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X CHROMOSOME INACTIVATION
IN
MOUSE EMBRYONAL CARCINOMA CELLS

by

Gary David Paterno

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

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

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ACKNOWLEDGEMENTS

I wish to thank Mike McBurney for the opportunity to work in his laboratory and for guiding me through the course of this study. His enthusiasm for science "rubbed-off" especially when things were not "golden". Who else could have convinced me that females were just as interesting at the cellular level.

I am grateful to the members of my research committee, Dave Brown, Donal Hickey and Doug Johnson for their advice and help during the course of this work.

I thank Verne Chapman and Janice Sanford for performing some of the DNA methylation experiments and to Verne for many helpful discussions.

Special thanks to all of the members of the McB lab; Liz Mary, Hoser, Big "J", Rude, Shake, Jane, Karen, Martha, Schmit, and Chantal for all your help, fun, friendship and coffee.

I thank Jacque Helie and George Ben-Tchavtchavadze for their expert help with the figures and photos in this thesis.

I thank the National Cancer Institute of Canada, the Government of Ontario and the University of Ottawa for awarding me the scholarships for this study.

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Abbreviations

EC	-	embryonal carcinoma
Xa	-	active X chromosome
Xi	-	inactive X chromosome
X^m	-	maternally derived X chromosome
X^p	-	paternally derived X chromosome
G6PD	-	glucose-6-phosphate dehydrogenase
6PGD	-	6-phosphogluconate dehydrogenase
PGK	-	phosphoglycerate kinase
HGPRT	-	hypoxanthine phosphoribosyl transferase
APRT	-	adenine phosphoribosyl transferase
alpha-GAL	-	Alpha galactosidase
Beta-GAL	-	Beta galactosidase
5-AC	-	5-azacytidine
p.c.	-	post coitum
Xce	-	X controlling element
EE	-	extraembryonicendoderm
8-AG	-	8-azaguanine
HAT	-	hypoxanthine-methotrexate-thymidine
hgprt	-	hypoxanthine phosphoribosyl transferase gene
RA	-	retinoic acid
PBS	-	phosphate buffered saline
DMSO	-	dimethylsulfoxide
BrdU	-	bromodeoxyuridine
HAT^+	-	growth in HAT medium
OCT	-	ornithine carbamoyl transferase
cAMP	-	cyclic adenosine monophosphate
hr	-	hours
min	-	minutes
sec	-	seconds
M	-	Molar

Abstract

An entire X chromosome is genetically inactive in the somatic cells of female mammals. The study of mechanisms which initiate and maintain this inactivation are important in understanding how gene expression is regulated during development. I have utilized embryonal carcinoma (EC) cells to study X chromosome inactivation. EC cells are analogous to early embryonic cells and diploid cells can possess one or two active X chromosomes. Thus, they provide an *in vitro* system for the study of X chromosome inactivation.

The experimental results demonstrated that retinoic acid induced the rapid and complete differentiation of P10 EC cells into a tissue resembling the extraembryonic endoderm of the embryo. This differentiation is accompanied by the rapid and synchronous inactivation of an X chromosome, in the majority of the cells, as determined by the 50% reduction in the specific activities of two X chromosome encoded enzymes and the appearance of a late replicating X chromosome in 55% of the differentiated cells. This synchronous system will provide the tool to study the mechanisms responsible for X inactivation. The P10 derived extraembryonic endoderm contained phosphoglycerate kinase-1 (PGK-1) electrophoretic and alpha-galactosidase (alpha-GAL) thermostability variants coded for by the maternal and paternally-derived X chromosomes. Thus, P10 cells did not preferentially inactivate the paternally-derived X chromosome like the analogous tissue in the normal embryo.

The inactivation of the maternal or paternal X chromosome in P10 cells may be influenced by the heterozygous X controlling element (Xce) alleles and suggests that the X inactivation process, in differentiating P10 cells, resembles that which occurs in normal embryonic, rather than, extraembryonic tissues. I propose a model of mitotic dilution to account for X chromosome imprinting during mammalian embryogenesis.

C86S1A1 and C100AG1 are female EC cell lines which possess an inactive X chromosome and a partially inactive X chromosome respectively and which are hgprt$^-$. The treatment of these cell lines with DNA demethylating agents, such as 5-azacytidine, resulted in a) the transient reactivation and expression of genes on the inactive X chromosome in almost all of the cells, b) the stable reactivation of the hgprt genes in 10-20% of the treated cells, and c) the isocyclic replication of both X chromosomes early in "S" phase of the cell cycle in C86S1A1 clones. C86S1A1(XO) cells, which have lost the inactive X chromosome, did not respond to 5-azacytidine. This strongly indicates that, in C86S1A1 cells, genes on the inactive X chromosome are being reactivated. C145FA12 and C145FAG11 are hgprt$^-$ clones of another female EC cell line, but these cells did not reactivate inactive X genes after exposure to 5-azacytidine. This suggests that they differ from C86S1A1 and C100AG1 in the states of their inactive X chromosomes. Differentiated derivatives of C100AG1 and C86S1A1 did not respond to

5-azacytidine, indicating that the ability to reactivate inactive X genes is dependent upon the undifferentiated state of the cell. The experimental evidence, suggests a model in which different female EC cell lines represent embryonic cells arrested at different stages of X chromosome inactivation and that DNA methylation may provide the initial mechanism for transcriptional inactivation of an X chromosome. It also suggests that there is a second inactivation mechanism linked to cellular differentiation which replaces, or is present in addition to, DNA methylation and is responsible for maintaining X inactivation in somatic cells.

Resume

Un chromosome X entier est génétiquement inactif dans les cellules somatique chez les mammifères femelles. L'élucidation des méchanismes qui commencent et maintient cette inactivation donne une sytemè pour l'etude de l'expression differentielle génétique. Les resultats demontrent que l'acide rétinoiique induit la différentiation rapide et complet des cellules P10 carcinomie embryonaire (EC) en un tissu qui resemble à l'endoderme extraembryonnaire de l'embryon. Cette différentiation est accompagnée par l'activation rapid and synchronisée de l'un des chromosomes X dans la majorié des cellules. L'inactivation est determinée par la reduction de 50% de l'activité spécifque de deux enzymes encodés du chromosome X. La différentiation est aussi accompagnée par l'apparition d'un chromosome X à replication tardive dans 55% des cellules différentiées. Ce system synchronisé servira d'outil afin d'étudier les méchansismes responsables de l'inactivation du chromosome X.

L'endoderme extraembryonnaire dérivé des cellules P10 contenait des variantes d'isoenzymes pour la PGK-1, suivant leur migration électrophorétiquè, et aussi pour l'alpha GAL, suivant leur degré de thermostabilité. Ces variantes sont encodées par chacun des deux chromosomes X, l'un maternel, l'autre paternel. Ainsi, l'endoderm extraembryonnaire derivé des cellules P10 n'a pas démontré l'inactivation du

chromosome X paternel tel qu'est le cas dans le tissu analogue chez l'embryon. L'inactivation du chromosome X maternel ou paternel chez les cellules P10 peut être influencée par les alleles "element de control" X (Xce) et suggère que le procédé d'inactivation du chromosome X chez le cellules P10 en étape de differentiation ressemble à celle qui survient chez les tissus embryonnaires. Je propose un modele de dilution mitotique afin d'expliquer de difference du chromosome X qui survient durant l'embryogenee chez les mammiferes.

Le traitement des lignées C100AG1 et C86S1A1 de cellules EC avec des agents de démethylation, tel que la 5-azacytidine, produit les effets suivants a) un reactivation transitoire et l'expression de genes sur le chromosome X inactif dans la quasi-totalité des cellules, b) la reactivation stable du gene hgprt dans 10-20% des cellules et c) la réplication des deux chromosomes X au début de la phase "S" de la cellule. Les cellules C86S1A1(XO) qui ont perdu le chromosome inactif ne répondent pas autraitement à la 5-azacytidine. C145FA12 et C145AG1 sont les autre lignée EC mais nonplus ne réactivent pas les genes X inactifs après le traitment à la 5-azacytidine, ce qui suggère qu'elles different quand à l'etat d'inactivation de leur chromosome X. Une fois différentiées, les cellules C100AG1 et C86S1A1 ne répndent plus a la 5-azacytidine. Ceci suggère que l'abilité de réactiver un chromosome X inactif dépend de l'etat non-differencié de la cellule. Les

résultats suggèrent un modèl ou des lignée différentes de cellules EC femelles représentent des cellules embryonnaires arrêtée a different stages d'inactivation du chromosome X et que la methylation de l'ADN peut fournirle méchanisme initial de l'inactivation de la transcription. Les résultats suggèrent aussi un autre méchanisme inconnu, relie à la différentiation cellulaire, qui remplace ou coexiste avec la methylation de l'ADN, et qui est responsable du maintient de l'etat inactif dans les cellules somatique.

CHAPTER ONE

INTRODUCTION

Our understanding of embryonic development and cellular differentiation requires the elucidation of the mechanisms which control the differential expression of each gene within the genome in a predictable temporal and spatial sequence. It is clear that different sets of genes must function at various times during embryonic development. Cells destined to form particular tissues must activate genes whose products are necessary for differentiation and repress genes that are no longer necessary, as well as genes which are not expressed in a particular differentiated cell type.

The study of development and differentiation also requires understanding the concepts of choice, commitment and stability. Any given embryonic cell must make a choice to differentiate along pathways characteristic of a given developmental lineage. Once this choice is made a cell is said to be committed, that is, it is destined to differentiate along a chosen developmental pathway and only that pathway. Once this commitment is made it is extraordinarily stable. At a later time a cell expresses the genes characteristic of the differentiated state of the committed cell lineage.

These developmental events must be regulated within

the cell in a temporal and spatial pattern in conjunction with other cells to give higher order structures comprising tissues and organs. These in turn must be regulated and patterned to give a functioning, multicellular organism. In all cases there must be levels of gene regulation which involve interacting cells, the intercellular environment, the intracellular environment and the previous developmental history of any given cell.

The subject of this thesis is mammalian X chromosome inactivation. X inactivation provides a unique, developmental model system to study a process which involves choice, commitment, the control of gene expression and stability. The study of the mechanisms controlling X chromosome inactivation may have a broader applicability in understanding other developmental regulatory mechanisms.

X CHROMOSOME INACTIVATION

The somatic cells of female, eutherian mammals possess two X chromosomes, while males possess only one X chromosome and a nonhomologous Y chromosome. The Y chromosome is the primary determinant of sex (Ohno, 1967). Early in embryogenesis, one of the two X chromosomes in each cell is genetically inactivated so that each somatic cell contains an active (Xa) and an inactive (Xi) X chromosome. The choice of which X chromosome will be inactivated is <u>random</u> (Lyon, 1961; Russell, 1961). Half of the embryonic cells at the

time of this event will express only the maternally derived X chromosome (X^m) and the other half express the paternally derived X chromosome (X^p).

The inactivation of the X chromosome is complete. There are no complementary active or inactive regions on both X chromosomes (Lyon, 1972; Gartler and Andina, 1976). However, there is a small region on the distal portion of the short arm of the human X chromosome which escapes, or partially escapes, inactivation and includes the genes for Xg, an embryonic blood group antigen (Gartler and Andina, 1976) and steroidal sulfatase (STS), an enzyme in which deficiencies result in X linked congenital icthyosis (Shapiro et al., 1978; Shapiro et al., 1979; Mohandas et al., 1978; Migeon et al., 1982). There is no homologous short arm on the murine X chromosome which is telocentric (Nesbitt, 1971) and the STS gene does not appear to be functional on the Xi (Gartler and Rivest, 1983). Thus, there may not be an equivalent region escaping inactivation on the mouse Xi chromosome.

The same X chromosome is maintained genetically inert in all mitotic descendents of a single somatic cell (Beutler et al., 1962; Davidson, et al., 1963; Rattazi and Cohen, 1972; Ray et al., 1972; Lyon, 1972). Spontaneous reactivation of individual Xi genes has been reported (Migeon, 1972), but it is a rare event. In addition, the Xi is refractory to attempts to reactivate it by various

physical and chemical means (Coming, 1966; Migeon, 1972; Romeo and Migeon, 1976; Wolf and Migeon, 1982). Thus X chromosome inactivation is heritable and it is stable.

The Xi is heterochromatic and has been observed in all somatic cells of female mammals that have been examined (Ohno et al., 1959; Lyon, 1972; Cattanach, 1975). The heterochromatic X chromosome in the interphase nucleus has also been termed the Barr body or the sex chromatin. The heterochromatic state of the Xi is consistant with the observation that heterochromatin is transcriptionally inactive (Cattanach, 1975).

Like most heterochromatic material, the Xi in all mammalian species replicates its DNA entirely during the late "S" phase of the cell cycle (late replicating) (Morishima et al., 1962; Nesbitt and Gartler, 1971; Mukerjee and Singh, 1964; Sharman, 1971). In systems where the products of the Xa and Xi can be distinguished, there is no expression of genes on the late replicating X (Rattazi and Cohen, 1976; Ray et al., 1972). Where both X chromosomes are genetically active, both X chromosomes replicate isocyclically early in 'S' phase (Takagi, 1974; Epstein et al., 1978; Issa et al., 1969) and late replication accompanies X inactivation, as determined by other criteria (Takagi and Martin, 1984; Epstein et al., 1978; McBurney and Strutt, 1980).

The Evolution of X Chromosome Inactivation

For the majority of eukaryotic gene products there is a proportional relationship between the number of copies of a particular gene and its level of protein product (Bartley and Epstein, 1980; Farber, 1973; Epstein et al., 1977; Westerveld et al., 1972; Marimo and Gainelli, 1975). Because female mammals are XX and males XY, there exists a gene dosage difference which would be reflected in increased levels of X encoded gene products. However, the levels of X encoded enzymes in male and female somatic cells are equal, due to the presence of a single Xa in female cells. X inactivation is thought to have evolved as a mechanism to insure the equivalent dosage of X encoded gene products in males and females, thus preventing variation for the action of natural selection between sexes (Lucchessi, 1978; Ohno, 1971). The genetic inactivation of one X chromosome is not detrimental to the female. There are mice which are karyotypically XO but are phenotypically normal, fertile females (Cattanach, 1962). This also demonstrates that a single X is all that is required for normal development.

The evolution of regulation of gene dosage levels by X inactivation in mammals probably provided a strong selective pressure for the retention of specific genes on the X chromosome (Ohno, 1967). All of the genes which have been mapped to the X chromosome in one mammalian species are X

encoded in a variety of other species (Ohno, 1973; Ricciuti and Ruddle, 1973; Shows and Brown, 1975; Chapman and Shows, 1976; Huertz and Hors-Cayla, 1978; Donald and Hope, 1981; Shimizu et al., 1981).

Within the animal kingdom there are several forms of dosage regulation of sex chromosomes which do not involve inactivation. In the family of gnats (Sciara), a complex mechanism involving X chromosome nondisjunction and elimination in male and female somatic and germ cells achieves dosage compensation between males and females (Cattanach, 1975; Rieffell and Crouse, 1966). In mealy bugs, there is a dosage compensation mechanism which inactivates the entire set of paternal chromosomes in female somatic cells (Brown and Nur, 1964; Brown, 1969). Drosophila possess an X-Y system of sex determination, however, sex is apparently influenced by the ratio of X chromosomes to autosomes and not the presence of a Y chromosome (Lucchessi, 1978). Instead of inactivation of one X chromosome in the female, there is a mechanism which reduces by one-half the expression of both X chromosomes in the female to the equivalent of the male level (Roehrdanz and Lucchesi, 1977; Lucchesi, 1978; Maroni and Lucchessi, 1980; Gadogkar et al., 1982). Other sex determining systems which involve ZZ-ZW sex chromosomes have apparently not evolved a similar mechanism for dosage compensation (Cock, 1964; Baverstock, 1982; Johnson and Turner, 1979; Ohno, 1969).

Turner, 1979; Ohno, 1969).

Within Mammalia, X inactivation is the rule, but there are some evolutionary exceptions involving certain species of moles, where giant X chromosomes are observed and the XO sex chromosome constitution is the normal female (Ohno, 1966; Ohno, 1969). In addition, X inactivation in marsupials is not random, but involves preferential inactivation of the paternally derived X chromosome (Cooper et al., 1971; Richardson et al., 1971; Sharman, 1971). There is some suggestion that random inactivation in eutherian mammals may have evolved from a system possessing preferential inactivation (Lifschytz and Lindsley, 1974; Cooper, 1971).

X CHROMOSOME ACTIVITY DURING MAMMALIAN DEVELOPMENT

The X Chromosome in Oocytes and Early Embryos of Mice

Investigation of the cytological manifestations of X inactivation (Austin, 1966; Issa et al., 1969; Takagi, 1974) and the extent of mosaicism in female mice heterozygous for X encoded coat colour alleles (Gardner and Lyon, 1971) determined that X inactivation occurred during embryological development but only beginning at the blastocyst stage, about the time of implantation. Since inactivation did not appear to occur until after several cleavages, several investigators looked for two-fold differences of X encoded enzymes in individual XX, and XO oocytes and in XX, XO and XY embryos at various developmental stages (Adler et al.,

1977; Chapman et al., 1978; Epstein et al., 1978; Kratzer and Gartler, 1978; Monk, 1978). Before X chromosome inactivation, female embryos with two Xa would have twice as much activity for X encoded enzymes as male embryos with one X chromosome.

The results from these studies demonstrated that XX oocytes possessed twice the specific activities of X encoded enzymes compared to those found in XO oocytes showing that both X chromosomes were active during oogenesis (Mangia et al., 1975; Monk and McLaren, 1981; Monk, 1978; Epstein, 1969; Epstein, 1972; Monk and Kathuria, 1977; Kozak et al., 1974).

The measurements of X encoded enzymes in embryos from the 8 cell stage until the early blastocyst (day 3 post coitum ; p.c.), clearly show that female (XX) embryos have twice the specific activities of HGPRT and alpha-GAL as those found in male (XY) embryos (Epstein et al., 1978; Monk and Harper, 1978; Kratzer and Gartler, 1978; Monk, 1978; Adler et al., 1977). The results for enzyme measurements prior to the 8 cell stage are masked by the presence of maternally inherited enzymes (Kratzer and Gartler, 1978).

Cytological investigations at early morula and early blastocyst stages demonstrate that both X chromosomes are euchromatic and early replicating, supporting the enzyme results (Takagi, 1974). Thus, at early embryonic stages,

both X chromosomes are genetically active.

A decrease in the specific activities of X encoded enzymes in XX embryos is interpreted as the time of initiation of X inactivation (Epstein et al., 1978; Monk and Harper, 1979; Monk, 1978). This decrease is first detected in the mouse at the early to mid-blastocyst transition (day 3 1/2-4 p.c.:40-50 cells), by measuring HGPRT and alpha-GAL specific activities. Estimates for the time of pgk-1 inactivation are later however, at about the egg cylinder stage (day 6 p.c.) (Kozak and Quinn, 1975). The enzyme activities of all X encoded enzymes are equal in female and male embryos by the egg cylinder stage (day 6 p.c.), suggesting that X inactivation has occured in all the cells of the female embryos (Monk, 1981) by this time.

The decrease in X encoded enzyme specific activities correlates temporally with cytological studies of inactivation (Takagi, 1974), but not with the estimates of the timing of X inactivation that are based upon mosaicism in female mice heterozygous for X encoded coat colour genes. (Gardner and Lyon, 1971). This discrepancy occurs because X inactivation occurs at different times in different tissues of the embryo.

X inactivation appears to occur concomitantly with the first differentiation events of each cell (Monk and Harper, 1979; Monk, 1981). Thus, inactivation occurs first in the

and the trophectoderm lineage which gives rise to mural trophoblast and chorionic ectoderm (West et al., 1977; Frels et al., 1979; Frels and Chapman, 1980, Papaioannou and West, 1981; Papaioannou et al., 1981; Takagi et al., 1978). The X^p or the X^m is randomly inactivated in the cells comprising the fetus, allantois, amnion and visceral yolk sac mesoderm, all of which are derived from the primitive ectoderm (epiblast lineage) (Papioannou et al., 1981).

The preferential inactivation in the trophectoderm and extraembryonic endoderm is accompanied by an initial replication of the Xi early in "S" phase of the cell cycle, with a shift to late replication at a later time (Takagi et al., 1982; Sugawara et al., 1983). This preferential inactivation of the X^p and its initial early replication pattern suggests the two events might be related and that there are differences in the X inactivation mechanism in certain extraembryonic tissues and the embryonic ectoderm (Takagi et al., 1982)

The relationship between the pattern of X inactivation and tissue differentiation during embryogenesis is summarized in Figure 1 which is adapted from Gartler and Riggs (1984) and Monk (1981).

The X Chromosome in Germ Cells

Since the oocyte possesses two Xa, either the germ line X chromosome must never undergo inactivation or the germ

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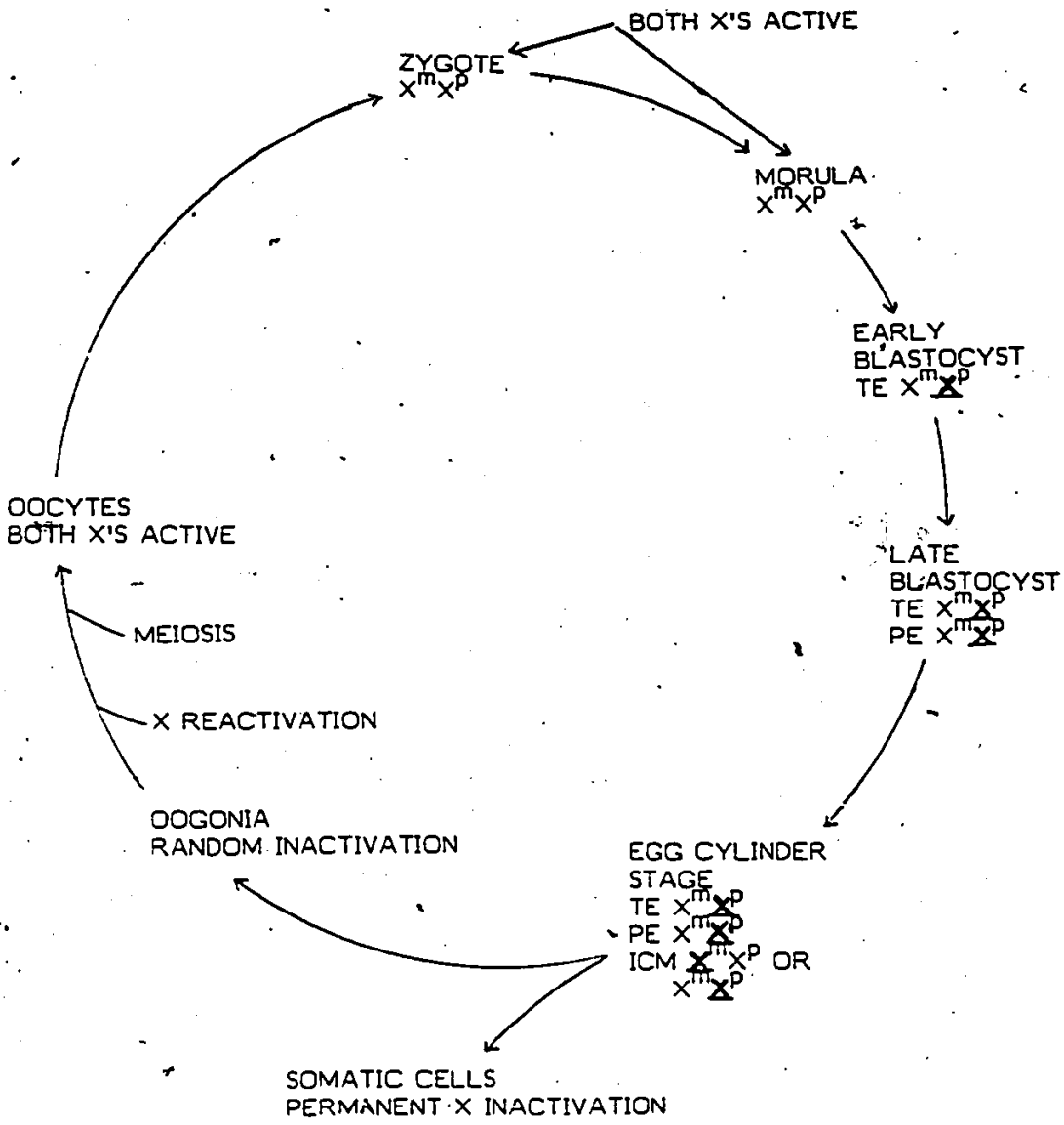
FIGURE ONE

X chromosome differentiation during mammalian embryogenesis. The X^m and X^p are the maternal and paternal X chromosomes respectively. The inactive state of a chromosome is indicated by bold-type. TE is trophectoderm; PE is primitive endoderm; ICM is inner cell mass.


BOTH X'S ACTIVE
ZYGOTE
X^mX^p
MORULA
X^mX^p
EARLY
BLASTOCYST
TE X^mX^p
LATE
BLASTOCYST
TE X^mX^p
PE X^mX^p
EGG CYLINDER
STAGE
TE X^mX^p
PE X^mX^p
ICM X^mX^p OR X^mX^p
SOMATIC CELLS
PERMANENT X INACTIVATION
OOGONIA
RANDOM INACTIVATION
X REACTIVATION
MEIOSIS
OOCYTES
BOTH X'S ACTIVE

cells giving rise to oocytes must reactivate Xi at sometime during their development. Witschi (1957) reported that migrating germ cells in human female embryos were sex chromatin positive suggesting they had an Xi. Later work involving the measurements of X encoded enzyme specific activities (Andina, 1978; Monk and McLaren, 1981), use of heterozygous electrophoretic variants of X linked enzymes (Johnston, 1980; Gartler et al., 1972; Gartler et al., 1973), and cytological determinations in mice (Gartler et al., 1980; Hartung and Stahl, 1977), humans (Ohno et al., 1962) and other mammals (Jagiello et al., 1982; Luciani et al., 1979) have demonstrated that female germ cells reactivate the Xi just prior to their entry into meiosis. Thus, germ cells possess cellular mechanisms which can entirely reactivate the previously Xi.

THE CONTROL OF X CHROMOSOME INACTIVATION

The most conspicuous feature of X inactivation is that it involves the repression of an entire X chromosome, while the other X chromosome in the same environment is not affected. Although X inactivation is a continuous process, it can be subdivided into certain components to make it easier to conceptualize. These events will be summarized below and include: 1) imprinting and recognition, 2) counting and 3) control centres of inactivation.

Chromosome Recognition and Imprinting

The preferential inactivation of the X^P in the somatic cells of marsupials and in the extraembryonic membranes of

eutherian mammals implies that the parental X chromosomes are chemically or structurally different, within the same cell. The differences between the X^m and the X^p are then recognized and acted upon by the X inactivation mechanism. This marking or imprinting is thought to occur during processes which accompany gametogenesis (Chandra and Brown, 1975). It is of interest that while the Xi chromosome is reactivated during oogenesis, the X chromosome is condensed and inactivated during spermiogenesis (Monesi, 1971; Vanha-Perttula et al., 1970). This difference in the state of the X chromosome in the sperm and egg may provide a basis for the distinction between the X chromosomes in the early embryo. This distinction between the X^p and the X^m in eutherian mammals is lost during embryogenesis since the cells of the epiblast demonstrate random inactivation.

