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REACTIVATION OF HPRT ON THE INACTIVE X CHROMOSOME
OF FEMALE EC CELLS
USING DNA DEMETHYLATING AGENTS

by

Amanda Jane Hockey

A thesis
presented to the University of Ottawa
in partial fulfillment of the
requirements for the degree of

Master of Science

© Amanda J. Hockey, Ottawa, Canada, 1989
To my parents
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CHAPTER 5. CONCLUSIONS.

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ABBREVIATIONS

SAC - 5-azacytidine
AG - azaguanine
aMEM - alpha minimal essential medium
BrdU - bromodeoxyuridine
BSA - bovine serum albumin
cDNA - DNA complementary to mRNA
EC - embryonal carcinoma
EDTA - ethylene diamine tetraacetic acid
EMS - ethyl methane sulfonate
FCS - fetal calf serum
G6PD - glucose-6-phosphate dehydrogenase
g6pd - gene encoding glucose-6-phosphate dehydrogenase
HAT - hypoxanthine-methotrexate-thymidine
HATf - grown in HAT medium
HPRT - hypoxanthine phosphoribosyl transferase
hpRT - gene encoding HPRT
HPRTm - mutant HPRT
hpRTm - gene encoding mutant HPRT
HPRTw - wild-type HPRT
hpRTw - gene encoding wild-type HPRT
HT - hypoxanthine-thymidine
MNU - methyl nitrosourea
mRNA - messenger RNA
PBS - phosphate buffered saline
PE - plating efficiency
PGK - phosphoglycerate kinase
pgk-1 - gene encoding the somatic cell isoform of PGK
POA - periodate-oxidized adenosine
RA - retinoic acid
SDS - sodium dodecyl sulfate
TCA - trichloroacetic acid
TG - thioguanine
$X_a$ - active X chromosome
Xce - X controlling element
$X_i$ - inactive X chromosome
ABSTRACT

Female mammalian somatic cells carry 2 X chromosomes, one of which is active; the other is inactive and late-replicating. The mechanisms responsible for X chromosome inactivation remain obscure, although there is increasing evidence that DNA methylation may play some role in the process. Study of the mechanisms responsible for the inactivation of an entire X chromosome may reveal control mechanisms which have broad applicability.

Female embryonal carcinoma (EC) cells provide an in vitro system for the study of X chromosome inactivation. The EC cell line C86S2 has two X chromosomes, one of which is genetically inactive (X_i). We have used MNU mutagenesis of C86 cells to isolate a mutant, C86AGM2, which carries a variant hprt allele on its active X chromosome. This variant allele encodes an HPRT enzyme which has only 0.8% of the activity of the normal enzyme, which is extremely thermolabile, and which has a slightly altered isoelectric point. Treatment of C86AGM2 cells with the DNA demethylating agents 5-azacytidine (5AC) and periodate-oxidized adenosine (POA) resulted in increased HPRT activity. This additional HPRT activity possessed thermodenaturation kinetics and electrofocusing patterns characteristic of wild type and heteromeric HPRT, indicative of co-expression of both hprt^m and hprt^w alleles.

Following 5AC or POA treatment, almost all cells in the population appeared to express some hprt^w product. However, the proportion of cells which stably expressed the hprt^w allele at levels able to sustain growth in HAT medium was very much lower, indicating that the inactive X chromosome
was expressed and subsequently repressed.

No such reactivation was observed in cells which were induced to differentiate prior to exposure to 5AC. The process of differentiation may therefore invoke stabilizing mechanisms of transcriptional repression which are refractory to reactivation attempts.

Treatment of C86AGM2 cells with POA or 5AC increased the frequency of HAT-resistant colonies by approximately 30 to 750 fold, respectively. All HAT-resistant clones isolated possessed HPRT enzyme with thermodenaturation and electrofocusing characteristics of heteromeric and wild-type HPRT, again consistent with co-expression of hprt alleles from both X chromosomes. The level of hprt mRNA was increased in all reactivants examined, and several clones also demonstrated elevated levels of pgk-1 mRNA. Furthermore, both X chromosomes replicated isocyclically with the autosomes.

In early female embryonic cells and certain lines of EC cells which possess 2 active X chromosomes, differentiation is accompanied by the inactivation of one of the X chromosomes. However, in the reactivant clones of C86AGM2 expressing the hprt genes on both X chromosomes and having 2 early-replicating X chromosomes, no evidence was seen for X inactivation following induced differentiation. Differentiated cells continued expression of both hprt™ and hprt™ alleles in each cell and no late-replicating X chromosome appeared. Thus it seems that the events of X chromosome inactivation and cellular differentiation can proceed independently.
CHAPTER 1
INTRODUCTION

1.1 INTRODUCTION

One of the unsolved challenges facing biologists today is the elucidation of the mechanisms which direct embryonic development. Although there are many genes whose products are necessary for the normal function of all cells and whose expression is shared by all tissues, a number of genes are activated in only one or a few cell types. In addition, certain genes are repressed once they have acted to the appropriate extent. In a mammalian cell, for example, only about 10 percent of the DNA is transcribed at any one time, and the particular gene sequences which are inactivated vary from cell to cell. Thus, differentiation in an organism is marked by quantitative and qualitative changes in gene expression, despite the identical genome existing within each individual cell.

Presently we have only a vague understanding of the molecular events causally responsible for the change in phenotype accompanying cell differentiation. It is clear, however, that temporal and tissue-specific regulation of gene expression in eukaryotes is accomplished by multiple control systems operating at several levels. One fundamental stage of control occurs at the level of DNA transcription, although a large body of evidence indicates that transcription is not simply regulated by a single mechanism. Still, models of gene regulation are studied in the hope of revealing control mechanisms which may have broad applicability. One such model is the mammalian X chromosome.
The subject of this thesis is the control of mammalian X chromosome activity. The X chromosome of female mammals provides an excellent opportunity to study gene regulation, as normal embryo development involves a near-permanent coordinate inactivation of thousands of genes on an entire X chromosome. This unique developmental event is interesting in itself, and an understanding of X chromosome regulation may improve our comprehension of other gene regulatory mechanisms.

1.2 THE MAMMALIAN X CHROMOSOME

Female mammals have two X chromosomes, one inherited from each parent, while males have a single X in addition to a Y chromosome. The Y chromosome is the primary determinant of sex (Ohno, 1967). The X and Y sex chromosomes are nonhomologous and heteromorphic, with the X being relatively large (comprising approximately 5% of the mammalian haploid genome), and the Y chromosome relatively small.

1.3 THE INACTIVE X CHROMOSOME

Early in embryo development of all eutherian mammals, at about the time the embryo implants in the uterine wall, one entire X chromosome in female cells is genetically inactivated and becomes condensed and inert. This process, termed X-inactivation (reviewed by Lyon, 1972; Martin, 1982), has evolved in mammals to compensate for sex differences in X chromosome dosage, and effectively equalizes between males and females the products of X-linked genes not involved in sex differentiation (Lyon, 1961). This process not only brings about dosage compensation between normal 2X females and 1X males, but it also compensates for any additional X chromosomes.
generated through meiotic or early mitotic errors. The inactivation process seems to inactivate, without limit, all but one X chromosome in a cell with X-chromosome aneuploidy but autosomal euploidy (Funderburk et al., 1981; Higeon et al., 1981; Willard and Breg, 1980).

The inactive X chromosome is characteristically heterochromatic and transcriptionally silent, and replicates its DNA entirely during the late S phase of the cell cycle (termed "late-replicating"). In contrast, the active X chromosome remains euchromatic, transcriptionally active, and replicates isocyclically with the autosomes (Epstein et al., 1978; Takagi, 1974; Monk and Harper, 1978; Graves and Cartler, 1983). During interphase, the inactive X is visible as a darkly-staining, peripheral nuclear structure called the sex chromatin or Barr body after its discoverer, the Canadian cytologist M.L. Barr. This inactive X has been observed in normal somatic cells of all female mammals examined. Each female somatic cell thus contains an active (X_a) and an inactive (X_i) X chromosome.

In eutherian mammals, the choice of which X will be inactivated in cells of the embryo proper is random. Either the paternally- (X_p) or maternally- (X_m) derived X chromosome can be inactivated such that the developing embryo and the resulting adult is a mosaic with respect to X chromosome expression (McMahon, Fosten and Monk, 1983).

Following X inactivation, the parental inactive state is maintained and stably inherited in all the mitotic descendants of somatic cells in vivo (Migeon, 1972; Gartler and Andina, 1976; deJonge et al., 1982; Graves and Young, 1982) and in culture (Davidson et al., 1963; Rattazi and Cohen, 1972; Ray et al., 1972). Only rare localized derepressions of discrete regions have been observed (Kahn and DeMars, 1975) despite a variety of treatments
aimed at reactivation. However, one major exception to the rule is 5-azacytidine treatment, which reactivates many genes on the X chromosome. This exception will be discussed in detail in section 1.9. Also, recently Wareham et al. (1987), using mice with Searle's X-autosome translocation, demonstrated that the silent gene encoding ornithine carbamoyl transferase (OCT) on the inactive normal X chromosome is reactivated during ageing.

A central feature of the inactivation mechanism is its chromosomal nature. All of the inactivated genes are on one X, while its homologue contains the active alleles; there are no complementary active or inactive regions on both chromosomes. However, as judged by cytological (Schempp and Meer, 1983) and biochemical (Race et al., 1975; Mohandas et al., 1979; Shapiro et al., 1979) evidence, there is a small region on the distal portion of the short arm of the human X chromosome which appears to escape inactivation. Three genes from this area have been shown to be nonrepressed: the Xg locus, which specifies an X-linked erythrocyte surface antigen and escapes X-inactivation in hematopoietic cells (Weatherall et al., 1970; Fialkow, 1970; Race and Sanger, 1975); the cell surface antigen MIC2X (Goodfellow et al., 1984); and steroid sulfatase (STS) (Mohandas et al., 1979, 1980; Shapiro et al., 1979), an enzyme important in hormone metabolism. This region may not entirely escape from the constraints of X inactivation, however, as recent work suggests that the inactive X may express less STS than its active homologue (Migeon et al., 1982; Lykkefeldt et al., 1984; Muller et al., 1980; Chance and Gartler, 1983; Ropers et al., 1981).
1.4 X CHROMOSOME ACTIVITY DURING EMBRYOGENESIS

Prior to the onset of X inactivation in the developing mammalian embryo, both X chromosomes are transcriptionally active and replicate isocyclically with the autosomes (Epstein et al., 1978; Monk and McLaren, 1981). Subsequent X inactivation, however, does not occur in all cells of the embryo at the same time (Epstein et al., 1978; Monk and Harper, 1979; Rastan et al., 1980; Monk 1981; Takagi et al., 1982), nor are the mechanisms by which it is achieved necessarily the same in all cell types.

X inactivation first occurs in cells of the trophectoderm and the primitive endoderm, concomitant with their differentiation at or prior to 3.5 and 4.5 days of gestation, respectively. In both of these cell populations, which contribute exclusively to extraembryonic tissues, the X chromosome of paternal origin is preferentially inactivated (Takagi and Sasaki, 1975; West et al., 1977; Harrison and Warburton, 1986; reviewed in Gartler and Riggs, 1983). Also, the inactive X in these cells initially replicates early in the S phase of the cell cycle, with a subsequent shift to late-replication (Takagi, 1974; Takagi et al., 1982). These two characteristics, preferential Xp inactivation and initial early replication, suggest that the two events may be related and that there are differences in the X inactivation mechanism between certain extraembryonic tissues and the embryonic ectoderm (Takagi et al., 1982; Migeon et al., 1985; Lock et al., 1987; Krumlauf et al., 1986; Migeon et al., 1986; Lock et al., 1986).

Preferential Xp inactivation is also seen in cells of female marsupials (reviewed in VandeBerg, 1983).

In the remaining cells of the embryo (the primitive ectoderm), X-inactivation occurs at 5.5 to 6.0 days of gestation, although at this time
most cells of the epiblast are undifferentiated. This population of cells gives rise to the fetus, though some descendants will contribute to the extra-embryonic tissues. In these cells, the choice of which X chromosome will be inactivated, that of maternal or paternal origin, is made at random, and the XI always displays a late replication pattern (Lyon, 1972; Takagi, 1974; Takagi et al., 1982).

1.5 CONTROL OF X INACTIVATION

Even the random process of X inactivation in embryonic lineages is under genetic control, however, since different X-linked mutants have been shown to be associated with different probabilities of inactivation. Classic genetic analyses on the mouse (Cattanach and Isaacson, 1967; Cattanach et al., 1969; 1970; Cattanach, 1970; Cattanach and Williams, 1972) provided evidence for the existence of a site responsible for controlling the X inactivation process. This site, named the X-chromosome controlling element, or Xce, has been mapped to a site in the distal region of the X chromosome between the linkage groups Ta and pgk-1 (Cattanach et al., 1970). Several different studies (Grahn et al., 1970; Krzanowska and Wabik, 1971; Falconer and Isaacson, 1972; Ohno et al., 1973) all map the X-controlling locus to the same site, indicating that it is a single element (Cattanach, 1975).

There are three known alleles at Xce which act in a cis dominant manner to determine the probability of an X chromosome containing them becoming inactivated. The Xce alleles do not dramatically alter the randomness of X inactivation. The variegation may extend from 50:50 in homozygotes to 70:30 in the Xce*/Xcec heterozygotes. It is not clear why
complete preferential inactivation is not observed in this system
(Cattanach, 1975; Gartler and Riggs, 1984).

Chromosomal rearrangements between the X chromosome and autosomes are
valuable for searching for controlling centres and studying the mechanisms
of the initiation of inactivation. When the rearranged X is inactivated
during the course of development, all of the loci lying close to the
heterochromatin are likely to be inactivated. This is called the spreading
effect (Lewis, 1950; Baker, 1964; Spofford, 1975). In X-autosome
translocations, the inactive state may spread into the autosome (Russell and
Montgomery, 1970; Russell, 1963; Ohno and Lyon, 1965), may fail to do so
(Eicher, 1970), or may "skip" proximal autosomal regions while spreading
into more distal ones (Keitges and Palmer, 1986). X chromosome inactivation
is probably initiated at a single site on the chromosome (Russel and
Cacheiro, 1978; Gartler and Riggs, 1983; Rastan and Robertson, 1985).

1.6 X CHROMOSOME ACTIVITY IN GERM CELLS

Although in somatic cells the inactive state is stable and heritable, a
reactivation event occurs in the germ line. As shown by cytogenetic,
allozyme expression, and gene dosage studies, the female germ cell lineage
undergoes a cycle of X-chromosome inactivation in the early embryo and
subsequent reactivation in oogenesis (just prior to entry into meiosis) such
that the mature mammalian oocyte possesses two active X chromosomes (Gartler
et al., 1975; Johnston, 1981; Kratzer and Chapman, 1981; reviewed by Monk,
1981, and Gartler and Riggs, 1983). Female germ cells therefore possess
cellular mechanisms which mediate reactivation of the entire previously
inactive X chromosome.
During spermatogenesis in males, the opposite situation is encountered. The single X chromosome in primary spermatocytes undergoes a precocious condensation and inactivation (Solari, 1974; Monesi, 1971) which is apparently essential for normal spermatogenesis (Lifschytz and Lindsley, 1972). DNA-mediated transfer experiments have suggested that X-inactivation in spermatocytes and extraembryonic lineages appears to be different at the molecular level from inactivation occurring in somatic cells (Kratzer et al., 1983; Venolia et al., 1984).

1.7 MODELS OF X CHROMOSOME INACTIVATION

X chromosome inactivation represents a unique kind of gene regulation in two respects. First, the active and inactive states of each gene are maintained in the same nucleus, in contrast to the typical tissue-specific genes in which both copies of a gene are either active or inactive. Secondly, the phenomenon involves not a battery of dispersed genes but most of the genes of a chromosome. The molecular principles responsible for this chromosomal regulation remain obscure, but are likely to be complex, possibly involving different mechanisms for inception of the event during development and for transmission of inactivity from one cell to its progeny.

It has been suggested that X inactivation takes place in at least three steps: (i) the initial event in which one of the two active X chromosomes in each cell is chosen for inactivation and the process is initiated; (ii) the spread of inactivation from the site(s) of the initial event along the length of the chromosome, encompassing all but the limited X-Y pairing and recombination region; and (iii) events that result in the heritable maintenance of repression of the genes on the inactivated X (Lyon,
1972; Gartler and Riggs, 1983).

Any model postulated for the X chromosome inactivation process must account for a number of observations. These have been summarized, and include:

1) stability and heritability of the inactive condition;
2) inactivation of the whole X chromosome except for a small distal portion, and presence of homologous active and inactive X chromosomes in the same nucleoplasm and cytoplasm;
3) mechanisms of imprinting, such that X's of paternal and maternal origin can be distinguished in marsupials and extraembryonic cells;
4) counting mechanisms, whereby only one X is active per diploid set of autosomes;
5) variant alleles of the X controlling element (XCA) which can affect the randomness of the inactivation choice in cells of the embryo proper;
6) reactivation in female germ cells in vivo and in somatic cells under certain conditions in vitro.

A number of models have been proposed, and these include episomal integration events (Crumbach et al., 1963) and the existence of inversion sequences (Gartler and Riggs, 1983), as well as models involving changes in DNA conformation (Gartler and Riggs, 1983) or binding of non-histone proteins (Ohno, 1969, 1973; Lyon, 1972). A simple model proposed by Comings in 1968 entails a single attachment site on the nuclear membrane for one X chromosome, and some evidence for this has been provided by recent high
voltage electron microscopy studies which indicate the possibility of a special nuclear envelope attachment region (Dyer et al., 1985).

The preferential paternal X inactivation occurring in the somatic cells of marsupials and in the extraembryonic membranes of eutherian mammals implies that the parental X chromosomes are chemically and/or structurally different within the same cell. This marking or imprinting is thought to occur during processes which accompany gametogenesis (Chandra and Brown, 1975; Surani et al., 1986). Recent work has provided evidence for heritable molecular differences between maternally and paternally derived alleles based on differential levels of DNA methylation (Sapienza et al., 1987; Reik et al., 1987). This distinction between $X_m$ and $X_p$ in eutherian mammals is believed to be transient and therefore lost during embryogenesis, as the cells of the epiblast (which differentiate late) demonstrate random inactivation. In addition, the imprinting phenomenon is thought to be independent of the mechanisms which initiate inactivation, as parthenogenetic embryos and ovarian teratomas, which possess only maternally-derived X chromosomes, demonstrate random X-inactivation even in tissues normally exhibiting preferential $X_p$ inactivation (Gartler and Riggs, 1984; Kaufman et al., 1978; Rastan et al., 1980).

It has become increasingly apparent that once initiated, the maintenance of the inactive state of an X chromosome is mediated by post-replication modification of the DNA structure. Using DNA transformation studies, Liskay and Evans (1980) demonstrated that DNA from the inactive X chromosome of somatic tissues will not function in DNA-mediated transformation of the hpert gene, whereas the same hpert gene from an active X does function (Liskay and Evans, 1980; Chapman et al.,
1982; Lester et al., 1982; Venolia and Cartler, 1983). This suggests that
the inactive X chromosome has been modified at the DNA level. In 1975,
Riggs, and independently, Holliday and Pugh, proposed that inactivation
could be carried out and stably maintained by a system which differentially
methylated the X chromosomes.

