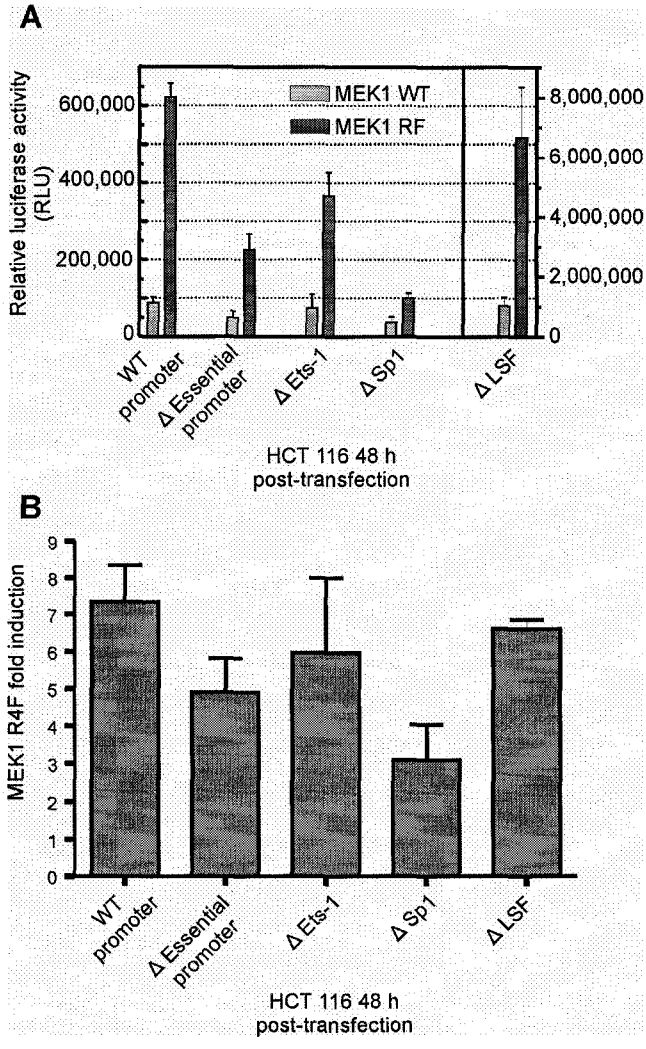

A
Mock
Ctrl si 50nM
ERK2 siRNA (nM)
50
100
200
ERK 1&2
p-ERK 1&2
TS
β-Actin
HCT116 48 h post-transfection
B
Relative Luciferase Activity (RLU)
300,000
200,000
100,000
0
Control
MEK1-ERK2 WT
MEK1-ERK2 LA

fusion increased luciferase activity by less than 1.5 fold compared to the cytoplasmic MEK1-ERK2 fusion (Fig. 3.11B). The extent of the TS promoter activation by ERK2 was far less than observed with the constitutively active MEK1 kinase. This suggests that MEK1 regulates the transcription of TS gene in an ERK2-independent fashion.

3.2.4 The Sp1 site in the essential TS promoter is required for the MEK1-dependent activation of TS

In order to map and identify the sequences and transcription factor binding sites responsible for the observed increase in TS promoter activity following expression of a constitutively active form of MEK1, we designed a series of promoter deletions (see Fig. 1.4). Based on previous reports showing the importance of the TS essential promoter region, we first engineered a series of constructs where the consensus binding sites for LSF, Ets-1 and Sp1 were deleted or where the whole essential promoter region was missing. As previously reported, deletion of the whole essential promoter region as well as deletion of Ets-1 and Sp1 binding sites led to a drop in basal activity (Fig. 3.12A) by a factor of 3 to 4-fold. In sharp contrast to previously reported data, deletion of the LSF consensus site led to a 10-fold *increase* in basal activity, suggesting that this region contains a strong negative element. To determine which of these sites might be responsible for the MEK1-dependent increase in TS expression, we also measured the ability of MEK1 R4F to transactivate these various constructs. The promoter constructs lacking the Ets-1 or the LSF sites exhibited a large increase in luciferase activity upon transfection with a constitutively active form of MEK1 and retained the ability to be transactivated by

Figure 3.12 Deletion of the Sp1 binding site in the TS essential promoter region impairs MEK1-dependent TS transcriptional activation.
(A) Relative luciferase activity of the promoter constructs where various sites in the essential promoter have been deleted in HCT116 cells 48 h post-transfection with the wild-type MEK1 or the constitutively active form of MEK1. Data shown here is the mean and SEM of three independent experiments. Luciferase activity was normalized to β-galactosidase activity used as a transient transfection efficiency control. Note the different scale between the left and right panels. **(B)** TS promoter fold induction following co-transfection with MEK1 R4F as compared to co-transfection with wild-type MEK1. Ratios were calculated from the data shown in (A).


A
Relative luciferase activity (RLU)
600,000
400,000
200,000
0
8,000,000
6,000,000
4,000,000
2,000,000
0
MEK1 WT
MEK1 RF
WT promoter
Δ Essential promoter
Δ Ets-1
Δ Sp1
Δ LSF
HCT 116 48 h post-transfection
B
MEK1 R4F fold induction
9
8
7
6
5
4
3
2
1
0
WT promoter
Δ Essential promoter
Δ Ets-1
Δ Sp1
Δ LSF
HCT 116 48 h post-transfection

MEK1 R4F (Fig 3.12B). However, deletion of the whole essential promoter region or deletion of the Sp1 binding site partially impaired the ability of MEK1 kinase to stimulate the transcription of the TS promoter. Importantly, since deletion of the Sp1 site only partially decreased MEK1 responsiveness, our data suggested that other site (s) within the TS promoter might also be responsive to MEK1 activity.

3.2.5 Sequences near the translational start of the TS gene are partially responsible for MEK1 dependent activation of the TS promoter.

To try to determine whether other MEK1 responsive elements are located in the TS promoter, we also engineered a number of promoter deletion constructs where regions flanking the 5' and 3'-end of the essential promoter were deleted (See Fig. 3.13). Deletion of the region flanking the 3'-end of the essential promoter led to an increase in the promoter basal activity (Fig. 3.14A). Similarly, deletion of the region flanking the 5'-end of the essential promoter (-132 bp/ +10 bp) led to an increase in basal activity, suggesting the presence of multiple negative elements in the regions flanking the essential promoter. Luciferase assays showed that co-transfection of each of these constructs together with the constitutively active form of MEK1 produced a transcriptional increase in both the -472 bp/-123 bp and the -132 bp/+10 bp promoter fragments (Fig 3.14B). However, the -472 bp/-123 bp construct was 3 to 4-fold less responsive to MEK1 R4F than the full length promoter, and 2-fold less responsive than the promoter fragment lacking the 5' upstream region. Our results seem to indicate that both the 5' and the 3' flanking regions of the promoter contains sites MEK1- responsive elements, but the region at the 3' end of the

Figure 3.13 Schematic representation of the various promoter deletions constructs engineered in this study. The full-length 500 bp human TS promoter is shown in blue and the important regions containing the tandem repeats, the inverted repeat and the essential promoter are indicated.