Recent work with parthenogenic embryos and ovarian teratomas, in which cases both X chromosomes are of maternal origin, have shown that inactivation proceeds normally, even in tissues normally having preferential X^p inactivation (Kaufman et al., 1978; Rastan et al., 1980). Thus, the concept of imprinting is thought to be independent of the mechanisms which initiate inactivation (Gartler and Riggs, 1984).

Counting

There is a mechanism which insures that only one X chromosome is active per diploid autosomal set of chromosomes. Thus, a cell possessing autosomal euploidy and

5X anueploidy will have four Xi (Funderburk et al., 1981). A tetraploid cell can have two Xa and two Xi, while triploid cells may have one or two Xi (Migeon et al., 1981; Willard and Breg, 1980). These data imply that the X/autosome ratio and autosomal genes are important components in a counting mechanism that insures the correct number of Xi in a cell.

Control Centres of Inactivation

Initiation of inactivation involves the silencing of one X chromosome and not the other. This type of inactivation is best explained by a single initiating or controlling site on the X chromosome (Gartler and Riggs, 1984) since multiple control centres should lead to mixtures of active and inactive regions on both X chromosomes. There are workers however, who feel that there is evidence supporting multiple control centres on the X chromosome (Eicher, 1970; Disteche et al., 1981; Nakagome, 1982).

Eicher first proposed the idea of multiple inactivation centres by studing a murine translocation where a piece of chromosome 7, carrying assayable markers, was inserted into an X chromosome. When this X chromosome became inactivated, cytological and genetic evidence suggested that the X chromosome segements on both sides of the insert were inactivated, but some regions of the autosomal insert remained genetically active. It was suggested that since both sides of an X chromosome that was separated by an active autosome insert were inactivated, there must be inactivation centres on both regions of the bisected X

chromosome.

Takagi's (1980) study of X replication patterns in 7 1/2 day embryos carrying balanced Searle's translocation (X, X^{16}, 16^{X}, 16) demonstrated that the intact X was late replicating in the majority of the cells, but in earlier stage embryos, a small proportion of cells showed 16^{X} late replicating. The X^{16} never appeared as late replicating in over 2500 embryonic cells that were examined. In addition, in unbalanced embryos (X^{16}/X;16/16) where X inactivation of X^{16} would result in "normal" embryos in terms of gene dosage, his studies showed no inactivation of the X^{16} or the normal X. These results suggest that there is a single inactivation centre on the distal region of the X chromosome

A less extensive study of Searle's translocation by Disteche et al (1979), using the same approach as Takagi, reached the opposite conclusion. In the cells of 9 day embryos and adults, they showed that the intact X was late replicating and either the X^{16} or 16^{X} (but never both) could be late replicating. They took this result as evidence for multiple inactivation centres. However, definitive evidence for multiple inactivation centres would require that both pieces of a reciprocally translocated X become inactivated.

The same kinds of results for human X chromosome abnormalities (Therman et al., 1974) and X-autosome translocations (Mattei, 1982; Gartler and Riggs, 1984) have been used to suggest a single inactivation control centre on the human X chromosome (Mattei, 1982; Gartler and Riggs,

1984).

Although this work is by no means complete, it suggests that there is a single inactivation centre present on the mouse and human X chromosome.

The spreading effect of inactivation observed for most mouse X-autosome translocations (Cattanach, 1974; Cattanach, 1975; Russell and Montgomery, 1970; Russell, 1972; Lyon, et al., 1964; Cachiero et al., 1973; Takagi, 1980; Eicher, 1970; Disteche et al., 1978) suggests that there is a site on the X chromosome where inactivation initiates and spreads into adjoining regions. Little is known about the spreading of inactivation, however, there is agreement that heterochromatinization is involved (Cattanach, 1975; Gartler and Riggs, 1984). It is thought that initiation of inactivation at a controlling site serves as a nucleation site for chromosome condensation, which is thought to be a highly cooperative process similar to crystallization (Weisbrod, 1982; Gartler and Riggs, 1984). Once the heterochromatic state is established, it may be self-propagating and heritable. However, there is growing evidence that the maintenance of the inactive state is mediated by post-replicational modifications of the DNA structure (Gartler and Riggs, 1984; Chapman et al., 1982; Riggs, 1975).

The presence of an inactivation centre has led to a search for heritable mutations affecting the randomness of X inactivation. One such mutation has been found in the mouse

which affects the probability of which X chromosome is inactivated (Cattanach and Issacson, 1967; Cattanach, 1975; Falconer et al., 1982; Johnston and Cattanach, 1981; Cattanach and Papworth, 1981). The X chromosome carrying the mutant allele has a greater probability of remaining the Xa in a cell after inactivation. This has been demonstrated by biased changes in coat colour variegation (Cattanach and Issacson, 1972; Falconer and Issacson, 1972) and skewed expression of heterozygous X encoded gene products (Cattanach and Papworth, 1981). This locus has be named the X controlling element (Xce) and maps to a site in the distal region of the X chromosome between the linkage groups Ta and pgk-1 (Cattanach et al., 1970; Johnston and Cattanach, 1981). There are three known alleles of Xce which have a graded effect upon the probability of inactivation (Johnston and Cattanach, 1981). The probability increased from 50:50 to approximately 70:30 in the most extreme allelic combination (Xce^c/Xce^a). It is not clear why complete preferential inactivation is not observed in this system (Cattanach, 1975; Gartler and Riggs, 1984). Thus, there appears to be a single site on the X chromosome which is important in the initiation of X inactivation. This inactivation is probably located in the distal region of the mouse X chromosome and may be Xce, or a locus similar to it.

MODELS OF X CHROMOSOME INACTIVATION

Before discussing some of the models of X chromosome inactivation it would be useful to summarize the

observations they must account for:

a. one of two homologous X chromosomes within a cell becomes genetically inactive early in embryogenesis and this inactivation is stable and heritable in somatic cells.

b. there is probably a single inactivation centre on the X chromosome where inactivation initiates and from which it spreads to inactivate most X chromosome genes.

c. reactivation of an Xi occurs in female germ cells. Therefore, inactivation cannot cause a permanent change in genetic information.

d. there is a mechanism to insure only one X chromosome is active per euploid autosomal set of chromosomes.

e. mechanisms of imprinting exist such that in extra-embryonic tissues the inactivation mechanism can distinguish between X chromosomes of paternal and maternal origin.

DNA Rearrangement Models

The episome integration model was the first to propose a molecular mechanism for X inactivation (Grumbach et al., 1963). It proposes that an episome integrates at an initiation site on an X chromosome to induce inactivation. This integration is thought to induce cellular changes which destroy additional episomes, inactivates or modifies them, or modifies insertion sites on the second X chromosome to prevent further inactivation. It is assumed that the initial integration event is slow but that the second inactivation step is rapid. Presumably, this episome is removed from the Xi sometime during oogenesis..

The episome integration model is plausible given the existence of genetic rearrangements and transposable elements in prokaryotes (Green, 1977), yeast (Green, 1977), Drosophila (Green, 1980) and possibly in Mammals (Jagadeeswaran et al., 1982). Transposable element insertion in Drosophila can lead to heritable changes in development (Bender et al., 1983) and insertion of retroviral sequences in mouse can result in mutations (Jaenisch et al., 1982). Endogenous retroviral-like sequences are present in the mouse genome and can be inherited through the germ line (Jaenisch, 1976).

The inversion cassette model identifies an initiation site on the X chromosome which is a defined sequence. Events leading to inactivation induce an inversion of this sequence in one X chromosome and results in its inactivation presumably due to sequence or coding disruption. As in the first model, this inversion somehow blocks the event in the second X chromosome at a rapid rate. There are switching mechanisms which control the synthesis of flagellar proteins in Salmonella which involve the reversable inversion of a 1.2 kb fragment of DNA (Zieg and Simon, 1980) and the switching of mating types in yeast is controlled by the specific insertion of unique, moveable DNA elements (Kushner et al., 1979).

DNA Conformation Models

The conformational changes of the DNA in the Z form are thought to occur at the X controlling site and initiate

inactivation. This conformational change may occur in conjunction with other mechanisms where, for example, the Z DNA conformation allows the binding of specific inactivation proteins. Z DNA probably exists under physiological conditions (Nordheim et al., 1982a; Singleton et al., 1982) and proteins have been found which bind specifically to Z DNA (Nordheim et al., 1982b).

Membrane Attachment Model

This simple model proposes that there is a single attachment site on the nuclear envelope for the binding of one X chromosome (Somings, 1966). Once an X chromosome is bound to the site, it is permanent and results in stable activation of this X. The unattached X is inactivated. It is suggested that the attachment site is coded for by autosomal genes so that there is one site per autosomal set of chromosomes, thus providing a mechanism for counting.

Protein Binding Models

The role of DNA binding proteins in inactivation was first proposed by Ohno (1968; 1973) and Lyon (1972). This model proposes that there are DNA binding proteins which are produced at defined levels by the autosomes. These proteins will bind at the initiation site for inactivation on an X chromosome. It is postulated that this initial binding is slow, but that once one protein is bound all others bind to this site because of high cooperativity and induce inactivation. In the activator version of the model, the cooperative binding of the proteins prevent a subsequent

inactivation mechanism from accosting this X, thus keeping it active. It has been argued that without a modification of the DNA the X DNA-protein interactions alone are not adequate to explain the stability of inactivation since proteins should be displaced during DNA replication and should therefore, occasionally accost the Xa (Riggs, 1975).

The majority of the models that are summarized are primarily concerned with the initiation and counting mechanism of inactivation. All the models are not completely adequate to resolve all of the characteristics of X inactivation, especially the spreading and maintenance of inactivation. In addition, the models are not mutually exclusive, so it is equally probable that all, a few, or none of the models are involved in X inactivation.

DNA METHYLATION

The characteristics of the Xi demand that there are mechanisms responsible for maintaining the active and inactive state. As previously mentioned, it is argued that these differences must involve heritable changes in the structure of the DNA, chromatin structure and organization, or a combination of the two (Riggs, 1975; Lyon, 1972; Gartler and Riggs, 1984; Chapman et al., 1982).

The methylation of cytosine bases in DNA is a post-transcriptional modification which has been implicated as a major mechanism controlling differential gene expression in eucaryotes (Cooper, 1983; Erlich and Wang, 1981; Felsenfeld and McGhee, 1982; Riggs, 1975; Riggs and Jones, 1983;

Holliday and Pugh, 1975; Scarano et al., 1977; Adams, 1970; Harrison, 1971; Comings, 1972; Jones and Taylor, 1980; Taylor and Jones, 1979) and has been assigned a role in the maintenence of X chromosome inactivation (Riggs, 1975; Gartler and Riggs, 1984; Graves, 1982; Mohandas et al., 1981; Lester et al., 1982; Jones et al., 1982; Venolia et al., 1982).

Presented here are some of the characteristics of DNA methylation which have implications for X inactivation (for reviews see Cooper, 1983; Doerfler, 1983; Riggs and Jones, 1983):

1. 5-methylcytosine is the only methylated base found in eucaryotic DNA and its' level is high, on the order of 2-4% of DNA cytosines.

2. The methylation of cytosines in DNA is accomplished by a cellular enzyme (only one found to date) which has the following properties: 1) it methylates cytosines in the 5 position such that the methyl group lies in the major groove of the DNA helix and does not hinder normal base pairing, 2) it preferentially recognizes and methylates 5'CG sequences symmetrically in both strands and 3) the enzyme has a higher affinity for half-methylated sites than for unmethylated sites. This differential affinity insures the heritability of DNA methylation after replication.

3. The foundation of the DNA methylation hypothesis in the control of gene expression rests upon the observation that the position of the methyl group in the major groove

alters the secondary structure of the DNA and strongly influences the binding of proteins to DNA. This is evident from the binding characteristics of the DNA methylase enzyme itself and the observations with other base analogs.

4. There is a negative correlation between the extent of methylation of a particular gene and its level of transcription. This has been observed for the expression of many autosomal genes in many systems. It is becoming clear however, that the extent of methylation is unimportant and the methylation of critical sites, such as in cellular promoters or enhancers, may be important for transcriptional regulation.

The model suggesting a role for DNA methylation in X chromosome inactivation was first proposed by Riggs (1975). The model suggests that the primary inactivation centres on both X chromosomes are unmethylated by passing through gametogenesis and both X chromosomes are active in the early embryo. A DNA specific methylase, coded for by the autosomes, recognizes the primary inactivation centre on the X chromosome and methylates critical sites de novo. This methylation activates an X linked function very rapidly which either inactivates the de novo methylase, modifies its activity or represses its synthesis so that the second inactivation centre is not accosted. The methylation of the inactivation centre induces structural changes in the DNA or influences the binding of an inactivation protein which mediates inactivation. The presence of a second maintainance

methylase or an altered de novo methylase ensures the heritability of the methylated inactivation centre and prevents the methylation of the second centre.

The model can also be reversed where the methylation of an inactivation centre preserves its activity and the unmethylated site on the second X is recognized by the inactivation mechanism.

There is growing evidence that de novo methylation occurs during early embryogenesis and in teratocarcinoma cells at a greater rate than observed in somatic cells (Jahner et al., 1982; Stewart et al., 1982). In addition, differences in the of DNA methylation beween the X^p and X^m early in embryogenesis may provide the basis for the preferential inactivation of the X^p in extraembryonic tissues. The de novo methylation of sequences during embryogenesis may obscure the differences between the two X chromosomes, leading to random inactivation at later stages. It is noted that sperm DNA is highly methylated for the majority of its sequences (Strum and Taylor, 1981; Kaput and Sneider, 1979; Erlich et al., 1982). However, changes in DNA methylation also occur during cellular differentiation in the embryo and extraembryonic tissues, which display preferential X^p chromosome inactivation, become undermethylated during differentiation, while embryonic tissues do not (Chapman et al., 1983). This undermethylation of DNA in these tissues may be involved in the preferential inactivation observed in these tissues.

Evidence for the Role of DNA Methylation in X Inactivation

The importance of DNA methylation in the control of differential gene expression in eucaryotes is based, in part, upon studies involving inhibitors of DNA methylation, including the base analog 5-azacytidine (5-AC). This base analog apparently inhibits DNA methylation by becoming incorporated into DNA. The nitrogen group in the 5 position cannot accept a methyl group (Jones and Taylor, 1980). Recent studies have also suggested that 5-AC directly inhibits the cellular methyltransferase enzyme (Creusot et al., 1982; Santi et al., 1983). The treatment of cultured cells with 5-AC causes a stable demethylation of the DNA (Christman et al., 1983; Creusot et al., 1982) and can result in the induction of cellular differentiation programs (Christman et al., 1983; Constandinides et al., 1977; Bodner et al., 1981; Sager and Kovac, 1982; Taylor and Jones, 1979; Jones and Taylor, 1980), the activation of endogenous retroviral genomes (Groudine et al., 1981; Niwa, et al., 1983; Stewert et al., 1982) and the expression of silent cellular genes (Harris, 1982; Ley et al., 1984; Delers et al., 1984).

Recent work with cultured interspecific somatic cell hybrids possessing an Xi have shown that DNA methylation may be involved in the maintenance of the inactive state of the X chromosome (Mohandas et al., 1981; Graves, 1982; Lester et al., 1982; Jones et al., 1982). In these studies, 5-AC was

shown to induce the high frequency reactivation of genes on the Xi. This reactivation apparently involved local regions on the X chromosome (Lester et al., 1982; Hors-Cayla et al., 1983). In addition, the majority of the X chromosome maintained its pattern of late replication (Jones et al., 1982; Hors-Cayla et al., 1983). Syntenic loci can also be reactivated but this is probably an independent event at each locus (Mohandas et al., 1981; Hors-Cayla et al., 1983). The expression of reactivated of genes on the Xi is stable, even in the absence of selective pressure. The loss of expression of the reactivated gene is always associated with the loss of the Xi (Graves, 1982; Lester et al., 1982).

Several recent experiments have suggested that the DNA from the Xi may be chemically distinct from Xa DNA. Liskay and Evans (1980) reported that a female mouse line carrying a wild type hgprt gene on the Xi did not function in DNA mediated gene transfer experiments, while the same hgprt gene on an Xa did function. Venolia and Gartler (1982) obtained the same results using clonal cell lines from a female heterozygous for a Lesch-Nyhan ($HGPRT^-$) mutation. The inefficiency of the Xi DNA in hgprt transformation experiments has been confirmed and extended by Chapman et al. (1982) using female mouse cells that were heterozygous for electrophoretic variants of the HGPRT enzyme. In addition, they later demonstrated that while somatic cell Xi DNA was inefficient in gene transfer, DNA from the preferentially inactivated X^p from visceral endoderm in the

embryo was functional in hgprt transformation (Kratzer et al., 1983). These results suggest that there is a difference in the inactivation mechanism which operates in extraembryonic tissues demonstrating preferential X inactivation and in somatic cells with random inactivation.

These DNA transformation results suggest that the hgprt gene from the Xi in somatic cells is significantly less efficient than the hgprt gene on the Xa in gene transfer, suggesting that there is some sort of modification of the DNA. There is, however, one report where Xi DNA from another Lesch-Nyhan female cell line did function in DNA transformation experiments (DeJonge et al., 1982)

The role of cytosine methylation as the primary mechanism of this modification is suggested from 5-AC studies with mouse-human cell hybrids. Cell hybrids carrying the human Xi did not function in DNA mediated gene transfer. After they were treated with 5-AC to induce hgprt reactivation, they did function in gene transfer experiments involving hgprt (Venolia et al., 1982; Lester et al., 1982). In other systems, methylation also decreases gene transfer efficiency (Razin and Riggs, 1980; Cristy and Scangos, 1984; Vardimon et al., 1982; Waechter and Baserga, 1982).

Molecular investigations of the patterns of methylation on the Xa and Xi have not been fruitful (Wolf et al., 1980; Wolf and Migeon, 1982). By using random probes of X chromosome specific sequences and Msp/Hpall restriction enzymes to assay for methylation, Wolf and Migeon (1982)

could find no obvious methylation differences between males and females. They also showed that the methylation patterns varied in clones of the same female cell. A later study using probes hybridizing to regions of the hgprt gene, also showed no obvious differences between male and female cells (Wolf et al., 1984). Although this method of assaying methylation is limited because it measures only a subset of possible methylated sites (Doerfler, 1983), it eliminates the possibility that the inactivation of the X is the result of global methylation of its sequences. This suggestion is supported by results using antibodies raised against 5-AC. Observations of chromosomes did not reveal a difference between the Xa and Xi in human and a monkey species (Miller, et al., 1982).

THE TERATOCARCINOMA SYSTEM

X inactivation occurs during early embryogenesis when the embryo consists of only a few cells, is inaccessible and difficult to work with experimentally in vitro. In addition, the temporal heterogeneity of X inactivation in embryos hampers its study. For these reasons EC cells have provided an alternative developmental system to study inactivation with a greater abundance of cells and the ease of experimental manipulation (McBurney and Strutt, 1980).

The EC Cell and Murine Teratocarcinomas

EC cells are the stem cells of malignant tumours called teratocarcinomas (Stevens, 1967; Pierce, 1967). These tumours, which normally arise in the gonads, are

characterized by the presence of a wide spectrum of differentiated cells within the tumour, which would normally be derived from all three germ layers of the embryo. In addition to the differentiated cell types, teratocarcinomas contain a morphologically undifferentiated cell type called embryonal carcinoma (EC). Injection of EC cells (Pierce and Dixon,1959; Pierce et al., 1960), or even a single EC cell,into appropriate mice produced teratocarcinomas containing the full range of differentiated cell types. Injection of differentiated cells did not result in tumour production (Pierce et al., 1960; Graham, 1977). Thus, EC cells are the stem cells of the tumour and, more importantly, they are developmentally pluripotent and capable of differentiating along multiple developmental pathways.

Teratocarcinomas can be propagated by serial transfer to appropriate animals by subcutaneous injection or they can form ascites tumours, clumps of cells which consist of a core of cells surrounded by a rind of differentiated, extraembryonic endoderm cells (Graham, 1977). These clumps of cells resemble the organization of normal embryos at about the sixth day of development (egg cylinder stage) and have been termed embryoid bodies (Stevens, 1960).

Origin Of EC Cells

Teratocarcinomas were first described as spontaneous testicular and ovarian tumours which resulted from the neoplastic transformation of a germ cell(s) (Stevens and

Little, 1954; Pierce and Beals, 1964; Stevens, 1967; Stevens and Varnum, 1974).

Teratocarcinomas can be induced experimentally in other strains of mice (e.g. C3H) by the transplantation of early embryos into ectopic sites, such as under the testes or kidney capsule (Stevens, 1970b; Damjanov et al., 1971; Illes, 1976). These foreign environments are thought to disrupt the embryo, resulting in teratocarcinomas. Some strains of mice are not permissive for teratocarcinoma production after embryo transplantation which has suggested that the host environment is important in their genesis (Solter and Damjanov, 1979).

The highest frequencies of teratocarcinoma production were obtained by transplanting 7 1/2-8 day embryos, although embryos are early as the two cell stage produced tumours (Stevens, 1968; Damjanov et al., 1971b; Graham, 1977).

There is a controversy regarding the origin of embryo devived teratocarcinomas. It has been suggested the these teratocarcinomas do not arise from germ cells but from pluripotent embryonic cells, possibly from the embryonic ectoderm (Mintz et al., 1978; Diwan and Stevens, 1976; Dewey et al., 1977; Evans et al., 1979). There is now evidence that pluripotent stem cells, with identical characteristics to EC cells, can be isolated directly from mouse embryos cultured in vitro (Evans and Kaufman, 1981; Martin, 1981; Axelrad and Bennett, 1982).

Thus, teratocarcinomas may arise spontaneously by the

neoplastic transformation of germ cells, or, in the case of embryo derived tumours, directly from a pluripotent embryonic cell, possibly the embryonic ectoderm. In addition, there is some evidence that different EC cell lines may be arrested at different stages of embryonic development. This is based primarily upon the restricted differentiation lineages demonstrated by some cell lines (Bernsteine et al., 1973; Martin and Evans, 1975b) and the state of activity of the X chromosomes in female EC cells (McBurney and Strutt, 1980; McBurney and Adamson, 1976).

Characteristics Of EC Cells

EC cells resemble the cells of the embryonic ectoderm of the embryo. Like embryonic cells, EC cells have sparse cytoplasms with relatively large nuclei and prominent nucleoli. They possess relatively few, spherical mitochondria, little endoplasmic reticulum or Golgi and have numerous free ribosomes (Pierce and Beals, 1964; Lo and Gilula, 1980). EC cells are also similar to embryonic cells using antigenic (Artzt et al., 1973; Jacob, 1977; Reisner et al., 1977; Solter and Knowles, 1978; Kemler et al., 1977; Harris et al., 1984; Boller and Kemler, 1982) and biochemical criteria (Bernsteine et al., 1973; Damjanov et al., 1971).

The best evidence for the hypothesis that EC cells are pluripotent embryonic cells comes from blastocyst injection experiments. When euploid EC cells (even a single EC cell) injected into a mouse blastocyst, they can contribute to

normal embryonic development. Their differentiated derivatives can be found in most differentiated tissues (including germ cells) of a normal, tumour free mouse (Brinster,1974; Mintz and Illmensee, 1975; Papaioannou et al., 1976; Dewey and Martin, 1980; Rossant and McBurney, 1982). Because EC cells can behave as embryonic cells, the study of EC cells provides a powerful in vitro model system for the study of many of the aspects of embryonic development and differentiation.

The EC Cell in Tissue Culture

Lines of EC cells can be adapted to tissue culture by plating dissociated cells from embryoid bodies or solid tumours, in the presence or absence of feeder cells (Kahan and Ephrussi,1970; Martin and Evans, 1975b; Iles, 1976; McBurney, 1976; Martin and Evans, 1975b; Rosenthal et al., 1970; Evans, 1972; Bernsteine, et al., 1973). Clusters of rapidly dividing EC cells grow out from these cells and can be identified, cloned and subcultured. Thus, homogenous clonal lines of EC cells can be obtained. These cells remain homogenous by frequent subculturing and maintain the ability to form fully differentiated teratocarcinomas when injected back into an animal at any time (Finch and Ephrussi, 1967).

EC cells can be induced to differentiate in vitro by culturing the cells in dense monolayers (Nicolas et al., 1975), in large attached clumps (McBurney, 1976) or as aggregates in suspension (Martin and Evans, 1975; Jones-Villeneuve et al., 1982). Most EC cells retain the

ability to differentiate into the full range of cell types in tissue culture, as found in the tumour (Martin and Evans, 1975; Nicolas et al., 1975; McBurney, 1976; Kahan and Ephrussi, 1970).

The first differentiated cell type observed in differentiating cultures are extraembryonic endoderm (EE) cells. This is followed by the appearance of many differentiated cell types including nerve, muscle (cardiac and skeletal), epithelium, cartilage, and adipose tissue (Martin and Evans, 1975b; McBurney, 1976). There are some EC cell lines which do not have or have lost the ability to differentiate under these conditions (Martin and Evans, 1975b; Martin, 1980).

The differentiation of EC cells in vitro can be monitored using morphological, biochemical and antigenic criteria (Martin and Evans, 1975b; McBurney, 1976; Jones-Villneuve et al., 1982; McBurney et al., 1982; Edwards, et al., 1983, Bernsteine et al., 1973; Graham, 1977; Solter and Knowles, 1978; Adamson, 1976; Strickland et al., 1980; Darmon et al., 1981; Paulin, 1982).

The difficulty in studying developmental events in this system is the heterogenous nature of the differentiation. Many workers have attempted to simplify and control the system by adding various drugs and compounds to differentiating EC cells, to direct the differentiation along limited developmental pathways. Strickland and Madavi (1978) first demonstrated that F9 EC cells treated in

monolayer with the vitamin A analog retinoic acid (RA), differentiated into a tissue resembling primitive EE of the embryo. Later work showed that coaddition of cAMP with RA led to the further differentiation of these cells into parietal EE (Strickland et al., 1980). Hogan et al., (1980) demonstrated that the aggregation of F9 cells in the presence of RA resulted in the differentiation of visceral rather than parietal EE. Thus the treatment of F9 EC cells with RA under different conditions is potentially useful for studying the determination events leading to the differentation of one cell type or another.

Jones-Villeneuve et al. (1982) demonstrated that the treatment of aggregates of P19 EC cells with high doses of RA resulted in the differentiation of cells along neuroectodermal lineages giving rise to only neurons, glial cells, and fibroblast-like cells. However, when P19 cells, C86S1 and C145A12 EC cells were exposed to high doses of RA in monolayer cultures, they did not differentiate along primitive endodermal or neural pathways but into fibroblast-like cells. Thus, the differentiation response to RA is dependent upon the cell type and culture conditions and provides the potential for studying many developmental events leading to different differentiation pathways.

Other drugs such as DMSO and low doses of RA induce the differentiation of muscle in aggregates of P19 cells (McBurney et al., 1982; Edwards and McBurney, 1983), while hexamethylene bis acetamide (HMBA) can induce the appearance

of fibroblasts and epithelial cells in monolayers or PCC4 EC cells (Speers et al., 1979). In addition, culturing of some EC cell lines in defined medium, in the absence of serum, induces differentiation into restricted cell types (Rizzino, 1983; Darmon et al., 1981).