1.8 DNA METHYLATION

Of several known modifications of DNA, methylation of the pyrimidine
base cytosine appears to be the only one to occur in mammals. This
post-replication methylation is a well analyzed phenomenon, and a number
of features render it an attractive system to mediate heritable patterns of
gene expression. Cytosine is the only methylated base found in DNA of
higher eukaryotes, and in mammals, between 2 and 7% of the total genomic
cytosine (depending on the species) is converted to 5-methylcytosine (5mC).
The enzymatic methylation takes place immediately following DNA replication
(Kautiainen and Jones, 1985) by the transfer of a methyl group from
S-adenosyl-methionine (SAM) to position 5 of a cytosine base in the newly
replicated strand of DNA (Shield et al., 1968).

In mammalian DNA, the distribution of 5mC is non-random, with
approximately 90% of these residues occurring in the dinucleotide sequence
CpG (Doskoci1 and Sorm, 1962; Salomon and Kay, 1970; Sneider, 1972; Gerlach
and Bedbrook, 1979), which is underrepresented in eukaryotic DNA (Josse et
al., 1961). This CpG doublet sequence is palindromic, thus allowing
symmetrical methylation of the complementary strand. Because the
methyltransferase enzyme acts preferentially on hemimethylated DNA strands
(Gruenbaum et al., 1982), and since DNA replication is semi-conservative,
the pattern of cytosine methylation on the parental DNA strand becomes a template for the methylation of the daughter DNA strand, and the distribution pattern of 5mC within the genome is therefore a stable and somatically heritable trait (Wigler et al., 1981). Methylation therefore represents a possible mechanism of genetic regulation at the level of individual genes or of genetic domains, or of whole chromosomes, such as the inactive X chromosomes (reviewed in Riggs, 1975; Holliday and Pugh, 1975; Razin and Riggs, 1980 and Doerfler, 1983).

In addition to methylation maintenance, patterns of methylation can be altered and repaired by sequence-specific de novo methylation; both de novo and maintenance activities appear to reside in the same enzyme (Bestor and Ingram, 1983; Sano et al., 1983; Pfeifer et al., 1983; Razin and Szyf, 1984). During vertebrate differentiation, decreases in DNA methylation as well as de novo methylation occur frequently (Erlich and Wang, 1981; Cooper, 1983; Gama-Sosa et al., 1983).

The conversion of cytosine to 5-methylcytosine introduces a methyl group into an exposed position in the major groove of the DNA helix. Methylation at this location does not interfere with normal base pairing; however it can influence binding of some proteins (Lin and Riggs, 1972; Huang et al., 1984). Thus, DNA methylation may exert its effect on gene transcription by affecting chromatin formation (Keshet et al., 1986), or by altering specific and/or nonspecific interactions between DNA and nuclear proteins. Recent findings suggest that the sequence specificity of the DNA methyltransferase enzyme may be conditional on the physical conformation of the DNA substrate (Kautiainen and Jones, 1985; Bestor, 1987).
Evidence indicating that the 5mC pattern present in DNA is a component of the multilevel control of gene expression has accumulated in many systems (Razin and Riggs, 1980; Ehrlich and Wang, 1981; Doerfler, 1983). In general, methylation of critical sites indicates gene inactivity (Busslinger et al., 1983; Lieberman et al., 1983; Stein et al., 1983, reviewed in Razin and Szyf, 1984, and Jaenisch and Jahner, 1984), although there are several exceptions to this rule (Grainger et al., 1983; Perry, 1983; Burch and Weintraub, 1983; Gerber-Huber et al., 1983). Methylation levels of 5' sequences has been shown to correlate most closely with gene activity (Busslinger et al., 1983). In addition, agents which induce DNA hypomethylation can induce activation of specific genes (Jones and Taylor, 1980; Compere and Palmiter, 1981; Groudine et al., 1981; Venolia et al., 1982; Jones, 1985).

A more direct link between DNA methylation and gene expression was obtained by DNA microinjection and transfection experiments. Studies using this strategy demonstrated that in vitro methylation of certain sites abolished the activity of several cloned cellular (Wigler et al., 1981; Stein et al., 1982) and viral (Fradin et al., 1982; Simon et al., 1983; Kruczek and Doerfler, 1983; Jaenisch and Jahner, 1984) genes when transferred into eukaryotic cells. These experiments suggest a cause and effect relationship between methylation at specific sites and gene repression.

However, some controversial results concerning the relationship between the DNA methylation pattern and gene activity have been reported showing either a good correlation (McGhee and Ginder, 1979; Vedel et al., 1983) or no correlation at all (Gerber-Huber et al., 1983; Vander Floeg and
Flavell, 1980; Wilks et al., 1984) between undermethylation and expression of a specific gene. Recent studies indicate that undermethylation of only certain critical sites located in the 5' region of genes coding for tissue-specific proteins is necessary (but not sufficient) for their expression (McKeon et al., 1982; Ott et al., 1982; Wilks et al., 1984). These observations suggest that methylation stabilizes, rather than causes, gene repression.

1.9 DNA METHYLATION AND THE X CHROMOSOME

Three lines of indirect evidence implicate DNA methylation in the control of X chromosome inactivation. First, as discussed previously, X\(_i\) DNA does not function efficiently in DNA transfection experiments. Secondly, treatment of cells with 5-azacytidine (5AC), which causes heritable hypomethylation of cytosine in the DNA, can result in reactivation of genes on the X\(_i\) (Mohandas et al., 1981; Lester et al., 1982; Graves, 1982; Hors-Gayla et al., 1983; Paterno et al., 1985; Lock et al., 1986). Moreover, DNA isolated from such 5AC-induced reactivants can now perform efficiently in transformation studies (Lester et al., 1982; Venolia et al., 1982). Third, certain regions and critical sites of mouse and human X-linked genes are differentially methylated when carried on the active versus inactive X chromosome (Yen et al., 1984; Wolf et al., 1984a, 1984b; Wolf et al., 1985; Toniolo et al., 1984; Riggs et al., 1985; Lock et al., 1986; Keith et al., 1986), and several of these regions are apparently conserved between mammalian species.

There are, however, some inconsistencies which suggest that X inactivation is maintained by more than just methylation. In extraembryonic
membranes, for example, the inactive X is apparently not regulated by DNA modification, as genes on the X, are capable of performing efficiently in DNA transformation (Kratzer et al., 1983). Also, in normal human diploid cells, 5AC does not appear to significantly reactivate the inactive X chromosome (Wolf and Migeon, 1982), whereas the frequency of 5AC induced reactivation in hybrid cells is $10^{-2}$ to $10^{-4}$ (Kahan and DeMars, 1975, 1980; Mohandas et al., 1981; Lester et al., 1982; Graves and Young, 1982; Hors-Cayla et al., 1983). It appears that the foreign environment of an interspecies hybrid (usually human-rodent) differs from that of normal human cells, as spontaneous and induced reactivation of loci on the inactive X occurs in the former, but not in the latter.

A similar observation has been made in this laboratory using mouse embryonal carcinoma cells, where 5AC increases the frequency of reactivation in some cell lines but not in others (Paterno, et al., 1985). These findings suggest that an additional restraint besides methylation is imposed on the inactive X in some, if not all tissues.

Recently, Lock et al. (1987) have shown that methylation of certain sequences on the inactive X does not take place until several days after inactivation has occurred. This indicates that methylation cannot play a significant role in the initiation or primary maintenance of X inactivation, and reinforces the suggestion that methylation of DNA acts as a secondary or "locking" mechanism.

A two step model of X inactivation was proposed by Cartler et al. in 1985. They postulated that 2 distinct and successive events occurred in X chromosome inactivation; the first event being heterochromatinization, the second, differential DNA methylation. The methylation acts as a "failsafe
"repressor" which stabilizes inactivation, although each individual step is sufficient for maintaining repression of X-linked genes. According to the model, sex chromatin formation is the only inactivation event taking place in extraembryonic cells, whereas heterochromatinization and methylation occur in cells of the embryo proper. This allows an explanation of the unusual behaviour of the inactive X from extraembryonic tissues, including incomplete dosage compensation, ability to function in DNA transformation assays, and complete reactivation upon fusion with cells of another species. Recent experiments indicate that, indeed, Inactive X DNA from extraembryonic tissue is less methylated than that from cells of the fetus (Wolf et al., 1984; Wolf et al., 1985).

This two step model also explains a number of other observations. The reactivatability of inactive X chromosomes retained in interspecies hybrids is postulated to be a result of the apparent absence of sex chromatin in these cells. If the heterochromatinization step of repression has been reversed, demethylation should induce frequent reactivation, but only at independent loci. Also, the rare reactivation events sometimes seen in normal diploid cells following treatment with 5AC may represent a localized destabilization of the locking mechanism, allowing an increased frequency of reactivation of genes on the inactive X.

All of these features, as well as the fact that 5AC treatment can activate genes in more than 30 different systems in addition to X-linked genes, and that some genes methylated in vitro were found to be inactive after their transfer into recipient cells, suggest that methylation very likely plays an important part in control of gene expression. Therefore, although this regulatory strategy is not unique to the X chromosome, it does
not rule out a significant role in the phenomenon of X inactivation. Experimental attempts to reactivate the inactive X chromosome may provide clues about the molecular mechanism(s) that are responsible for maintaining the inactive state.

1.10 REACTIVATION OF THE INACTIVE X CHROMOSOME

The mechanisms which have evolved to maintain the inactive X have resulted in a process which is remarkably stable. Except in germ cells, where reactivation occurs normally, and in extraembryonic tissue where partial reactivation has been demonstrated (Migeon, 1985), inactivation is highly stable in normal somatic cells derived from the embryo proper. Within an organism, somatic cell clones have shown no evidence for any reactivation of X-linked genes (for reviews see Cartler and Riggs, 1983; Graves, 1983), and in culture, cells have resisted reactivation of their silent X chromosome by a number of various chemical and physical treatments (Comings, 1966; Migeon, 1972; Romeo and Migeon, 1975; Kahan, 1978; Migeon et al., 1978).

However, as discussed in the previous section (1.9), treatment of certain cell lines with DNA demethylating agents can induce the reactivation of genes of the inactive X chromosome. Cells subjected to these drugs undergo generalized DNA demethylation and may reactivate genes carried on the inactive X in up to 10% of surviving cells (Paterno et al., 1985). Once reactivated by 5AC treatment, the active transcriptional state for these X-linked genes is remarkably stable, even after removal of the drug.

In 1981, Mohandas et al first demonstrated that 5-azacytidine treatment of a mouse-human cell hybrid resulted in a 1,000 fold increase in
the frequency of expression of the wild-type hypoxanthine phosphoribosyl transferase (hprt) allele on the inactive human X chromosome. Expression of this allele in the normally HPRT-deficient cell line enabled the cell to form HAT-resistant colonies. In addition, two unselected human X-linked markers, glucose-6-phosphate dehydrogenase (G6PD) and phosphoglycerate kinase (pgk-1), were expressed in a small proportion of the HPRT⁺ cells. Similar studies using HPRT-deficient human-mouse hybrids (Lester et al., 1982; Hors-Cayla et al., 1983) and HPRT-deficient Mus musculus × M. caroli lines (Graves, 1982) confirmed that 5-azacytidine treatment increased the appearance of HPRT⁺ colonies and that occasionally reactivants expressing all four human markers, g6pd, pgk-1, alpha-gal, and hprt could be recovered (Hors-Cayla et al., 1983; Graves, 1983). In each of these cases, however, the reactivation was not a whole chromosome event, nor did the reactivants carry two synchronously-replicating X chromosomes.

The reactivants did show variable levels of expression of gene products (Lester et al., 1982), indicating perhaps an influence of varying degrees of demethylation in and around the reactivated gene itself or in neighboring regions which are themselves responsible for maintaining genetic inactivity of localized segments of the inactive X chromosome.

In this laboratory, Paterno et al. (1985) demonstrated reactivation of the inactive X chromosome in an HPRT-deficient female embryonal carcinoma cell line named C86S1A1. Treatment of these cells with 5-azacytidine resulted in the transient expression in almost all cells of elevated levels of HPRT and three other enzymes encoded by X-linked genes, and stable expression of HPRT in up to 5 to 20% of surviving cells. Furthermore, in most of the clones which stably expressed HPRT, both X chromosomes
replicated in early $S$ phase. This total reversal of the heterochromatic pattern of the inactive X was not observed in the above described cases. Even without selection, 5-azacytidine-induced X-chromosome reactivation appears to be extremely stable, as loss of HPRT$^+$ activity was always associated with the loss of the X chromosome carrying the reactivated $hp$rt allele (Mohandas et al., 1981; Graves, 1982; 1983; Lester et al., 1982; Paterno et al., 1985).

In addition to interspecific cell hybrids and cultures of mammalian tissues then, embryonal carcinoma cells can be used to study reactivation events. Certain cell lines of these undifferentiated cells have been shown to reactivate loci on their inactive X, both spontaneously and with treatment, without the prior requirement of interspecific cell fusion. The observed reactivation in some embryonal carcinoma cells may reflect a similarity of these cells to early (6 to 7 day post coitum) cells of the differentiating embryo, or to cells committed to differentiate into extraembryonic-type cells.

1.11 EMBRYONAL CARCINOMA CELLS

Studies of the details and molecular events associated with X chromosome inactivation are hampered by the inaccessibility and small size of the embryo (16-cell morula stage) at the time X inactivation occurs, as well as the asynchrony of the X inactivation process within different embryo tissues. For these reasons, it is useful to have a more convenient system with which to study events involved in regulation of X chromosome expression. Embryonal carcinoma cell lines have provided such an alternative developmental system due to their embryonic-like developmental
age, ease of manipulation and ability to grow in large numbers.

Embryonal carcinoma (EC) cells are the undifferentiated stem cells of malignant tumours known as teratocarcinomas (Stevens, 1967; Pierce, 1967). These tumours arise spontaneously in the gonads of a variety of species, or can be induced experimentally by transplantation of an early embryo to an extrauterine site. The subsequent disorganized growth of the embryo forms the tumour, which is composed of a wide variety of differentiated cells in addition to a core of pluripotent stem cells.

That EC cells resemble early embryonic cells, and therefore represent a valid system for the study of developmental events, has been demonstrated by the common morphological (Pierce and Beals, 1964; Lo and Gilula, 1980), biochemical (Damjanou et al., 1971; Bernsteine et al., 1973), and antigenic (Artzt et al., 1973; Jacob, 1977; Reisner et al., 1977; Solter and Knowles, 1978; Kermeler et al., 1977; Boller and Kemler, 1982; Harris et al., 1986) characteristics shared by both cell types. In addition, EC cells are developmentally pluripotent and as such are capable of differentiation along multiple developmental pathways (Kahan and Ephrussi, 1970; Martin and Evans, 1975; Nicolas et al., 1975; McBurney 1976). Indeed, when individual EC cells are injected into a blastocyst, they participate in normal development of the embryo and can be found in most types of differentiated tissues in the resultant chimeric adult (Brinster, 1974; Papaioannou et al., 1976; Dewey and Martin, 1980; Rossant and McBurney, 1982). However, some lines of EC cells retain only restricted capacity to differentiate, forming only cells of certain lineages, and therefore may be arrested at different stages of embryonic development.
The feature which renders EC cells of most use is their adaptability to tissue culture. In vitro, these cells retain their pluripotentiality and can be grown easily in large numbers to provide a virtually limitless source of experimental material. Moreover, under appropriate culture conditions (such as aggregation or exposure to drugs such as retinoic acid or dimethylsulfoxide), EC cells can be induced to differentiate into most, if not all, of the different cell types initially present in their tumour of origin (Martin et al., 1978; McBurney and Strutt, 1980; Jones-Villeneuve et al., 1982; Takagi and Martin, 1984). In addition, as discussed below, EC cells retain the major properties of X chromosome dosage compensation that occur in embryological development.

1.12 THE X CHROMOSOME IN EC CELLS

The resemblance of EC cells to early embryonic cells led to the suggestion that female EC cells may possess two active X chromosomes, and that one would be inactivated upon differentiation of the cells in culture (McBurney and Adamson, 1976; Martin et al., 1978; McBurney and Strutt, 1980). Biochemical and cytogenetic studies of a female EC cell line (P10) heterozygous for pgk-1 showed that all clonal populations of these cells contained equal levels of both PGK-1 isozymes (PGK-1a and b) suggesting that these cells have two active X chromosomes. These cells also possessed two synchronously replicating X chromosomes (McBurney and Strutt, 1980). Martin et al. (1978) and Takagi and Martin (1984) described similar results for another EC cell line. Upon differentiation of these cells in culture, 50% decreases in the level of X chromosome gene products (Martin et al., 1978) and the appearance of allocyclic X chromosomes in the majority of
differentiated cells (McBurney and Strutt, 1980; Takagi and Martin, 1984; Paterno et al., 1985) indicated differentiation in these cells is accompanied by the inactivation of one X chromosome in each cell.

Different lines of female EC cells have been shown to represent a number of different developmental stages in the process of X inactivation. In this laboratory, four lines of female EC cells have been characterized which each possess unique X chromosome characteristics. One cell line (P10) carries 2 active X chromosomes, both of which are early replicating, and upon differentiation of these cells one X becomes inactive. Three other cell lines (C100, C86, and C145) each possess only 1 active X chromosome, but the inactive X is late replicating in only two of these cell lines (C86 and C145). Furthermore, treatment of cells C100 and C86 with 5-azacytidine results in the reactivation of the inactive X chromosome, whereas C145 is apparently unable to reactivate the inactive X. These characteristics indicate that these four female EC lines may represent embryonic cells arrested at various intermediate developmental stages in the X inactivation process (Paterno et al., 1985). Embryonal carcinoma cells, then, are an experimentally valuable system for studying the regulation of X chromosome differentiation during development.

1.13 THE THESIS PROJECT

The inactivation of an X chromosome in all cells of the female mammalian embryo is a unique and important developmental event. Elucidation of the mechanisms which direct this process would solve a 30-year-old dilemma, and may provide an insight into other aspects of regulation of gene expression. We therefore propose to study the control mechanisms underlying
X inactivation by inducing reactivation of the inactive X chromosome in female murine embryonal carcinoma cells.

Although interspecific hybrids are frequently used for such reactivation experiments, we have chosen the EC cell system for two reasons. First, EC cells more closely resemble the normal cellular environment in which X inactivation takes place, whereas interspecific hybrids may or may not retain all of the appropriate regulatory mechanisms present in a normal cell. Secondly, unlike hybrid cells, EC cells can be induced to differentiate, allowing the study of the effects of differentiation on a reactivated X chromosome.

Because the murine EC cell lines available to us which are capable of reactivating their inactive X chromosome do not possess any heterozygous X-linked markers, our approach has been to develop a cell line expressing a qualitatively distinguishable marker which allows distinction of each X chromosome. The target enzyme selected for these studies was the X-linked hypoxanthine-guanine phosphoribosyl transferase (HPRT). This enzyme converts hypoxanthine (Hx) or guanine to its corresponding nucleotide, IMP or GMP, and thus channels these purines into nucleic acid synthesis. There is a single HPRT structural gene in the mammalian X chromosome (Seegmiller et al., 1967; Miller et al., 1971; Chapman and Shows, 1976).

HPRT was the X-linked marker of choice because its presence is readily assayable either directly in cell-free extracts or indirectly in intact cells, and because of the availability of selective methods for isolating variants having lost or regained this enzymatic activity. Szybalski, Szybalska and Ragni (1962) demonstrated that human D98 cells that were partially or completely deficient in HPRT activity could be isolated by
selecting cells that were resistant to toxic purine analogues such as 8-azaguanine (8AG) or 6-thioguanine (6TG). Such variants remain viable because purine nucleotides can be synthesized by de novo pathways in the absence of HPRT.