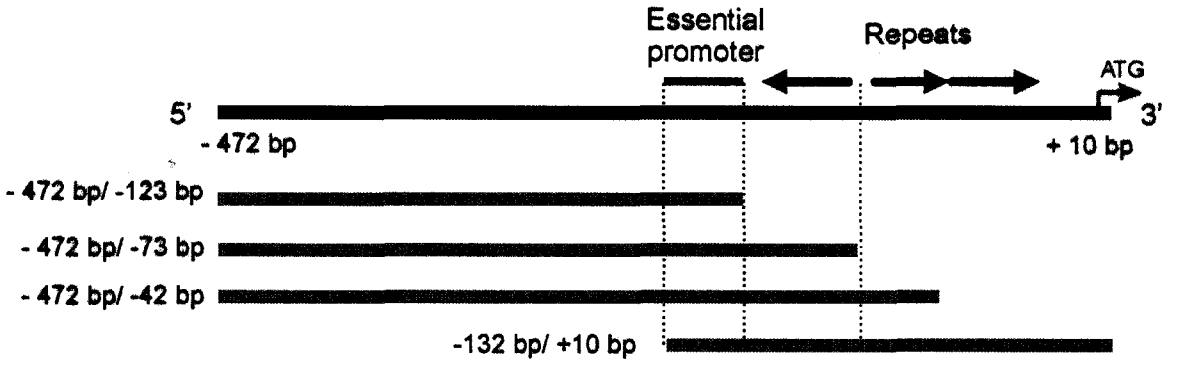
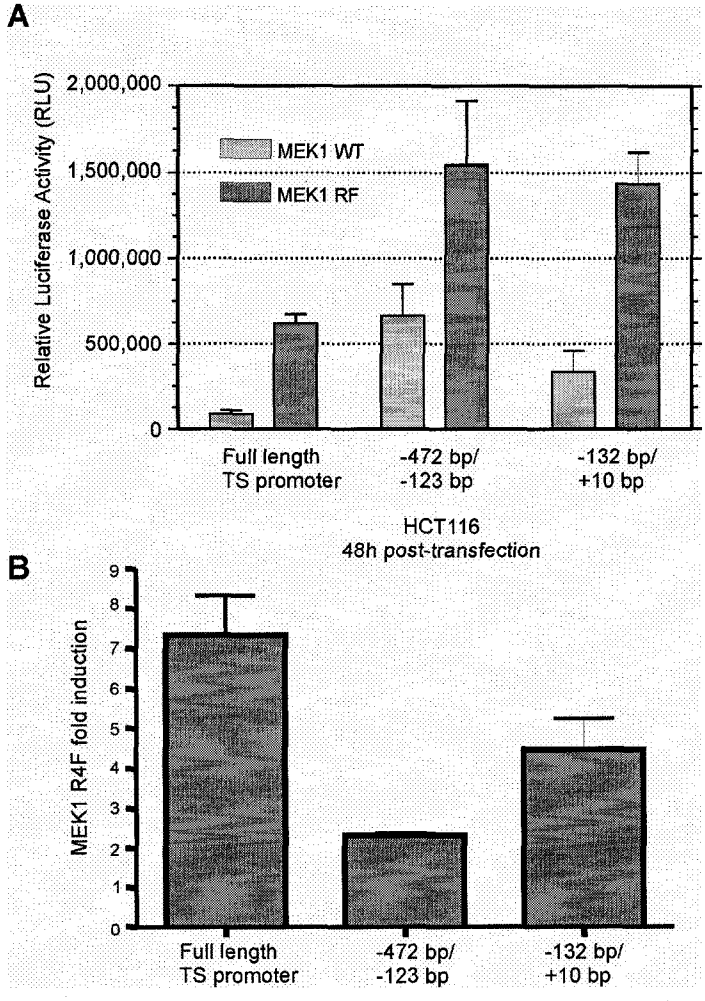
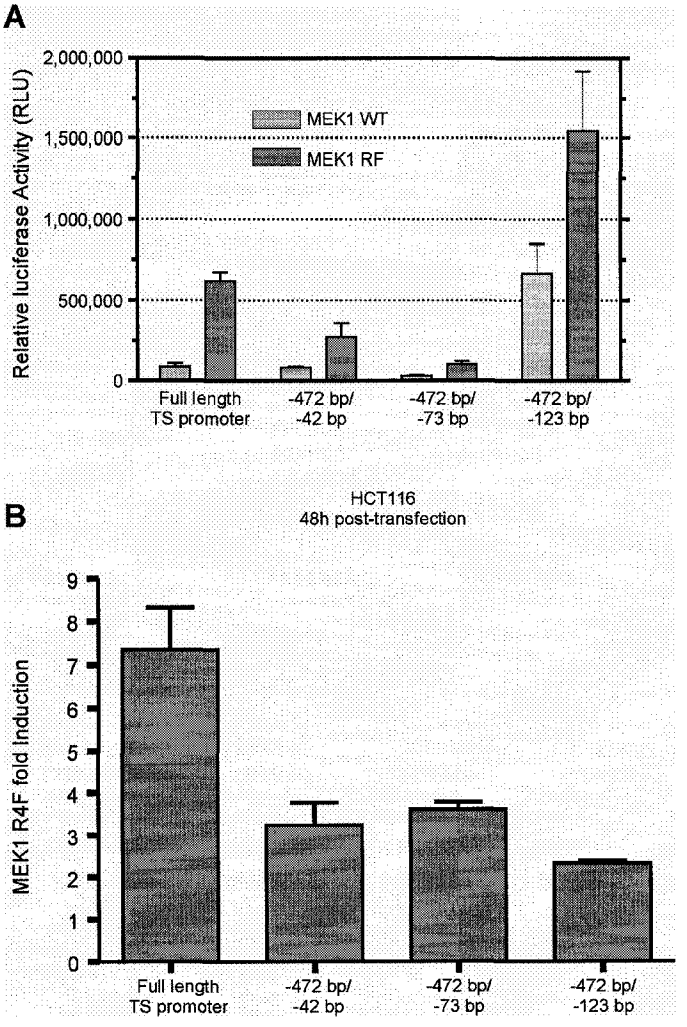

Essential
promoter
Repeats
ATG
5'
3'
- 472 bp
+ 10 bp
- 472 bp/ -123 bp
- 472 bp/ -73 bp
- 472 bp/ -42 bp
-132 bp/ +10 bp

Figure 3.14 Effect of deletion of the sequences flanking the 3' and the 5' end of the promoter on MEK1 responsiveness. (A) (A) Relative luciferase activity of the promoter deletions constructs in HCT116 cells 48 h post-transfection with the wild-type MEK1 or the constitutively active form of MEK1. Data shown here is the mean and SEM of three independent experiments. Luciferase activity was normalized to β-galactosidase activity used as a transient transfection efficiency control. **(B)** TS promoter fold induction following co-transfection with MEK1 R4F as compared to co-transfection with wild-type MEK1. Ratios were calculated from the data shown in (A).


A
Relative Luciferase Activity (RLU)
2,000,000
1,500,000
1,000,000
500,000
0
MEK1 WT
MEK1 RF
Full length
TS promoter
-472 bp/
-123 bp
-132 bp/
+10 bp
HCT116
48h post-transfection
B
MEK1 R4F fold induction
9
8
7
6
5
4
3
2
1
0
Full length
TS promoter
-472 bp/
-123 bp
-132 bp/
+10 bp

essential promoter contains a site that, when absent, leads to a more marked decrease in MEK1 responsiveness. The region of the TS gene flanking the 3' end of the essential promoter has been shown to contain two or more direct 28 bp repeats and one inverted repeat that can affect both promoter transcription and translation (Mandola et al., 2003; Takeishi et al., 1989). In order to try to identify the MEK1 responsive site in the 3'-end of the promoter, we performed another series of promoter deletions, where the various repeats where progressively deleted. Deletion of the first direct repeat along with the translational start of the TS gene (-472 bp/-42 bp) had no influence on the promoter basal activity (Fig 3.15A). Deletion of the second direct repeat (-472 bp/ -73bp construct) led to a decrease in the basal promoter activity. This is consistent with the presence of a previously described USF site found in this repeat. Deletion of sequences upstream of the second direct repeat (-472 bp/ -123 bp) led to an increase in promoter activity, indicating that this region contains negative elements. Since this region contains the 28 bp inverted repeat, our data indicate that this inverted repeat, unlike the first direct repeat, contains elements that negatively control TS expression. Comparison of promoter activity of these constructs following co-transfection with MEK1 R4F, demonstrated that all of these constructs retained their ability to be transactivated by MEK1 (Fig 3.15A, 3.15B). However, MEK1 R4F only partially increased the transcription these three constructs when compared to the full length promoter. Deletion of the region containing the first direct repeat and the TS translational start significantly decreased the ability of MEK1 to increase the promoter transcription rate. Therefore, we conclude that a MEK1-responsive site is located in the 3'-end of the promoter within these 40 nucleotides. We have shown that the human TS promoter has multiple

Figure 3.15 Effect of deletion of the direct and inverted repeats found in the 3' end of the TS promoter on MEK1 responsiveness. (A) Relative luciferase activity of the promoter deletions constructs in HCT116 cells 48 h post-transfection with the wild-type MEK1 or the constitutively active form of MEK1. Data shown here is the mean and SEM of three independent experiments. Luciferase activity was normalized to β-galactosidase activity used as a transient transfection efficiency control. **(B)** TS promoter fold induction following co-transfection with MEK1 R4F as compared to co-transfection with wild-type MEK1. Ratios were calculated from the data shown in (A).


A
2,000,000
1,500,000
1,000,000
500,000
0
Relative luciferase Activity (RLU)
MEK1 WT
MEK1 RF
Full length TS promoter
-472 bp/ -42 bp
-472 bp/ -73 bp
-472 bp/ -123 bp
B
HCT116
48h post-transfection
MEK1 R4F fold induction
9
8
7
6
5
4
3
2
1
0
Full length TS promoter
-472 bp/ -42 bp
-472 bp/ -73 bp
-472 bp/ -123 bp

MEK1-responsive sites; one of these is the Sp1 site located in the essential promoter region and the other is found in the 40 bp region just upstream of the TS gene translational start site.

3.3 Role of LSF and GABP transcription factors in the transcriptional regulation of human TS

LSF, Sp1 and GABP transcription factors have all been previously shown to play an important role in the regulation of the TS gene expression in murine cells. The ets transcription factor, GABP, along with the Sp1 transcription factor was reported to control the transcription of the mouse TS gene in a synergistic fashion (Rudge and Johnson, 2002). LSF was shown to be critical for S-phase dependent induction of TS in NIH3T3 cells and was thus proposed to be a transcription factor involved in G_1/S transition (Powell et al., 2000). However, since most of these studies were performed in rodent cells, we decided to determine if LSF and GABP transcription factors also play a central role in the regulation of human TS expression.

3.3.1 Effect of inhibition of MEK1/2 by UO126 on LSF expression pattern as determined by 2D SDS PAGE

Our data (section 3.2.1) indicated that pharmacological inhibition of MEK1/2 was associated with a decrease in TS levels. Based on previous evidence showing that LSF is a downstream target of the MAP kinase signalling pathway in human lymphocytes (Volker et al., 1997), we identified LSF as a likely downstream target of MEK1 activity. To determine if MEK1 activity could affect LSF phosphorylation status, we performed 2D electrophoresis of HCT116 cell extracts treated for 2 h with DMSO, 20 μM UO126 or 10 μM SB203580. The LSF transcription factor has a predicted isoelectric point of 5.8. In DMSO or SB203580 treated cells, following 2D SDS PAGE separation of the protein extract, the LSF specific antibody recognized

two different series of peptides: the first signal was found in the region between pH 5 and 6 at an approximate molecular weight of 65 KDa, whereas the second was detected as three distinct dots around pH 9 at an approximate molecular weight of 70 kDa (Fig. 3.16). In contrast, treatment of HCT116 cells with 20 μM UO126 for 2 h dramatically changed the pattern of LSF expression on 2D gels. The intensity of the three spots picked up in the region corresponding to pH 9 was largely decreased, and was comcomittant with an increase in signal intensity in the region corresponding to pH 6. Theoretically, phosphorylation of amino acid residues in a polypeptidide is associated with a decrease of the isoelectric point of the protein. Based on the 2D expression pattern of LSF, our data suggested that the signal detected in the pH 9 range was the unphosphorylated form of the protein, which was inconsistent with the predicted pI of the LSF transcription factor. However, further 2D experiments showed that a similar expression pattern of LSF was observed in a number of human cell lines (data not shown). To determine if the two signals detected by the LSF antibody on 2D gels were both specific, we performed siRNA knock-down of LSF mRNA in HCT116. 48 h post-transfection with 50 nM of a control siRNA or a siRNA targeting LSF, extracts were resolved by 2D electrophoresis and probed for LSF. Extracts from cells transfected with the control siRNA exhibited the typical LSF expression pattern with two distinct regions (Fig. 3.17). However, extracts from cells treated with the LSF siRNA showed a marked decrease in the intensity of the signal around pH 6, with no observable change in the intensity of the signal in the pH 9 range. We conclude from this experiment that the signal recognized by the LSF antibody in the high pH range was likely due to non-specific detection of a different protein.