The X Chromosome in EC Cells

The resemblance of EC cells to early embryonic cells led to the suggestion that EC cells may possess two Xa. McBurney and Adamson (1976) first showed that female EC cell lines derived from embryo-induced teratocarcinomas, may differ in the states of their X chromosomes. They used three independent criteria for determining the activity of the X chromosomes: replication patterns of the X chromosomes, specific activities of enzymes encoded by the X chromosome genes and the frequency of recovery of mutants resistant to the nucleotide analog 8-azaguanine, which should be dependent upon the number of active copies of the X encoded gene, hgprt.

Their results demonstrated that C86S1 EC cells have all the characteristics of possessing an Xi while C100 EC cells had some characteristics, but not others (late replication and 2x alpha-GAL specific activity) suggesting it may possess a partially inactive Xi.

Martin et al. (1978) have described a female, EC cell line, (LT) derived from a spontaneous ovarian teratocarcinoma which, on the basis of specific activities of X encoded enzymes, demonstrated dosage differences

between XX and XO cells consistent with the expression of both X chromosomes. Furthermore, they showed that the expected 50% reductions in the specific activities of these enzymes occurred during the induced differentiation of these cells *in vitro*. Later work demonstrated that in LT EC cells both X chromosomes replicated isocyclically early in "S" phase of the cell cycle and the shift to late replication of one X chromosome occurred, in approximately 50% of the cells, during differentiation (Takagi and Martin, 1984).

McBurney and Strutt (1980) described an embryo derived EC cell line, called P10, which was heterozygous for electrophoretic variants of *pgk-1* alleles, thermostability variants of *alpha-gal* alleles and the X controlling element alleles (*Xce*). Many previous studies have demonstrated the value of allelic variants of X encoded genes for studies involving X inactivation (Beutler *et al.*, 1962; Ray *et al.*, 1972; West *et al.*, 1977). This cell line was found to possess a euploid, female karyotype and all clonal populations of EC cells contained both PGK-1 isozymes. In addition both X chromosomes replicated in synchrony with the autosomes during early "S" phase. These data demonstrated that P10 EC cells contained two Xa. When P10 cells were induced to differentiate, the majority of the cells contained an Xi chromosome after 2-3 weeks in culture, as detected by X chromosome replication patterns.

Thus, EC cells provide an ideal system for the investigations into mechanisms of X chromosome inactivation.

Various female EC cell lines differ with respect to the activity status of their X chromosomes and may represent embryonic cells arrested at different stages of X inactivation. X inactivation occurs in culture when EC cells possessing two Xa are induced to differentiate.

THE THESIS PROJECT

X chromosome inactivation is a developmental event occurring in all cells of the female embryo. It involves mechanisms which result in the heritable, transcriptional silencing of an entire X chromosome. Thus, the study of X chromosome inactivation provides a system for studying differential gene expression in eucaryotes. Elucidation of mechanisms controlling X inactivation may have a wider application for the understanding of gene regulation in its entirety.

The Aim of the Thesis

The work in this thesis is comprised of two chapters. The first chapter involves the synchronization and characterization of an *in vitro* system for the study of X chromosome inactivation using P10 EC cells. I attempted to simplify this system by inducing the differentiation of P10 cells by exposing them to RA. The exposure of P10 cells to RA resulted in the rapid and complete differentiation into cells resembling the EE of the embryo and was accompanied by rapid and synchronous X chromosome inactivation. The induction of X inactivation in a single, differentiated cell type provides an experimental system more amenable for the

investigation of the mechanisms involved in X chromosome inactivation.

The second chapter of the thesis deals with attempts to define a molecular mechanism for X chromosome inactivation, by inducing the reactivation of the Xi in female EC cell lines. By attempting to reactivate Xi in these cells, by treating them with agents of known modes of action, I have provided evidence for the role of DNA methylation as one of the mechanisms responsible for the transcriptional inactivation of the Xi during X chromosome inactivation. In addition, the different responses of different EC cell lines to DNA demethylating agents suggest that they may be arrested at different stages of X chromosome inactivation.

CHAPTER TWO

METHODS AND MATERIALS

Cell Lines

All embryonal carcinoma cell lines described in this thesis were derived from teratocarcinomas produced in C3H/He strain of mice by embryo transplantation (McBurney and Adamson, 1976; McBurney, 1976; McBurney and Strutt, 1980). All cell lines are rigourously clonal in origin. The X chromosome content of each cell line was characterized by G banding of chromosomes. P10(XO) clones were also identified by the expression of a single PGK-1 isozyme. The HGPRT$^-$ cell lines were selected by exposure of cells to 10 ug/ml 8-azaguanine (8-AG). Clones of cells reactivated by exposure to 5-azacytidine (5-AC) are identified by the addition of AZA#n where # identifies the concentration of 5-AC cells were exposed to and n identifies the clone. The relevant characteristic of all cell lines used in this study are summarized in Table 1.

Culture of Embryonal Carcinoma Cells

All cells were cultured in alpha minimal essential medium (Stanners et al., 1971) (Gibco, Long Island, N.Y.) containing 50ug/ml each of streptomycin and penicillin and supplemented with 7.5% bovine calf serum and 2.5% fetal bovine serum (Canadian Veterinary Supplies, Perth, Ont.). Cells were routinely subcultured every two days by treating them with Ca^{++} and Mg^{++}-free phosphate buffered saline (PBS) containing 0.025% trypsin and 1mM EDTA to remove them from

TABLE ONE

SOME CHARACTERISTICS OF EMBRYONAL CARCINOMA CELL LINES

CELL LINE	ORIGIN	SEX CHROMOSOMES	LATE REPLICATING	CHARACTERISTICS
P10	C3H/He 7.5 day embryo	XX	no	pgk-1b,alpha-gal^{r},Xce^{a} /pgk-1a;alpha-gal^{s},Xce^{c}
P10(X0)n	"	X0	no	pgk-1b, alpha-gal^{r},Xce^{a} or pgk-1a, alpha-gal^{s},Xce^{c}
C86S1	C3H 7.5 day embryo	XX	yes	no heterozygous X linked markers.
C86S1A1	"	XX	yes	derived from C86S1 AG^{r}
C86S1A1(X0)n	"	X0	no	derived from C86S1A1 AG^{r}
C145a	C3H 6.5 day	XX	no	no heterozygous X linked markers
C100AG1	"	XX	no	derived from C145a AG^{r}
C145F	C3H 6.5 day	XX	yes	no heterozygous X linked markers
C145FAGn	"	XX	yes	derived from C145F AG^{r}
C86S1A1AZAn	"	XX	no	derived from C86S1A1 by 5-AC treatment AG^{s}
P19	C3H 7.5 day	XY	no	no heterozygous X linked markers

* n indicates a number of clonal isolates.
AG^{r} - azaguanine resistant. AG^{s} - Azaguanine sensitive.
pgk-1- phophoglycerate kinase gene. alpha-gal- alpha galactosidase gene.
Xce- X controlling element gene.

the surface of tissue culture vessels. Dispersed cells were then replated into fresh medium at the required cell density. P10 cells were subcultured into tissue culture vessels containing mitomycin-C treated STO mouse fibroblasts as feeder cells (McBurney, 1976).

All cell lines were replaced by frozen stocks at approximately monthly intervals or when karyotype abnormalities appeared during frequent G banding. Clonal lines of all cells were established by picking single cells into separate culture vessels.

Retinoic Acid Induced Differentiation

Differentiation of EC cells was initiated by plating $5x10^5$ or $1x10^6$ cells into gelatin coated 60mm or 100mm tissue culture dishes (Fisher, Toronto, Ont.). All-trans retinoic acid (RA) (Sigma, St. Louis., Mo.) at $1x10^{-7}$ M was prepared as a 10^{-2} M stock solution in DMSO and diluted into the medium. The medium was replaced at 2 day intervals and RA was present continuously. Before exposure to RA, P10 cells were cultured for 24 hr in the absence of STO feeders.

Chromosome Preparations

G banding of EC cells chromosomes was carried out as previously described (McBurney, 1976). Chromosome replication patterns were monitored as previously described (Alves and Jonassen, 1978; McBurney and Strutt, 1980). Briefly, cells were exposed to 10^{-4}M 5-bromodeoxyuridine (BrdU) for 5-8 hr. before the addition of Colcemid (6ug/ml) (Sigma). After 1 hr the cells were harvested and chromosome

preparations were made. Slides were stored in the dark for 5-7 days and then stained with 2% Giemsa (Fisher) in 0.3M Na_2HPO_4 (pH 10.4) for 20-40 min.

Cell cycle times were determined by continuous exposure of differentiating cells to 10^{-5}M BrdU (Alves and Jonassen, 1978). Every 24 hr Colcemid (6ug/ml) was introduced into parallel cultures for 2 hr before cells were harvested for chromosome preparations. Slides were kept in the dark for 2-5 days before staining in 2% Giemsa in Na_2HPO_4 (pH 10.4-10.6) for 5-12 min. The numbers of cell cycles were scored by the pattern of differential staining of the BU-substituted chromatids. Approximately 100 metaphases were scored for each time point.

Immunofluorescence

Cells were prepared for immunofluorescence by growing and treating them on gelatin-coated 22mm coverslips as previously described (Edwards and McBurney, 1983; Jones-Villeneuve et al., 1982). Alternatively, cells were harvested in 1mM EDTA in PBS and replated at the required cell density on poly-L-lysine (1mg/ml in H_2O) (Sigma) coated 22mm coverslips in medium approximately 20-30 min before fixation in cold 95% 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.

PGK Electrophoresis

Extracts for PGK electrophoresis were prepared by suspending the cells in 3 volumes of water and disrupting by 2 rounds of freezing (-70°C) and thawing and stored at -70° or used immediately. Prior to electrophoresis samples were centrifuged a 7000 x g to remove cell debris.

Electrophoresis was carried out on cellulose acetate membranes (Gelman, Oakville, Ont.) essentially as described by Bucher et al. (1980) using 1 ul samples. The electrophoresis buffer was modified and consisted of 20mM Tris, 10mM sodium citrate, 5mM $MgSO_4$, and 2mM EDTA (pH 8.2). Gels were stained as described (Bucher et al., 1980) and examined under a UV transilluminator (UV products, San Gabriel, Cal). The gels were photographed at 30 sec intervals through a Kodak CPBY UV and 5RY blue light filters using Polaroid type 57 film. The fluorescent intensity of each PGK band increased in a linear manner for at least 15 min under these conditions. Densitometric tracings of photographic negatives were performed with a Beckman DU 8 spctrophotometer equipped with the Gel Scan programme.

Exposure of Cells To 5-Azacytidine

Cells were exposed to 5-AC essentially as described by Mohandas et al. (1982). Cells were seeded at $3x10^5$ into 60mm tissue culture dishes. After 24 hr freshly prepared 5-AC (Sigma St. Louis Mo.) was added to the cultures. 24 hr later the drug was removed and the monolayer was washed twice and

the cells propagated for an additional 24 hr The cells were then harvested by trypsinization and plated into medium containing 15.0ug/ml hypoxanthine, 0.1 ug/ml methotrexate and 15.0ug/ml thymidine (HAT medium) (Littlefield,1964). A known number of cells were also plated into alpha medium to determine the plating efficiency of cells after 5-AC treatment. 2-mercaptoethanol at $1x10^{-4}$M was added to all media to improve plating efficiencies (Oshima, 1978).

The medium containing HAT was replaced at 3 day intervals and on day 8, the cultures were scored for colonies by staining in a 2% Giemsa solution in 95% methanol for 15 min. after which the plates were washed in water and air dried. In each experiment several HAT resistant colonies were picked and propagated in medium containing 15ug/ml hypoxanthine and 15ug/ml thymidine (HT medium). Only one colony was picked from each plate to avoid the possibility of picking sister colonies.

Autoradiography

Cells for autoradiography were plated at $1x10^5$ into 35mm tissue culture dishes containing a single 30mm diameter coverslip that had been freshly coated with poly-L-lysine. Cultures were incubated for one hour at 37°C to allow the cells to attach before ^{3}H-hypoxanthine (Amersham 1 Ci/mmol) or ^{3}H-guanine (NEN 8.3 Ci/mmol) were added at 2 uCi/ml. After 1-3 hr incubation the coverslips were washed in PBS and fixed in ice cold absolute methanol for 10 min. After rinsing in cold methanol 5x, the cells were rehydrated in

water and extracted with 5% TCA for 5 min. The coverslips were washed 2x in water and 3x in methanol before air drying. The coverslips were dipped in Kodak NTB-2 emulsion and stored in the dark under dessication at 4°C for 1-7 days before development with Kodak D19 developer. The coverslips were stained for 1 min in 2% Giemsa in phosphate buffer (pH 6.86) and viewed using a Leitz Ortholux II Photomicroscope using bright field optics.

Determination of Enzyme Specific Activities

Crude enzyme extracts were prepared by suspending cells in three volumes of distilled water and disrupted by two rounds of freezing and thawing (-70°C). The samples were then centrifuged at 7000xg for 15 min before the supernatants were stored at -70°C. Samples were thawed and kept on ice for no longer than 2 hr before use. Protein concentrations of the extracts were determined as described by Hartree (1972).

Glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGD, E.C. 1.1.1.44) activities was measured at 25°C and pH 7.4 by the method of Rosenstraus and Chasin (1975). Phosphoglycerate kinase (PGK, E.C. 2.7.2.3) was assayed at 25°C and pH 8.0 according to the method of Chen et al. (1971). The rates of reaction of these enzymes were measured at 340 nm on a Beckman DU 7 spectrophotometer using the Kinetics II program. Alpha-galactosidase (alpha-GAL, E.C. 3.2.1.22) activities were determined using 5mM p-nitrophenyl-alpha-galactopyranoside

(Sigma) in 0.1M sodium citrate-citric acid buffer (pH 4.2) at 37°C for 60 min. Hypoxanthine guanine phosphoribosyl transferase (HGPRT, E.C. 2.4.2.8.) and adenine phosphoribosyl transferase (APRT, E.C. 2.4.2.7.) were determined simultaneously as described by McBurney and Adamson (1976) using ^{3}H-hypoxanthine and ^{14}C-adenine as substrates.

For thermodenaturation determinations of alpha-GAL, samples were diluted in .02M imidizole buffer (pH 7.2) and 50 ul aliquots were heated in sealed tubes at 52°C. (Lusis and West, 1976).

CHAPTER THREE

RESULTS

X CHROMOSOME INACTIVATION IN P10 CELLS

The Stability of the Two X Chromosomes in P10 Cells

McBurney and Strutt (1980) have previously shown that the two X chromosomes in P10 cells were genetically active. The two X chromosomes replicated isocyclically early in "S" phase of the cell cycle and all subclones of P10, which contained two X chromsomes, expressed both electrophoretic variants of PGK-1.

However, Featherstone (M.Sc. thesis, University of Ottawa) found that in P10 cells, X encoded enzyme specific activities were not twice those found in male EC cells (XY), female EC cells possessing a cytologically inactive X chromosome or P10 cells containing a single X chromosome. There appeared to be considerable variation in enzyme activities in P10 cell lines that were unrelated to the number of X chromosomes and considerable variation between different EC cell lines.

Early in the course of my work, it was noted that the two Xa in P10 cells was unstably maintained. Long term cultures invariably became overgrown by clones which contained a single X chromosome, and may or may not have acquired other chromosome abnormalities. Either X chromosome could be lost (McBurney and Strutt, 1980). This instability seemed to be a consequence of both X chromosomes being active, since other EC cell lines carrying an Xi are,

karyotypically, very stable (McBurney and Adamson, 1976). In addition, this instability is not a result of the mouse strain that P10 is derived from or because of euploidy since an EC cell line derived from the same strain, with a euploid male karyotype, is chromosomally very stable (McBurney and Rogers, 1982). Pluripotent stem cells derived from female embryos also have unstable X chromosomes (Robertson et al., 1983).

I attempted to obtain P10 cells that had more stable X chromosomes. Single P10 cells were cloned and grown up to a colony of 50-100 cells. At this time, half of the colony was isolated and subjected to PGK-1 electrophoresis. A colony that possessed both PGK-1 isozymes was identified and single cells from the remaining half of the colony were recloned. This procedure was repeated five times in an attempt to obtain clones in which both X chromosomes were stably retained. Unfortunatly, I did not recover this desired clone. Table 2 shows the history of X chromosome stability in one such clone over time.

Because of the problem of X instability, all experiments reported in this thesis were carried out on recently cloned cultures whose chromosomes had been carefully G banded before (and often during and after) each experiment to ensure the presence of two X chromosomes in all (>90%) of the cells.

X Encoded Enzyme Specific Activities in P10 Cells

I have measured the specific activities of X encoded

TABLE TWO

INSTABILITY OF THE TWO X CHROMOSOMES IN A P10 CLONAL LINE

TIME (DAYS)	PERCENT OF XX CELLS
0	92
3	94
10	82
14	71
21	68
28	57
40	52

The P10 clone was isolated from a single cell and grown up to approximately 100 cells (11 days) before half of the colony was isolated for PGK-1 electrophoresis. The second half of the colony as grown up to 5×10^5 cells before G banding was performed (day 0). Cells were subcultured every 2 days on STO feeder cell and G banded on the days indicated.

enzymes in clonal lines of P10 cells possessing one or two X chromosomes. Clones of P10 cells containing two X chromosomes generally had higher activities of the X encoded enzymes G6PD, PGK, HGPRT and alpha-GAL, but there was no consistent two-fold differences as one might have expected and has been reported (Martin et al., 1978) (Figure 2). Functionally related autosomal enzymes had similar activities in P10 XX and XO cells. There were considerable clone-to-clone variabilities of X encoded specific activities in P10 cells with two X chromosomes (Figure 3a and b). Less variablity was observed between XO clones of P10 cells (Figure 3c), regardless of whether the clone carried the original maternal or paternal X chromosome.

When X encoded enzyme activities were adjusted for differences in metabolism, by taking into account the activities of functionally related autosomal enzymes in XX and XO clones, there was still no two fold differences in X encoded specific activities.

Thus, the specific activities of X encoded enzymes in P10 XX cells demonstrate clonal differences which are not proportional to the number of Xa. X-encoded specific activities can also be influenced by mechanisms, in addition to X inactivation, such as developmental age, autosomal regulatory genes, tissue type and in vitro culture (Monk, 1979; Steele and Owens, 1973; Steele and Migeon, 1973).

FIGURE TWO

Relative specific activities of some X-encoded and autosomal enzymes in XX (open bars) and XO (stipled bars) P10 EC cells. These values represent the averages of 4-6 independent experiments. Specific activities and standard deviations of these values are listed in Appendix One.

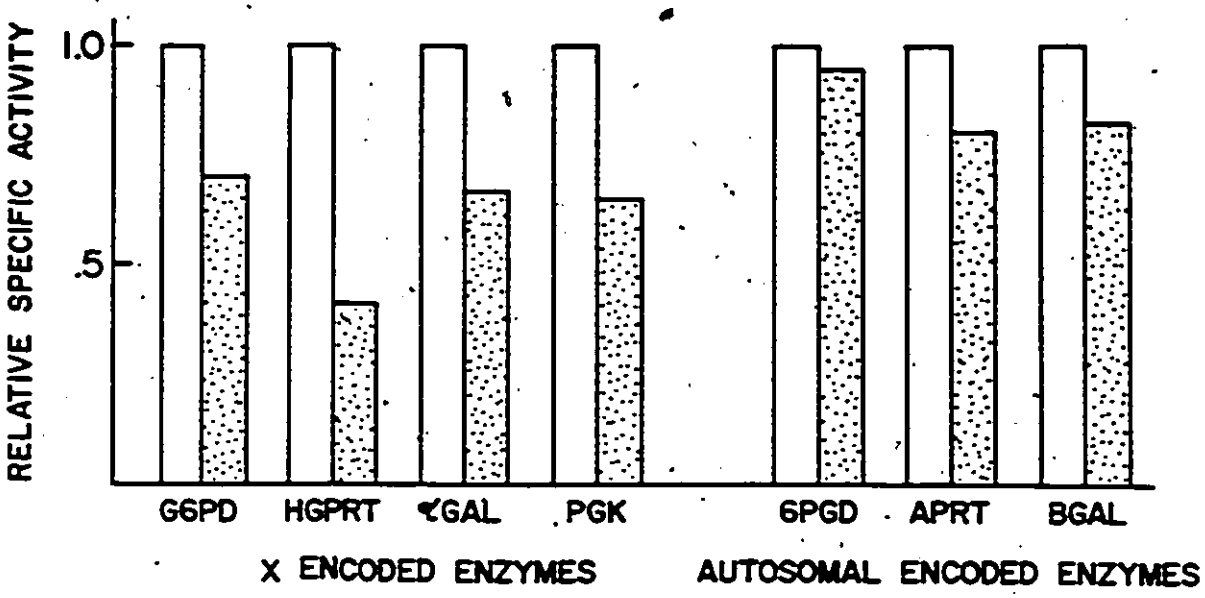
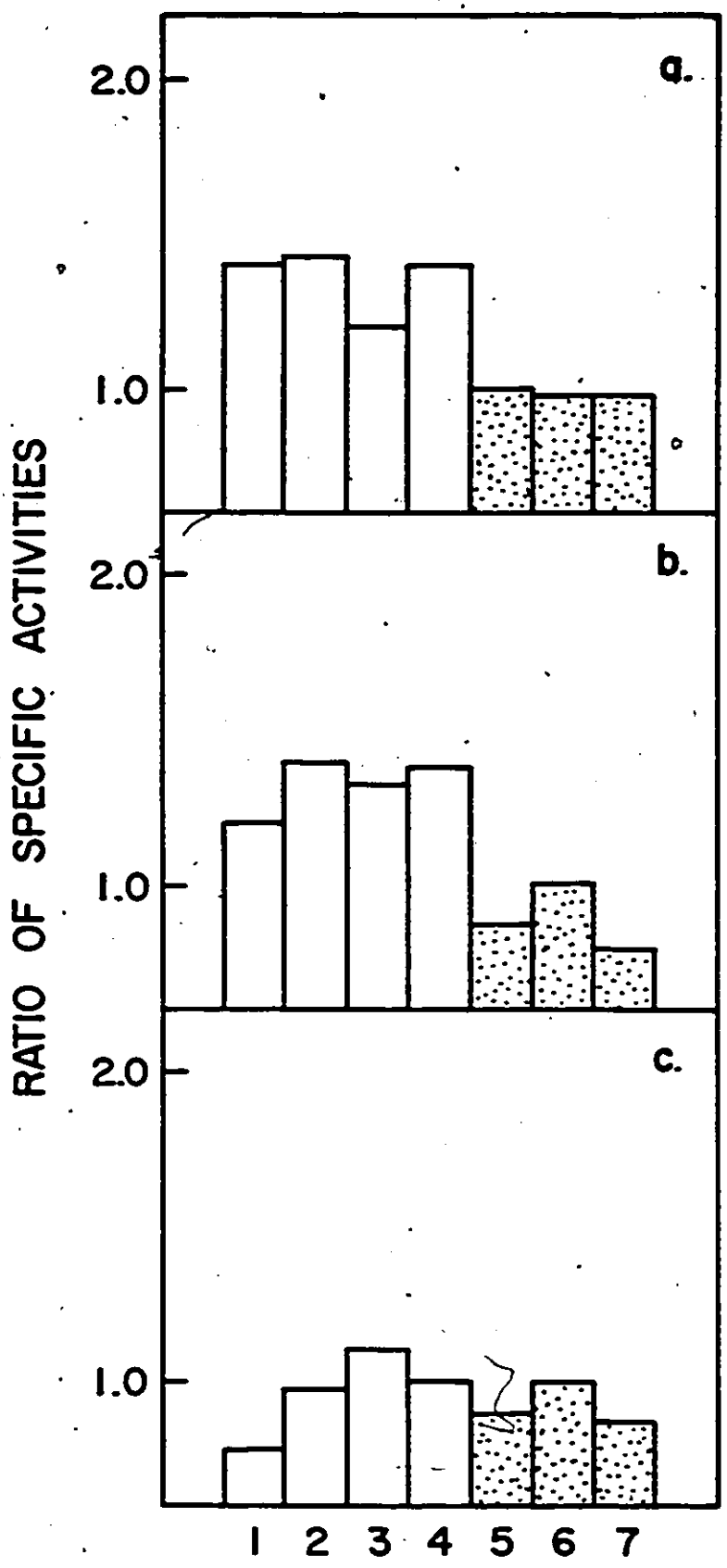

RELATIVE SPECIFIC ACTIVITY
1.0
.5
G6PD
HGPRT
αGAL
PGK
6PGD
APRT
BGAL
X ENCODED ENZYMES
AUTOSOMAL ENCODED ENZYMES

FIGURE THREE

The ratio of specific activities of some X-encoded (open bars) and autosomal (stippled bars) enzymes in clones of XX and XO P10 cells. a. P1042(XX)/P1041S7(XO) - b. P1042112(XX)/P1041S9(XO) c. P1041S7(XO)/P1041S9(XO). 1, G6PD; 2, PGK; 3, HGPRT; 4, alpha-GAL; 5, 6PGD; 6, APRT; 7, beta-GAL.


a.
b.
c.
2.0
1.0
2.0
1.0
2.0
1.0
RATIO OF SPECIFIC ACTIVITIES
1 2 3 4 5 6 7

Retinoic Acid Induced Differentiation

Retinoic acid (RA) induces the F9 line of EC cells to differentiate into a cell type resembling primitive extraembryonic endoderm (EE) of the embryo (Hogan et al., 1981; Strickland et al., 1980) which can subsequently develop into either parietal or visceral EE, depending upon the culture conditions (Hogan et al., 1981; Strickland and Mahdavi, 1978; Strickland et al., 1980).

When P10 cells were cultured as monolayers in the presence of 10^{-7}M RA, they too differentiated into a cell resembling primitive EE which spontaneously developed into cells of parietal EE morphology (Figure 4 a-f). Strickland et al. (1980) reported that the addition of 10^{-5}M dibutyryl cAMP after RA exposure facilitated the differentiation of F9 cells into cells with parietal EE characteristics. The addition of dibutyryl cAMP to differentiating P10 cells did not increase the proportion of cells with parietal EE morphology. In addition, the RA could be removed after 72 hr and the cells differentiated to the same extent. The differentiation of P10 cells was also dependent upon culture conditions. When aggregates of P10 cells were treated with RA, they differentiated along neuroectodermal pathways (Jones-Villeneuve et al., 1982).

Cells with EC morphology had disappeared by 4 days and by this time the cells had lost the cell surface antigen recognized by the monoclonal antibodies AEC3A1-9 (Harris et al., 1984) (Figure 5) and SSEA-1 (Solter et al., 1981) (data

FIGURE FOUR

Phase contrast micrographs of P10 cells treated with 1 x 10^{-7} M retinoic acid. P10 cells were cultured in the absence of STO feeders for 24 hr and seeded at 5 x 10^{5} cells into gelatin-coated 60mm tissue culture plates. Cells were continuously exposed to retinoic acid and photographed at daily intervals. a. P10 EC cells (x112) b. Day 1 (x112) c. Day 2 (x112) d. Day 3 (x112) e. Day 4 (x112) f. Day 7 (x112).