Cells which have subsequently regained their HPRT activity can be selected by blocking de novo purine biosynthesis with aminopterin and simultaneously supplying hypoxanthine. HAT (hypoxanthine, aminopterin, thymidine) medium has been developed for this purpose (Szybalski et al., 1962). Sharp, Capecchi and Capecchi (1973), and Fenwick and Caskey (1975) have described revertants of HPRT-deficient mouse cells that have enzymes which differ from that of the wild-type cells in heat inactivation rates or kinetic properties.

Another advantage of the use of the HPRT enzyme as a marker for reactivation is its multimeric structure. Under physiological conditions, HPRT exists as a tetramer (Holden and Kelley, 1978; Johnson et al., 1979), although dissociation to form dimers is seen in solutions of low ionic strength (Johnson et al., 1979). Study of a multimeric X-linked marker enzyme in reactivation experiments is valuable because should different enzyme subunits be produced within the same cell, heteropolymers will be formed. If the marker HPRT possesses characteristics qualitatively distinguishable from the wild-type, a heteropolymer should retain characteristics intermediate to the mutant and WT homopolymers.

Previous reactivation experiments on EC cells in this laboratory have made use of quantitative differences in HPRT activity to assess the active state of each X chromosome (see section 1.10). Evidence that the quantitative change in HPRT activity levels seen in C86S1A1 cells following
5AC treatment was due to reactivation of the X, was derived partially from studies which indicated that cells which had lost the inactive X chromosome did not respond in a similar manner. However, due to the lack of any X-linked markers, the possibility of a reversion event or other phenomena resulting in increased HPRT levels could not be ruled out.

This thesis attempts to provide definitive proof for the reactivation of the inactive X chromosome in C86 EC cells via the isolation of, and investigation of reactivation attempts on, a cell line which possesses a qualitatively distinguishable X-linked marker. Further studies, including the effects of differentiation on reactivants, are also discussed.

This first chapter of my thesis has attempted to provide some salient background information and to give an overall introduction to the experimental work that was performed. Chapter 2 details the procedures and techniques used for these experiments. Chapter 3 describes the derivation and isolation of several mutant cell lines possessing qualitatively altered HPRT enzymes, and Chapter 4 gives the results of reactivation experiments performed using one of these mutant cells. The latter two chapters also include a discussion of the experimental results. Chapter 5 presents conclusions drawn from this work and suggestions for further studies.
CHAPTER 2
MATERIALS AND METHODS

2.1 TISSUE CULTURE TECHNIQUES

2.1.1 CELL LINES

All cell lines used for these studies are female embryonal carcinoma (EC) cells derived from teratocarcinomas of C3H/He strain mice. The C86 line was initiated from cells of the C3H tumour 86 originally produced by the extrauterine grafting of 7.5 day old C3H embryos (Iles, 1976). C86S1 and C86S2 are two clonal EC cell lines grown from single cells of the C86 cell population (McBurney, 1976). These clonal lines are slightly aneuploid, containing 41 chromosomes including a trisomy for chromosome 11 and an XX sex chromosome constitution. Initial feeder dependance of C86S2 diminished with time in culture.

In this report, clones are named according to the manner in which they were isolated. Clones with the prefix AG or TG are AG- or TG-resistant, respectively. A suffix of Mn or ηn indicates the cell was originally mutated by either methyl nitrosourea (MNU) or 6-irradiation respectively, where n is an arbitrary clone number. Clones selected for reversion to HAT² after mutagen treatment are indicated "rv", whereas clones reactivated spontaneously, or by exposure to 5-azacytidine (5AC) or periodate-oxidized adenosine (POA), are termed re5Pn, reACn, or rePOAn, respectively. All cell lines possess similar differentiation capabilities to the parental C86 EC cell line.
2.1.2 CULTURE CONDITIONS

All cells were routinely cultured in plastic tissue-culture grade petri dishes (Falcon) in culture medium (CM) consisting of alpha minimal essential medium (aMEM) (Gibco, Long Island N.Y.) supplemented with 10% fetal bovine serum (Bocknek). Cultures were maintained in a humidified atmosphere (5% CO₂, 95% air) at 37°C unless otherwise stated.

For the routine passage of cells, cultures were treated for 5 to 10 minutes with a Ca⁺⁺ and Mg⁺⁺ -free phosphate buffered saline (PBS) containing 0.025% trypsin and 1 mM ethylene diamine tetraacetic acid (EDTA) (Dulbecco and Vogt, 1954) to facilitate detachment from the plate surface. Subsequent vigorous pipetting resulted in a single cell suspension which was used to seed new cultures. EC cells were generally maintained at relatively high cell density and subcultured at 2 day intervals.

In plating efficiency (PE) experiments, single cells in suspension were counted using a Coulter Counter and Channelyser (Coulter Electronics Inc., Hialeh, Florida) and known numbers of cells were plated in the presence of 10⁻⁴ M beta mercaptoethanol (Oshima, 1978). All cell lines were replaced from frozen stocks at regular intervals.

2.1.3 RETINOIC ACID-INDUCED DIFFERENTIATION

Differentiation of EC cells was induced by plating a monolayer of 10⁶ cells per 100 mm dish in the continuous presence of 10⁻⁷ M all-trans retinoic acid (RA). The RA-containing medium was replaced every two days, and cultures were not otherwise disturbed for 10 to 15 days.
2.1.4 EXPOSURE OF CELLS TO MUTAGENS

Cells to be treated with mutagens were generally plated (at 10^5 cells/mL) one day prior to treatment to ensure exponential growth phase of attached cells. Methyl nitrosourea at various concentrations was dissolved in 70% ethanol immediately before use and kept on ice. Treatment with MNU was for 2 hours in medium containing 5% FCS; the ethanol concentration was never greater than 1% during this treatment period. After treatment, the medium was changed, and the cultures were incubated and subcultured as usual for 5 days to allow growth and expression of any induced mutations (Erickson et al., 1978; Bradley et al., 1980).

Ethyl methanesulfonate (EMS; Eastman) was added directly to the culture medium at between 100 and 300 ug/mL (as described) and the cells incubated for 15 hours. Following EMS exposure, treated cells were washed 3 times in serum-free medium and fresh culture medium was added; cells were allowed to recover for 48 hours before selection was applied (Brown and Thacker, 1984; Brown et al., 1985).

γ-irradiation (300 Rads) was administered using a 2000 Ci Cesium-137 source (CNRL) delivering approximately 71.2 R/minute. Exponentially growing cells were trypsinized and irradiated while in a single cell suspension in serum-containing medium. Irradiated cells were replated immediately following treatment and the medium was changed approximately 6 hours after cells had attached. Cells were plated for selection experiments after a 3 day recovery and expression period (Brown and Thacker, 1984; Brown et al., 1985).

Mutagen toxicity was evaluated either by plating cells immediately after treatment to determine plating efficiency (PE) relative to untreated
cells, or by treating a known number of cells with mutagen after they were placed and allowing colonies to form undisturbed. Similar results were obtained using both procedures. The plating efficiency of control cells varied between 8 and 50%.

2.1.5 EXPOSURE OF CELLS TO DNA DEMETHYLATING AGENTS

Cells were exposed to 5-azacytidine (5AC) essentially as described by Mohandas et al. (1981). 10^5 cells per mL were plated into tissue culture grade dishes, and 24 hours later prewarmed medium containing freshly prepared 5AC was added at the concentration specified (usually 3 or 5 μM). Following a 24-hour exposure to the drug, the medium was removed and the cells washed twice with serum-free medium before fresh serum-containing medium was added. Cells were maintained in normal growth medium and allowed to recover for the indicated amount of time (usually 24 hours) before they were prepared for plating or harvesting.

Cells treated with periodate-oxidized adenosine (adenosine-2',3'-dialdehyde) were plated in a similar manner to that described above. The concentration of POA was usually 1 μM and treatment time varied from 1 to 4 days, as indicated ( Litecoino and Kerbel, 1986).

2.1.6 SELECTION PROCEDURES

For selection, known numbers of cells (usually 10^6) were plated into 100 mm diameter tissue culture dishes containing 15 mL of culture medium supplemented with drug. Ouabain-resistant cells were selected in 3 mM ouabain diluted directly into the medium. Cells to be selected for HPRT- phenotype were plated in the toxic guanine analogues 8-azaguanine (10
ug/mL) or 6-thioguanine (2-amino-6-mercaptopurine) (5 ug/mL). Revertants or reactivants selected for HPRT\textsuperscript{+} phenotype were grown in HAT medium (containing hypoxanthine (15 ug/mL), methotrexate (0.1 ug/mL) and thymidine (15 ug/mL) (Littlefield, 1964). Methotrexate blocks \textit{de novo} synthesis of purines; only those cells expressing the purine salvage enzyme HPRT will survive. All selection media contained 10\textsuperscript{-4} M \textit{N}-mercaptoethanol.

All selection plates were incubated for 7 to 10 days before clones were picked into separate culture vessels, or screened for colonies by staining with 1% methylene blue in 70% ethanol. During the period of colony growth, the medium was not changed in order to avoid problems of colony splitting. All drugs remained active during this period.

A colony was defined as containing greater than 50 cells, and the mutant or reactivant frequency per colony-forming unit (or per surviving cell) was calculated by dividing the observed colony frequency by the plating efficiency of cells not exposed to selective agents. The plating efficiency was always determined at the same time as the mutant frequency.

Picked colonies were grown to 5 x 10\textsuperscript{5} cells in their respective selective media, at which time they were cultured in regular medium, except for those colonies selected in HAT. Prior to growth in culture medium, HAT\textsuperscript{c} colonies were grown for 3 passages in hypoxanthine (15 ug/mL) - thymidine (15 ug/mL) (HT) - containing medium.

2.2 CHEMICALS AND PREPARATION OF DRUGS

All chemicals used in this study other than radio-labelled chemicals (Amersham) were obtained from Sigma Chemical Company unless otherwise stated. Where indicated, drugs were prepared fresh before use; others were
prepared from stock solutions.

Retinoic acid was prepared as a $10^{-2}$ M stock solution in 95% ethanol and stored in the dark at 4°C. Working concentrations were achieved from dilutions directly into medium before use from a stock solution of $10^{-5}$ M prepared fresh weekly. 8-Azaguanine (AG) was stored as a 10 mg/mL solution in dimethyl sulfoxide (DMSO). 6-Thioguanine was kept at 4°C as a 5 mg/mL solution in PBS. Beta-mercaptoethanol was prepared fresh before use at $10^{-2}$ M in PBS. HAT medium, containing 15 ug/mL hypoxanthine, 0.1 ug/mL methotrexate and 15 ug/mL thymidine, was prepared from a 100x stock.

5-Azacytidine (5AC) was prepared immediately before use as a 1000x stock in serum-free medium, as was periodate-oxidized adenosine (POA). Ethyl methanesulfonate (EMS) and methyl nitrosourea (MNU) were prepared fresh before use at the concentrations specified.

2.3 IMMUNOFLUORESCENCE

Cells to be studied via indirect immunofluorescence were prepared essentially as described previously (Edwards and McBurney, 1983, Jones-Villeneuve and McBurney, 1982). Briefly, cells were plated onto gelatin-coated 22 mm² coverslips, usually one day prior to examination, and attached cells were washed in phosphate-buffered saline and fixed in ice-cold methanol. For AEC3A1-9 (Harris et al., 1984), and SSEA-1 (Solter et al., 1978) antibodies, 40 uL of a 1/500 dilution (in PBS) were used. TROMA-1 and TROMA-3 (Boller and Kemler, 1982) monoclonal antisera were used undiluted as culture supernatants. Cells were scored and counted using a Leitz Ortholux II microscope and a Leitz 12 filter cube for fluorescein.
2.4 CHROMOSOME PREPARATIONS

2.4.1 PREPARATION OF METAPHASE SPREADS

Chromosomes were prepared for viewing in the following manner. Exponentially growing cultures of cells were exposed to 0.06 ug/mL colcemid for 45 minutes (if chromosomes were to be G-banded) or 1 hour (following BrdU exposure if replication patterns were to be studied) before addition of trypsin-EDTA and dispersal into a single cell suspension. Cells were then allowed to swell in 0.56% KCl for 7-8 minutes at room temperature before fixing in several changes of freshly prepared 3:1 methanol-acetic acid. Fixed cells were then air-dried onto slides.

2.4.2 KARYOTYPING

G-banding of prepared chromosome was carried out using the trypsin technique of Seabright (1971). Briefly, prepared slides were dipped into 0.025% trypsin (Bacto Difco) in 0.15 M NaCl and 0.01 M Tris HCl pH 8.0 for 15 to 150 seconds. Slides were then rinsed, dried and stained in 2% Giemsa (Fisher) in pH 6.8 buffer for 2 to 5 minutes. Chromosome identification was made by comparison with a standard mouse karyotype (Nesbitt and Francke, 1973).

2.4.3 DETERMINATION OF CHROMOSOME REPLICATION PATTERNS

Chromosome replication patterns were studied by exposure of cells to 10^{-4} M 5-bromodeoxyuridine (BrdU) for 5 - 6 hours prior to addition of colcemid (Alves and Jonassen, 1978; McBurney and Strutt, 1980) and harvesting as described above. Slides were stored in the dark for 5 to 15 days before staining with 2% Giemsa in 0.3 M Na_{2}HPO_{4} (pH 10.4) for 20-40
minutes. Approximately 70 metaphases were scored for each point.

2.5 BIOCHEMICAL DETERMINATION OF HPRT ENZYME ACTIVITIES

2.5.1 PREPARATION OF CELL EXTRACTS

Crude enzyme extracts were prepared by suspending washed cells in 25 mM phosphate buffer (pH 7.4) containing 5 mM MgCl₂ to give a concentration of 3 x 10⁷ cells/mL, and disruption by 3 rounds for freezing and thawing followed by sonication on ice for 2 minutes at 70% maximum output (Fisher sonific dismembrator). The samples were then centrifuged at 1200g for 15 minutes at 4°C, and the supernatants stored at -70°C in small aliquots until further use. Protein concentrations were determined by TCA precipitation method (Coming and Tack, 1972) and were generally in the range of 2-3 mg/mL.

2.5.2 HPRT ENZYME ASSAY

Hypoxanthine-guanine phosphoribosyl transferase (HPRT, EC 2.4.2.8) activity was assayed in vitro using labelled hypoxanthine as the substrate. Reaction mixtures (200 uL volume) contained 25 mM phosphate buffer (pH 7.4), 5 mM MgCl₂, 2 mM phosphoribosyl pyrophosphate (PRPP) (prepared immediately before use), 9.643 uM hypoxanthine and 0.357 uM ³H-hypoxanthine (2.8 Ci/mmol) and 30-60 ug protein. The mixture was incubated at 37°C and the reaction was initiated by addition of enzyme extract. At various time intervals (from 3 to 240 minutes depending on enzyme activity), 50 uL of the reaction mixture was removed and added to 1.0 mL of ice cold 0.1 M lanthanum chloride (Fisher) to precipitate the phosphorylated product (inosine monophosphate, IMP) as an insoluble lanthanum salt. The
precipitated IMP salt was collected by suction filtration onto glass fiber filters (Whatman, GF/C) which were then washed with 40 mL of water and 5 mL of cold ethanol, dried, and added to 7 mL of toluene-based scintillation fluid (BDH) for determination of radioactivity using a liquid scintillation counter (Beckman).

2.6 HEAT DENATURATION OF HPRT

Cell extracts prepared as described above were assayed for the rate of heat denaturation of the HPRT enzyme as follows. Aliquots of 20 to 40 uL of extract were place in sealed 100 uL eppendorf tubes and heated in an 80°C circulating water bath (Haake) for 1 to 60 minutes. Following heating, extracts were quickly cooled on ice and centrifuged (1200g) at 4°C to pellet any precipitated protein. The extract supernatant was then assayed for HPRT activity as described, which was expressed as a percentage of initial activity present before heating.

2.7 ISOELECTRIC FOCUSING OF HPRT

2.7.1 PREPARATION OF POLYACRYLAMIDE GELS

Glass plates (125 mm x 256 mm x 1 mm) for polyacrylamide gel casts were prepared by thorough washing and degreasing, followed by treatment with either silane A-174 (30 uL silane A-174 plus 30 uL acetic acid in 10 mL 95% ethanol) to bind the acrylamide, or repel-silane (dimethyldichlorosilane; approximately 2% solution in 1,1,1-trichloroethane (BDH)) to repel the gel. The prepared glass plates were mounted (treated sides together) on metal clamps using a 1 mm thick rubber gasket as a spacer. To remove oxygen, the resultant mold was flushed with nitrogen.
Stock solutions of acrylamide monomer contained 24.25 g acrylamide ('electran'; BDH) and 0.75 g bis (NN'-methylenebis- acrylamide) (BDH) in 250 mL of distilled water. The solution was treated with Amberlite monobed resin MB-1 (2.5 g) (BDH) and mixed for 1 hour to remove acrylic acid before filtration and storage at 4°C. Acrylamide stock solutions were used within 2 weeks of preparation.

Polyacrylamide slab gels (5% total acrylamide, 3% crosslinker) were mixed according to Pharmacia instructions. A quantity sufficient for 3 gels (115 x 230 x 1 mm) was prepared each time to ensure batch reproducibility, and contained 50 mL stock acrylamide solution, 17 mL distilled water, 26.7 mL 50% glycerol, and 6.33 mL ampholytes pH 6-8 (Pharmacia). This solution was mixed on ice and deaerated for approximately 5 minutes before polymerization was initiated by the addition of 1.0 mL freshly made ammonium persulfate (22.8 mg/mL) and 33.3 uL of N,N,N',N'-tetramethylethylenediamine (TEMED) with gentle but rapid and thorough mixing. The polyacrylamide solution was then quickly poured into precooled (4°C) prepared gel cassettes. Polymerization was complete within 1 hour at room temperature. Gels could be stored at 4°C covered with plastic wrap for at least several weeks.

2.7.2 HPRT ISOELECTRIC FOCUSING

Cell extracts for isoelectric focusing (IEF) were prepared as described for HPRT enzyme assays except that cells were suspended in 1 or 3 volumes (depending on cell type) of IEF buffer (10 mM sodium phosphate buffer pH 6.8, 0.10 M NaCl, 5 mM KCl, 0.1% Triton-X 100, 15% sucrose, 10 mM dithiothreitol (DTT) and 1 mg/mL bovine serum albumin; Chasin and Urlaub,
prior to disruption and centrifugation. Just prior to IEF, samples were preincubated at 37°C for 2 hours to reduce the occurrence of multiple banding (Johnson, 1977).

Prepared gels were focused using an LKB Multiphor apparatus and focusing was across the long (230 mm) dimension of the gel. The gel was cooled to 10°C by water from a circulating water bath (Haake) pumped through a glass cooling plate beneath the gel. The anode solution was 0.04 M glutamic acid and the cathode solution was 0.1 M NaOH. The gels were prefocused at 10 W constant power for 500 V.h before samples were applied directly to the gel surface in volumes up to 20 µL within the guidelines of a plastic application mask (5 x 10 mm). The samples were run into the gel slowly at low constant voltage (200 volts for 1/2 hour, then 500 volts for 1/2 hour) before the power supply was switched to constant power (12 watts) for approximately 16 hours. The electrode strips were not changed during the run or following prefocusing. Following the gel run, the pH gradient was determined using a surface pH electrode.