Figure 3.16 Expression pattern of LSF on 2D SDS PAGE following treatment with DMSO, UO126 and SB203580. HCT116 cells were treated for 2 h with DMSO, 20 μM UO126 or 10 μM SB203580. pH 3-10 IPG strips were rehydrated with 125 μg of extract and samples were resolved by 2D SDS PAGE. Following transfer on PVDF, membranes were probed with an anti-LSF antibody (BD Biosciences)

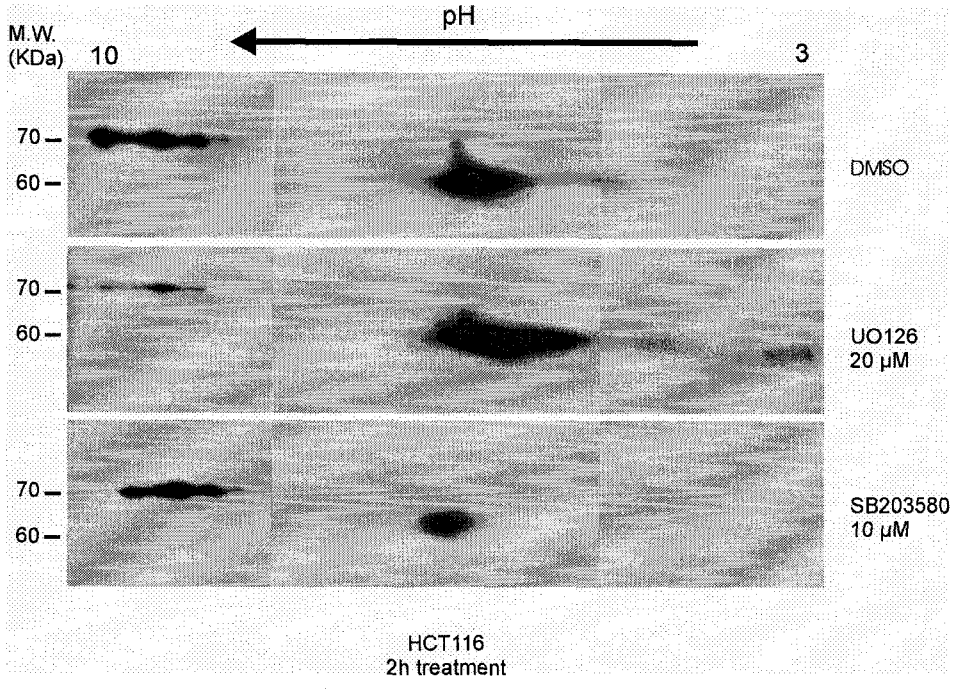
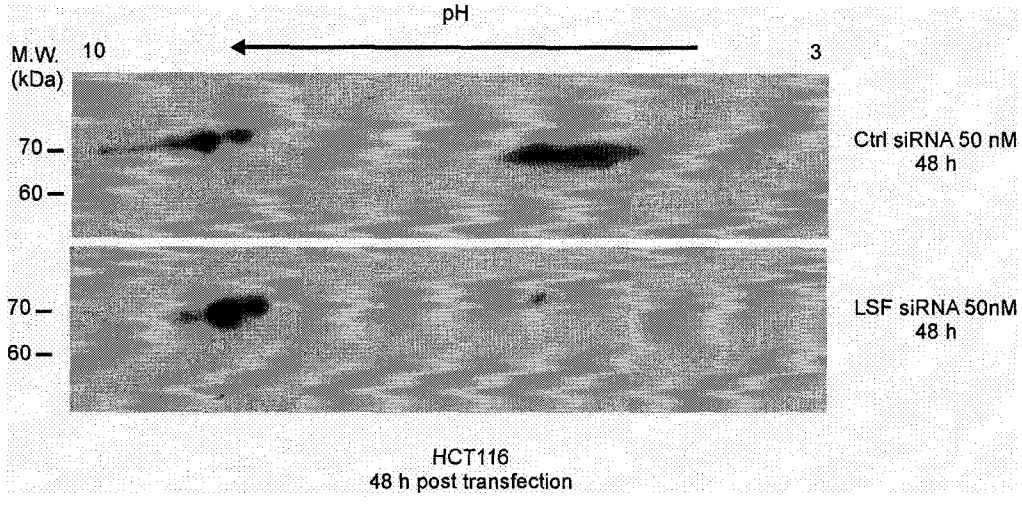

pH
M.W.
(KDa)
10
3
70
60
DMSO
70
60
UO126
20 μM
70
60
SB203580
10 μM
HCT116
2h treatment

Figure 3.17 LSF 2D SDS PAGE expression pattern following LSF mRNA knock-down. HCT116 cells were treated transfected for 48 h with 50nM of a control non-targeting siRNA or 50nM of a siRNA targeting LSF messenger RNA. 125 µg of extracts were rehydrated on pH 3-10 IPG strips and samples were resolved by 2D SDS PAGE. Following transfer on PVDF, membranes were probed with an anti-LSF antibody (BD Biosciences).

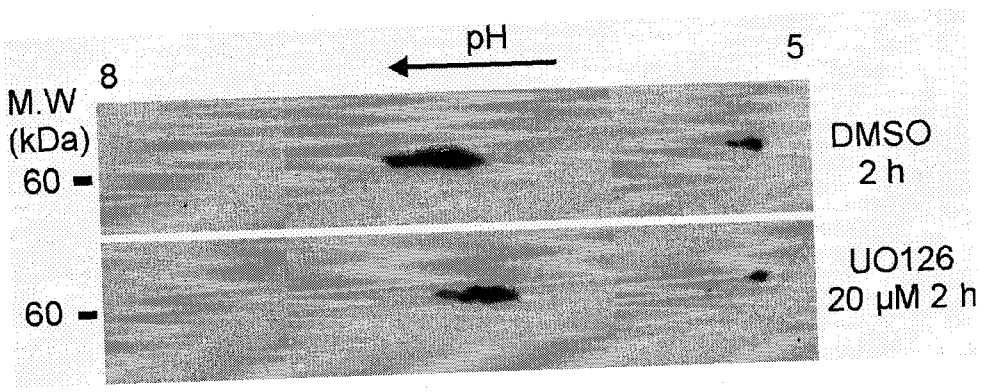

pH
M.W.
(kDa)
10
3
70
60
Ctrl siRNA 50 nM
48 h
70
60
LSF siRNA 50nM
48 h
HCT116
48 h post transfection

To determine if MEK1/2 activity affects the phosphorylation status of LSF, we also performed 2D electrophoresis of HCT116 cells extracts treated with DMSO or with UO126 on pH 5-8 IPG strips. The use of IPG strips with a narrower pH range gave a much better resolution of the LSF peptides found in the pH 6 region. In extracts from DMSO treated cells, we could distinguish a total of about 5 spots in the region corresponding to pH 6 as well as a unique spot around pH 5.5 (Fig.3.18). This pattern is characteristic of phosphoproteins and is consistent with the fact that LSF has been reported to be phosphorylated on multiple serine residues (Ylisastigui et al., 2005). Thus, the unique spot observed around pH 5.5 could correspond to a LSF protein where multiple serines are phosphorylated. However, contrary to our expectations, the pattern of LSF expression on 2D gel was unaffected by treatment of HCT116 cells with 20 μM UO126 for 2 h. Therefore, despite previous evidence that LSF phosphorylation is affected by the MAP kinase pathway in lymphocytes, this appears not to be the case in HCT116 cells, which are of epithelial origin. Since the observed LSF expression pattern by 2D electrophoresis is consistent with a phosphoprotein, we believe that LSF must be phosphorylated by other cellular kinases in HCT116 cells.

3.3.2 Basic peptides recognized by the LSF antibody in the high pH range belong to a LSF related transcription factor: TFCP2L2.

Although they appeared to belong to unrelated proteins, the intensity of the signals detected up by the LSF antibody in the pH 6 and pH 9 regions on 2D gels was quite similar. We decided to investigate whether the two proteins were related. We performed mass spectrometry analysis of the peptides recovered from the two

Figure 3.18 Inhibition of MEK1/2 does not affect LSF phosphorylation. HCT116 cells were treated for 2 h with DMSO or 20 µM UO126. pH 5-8 IPG strips were rehydrated with 125 µg of extracts and samples were resolved by 2D SDS PAGE. Following transfer on PVDF, membranes were probed with an anti-LSF antibody (BD Biosciences)


pH
8
5
M.W
(kDa)
60
DMSO
2 h
60
UO126
20 μM 2 h

regions in order to identify the protein present around pH 9, as well as to confirm the presence of LSF in the pH 6 region. Protein extracts from HCT116 cells were resolved by 2D electrophoresis on 17 cm 3-10 IPG strips (Bio-Rad). The two regions containing peptides reacting with the LSF antibody were located and cut out from a Coomassie-stained gel and sent for analysis by nano liquid chromatography MS/MS. The results of the analysis are shown in Table 3.1. The presence of LSF (also called Cp2, LBP1c) was confirmed in the region corresponding to pH 6, consistent with the theoretical isoelectric point of the protein. No peptides belonging to LSF were identified in the pH 9 region. However, a LSF-related transcription factor, TFCP2L2, was identified in the pH 9 region. Since TFCP2L2, like LSF, belongs to a large family of transcription factors related to Drosophila grainyhead, we hypothesized that it might be involved in the regulation of TS. Therefore, we cloned a cDNA encoding this cellular protein into the mammalian expression plasmid pcDNA3; HCT116 cells were then transfected with increasing amounts of this TFCP2L2 expressing plasmid. 48 h post-transfection, we confirmed by real-time PCR that TFCP2L2 mRNA was overexpressed by a factor of more than 20-fold compared to mock transfected cells or cells transfected with a plasmid encoding GFP (data not shown). Analysis of HCT116 extracts transfected with TFCP2L2 by western blot gave no indication of any significant changes in the TS upon ectopic expression of this transcription factor. However, overexpression of TFCP2L2 at the protein level was not confirmed due to the lack of an appropriate antibody. We also performed luciferase assays to determine the effect of TFCP2L2 on TS transcription. Again, co-transfection of the TFCP2L2 transcription factor with the TS reporter construct had no significant effect on the TS promoter activity (data not shown).