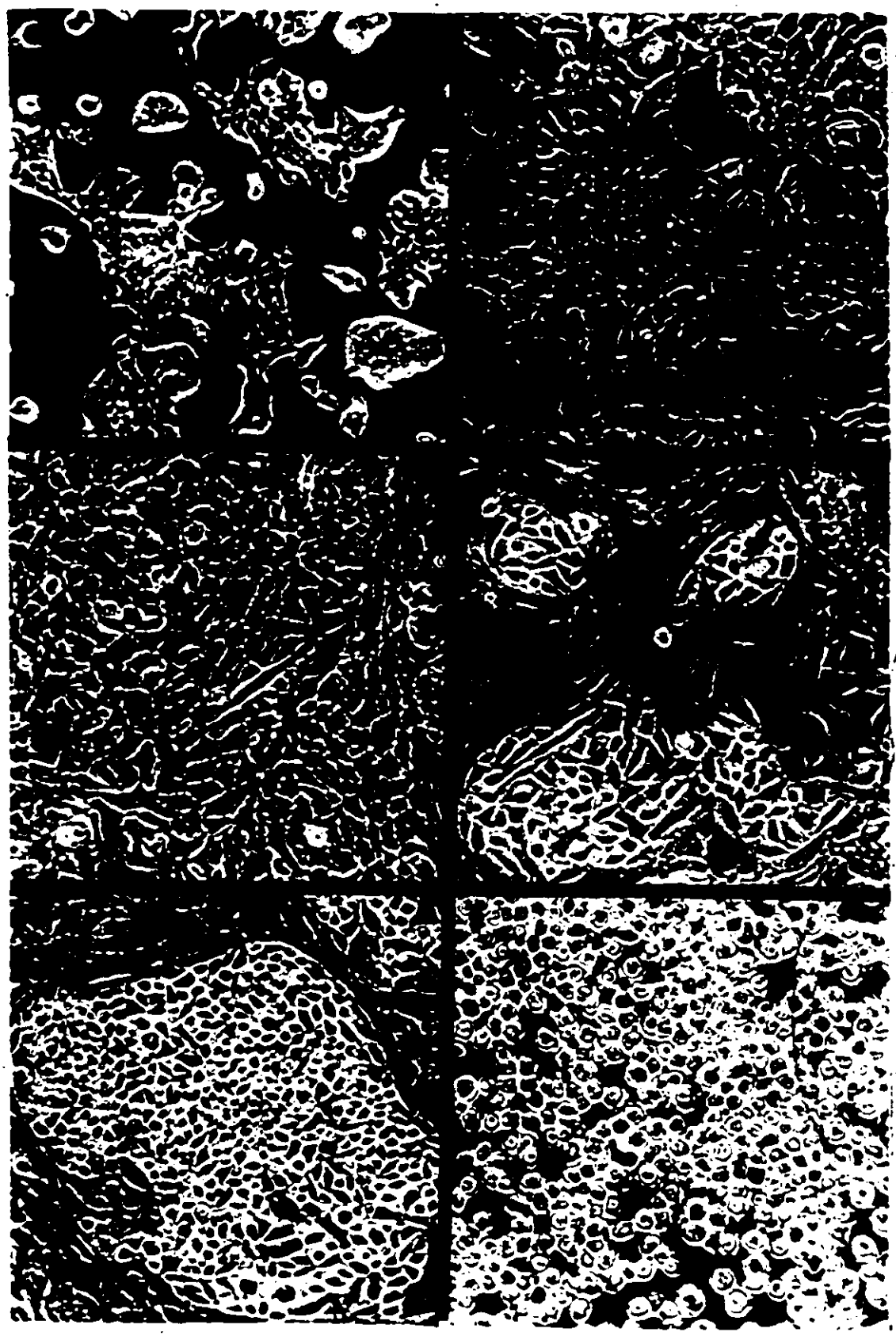
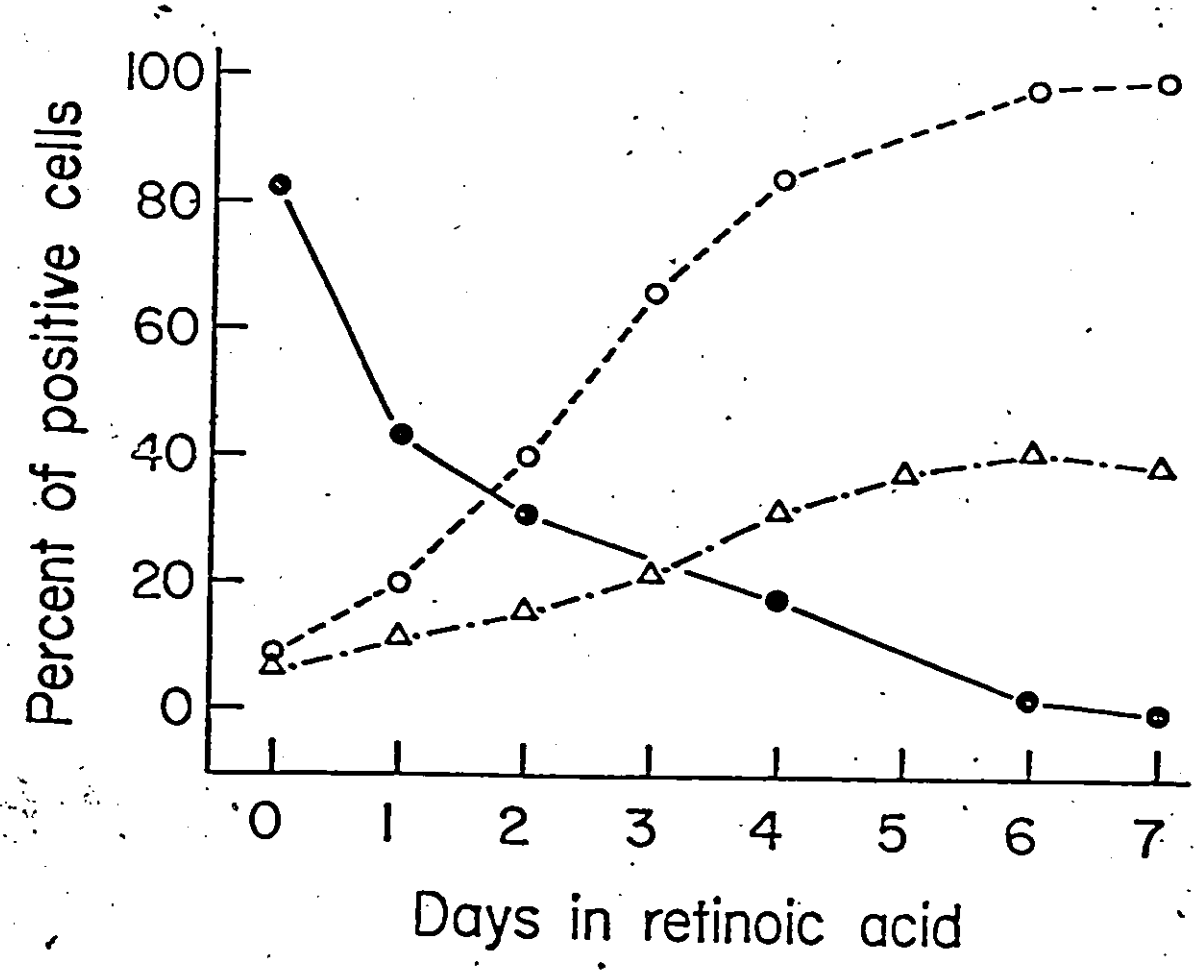


FIGURE FIVE

Percentage of differentiating P10 cells carrying antigens reactive with three monoclonal antibodies. a. (●——●) AEC3A1-9 is specific for a cell surface antigen (SSEA-1 antigen) on EC cells. b. (○- - - -○) TROMA-1 recognizes intermediate filaments present in visceral and parietal extraembryonic endoderm cells. c.(△-·—·—·—△) TROMA-3 recognizes intermediate filaments present only in parietal endoderm. A total of 500-700 cells were scored for each time point.


Percent of positive cells
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Days in retinoic acid

not shown). These antibodies recognize a cell surface antigen called the stage specific embryonic antigen (SSEA-1) which is present on the surface of early embryo cells, EC cells and sperm cells, but not most other differentiated cell type.

In addition to losing these markers of undifferentiated EC cells, all RA-treated cells acquired intracellular cytoskeletal antigens recognized by the monoclonal antibody TROMA-1 (Figure 5), a marker for EE and several other epithelial cell types (Boller and Kemler, 1982). Approximately 40% of the differentiated cells were also positive for the TROMA-3 antibody, a marker for parietal EE (Boller and Kemler, 1982). The proportion of cells recognized by TROMA-1 and TROMA-3 antibodies are similar to those observed in F9 EC cells treated with RA and dibutyryl cAMP (Boller and Kemler, 1982). TROMA-1 and TROMA-3 recognize antigens of the cytokeratin class of intermediate filaments which are only observed in the EE and trophoblast cells of the early embryo (Boller and Kemler, 1982; Paulin et al., 1981).

Another characteristic of the EE cell lineages is that their repetitive DNA sequences contain less methylated cytosines than the DNA of cells from embryonic cell lineages (Chapman et al., 1984). RA-treated P10 cells also have undermethylated repetitive sequences (J. Sanford, personal communication). Thus, RA-treated P10 cells possess another characteristic of EE of the embryo.

The response of P10 cells to RA appears to be similar to that described for the F9 line of EC cells (Strickland et al., 1980). The cells rapidly and irreversibly develop into a cell type closely resembling parietal EE.

X Chromosome Status During Differentiation

Transcriptionally active chromatin generally replicates during the early part of "S" phase of the cell cycle, whereas inactive chromatin, including the Xi, replicates during the late "S" phase. A 5-bromodeoxyuridine (BrdU)-Giemsa staining procedure was applied to differentiating P10 cultures to investigate the replication patterns of their X chromosomes (McBurney and Strutt, 1980). During the early stages of RA exposure, the two X chromosomes replicated in synchrony (isocyclically) with the autosomes primarily in the early part of "S" phase. However, by day 3, a significant proportion of metaphase spreads (in 3 experiments an average of 28%) contained one X chromosome which stained uniformly dark indicating that it had replicated its DNA entirely during the late "S" phase (Figure 6). By day 4, approximately 55% of metaphase spreads contained a late replicating X chromosome (Figure 7). The proportion of cells possessing a late replicating X chromosome remained at this level for at least 3 weeks in culture.

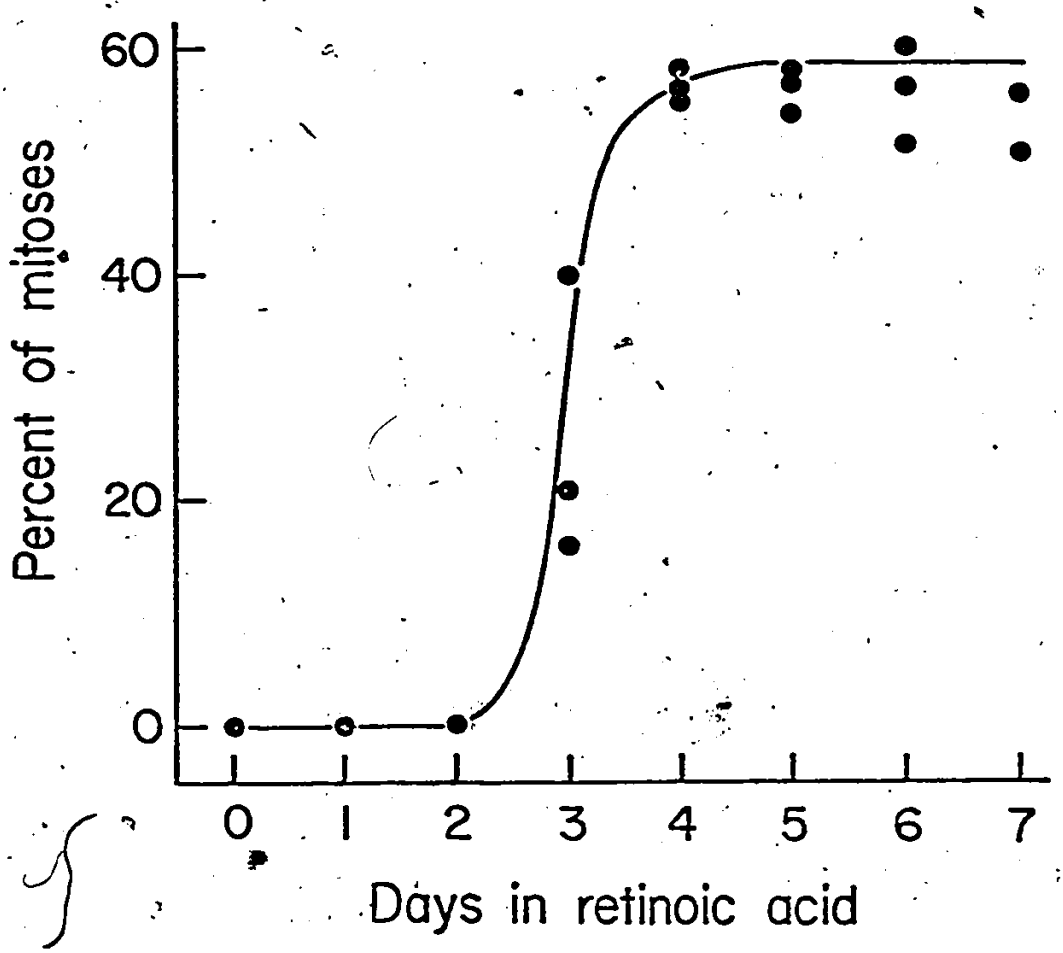
It was of interest to determine the number of cell cycles the P10 cells had traversed between the time of exposure to RA and the first appearance of a late

FIGURE SIX

Chromosome replication patterns in a differentiated P10 cell. Differentiated cells were exposed to BrdU for 7 hr before metaphases were collected and stained as described in the Method and Materials. The inactive X chromosome (thin arrow) stains uniformly dark indicating that it replicated entirely during the late "S" phase. The active X (thick arrow) shows a characteristic banded appearance.

FIGURE SEVEN

Appearance of a late replicating X chromosome during differentiation of P10 cells. Percentage of mitoses containing a late replicating X chromosome was determined by the BrdU incorporation procedure. Each point represents 50-70 metaphases each containing 40 chromosomes. The results of 3 independent experiments are shown and the points at Days 0, 1 and 2 are superimposed all with 0 % mitoses possessing a late replicating X chromosome.


60
40
20
0
Percent of mitoses
0
1
2
3
4
5
6
7
Days in retinoic acid

replicating X chromosome. The late replicating X chromosome first appeared in metaphases collected 72 hr after RA exposure. I used a procedure (Alves and Jonnason, 1978) of continuous exposure to BrdU to assess the number of cell cycles completed by 72 hr (Table 3). Exposure to RA did retard the rate of proliferation of P10 cells, as it does in other EC cell lines (Rosenstraus et al., 1982), such that by 72 hr 40% of the labelled mitoses were completing their third replication cycle.

Thus, it seems likely that the X chromosome first became late replicating during the third "S" phase after RA exposure.

Kinetics of X Inactivation in P10 Cells

I have measured the specific activities of X encoded enzymes during the differentiation of RA-treated P10 cells. These measurements are based on the assumption that the variations in X encoded specific activities observed in EC cells are the result of clonal differences and that any reductions of enzyme activity during differentiation are the result of X inactivation. Since the studies reported in this section were carried out on clonal populations, the problem of clonal variability is eliminated.

The changes in the specific activities of X encoded enzymes during differentiation of P10 cells are documented in Figure 8. The specific activities of G6PD (panel a) and PGK (panel c) decreased during differentiation to approximately 50% of their value in EC cultures. A subclone

TABLE THREE

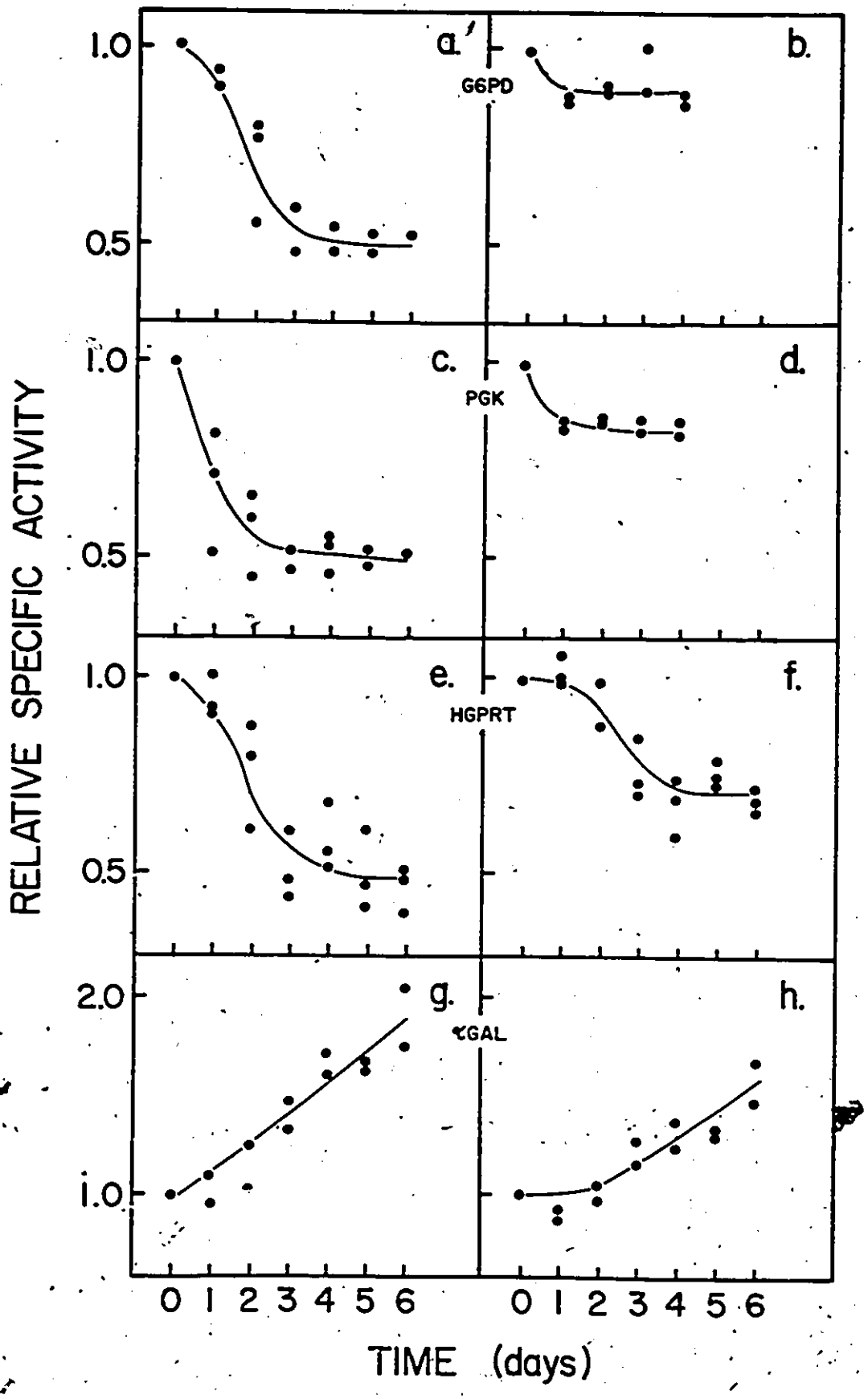
Rate of Cell Division during Retinoic Acid Induced Differentiation of P10 Cells*

Time (hr)	RA Treatment (M)	% of metaphases from cells having traversed 1-4 cycles*			
		1	2	3	4
24	0	28	72	0	0
	10^{-7}	26	74	0	0
72	0	0	8	48	52
	10^{-7}	0	61	39	0

*Cultures were continuously exposed to BrdU and metaphase spreads prepared and differentially stained to reveal fully BU-substituted DNA. On the basis of the chromatid staining pattern, each metaphase was classified as being from a cell which had traversed 1, 2, 3, or 4 cell cycles in the presence of BrdU (1).

FIGURE EIGHT

Relative specific activities of some X encoded enzymes during the differentiation of XX and X0 P10 EC cell. Cells were continuously exposed to 1 x 10^{-7}M retinoic acid to induce differentiation. Cells were harvested at daily intervals for enzyme determinations. The results represent 2-4 independent experiments which have been normalized to the Day 0 value in each experiment. a. G6PD in P10(XX). b. G6PD in P10(X0). c. PGK in P10(XX). d. PGK in P10(X0). e. HGPRT in P10(XX). f. HGPRT in P10(X0). g. alpha-GAL in P10(XX). h. alpha-GAL in P10(X0).


a.
G6PD
b.
c.
PGK
d.
e.
HGPRT
f.
g.
αGAL
h.
1.0
0.5
2.0
1.0
0 1 2 3 4 5 6
0 1 2 3 4 5 6
RELATIVE SPECIFIC ACTIVITY
TIME (days)

of P10, containing only one X chromosome, was measured in parallel experiments and only a slight decrease in the specific activity of these enzymes was observed (panels b and d). HGPRT activites also decreased by almost 50% during differentiation of P10 XX cells, however, HGPRT specific activites also decreased in P10 XO clones by approximately 30%. The specific activities of alpha-GAL rose markedly in differentiating cultures of both XX and XO P10 lines and any reductions in activity, that may be due to X inactivation, were masked by the increases in activity which resulted from the change of cell type (panels g and h).

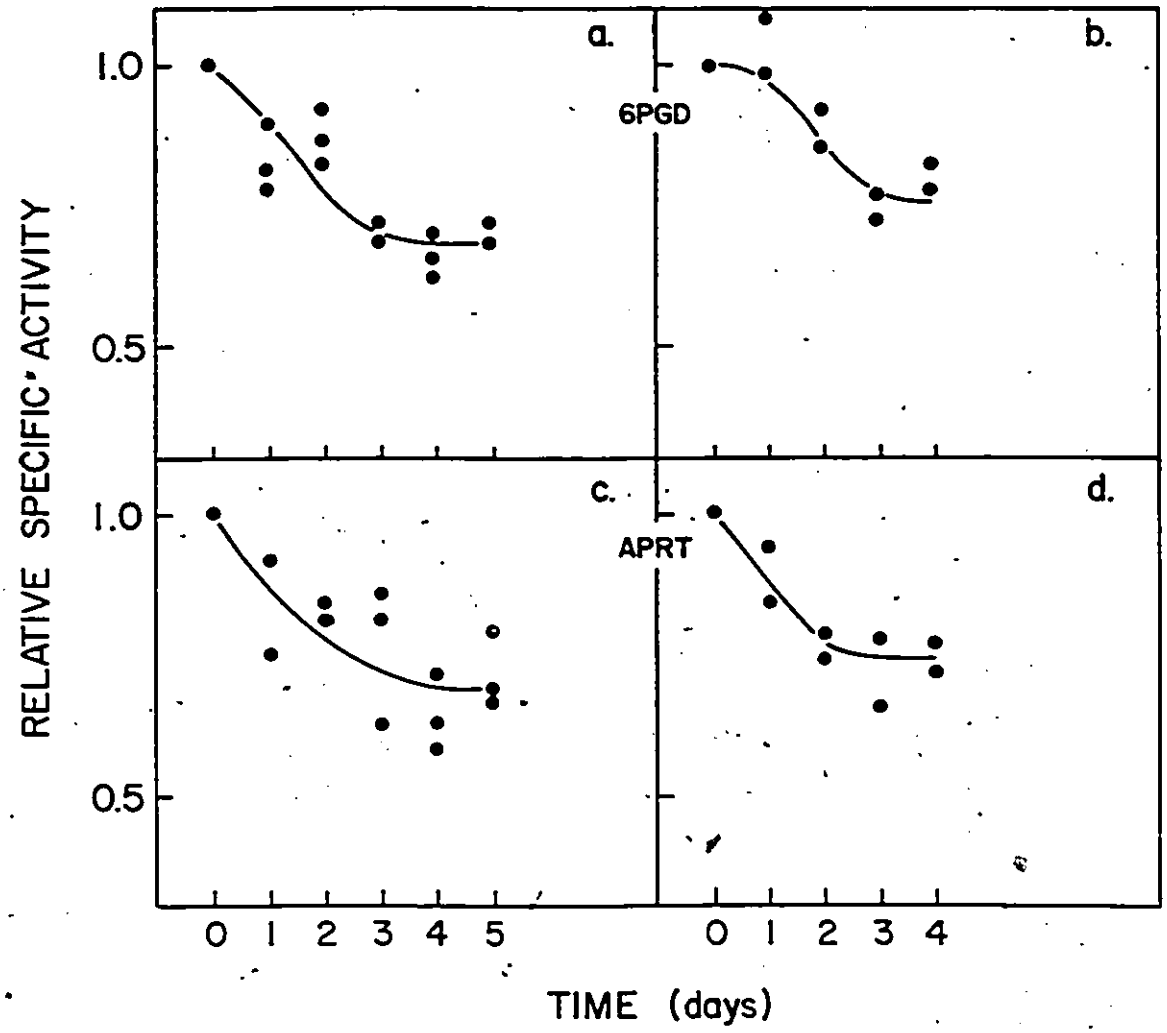
I have also measured the specific activities of the autosomally encoded enzymes 6PGD and APRT (Figure 9). 6PGD activities are reduced by approximately 30% of their values in both XX and XO P10 cell lines (panels a and b). This was also true for the specific activities of APRT during differentation (panels c and d).

Inactivation Preference

In the mouse embryo, the X^P is preferentially inactivated in all derivatives of the primitive EE (Takagi and Sasaki, 1975; West et al., 1977). Because RA-treated P10 cells closely resemble parietal EE, I investigated whether the X^P was preferentially inactivated in these cells. I made use of the fact that P10 cells are heterozygous for the X-linked genes encoding pgk-1 and alpha-gal. The pgk-1a gene product can be distinguished from the product of the X^m-linked pgk-1b by electrophoresis (Nielson and Chapman,

FIGURE NINE

Changes in specific activities of two autosomally encoded enzymes during differentiation of XX and X0 P10 EC cells. a. 6PGD in P10(XX). b. 6PGD in P10(X0). c. APRT in P10(XX). d. APRT in P10(X0).


a.
b.
6PGD
c.
d.
APRT
1.0
0.5
1.0
0.5
RELATIVE SPECIFIC ACTIVITY
0 1 2 3 4 5
0 1 2 3 4
TIME (days)

1977. The alpha-GAL activities have different thermolabilities (Lusis and West, 1976).

Figure 10 shows the PGK-1 isozyme profiles of RA-treated P10 cultures at various times after the addition of the drug. Both forms of PGK-1 remained present in populations of P10 cells, even after differentiation when the majority of the cells had reduced specific activities of PGK-1 and contained a late replicating X chromosome. Both isozymes were present with roughly equal activities in untreated cultures (McBurney and Strutt, 1980), but in most experiments, the PGK-1A activity exceeded that of PGK-1B after the completion of differentiation. Densitometric traces of photographic negatives of PGK-1 zymograms taken at different development times revealed that the activity of PGK-1A exceeded PGK-1B by approximately 10-15% (Table 4). Since PGK-1B is the product of the X^m, this result argues against preferential inactivation of the X^p.