HPRT activity within the gel was developed in situ by applying a concentrated reaction mixture with a bent glass rod, at a ratio of 12 µL/cm² of the gel surface. The reaction mixture contained 55 mM Tris (pH 7.4), 0.186% MgCl₂, 6.5 mM PRPP and 10 µM [¹⁴C]-hypoxanthine (50.9 mCi/mmol). The wetted gel was then incubated at 37°C for 5 minutes while a plastic-backed cellulose-polyethylene imine (PEI) thin-layer chromatography sheet (Sigma) was soaked in water and blotted to remove excess moisture. The PEI paper was then applied to the surface of the gel (in a rolling motion to minimize the entrapment of air bubbles), and the incubation was continued for a further 60 minutes. This procedure transfers the
radioactive IMP formed by the focused HPRT enzyme to the PEI sheet, and is a modification of the method of Shin et al. (1971). Following incubation, the cellulose-PEI paper was carefully removed from the gel, washed for 10 minutes in slowly running cold tap water, and dried in 50°C oven. The dried sheet was exposed to a Kodak XAR-1 X-ray film at -70°C for 1-14 days (depending on the activity) before being developed.

2.8 DETERMINATION OF DNA 5-METHYLCYTOSINE CONTENT

2.8.1 PREPARATION, ISOLATION AND HYDROLYSIS OF DNA

The procedure used to quantitate the 5-methylcytosine (5MC) content of cellular DNA was based on that of Plateau et al. (1983). Cells (plated at 10⁵/mL) were incubated (in the presence or absence of demethylating drugs) with 6-³H-uridine (4 uCi/mL) for 24 hours. The labelled cells were then washed, collected and pelleted, and dissolved in 1.0 mL of 0.3 N NaOH in 0.5% SDS at 37°C overnight. The lysate was neutralized with 150 uL of 2 N HCl, and proteins were digested at 37°C for 24 hours with 150 uL of freshly made proteinase K solution (1.0 mg/mL) and 250 uL of 0.5 Tris/HCl pH 7.6 buffer. Samples were then chilled on ice, and 50 ug salmon sperm carrier DNA and 500 uL of 50% TCA was added. The precipitated DNA was washed and dried, and hydrolyzed to completion in formic acid at 170°- 180°C for 30 minutes.

2.8.2 SEPARATION AND QUANTITATION OF BASES BY HPLC

The bases remaining in the hydrolyzed DNA preparation were prepared for high performance liquid chromatography (HPLC) by dissolution in HPLC buffer (10 mM KH₂PO₄, pH 2.5 plus 3% methanol). The residues were then
separated at room temperature using an LKB HPLC system fitted with a Whatman Partisil-SCX column using isocratic elution with HPLC buffer under low pressure at a flow rate of 2 mL/minute. Known quantities of the five bases were run on the column to identify the elution position of each base and to calibrate the detection system. Cytosine (C) and 5-methylcytosine peaks were well separated from all other absorbing material, and these fractions (approximately 28 aliquots of 1/2 mL each) were collected and assayed for radioactivity using a liquid scintillation counter. 5-Methylcytosine as a percentage of DNA cytosine bases was calculated as follows:

\[
%5MC = \frac{\text{cpm (5MC)}}{\text{cpm (5MC)} + \text{cpm (C)}} \times 100
\]
CHAPTER 3

ISOLATION OF MUTANTS POSSESSING ALTERED HPRT

C86 cells are female embryonal carcinoma cells containing 2 X chromosomes. In these cells, one X is active (X_a) and early-replicating; the other is inactive (X_i) and late-replicating (McBurney, 1976; McBurney and Adamson, 1976). C86S2C1 cells are a clonal cell line derived from C86 cells. It has been shown previously that demethylation of the DNA in C86 EC cells using 5-azacytidine results in the reactivation of genes on the inactive X chromosome, as indicated by an increase in the cellular activity of several X-linked genes (Paterno, et al., 1985). It was concluded that this increase in activity of X-linked genes was a result of reactivation of the inactive X chromosome as cells which had lost the inactive X did not respond to 5AC. Due to the lack of heterozygous X-linked markers in this cell line, it was not possible to demonstrate unequivocally that reactivation had indeed occurred. The purpose of these experiments was to provide such evidence.

Several techniques were employed with the purpose of producing a mutant cell line from C86 cells which possesses an X-linked marker; in this case, an altered HPRT enzyme encoded by the hpri allele on the active X chromosome. If a cell line was isolated such that the protein product of this mutant allele (hpri) was qualitatively distinguishable from that of the normal, wild-type allele on the X_i (hpri), this cell line would be a valuable system for studying reactivation of the X_i. That is, appearance of HPRT activity with characteristics of the wild-type (HPRT) enzyme, in addition to the retention of mutant HPRT in these heterozygous C86 cells,
would be unequivocal proof of reactivation of the inactive X chromosome. This chapter describes the isolation of such a clone.

3.1 RESULTS

3.1.1 CYTOTOXICITY OF MUTAGENS AND INDUCTION OF MUTATION

EMS, MNU, and \( \gamma \)-radiation were compared as mutagens for the hpRT locus of the active X chromosome of C86S2Cl cells. The relative mutagenicity of each drug was determined by measuring the frequency of colonies arising following treatment which were resistant to toxic concentrations of azaguanine (AG), thioguanine (TG), or ouabain (OUA). Cytotoxicity was measured concurrently with cell survival following drug treatment, as described. The results of these experiments are summarized in Table 3.1. EMS and \( \gamma \)-irradiation appeared to be relatively poor mutagens in this system; in contrast, MNU treatment induced a 75 fold increase in OUAF cells and a greater than 600 fold increase in TG- and AG-resistant cells over controls.

To determine if the EMS and MNU concentrations chosen were optimal, the relative cytotoxicity and mutagenicity (expressed as resistance to TG) of different concentrations of EMS and MNU were determined (Figure 3.1). EMS treatment (of up to 300 \( \mu \)g/mL, with cytotoxicity of up to 60\%) caused a low and variable frequency of 6TG-resistant mutants which was not significantly higher than spontaneous rates, and did not obviously increase with increased mutagen concentration. MNU, however, was found to be highly mutagenic in these cells at relatively low toxicity, and a dose-dependent increase in mutation induction was observed. At concentrations of MNU causing approximately 50\% cell death (1200 \( \mu \)M; 2 hour treatment), mutations
Table 3.1

Mutation induction and cytotoxicity of three mutagens on C86S2C1 EC cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>% Cell Survival</th>
<th>6TG</th>
<th>8AG</th>
<th>OUA</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>--</td>
<td>100</td>
<td>2±2</td>
<td>3±1</td>
<td>&lt;0.6</td>
</tr>
<tr>
<td>EMS</td>
<td>300 ug/mL</td>
<td>48±8</td>
<td>6±1</td>
<td>7±1</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>ɣ-irradiation</td>
<td>300 Rads</td>
<td>15±5</td>
<td>9±2</td>
<td>26±4</td>
<td>1.3±1</td>
</tr>
<tr>
<td>MNU</td>
<td>1200 uM</td>
<td>51±4</td>
<td>1220±225</td>
<td>1975±75</td>
<td>45±6</td>
</tr>
</tbody>
</table>

Exponentially growing cells were treated with ethyl methanesulfonate (EMS), ɣ-irradiation, or methyl nitrosourea (MNU) at the dose indicated. Treated cells were allowed to recover for 2 to 5 days (as described in Materials and Methods) before plating in selective media (5 ug/mL 6-thioguanine (6TG), 10 ug/mL 8-azaguanine (8AG), or 3 mM ouabain (OUA)). The number of colonies resistant to each selective medium has been corrected for plating efficiencies. % Cell Survival is the percentage of cells surviving treatment (assayed immediately after the drug insult) relative to untreated controls. Results are an average of 2 (ɣ-irradiation), 3 (EMS), or 4 (MNU) independent experiments.
Figure 3.1

Cytotoxicity and mutation induction response of C86S2Cl cells to EMS and MNU.

Cells were exposed to various doses of EMS (a) or MNU (b) as described in Materials and Methods. Filled symbols (solid lines) on each graph represent the mutation frequency expressed as the number of colonies resistant to 6-thioguanine (5 μg/mL) per surviving cell. The relative survival (open symbols, dashed lines; determined at the same time as the mutation frequency) indicates the percentage of cells surviving drug treatment relative to untreated controls. The absolute cloning efficiency of these cells before treatment was 17%.
to TG-resistance were approximately 200 fold higher than at EMS doses of similar cytotoxicity, and 600 fold higher (1-2 \times 10^{-3}) than controls (spontaneous frequency of approximately 2 \times 10^{-6}).

3.1.2 SELECTION OF HPRT VARIANTS

Mutants altered at the hprt locus were isolated by selecting for resistance to toxic guanine analogues after exposure of cells to various mutagens. In attempts to produce at least one cell with a qualitatively altered HPRT, three separate sets of such clones were isolated from C86S2Cl cells by different selective procedures. "Group 1" clones were treated with one of two mutagens and selected for resistance to high levels of AG or TG. "Group 2" clones were selected from a subset of Group 1 (HPRT') clones for reversion to an HPRT' phenotype; and "Group 3" mutants were grown in low doses of AG in attempts to produce clones with reduced (but relatively high) levels of HPRT activity.

Group 1 clones were isolated during the course of the mutagen studies on C86S2Cl cells described above; sixteen MNU- and \gamma-ray-derived AG' and TG' (HPRT') clones were established as cell lines for further characterization.

Due to the observed high efficiency of MNU as a mutagen in this system and its relatively low toxicity, it was chosen to induce both reverse mutations in the HPRT' cells (Group 2 clones), and resistance to low doses of AG in C86S2Cl cells (Group 3 clones), as described below.

Group 2 clones were isolated from several AG' and TG' Group 1 clones which displayed low spontaneous rates of reversion to HAT'. Six of these HPRT' clones were re-treated with 1200 \muM MNU and back-selected in HAT-containing medium, at either 37°C or 34°C incubation temperatures.
Because of the point mutation mode of action of MNU (alkylation of guanine, which is subsequently removed by DNA repair mechanisms (Olsson and Lindahl, 1980; Lindahl et al., 1980; Naslund et al., 1986)), any HPRT<sup>+</sup> cells arising from a second MNU treatment must be a result of further mutation in the HPRT enzyme restoring at least partial enzyme activity. It is therefore possible that this restored HPRT enzyme is qualitatively distinguishable from the wild-type enzyme. Colonies were grown at 34°C to increase the chance of isolating a temperature-sensitive HPRT. The results of this back selection experiment are shown in Figure 3.2. At 37°C, MNU treatment increased the frequency of HAT<sup>+</sup> cells appreciably in only 2 of 6 clones (AGY1 and AGM2), and at 34°C, MNU treatment had little or no effect on the number of HAT<sup>+</sup> colonies arising. However, in 5 of 6 cases, the lower temperature increased the frequency of HAT<sup>+</sup> cells arising from control and MNU-treated cultures. In fact, culturing untreated AGM2 cells at 34°C increased the frequency of HAT<sup>+</sup> colonies by over 300 times. Twelve of these HAT<sup>+</sup> "Group 2" colonies representing three parental clones were picked and grown for further characterization.

For Group 3 mutants, MNU-treated C86S2Cl cells were sparsely dispersed in various non-toxic concentrations of AG, and 13 actively growing colonies were isolated. The results of this experiment are shown in Figure 3.3. At 1 μg/mL AG (the concentration of AG chosen for growth of colonies with reduced HPRT levels), more than twice as many MNU-treated cells survived as control cells.
Figure 3.2

Frequency of reversion to HAT\textsuperscript{f} of several HPRT\textsuperscript{−} clones.

Several azaguanine- (AG) or thioguanine- (TG) resistant clones were treated with 1200 \textmu M MNU as described, and allowed to recover for 3 days before plating in HAT medium at either 37\textdegree C or 34\textdegree C. HAT\textsuperscript{f} colonies are expressed as a fraction of surviving cells. White and light grey bars represent control cells grown at 37\textdegree C and 34\textdegree C respectively, and black and dark grey bars represent MNU-treated cells grown at 37\textdegree C and 34\textdegree C, respectively. Arrows on bars represent values less than or greater than those values given, as indicated. Twelve of the resultant HAT\textsuperscript{f} colonies (representing three parental clones) were designated "Group 2" clones and isolated and grown for further characterization of HPRT.
Figure 3.3

Effect of MNU treatment on survival of C86S2C1 cells in low doses of azaguanine.

Cells were treated with 1200 μM MNU as described followed by a recovery and expression period of 5 days before plating in various doses of 8-azaguanine. Colonies were grown for 8 days before being stained and counted. Open bars represent untreated control cells; stippled bars represent MNU-treated cells. The arrow represents the azaguanine concentration at which colonies were grown in subsequent selection of MNU-treated cells resistant to low doses of AG ("LAGM"; Group 3 clones).
3.1.3 CHARACTERIZATION OF HPRT VARIANT CLONES

3.1.3.1 HPRT ENZYME LEVELS

The HPRT specific activities (Table 3.2, column A) for clones from each group of mutants were measured in vitro as described in Materials and Methods. As expected, AG- and TG-resistant clones possessed reduced levels of HPRT activity. Group 1 clones resistant to 10 μg/mL AG retained HPRT specific activities ranging from 0.8 to 12% of parental C86S2Cl (wild-type) levels, and TG$^c$ clones showed HPRT activities of 2-3% of wild-type levels. Both MNU and $\gamma$-irradiation-derived mutants showed a similar range of levels of HPRT activity. Surprisingly, Group 2 clones back-selected in HAT show only a slight increase in HPRT activity over parental cells, indicating that high levels of HPRT activity are not essential for growth in HAT-containing medium. Indeed, one Group 1 clone (AG$^y2$; 12% WT activity), was found to be resistant to both AG and to HAT. HPRT levels in Group 3 clones (selected in 1 μg/mL "low dose" AG) ranged from 35 to 78% of wild type.

3.1.3.2 EFFECTS OF TEMPERATURE ON GROWTH OF AGM2 CELLS IN HAT

During the back selection experiments, it was found that one particular clone (AGM2), which was sensitive to killing by HAT at 37°C, was comparatively HAT-resistant at 34°C. This phenomenon was further investigated, and the results of several plating efficiency experiments are summarized in Table 3.3. At 37°C, AGM2 cells are AG$^s$ and HAT$^s$, yet at 34°C, with little change in the ability of cells to grow in AG, an increase of between $10^3$ and $10^4$ fold more AGM2 cells are able to survive in HAT. One possible explanation for this phenomenon is that the mutant HPRT is
Table 3.2

HPRT enzyme characteristics of isolated clones.

HPRT specific activities, heat stabilities and isoelectric points were determined for various independently isolated clones. HPRT specific activities (column A) were measured in vitro, and are expressed as a percentage of the parental (C86S2C1) wild-type (WT) activity (0.563 nmol IMP/min/mg protein). The Relative Heat Stability of each clone (column B) is the amount of HPRT enzyme activity remaining after heating cell extracts, expressed as a percentage of the amount of activity expected at the normal (wild-type; C86S2C1) rate of thermodenaturation. To determine the HPRT heat stability, cell extracts were heated to 80°C for 10 minutes, cooled, and the supernatant assayed for residual HPRT activity. Under these conditions, the WT HPRT decreases to 90% of its initial activity. The isoelectric point (pI; column C) was established following focusing of the HPRT in acrylamide gels (pH 6-8, Pharmacia). "YES" indicates clones possessing HPRT with an altered pI; "NO" indicates clones tested showing no difference in HPRT pI. All HPRT specific activity measurements and heat inactivation results are an average of at least two independent experiments. The clones tested were isolated in three different manners as described in the text. Briefly, Group 1 clones were isolated in azaguanine (AG) (10 ug/mL) or thioguanine (TG) (5 ug/mL) following treatment with MNU (M) or γ-irradiation (γ). Group 2 clones include mutagen-induced revertants (rv) of 3 clones (AGM2, AGM3, or AG31, as indicated) to HAT-resistance (HAT+) at either 37°C (37-n) or 34°C (34-n), where n represents clone number. Group 3 clones are resistant to 1 ug/mL (low dose) azaguanine (LAg) following MNU (M) treatment. -- = Not Done.
<table>
<thead>
<tr>
<th>Name</th>
<th>Phenotype</th>
<th>HPRT Activity (% WT)</th>
<th>Heat Stability at 80°C</th>
<th>Altered pI?</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>S2C1</td>
<td>HATr</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td>AGM1</td>
<td>AGr</td>
<td>11</td>
<td>90%</td>
<td>ND</td>
</tr>
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<td>AGM2</td>
<td>AGr</td>
<td>0.8</td>
<td>7%</td>
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</tr>
<tr>
<td>AGM3</td>
<td>AGr</td>
<td>2</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>AGM4</td>
<td>AGr</td>
<td>3</td>
<td>100%</td>
</tr>
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<td>R</td>
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<td>5</td>
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</tr>
<tr>
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</tr>
<tr>
<td>U</td>
<td>AGY3</td>
<td>AGr</td>
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</tr>
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<td>1</td>
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<td>TGr</td>
<td>2</td>
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</tr>
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<td>R</td>
<td>rv34-2</td>
<td>-</td>
<td>3</td>
<td>76%</td>
</tr>
<tr>
<td>O</td>
<td>rv34-3</td>
<td>-</td>
<td>3</td>
<td>48%</td>
</tr>
<tr>
<td>U</td>
<td>AGM3</td>
<td>rv37-1</td>
<td>12</td>
<td>36%</td>
</tr>
<tr>
<td>P</td>
<td>rv34-1</td>
<td>HATr at 34°C</td>
<td>8</td>
<td>31%</td>
</tr>
<tr>
<td>&quot;</td>
<td>rv34-2</td>
<td>-</td>
<td>5</td>
<td>48%</td>
</tr>
<tr>
<td>&quot;</td>
<td>rv34-3</td>
<td>-</td>
<td>5</td>
<td>36%</td>
</tr>
<tr>
<td>AGY1</td>
<td>rv37-1</td>
<td>HATr at 37°C</td>
<td>6</td>
<td>100%</td>
</tr>
<tr>
<td>&quot;</td>
<td>rv34-1</td>
<td>HATr at 34°C</td>
<td>5</td>
<td>100%</td>
</tr>
<tr>
<td>&quot;</td>
<td>rv34-2</td>
<td>-</td>
<td>4</td>
<td>100%</td>
</tr>
<tr>
<td>LAGM 1a</td>
<td>LAGr</td>
<td>70</td>
<td>41%</td>
<td>-</td>
</tr>
<tr>
<td>LAGM 1b</td>
<td>LAGr</td>
<td>74</td>
<td>45%</td>
<td>-</td>
</tr>
<tr>
<td>LAGM 1e</td>
<td>LAGr</td>
<td>48</td>
<td>49%</td>
<td>ND</td>
</tr>
<tr>
<td>LAGM 2a</td>
<td>LAGr</td>
<td>60</td>
<td>69%</td>
<td>-</td>
</tr>
<tr>
<td>LAGM 2b</td>
<td>LAGr</td>
<td>78</td>
<td>66%</td>
<td>-</td>
</tr>
<tr>
<td>LAGM 2d</td>
<td>LAGr</td>
<td>35</td>
<td>62%</td>
<td>ND</td>
</tr>
<tr>
<td>LAGM 3a</td>
<td>LAGr</td>
<td>65</td>
<td>62%</td>
<td>-</td>
</tr>
<tr>
<td>LAGM 3b</td>
<td>LAGr</td>
<td>55</td>
<td>60%</td>
<td>-</td>
</tr>
<tr>
<td>LAGM 3c</td>
<td>LAGr</td>
<td>50</td>
<td>50%</td>
<td>ND</td>
</tr>
<tr>
<td>LAGM 3d</td>
<td>LAGr</td>
<td>58</td>
<td>71%</td>
<td>-</td>
</tr>
<tr>
<td>LAGM 4a</td>
<td>LAGr</td>
<td>66</td>
<td>60%</td>
<td>-</td>
</tr>
<tr>
<td>LAGM 4b</td>
<td>LAGr</td>
<td>66</td>
<td>56%</td>
<td>-</td>
</tr>
<tr>
<td>LAGM 4c</td>
<td>LAGr</td>
<td>41</td>
<td>52%</td>
<td>ND</td>
</tr>
</tbody>
</table>
Table 3.3

Effect of temperature on the frequency of AGM2 colony formation in selective media.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Selective Medium</th>
<th>37°C</th>
<th>34°C</th>
<th>34°C vs 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>--</td>
<td>84</td>
<td>85</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>8AG</td>
<td>&lt;0.0013</td>
<td>8.4</td>
<td>&gt;6500</td>
</tr>
<tr>
<td></td>
<td>HAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>--</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8AG</td>
<td>112</td>
<td>63</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>HAT</td>
<td>0.0044</td>
<td>5.4</td>
<td>1230</td>
</tr>
<tr>
<td>3</td>
<td>--</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8AG</td>
<td>89</td>
<td>65</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>HAT</td>
<td>&lt;0.0025</td>
<td>25</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>4</td>
<td>--</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8AG</td>
<td>--</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HAT</td>
<td>0.0045</td>
<td>5</td>
<td>1100</td>
</tr>
</tbody>
</table>

AGM2 cells were grown and subcultured normally at 37°C or 34°C (as indicated) for five days prior to plating. Known numbers of cells were then plated into selective or control media, and cells were further incubated at the former temperature undisturbed for one week before colonies were stained and counted. Cell survival is expressed relative to the number of cells surviving in untreated controls at the given temperature.
extremely thermolabile, and does not function well at $37^\circ$C. Thermosensitivity is a common characteristic of residual HPRT enzyme activity in cells selected for resistance to 8AG or 6TG (Sharp et al., 1973; Fenwick and Caskey, 1975; Epstein et al., 1977). Measurements of the HPRT specific activity of extracts of cells grown at each temperature showed that in $34^\circ$C-grown cells, the HPRT specific activity was 3 to 5 fold higher than in cells grown at $37^\circ$C (0.017 and 0.005 nmol IMP/min/mg protein, respectively).