Table 3.1 Proteins identified by mass spectrometry following analysis of the regions reacting with the anti-LSF antibody. Proteins detected in the medium pH range (pH 6) are shown in the first part of the table and include the LSF transcription factor (CP2). Proteins detected in the high pH range of the gel (pH 9) are shown in the bottom part of the table and include TFCP2L2 transcription factor, related to CP2.

Medium pH range				
Proteins detected	GI number	MW (kDa)	pI	Number of peptides detected
DnaJ (Hsp40) homolog, subfamily C, member 3 [Homo sapiens]	54781373	57.6	5.83	17
heat shock 70kDa protein 8 isoform 1 [Homo sapiens]	5729877	70.9	5.37	15
vimentin	340219	53.7	5.03	14
alpha-globin transcription factor CP2 - human	283976	57.2	5.53	3
High pH range				
proteasome subunit p58 [Homo sapiens]	2656092	61.0	8.47	17
cytochrome P450, family 2, subfamily A, polypeptide 7 isoform 1 [Homo sapiens]	15147330	56.4	7.69	12
aldehyde dehydrogenase 6A1 precursor [Homo sapiens]	11095441	57.8	8.72	10
chaperonin containing TCP1, subunit 6B (zeta 2) [Homo sapiens]	5729761	59.5	7.57	10
fibrinogen beta chain [Homo sapiens]	7924018	55.9	8.54	5
glucuronosyltransferase (EC 2.4.1.17) - human	106109	60.0	6.88	2
TFCP2L2 protein [Homo sapiens]	45709470	57.8	7.61	1

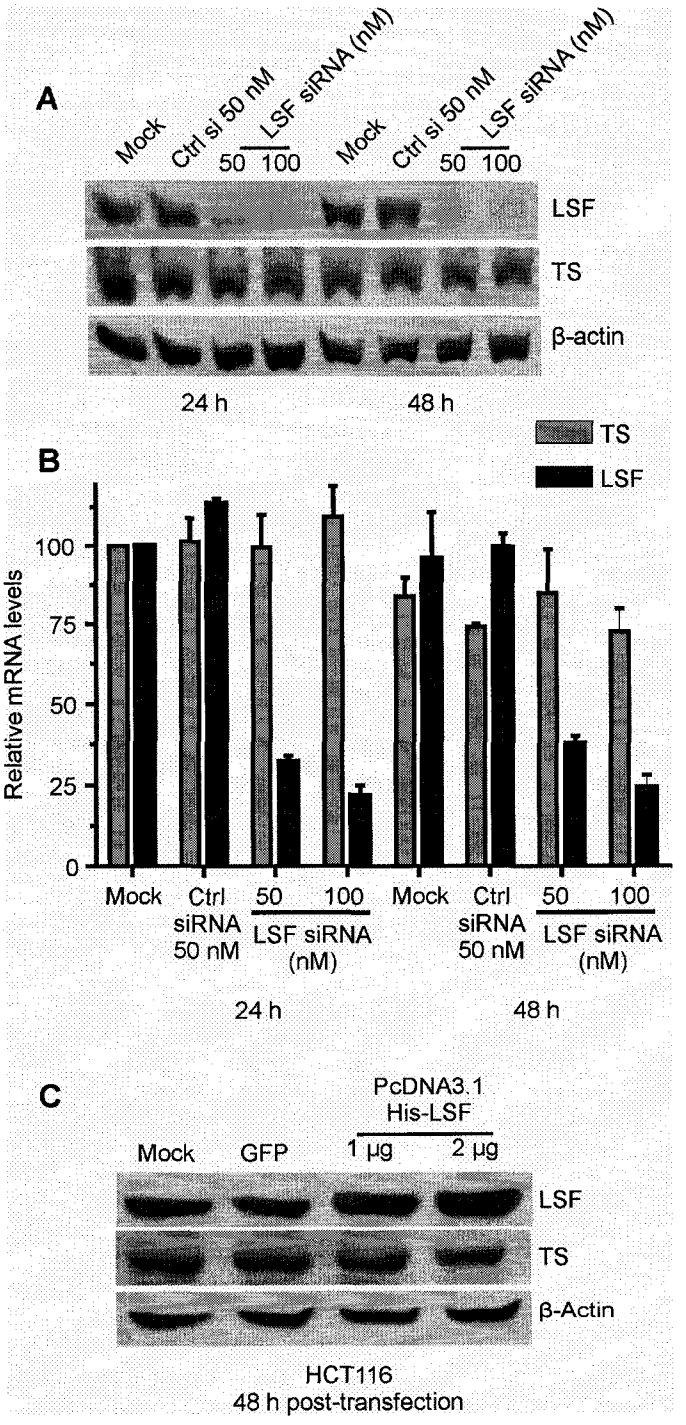
3.3.3 Expression levels of the LSF transcription factor do not affect TS expression

To further investigate the putative role of LSF in the transcriptional regulation of human TS, we performed an siRNA knock-down of LSF in HCT116 cells. 24 h and 48 h following transfection of HCT116 cells with an increasing concentration an LSF siRNA, LSF levels were drastically decreased, both at the protein and mRNA levels (Fig 3.19A, 3.19B). However, under the same conditions, TS protein and mRNA levels remained largely unchanged. Similar results were obtained in MCF-7 cells (data not shown). Additionally, transfection of HCT116 cells with a plasmid encoding a His-tagged LSF protein did not produce any significant change of TS protein levels (Fig. 3.19C). We therefore conclude that in HCT116 and MCF-7 cells, TS expression is independent of LSF.

3.3.4 LSF does not affect TS transcription in HCT116 cells

To investigate the significance of the putative LSF binding site in the TS promoter, we analyzed the human TS promoter for transcription factor binding sites using Match (http://www.gene-regulation.com/pub/programs.html). The *accccgccgag* sequence, immediatly 5' to the essential promoter region was identified by the algorithm as a weak LSF binding site. However, the position of the LSF site was different than previously reported since it did not overlap the Ets-1 and Sp1 sites of the essential promoter region (see Fig. 1.4). To determine whether this site played a role in the transcriptional regulation of the TS promoter, we engineered promoter constructs where the LSF site identified by Match was deleted or mutated by site-directed mutagenesis.

Figure 3.19 Effects of downregulation and overexpression of LSF on TS expression levels. (**A**) Western blot analysis of HCT116 cells transfected for 24 h or 48h with increasing concentrations of LSF specific siRNA. (**B**) Relative mRNA expression levels of TS, and LSF mRNA 24 and 48 h post-transfection with increasing concentration of an siRNA targeting LSF. mRNA levels were normalized to β-2-microglobulin mRNA levels. The mean and SEM of three independent experiments is shown. (**C**) Western blot analysis of HCT116 cells transfected for 48h with increasing amounts of a His-LSF expression plasmid. The GFP expression plasmid was used as a control.


A
Mock
Ctrl si 50 nM
LSF siRNA (nM)
50 100
Mock
Ctrl si 50 nM
LSF siRNA (nM)
50 100
LSF
TS
β-actin
24 h
48 h
B
TS
LSF
Relative mRNA levels
100
75
50
25
0
Mock
Ctrl siRNA 50 nM
50 100
LSF siRNA (nM)
Mock
Ctrl siRNA 50 nM
50 100
LSF siRNA (nM)
24 h
48 h
C
PcDNA3.1 His-LSF
Mock
GFP
1 μg
2 μg
LSF
TS
β-Actin
HCT116
48 h post-transfection

Surprisingly, both constructs where the putative LSF site was modified exhibited a 8- to 10- fold *increase* in promoter basal activity higher compared to the wild-type promoter (Fig 3.20A). However, no change was observed in TS promoter activity following co-transfection of a His-tagged LSF protein with the full length TS promoter construct (Fig. 3.20B). Taken together, these results strengthen our conclusion that LSF is not involved in the transcriptional activation of TS in HCT116 cells. The putative LSF site that we identified the human TS promoter was different than previously reported and in fact appeared to be a strong negative element. Furthermore, overexpression of LSF did not affect the TS promoter activity in reporter assays. Our data do not support the notion that LSF can act as a transcriptional activator of TS but are consistent with the role of LSF as a repressor in certain promoter context (Ylisastigui et al., 2005).

3.3.5 GABP expression does not affect TS expression in human cells

GABP transcription factors functions normally as heterodimers, with the GABPα recruiting the GABPβ protein (Brown and McKnight, 1992), which contains the transactivation domain, to Ets binding site. Since GABP plays an important role in the regulation of the mouse TS promoter, we tested the ability of GABP transcription factors to stimulate the activity of the human TS promoter. Overexpression of GABPα, GABPβ1 or GABPβ2 alone had no effect on the TS promoter activity in luciferase assays (Fig 3.21). Given the possibility that GABPα or GABPβ1 or β2 alone might not be able to induce a transcriptional response, we also performed co-transfections of GABPα along with GABPβ1 or β2 and measured their effect on TS promoter activity. Co-expression of GABPα together with its

Figure 3.20 Role of LSF in the control of the human TS gene expression. **(A)** Comparison of the relative luciferase activity of the TS wild-type promoter, and promoters where the LSF site has been mutated by SDM or deleted. Cells were co-transfected with pGL2B-TS, pSV-β-gal the various GABP expression plasmids for 48 h. Data shown here is the mean and SEM of three independent experiments. Luciferase activity was normalized to β-galactosidase activity used as a transient transfection efficiency control. **(B)** Effect of LSF overexpression on TS promoter activity. Cells were co-transfected with pGL2B-TS, pSV-β-gal the various and a pcDNA3.1 His-myc-LSF or pCDNA3.1 His-myc alone for 48 h. Data shown here is the mean and SEM of three independent experiments. Luciferase activity was normalized to β-galactosidase activity used as a transient transfection efficiency control.