The thermolability profiles of the alpha-GAL activities in undifferentiated cultures of P10 cells were intermediate between that of cells which contained one X chromosome and either the alpha-gal^s or the alpha-gal^r alleles (Figure 11). The profile was identical to that obtained from a 1:1 mixture of extracts containing only alpha-GAL^s or alpha-GAL^r activities. The thermal denaturation characteristics of murine alpha-GAL suggest that it may be a dimer or a multimer (Lusis and West, 1976). It is not clear with what kinetics the alpha-GAL heterodimers would denature, but the

FIGURE TEN

PGK-1 isoenzyme profiles during retinoic acid-induced differentiation of XX P10 cells. At daily intervals, samples containing equal amounts of proteins were electrophoresed on cellulose acetate membranes and stained for PGK-1 activity. Lanes 1-7 are from samples prepared 1-7 days after retinoic acid exposure.

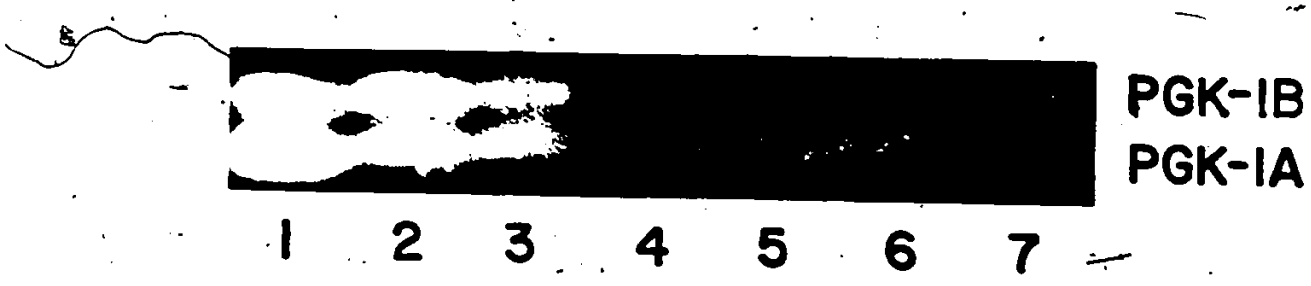

PGK-1B
PGK-1A
1 2 3 4 5 6 7

TABLE FOUR

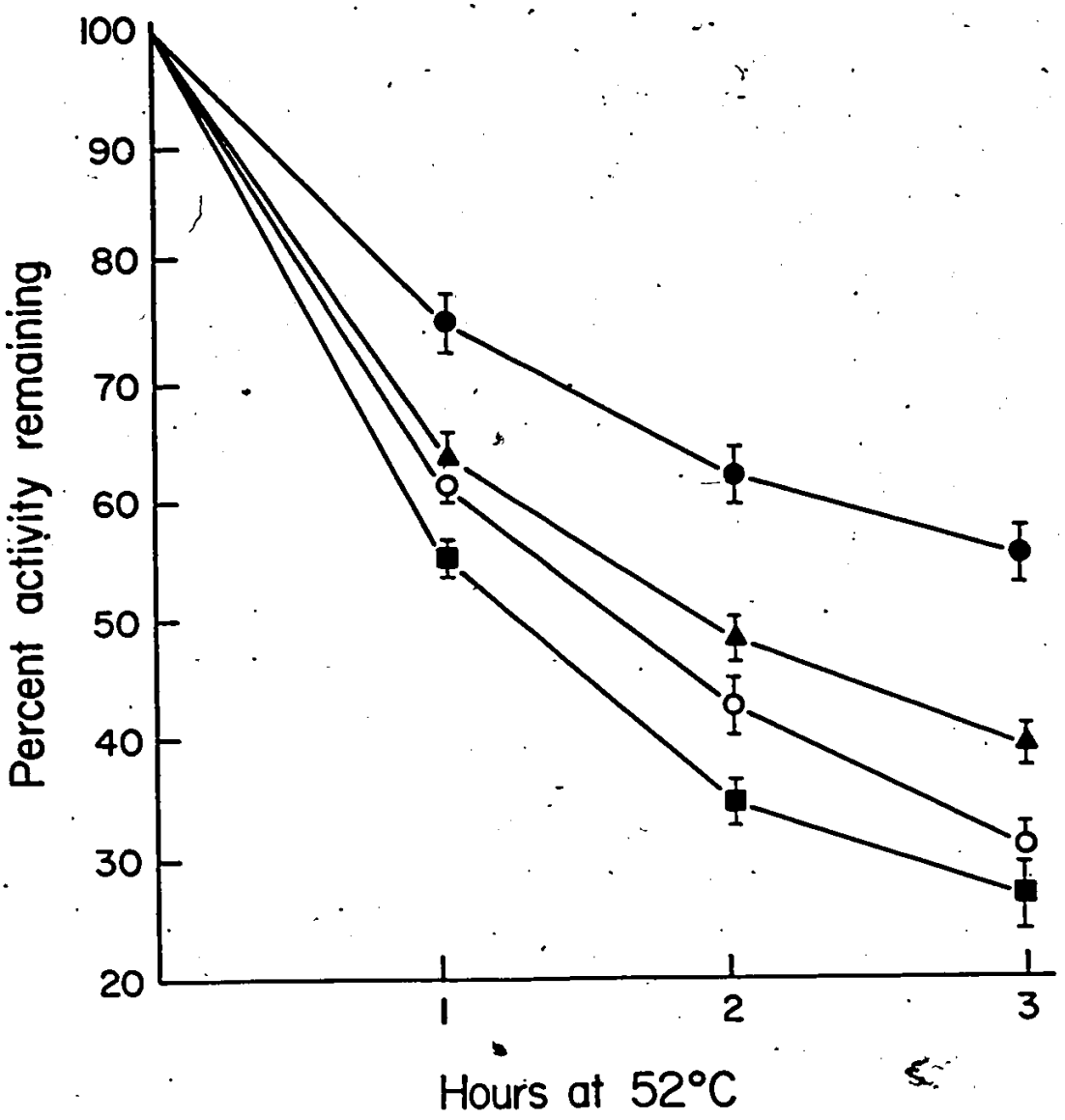
The Relative Proportions of PGK-1a and PGK-1b Isozymes in Differentiated P10(XX) Cells

	Percent of Activity	
	PGK-1b	PGK-1a
1.	42	58
2.	46	54
3.	38	62
4.	40	60

Densitometric scans of photograghic negetives of PGK-1 zymograms were performed as described in the Methods and Materials. Each set represents an independent experiment involving P10(XX) cells exposed to RA for 7 (1. and 2.) or 12 (3. and 4.) days.

FIGURE ELEVEN

Thermodenaturation profiles of alpha-GAL in P10 EC cells. Extracts of clones of P10 cells were heated to 52°C. for various times and assayed for alpha-GAL activity. Clones of X0 P10 cells carrying the alpha-gal^{r} or alpha-gal^{s} allele were identified by assaying the PGK-1 isoenzyme expression. The pgk-1a gene is linked to the alpha-gal^{s} gene. (●——●) X0 segregant of P10 containing only the alpha-gal^{r} allele. (■——■) X0 segregant of P10 carrying only the alpha-gal^{s} allele. (▲——▲) Undifferentiated P10 cells. (○——○) P10 cells exposed to 1 x 10^{-7} M retinoic acid for 7 days.


100
90
80
70
60
50
40
30
20
Percent activity remaining
1
2
3
Hours at 52°C

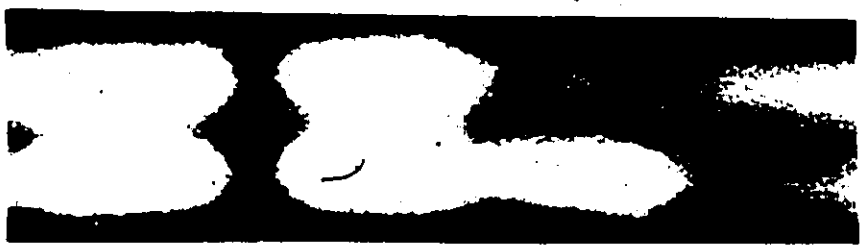
results here appear consistent with the presence of active copies of both alpha-gal alleles.

Following RA-induced differentiation, the P10 cultures had alpha-GAL thermolability profiles suggesting a higher proportion of alpha-GAL^s than alpha-GAL^r. Since alpha-gal^r is present on the X^m, this result again argues for the absence of preferential inactivation of the X^p.

EE-like cells can develop from many EC cell lines by aggregation of cells in the absence of drugs (Martin and Evans, 1975a). With P10 cells a layer of EE cells did develop around the outside of each aggregate and it was surgically removed for PGK-1 analysis. Figure 12 shows that both PGK-1 isozymes were also present in EE differentiated in this way, although the proportions of each isozyme varied considerably in EE removed from individual aggregates. Although I could not obtain evidence that X inactivation had occurred in this tissue, if this were the case then my results show that inactivation was random. The variation in the proportions of PGK-1A and PGK-1B is probably the consequence of a relatively small number of stem cells that contain an Xi and give rise to all of the EE cells around one aggregate.

FIGURE TWELVE

PGK-1 isoenzyme profiles of extraembryonic endoderm cells from aggregation induced P10 cell differentiation. Lanes 1 and 2. EE samples collected from around individual aggregates 7 days after aggregation. Lanes 3 and 4. EE samples from around individual aggregates 9 days after aggregation.



PGK-1B

PGK-1A

1 2 3 4

DISCUSSION

Retinoic acid induces the rapid and complete differentiation of P10 cells into a single cell type. This differentiation is accompanied by the rapid and synchronous appearance of a late replicating (inactive) X chromosome in the differentiating cells. McBurney and Strutt (1980) also observed the appearance of late replicating X chromosomes in P10 cells induced to differentiate by aggregation, but only after 3 weeks in culture. The temporal pattern of differentiation by this method of induction is asynchronous and results in multiple differentiated cell types. Thus, the induction of late replication by RA in a single differentiated cell type provides a more amenable system to investigate X inactivation.

The appearance of the late replicating X chromosome is also correlated with the 50% reduction in specific activities of the X encoded enzymes G6PD and PGK in XX P10 cells, but not in XO P10 cells. These results are consistent with X inactivation accompanying differentiation.

HGPRT specific activity also decreased by 50% during the differentiation of P10 XX cells. However, HGPRT activities in P10 XO cells decreased by 30%, suggesting that there are other regulatory mechanisms, in addition to X inactivation, controlling the levels of this enzyme. Measurements of the functionally related autosomal enzyme, APRT, also show a reduction in specific activity in both XX and XO P10 clones. Since there is a reduction in rate of

cellular proliferation of P10 cells during RA-induced differentiation, the decreases in the activities of these enzymes may be due to metabolic regulation.

The two-fold decreases in PGK and G6PD activities were complete by 2-3 days, whereas the late replicating X was detectable only after 3-4 days. Although the half-lives of PGK-1 and G6PD mRNAs and proteins are not known, this result suggests that the appearance of a late replicating X chromosome lags behind transcriptional inactivation and is a relatively late event in the genesis of an Xi.

Only about 55% of informative metaphases from differentiated P10 cells had a clearly identifiable late replicating X chromosome. Since virtually all the cells were differentiated, this result may indicate that 45% of differentiated EE cells had two early replicating, and therefore active, X chromsomes. I think this is unlikely. In female mouse embryonic fibroblasts, the proportion of metaphases identified with a late replicating X chromosome is always less than 100% and depends upon the interval between the addition of BrdU and colcemid (McBurney and Strutt, 1980). My interpretation of the relatively low detection efficiency seen in RA-treated P10 cells is that the Xi initiated replication in the second half of "S" phase but completed replication well before the end of "S", that is before the last of the heterochromatin replicated. Thus the cytogenetic procedure underestimates the proportion of late replicating X chromosomes.

Takagi and Martin (1984) also showed that late replication was only observed in approximately 55% of differentiated LT-1 EC cells but that reductions in X encoded specific activities were consistent with X inactivation occurring in the entire population.

Direct evidence for the presence of only one active X chromosome in each cell could be obtained by cloning the differentiated cells and assaying their PGK-1 and alpha-GAL isozyme expression. Unfortunately, differentiated P10 cells did not clone at all. My attempts to improve this efficiency by the addition of supplementary growth factors (EGF, conditioned medium) and the introduction of transforming genes (SV40 large T antigen) into EC and differentiated cells were not successful.

The RA-treated P10 cells possess a number of markers characteristic of parietal EE but, unlike the situation in the normal embryo, the P10 derived EE did not contain preferentially inactivated X^ps. The preferential inactivation of the X^p in normal EE cells suggests that the X^p is chemically distinguishable from the X^m when EE cells inactivate their X chromosomes and that these EE cells have an inactivation mechanism which can recognize and inactivate the X^p. Recent evidence suggests that the inactive X^p DNA in EE is chemically distinct from Xi DNA in embryonic somatic tissues (Chapman et al., 1982; Kratzer et al., 1982; Chapman et al., 1984). Preferential X^p inactivation occurs in EE before X inactivation occurs in cells destined to develop

into embryonic tissues (Monk, 1981). Therefore, it seems possible that a chemical marking or imprinting of the X^p (or X^m) occurs during gametogenesis and this marking is lost, or drops below a threshold, between the times that the EE and embryonic cells inactivate their X chromosomes. My results can be interpreted to be consistent with this "Mitotic Dilution" model because a large number of mitoses have occurred since the X chromosomes of P10 cells could have been "marked" by gametogenesis.

It would be of interest to look at the inactivation preference of P10 cells in the EE in vivo. P10 cells efficiently participate in embryogenesis following injection into mouse blastocysts (Rossant and McBurney, 1983). Since the environment of the normal embryo is thought to "normalize" the behavior of EC cells, we have attempted to assess the X chromosome status of P10-derived EE formed in the embryo. Although P10 cells efficiently contribute to a variety of differentiated tissues, their derivatives have not been found in the EE formed in vivo (J. Rossant, pers. comm.). It is unclear whether this failure to colonize the EE is related to the inability to these P10 cells to preferentially inactivate their X^p.

It has been suggested that the difference in DNA methylation patterns between the X^m and X^p might provide a basis for the recognition of the X^p since sperm DNA is highly methylated (Strum and Taylor, 1981). Changes in methylation patterns accompanying embryogenesis, including

de novo methylation (Jahner *et al.*, 1981), may dilute out these differences with time. It is also noted that the DNA of the EE is undermethylated when compared to embryonic DNA (Chapman *et al.*, 1984) and this difference may be involved in preferential X^P inactivation. However, undermethylation cannot be a signal for preferential X^P inactivation since P10 derived EE also has undermethylated DNA sequences.

Takagi *et al.* (1982) and Sugawara *et al.* (1983) have shown that the first cytogenetic consequence of X inactivation in embryo-derived EE is the early or precocious replication of the Xi. This early replicating X shifts to late replication at later developmental stages. A small proportion of differentiated LT-1 teratocarcinoma cells possess an early replicating X chromosome and it was suggested that these may be obtained from differentiated EE cells (Takagi and Martin, 1984). I did not observe precocious X replication in differentiating P10 cultures at any time. This is another difference between the normal embryo and the P10 culture system and is consistent with the involvement of precocious replication with preferential X^P inactivation.

In the RA treated cultures of P10, the X^P linked *pgk-1a* and alpha-*gal*^{s} alleles appeared to be 10-15% more active than the X^m-linked alleles. The locus, called *Xce*, has been identified on the mouse X chromosome and has been shown to determine the probability of an X chromosome becoming inactivated in mouse fetal tissues (Johnston and Cattanach,

1981; Cattanach and Papworth, 1981). In females bearing the Xce^{c} and Xce^{a} alleles on different X chromosomes, the Xce^{c}-bearing chromosome has a higher likelihood of being the Xa in somatic tissues (Cattanach and Papworth, 1981; Johnston and Cattanach, 1981). In EE cells of the embryo however, preferential X^{p} inactivation always occurs regardless of the Xce genotypes of the X chromosomes (Papioannou and West, 1981). The mouse which contributed the X^{p} to P10 cells bore the pgk-1a, alpha-gal^{s} and Xce^{c} alleles while the X^{m} genotype is pgk-1b, alpha-gal^{r} and Xce^{a}. Thus, it is possible that X inactivation in P10 derived EE is influenced by the heterozygous Xce alleles and occurs according to processes characteristic of tissues derived from the embryonic ectoderm.

Conclusions

The exposure of P10 cells to RA induced their rapid and complete differentiation into cells resembling the EE of the embryo. This differentiation was accompanied by the inactivation of one X chromosome, in the majority of the cells, as determined by the reduction in specific activities of X encoded enzymes and the appearance of a late replicating X chromosome. In contrast to the situation in the embryo, differentiated P10 cells did not preferentially inactivate their X^{p}.

CHAPTER FOUR

REACTIVATION OF THE INACTIVE X CHROMOSOME IN EC CELL

RESULTS

It has been shown previously that DNA demethylating agents, such as 5-AC, could induce the reactivation of the hgprt locus on the Xi in somatic cell hybrids at high frequencies (Lester et al., 1982; Mohandas et al., 1981; Graves, 1982). This suggests that DNA methylation may be involved in the maintenence of X chromosome inactivation. Female EC cell lines resemble embryonic cells at about the time X chromosome inactivation occurs and thus they provide a system for the study of the initial events of X inactivation rather than its maintenance. I have studied the role of DNA methylation in the control of X chromosome inactivation by attempting to reactivate the expression of the Xi in various EC cell lines.

The Effect of 5-AC on the Appearance of HAT^{+} Colonies in 8-AG^{r} Cultures

I have utilized female EC cell lines that contained an Xi and were HGPRT^{-} mutants. These cells lines were presumed to be heterozygous for the HGPRT deficiency where the wild-type allele was present, but silent on the Xi. Mutant cell lines had minimal (10% of wild-type) HGPRT activity and did not survive in medium containing HAT (Littlefield, 1964). Thus, if 5-AC induced reactivation of the hgprt locus on the Xi, I would expect an increase in the number of cells able to survive in HAT medium and an increase in the HGPRT

specific activity in these cells.

To rule out the possibility that 5-AC may induce the high frequency reversion of the hgprt mutation on the Xa, I isolated spontaneous XO segregant cell lines from C86S1A1. These cell lines had lost the late replicating X chromosome but retained the Xa bearing the hgprt$^-$ gene. By comparing XX and XO EC cells, the effect of 5-AC on the hgprt locus of the Xa and Xi could be distinguished.

5-AC was equally toxic to all EC cell lines. Plating efficiency experiments for all cell lines treated with 5-AC were similar (Figure 13). The number of surviving cells decreased progressively after exposure to increasing concentrations of 5-AC.

Figure 14 (a and b) shows the massive rise in the number of C86S1A1 and C100AG1 colonies able to survive in HAT medium (HAT$^+$). This increase occurred in a dose dependent manner such that at 5-AC concentrations of 5uM approximately 5-20% of the surviving cells are HAT$^+$.

When C86S1A1XO cells were treated with 5-AC, no HAT$^+$ colonies were recovered even when up to $5x10^6$ were selected in HAT medium (Figure 14c). To rule out the possibility that this nonresponsiveness was unique to the hgprt mutation in C86S1A1XO clones we tested the ability of 5-AC to induce the HAT$^+$ phenotype in another independent XO clone derived from the EC cell line called P10. No colonies were recovered in HAT at any 5-AC concentration tested (data not shown).

When I treated two other EC cell lines containing an Xi

FIGURE THIRTEEN

Relative plating efficiencies of EC cell lines exposed to various concentrations of 5-AC. a. C86S1A1(XX). b. C100AG1(XX). c. C86S1A1(X0). d. C145FAG11(XX).

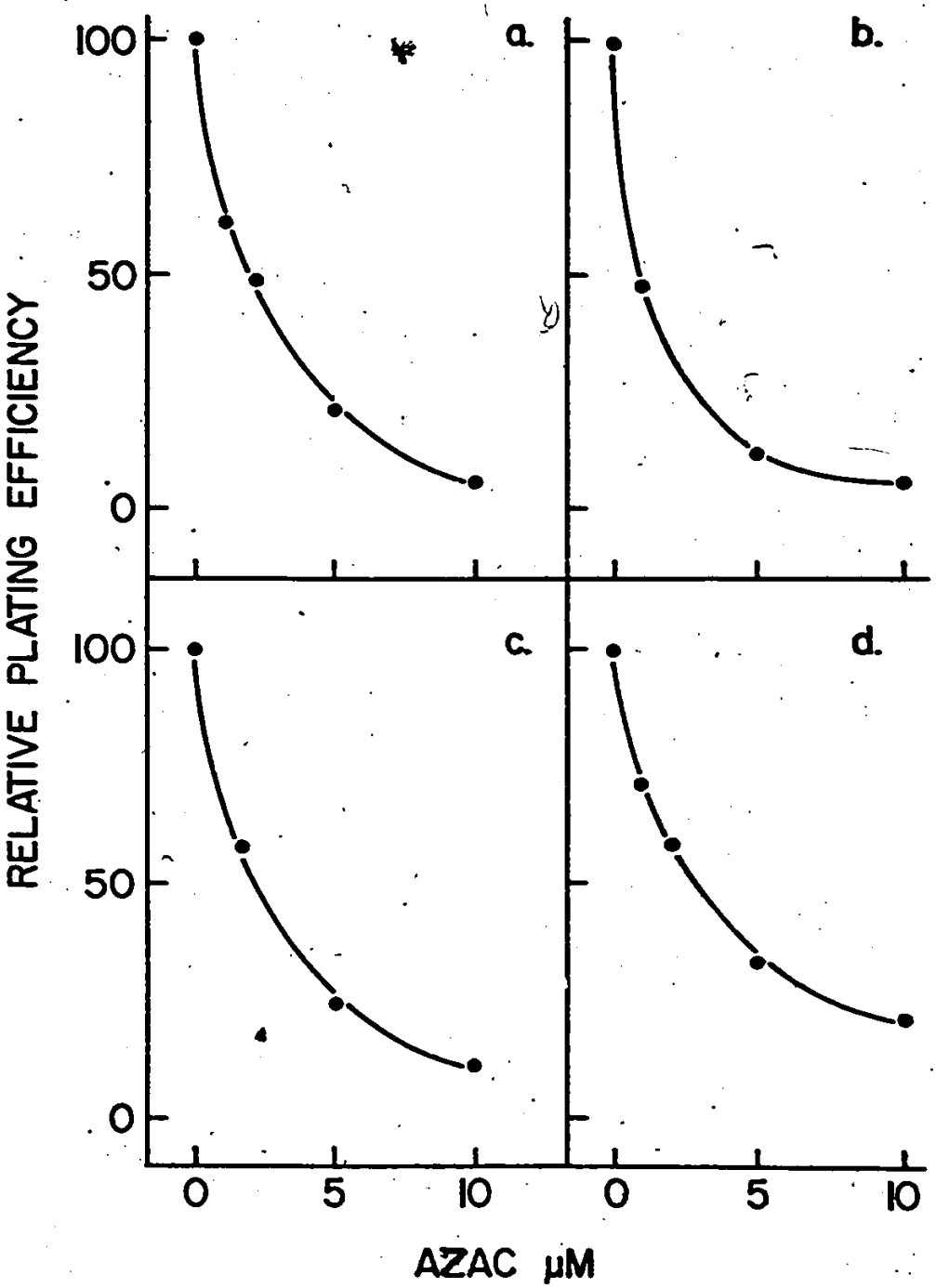
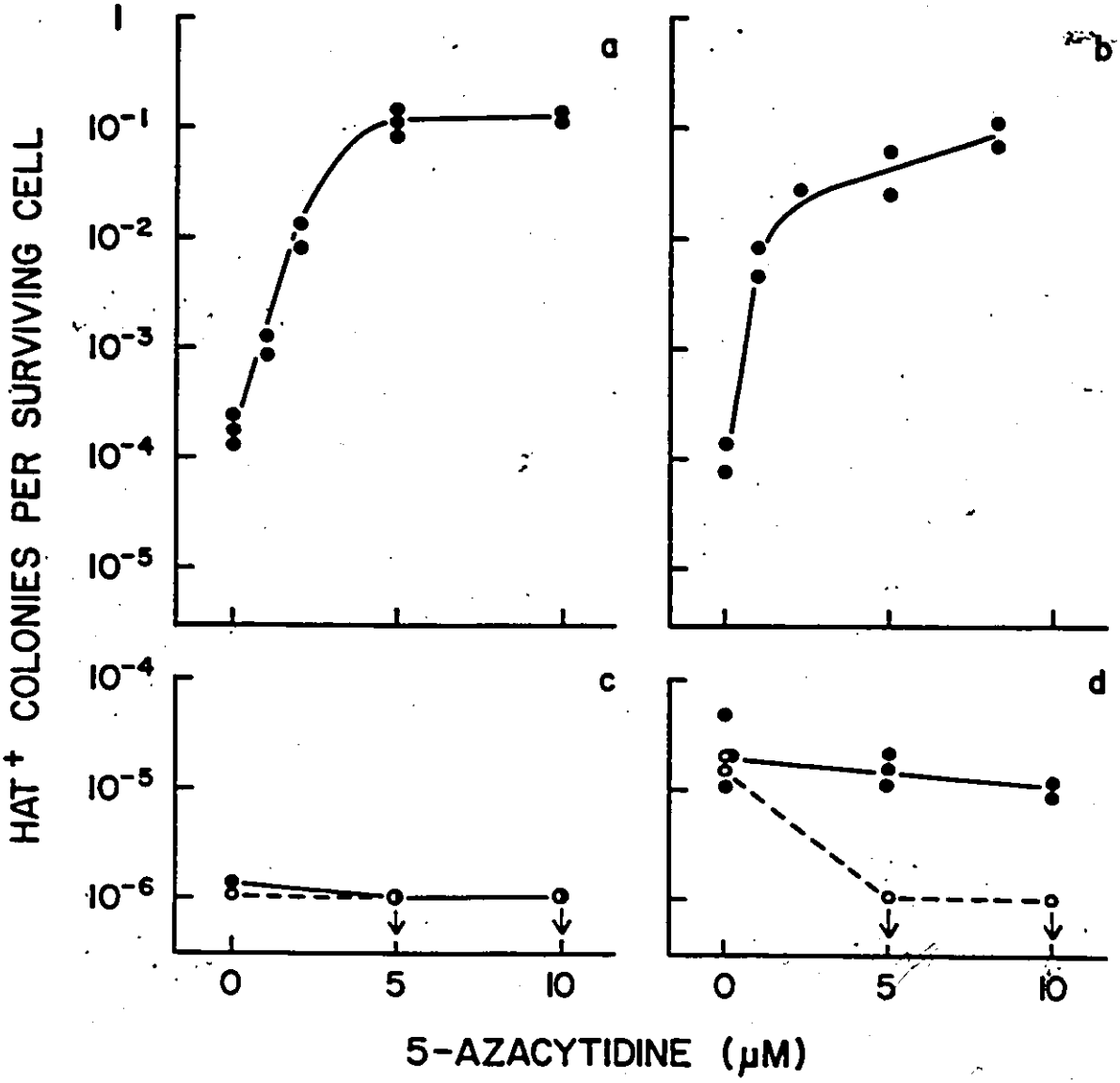

a.
b.
c.
d.
100
50
0
RELATIVE PLATING EFFICIENCY
0
5
10
AZAC μM

FIGURE FOURTEEN

HAT^+ colonies induced by treatment of EC cells lines with 5-AC. Cells were exposed for 24 hr to various concentrations of 5-AC before removal of the drug and cultured for an additional 24 hr. Cells were harvested and known numbers of cells were plated in media with and without HAT. a. C86S1A1(XX). b. C100AG1(XX). c. (●——●) C86S1A1(XO) clone 1, (○----○) C86S1A1(XO) clone 2. d. (○----○) C145FAG11, (●——●) C145FA12.


a
b
c
d
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-4}
10^{-5}
10^{-6}
HAT+ COLONIES PER SURVIVING CELL
0
5
10
0
5
10
5-AZACYTIDINE (μM)

(C145fAG11 and C145fA12) with 5-AC, there was no increase in the HAT^+ frequency at any concentration tested (Figure 14d).