3.1.3.3 **HPRT HEAT INACTIVATION RATES**

In order to determine if this temperature-dependent behaviour of AGM2 was due to a mutation affecting the heat stability of the HPRT enzyme, the heat inactivation kinetics of this clone and several others was determined (Table 3.2, column B). Briefly, cell extracts were heated to $80^\circ$C for 10 minutes, quickly cooled, and subsequently assayed for remaining HPRT activity.

Relative to the wild-type HPRT (which is a reasonably stable enzyme having a half-life of 42 minutes at $80^\circ$C), three of seven assayed Group 1 AG$^c$ clones showed decreased HPRT thermostability. Two of these (AGM2 and AG$^c2$) were significantly altered, with only 7% and 24% of the expected HPRT activity remaining, respectively. Many Group 2 clones (7 of 12) and all of the Group 3 clones also showed HPRT activity with reduced heat stabilities. In contrast, none of 5 TG$^c$ (Group 1) clones demonstrated altered heat stabilities.

Figure 3.4 illustrates the heat inactivation kinetics at $80^\circ$C of the HPRT enzyme from wild-type cells and from two isolated clones. HPRT from
Figure 3.4

Heat inactivation rates of HPRT enzyme from various clones.

The rate of heat inactivation of HPRT was determined by heating cell extracts to $80^\circ$C for various times before cooling and assaying the supernatant for residual HPRT activity. The activity at different time points is expressed as a percentage of the activity present before heating. Shown here are HPRT inactivation kinetics of wild-type cells (C86S2Cl) (triangles), AGM2 cells (circles), and AGM3 rv37·1 cells (squares).
AGM2 is inactivated extremely rapidly (with a half life of approximately 3 minutes). HPRT from AGM3 rv37-1 is inactivated about 3 times as rapidly as WT enzyme, with a half life of 15 minutes at 80°C.

A mixing experiment demonstrated that the increased sensitivity of AGM2 HPRT to heating was not due to some property of the enzyme preparation used for the heat denaturation experiment, but to a difference in the enzyme itself (Figure 4.4, panel a). Before heating, wild-type (C86S2C1) and AGM2 cell extracts were mixed to produce a solution containing 45% wild-type and 55% mutant HPRT activity. As shown, the mixed extract demonstrated the expected biphasic curve (possessing both wild-type and AGM2 inactivation kinetics) that could be extrapolated to show that 45% of the original mixture possessed wild-type heat denaturation kinetics.

3.1.3.4 HPRT ISOELECTRIC POINT

In addition to assaying the HPRT heat denaturation rates of several clones, the isoelectric point of a few select mutants was determined (Table 3.2, column C, Verne Chapman, personal communication). Of 8 clones examined, 2 mutants possessed HPRT with an altered isoelectric point (Figure 3.5).

A sensitive surface electrode pH meter was used to measure the pH gradient within the isoelectric focusing (IEF) gel, and it was determined that the pI of the major band of AGM2 HPRT is 6.81, while AGMY HPRT exhibits a pI of 7.02. These represent a shift of 0.08 and 0.29 pH units from wild-type (pI = 6.73), respectively. Interestingly, these two clones also possessed the least heat stable HPRT.
Figure 3.5

Isoelectric focusing of HPRT enzyme.

Cell extracts from wild-type cells and two mutant clones were focused on an acrylamide gel containing pH 6-8 ampholines (Pharmacia). HPRT activity was assayed in situ by overlaying the gel with a reaction mixture containing $^{14}$C-labelled substrate and a cellulose polyethylene imine (PEI) paper. After washing and drying, the PEI paper was exposed to X-ray film for 25 hours. Lanes 1 and 4, C86S2C1; lane 2, AGM2; lane 3, mixture of C86S2C1 and AGM2; lane 5, AG½2; lane 6, mixture of C86S2C1 and AG½2. The amounts of protein loaded were: C86S2C1, 25 μg (5 μL cell extract); AGM2, 195 μg (20 μL of cell extract); AG½2, 150 μg (15 μL of cell extract). The anode is at the top.
As has been observed by others (Bakay and Nyhan, 1971; Arnold and Kelley, 1971; Johnson et al., 1979; Chasin and Urlaub, 1976; Chapman et al., 1983; Johnson et al., 1985), the HPRT activity (of mutant and wild-type cells) was found in multiple electrophoretic bands. Since the isoelectric focusing was conducted under conditions which should eliminate pseudo-isoenzymes (i.e. preincubation of cell extracts before heating, etc (Johnson, 1977)), and since all of the bands are usually affected in mutants with altered or deficient HPRT (Chasin and Urlaub, 1976), the multiple bands presumably represent physiologically-modified forms of the same gene product (Arnold and Kelley, 1971; Chasin and Urlaub, 1976; Chapman et al., 1983). In any case, although it is unfortunate that AGM 2 has an HPRT sub-band which apparently co-migrates with the wild-type HPRT activity, it is most likely that this band represents a physiologically modified HPRT molecule, and not the presence of wild-type HPRT enzyme in the mutant cells.

3.1.4 REACTIVATION STUDIES USING ISOLATED HPRT VARIANT CLONES

In order to ensure that the mutant clones of C86S2Cl cells isolated in this report still retained the ability to reactivation their inactive X chromosome, the frequency of "reactivation" to HAT<sup>c</sup> of several HAT<sup>s</sup> clones was determined following DNA demethylation. Cells were treated with 3 μM 5AC for 24 hours or 1 μM POA for 4 days, followed by a 24 hour recovery and expression period in the absence of drug. The frequencies of HAT<sup>c</sup> colonies arising in cultures before and after treatment are shown in Table 3.4.

Of the MNU-derived clones, 8 of 8 showed an increase over controls in the number of HAT<sup>c</sup> colonies arising following 5AC or POA treatment. Of the
Table 3.4

Formation of HAT^f colonies of AG- and TG-resistant clones following 5AC or POA treatment.

Cells were exposed to 3 μM 5AC for 24 hours or 1 μM POA for 4 days, followed by a further 24 hours incubation in the absence of drug. Known numbers of cells were then plated in HAT-containing medium. P.E. represents the plating efficiency (number of colonies formed per cells plated). The reactivation frequency is calculated as the number of HAT^f colonies formed per plated cell divided by the plating efficiency. Fold increase indicates the increase in number of HAT^f colonies arising following treatment over spontaneous HAT^f cells arising from untreated controls.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>P.E.</th>
<th># HAT Resistant Colonies / 10^5 Cells Plated</th>
<th>Reactivation Frequency (x 10^-4)</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGM1</td>
<td>--</td>
<td>25%</td>
<td>61</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>POA</td>
<td>26%</td>
<td>263</td>
<td>101</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>27%</td>
<td>2680</td>
<td>993</td>
<td>41</td>
</tr>
<tr>
<td>AGM2</td>
<td>--</td>
<td>35%</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>POA</td>
<td>5%</td>
<td>10</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>7%</td>
<td>125</td>
<td>179</td>
<td>250</td>
</tr>
<tr>
<td>AGM3</td>
<td>--</td>
<td>49%</td>
<td>14</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>9%</td>
<td>222</td>
<td>247</td>
<td>86</td>
</tr>
<tr>
<td>AGM4</td>
<td>--</td>
<td>49%</td>
<td>49</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>11%</td>
<td>428</td>
<td>389</td>
<td>35</td>
</tr>
<tr>
<td>TGM1</td>
<td>--</td>
<td>14%</td>
<td>0</td>
<td>&lt;0.36</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>POA</td>
<td>16%</td>
<td>3</td>
<td>2</td>
<td>&gt;5</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>7%</td>
<td>7</td>
<td>10</td>
<td>&gt;28</td>
</tr>
<tr>
<td>TGM2</td>
<td>--</td>
<td>53%</td>
<td>1</td>
<td>&lt;0.1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>11%</td>
<td>35</td>
<td>32</td>
<td>357</td>
</tr>
<tr>
<td>TGM3</td>
<td>--</td>
<td>37%</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>20%</td>
<td>42</td>
<td>21</td>
<td>39</td>
</tr>
<tr>
<td>TGM4</td>
<td>--</td>
<td>7%</td>
<td>0</td>
<td>&lt;0.7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>POA</td>
<td>10%</td>
<td>18</td>
<td>18</td>
<td>&gt;26</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>19%</td>
<td>7</td>
<td>4</td>
<td>&gt;5</td>
</tr>
<tr>
<td>AGM1</td>
<td>--</td>
<td>18%</td>
<td>0</td>
<td>&lt;0.28</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>POA</td>
<td>5%</td>
<td>5</td>
<td>10</td>
<td>&gt;36</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>8%</td>
<td>110</td>
<td>138</td>
<td>&gt;496</td>
</tr>
<tr>
<td>AGM3</td>
<td>--</td>
<td>19%</td>
<td>0</td>
<td>&lt;0.26</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>POA</td>
<td>10%</td>
<td>9</td>
<td>9</td>
<td>&gt;34</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>23%</td>
<td>164</td>
<td>71</td>
<td>&gt;271</td>
</tr>
<tr>
<td>AGM4</td>
<td>--</td>
<td>49%</td>
<td>0</td>
<td>&lt;0.10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>6%</td>
<td>0</td>
<td>&lt;0.78</td>
<td>--</td>
</tr>
<tr>
<td>TGM1</td>
<td>--</td>
<td>43%</td>
<td>0</td>
<td>&lt;0.12</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>POA</td>
<td>11%</td>
<td>0</td>
<td>&lt;0.43</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>11%</td>
<td>0</td>
<td>&lt;0.43</td>
<td>--</td>
</tr>
<tr>
<td>TGM2</td>
<td>--</td>
<td>34%</td>
<td>0</td>
<td>&lt;0.15</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>POA</td>
<td>4%</td>
<td>0</td>
<td>&lt;1.18</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>5%</td>
<td>0</td>
<td>&lt;0.94</td>
<td>--</td>
</tr>
<tr>
<td>TGM3</td>
<td>--</td>
<td>39%</td>
<td>0</td>
<td>&lt;0.13</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>POA</td>
<td>15%</td>
<td>2</td>
<td>1.33</td>
<td>&gt;10</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>12%</td>
<td>0</td>
<td>&lt;0.39</td>
<td>--</td>
</tr>
<tr>
<td>TGM4</td>
<td>--</td>
<td>30%</td>
<td>90</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>POA</td>
<td>9%</td>
<td>98</td>
<td>109</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5AC</td>
<td>9%</td>
<td>415</td>
<td>461</td>
<td>15</td>
</tr>
</tbody>
</table>
\( \gamma \)-irradiated clones, only 4 of 7 demonstrated an increase under similar conditions. Of these, one (TG33) responded to POA treatment, but not to 5AC. Assuming that the hppt locus on the inactive X has not been altered, it thus appears that 3 of the \( \gamma \)-radiation-derived clones no longer reactivate their inactive X chromosome under these conditions, at least at the hppt locus. As indicated by karyotypic analysis, at least two of these clones (AG34 and TG51) retain 2 X chromosomes (data not shown). In addition, lack of reactivatability was not due to non-incorporation of 5AC, as these clones were shown to be demethylated (Figure 3.6) to the same or greater extent than treated AGN2 cells, which are able to reactivate their inactive X chromosome (see Chapter 4).

As shown in control experiments, the spontaneous frequency of HAT\( ^{c} \) colonies amongst different clones varied significantly, from \( <1 \times 10^{-5} \) (for 9 of 15 clones) to approximately \( 3 \times 10^{-3} \) (for 2 of 15 clones). In addition, the magnitude of the induced reactivation event also varied clonally. Overall, POA treatment resulted in increases in the frequency of HAT\( ^{c} \) colonies of approximately 4 to \( >36 \) times, and 5AC treatment produced increases ranging from 5 to \( >500 \) fold over control cells.

5AC and POA appear to be equally toxic to these cells under these conditions, causing a decrease in plating efficiency immediately following treatment of an average of 70\% (ranging from 50 - 90\%). However, 3 of 15 clones had plating efficiencies higher than controls after POA (4 to 43\% higher), or 5AC (8 - 171\% higher) treatment; two of these clones were the same in both instances. This may reflect an insensitivity of these clones to the cytotoxic effects of these drugs or, more likely, since in each case reactivation appeared to have occurred, an erroneously low plating efficiency measured for the control cells.
Figure 3.6

Demethylation of DNA in isolated clones following 5AC treatment.

Cells were treated with 3 μM 5AC for 24 hours followed by a 24 hour recovery period before DNA from control and 5AC-treated ACM2, AG®4, and TG®1 cells were digested and analyzed for 5-methylcytosine (5MC) content as described. % 5-Methylcytosine represents the percentage of total cytosine bases which are methylated. A significant decrease (60, 69, and 75%, respectively) in 5MC content of each clone is seen following 5AC treatment. As determined by the numbers of HAT® colonies arising following 5AC or POA treatment (Table 3.4), clone ACM2 is able to reactivate its inactive X chromosome, whereas, under these circumstances, clones AG®4 and TG®1 are apparently not.
3.2 **DISCUSSION**

In these experiments, we have used an efficient mutagen, MNU, as well as \( \gamma \)-irradiation to induce mutations in the hpert locus of the active X chromosome of C86S2C1 cells. Using these procedures, we have isolated several mutants which possess a quantitatively and/or qualitatively altered, HPRT enzyme.

All of the clones isolated in this report possess reduced levels of HPRT specific activity. The HPRT enzyme of many of the selected mutants also exhibit decreased thermostability relative to wild-type HPRT, and 2 of these clones (AGM2 and AG/2) possess HPRT with an altered isoelectric point. The HPRT enzyme of these mutants can therefore be distinguished from wild-type HPRT using one or more of three different criteria: specific activity, heat inactivation kinetics, and isoelectric point. HPRT from clone AGM2 retains 0.8% of WT activity, is very heat labile with a half-life of 3 minutes at 80°C, and has a slightly altered pI (0.08 pH shift). AG/2 HPRT possesses 12% of WT activity, is slightly less heat stable than WT, and exhibits a relatively large pI shift of 0.29 pH units.

Assuming that the hpert allele on the inactive X chromosome of these cells remains unaltered, these mutant cell lines can be used to study reactivation events at this locus. Appearance of HPRT activity with characteristics of the wild type enzyme, in addition to retention of the mutant HPRT in these heterozygous cells, would provide unequivocal proof of X\(_1\) reactivation. Preliminary experiments described in this Chapter indicate that many of these clones indeed appear able to reactivate their inactive X chromosome, at least at the hpert locus. That is, following treatment with DNA-demethylating agents, HAT-sensitive cells regain their
HAT-resistance (presumably through the production of wild-type HPRT enzyme from the X). 

3.2.1 LOSS OF REACTIVABILITY IN CERTAIN CLONES

Of the clones tested, all (8 of 8) MNU-derived HAT³ clones were reactivatable to varying extents (to a maximum of 500 fold increase over spontaneous), and also exhibited differences in their spontaneous reactivation rates (from < 1 x 10⁻⁵ to >2 in 1000. Several X-irradiation-derived clones (3 of 7; AG64, TG61 and TG62) appeared unable to reactivate their X under the conditions used, as assayed by inability to grow in HAT medium following SAC or POA treatment. This phenomenon was not due to lack of incorporation of SAC, as DNA demethylation was shown to occur in at least two of these clones. G-banding of chromosomes from these cells indicated that these two clones retained two X chromosomes.

A number of explanations are possible for this apparent loss of reactivatability. i) Reactivation may have occurred only at X-linked loci other than the hppt allele, therefore preventing growth in HAT medium; ii) reactivation of the hppt allele may have taken place, but resulted in only very low levels of transcription of hppt (see Chapter 4 for further evidence of variable levels of hppt transcription); or iii) the mutagen insult may have altered the hppt allele on the X such that the hppt protein product is no longer functional. It is possible that these clones have entirely lost their ability to reactivate their X, perhaps as a result of the mutagen treatment used for their isolation. For example, a gene encoding an activator protein involved in X chromosome reactivation (as proposed by Monk, 1986) may have been altered or deleted. Or perhaps a
further step in the developmental X inactivation "locking" process may have been triggered, resulting in a cell such as C145 (see section 1.12) which is refractory to reactivation attempts. It may be relevant that all of the non-reactivable clones were produced following γ-irradiation treatment, whereas each of the MNU-derived clones isolated demonstrated an ability to reactivate its X<sub>1</sub>. Further studies on these apparently unreactivable clones may yield valuable information about the mechanisms of X chromosome inactivation.

It may also prove interesting to investigate the clone TG83, which apparently responds to treatment with POA but not SAC. DNA methylation levels in this clone before and after treatment were not determined, but perhaps closer examination of this clone will provide details about the mode of action of POA relative to SAC. Another clone, TGN4, may also reactivate preferentially with POA.

3.2.2 COMPARISON OF MUTAGENS

The mutagen MNU has proven to be an appropriate choice for use in isolation of HPRT<sup>−</sup> cells, as, in addition to the fact that all of the MNU-derived clones isolated apparently retain their ability to reactivate the X<sub>1</sub>, MNU is an extremely efficient mutagen. Under the conditions tested, a 1200 μM dose of MNU was found to be a significantly more effective mutagen than both γ-irradiation (300 Rads) and EMS (300 μg/mL), while being concomitantly less cytotoxic. These results are similar to other reports which indicate that MNU is also highly mutagenic at low toxicities in V79 Chinese hamster cells (Bradley et al., 1980; Erickson et al., 1978). The low mutation frequency observed with EMS in C86 cells is comparable to
results obtained by McBurney et al (1976) in this and in other lines of EC cells.