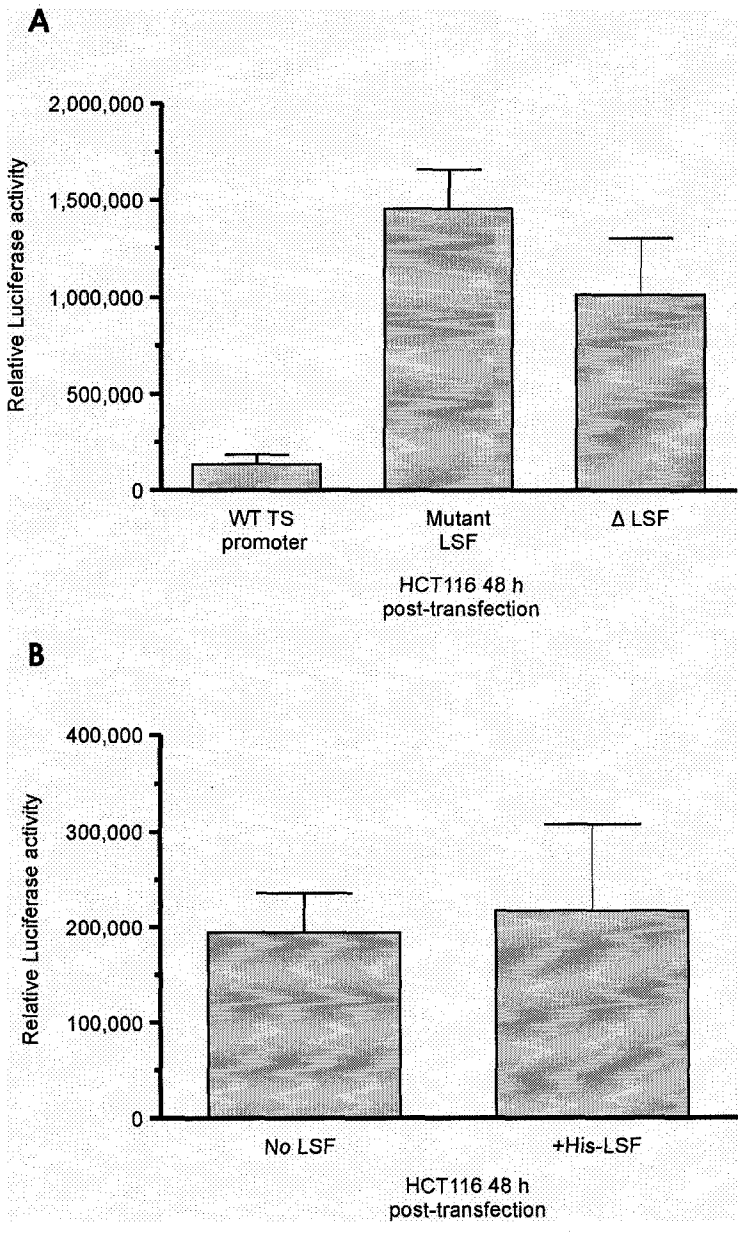
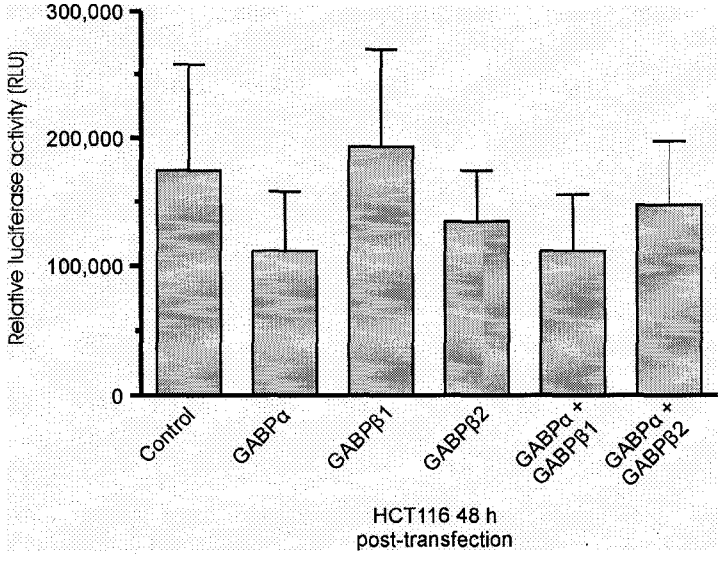

A
2,000,000
1,500,000
1,000,000
500,000
0
Relative Luciferase activity
WT TS promoter
Mutant LSF
Δ LSF
HCT116 48 h post-transfection
B
400,000
300,000
200,000
100,000
0
Relative Luciferase activity
No LSF
+His-LSF
HCT116 48 h post-transfection

Figure 3.21 Effect of the expression of GABPα, GABPβ1 and GABPβ2 on the TS promoter transcription. Relative luciferase activity of the full length TS promoter following expression of various GABPα and GABPβ1 or β2 proteins. Cells were co-transfected with pGL2B-TS and pSV-β-gal and incubated for 24 h before addition of the drug. Data shown here is the mean and SEM of three independent experiments. Luciferase activity was normalized to β-galactosidase activity used as a transient transfection efficiency control.


300,000
200,000
100,000
0
Relative luciferase activity (RLU)
Control
GABPα
GABPβ1
GABPβ2
GABPα + GABPβ1
GABPα + GABPβ2
HCT116 48 h post-transfection

transcriptional co-activators had little or no effect on luciferase activity. Therefore, we conclude that, unlike in rodent cells, GABP transcription factors do not affect the rate of TS gene expression in human cells.

Chapter 4: DISCUSSION

Despite the central role of TS in nucleic acid metabolism and the relatively large number of chemotherapeutics drugs that target TS activity, very little is known about the molecular pathways controlling human TS expression. Most studies to date looking at the transcriptional control of the TS gene have been performed in rodent models, based on the assumption that, since the regulatory sequences are highly conserved in the rat, mouse and human genes, they share common regulatory pathways. However, there is accumulating evidence that the mouse and the human gene might not share common regulatory features. The present study was undertaken to uncover the mechanisms underlying the transcriptional regulation of the human thymidylate synthase gene. We investigated both the cell-cycle dependency of TS in human cells and also explored the role of growth factor signalling pathways in human TS expression. In addition we assessed the role of LSF and GABP transcription factors in the transcriptional control of the human TS gene.

4.1 Human TS and the cell cycle

4.1.1 Classical view of TS: an S-phase dependent enzyme.

The widespread assumption that TS is a cell-cycle dependent enzyme has come from studies that, for the most part, have used rodent models. In early studies using synchronized murine cells, both TS mRNA and TS activity increased as cells reached S-phase (Nagarajan and Johnson, 1989; Navalgund et al., 1980). The key transcriptional regulators of the G_1/S transition are the E2F transcription factors (Fan and Bertino, 1997), which are well known to activate the expression of a number of

genes required for DNA synthesis including TS (DeGregori et al., 1995). Later studies using microarray analysis showed an accumulation of TS transcripts following ectopic overexpression of various E2F transcription factors (Ishida et al., 2001; Kalma et al., 2001; Polager et al., 2002). These studies reinforced the hypothesis that TS is a S-phase dependent enzyme and also established E2F transcription factors as determinant regulators of the TS gene expression. Also, one might intuitively expect that high TS levels are only required when a cell is actively replicating its genome, since TS activity is needed during S-phase to provide enough dTMP for the DNA replication machinery to function properly.

There are, however, a number of studies that do not support this hypothesis. For example, in asynchronously growing human cancer cells, TS levels were shown to be high in cycling cells (largely independent of the phase of the cell cycle) and low in confluent cells (Pestalozzi et al., 1995). There is scientific agreement that non-dividing (G_0) cells have low TS levels, which increase when cells are stimulated to grow. Proliferating cells need balanced levels of the four deoxyribonucleotides for normal DNA replication. The four deoxyribonucleotides are directly synthesized from the corresponding ribonucleotides by the enzyme ribonucleotide reductase (RNR); however, only dTMP requires an additional step, catalyzed by thymidylate synthase, to complete its synthesis. Until recently, RNR was considered to be a prime example of a cell cycle-dependent enzyme. RNR is a dimeric enzyme composed of two non-identical subunits, R1 and R2, encoded by separate genes. R2 is rate-limiting for the enzymatic activity of RNR and its level is cell-cycle dependent (Chabes and Thelander, 2000). However, p53R2, an analog of R2, has recently been found to be independent of the cell-cycle (Guittet et al., 2001;

Nordlund and Reichard, 2006). p53R2 can substitute for R2 to form an active RNR enzyme capable of generating deoxyribonucleotides in resting cells or following DNA damage. A factor not widely appreciated is that RNR and TS must be coordinated. Without adequate levels of TS, dUMP could rise to a high enough level to allow its incorporation into DNA in place of dTMP. This is potentially a mutagenic event (leading to AT → GC transition) since uracil can be recognized, excised, and replaced by cytosine by a base-excision DNA repair pathway designed to recognize spontaneous deamination of cytosine (converting it to uracil) in DNA (Lindahl, 2000). Given the danger to the integrity of the genome should dUMP be incorporated into DNA, one might *a priori* assume the necessity of having adequate levels of TS available whenever deoxynucleotides are synthesized by RNR. Based on recent insight that RNR activity can be independent of S-phase, there is therefore sufficient reason to expect that TS activity should also be independent of the cell-cycle.