The spontaneous frequency of HAT^+ colonies in C86S1A1 and C100AG1 cells was high, in the order of 10^{-4}, suggesting that both populations already had a significant number of HAT^+ cells. I therefore recloned C86S1A1 by picking single cells into separate culture vessels and growing them up to approximately $2x10^6$ cells before exposing them to 5-AC. All three clones that I tested showed a high, spontaneous incidence of HAT^+ colonies between $5x10^{-4}$ and $1x10^{-5}$ and all showed dramatic increases in HAT^+ colonies when treated with 5uM 5-AC (Figure 15).

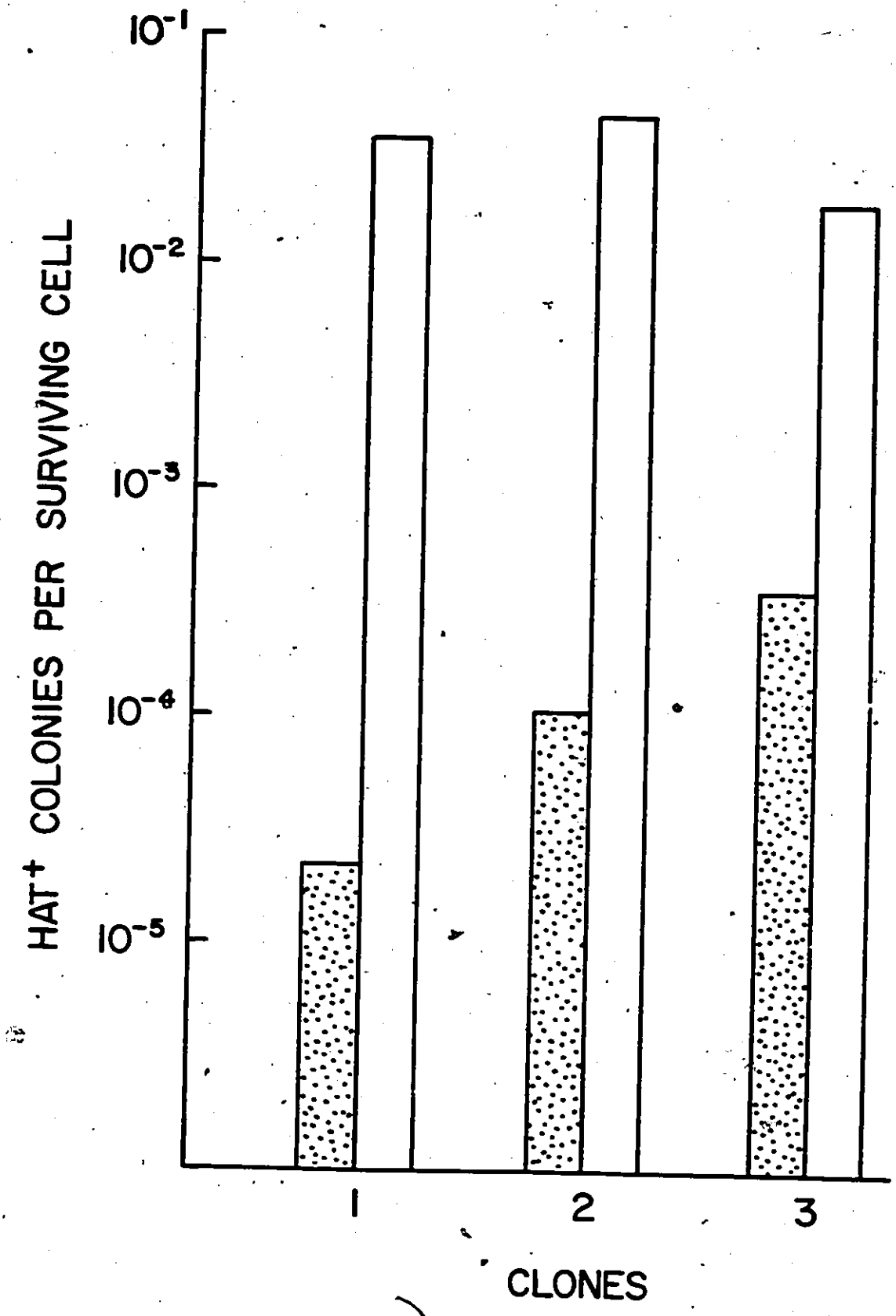
Plating efficiencies of the EC cell lines C86S1 and P19, both possessing wild-type HGPRT activities, were similar to C86S1A1 (data not shown). Thus, $HGPRT^+$ cells do not have a selective advantage for survival after 5-AC treatment.

The high frequency of HAT^+ colonies suggests that 5-AC is not inducing mutational events. However, I tested the ability of 5-AC to act as a mutagen by selecting for the forward mutation to the $8\text{-}AG^r$ phenotype in C86S1 and P19 cells. No colonies were recovered when $3x10^6$ cells were selected in 10ug/ml 8-azaguanine or 5ug/ml 6-thioguanine. Therefore, 5-AC does not appear to be a potent mutagen in this system.

The incorporation of 5-AC into DNA induces demethylation of the DNA. This has been documented in may

FIGURE FIFTEEN

Spontaneous and 5-AC-induced HAT^+ colonies in clones of C86S1A1(XX) EC cells. Single cells of C86S1A1(XX) were isolated and grown up to approximately 2×10^6. One half of the population was exposed to 5uM 5-AC for 24 hr and allowed to recover for an additional 24 hr. The untreated population was cultured in the same way in the absence of 5-AC. Known numbers of treated (stippled bars) and untreated cells (open bars) were plated into media with or without HAT.


10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
HAT+ COLONIES PER SURVIVING CELL
1
2
3
CLONES

systems (Jones and Taylor, 1980; Christman et al., 1983; Creusot and Christman, 1982). C86S1A1 cells were exposed to 5-AC for 24 hr. and the DNA was extracted from populations of cells at various times after treatment. The DNA was digested with MspI and HpaII restiction endonucleases and analysed by Southern blotting for differences in methylation at CCGG sequences. The blots were probed with cloned DNA sequences of Major Satelite DNA and Major Interspersed Fragment DNA (MIF). Major Satelite is a highly repetitive sequence associated with mouse heterochromatin and MIF is a moderately repetitive sequence found interspersed throughout the mouse genome (Chapman et al., 1983).

The treatment with 5-AC induced significant demethylation at CCGG recognition sites in both of these sequences, by the first day after treatment. Populations of cells at day 14 after treatment appeared to have remethylated these sequences to variable extents. There was little difference in the methylation pattern of these sequences in 5-AC reactivated clones (AZA clones) and untreated C86S1A1 cells (Janet Sanford, personal communication)

Thus, the treatment of C86S1A1 and C100AG1 EC cells with 5-AC induces the appearance of HAT^+ cells at high frequencies. 5-AC does not induce HAT^+ cells in populations of C86S1A1X0 cells. This strongly suggests that 5-AC is reactivating the *hgprt*$^+$ gene on the Xi.

The inability of 5-AC to induce reactivation in

C145FAG11 and C145FA12 suggests that they differ in the states of their X chromosomes such that 5-AC has no effect.

Characteristics of 5-AC Reactivated Clones

Morphology

All HAT$^+$clones of C86S1A1 and C100AG1 were judged to be EC cells by morphological criteria, presence of the EC specific SSEA-1 antigen and their ability to differentiate in vitro. Aggregation of these cells under conditions conducive to differentiation resulted in the appearance of a spectrum of cell types, including extraembryonic endoderm, neurons and cardiac muscle (data not shown). These cells also differentiated as monolayers in the presence of $1x10^{-7}$M RA in a manner identical with their C86S1A1 parent. The cells acquired a fibroblastic morphology and rapidly lost the cell surface antigen recognized by the monoclonal antibody AEC3A1-9 (Harris et al., 1984; Jones-Villeneuve et al., 1982)(Figure 16a and b).

Enzyme Levels

I have measured the specific activities of G6PD, PGK and alpha-GAL in reactivated clones of C86S1A1 cells. The results presented in Table 5, demonstrate that the specific activities of G6PD, PGK and alpha-GAL in five reactivated clones were not twice those found in C86S1A1. clones. HGPRT specific activities, however, were equal 3-10 higher than those found in C86S1A1.

Comparisons of Southern blots of DNA from C86S1A1, C86S1A1XO and reactivated clones revealed that the

FIGURE SIXTEEN

Percentage of cells carrying cell surface antigens reactive with SSEA-1 antibodies. Cells were induced to differentiate by continuous exposure to 1×10^{-7}M retinoic acid. Cells were processed for SSEA-1 immunofluorescence as described in the Methods and Materials at daily intervals. a. C86S1A1(XX). b. C86S1A1AZA1B(XX).

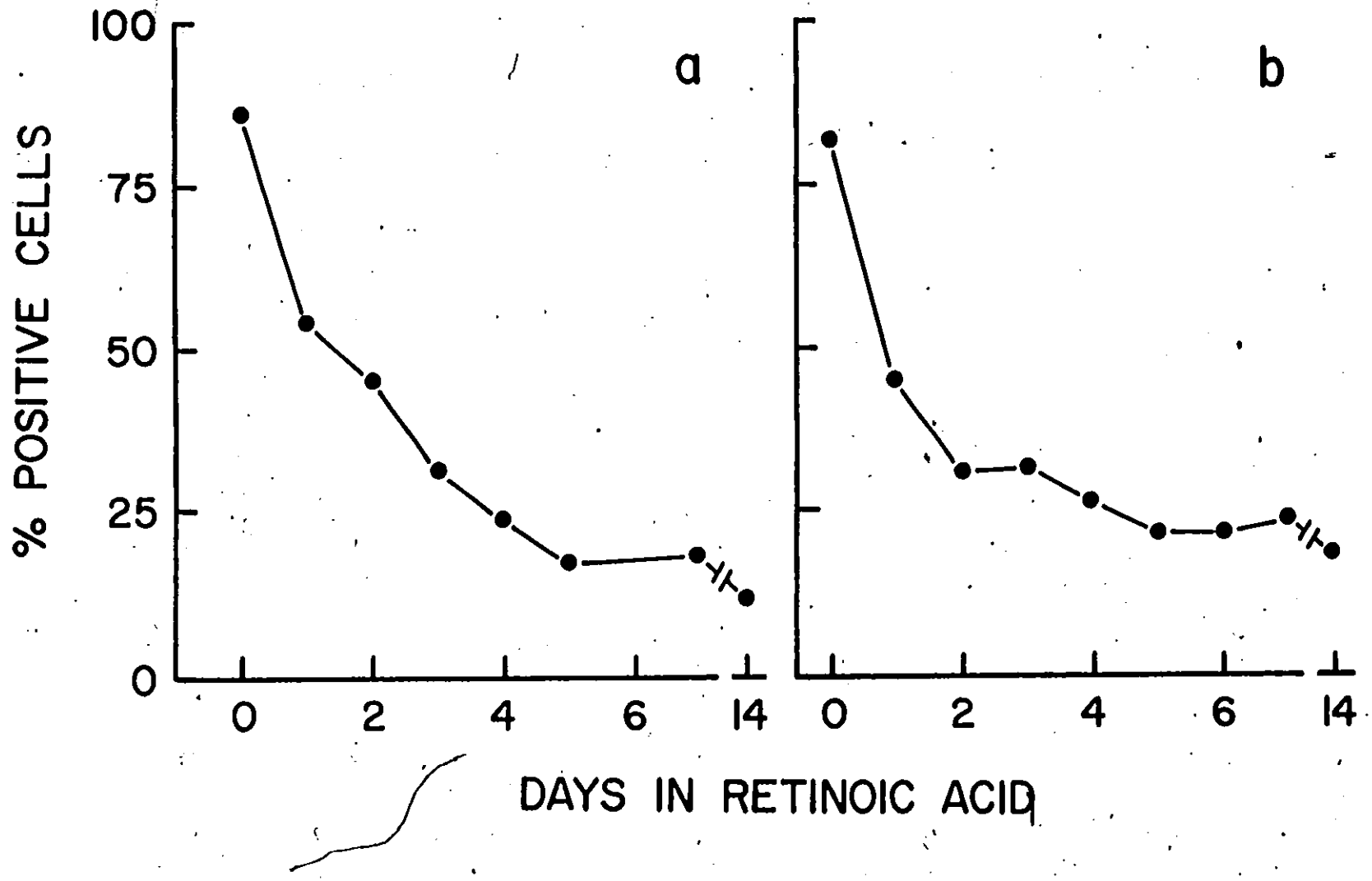

100
75
50
25
0
% POSITIVE CELLS
a
b
0
2
4
6
14
0
2
4
6
14
DAYS IN RETINOIC ACID

TABLE FIVE

SPECIFIC ACTIVITES OF SOME X ENCODED ENZYMES IN C86S1A1 CLONES

Cell Line	HGPRT	G6PD	PGK	alpha-GAL	APRT	6PGD
C86S1	1.6±.3	40±4	330±70	0.7±.1	0.9±.1	32±5
C86S1A1	0.2±.1	33±9	540±30	0.6±.1	1.2±.1	25±2
AZA0A	1.2±.1	24±3	360±80	0.1±.1	0.6±.1	15±1
AZA0B	1.0±.1	15±2	500±100	0.3±.1	-	-
AZA1A	0.8±.2	16±2	630±80	-	-	-
AZA1B	1.5±.2	22±2	380±60	0.3±.1	0.9±.2	19±2
AZA5A	2.0±.2	17±4	430±50	0.2±.1	-	-

Specific activities are expressed in nmoles of substrate converted per minute per mg. protein. The autosomal enzymes 6PGD and APRT are functionally related to the X encoded enzymes G6PD and HGPRT respectively. They were assayed to control for changes due to differences in cell metabolism.

reactivated clones possessed two copies of the hgprt gene (C. Adra per. comm.).

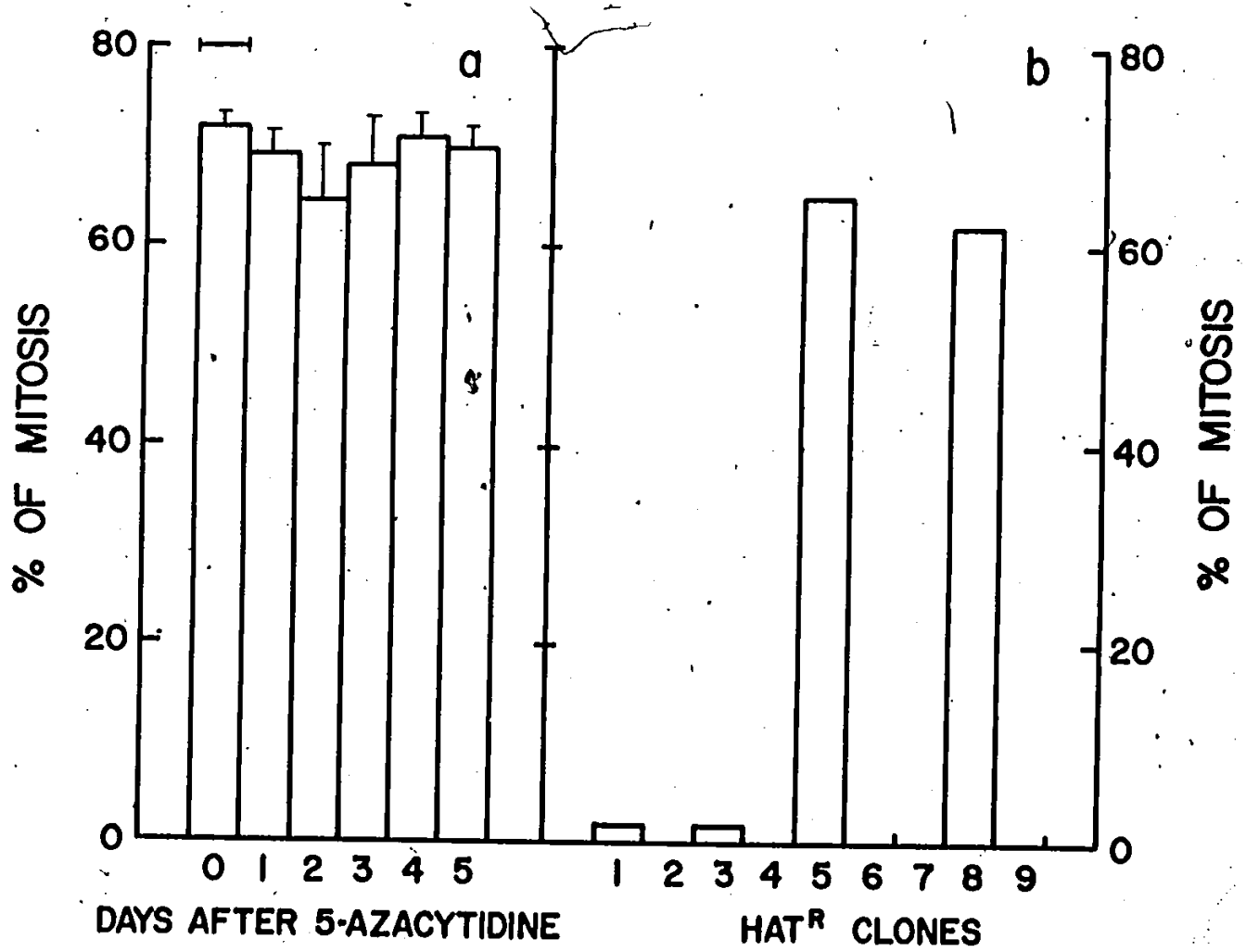
Chromosome Replication Patterns

Like most heterochromatic material, the Xi replicates its DNA entirely during the late "S" phase of the cell cycle (Morishima, et al., 1972; Lyon, 1968; Ray et al., 1972). To investigate further the genetic activities of the X chromosomes in cells treated with 5-AC, I analyzed the X chromosome replication patterns of these cells using a BUdR incorporation procedure (Alves and Jonassen, 1978; McBurney and Strutt, 1980). Previously, it was shown that C86S1 and C145F cells possessed a late replicating X chromosome, whereas C100 did not (McBurney and Adamson, 1976). The results of McBurney and Adamson (1976) were obtained using the ^{3}H-thymidine incorporation procedure of Nesbitt and Gartler (1970). I have confirmed these observations using the BUdR incorporation procedure. Under standard conditions 72% (81/114) of informative metaphase spreads of C86S1A1 cells and 81% (47/58) of C145fAG11 cells contained an identifiable late replicating X chromosome. No late replicating X chromosome was observed in any informative spread of C100AG1 cells (0/47).

After 5-AC treatment, I observed only a slight, transient decrease in the proportion of cells containing a late replicating X chromosome (Figure 17a). However, when I scored C86S1A1AZA clones, these cells demonstrated different X chromosome replication patterns (Figure 17b). In the

FIGURE SEVENTEENN

X chromosome replication patterns in C86S1A1(XX) cells exposed to 5-AC. a. The percentage of mitoses possessing a late replicating X chromosome were identified in populations of cells at daily intervals after exposure to 5uM 5-AC for 24 hr (⊢——⊣). b. Percentage of mitoses containing a late replicating X chromosome in C86S1A1AZA clones. Chromosome spreads were made at daily intervals after BrdU exposure as described in the Methods and Materials. Between 30-60 metaphase spreads were scored for each time point in two experiments.


a
b
80
60
40
20
0
% OF MITOSIS
0 1 2 3 4 5
DAYS AFTER 5-AZACYTIDINE
1 2 3 4 5 6 7 8 9
HAT^R CLONES

majority of clones, the previously late replicating X chromosome in C86S1A1 (Figure 18a) now replicated isocyclically with the Xa early in "S" phase (Figure 18b). G banding revealed that these clones still contained two X chromosomes (Figure 18c). Only two clones still possessed late replicating X chromosomes; however, these clones possessed an X chromosome which appeared to replicate earlier in "S" phase than the parent C86S1A1 cell.

Stability of HGPRT Reactivation

To assess the stability of the HGPRT$^+$ phenotype in the absence of selection, I grew C86S1A1AZA clones in the absence of HAT medium for various times and then assessed their ability to grow in medium containing 10 ug/ml 8-AG. There was a steady increase in the number of cells able to form colonies in this medium, such that at day 22 (44 generations) 4% of the cells could form colonies. Of the nineteen clones I examined, all possessed a single X chromosome (data not shown). This suggests that the increase in the number of 8-AG^r cells is not due to a high frequency reinactivation of the hgprt$^+$ gene but rather a loss of the entire X chromosome carrying this gene.

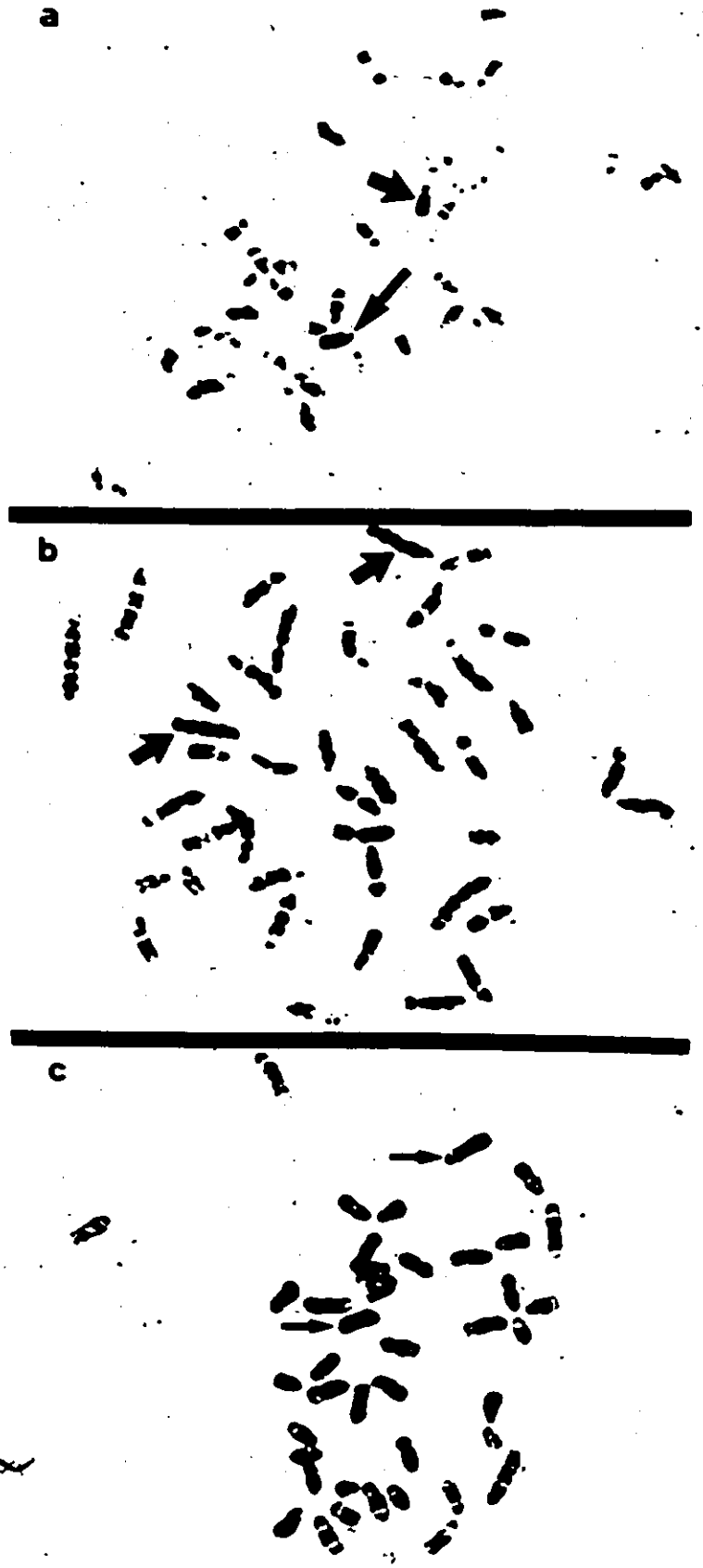
Kinetics of the Reactivation Event at the hgprt Locus

I wished to know how quickly the reactivation event occurred at the hgprt locus in C86S1A1 and C100AG1 cells and secondly, whether it occurred in all the cells or just a subpopulation of competant cells.

Measurements of the changes in HGPRT specific activity

FIGURE EIGHTEEN

X chromsome replication patterns in C86S1A1(XX) and C86S1A1AZA(XX) EC cells. a. C86S1A1(XX) before 5-AC treatment. The inactive X (thin arrow) stains uniformly dark indicating that it is replicated entirely during the late "S" phase. The active X (thick arrow) shows a characteristic banded appearance. b. C86S1A1AZA1B reactivated clone. Both X chromsomes (thin arrows) replicate isocyclically primarily during the early "S" period. c. G banded karyotype of C86S1A1AZA1B demonstrating the presence of two X chromosomes (arrows).


a
b
c

in C86S1A1 cells after 5-AC treatment, in the absence of HAT selection, are shown in Figure 19a. There was an increase in HGPRT specific activity beginning 1 day after the addition of 5-AC, which reached a maximum on day 3 after which the activity declined. The pattern of specific activity changes was identical for C100AG1 cells (Figure 19b).

There was little change in HGPRT specific activity in C86S1A1XO (Figure 19c), C145fAG11 or C145fA12 (Figure 19d) cell lines at any time after 5-AC treatment.

Figure (20a-f) shows the rate of ^{3}H-hypoxanthine incorporation into macromolecules in C86S1A1 cells after 5-AC treatment. The pattern of ^{3}H-hypoxanthine incorporation was similar to that observed for HGPRT specific activity. The majority of the cells showed increases in incorporated ^{3}H-hypoxanthine beginning 1 day after 5-AC treatment and reaching a maximum at 2 days. Approximately 75-80% of the cells at day 2, possessed grain counts within the range determined for the parent C86S1 cell possessing wild-type HGPRT activities. Beginning at day 3, the majority of the cells had reduced grain counts till by day five, the majority of the cells possessed levels of ^{3}H-hypoxanthine incorporation present in the cells before 5-AC treatment (Figure 21). Approximately 15-20% of the cells at day five retained ^{3}H-hypoxanthine incorporation levels, comparable or higher than, C86S1 possessing wild-type HGPRT activities.

When C100AG1 cells were treated under the same conditions they responded in the same manner as C86S1A1

FIGURE NINETEEN

Changes in HGPRT specific activities in EC cell lines exposed to 5-AC. Cells were exposed to 5uM 5-AC for 24 hr (|——|) and cells were harvested for HGPRT determinations at daily intervals. These results are the average of 3 or 4 independent experiments and the vertical bars indicate the standard deviation. a. C86S1A1(XX). b. C100AG1(XX). c. C86S1A1(X0). d. (O———O) C145FA12(XX), (●———●) C145FAG11(XX).