MNU acts as a DNA alkylating agent which causes point mutations resulting from depurination of DNA (especially removal of guanine sites; O\textsuperscript{6}-methylguanine produced by MNU is removed by DNA repair mechanisms (Olsson and Lindahl, 1980; Lindahl et al., 1982)). EMS also produces small (presumably point) mutations (Nalbantoglu et al., 1983), while \textdelta-rays irradiation results in large deletions in the genome (Thacker, 1986; Brown et al., 1986). It is therefore likely that the reduced HPRT activity seen in MNU-treated cells, as well as the large number of MNU-treated clones isolated in this report which have altered HPRT thermostability (22/33) is due to altered amino acid sequences in the enzymes resulting from one or more missense mutations in the corresponding structural gene. These mutations decrease the enzyme activity without abolishing it, and frequently render the enzyme less heat stable. Of the \textdelta-rays-irradiated clones tested, only 1 of 4 demonstrated altered thermostability.

3.2.3 **EFFECT OF TEMPERATURE ON GROWTH OF AGM2 CELLS IN HAT**

During the course of these experiments, it was discovered that AGM2 cells exhibit a temperature-dependent ability to grow in HAT-containing medium. Given the identical heat denaturation rates and isoelectric focusing patterns of HPRT from cells grown at each temperature, it is probable that the same HPRT enzyme species is being produced at 34° and 37° C. The temperature-sensitive phenotype may be explained by the fact that the HPRT enzyme isolated from these cells is heat labile \textit{in vitro}. It has also been shown that abnormal proteins are selectively degraded (Capecchi
et al., 1974); it is likely that the rate of this process is reduced at 34°C relative to 37°C, resulting in an increased half-life of HPRT protein at the lower temperature.

3.2.4 CHOICE OF MUTANT FOR FURTHER STUDIES

Of the mutant clones isolated and described in this chapter, two (AGM2 and AG82) possess HPRT which is quantitatively and qualitatively distinguishable from the wild-type enzyme using at least three criteria (specific activity, thermodenaturation kinetics and pI). For further studies on reactivation of the inactive X chromosome, it is important to study both transient reactivation events and events related to stable expression of genes from the Xₐ. As shown earlier, clones which stably express elevated levels of HPRT can be selected in HAT-containing medium.

Unfortunately, the level of residual HPRT activity in AG82 (12% of wild-type levels) allows this cell line to grow in HAT-containing medium without requirement for expression from the hprt allele on the Xₐ.

However, the clone AGM2 is HAT⁺, allowing the use of HAT medium to select for cells which stably express elevated HPRT levels. The following chapter describes the results of reactivation experiments performed using AGM2 cells.

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CHAPTER 4

REACTION OF THE INACTIVE X CHROMOSOME IN FEMALE EC CELLS.

Previous studies in this laboratory have indicated that treatment of female EC cells with 5AC and other DNA demethylating agents results in the high frequency reactivation of the inactive X chromosome (Paterno et al., 1985). In the previous chapter, we have described the isolation of several HAT* clones and the results of reactivation assays on these cells. Demethylation of cellular DNA was achieved using the DNA demethylating agents 5-azacytidine (5AC) and periodate-oxidized adenosine (POA). 5AC directly inhibits DNA methylation via incorporation into DNA and subsequent inhibition of the methyltransferase enzyme (Creusot et al., 1982), whereas POA decreases methylation indirectly by its inhibition of the S-adenosyl homocysteine (SAHC) hydrolase enzyme, causing an increase in cellular SAHC levels and therefore end product inhibition of the methylation reaction (Bartel and Borchardt, 1984; Patel-Thombre and Borchardt, 1985).

The high frequency of HAT* colonies arising following treatment of several clones suggests that 5AC and POA are not inducing mutational events. However, in order to provide further evidence that the incidence of HAT* colonies is indeed due to a reactivation of the hpert locus on the inactive X and not a reversion of the mutated hpert, we have chosen to more closely examine the clone AGM2. In addition to the relatively low rate of spontaneous reactivation of AGM2 and its high reactivation rates with both 5AC and POA, this clone possesses an altered HPRT (HPRT°) which is quantitatively and qualitatively distinguishable from the wild-type HPRT (HPRT°).
To provide definitive evidence for reactivation, in addition to assaying for quantitative changes in HPRT activity as before (Chapter 3, this thesis; Paterno et al., 1985), we can now examine the characteristics of reactivant HPRI enzyme to determine the locus of origin of the HPRT protein. Assuming an unaltered hprt locus exists on the inactive X chromosome, any expression from the hprt locus on the X would result in the appearance of HPRT activity with wild-type characteristics: increased HPRT specific activity, wild-type heat denaturation kinetics, and normal (wild-type) isoelectric point. Retention of HPRT in these treated heterozygous cells would prove that the presence of HPRT was not due to reversion of the mutated allele. Furthermore, as HPRT is a multimeric enzyme, detection of HPRT protein with characteristics intermediate to the mutant and wild-type enzymes would suggest that heteromeric HPRT molecules were being produced as a result of expression of both hprt alleles within a cell. This chapter describes the experiments performed to provide definitive proof of reactivation of the inactive X chromosome in C86AGM2 cells.

4.1 RESULTS

4.1.1 EFFECTS OF 5AC AND POA ON AGM2 EC CELLS

4.1.1.1 CYTOTOXICITY

DNA demethylation of AGM2 cells was achieved by exposure to 3 uM 5AC for 24 hours or 1 uM POA for 4 days. The effects of different concentrations of these drugs on the survival of AGM2 cells are illustrated in Figure 4.1. Although this figure only illustrates the effects of 1 day exposures of POA, the cytotoxic effects of 4 day POA treatments were not
To determine the cytotoxicity of 5AC and POA to ACK2 cells, known numbers of exponentially growing cells were plated into tissue culture dishes and allowed to attach for 10 hours before addition of various concentrations of 5AC (squares) or POA (triangles). After 24 hours, the drug was removed, the cells washed carefully, and colonies were allowed to form undisturbed for one week before staining and counting. Cell survival is expressed as a percentage of cells surviving treatment relative to untreated controls. Results are an average of 2 to 3 independent experiments; plating efficiencies at each concentration did not vary by more than 5% between experiments. Arrows indicate the drug concentrations used in reactivation experiments. Experiments measuring the cytotoxicity of 4 day exposures of 1 μM POA consistently gave results not significantly different from 1 day exposure periods to POA (73.5% and 66.6% relative plating efficiencies, respectively).
significantly different (66.6% and 73.5% relative plating efficiencies for 1 μM POA, 1 and 4 day treatment periods, respectively).

It is evident that under these circumstances POA is significantly less cytotoxic than 5AC. That is, when cells are exposed to these drugs in culture and left undisturbed to form colonies, only 6% of cells survive 5AC treatment (3 μM, 24 hours) whereas more than 73% cell survival occurs with POA (1 μM, 4 days). However, as seen in Chapter 3 (Table 3.4), POA is apparently more cytotoxic than 5AC under plating conditions used in reactivation experiments. If cells are trypsinized and replated following treatment, the plating efficiency for POA treated cultures is greatly reduced. Figure 4.1 indicates that 5AC is very toxic; however, cells which survive treatment possess a relatively high plating efficiency.

In experiments using parental (C86S2Cl) cells, 5AC and POA were found to be approximately equally toxic to these cells as to AGM2.

4.1.1.2 DNA DEMETHYLATION

It is apparent that 5AC and POA treatment differ markedly in their efficiencies of reactivation (Table 3.4). This effect may be due to the different modes of action of each of these drugs, and/or to different efficiencies as DNA demethylating agents.

To determine the extent of DNA demethylation occurring in treated cells, the 5-methylcytosine (5mC) content of cellular DNA was measured at daily intervals following treatment. The results, illustrated in Figure 4.2, indicate that both treatments induce DNA-demethylation, but to different degrees. Following 24 hours of 5AC treatment, genomic levels of 5mC decreased by 61%. POA treatment however, resulted in a reduction in 5mC levels of only
Figure 4.2

DNA demethylation in cells treated with 5AC or POA.

AGM2 cells were exposed to 3 μM 5AC or 1 μM POA as described (represents treatment period) and collected at daily intervals for measurement of 5-methylcytosine content by DNA hydrolysis and HPLC. Results are expressed as the percentage of total cytosine residues which are methylated. Error bars represent standard deviations over 3 to 6 separate determinations.
18% within one day, continuing slightly to a total 22% decrease in control 5mC levels over 4 days of treatment. Although the 5mC content of POA-treated cells is not significantly lower in cells treated with POA for 4 days than that in cells treated for 1 day, the 4 day treatment period for reactivation experiments was chosen from results obtained using this drug in other laboratories (Lipeplo and Kerbel, 1986).

Following removal of 5AC from treated cells, rapid and significant remethylation occurs. 5mC levels return to 54% and 67% of original levels 1 and 3 days following removal of 5AC, respectively. This effect has been studied previously (Lay et al., 1984). The rate of remethylation of POA-treated cultures following removal of the drug was not determined.

4.1.2 REACTIVATION OF AGM2: TRANSIENT STUDIES

4.1.2.3 HPRT SPECIFIC ACTIVITIES

Treatment of AGM2 cells with DNA demethylating agents results in an increase in the frequency of cells surviving in HAT medium (see section 3.1.4). To determine if this was due to an increase in HPRT specific activity within the cells, cell extracts were collected from control and 5AC- and POA-treated cultures and assayed for HPRT specific activity (Table 4.1). In cells harvested 1 day following removal of 5AC, a 7-fold increase in HPRT specific activity was observed in AGM2 cells (to 0.034 nmol IMP/min/mg protein) whereas POA treatment results in a slightly smaller increase of approximately 5-fold (to 0.024 nmol IMP/min/mg protein) over untreated control cells (0.005 nmol IMP/min/mg protein).

Previous work in this laboratory (Paterno et al., 1985) has shown that the increase in HPRT activities seen in certain female EC cell lines
Table 4.1

HPRT Specific Activities of 5AC and POA Treated AGM2 Cells.

<table>
<thead>
<tr>
<th>Cell Name</th>
<th>Treatment</th>
<th>HPRT Specific Activity</th>
<th>% WT HPRT Activity</th>
<th>Fold Increase over Untreated Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>C86S2C1</td>
<td>--</td>
<td>0.563</td>
<td>100</td>
<td>--</td>
</tr>
<tr>
<td>AGM2</td>
<td>--</td>
<td>0.005</td>
<td>0.8</td>
<td>1</td>
</tr>
<tr>
<td>AGM2</td>
<td>POA</td>
<td>0.024</td>
<td>4.2</td>
<td>5</td>
</tr>
<tr>
<td>AGM2</td>
<td>5AC</td>
<td>0.034</td>
<td>6.0</td>
<td>7</td>
</tr>
</tbody>
</table>

Cells were exposed to 3 μM 5AC (24 hours) or 1 μM POA (4 days) followed by 24 hours incubation in the absence of drug prior to harvesting and preparation of cell extracts for in vitro assay of HPRT specific activity. Specific activities are expressed as nmol IMP formed/min/mg of protein. Fold increase represents the change in HPRT activity of treated cells over untreated control cultures. "WT" indicates wild-type HPRT.
following 5AC treatment is transient. As shown in Figure 4.3, the HPRT specific activity of AGM2 cells exposed to 5AC also showed a transient increase: the maximum level of HPRT activity was found to occur at 3 days following treatment. This 'delayed' appearance of elevated HPRT levels (maximum cellular DNA demethylation occurred 1 day following 5AC treatment) may indicate that it is not demethylation per se which turns on the hprt gene. Perhaps the 'target' of 5AC is not hprt itself, but another gene whose product activates the hprt allele.

Although the peak of HPRT activity occurred at 3 days following 5AC exposure, for all transient assay experiments described in this chapter, 5AC-treated AGM2 cells were harvested only 1 day following treatment (when HPRT levels reached approximately 70% of maximum). A similar experiment for POA-treated cultures was not performed.

The level of hprt mRNA in 5AC-treated cells was also measured (Figure 4.12a, lane 3; Table 4.3). In AGM2 cells, the amount of hprt mRNA is only 60% of that in C86 parental cells. (Reduced levels of hprt mRNA have been previously noted in other 8AG-resistant cells (Paterno et al., 1985)). Following 5AC treatment, the level of hprt mRNA increased by 70%, indicating that the increased HPRT specific activity seen in extracts of these cells was not simply due to elevated levels of protein synthesis. However, no such increase was seen in pgk-1 mRNA levels in the 5AC-treated cells (Figure 4.12b, lane 3; Table 4.3).
Changes in HPRT specific activity in AGM2 cells exposed to 5AC.

Cells were exposed to 3 uM 5AC for 24 hours (→) and harvested at daily intervals for determination of HPRT specific activity in vitro as described. Arrow indicates time at which cells were harvested for all other experiments.
4.1.2.2 HPRT HEAT DENATURATION RATES

AGM2 HPRT is considerably more thermolabile than the parental wild-type HPRT (section 3.1.3.3). To determine if the increase in HPRT specific activities seen following 5AC and POA treatment resulted from the expression of the hprt<sup>+</sup> allele on the X<sub>1</sub>, the thermodenaturation kinetics of HPRT from 5AC-treated C86AGM2 cells was measured (Figure 4.4b). As seen in the heat denaturation curve, the HPRT activity from 5AC-treated C86AGM2 cells was considerably more heat stable than that from untreated AGM2 cultures (dashed lower line). Extrapolation of the curve indicates that approximately 37% of the HPRT activity from these treated cells had thermodenaturation kinetics similar to that of the wild-type enzyme from C86 parental cells. Panel a shows the result of a reconstruction experiment containing a mixture of extracts of AGM2 and wild-type (C86S2Cl) cells (extracts mixed prior to heating). The biphasic curve obtained exhibits the appropriate two distinct slopes corresponding to the denaturation rates of the two types of HPRT. In addition, the ratio at which the two extracts were mixed can be predicted from extrapolation of the curve.

An important difference exists between the heat denaturation kinetics of the 5AC-treated cultures and the mixture of wild-type and AGM2 cell extracts. In 5AC-treated cells (Figure 4.4b), in addition to the HPRT enzyme with wild-type thermodenaturation kinetics, the thermosensitive portion of the biphasic curve demonstrates a slope which is significantly less than that of the HPRT enzyme from untreated AGM2 cells. That is, the extract from 5AC-treated cells possessed a portion of HPRT molecules with heat stability intermediate to the wild-type and mutant HPRTs. This
Figure 4.4

Heat inactivation of HPRT enzyme in 5AC-treated cultures of AGM2.

35 μL aliquots of extracts from 5AC-treated AGM2 cells (b) were incubated in tightly closed vials at 80°C for various times, cooled and the supernatant assayed for remaining enzyme activity. The enzyme assay was performed at 37°C for 30, 60, 90, and 120 minutes reaction time following the addition of 3H-Hx and other components of the reaction mixture as described. HPRT activity is expressed as a percentage of the initial activity present before heating, and was 1973 cpm of 3H-IMP after 60 minutes for a 5 μL (24 μg protein) sample of 5AC-treated cells. For comparison, a mixture of AGM2 and WT (C86S2C1) cell extracts was also assayed following identical preparation and heating procedures (a). The mixture contained 96 volumes of AGM2 (572 μg protein) and 4 volumes of wild-type C86S2C1 (14 μg protein) cell extract, and its initial activity (3575 cpm in 60 min for a 5 μL sample (29 μg protein)) was the sum of the activities from the individual component extracts. Each point in the figure represents the mean activities for each time period in the reaction from two separate experiments. Upper solid (-----) lines and lower dashed (---) lines on each graph represent wild-type and mutant (AGM2) heat denaturation curves, respectively, for comparison (see Figure 3.4).
intermediate slope is likely due to the presence of HPRT heterotetramers, composed of both HPRT° and HPRT* monomers.

This intermediate slope is not seen in the reconstruction experiment where wild-type and mutant cell extracts were mixed (Figure 4.4a), indicating that these heteropolymers only arise under conditions when both mutant and wild-type HPRT monomers are being produced and assembled within the same cell.

It is also evident from the heat inactivation graph that the 5AC-treated cultures possess little or no HPRT enzyme with mutant (AGM2) heat inactivation kinetics. The absence of a slope equivalent to the AGM2 HPRT suggests that expression of some wild-type HPRT occurs in almost all cells. This is consistent with results obtained by Paterno et al. (1985).

4.1.2.3 HPRT ISOELECTRIC FOCUSING

To confirm the presence of wild-type HPRT activity indicated by the heat inactivation experiments performed above, extracts of 5AC- and POA-treated cells were collected for HPRT isoelectric focusing (Figure 4.5 lanes 4, 5 and 6). In addition to the band of HPRT activity present in C86ACM2 cells, both 5AC- and POA-treated cultures showed the presence of a strong band of HPRT activity at the location of the wild-type C86 enzyme. A less intense band of HPRT activity at a position intermediate to the wild-type and mutant bands was also present (Figure 4.5, arrow). This intermediate band is absent from IEF gels of mixtures of WT and AGM2 extracts (Figure 4.5, lane 3). These two additional bands of HPRT activity in 5AC- and POA-treated cultures confirm the presence of HPRT wild-type homomers and wild-type/mutant heteromers.
Figure 4.5

Isoelectric focusing of HPRT from 5AC- and POA-treated EC and differentiated AGM2 cells.

Cell extracts were collected from untreated control cultures of AGM2 and cells treated with 3 μM 5AC or 1 μM POA in the usual manner. 15 to 150 μg protein (as indicated) were loaded onto acrylamide gels containing pH 6-8 ampholytes (Pharmacia) and focused overnight at 12 W constant power. Following overlaying with a reaction mixture containing labelled substrate and with a cellulose PEI paper, the HPRT activity was viewed in situ after exposure of the PEI paper to X-ray film for 27 hours.

Between 11 and 21 uL of cell extract were loaded in each lane, except where indicated. Lane 1, C86S2Cl (15 μg protein; 3 uL cell extract); lane 2, AGM2 (150 μg protein); lane 3, mixture of C86S2Cl and AGM2 (15 and 150 μg protein, respectively); lanes 4 and 5, 5AC-treated AGM2 (150 and 85 μg protein) (separate experiments); lane 6, POA-treated AGM2 (150 μg protein). Lane 7 is an extract from differentiated AGM2 cells and lane 8 is from 5AC-treated differentiated AGM2 cells (both 150 μg protein). AGM2 cells were differentiated in monolayer by exposure to 10^{-7} M retinoic acid for 12 days prior to 5AC treatment in the usual manner. Cells were harvested following a 24 hour recovery period, and extracts were prepared and focused as described previously. No significant difference in HPRT specific activity was found in differentiated AGM2 cells after 5AC treatment (see text). The anode is at the top. The arrow indicates a band of HPRT activity from the HPRT wild-type/mutant heterodimer. This band was seen in all IEF gels of HPRT from 5AC- and POA-treated EC (undifferentiated) AGM2 cells.

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Although HPRT is a tetramer under normal physiological conditions (Holden and Kelley, 1978; Johnson et al., 1979), in solutions of low ionic strength (such as this isoelectric focusing buffer), the HPRT tetramer dissociates to form two dimers (Johnson et al., 1979). It is for this reason that only a single intermediate band is seen in the IEF gels -- representing the HPRT<sup>+</sup>/<sup>-</sup> heterodimer. A densitometric scan of part of this IEF gel is shown in Figure 4.6.

Thus, evidence from isoelectric focusing and thermodenaturation experiments each support the conclusion that both the hp<sup>+</sup> and hp<sup>-</sup> alleles are transcribed in 5AC- and POA-treated C86AGM2 cells. However, the relative intensities of the HPRT bands seen in the IEF gels do not directly reflect the levels of expression of each allele because the enzymatic activity of the hp<sup>-</sup>-derived monomer is considerably less than that of the hp<sup>+</sup>-derived monomer.