4.1.2 Human TS expression is independent of E2F activity

The present report provides additional supporting evidence that TS expression in human cells is not closely linked to S-phase progression and also that it is not dependent on E2F activity. To have a better understanding of the exact timing of TS expression in synchronized cells, we compared the timing of TS expression with cyclin E (a known direct target of E2F transcription factors). When serum-deprived HCT116 cells were stimulated to enter the cell cycle, both TS and cyclin E started to increase several hours after addition of serum (G_1 and early S phase). However, TS and cyclin E differed in that the increase in TS mRNA and TS protein was more gradual than the increase in cyclin E and occurred a few hours

later. Moreover, as cells progressed through the cell-cycle, TS mRNA and TS protein levels remained high while cyclin E declined. Expression of TS and cyclin E was also followed in exponentially-growing cells subjected to serum deprivation. Again, the patterns of cyclin E and TS expression showed distinct differences. TS protein and mRNA levels declined almost linearly over a 6 day period whereas cyclin E mRNA decreased sharply in the first day of serum deprivation. Additionally, cyclin E mRNA correlated with the faction of cells in S-phase, but TS did not. Our data clearly show that, in human cells synchronized by serum-starvation, TS and cyclin E are both activated during the G_1 phase of the cell-cycle and reach a maximum level of expression in early S-phase. However, the E2F-responsive cyclin E is upregulated before TS, suggesting that they are not controlled by the same regulatory pathways.

To assess more directly the role of cellular proteins involved in the G_1/S transition on TS expression, we also overexpressed E2F1, Dp1 and cyclin E in human HCT116 and MCF-7 cancer cell lines as well as in GM38 normal fibroblasts. Ectopic expression of these proteins had no discernible effect on endogenous TS expression in any of the cell lines studied, indicating that neither E2F1 nor cyclin E significantly affect TS expression in human cells. Notably, in normal human fibroblasts, expression of E2F1 and E2F1+Dp1 led to a strong accumulation of endogenous cyclin E due to increased E2F1 activity, but no change in TS protein expression was observed. *Our results, therefore, do not support a role for E2F in TS expression.* In the past, the level of expression of E2F1 has been used as a prognostic marker for response to anti-TS drugs on the assumption that TS is an S-phase enzyme (Kasahara et al., 2000; Sowers et al., 2003a; Banerjee et al., 2000).

The evidence presented in this report clearly indicates that, in human cells, TS is strictly expressed in early-mid G_1 and is not regulated by E2F1 activity. Therefore, levels of E2F may not be a valid predictive marker for tumour response to anti-TS drugs as previously suggested (Belvedere et al., 2004).

4.1.3 Role of CDK4 and CDK2 activity in TS expression

The main cellular components driving G1 phase progression are the cyclin D-CDK4/6 and cyclin E-CDK complexes (Boonstra, 2003). To try to better understand the events controlling TS expression during G_1, we next used pharmacological inhibitors of CDK4 and CDK2 to block cell cycle progression. Treatment of HCT116, MCF-7 and PC3 cells with roscovitine and CDK2 inhibitor led to an accumulation of cells in G_2/M without an observable effect on endogenous TS levels. In contrast, treatment with a small molecule inhibitor of CDK4, blocked cell-cycle progression in G1 and was associated with a marked decrease in TS expression of in all three cell lines used in this study. Previous studies have shown that treatment of cancer cells with the CDK inhibitors UCN-01 and flavopiridol led to a downregulation of TS levels and sensitized cells to 5-fluorouracil (Abe et al., 2000; Hsueh et al., 1998). UCN-01 is a broad specificity kinase inhibitor, known to inhibit not only CDKs but also PKC and Chk1 (Akinaga et al., 1991; Busby et al., 2000). Flavopiridol is a flavonoid that exerts strong inhibitory activity against CDK2, CDK4 as well as CDK1 (Carlson et al., 1996). In the present study, we observed a marked decreased in TS levels following inhibition of CDK4 but not CDK2. We believe that the observed decrease in TS expression following treatment with UCN-01 or flavopiridol was solely due to

inhibition of CDK4, and not due to inhibition of other CDKs such as CDK2 and CDK1.

4.1.4 Role of p16INK4A and p27KIP1 in TS regulation

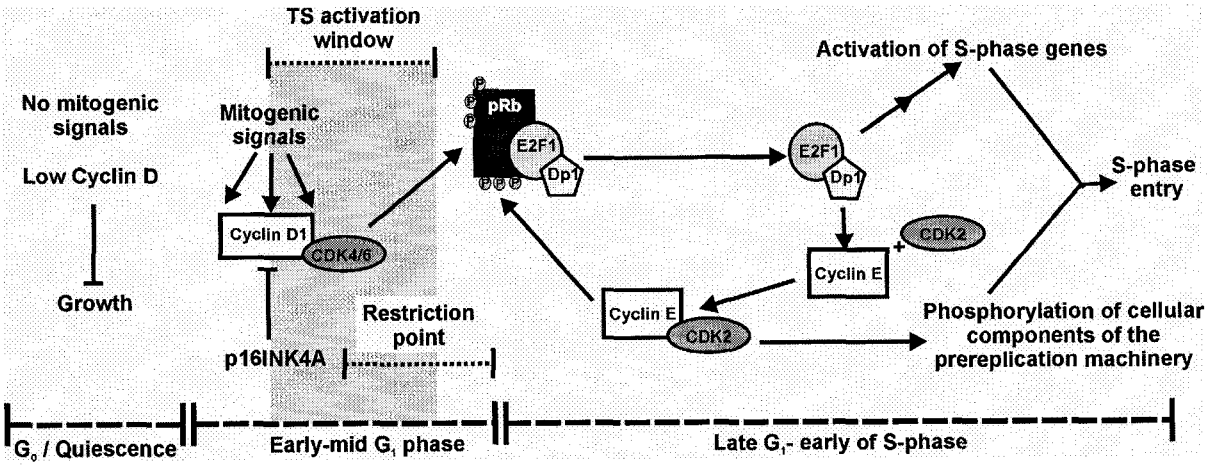
To provide independent evidence that CDK4 activity is indeed linked to TS expression, we carried out experiments using adenovirus-mediated expression of p16INK4A and p27KIP1. p16INK4A is known for its ability to specifically bind and inhibit CDK4 and CDK6 whereas p27KIP1 has the ability to inhibit CDK4 and CDK6 as well as CDK2 (Vidal and Koff, 2000). Consistent with the data obtained following treatment with the CDK4 inhibitor, overexpression of p16INK4A blocked MCF-7 and HCT116 cells in G_0/G_1 and produced a clear decrease in TS levels. Differences were observed in MCF-7 and HCT116 cells overexpressing p27KIP1 as compared to the same cells following treatment with roscovitine or the CDK2 inhibitor. Upon expression of p27, cells did not accumulate in G_2/M but were instead blocked in G_0/G_1 and S-phase. High p27 levels were associated with a strong decrease of TS levels in MCF-7 but the decrease was only marginal in HCT116 cells. p27 is primarily characterized for its ability to bind and inhibit cyclin E-CDK2 complexes, but its ability to block cyclin D/CDK4 complexes remains controversial; p27 has been shown to be required for the proper assembly of active cyclin D-CDK4 (Dong et al., 1998; Olashaw et al., 2004) In our study, since both p16 and p27 led to an accumulation of cells in G_0/G_1, we believe that both proteins were able to block CDK4 activity, and that the difference observed between the two cell lines might be due to different expression levels of cyclin D-CDK4 and cyclin E-CDK2 complexes in HCT116 and MCF-7. Nevertheless, ectopic expression of p16 produced a clear

decrease in TS, confirming the role of CDK4 in the control of TS expression during G_1. p16 expression is increased following oncogene activation and constitutes a safeguard mechanism for cellular transformation (Taylor et al., 2004). The p16INK4A gene is a tumour suppressor (Serrano et al., 1996) and its locus is often inactivated in tumours (Brown et al., 2004; Lee et al., 2006). Based on our findings, inactivation of p16INK4a is expected to lead to an increase in CDK4 activity and an increase in TS levels. Since TS itself has been recently described as an oncogene (Rahman et al., 2004), loss of p16INK4A would constitute a "double hit" with loss of a tumour suppressor and concomitant activation of an oncogene. A recent paper showed that following p16INK4A expression in human cells the TS, DHFR and RNRII promoters were deacetylated following recruitement of HDACs (histone deacetylases) and the transcriptional corepressor mSin3B (Gunawardena et al., 2007). Interestingly, the recruitment of both HDACs and Sin3B was dependent on the SWI/SNF chromatin remodelling complex. The SWI/SNF remodelling complex is involved in both transcriptional repression and transcriptional activation (Klochendler-Yeivin et al., 2002). It would be interesting to determine if the decrease we observed in TS expression following inhibition of CDK4 is dependent on the SWI/SNF complex activity. The SWI/SNF chromatin remodelling complexe activity could possibly be controlled by CDK4 activity, independently of pRb-E2F.

4.1.5 Expression of TS in human cells is an early-mid G_1 event taking place before the restriction point

The cell cycle is a highly complex process that is only partially understood. Dysregulation of cell-cycle proteins is often found in malignant cells, and

Figure 4.1 Model of the G_1 phase of the cell cycle showing the Restriction Point and the postulated TS activation window. In the absence of mitogenic signals, cells are in a resting (G_0) state. Upon stimulation with growth factors, cells enter G_1 and cyclin D levels increase. As G_1 progresses, cyclin D-CDK4/6 complexes phosphorylate pRb leading to the release of E2F/Dp1 complexes. E2Fs then activate the transcription of a series of S-phase genes and cyclin E. Once Cyclin E is synthesized, it associates with CDK2 and drives S-phase entry. The TS activation window is proposed to coincide with the period that CDK4 is active.