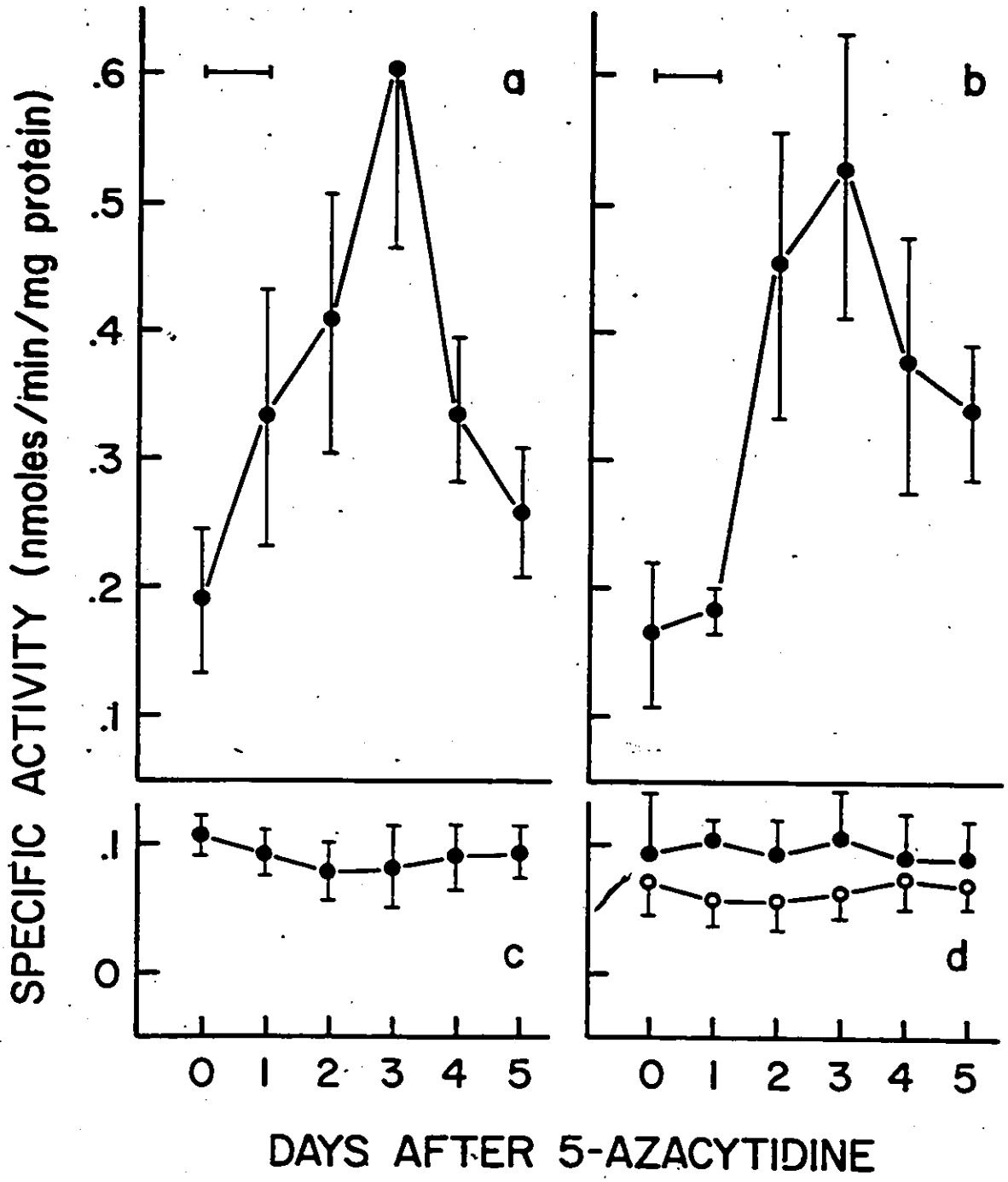

SPECIFIC ACTIVITY (nmoles/min/mg protein)
.6
.5
.4
.3
.2
.1
a
b
.1
0
c
d
0 1 2 3 4 5
0 1 2 3 4 5
DAYS AFTER 5-AZACYTIDINE

FIGURE TWENTY

Changes in ^{3}H-hypoxanthine incorporation into EC cell lines after exposure to 5-AC. Cells were exposed to 5uM 5-AC for 24 hr and cells were processed for autoradiography as described in the Method and Materials at daily intervals. Autoradiographic grain were counted over individual cells in randomly selected fields. At least 500 cells were counted for each time point. a. C86S1 ($HGPRT^{+}$) not treated with 5-AC(stipled bars), C86S1A1 ($HGPRT^{-}$) not treated with 5-AC (open bars). b. C86S1A1 1 day after 5-AC addition. c. day 2. d. day 3. e. day 4. f. day 5. g. C86S1A1(XO) 2 days after 5-AC addition. h. C145FAG11 2 days after 5-AC addition.

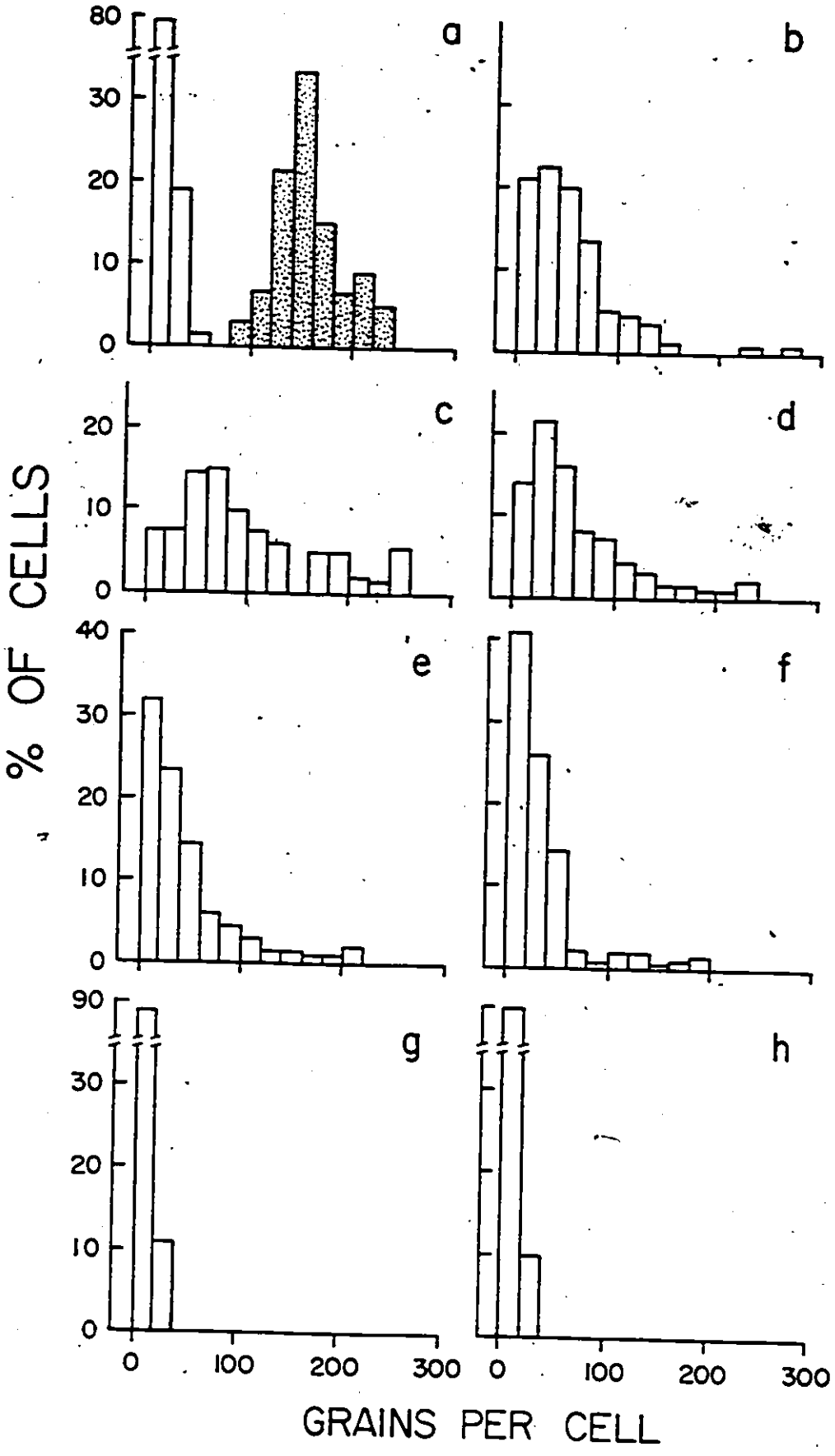
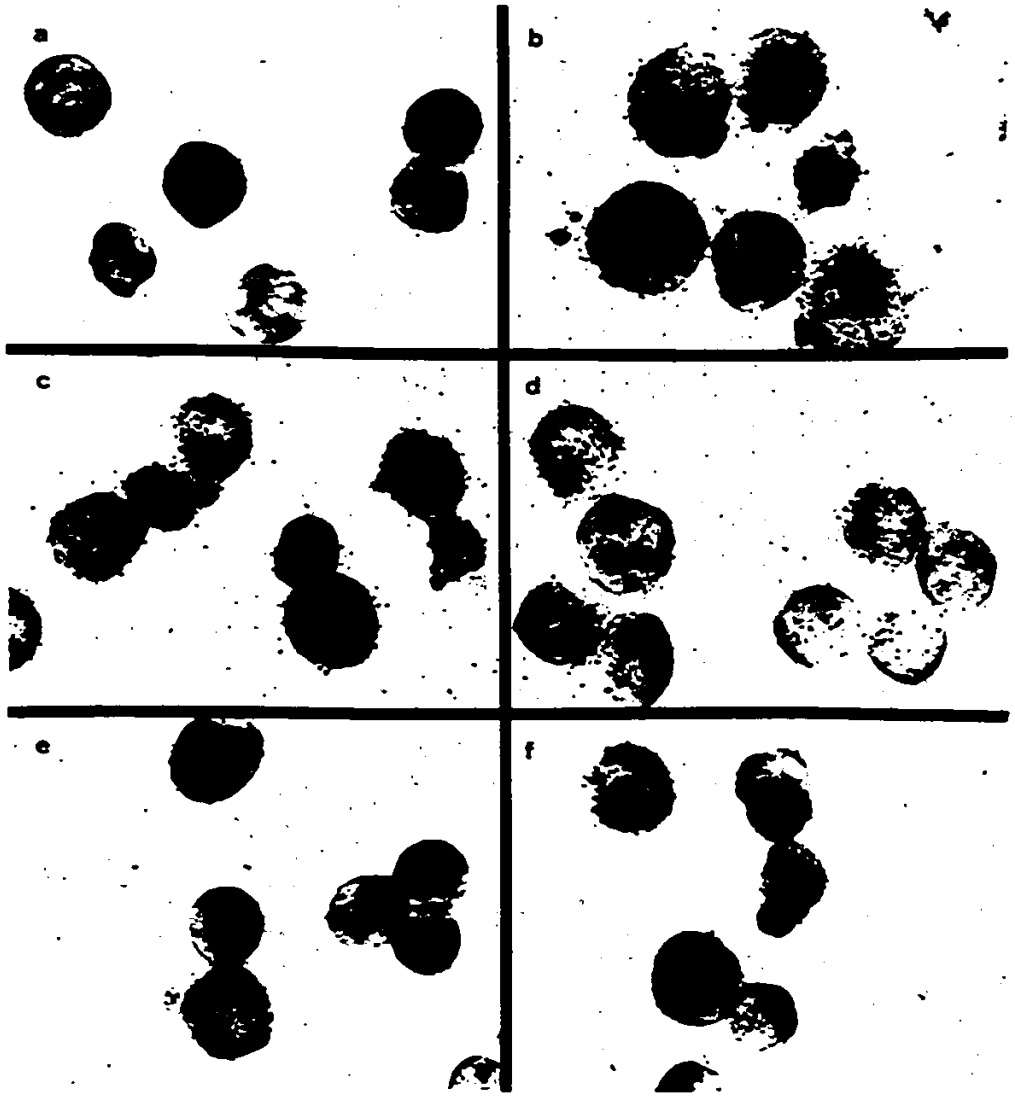

a
b
c
d
e
f
g
h
80
30
20
10
0
20
10
0
40
30
20
10
0
90
30
20
10
0
0
100
200
300
0
100
200
300
% OF CELLS
GRAINS PER CELL

FIGURE TWENTY-ONE

Bright field micrographs of ^{3}H-hypoxanthine incorporated in C86S1A1(XX) EC cells treated with 5-AC. Cells were exposed to 5uM 5-AC and processed for autoradiography as described. a. C86S1A1(XX) untreated. b. C86S1A1(XX) Day 1 after 5-AC addition. c. Day 2. d. Day 3. e. Day 4. f. Day 5. (X3200)


a
b
c
d
e
f

cells (data not shown).

In contrast, when I measured ^{3}H-hypoxanthine into C86S1A1(XO) cells or C145fAG11 cells, there was no increase in grain counts at any time after 5-AC treatment. Figure (20g and h) show the grain counts for these two cell lines on day 2.

Thus, the treatment of C86S1A1 and C100AG1 with 5-AC induced a rapid increase in HGPRT specific activities in almost all the cells, beginning 1 day after exposure to 5-AC and reaching a maximum between day 2 and day 3, when approximately 80% of the cells have increased HGPRT levels. The reduction of HGPRT activities observed between day 3 and day 4 may represent cells which have transiently increased their activities. Alternatively, it may be due to a reduction in the number of healthy, viable cells, due to 5-AC toxicity. The 10-20% of the cells which retain elevated HGPRT activities is in close agreement with the proportion of viable cells able to survive in HAT medium after 5-AC treatment.

Reactivation of Other X-Linked Loci

The measurements of specific activities of G6PD, PGK and alpha-GAL in C86S1A1AZA clones did not demonstrate elevated enzyme levels consistent with the expression of two active copies of their respective genes, despite the presence of two isocyclically replicating X chromosomes. My previous kinetic experiment with HGPRT demonstrated that the majority of C86S1A1 and C100AG1 cells only transiently

increased their activities in response to 5-AC. Thus, I wished to determine if other X-encoded enzymes also showed these transient increases in activity.

In the absence of heterozygous markers for structural genes on the X chromosomes in any of these cell lines, I have measured the specific activities of G6PD, PGK and alpha-GAL at intervals of time after 5-AC treatment.

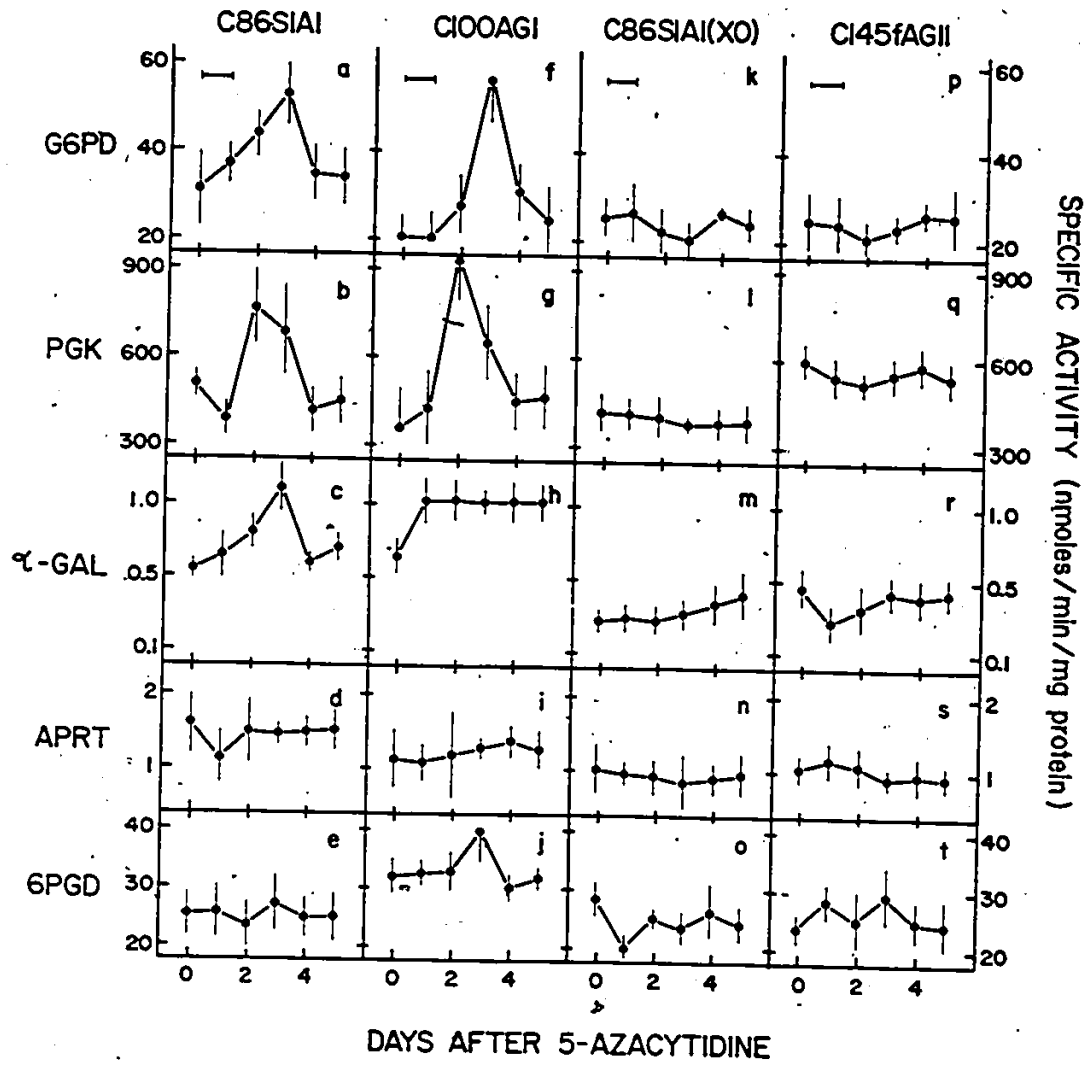
All three enzymes showed increases in specific activity in 5-AC treated C86S1A1 cells as was observed for HGPRT (Figure 22 a-c). G6PD and alpha-GAL increased in activity beginning on day 1, reaching a maximum on day 3 and a reduction on day 4. The specific activity of PGK however, peaked on day 2 and declined beginning on day 3. There was little change in the activities of APRT (Figure 22d) and 6PGD (Figure 22e) during this time, suggesting that these changes may be specific to the X chromosome encoded genes.

Increases in G6PD (Figure 22f) and PGK (Figure 22g) were observed in 5-AC treated C100AG1 cells, after which the activities declined in a manner similar to the activities in C86S1A1 cells. However, alpha-GAL activities (Figure 22h) increased to a maximum on day 1 and remained at this level of activity.

There was no significant change in the specific activities of G6PD, PGK, alpha-GAL or the autosomally encoded enzymes APRT and 6PGD in C86S1A1X0 cells (Figure 22k-o) or C145fAG11 cells (Figure 22p-t) at any time after 5-AC treatment.

FIGURE TWENTY-TWO

Changes in specific activities of some X encoded and autosomal enzymes in EC cell line exposed to 5-AC. Cells were exposed to 5uM of the drug for 24 hr (⊢—⊣) and cells were harvested at daily intervals for enzyme determination. The cells lines are indicated at the top of the figure and the enzymes along the side of the figure. These results are the average of 3 or 4 independent experiments.


C86SIAI
C100AGI
C86SIAI(XO)
C145fAGII
G6PD
PGK
α-GAL
APRT
6PGD
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
60
40
20
900
600
300
1.0
0.5
0.1
2
1
40
30
20
0
2
4
SPECIFIC ACTIVITY (nmoles/min/mg protein)
DAYS AFTER 5-AZACYTIDINE

The transient increases in specific activity of all three X encoded enzymes, in C86S1A1 and C100AG1, suggests that these syntenic loci on the Xi have been reactivated along with hgprt. Thus, the reactivation appears to involve the entire X chromosome and is not simply a local derepression at the hgprt locus. The magnitude of the transient increases in specific activity suggests that the reactivation occurs in the majority of the cells. However, C86S1A1AZA clones do not possess twice the specific activity that would be expected if both X chromosomes were active. This suggests that the stability of reactivation is an independent event at each locus in each cell.

Reactivation in Differentiated EC Cells

The reactivation frequency of the Xi is very high in our EC cells but it is a rare event in somatic cells (Migeon, 1982; Wolf and Migeon, 1982). We, therefore, determined if differentiated C86S1A1 and C100AG1 cells retained their ability to reactivate Xi genes after 5-AC treatment.

To induce cell differentiation, we continuously treated monolayers of these cells with $1x10^{-7}$M retinoic acid for 10-14 days (Strickland and Mahdavi, 1978; Strickland et al., 1980).

Unfortunately, these differentiated cells did not clone efficiently and I was unable to quantitate colony formation in HAT. I therefore investigated the ability of 5-AC to reactivate Xi genes by measuring the enzyme specific

activities, ^{3}H-hypoxanthine incorporation and changes in X chromosome replication patterns in these differentiated cells.

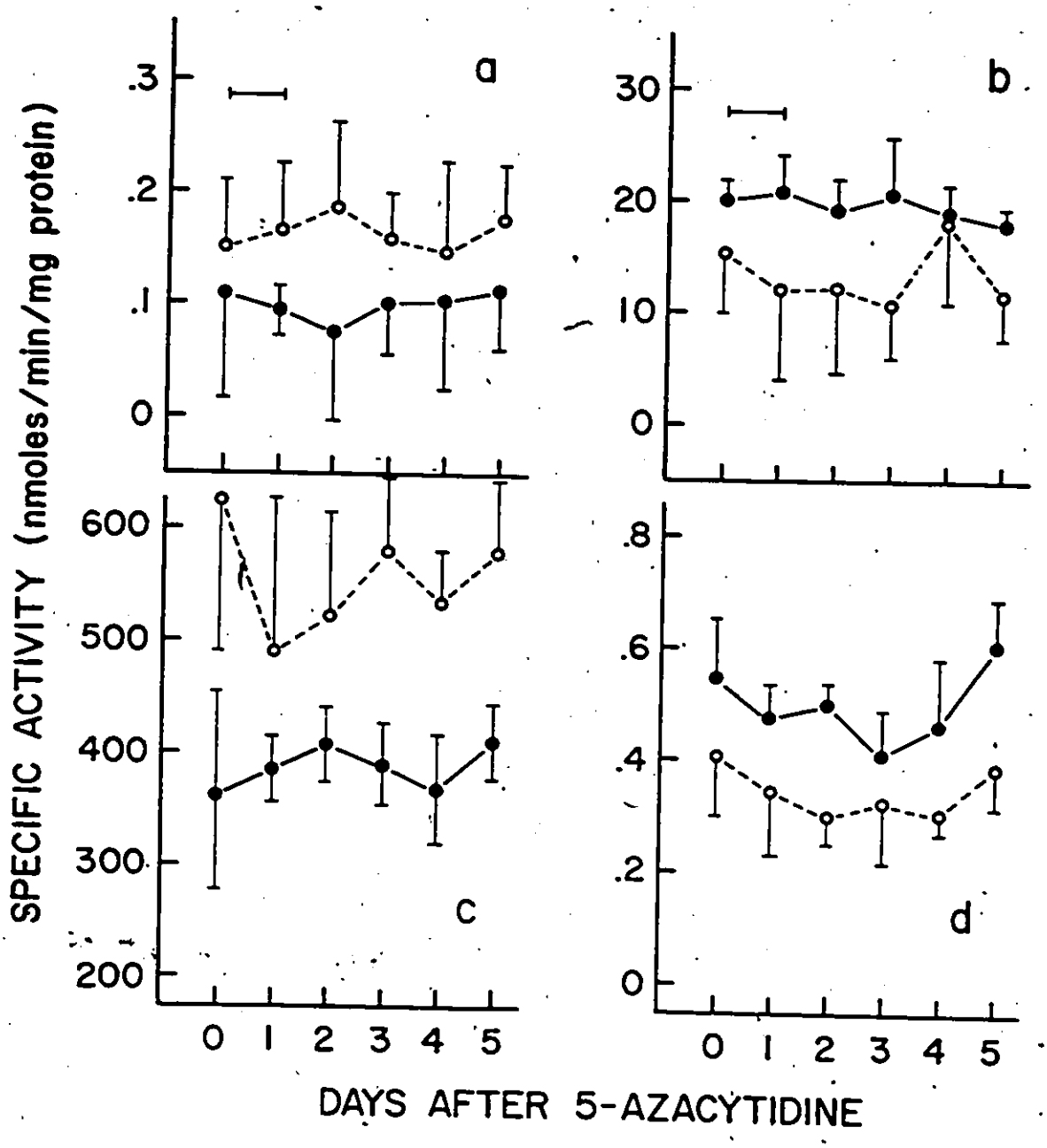
When I treated differentiated C86S1A1 or C100AG1 cells with 5-AC and monitored the HGPRT activity in time course experiments, I observed no change in activity either by ^{3}H-hypoxanthine incorporation into cells by autoradiography (data not shown) or by measurements of specific activities in vitro (Figure 23a). The specific activities of PGK (Figure 23b), G6PD (Figure 23c) and alpha-GAL (Figure 23d) also showed no change. Higher concentrations of 5-AC (up to 30 uM) were also not effective (data not shown).

Approximately 72% (51/70) of the differentiated cells derived from C86S1A1 possessed a late replicating X chromosome before 5-AC treatment and this proportion did not change at any time after 5-AC treatment (data not shown). Curiously, the induction of cell differentiation in C100AG1 did not result in the late replication of one X chromosome in these cells. This is in contrast to the induction of late replication accompanying differeniation in other EC cells possessing two isocyclically replicating X's (McBurney and Strutt, 1980; Takagi and Martin, 1984).

Autoradiography of ^{3}H-thymidine incorporation showed that 51% of differentiated C86S1A1 cells were replicating DNA in the presence (253/500) or absence (231/447) of 5-AC during the 24 hr treatment period. It is therefore unlikely that 5-AC is not effective because the majority of the cells

FIGURE TWENTY-THREE

Changes in the specific activities of some X encoded enzymes in differentiated cells after exposure to 5-AC. C86S1A1(XX) (●——●) and C100AG1 (○----○) were differentiated in the presence of 1 x 10^{-7} M retinoic acid for 14 days. Differentiated cells were then exposed to 5uM 5-AC for 24 hr (⊢——⊣) and cells were harvested for enzyme determinations at daily intervals. These results are the average of 2 or 3 independent experiments. a. HGPRT. b. G6PD. c. PGK. d. alpha-GAL.


a
b
c
d
.3
.2
.1
0
30
20
10
0
600
500
400
300
200
.8
.6
.4
.2
0
0 1 2 3 4 5
0 1 2 3 4 5
SPECIFIC ACTIVITY (nmoles/min/mg protein)
DAYS AFTER 5-AZACYTIDINE

are not cycling or because 5-AC inhibits DNA replication.

Thus, the differentiated progeny of C86S1A1 and C100AG1 EC cells apparently lost their ability to respond to 5-AC to induce the reactivation of genes on the Xi and induce early replication the Xi. In the case of C100AG1, the loss of the reponse to 5-AC with differentiation was not accompanied by late replication of an X chromosome. This suggests that late replication is not required for transcriptional inactivation.