4.1.3 DIFFERENTIATED AGM2 CELLS FAIL TO REACTIVATE

In 1985, Paterno et al. demonstrated that when C86S1Al cells were induced to differentiate prior to exposure to 5AC, reactivation did not occur. To determine if differentiated AGM2 cells are able to reactivate their inactive X chromosome, these cells were induced to differentiate in the presence of 10<sup>-7</sup> M RA for 10 to 15 days prior to DNA demethylation. Retinoic acid treatment induced these cells to differentiate into several cell types, including neurons, fibroblast-like cells and epithelium, as judged by morphology, reactivity with antisera specific for these cell types, and loss of the AEC3Al-9 antigen characteristic of EC cells (data not shown). In addition, differentiated cells lost their immortality.
Figure 4.6

Distribution of HPRT activity in the isoelectric gel.

Densitometric tracings (from cathode to anode) of part of the autoradiogram shown in Figure 4.5 are illustrated here. The relative intensities of the HPRT activity localized in each band were determined using an LKB laser densitometer. The gel lane numbers are indicated in parentheses: a) C86S2C1 (1); b) AGM2 (2); c) POA-treated AGM2 (6); d) 5AC-treated AGM2 (4).
Following differentiation in this manner, cells were exposed to 3 μM 5AC for 24 hours. After a 24 hour expression period, the differentiated cells were harvested and assayed for HPRT specific activity. No significant increase in HPRT activity was seen in these cells following 5AC treatment (0.009 nmol IMP/min/mg protein for differentiated AGM2 cells; 0.010 nmol IMP/min/mg protein for differentiated AGM2 cells following 5AC treatment).

Following this differentiation and 5AC treatment, AGM2 cells were also collected for isoelectric focusing (Figure 4.5, lanes 7 and 8). As seen in the gel, treatment of these differentiated cells with 5AC did not result in the appearance of any HPRT activity possessing wild-type isoelectric point, nor any intermediate bands of HPRT activity (lane 8).

The failure of differentiated AGM2 cells to express the hprt mutant allele was not a result of the lack of DNA demethylation by 5AC. Although differentiated cells already possessed reduced 5mC levels (3.6 ± 0.3 %5mC relative to 4.4 ± 0.1 %5mC for undifferentiated cells), treatment with 5AC resulted in a further decrease in 5mC levels by approximately 50% (to 1.8 ± 0.4 %5mC).

### 4.1.4 STABLE REACTIVANTS OF AGM2

#### 4.1.4.1 FREQUENCY OF REACTIVATION

C86AGM2 cells spontaneously form colonies in HAT medium at frequencies of 1 - 7 x 10⁻³. Treatment of AGM2 cells with 5AC or POA significantly increased the proportion of HAT-resistant cells in these cultures to 1 - 2 x 10⁻² and 1 - 2 x 10⁻³, respectively. To further investigate the characteristics of 5AC- and POA-induced reactivated clones
of AGM2, reactivants which stably expressed elevated levels of HPRT were selected in HAT-containing medium and clonal populations were grown for further examination.

The results of several reactivation experiments using AGM2 cells are illustrated in Figure 4.7. Rates of reactivation following 5AC treatment range from 220 to 750 fold over spontaneous rates. In an attempt to reduce the spontaneous reactivation frequency, AGM2 cells were grown in 8-azaguanine for 1 week immediately prior to treatment with 5AC (Figure 4.7 panel b). This "pre-selection" in AG reduced the number of spontaneous HAT<sup>e</sup> colonies arising by 7 fold (7 x 10<sup>-5</sup> to 1 x 10<sup>-5</sup>) while concomitantly reducing the reactivation frequency by less than 4 fold (1.8 x 10<sup>-2</sup> to 4.6 x 10<sup>-3</sup>). However, within less than 1 week of return of AGM2 cells to normal growth medium, the rate of spontaneous HAT<sup>e</sup> cells arising returned to levels equal to or higher than those prior to AG selection (1.8 x 10<sup>-4</sup>) (Figure 4.3c).

4.1.4.2 HPRT SPECIFIC ACTIVITIES

Several HAT<sup>e</sup> colonies which arose from AGM2 cells either spontaneously or following 5AC or POA treatment were isolated and established as clones for further characterization (named "reSP", "reAC", and "rePOA", respectively). All reactivants retained EC-like morphologies.

The HPRT specific activities of 3 spontaneous, 2 POA- and 7 5AC-induced HAT<sup>e</sup> clones were measured and are illustrated in Figure 4.8. All reactivants exhibited increased levels of HPRT specific activity over AGM2 levels but less than that of C86 cells, ranging from a 9 to 73-fold
Frequency of HAT$^+$ colonies arising in AGM2 cells following 5AC or POA treatment.

AGM2 cells were exposed to POA or 5AC as described and tested for their ability to grow in HAT. (a) Cells cultured in normal growth medium prior to reactivation experiment. (b) Cells cultured in 10 μg/mL AG for one week immediately prior to start of experiment. (c) Cells grown in AG for one week followed by culturing under non-selective conditions for 5 days prior to reactivation experiment. Open bars represent untreated controls (spontaneous HAT$^+$ frequency); light grey bars represent cells treated with POA, and dark grey bars represent 5AC-treated cultures.
**Figure 4.8**

**HPRT specific activities of reactivants.**

Cell extracts from wild-type (C86S2C1) cells (solid bar), parental (AGM2) cells (open bar), and reactivants (induced by 5AC ("reAC", dark grey bars), POA ("rePOA", light grey bars), or spontaneously ("reSP", cross hatched bars)) were assayed for HPRT activity. The HPRT specific activities of reactivants ranged from 0.042 to 0.367 nmol IMP/min/mg protein (7% to 65% of wild-type C86S2C1 activity: rePOA2c and reAC2a, respectively). Clones illustrated here (with HPRT specific activities expressed in nmol IMP/min/mg protein in parentheses) are: 1, C86S2C1 (0.563); 2, AGM2 (0.005); 3, rePOA2c (0.042); 4, reAC1b (0.053); 5, reAC1a (0.057); 6, reSP3a (0.073); 7, reAC3b (0.075); 8, reAC1d (0.090); 9, reAC2c (0.136); 10, rePOA3c (0.156); 11, reAC2b (0.168); 12, reSP1c (0.181); 13, reSP2a (0.205); 14, reAC2a (0.367). Results represent an average of 2 to 3 determinations.
increase over AGM2 (7 to 65% of wild-type C86 levels; rePOA2c and reAC2a, 0.042 and 0.367 nmol IMP/min/mg protein, respectively). No correlation was observed between levels of HPRT specific activity and the manner in which reactivants were produced.

4.1.4.3 HPRT HEAT DENATURATION RATES

The rates of heat denaturation of HPRT from 4 reactivants were measured and are shown in Figure 4.9. Each reactivant assayed indicated the presence of HPRT activity with stability similar to that of the wild-type enzyme. In addition, as seen in the transient assays, the reactivants possessed HPRT with heat stabilities intermediate to the mutant and wild-type HPRT, indicating the presence of HPRT+/HPRT- heterotetramers within each cell of the clonal population. This intermediate slope was different for each reactivant, indicating the proportions of the HPRT+/HPRT- heterotetramers in each clone were unique. The intermediate slope was inversely proportional to the measured HPRT specific activities of each reactivant. Also, as observed in the heat inactivation experiments using extracts from 5AC-treated AGM2 cells, little or no HPRT appeared to possess the inactivation kinetics of the mutant enzyme.

4.1.4.4 HPRT ISOELECTRIC FOCUSING

The HPRT isoelectric focusing pattern of each reactivant was examined on a pH 6-8 isoelectric focusing gel (Figure 4.10) and several lanes of the gel were scanned using a laser densitometer to determine relative intensities (Figure 4.11). In each reactivant, the gels revealed the presence of a strong band of HPRT activity with the pI of the wild-type
Figure 4.9

Rate of heat inactivation of HPRT enzyme from four reactivants.

Cell extracts were heated at 80°C for various times before HPRT activity was measured as described previously. HPRT activities are expressed as a percentage of the initial HPRT activity present in the cell extract before heating. Lower and upper dashed lines on each graph represent the heat denaturation rates of the parental (AGM2) and the wild-type (C86S2Cl) HPRT, respectively. (a) reAC1d; (b), reAC1b; (c) rePOA2c; (d) reAC2a.
Figure 4.10

Isoelectric focusing of HPRT enzyme of reactivants.

Cell extracts were harvested and prepared in IEF buffer as described before focusing on a pH 6-8 acrylamide gel. HPRT activity was viewed in situ by staining with a labelled reaction mixture and transfer to a cellulose PEI paper before being exposed to X-ray film for 1 to 2 days. Lane 1, mixture of C86S2Cl and AGM2 cell extracts; lane 2, reACla; lane 3, reAClb; lane 4, reACld; lane 5, reAC2a; lane 6, reAC2b; lane 7, reAC3b; lane 8, rePOA2c; lane 9, rePOA3c; lane 10, reSP1c; lane 11, reSP2a. 50 ug protein (between 4 and 12 uL cell extract) were loaded onto each lane except for lane 1 (15 ug protein/3 uL cell extract for C86S2Cl; and 150 ug protein/15 uL cell extract for AGM2) and lane 11 (135 ug protein). The anode is at the top.
Figure 4.11

Distribution of HPRT activity of reactivants in the isoelectric focusing gel.

Densitometric tracings (from cathode to anode) of part of the autoradiogram shown in Figure 4.10 are illustrated here. The relative intensities of the HPRT activity localized in each band were determined using an LKB laser densitometer. Numbers in parentheses indicate the lane numbers of the gel in Figure 4.10. (a) mixture of AGM2 and C86S2C1 (1); (b) reAC2b (6); (c) rePOA2c (8).
enzyme in addition to a less intense band of HPRT activity intermediate to wild-type and mutant pIs. These bands represented the HPRT<sup>W/W</sup> homodimer and the HPRT<sup>W/A</sup> heterodimer, respectively. (The HPRT tetramer dissociates to form dimers in solutions of low ionic strength.) The presence of an HPRT heterodimer band on isoelectric focusing gels and the HPRT activity with intermediate heat stability is evidence for the co-expression of both the hprt<sup>W</sup> and the hprt<sup>A</sup> alleles in each cell of these clonal populations.

Within these reactivants, the HPRT<sup>W/A</sup> heterodimer band never exceeded that of the HPRT<sup>W/W</sup> homodimer band, which, in turn, was always greater than that of the HPRT<sup>W/A</sup> mutant band.

Consistent with the diverse HPRT specific activities and distinct heat inactivation kinetics of the reactivants, each clone possessed a unique ratio of dimer bands. Table 4.2 summarizes the relative ratios of the bands of HPRT activity within each reactivant. The relative sum of the intensities of the bands in each lane is directly proportional to the measured HPRT specific activity for each reactivant.

4.1.4.5 HPRT AND PGK-1 mRNA LEVELS

Further evidence for the reactivation of the inactive X hprt can be provided by measurement of the levels of hprt mRNA in reactivants. RNA collected from 5AC-treated unselected populations of AGM2 and reactivants was analyzed by C.N. Adra by Northern blotting and probing with an hprt cDNA. (Figure 4.12a). The levels of hprt mRNA in reactivants were higher than AGM2 levels in all cases, ranging from over 2 fold (in reAC2b cells) to almost 5 fold (in reAC2a cells) (Table 4.3). The results are consistent with the biochemical analyses of increased HPRT specific activities, and
Table 4.2

Relative ratios of HPRT heterodimer and homodimer bands in isoelectric focusing gels of reactivants.

<table>
<thead>
<tr>
<th>Cell Name</th>
<th>m/m</th>
<th>w/m</th>
<th>w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGM2</td>
<td>1 - 0</td>
<td>(0.2)</td>
<td></td>
</tr>
<tr>
<td>reAC1a</td>
<td>1 - 1.7</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>reAC1b</td>
<td>1 - 1.1</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>reAC1d</td>
<td>1 - 2.1</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>reAC2a</td>
<td>1 - 2.7</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>reAC2b</td>
<td>1 - 2.6</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>reAC3b</td>
<td>1 - 2.5</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>rePOA2c</td>
<td>1 - 1.1</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>

The relative quantities of activity in the HPRT homo- and heterodimer bands were estimated by integration of the peak area produced by laser densitometer scans of IEF gel autoradiograms. "m/m" indicates the HPRT mutant homodimer band, "w/m" indicates the HPRT wild-type/mutant heterodimer band, and "w/w" indicates the HPRT wild-type homodimer band. The isoelectric focusing pattern of AGM2 shows the presence of a small band of HPRT activity which apparently co-migrates with the wild-type HPRT homodimer (see Figure 3.5 and 4.5). This species is believed to be an isoenzyme. The percentage of activity in the different dimer species did not change when these cell extracts were analyzed on other gels, nor when the concentration of the lysate was varied by a factor of 2. Focusing patterns from cell extracts isolated at different times (separate experiments) were also not significantly different.
Figure 4.12

Level of hprt and pgk-1 mRNA in reactivants of AGM2 and parental cells.

The RNA from various EC cells and stable reactivant clones of AGM2 were analyzed by C.N. Adra by Northern blotting and probing with cDNAs to two X-linked genes: hprt (a) and phosphoglycerate kinase (pgk-1) (b). Total RNA was purified, fractionated on a 1% formaldehyde gel, blotted on a nylon membrane, then hybridized to (a) an hprt probe, pHPT5 (Konecki et al., 1982), or (b) a mouse pgk-1 cDNA insert (largely 3’ untranslated region) (Adra et al., 1987). The filters were washed at 50°C in 1 x SSC + 0.1% SDS for 1 hour. Equal amounts (50 ug) of total RNA of the following samples were loaded in each lane: C86S2C1 (lane 1), AGM2 (lane 2), 5AC-treated AGM2 (lane 3), reAC2b (lane 4), reAC3b (lane 5), reAC1a (lane 6), reAC2a (lane 7), reAC1b (lane 8), reAC1d (lane 9), BJK88p18 (a Chinese hamster cell line with a deleted hprt (Fuscoe et al., 1983)) (lane 10). Chinese hamster pgk-1 is not recognized by the mouse pgk-1 probe used here.
Table 4.3

Comparison of relative levels of hprt and pgk-1 mRNA in reactivants.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Cell</th>
<th>mRNA Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>hprt</td>
</tr>
<tr>
<td>1</td>
<td>C86S2G1</td>
<td>100%</td>
</tr>
<tr>
<td>2</td>
<td>AGM2</td>
<td>57%</td>
</tr>
<tr>
<td>3</td>
<td>AGM2 (5AC)</td>
<td>128%</td>
</tr>
<tr>
<td>4</td>
<td>reAC2b</td>
<td>133%</td>
</tr>
<tr>
<td>5</td>
<td>reAC3b</td>
<td>160%</td>
</tr>
<tr>
<td>8</td>
<td>reAC1b</td>
<td>170%</td>
</tr>
<tr>
<td>6</td>
<td>reAC1a</td>
<td>170%</td>
</tr>
<tr>
<td>9</td>
<td>reAC1d</td>
<td>207%</td>
</tr>
<tr>
<td>7</td>
<td>reAC2a</td>
<td>274%</td>
</tr>
</tbody>
</table>

Relative mRNA levels were calculated from laser densitometric scans of the preceding Northern blot (Figure 4.11). Percentages represent relative intensities of the bands as determined by integration of peak height areas produced by an LKB laser densitometer. Levels are expressed relative to wild-type (C86S2G1) levels of hprt and pgk-1 mRNA (lanes 1 of panels A and B, respectively).
indicate that all reactivants undergo a concomitant increase in hprt mRNA expression, although the extent of hprt mRNA increase did not always correlate well with the increased HPRT activities.

Levels of mRNA from another X-linked gene (pgk-1) were also examined by C.N. Adra by reprobing the same RNA blot with a pgk-1 cDNA probe (Figure 4.12b). In four of 7 clones assayed (reAC2b, reAC3b, reAC1a, and reAC2a), levels of pgk-1 mRNA were higher than those of C86AGM2 cells. However, there appeared to be no correlation between the levels of hprt and pgk-1 mRNA in individual clones; that is, clones which demonstrated increased levels of pgk mRNA were not necessarily those with the highest levels of hprt mRNA.

4.1.4.6 CHROMOSOME REPLICATION PATTERNS

A main feature of the X1 is its timing of replication; like most heterochromatic material, the inactive X chromosome replicates its DNA in the second half of the S phase of the cell cycle. To further investigate the extent of reactivation in the clones isolated, the chromosome replication patterns of several reactivants were studied using cytogenetic procedures (Alves and Jonassen, 1978).

Figure 4.13 shows metaphase spreads of AGM2 cells and those of a reactivant. AGM2 cells possess a uniformly-dark staining X chromosome characteristic of late replication (Figure 4.13 panel a; McBurney and Adamson, 1976), while reactivant reAC1d exhibited the characteristic banded pattern of two isocyclically-replicating X chromosomes (Figure 4.13 panel b). In over 60 metaphases scored for each of 5 reactivants examined (reAC1b, reAC1d, reAC2a, rePOA2c, and reSP1c), no evidence was found for a
Metaphases showing chromosome replication patterns in AGM2 cells and in reactivants.

Cells were exposed to $10^{-4}$ M BrdU for 6 hours before metaphases were collected and stained for viewing as described. Using this procedure, late replicating chromosomal regions appear dark. The inactive X chromosome (large arrow) stains uniformly dark indicating that it is replicated only during the late S phase. The active (isocyclic) X chromosome (small arrows) demonstrates a characteristic banded appearance. (a) AGM2; (b) reACld. All reactivants possessed 2 X chromosomes (as identified by G-banding procedures) which replicated isocyclically with the autosomes, primarily during the early S phase. In all reactivant metaphase preparations studied, no late-labelling X chromosome was observed.
late replicating X chromosome in any of these cells. The presence of 2 X chromosomes in each reactivant examined was verified by G-bANDING.

4.1.4.7 METHYLATION LEVELS

To determine the overall methylation levels of reactivants, the % 5-methylcytosine content of 7 reactivants was determined as described previously. All clones analyzed possessed 5mC levels not significantly different from parental AGM2 5mC levels (4.4% ±0.1%) (reAC1a, 4.2% ±0.07%; reAC1d, 4.5%; reAC2a, 4.0% ±0.22%; reAC2b, 4.7% ±0.08%; reAC3b, 4.5% ±0.09%; reSP1c, 4.3% ±0.07%; and reSP2a, 4.3% ±0.05%. Results are an average of 2 to 4 determinations). It is interesting to note that the reactivant with the lowest methylation level has the highest HPRT specific activity, although none of these reactivants appear to contain genomic DNA which was significantly hypomethylated relative to C86AGM2 cells.

4.1.5 EFFECTS OF DIFFERENTIATION ON THE ACTIVE X CHROMOSOMES OF REACTIVANTS

The results discussed in the previous sections suggest that HATr clones of C86AGM2 stably express the hp rt allele from the previously inactive X chromosome, and that this chromosome is no longer late-replicating. It is therefore likely that this reactivation phenomenon represents a whole chromosome event. Previous studies in this laboratory using C86 cells also suggest that reactivation of the Xr occurs as a whole chromosome event (Paterno et al., 1985).
It has been shown that differentiation of EC cells possessing 2 active X chromosomes results in the concomitant inactivation of one X (Paterno and McBurney, 1985). To determine if differentiation has any effect on transcription from, or early replication of, the reactivated X chromosome of reactants, clones were differentiated in monolayer by exposure to RA acid for 10 to 15 days as described. Following differentiation, cells were harvested for measurement of HPRT specific activities, isoelectric focusing, and determination of chromosome replication patterns. Studies of the antigenic and morphologic characteristics of these cells indicated that they differentiated into the same spectra of cell types seen in differentiated AGM2 cultures (data not shown).