TS activation window
No mitogenic signals
Low Cyclin D
Growth
Mitogenic signals
Cyclin D1
CDK4/6
p16INK4A
Restriction point
pRb
E2F1
Dp1
Activation of S-phase genes
E2F1
Dp1
S-phase entry
CDK2
Cyclin E
Cyclin E
CDK2
Phosphorylation of cellular components of the prereplication machinery
G0 / Quiescence
Early-mid G1 phase
Late G1- early of S-phase

overexpression of cyclin D1 has been shown to occur frequently in tumours (Bartkova et al., 1994; Bartkova et al., 1995), including colon cancer (Kong et al., 2000). Stimulation of resting cells by addition of growth factors leads to the transcriptional activation of cyclin D (Fig 4.1). As G_1 progresses, cyclin D associates with CDK4/6 and there is a first wave of pRb phosphorylation, leading to the release of E2F transcription factors. Cyclin E expression is induced by E2F and newly synthesized cyclin E then associates with CDK2 to drive further G_1 progression. In cycling cells, E2F1 activity and cyclin E expression normally reach a maximum in late G_1/early S-phase (Dulic et al., 1992) after transit through the "Restriction Point". The early part of G1 is dependent on the presence of growth factors and this dependency on extracellular signals ends with the onset of pRb phosphorylation (Blagosklonny and Pardee, 2002). Experiments in rat cells showed that expression of a constituvely active form of pRb resulted in an increase expression of enzymes involved in nucleic acid metabolism, including TS, DHFR and RNR-R2 (Angus et al., 2002). Our experiments clearly demonstrate that TS expression in human cells differs significantly from rodent cells in that it is independent of E2F1 and cyclin E, whose activity is regulated by pRb status. However, we showed that inhibition of CDK4 leads to a decrease in TS protein. This suggests that there exists a TS activation window in early-mid G_1 prior to the phosphorylation of pRb and activation of cyclin E-CDK2 that drive G_1/S transition (Fig. 4.1). Therefore, TS expression in human cells is dependent on events taking place in early-mid G_1, in the same time frame as the Restriction Point. Also, since TS levels are low in non-dividing cells and are elevated in proliferating cells (largely independent of the phase of the cell-

cycle), we propose that TS could be used as a marker for cell proliferation as previously suggested by Pestalozzi et al (1995), similar to Ki-67 and PCNA.

4.2 TS expression is regulated by the MAP kinase signalling pathway

4.2.1 TS expression is regulated by MEK1

We investigated the role of growth factor signalling in the regulation of TS. Our results showed that inhibition of MEK 1/2 by UO126 led to a decrease in TS protein and mRNA levels. Luciferase assays confirmed that introducing a constitutively active form of MEK1 in HCT116 cells stimulated the transcription of the TS gene by a factor of 8 to 10-fold. Other experiments using promoter deletion assays identified several sites within the TS promoter that are responsive to MEK1 activity. Deletion of the Sp1 consensus sequence found in the essential promoter and as second region near the translational start of the gene greatly reduced the ability of MEK1 to transactivate the TS promoter. *Taken together, our results show for the first time that the MAP kinase pathway controls TS expression in human cells.* Interestingly, it is now well established that the MAP kinase pathway plays an important role in G_1 progression (Torii et al., 2006; Massague, 2004). Accumulation of cyclin D is dependent on the presence of growth factors and ERK 1/2 have been shown to directly participate in the induction of cyclin D levels (Lavoie et al., 1996). The fact that TS levels are dependent on MEK activity also nicely confirms our observation that the transcriptional activation of the TS gene takes place during the growth factor-dependent part of G_1, prior to the Restriction Point.

An interesting observation was made when we compared the effects of siRNA knock-down of MEK1 versus MEK2 on TS expression levels. Whereas a decrease

in MEK1 levels was clearly associated with a decrease in TS levels, knock-down of MEK2 had the opposite effect. Although MEK1 and MEK2 have long been considered to have overlapping functions, more and more evidence is accumulating to suggest that MEK1 and MEK2 have distinct roles (Belanger et al., 2003; Giroux et al., 1999). A recent paper aiming to unravel the specific roles of these two proteins showed that inhibition of MEK1 was associated with decreased proliferation and with cellular senescence, whereas depletion of MEK2 lead to an accumulation of cyclin D and CDK4/6 activation (Ussar and Voss, 2004). If the primary function of MEK1 is related to the control of cellular proliferation, it is therefore not surprising that specific knock-down of this kinase is associated with a decrease in TS expression. Also, the increase we observed in TS levels following siRNA knock down of MEK2 could be linked to an increase cyclin-CDK4 activity triggered by MEK2 deficiency.

4.2.2 TS expression is MEK1-dependent but ERK2 independent

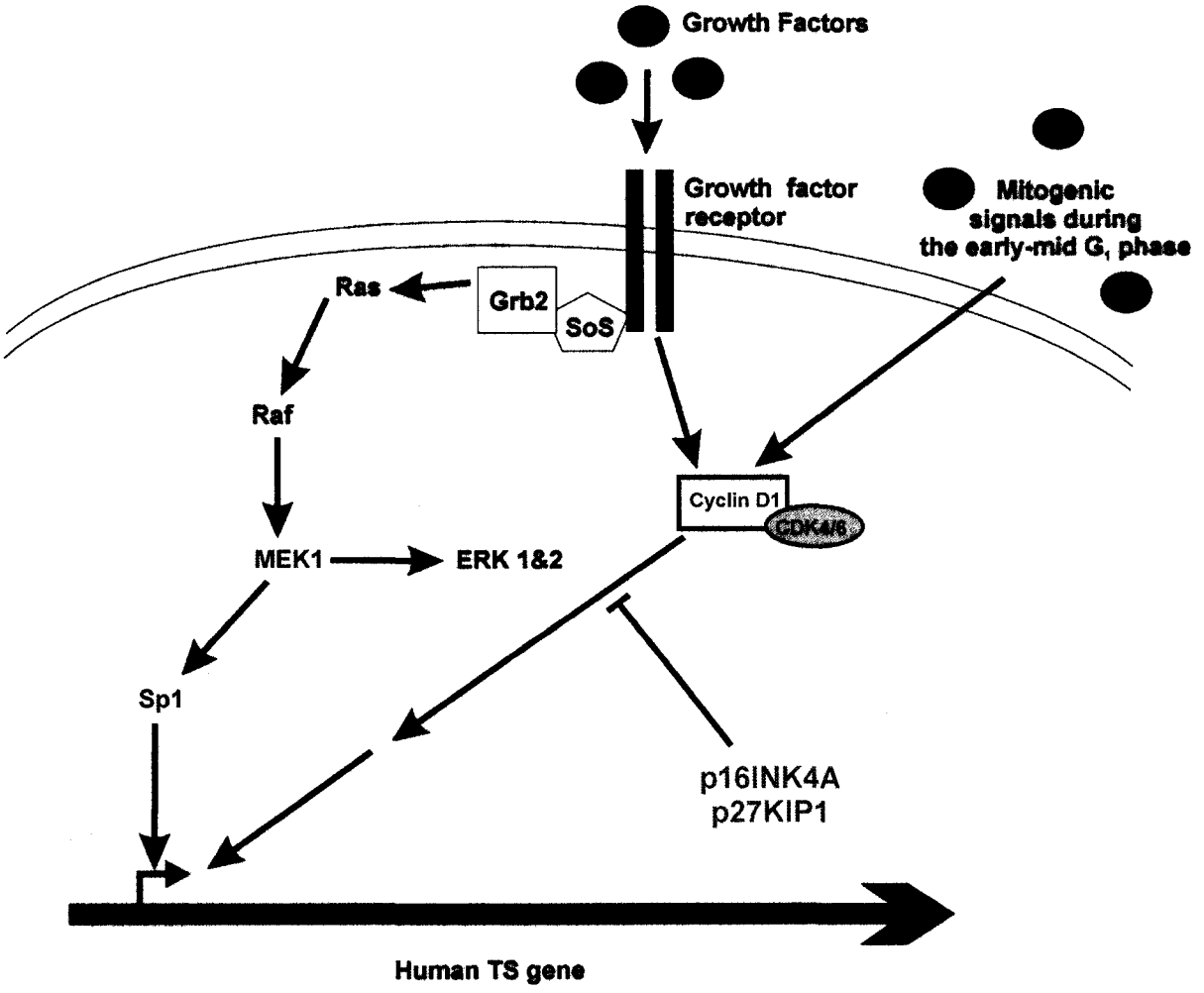
The canonical MAP kinase pathway typically involves a well established signalling cascade where activated MEK kinase phosphorylates ERK 1 and 2, which can then translocate to the nucleus to activate of number of transcription factors and nuclear targets (Turjanski et al., 2007). Surprinsingly, our data suggest that MEK1 controls TS expression in human cells in an ERK-*independent* fashion. Since MEK 1/2 are strictly cytoplasmic proteins, our model implies that the MAP kinase pathway is not always linear and that MEK 1/2 could have downstream targets other than ERK 1/2. To date, the only known downstream targets of MEK 1/2 are ERK 1 and 2 kinases. However, a number of studies have reported cellular events that are MEK-dependent, yet ERK-independent. For example, phosphorylation of the cell

signalling proteins SOS and Shc has been shown to be dependent on MEK but not ERK activity (Holt et al., 1996; Kao et al., 1997). Thus, MEK kinases might play a more complex role than previously understood. Based on the exclusively cytoplasmic localization of MEK 1 and 2, we postulate they could either directly phosphorylate a transcription factor that would then translocate to the nucleus, or activate a downstream kinase other than ERK that would in turn translocate to the nucleus. Further experiments are required to better understand this observation.

4.2.3 Putative role of the Sp1 site in the TS essential promoter

Our results suggest that the Sp1 consensus site present at the 3' end of the essential promoter region is at least in part responsible for MEK1-dependent increase in TS. Although it does not appear to be the only site responsive to MEK1, deletion of this site decreases the TS promoter responsiveness to MEK1 R4F by a factor of 3 to 4-fold. A recent study showed that Sp1 is preferentially transcribed in G_1 and is expressed at high levels during the early part of the cell cycle (Grinstein et al., 2002). This study also showed that expression of a dominant negative form of Sp1 led to an accumulation of cells in G_1 and was associated with a decrease in cyclin D levels as well as an increase in p27KIP1. The cyclin D promoter contains several Sp1 binding sites (Motokura and Arnold, 1993) and Sp1 has been shown to be involved in the regulation of cyclin D expression (Lin et al., 2002; Nagata et al., 2001). Taken together, this evidence suggests that Sp1 is an important G_1 regulator that is active in cells before transit through the Restriction Point. Interestingly, Sp1 interaction with the transcriptional co-repressors SMRT and NcoR has recently been shown to be modulated by MEK activity (Lee et al., 2005). Also VEGF-A induction

Figure 4.2 Schematic model of the cellular pathways involved in the regulation of the human TS gene. Following activation of growth factor receptors at the plasma membrane, the MAP kinase pathway is activated and lead to the activation of MEK1. MEK1 then modulate the Sp1 transcription factor activity to regulate TS transcription. During the growth factor dependent part of G_1, growth factor signalling pathways induce expression of cyclin D which associates with CDK4. Cyclin D-CDK4 complexes activate TS transcription through an unknown mechanism. The cell cycle inhibitors p16INK4A and p27KIP1 can disrupte CDK4 fonction and block TS activation.