4.1.5.1 HPRT SPECIFIC ACTIVITIES

The HPRT specific activities of reactants and their differentiated progeny are presented in Figure 4.14. If differentiation is accompanied by inactivation of an X chromosome, a decrease in levels of HPRT activity would be expected. Differentiation of almost all reactants, as well as parental AGM2 cells, resulted in little or no significant change in HPRT specific activities. However, in two of ten cases (reAC1b and reSP3a), differentiation resulted in a significant (60%) decrease in HPRT activity (from 0.053 to 0.020 and 0.074 to 0.029 nmol IMP/min/mg protein, for reAC1b and reSP3a, respectively). To investigate if the decrease in HPRT activity observed in these cells was due to the inactivation of an X chromosome, the isoelectric focusing pattern of several reactants was determined.
Figure 4.14

HPRT specific activities of differentiated reactivants.

Reactivant cells were differentiated in monolayer by exposure to $10^{-7}$ M retinoic acid for 10 to 15 days before harvesting and measurement of HPRT specific activity. Open bars represent HPRT activities of reactivants before differentiation; stippled bars denote HPRT activities after RA treatment. Clones are: 1, reAC1a; 2, reAC1b; 3, reAC1d; 4, reAC2a; 5, reAC2b; 6, reAC2c; 7, reAC3b; 8, rePOA2c; 9, reSP1c; and 10, reSP3a. Each bar represents a separate experiment.
Hpr of Specific Activity (nmol IMP/min/mg protein)
4.1.5.2 HPRT ISELEETRIC FOCUSING

Figure 4.15 shows the isoelectric focusing pattern of several reactivants and their differentiated progeny. In up to four separate differentiation experiments for each clone, no decrease in the homo- or heterodimer HPRT bands was observed, nor did the relative ratio of any band appear to change for 9 of 11 reactivants. However, differentiation of reAChb (data not shown) and rePOA2c (Figure 4.15 lane 5 and 6) appeared to induce a change in the HPRT isoelectric focusing profile. In reAChb cells, a decrease in the intensity of each band was observed, although the relative ratio of each dimer species remained constant. In contrast, HPRT from rePOA2c cells showed a change in the relative intensities of the homo- and heterodimer bands such that the HPRT\textsuperscript{\textw} homodimer increased in intensity and the HPRT\textsuperscript{\texth} homodimer band decreased significantly in intensity relative to their ratios prior to differentiation. This change in HPRT IEF pattern may be a result of partial inactivation of the hprt\textsuperscript{\texth} allele.

In lanes 7 and 8 of Figure 4.15, the bands of HPRT activity of reSP3a appear to decrease in intensity following differentiation, seemingly in correlation with the HPRT specific activity levels reported in the previous section. Unfortunately, this is primarily due to the amount of cell extract loaded onto this lane (only 40% of the preceding lane), making direct comparisons difficult. However, it appears that there is not a significant change in the relative ratios of the different HPRT hetero- and homodimers.
Figure 4.15

Isoelectric focusing of HPRT from differentiated reactivants.

Reactivants were differentiated in retinoic acid as described previously for 10 to 15 days before harvesting and preparation of cell extracts. HPRT activity in these extracts was then focused using pH 6-8 acrylamide gels and viewed in situ as described in Materials and Methods. 100 ug of protein (between 8 and 13 uL cell extract) were loaded for each sample except where indicated. Cell extracts are as follows: lane 1, reAC3b; lane 2, reAC3b following RA treatment for 12 days; lane 3, reAC1a (120 ug protein); lane 4, reAC1a following RA treatment for 15 days (120 ug protein); lane 5, rePOA2c; lane 6, rePOA2c following RA treatment for 11 days; lane 7, reSP3a; lane 8, reSP3a following RA treatment for 10 days (40 ug protein only; 5.8 uL cell extract); lane 9, reAC2a; lane 10, reAC2a following RA treatment for 11 days. The anode is at the top.
4.1.5.3 CHROMOSOME REPLICATION PATTERNS

As discussed in section 4.1.4.6, all reactivants apparently possessed 2 early replicating X chromosomes. To determine if differentiation of reactivants affected the replication pattern of the X chromosomes, three reactivants were prepared for examination of chromosome replication patterns following their differentiation. In each differentiated reactivant examined (reAC1d, reAC2a, rePOA2c), no evidence for a late replicating X chromosome was found. Over 70 metaphase spreads were examined for each reactivant, which retained a mean of 41 chromosomes, including 2 X chromosomes.
4.2 DISCUSSION

In this report, we investigated the reactivation of the inactive X chromosome in the clone C86S2C1 AGM2. This clone possesses a mutant hprt (hprt<sup>+</sup>) which encodes an enzyme that is qualitatively distinguishable from the wild-type enzyme in its specific activity, heat inactivation kinetics and isoelectric focusing point. Treatment of C86AGM2 cells with the DNA demethylating agents 5AC and POA resulted in increased HPRT activity. This increased HPRT activity possessed heat denaturation kinetics and isoelectric focusing characteristics consistent with the expression of both hprt<sup>+</sup> and hprt<sup>+</sup> alleles within the same cell. Thus, in C86AGM2 cells treated with DNA demethylating agents, increased levels of HPRT activity were the result of activation of the hprt<sup>+</sup> allele on the inactive X chromosome.

4.2.1 REACTIVATION IS TRANSIENT AND EXTENSIVE

In unselected cell populations treated with 5AC, the increase in HPRT activity was found to be transient, reaching a maximum about 3 days following removal of the drug. In these 5AC-treated cultures, virtually all of the HPRT activity was less heat labile than the enzyme from untreated AGM2 cells (Figure 4.4) implying expression from the hprt<sup>+</sup> allele in almost all of the cells in the treated population. Increased levels of hprt mRNA in these cells (Table 4.3) was also consistent with elevated transcription from perhaps 70% of the population. Studies of [<sup>3</sup>H]-hypoxanthine uptake in individual C86 cells following 5AC treatment by Paterno et al. (1985) resulted in similar conclusions. Treatment of AGM2
cells with POA also apparently resulted in the transient expression of the hprt\textsuperscript{*} allele in most cells.

4.2.2 \textbf{VARIABLE HPRT TRANSCRIPTION IN STABLE REACTIVATE\\NTS}

The proportion of cells in C86ACM2 cultures which subsequently stably expressed the hprt\textsuperscript{*} allele at levels sufficient to sustain growth in HAT medium was much lower (approximately 1 - 2% in 5AC-treated cultures and 0.1 - 0.2% in POA-treated cultures). Thus, the hprt\textsuperscript{*} allele on the inactive X chromosome was activated and subsequently repressed in the vast majority of these cells. If expression of the hprt\textsuperscript{*} allele were a direct consequence of DNA demethylation, then remethylation of the genome which occurs after removal of 5AC and POA may be responsible for the repression event.

Amongst the HAT-resistant clones which stably express the hprt\textsuperscript{*} allele, the level of genomic methylation was variable, but similar to that of the parental cells. It is possible that a few critical sites of DNA remained unmethylated in these cells allowing continued expression of the hprt\textsuperscript{*} allele.

Interestingly, the level of transcription of the hprt\textsuperscript{*} allele was variable from clone to clone (Table 4.3). Levels of hprt mRNA were lower than that predicted for transcription from 2 hprt loci in one reactivant (reAC2b), while three reactivants (reAC3b, reAC1b, and reAC1a) exhibited "appropriate" levels of hprt mRNA, and two other reactivants (reAC1d and reAC2a) possessed levels of hprt mRNA consistent with "hyper-transcription" from one or both loci. This clonal variation in expression of genes on the X\textsubscript{i} following 5AC treatment has been observed by others, including partial
expression (Migeon et al., 1982; Lester et al., 1982; Mohandas et al., 1984; Wolf et al., 1984), complete reactivation (Lester et al., 1982), and even hypertranscription (Migeon et al., 1985). This variable expression of gene loci is perhaps attributable to changes in methylation at sites, whether within the locus or elsewhere, that influence the level of HPRT expression. The target for 5AC and POA action may not be the genes on the inactive X chromosomes themselves but another gene whose product activates these X-linked genes. Perhaps the extent of demethylation resulted in changes in the chromatin conformation such that genes are transcribed at varying levels. In any event, it seems that 5AC treatment can influence not only the expression of the locus on the inactive X chromosome but also the amount of product that is made.

4.2.3 HPRT POLYMERS IN REACTIVANTS AND LEVELS OF ACTIVITY

Despite apparent hprrt hypertranscription observed in some reactivants, these clones do not exhibit HPRT specific activities approaching the wild-type cells. A likely explanation is derived from the tetrameric nature of the native HPRT enzyme (Holden and Kelley, 1978; Johnson et al., 1979). Generally, heteromeric molecules (in this case, those carrying one or more HPRT<sup>a</sup> monomers with one or more HPRT<sup>+</sup> monomers) have activity corresponding to the arithmetic mean of the activities of the contributing monomers. If we assume random association of HPRT monomers, we can calculate the probability of the presence of each HPRT species (ie one each of HPRT<sup/>/</sup> and HPRT<sup>/</sup> homotetramers and three species of HPRT<sup>/</sup> heterotetramers) and multiply each by the relative activity of that HPRT
molecule, to get an estimate of the relative HPRT activity of the cell under those conditions. We have already determined (see Chapter 3) that the HPRT<sup>o/m</sup> homotetramer has approximately 1% activity relative to the HPRT<sup>+</msup/> homotetramer (100% activity); therefore, if we use the arithmetic mean assumption, the HPRT heterotetramers have relative activities corresponding to the mean of the activities of its component monomers.

From these calculations it can be demonstrated that if both hpri<sup>−</sup> and hpri<sup>+</sup> loci are expressed at equal rates, the expected HPRT activity in that cell would be approximately 50% of wild-type levels. Even in cells where the hpri<sup>−</sup> allele is expressed at twice the rate of hpri<sup>+</sup> (as may be the case for reAC2a), the expected HPRT activity in these cells only increases to 67% of normal HPRT activity levels (which, interestingly, is close to the measured HPRT specific activity of reAC2a).

It follows from these assumptions, then, that isolation of reactivants with HPRT specific activities below those of the C86 wild-type cells by no means indicates that the reactivants have not fully reactivated their hpri<sup>−</sup> allele. This explains why reactivants isolated in this study appear to have low levels of HPRT activity relative to the amounts of HPRT mRNA they contain. This hypothesis also predicts that the reactivation frequency of AGM2 cells is actually higher than that calculated from the number of HAT<sup>+</sup> cells arising in treated cultures, as only cells which stably express exaggerated levels of hpri<sup>−</sup> will produce sufficient functional HPRT to survive in HAT medium.

To further complicate the situation, there is evidence to suggest that the heteromeric HPRT molecules may have less activity than the
arithmetic means of the component monomers (i.e. the HPRT<sup>+</sup> monomer may exert a negative effect on the activities of the HPRT<sup>-</sup> monomer(s) in a multimeric enzyme). In isoelectric focusing gels (where the HPRT enzyme exists as a dimer), the activities associated with the HPRT<sup>+/+</sup> heterodimer were almost always less than those seen in the corresponding HPRT<sup+)/+</sup> homodimer. If the HPRT<sup>+/+</sup> heterodimer activity was equal to the arithmetic mean of the activities of its component subunits (100% for HPRT<sup+)/+</sup> and 4% for HPRT<sup>-/-</sup>, resulting in HPRT<sup>+/+</sup> with approximately 50% activity), and the levels of transcription of hprt<sup>+</sup> and hprt<sup>-</sup> were approximately equal within a cell, then the band of HPRT<sup>+/+</sup> heterodimer activity seen in the gels should be as intense as that of the HPRT<sup+)/+</sup> homodimer. However, calculations using the measured ratio of the bands of HPRT activity seen in the IEF gels of reactivants indicate that the activity of HPRT<sup>+/+</sup> is approximately 10-15% that of HPRT<sup+)/+</sup>. These calculations can only be made loosely, as the influence of factors such as non-random association of monomers and selective degradation of abnormal HPRT protein (Capecchi et al., 1974) cannot be disregarded.

4.2.4 REACTIVATION IS DEPENDENT ON THE UNDIFFERENTIATED STATE

In G86AGM2 cells induced to differentiate prior to 5AC treatment, transient activation of the hprt<sup>+</sup> did not occur. 5AC was effective in inducing DNA demethylation of these differentiated cells, but this was not accompanied by expression of hprt<sup>+</sup>. No evidence was found for any change in HPRT activity or appearance of HPRT<sup>+</sup>. As EC cells lose their immortality upon differentiation, we could not select for stable
reactivants in HAT medium. In similar experiments, Paterno et al. (1985) demonstrated that expression of X-linked genes other than hprr also did not occur. These observations are consistent with the proposal that there are multiple mechanisms responsible for repression of X-linked genes in the X-inactivation process. The process of differentiation appears to invoke a further mechanism of control of transcriptional repression of the Xq. For reactivation to occur in these cells, DNA demethylation may still be necessary, but no longer sufficient. Differentiation of C86AGM2 cells may induce a change in the chromatin conformation of the inactive X, rendering it more stably repressed than in undifferentiated cells. 5AC treatment may therefore no longer induce frequent reactivation events which involve the entire X chromosome, although undetected localized derepression events may have occurred.

4.2.5 DIFFERENTIATION OF REACTIVANTS

Certain lines of female EC cells have been shown to possess two active X chromosomes, one of which inactivates upon cellular differentiation (Martín et al., 1978; McBurney and Strutt, 1980; Paterno and McBurney, 1985). However, reactivants of C86AGM2 cells which possess two isocyclically-replicating X chromosomes and which express hprr genes on both X chromosomes showed no evidence of X inactivation following induced differentiation. Expression of both hprr and hprr alleles continued in each cell, and neither X chromosome became late-replicating.

However, in the reactivant rePOA2c, electrofocusing revealed a change in the ratio of HPRT dimer bands following differentiation. The HPRT
homodimer band showed reduced levels relative to the HPRT™ homodimer band, indicating that transcription of the hprr represents allele may have decreased as a result of differentiation. If partial inactivation is indeed responsible for this change in HPRT isoelectric profile, it is interesting to note that it is the previously active hprr allele which is being repressed, and not the allele on the originally inactive X chromosome. Thus, the previously inactive X chromosome apparently does not demonstrate preferential re-inactivation, and may not be "imprinted" in any way.

The spectrum of differentiated cell types formed by these reactants was similar to that seen upon differentiation of parental C86 cells, yet the differentiated cells formed by these reactants apparently retained 2 active X chromosomes. This lack of re-inactivation upon differentiation is consistent with observations of Paterno et al. (1985), although in the latter studies a 50% decrease in levels of HPRT activity was observed in the differentiated cells.

Although X inactivation normally occurs in embryos concomitantly with cellular differentiation (Monk and Harper, 1979), it seems that at the cellular level, the events of X chromosome inactivation and cellular differentiation can proceed independently. Perhaps X inactivation is a one-time only event, or perhaps re-inactivation can occur only in chromosomes which are not already partially inactive (i.e., the inactivation event is a whole chromosome event). Escape of the hprr gene from re-inactivation may also indicate that the process of X inactivation is a sequential one, in which DNA methylation must occur prior to the imposition of further mechanisms of X1 maintenance (Monk, 1986). Although
several studies indicate that methylation of genes on the inactive X chromosome occurs after the inactivation event has taken place (Lock et al., 1987; Adra et al., in preparation), models of the X inactivation process have been proposed that predict perhaps two stages of DNA methylation, occurring both prior to and following a conformational event which further stabilizes the X inactive state (Monk 1986).
CHAPTER 5

CONCLUSIONS

The primary objective of this thesis was to develop a system that would provide unequivocal proof of reactivation of the inactive X chromosome in C86 female EC cells following treatment with DNA demethylating agents. The isolation of C86AGM2, which possesses a qualitatively altered hprt allele on the X, and the identification of heteromeric HPRT enzyme in cultures of AGM2 cells treated with 5AC and POA have provided such proof.

In the process of investigating the reactivation event in these cells, we have observed a number of features of reactivants which may be relevant to our study of the mechanisms of the X inactivation process.

The variable levels of hprt transcription observed between different HAT-resistant clones indicates that reactivation of this locus is not simply an "on" or "off" event. It seems unlikely that relative levels of DNA methylation directly affect transcription rates of nearby loci; however it is possible that varying degrees of demethylation in and around the reactivated gene itself (or in neighbouring regions which are responsible for maintaining genetic inactivity of localized segments of the X) may influence transcription. It may prove interesting therefore to examine the methylation pattern in sequences close to the hprt locus in clones which exhibit only partial expression of the hprt allele relative to that of clones which hypertranscribe the hprt. Of course, any "activator" gene for X-linked loci need not be located on the X chromosome.
Expression of the hprrt allele from the inactive \( X \) in most cells following treatment was found to be transient; the allele was expressed and subsequently repressed. The important detail concerning this phenomenon might not be the fact that the majority of the treated AGM2 cells reactivated their \( X_1 \), but that the majority of cells also \textit{inactivated} their \( X_1 \). In sharp contrast, none of the clones isolated which stably expressed the hprrt" appeared able to inactivate their \( X_1 \), even after differentiation was induced. This may indicate that the mechanism(s) responsible for "short-term" activation of repressed genes is not related to that required for stable reactivation. This may explain why 5AC and POA induced similar increases in hprrt levels in AGM2 cells in transient reactivation assays, yet POA was apparently ten-fold less efficient at inducing stable reactivation. 5AC and POA induce DNA demethylation by two different modes of action.

It is important to continue studies of reactivation events in the C86AGM2 cells we have isolated. For example, further attempts should be made to induce inactivation of the reactivated \( X \) chromosome in the HAT-resistant clones we have isolated. \textit{In vitro} or \textit{in vivo} methylation may or may not induce a re-inactivation event. These studies may yield important observations about the mechanisms involved in maintaining transcriptional activity in these cells, as well as the apparent separation of the events of \( X \) chromosome inactivation and cellular differentiation.

The observations we have made in this thesis support a model for \( X \)-chromosome inactivation proposed by Monk in 1986. Monk postulated that the inactivation process involves four events, including a) initial choice of a single active \( X \) chromosome, b) methylation of key site sequences which
inactivate en bloc the X chromosome not chosen to be active, c) a conformational event, which demarcates the inactive X chromosome into segments of inactivity, and occurs upon differentiation of cells, and d) further methylation of the inactive genes on the X in somatic tissues.

It is proposed that embryonal carcinoma cells exhibit a level of X chromosome control consistent with stage b) in the inactivation process, as 5AC treatment results in reactivation of the whole X chromosome (Monk, 1986). I believe that C86AGM2 cells may be at this stage in the X inactivation process. According to the model, differentiation of cells induces the DNA conformation change described in stage c), whereby 5AC-induced reactivation is restricted. Similarly, differentiated AGM2 cells do not appear to respond to 5AC. However, reactivants of AGM2 apparently do not undergo this conformational event upon differentiation. The model allows a simple explanation for this phenomenon -- the conformational change (stage c) may be dependent on key site methylation (stage b). I believe that the HAT-resistant clones isolated in this study lost this "key site" methylation when reactivation occurred following 5AC treatment. Therefore, these reactivants are unable to inactive their X chromosomes until methylation of these key sites reoccurs.

C86AGM2 cells and their reactivants thus may prove to be extremely useful for further studies of the mechanisms involved in the X chromosome inactivation process.
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