Growth Factors
Growth factor receptor
Mitogenic signals during the early-mid G, phase
Ras
Grb2
SoS
Raf
MEK1
ERK 1&2
Cyclin D1
CDK4/6
Sp1
p16INK4A
p27KIP1
Human TS gene

was shown to be regulated by Sp1 and Sp3 transcription factors in a MEK/ERK-dependent fashion (Strowski et al., 2004).

Thus, there is increasing evidence that Sp1 might be an important transcription factor involved in the regulation of TS during the G1 phase, based both on our evidence as well as that of others. However, further work is needed to confirm the exact function of Sp1 in the transcriptional control of TS. Based on our evidence, we propose that TS expression during G_1 is controlled by Sp1 in a MEK1-dependent but ERK 2-independent fashion (Fig 4.2). In parallel, also during the G_1 phase, cyclin D-CDK4 complexes also regulate TS expression through a transcription factor yet to be determined.

4.3 TS regulatory mechanisms are not conserved across species

The LSF transcription factor has been shown to be a positive regulator of TS in mouse cells and to be critical for proper S-phase expression of the enzyme (Powell et al., 2000). Also, Fe65, a ligand of APP (amyloid precursor protein) that can interact with LSF (Zambrano et al., 1998), was shown to prevent LSF-dependent activation of TS expression (Bruni et al., 2002) in human cell lines. However, our results strongly argue against a role for LSF as regulator of TS levels in human cells. Our data show that neither overexpression nor downregulation of LSF affect TS expression in HCT116 cells. Surprisingly, point mutation or complete deletion of the LSF site found in the human TS promoter led to a major *increase* in the TS promoter basal activity. Downregulation or overexpression of LSF transcription factor failed to affect TS expression in HCT116 cells, yet modifying the LSF consensus binding site strongly enhanced the promoter activity. It is interesting

to note that LSF has been shown to act either as a transcriptional repressor or as a transcriptional activator, depending on the promoter context (Ylisastigui et al., 2005; Kim et al., 1987). Therefore, the differences between our observations and previously reported data could be due to differences in the tissue origin of the cell line used in the present report. In the future, it would be interesting to test the relative activity of the LSF promoter mutant in different cell lines to determine if the LSF motif found in the TS essential promoter region can act either as a repressor or as an activator depending on the cell lineage.

The role of the GABP transcription factor in the regulation of TS was also investigated in this study. Despite the fact that GABP was previously shown to bind to the Ets-1 site in the mouse TS essential promoter and to stimulate its activity (Rudge and Johnson, 2002), our results argue against the role of GABP in the regulation of the human TS promoter. Using luciferase assays, we were able to demonstrate that transfection of GABP α alone or along with its binding partners (GABP β1 and GABP β2) failed to increase TS promoter activity. However, luciferase assays following deletion of the Ets-1 site in the essential promoter region led to a 3-fold decrease in the basal activity. This experiment clearly confirmed the role of the Ets-1 site as a positive regulator of the TS gene expression. However, it appears that in human cells the GABP transcription factor does not bind to this site. This does not rule out the possibility that other factors from the Ets family might bind to this site in human cells. A recent report showed that the GABPα transcription factor is involved in cell-cycle progression and that disruption of the GABPα is associated with decreased expression of a number genes required for DNA

synthesis including TS (Yang et al., 2007). However, this study was performed in mouse embryonic fibroblasts (MEFs) and not in cells of human origin.

As aforementioned, we have also shown that adenovirus-mediated expression of E2F1 or E2F1 along with its transcriptional co-activator Dp1 failed to increase TS levels in the three human cell lines used in this study. Remarkably, in normal human fibroblasts, ectopic expression of E2F1 and E2F1+Dp1 was associated with an important increase in E2F activity (as demonstrated by the strong increase in cyclin E levels), yet TS levels remained unchanged. We therefore conclude that TS expression is largely independent of E2F activity. Our results are consistent with previous observations showing that MCMV infection upregulated TS in an E2F-depedendent fashion in mouse cells (Gribaudo et al., 2000), whereas in human cells infected with CMV, TS was upregulated indepedendently of E2F transcription factors (Gribaudo et al., 2002).

One important conclusion from the evidence presented in the present study is that there are major differences in the mechanisms that control TS expression between rodent and human cells. This implies that different transcription factors might be involved in the regulation of the TS gene in different species. Despite the apparent similarity of regulatory sequences in the TS promoter of the rat, mouse and human genes, it appears that regulation of TS is significantly different in these organisms. Therefore, future studies looking at the regulation of the human TS gene should use cells of human not rodent origin.

4.4 Rationale for new anticancer combination therapies

Thymidylate synthase is the target of a number of cancer chemotherapeutic drugs. Overexpression of TS is one mechanism by which tumours may develop resistance to such drugs (Johnston et al., 1995). High levels of TS have been shown to occur in a number of tumours (Haqqani et al., 1999; Mizutani et al., 2003) and are associated with poorer overall prognosis (Shintani et al., 2003; Suzuki et al., 1999). These clinical studies are consistent with the fact that TS was recently described as an oncogene (Chen et al., 2007; Rahman et al., 2004). Therefore, inhibition of the cellular pathways controlling TS expression may be expected to decrease TS levels, decrease the tumorigenic potential of cancer cells and increase the sensitivity of the tumour to drugs targeting TS activity.

The role of the MAP kinase pathway in the development of cancer is well established. The Ras kinase is a potent proto-oncogene that has been shown to be mutated in 30% of tumours; the incidence of Ras mutations in colon cancer is 50% (Malumbres and Barbacid, 2003). Its downstream target, B-Raf, has also been shown to be mutated frequently in number of cancer (Davies et al., 2002). Gain of function mutations of Ras or Raf protein kinases lead to the constitutive activation of the MAP kinase pathway, even in the absence of extracellular signals and have been shown to promote cancer. Given the critical role of these signalling proteins in oncogenesis, a number of compounds targeting the activity of Ras, Raf and MEK have been designed for the treatment of cancer (Roberts and Der, 2007). Sorafenib and PD0325901 are small molecule inhibitors of Raf and MEK 1/2, respectively, that are currently under clinical development (Gollob et al., 2006; Wang et al., 2007). We have shown in the present study that MEK1 activity controls the level of

expression of the TS enzyme. Therefore, we propose that inhibitors of the MAP kinase pathway and drugs targeting dTMP synthesis will act synergistically for the treatment of cancer. Inhibition of the MAP kinase pathway would not only decrease the proliferation of cancer cells but also block TS expression, rendering tumour cells more sensitive to drugs targeting TS activity.

In recent years, a number of new chemotherapeutics compounds targeting the cell cycle have been developed to inhibit the growth of cancer cells (Collins and Garrett, 2005). PD 0332991, a specific inhibitor of CDK4/6, showed antitumour activity in xenografts (Fry et al., 2004). As shown in this study, TS expression is decreased both by small molecule inhibitors of CDK4 and following overexpression of p16INK4A. Interestingly, it has previously been shown that tumour tissue from patients who responded to fluoropyrimidine-based treatment expressed high levels p16INK4A (Kamoshida et al., 2004). Since we have shown that CDK4 regulates TS expression, high levels of p16INK4A would be expected to decrease TS levels and to render cells more sensitive to anti-TS drugs. The data presented in this thesis provides a rationale for evaluating combination therapy of anti-TS drugs and CDK4 inhibitors, since these two classes of drugs are expected to act synergistically.

4.5 Concluding remarks

Thymidylate synthase is a central enzyme responsible for the production of dTMP, a nucleotide required for DNA synthess and repair. The key role of this cellular protein has made it the target of a number of chemotherapeutic drugs that block TS activity and deplete dTMP pool, ultimately leading to apotosis of the cells. Most of the drugs targeting TS activity have been widely used for the clinical treatment of colon

cancer. However, resistance to anti-TS drugs may arise in tumours following treatment with these inhibitors, perhaps because of increase TS levels. We undertook this project to better understand the mechanisms underlying the regulation of TS in human cells, since unveiling the molecular pathway involved in the regulation of this enzyme might provide alternative therapeutic options for the treatment of colon cancer as well as other types of cancer where drugs targeting TS are widely used. In the present study, we showed that in human cells TS levels are dependent on molecular events taking place during the early-mid part of G_1. TS levels are controlled by CDK4 activity and p16INK4A levels, before passage of the cells through the Restriction Point. Interestingly, this part of the cell cycle is known to be dependent on the presence of extracellular growth factors. We have also demonstrated that TS transcription is regulated by MAP kinase pathway, a prototypic growth signalling cascade. Inhibition of MEK1 but not ERK 2 decreased TS expression levels, whereas expression of a constitutively active form of MEK 1 stimulated the activity of the TS promoter by a factor of 10. MEK1 dependent stimulation of TS transcription occurs through several sites including the Sp1 consensus site found in the TS essential promoter region. Inhibition of both CDK4 and MEK1 is associated with a significant decrease in TS levels, and they could represent interesting new targets for combination therapy with thecommonly used TS inhibitors. This study also clearly demonstrated that there are major differences in the regulatory mechanisms controlling TS expression in rodent and human cells. Despite having been shown to be important regulator of TS in rodent models, LSF E2F and GABP transcription factors do not affect TS expression in human cells.

Therefore, future studies of the regulatory mechanism controlling TS expression need to be performed in cells of human origin.

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