State of the X Chromosome in C86S1A1AZA Clones after Differentiation

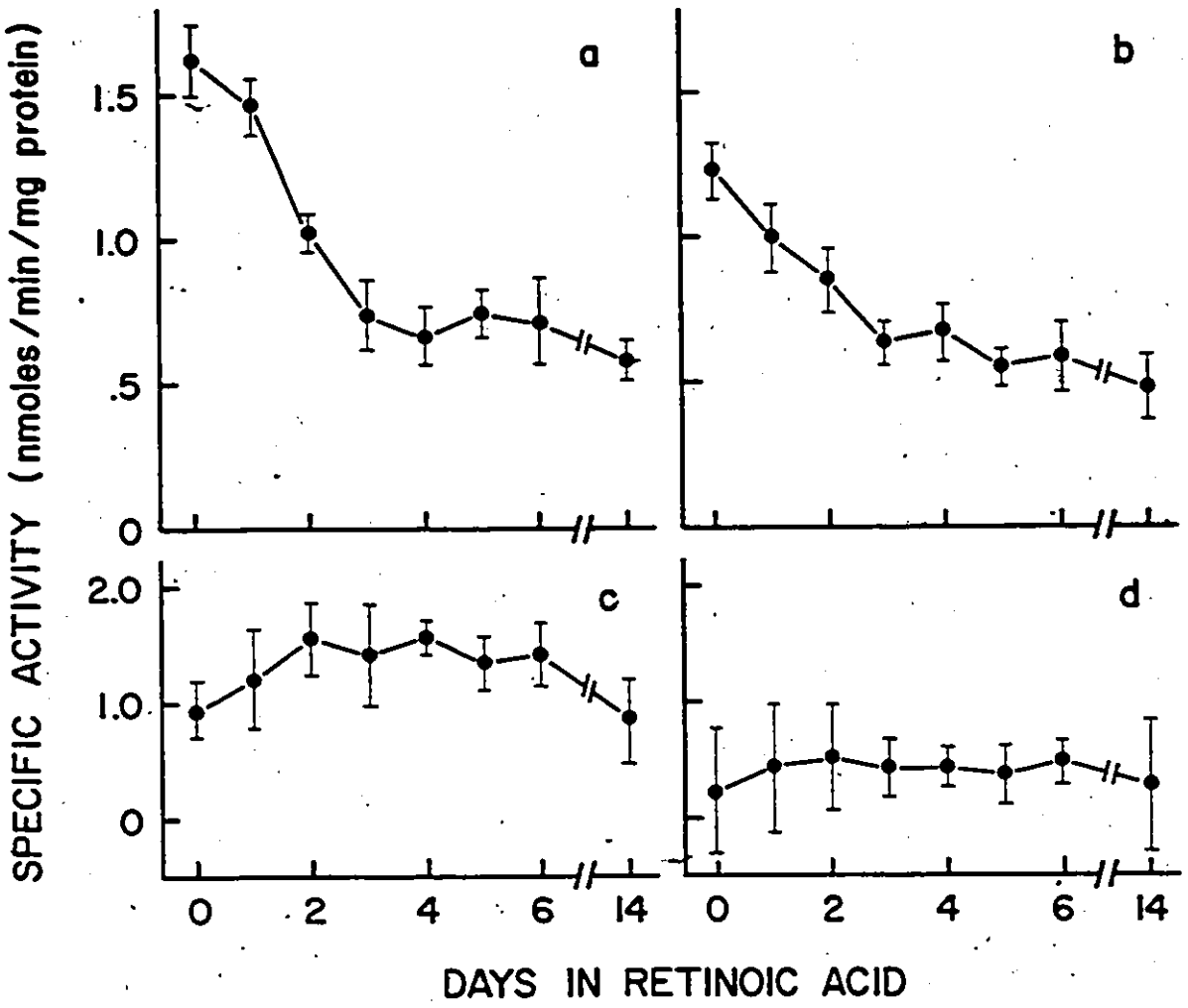
I have induced the differentiation of C86S1A1AZA lines in an attempt to induce reinactivation of an X chromosome. I wished to know if the reactivated clones would reinactivate an X chromosome after differentiation in retinoic acid (RA) and, if so, would they reinactivate the previously inactive X or demonstrate random inactivation.

No late replicating X chromosomes were observed in any informative metaphase spread of differentiated C86S1A1AZA clones at any time after induction of differentiation (data not shown).

Measurements of HGPRT specific activities during RA treatment of two clones of C86S1A1AZA revealed that the enzyme activities decreased by approximately 50% by day 4 and remained stable at this level for at least 10 more days (Figure 24a and b). Measurements of APRT showed no decrease in enzyme activity (Figure 24c and d). The specific

FIGURE TWENTY-FOUR

Changes in HGPRT and APRT specific activities during the differentiation of 5-AC reactivated clones of C86S1A1(XX) EC cells. Cells were induced to differentiate by exposure to 1 x 10^{-7}M retinoic acid. Cells were harvested at daily intervals for enzymes determinations. These results are the average of 3 independent experiments. a. HGPRT in C86S1A1AZA1B. b. HGPRT in C86S1A1AZA0A. c. APRT in C86S1A1AZA1B. d. APRT in C86S1A1 AZA0A.


a
b
c
d
1.5
1.0
.5
0
2.0
1.0
0
0
2
4
6
14
SPECIFIC ACTIVITY (nmoles/min/mg protein)
DAYS IN RETINOIC ACID

activities of other X-linked enzymes showed either no change or they increased over time (Figure 25).

Autoradiography of ^{3}H-hypoxanthine incorporation showed that 76% (391/500) of the cells retained lower, but significant levels, of ^{3}H-hypoxanthine incorporation up to 14 days after RA exposure (Figure 26a and b). The large proportion of HGPRT^{-} cells are probably dead cells since approximately 20% of the cells did not exclude trypan blue at the initiation of the experiments. These large numbers of dead cells probably resulted from the harsher methods employed to obtain a single cell suspension of differentiated cells.

Effects of Other Agents on HGPRT Reactivation

The results that were obtained by testing other agents to induce HGPRT expression confirm that DNA demethylation is the probable mode of action for Xi reactivation. I have assayed a number of compounds for their ability to reactivate the hgprt locus on the Xi in C86S1A1 cells. Because these compounds differ in their mode of action, metabolism in the cell and in their cytotoxicity, I have assayed the ability of these compounds to stimulate ^{3}H-hypoxanthine incorporation in a standard assay.

The cells were exposed to the agent for 24 hr after which time the medium was removed, the cells washed and incubated in normal medium for an additional 24 hr. At this time they were exposed to 2 uCi/ml ^{3}H-hypoxanthine for 2 hr A known number of cells were resuspended in 10% TCA,

FIGURE TWENTY-FIVE

Changes in specific activities of some X encoded enzymes during differentiation of 5-AC reactivated clones of C86S1A1(XX). a. G6PD in C86S1A1AZA1B. b. G6PD in C86S1A1AZA0A. c. PGK in C86S1A1AZA1B. d. PGK in C86S1A10A. e.alpha-GAL in C86S1A1AZA0A. f. alpha-GAL in C86S1A1AZA1B.

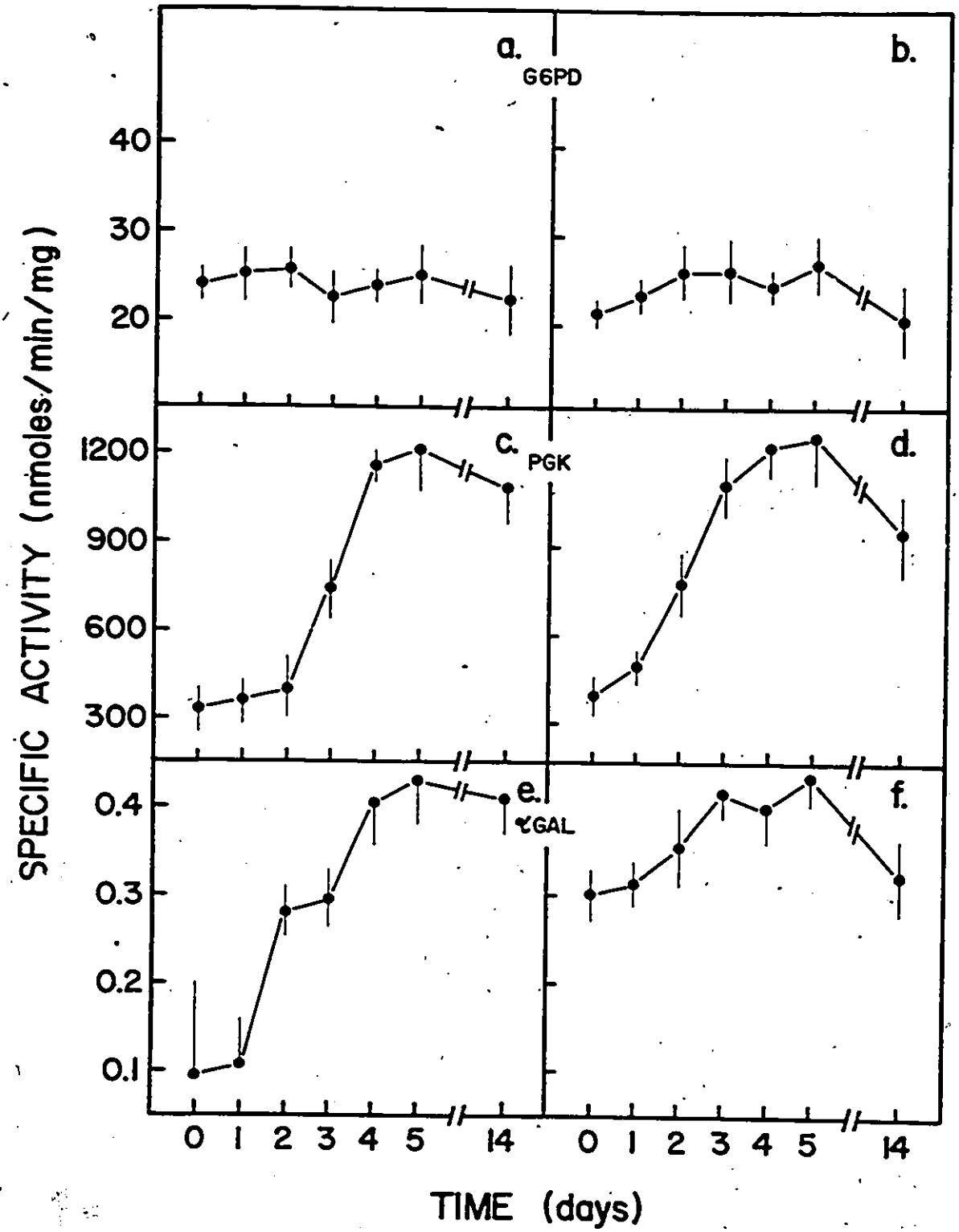
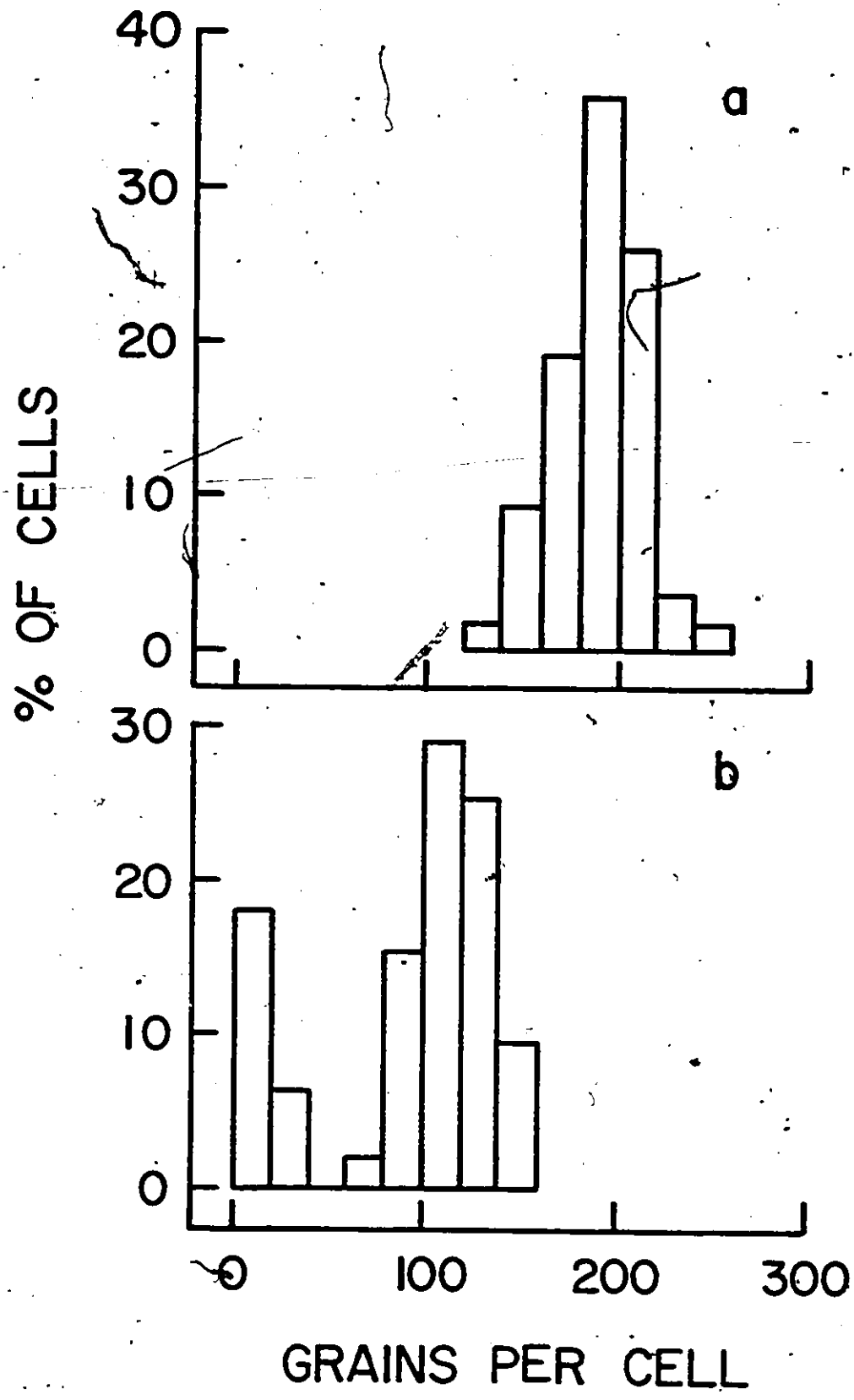

a.
G6PD
b.
40
30
20
c.
PGK
d.
1200
900
600
300
e.
αGAL
f.
0.4
0.3
0.2
0.1
0 1 2 3 4 5 14
0 1 2 3 4 5 14
SPECIFIC ACTIVITY (nmoles/min/mg)
TIME (days)

FIGURE TWENTY-SIX

Changes in ^{3}H-hypoxanthine incorporation after differentiation of 5-AC reactivated clones of C86S1A1(XX). a. undifferentiated C86S1A1AZA1B cells. b. C86S1A1AZA1B cells after 14 days exposure to 1×10^{-7}M retinoic acid.


40
30
20
10
0
a
% OF CELLS
30
20
10
0
b
0
100
200
300
GRAINS PER CELL

filtered and counted by liquid scintillation. The concentrations reported for each compound are the maximum used which still resulted in cell viability in colony assays. The results of these assays are reported in Table 6.

They demonstrate that 5-aza-2-deoxycytidine is as effective as 5-AC at one-tenth the concentration, probably reflecting the ability of this base analog to be incorporated directly into DNA (Jones et al., 1982). The inability of 2-deoxycytidine to stimulate ^{3}H-hypoxanthine incorporation demonstrates that the effect is not simply due to a pertubation of cytosine pools. Since cytidine apparently competes out 5-AC, this base analog probably works through incorporation from these pools. The presence of the nitrogen group in the 5' position is essential since 6-azacytidine has no effect. Although 5-bromo 2-deoyxcytidine apparently has no effect this may be due to to a difference in cell metabolism of this compound as has suggested for the inability of 5-fluoro-2-deoxycytidine to induce reactivation of hgprt in other systems (Jones et al., 1982). Bromodeoxyuridine does not stimulate ^{3}H-hypoxanthine incorporation, although it is known to affect DNA secondary structure in other systems (Lin and Riggs, 1972; 1976). It has also been shown to reactivate endogenous MuLV retroviruses in certain EC cell lines (Niwa et al., 1983) and EC-Friend cell hybrids at high frequencies (Asche, et al., 1984). Both DMSO and sodium butyrate have been shown to be possible demethylating agents

TABLE SIX

EFFECTS OF OTHER AGENTS ON HGPRT REACTIVATION IN C86S1A1 CELLS

Agent	CPM/10^5 Cells
Control	396+102
5-azacytidine (1uM)	642+67
" (5uM)	4324+475
" (10uM)	5287+527
5-aza-2-deoxycytidine (.1uM)	1822+672
" (.5uM)	4380+507
2-deoxycytidine (10uM)	452+108
" (100uM)	432+27
5-azacytidine (5um) + 2-deoxycytidine (20uM)	627+101
6-azacytidine (1uM)	482+57
" (20uM)	307+152
" (100uM)	342+77
5-bromo-2-deoxycytidine (20uM)	477+107
5-bromodeoxyuridine (1uM)	417+170
" (10uM)	322+127
" (100uM)	347+60
Dimethyl sulfoxide (100mM)	410+182
" (500mM)	467+103
Sodium Butyrate (100uM)	510+62
" (500uM)	232+121
s-adenosylhomocysteine (10.0uM)	547+62
" (1.0mM)	2721+547
" (5.0mM)	4262+273

(Lester, et al., 1982; Harris, 1982). They have been shown to induce HGPRT reactivation in mouse-human hybrids (Lester, et al., 1982) and the reversal of the TK^- phenotype in V79 Chinese hamster cells (Harris, 1982), but at a much lower frequencies than 5-AC. In both of these studies, these compounds were only effective after long exposure times. Thus the inability of these compounds to stimulate ^{3}H-hypoxanthine incorporation in my assay system may be a result of the short exposure time.

The treatment of cells with high concentrations of s-adenosyl homocysteine resulted in the incorporation of ^{3}H-hypoxanthine to levels equivalent to those observed with 5-AC. This compound has been shown to interfere with methyl transfers within the cell involving s-adenosyl methionine by product feed-back inhibition (Razin et al., 1984).

DISCUSSION

The Role of DNA Methylation in X Inactivation

Inactive X Genes

My results provide additional evidence for the role of DNA methylation as a primary mechanism for transcriptional suppression of genes on the Xi. Agents which are known to induce DNA methylation (5-azacytidine, 5-aza-2-deoxycytidine, s-adenosylhomocysteine) induce the expression of genes on the Xi in C86S1A1 and C100AG1 EC cells. The treatment of these cells with 5-AC results in a) the rapid and extensive demethylation of cellular DNA, b) the transient increases in the specific activities of X encoded enzymes and c) the stable reactivation of HGPRT expression in 10-20% of the surviving cells. 5-AC has no effect on C86S1A1(X0) cells; this strongly suggests that 5-AC reactivates the expression of genes on the Xi.

The kinetic data for the increases in HGPRT specific activity and ^{3}H-hypoxanthine incorporation demonstrate that the response to 5-AC in C86S1A1 and C100AG1 occurs rapidly (within two cell cycles) and that almost all the cells increase their levels of HGPRT. Measurements of the specific activities for other X encoded enzymes suggest that these syntenic loci are reactivated in the majority of the cells. Thus, the reactivation is not unique to the hgprt gene but probably involves the entire X chromosome. 5-AC did not have an effect on the specific activities of autosomally encoded enzymes, suggesting that the expression of these enzymes are

not regulated by a mechanism involving DNA methylation.

The reason for the transient response to 5-AC is not known, but the decreases in the X encoded specific activities may be due to a rapid remethylation of Xi DNA sequences. The changes in the X encoded enzyme activities are similar to the kinetics of global demethylation and remethylation of mouse erythroleukemia cell DNA after 5-AC treatment (Ley et al., 1984). Alternatively, this decrease may be due to the death of cells incorporating high levels of 5-AC and the overgrowth of cells which have incorporated little or no 5-AC during the treatment period and, therefore, do not reactivate Xi genes.

The 10-20% of C86S1A1 and C100AG1 cells which appear to retain increased levels of HGPRT is in close agreement with the proportion of viable cells able to survive in HAT medium after 5-AC treatment. The frequency of stable hgprt reactivation, after 5-AC treatment, are 10-1000 fold higher than was observed in somatic cell hybrids (Lester et al., 1982; Mohandas et al., 1981; Hors-Cayla et al., 1983) and Xi reactivation is not observed in diploid somatic cells (Wolf and Migeon, 1982). This suggests that DNA methylation may be the primary mechanism of X inactivation in EC cells and that there are additional mechanisms for X inactivation in somatic cells.

Cells which have been selected for hgprt expression are stable in the absence of selective pressure. However, C86S1A1AZA clones do not contain twice the specific activities of G6PD, PGK and alpha-GAL when compared to

C86S1A1 cells. This demonstrates that the reactivation of these loci is not stable and they are transcriptionally inactivated. This may also suggests that the stable expression of each Xi gene is an independent event in each cell. This may involve reinactivation of these loci, perhaps by DNA remethylation, and suggests that small regions on the Xi escape reinactivation to remain transcriptionally active. However, in the absence of heterozygous markers for these X encoded enzymes I cannot eliminate the possibility that these loci are reactivated and that the alleles on the Xa and Xi are one-half as active.

X Chromosome Replication Patterns

The treatment of C86S1A1 cells with 5-AC also induces a response at the level of the chromosome resulting in the isocyclic replication of both X's early in "S" phase of the cell cycle. The treatment of various other cells with 5-AC has been shown to induce the undercondensation of heterochromatin and alter the timing of DNA replication on all chromosomes (Schmid and Haaf, 1984; Viegas-Pequignot and Dutrillaux, 1981; Shafer and Priest, 1984). In addition, the concentration of 5-methyl cytosines is much greater in constituetive heterochromatin than in euchromatin, suggesting that methylation is involved in inducing or maintaining this chromatin state (Razin and Cedar, 1977).

However, I found that there was no absolute correlation between the reactivation of hgprt expression and the early replication pattern of the X chromosomes. Hors-Cayla et al.

(1983) also found no correlation between the time of replication and the expression or nonexpression of Xi genes on the human Xi in hamster-mouse somatic cell hybrids treated with 5-AC.

In the case of C100AG1, this cell already contains two early replicating X chromosomes and by this criteria, both X's are active. However, based upon the frequency of 8-AG^r cells and X encoded enzyme activities, McBurney and Adamson (1976) suggested that this cell may have a partially inactive X. The treatment of the HGPRT$^-$ mutant, C100AG1, with 5-AC resulted in hgprt reactivation at a frequency comparable to C86S1A1 cells. Although I have not been able to isolate the appropriate XO segregant from these cells, the data suggests that I am reactivating Xi genes. Thus; this X chromosome is transcriptionally suppressed for almost all gene loci but is not late replicating, suggesting that late replication is a late event in the genesis of an Xi.

Alternative Possibilities to X Chromosome Reactivation

Although my results can be positively correlated with 5-AC induced demethylation of the DNA and expression of the Xi, it is useful to eliminate other explanations for these results. These possibilities include:

i. Reversion mutation. 5-AC induces the high frequency reversion of the hgprt mutation on the Xa. This is probably not the case in my system, since C86S1A1(XO) cells, which retain the X chromosome bearing the mutant hgprt gene, do not revert. In addition, the frequency of reactivation that I

observe is very high for a mutational event and 5-AC is not a potent mutagen (Landolf and Jones, 1982).

ii. Gene amplification. 5-AC induces the amplification of the mutant gene, so that increased transcription of the mutant mRNA produces increased levels of the mutant enzyme. These increased levels of functionally inefficient HGPRT allow the cell to function normally. Reversions of the HGPRT$^-$ phenotype in some Chinese hamster fibroblasts has been shown to occur by amplification of the mutant gene (Fenwick et al., 1984). Southern blots of C86S1A1AZA clones suggest that there is no amplification of the hgprt genes. In addition, this amplification should also occur in C86S1A1(XO) cells with the same frequency.

iii. Loss and Reduplication. This phenomenon involves the loss of the X chromosome carrying the hgprt$^-$ gene and the reduplication of the Xi, presumably through mitotic nondisjunction. This phenomenon has been observed in the expression of the retinoblastoma gene (Cavenee et al., 1983). C86S1A1AZA clones segregated XO clones which were 8-AG^r, indicating that the chromosome bearing the hgprt$^-$ gene was not eliminated.

iv. Regulatory gene mutation. It is possible that the hgprt mutation in my cells does not involve the hgprt gene, but is in a regulatory gene which controls hgprt transcription. This regulatory gene may be activated by 5-AC and the gene may be on any chromosome and its product functional in trans. There is some evidence that a separate

regulatory gene for hgprt may exist (Kadouri et al., 1978). However, C86S1A1(XO) cells do not reactivate hgprt expression, making this possibility unlikely.

Xi Reactivation is Dependent upon the Undifferentiated State

The ability of 5-AC to reactivate Xi genes in C86S1A1 and C100AG1 cells is lost after they are induced to differentiate by exposure to RA. These data suggest that there is a change in the state of the inactive X which accompanies cellular differentiation and also suggest that there are different mechanisms which are responsible for controlling X inactivation in the undifferentiated and differentiated states. X inactivation accompanies cellular differentiation in the embryo (Monk and Harper, 1979; Monk, 1981) and when EC cell lines bearing two Xa are induced to differentiate (McBurney and Strutt, 1980; Martin et al., 1978; Takagi and Martin, 1984). These observations are consistent with changes in the state of the Xi being associated with cellular differentiation.

There are several examples where mechanisms controlling gene expression are dependent upon the differentiated state of the cell. Many workers have utilized viruses as probes of the state of genetic regulation in EC cells (Teich et al., 1977; Segel and Khoury, 1979; Niwa et al., 1983; Gautsch and Wilson, 1983; Stewart et al., 1982). In general retroviruses and papovaviruses can enter EC cells but are blocked from expression by a cellular control mechanism. However, both are

infective in differentiated cells. Stewart et al. (1982) found a positive correlation between the block of Moloney leukemia virus (MuLV) and the methylation of the infective virus in F9 EC cells. In addition, 5-AC could induce the expression of the virus in these cells after differentiation. However, Niwa et al. (1983) and Gautsch and Wilson (1983) found that the MuLV virus was not expressed in EC cells but that methylation of the viral genome occurred 8-10 days after viral infection. They also found that 5-AC did not induce viral gene expression in EC cells but could induce viral exression in these cells after differentiation. These data suggest that DNA methylation is an important regulator of gene activity but there are other mechanisms which can regulate virus expression in EC cells and in differentiated cells. Changes in these regulation mechanisms are dependent upon the state of cellular differentiation.

The inability of 5-AC to reactivate genes on the Xi in two other EC cell lines, C145FAG11 and C145FA12, suggests that these EC cells lack the mechanism to respond to 5-AC or that they differ in the states of inactivation of their X chromosomes. These cells are similar to C86S1A1 in the late replication pattern of an X chromosome, cell morphology, the presence of SSEA-1 surface antigens (unpublished results) and their capacity for pluripotent differentiation (McBurney, 1976). These cells may be more "advanced" in terms of the differentiated state of their Xi, suggesting a second mechanism for the maintenance of X inactivation which is

stable, irreversible, and occurs after transcriptional suppression of the Xi by DNA methylation. The expression of this second inactivation mechanism is probably linked to cellular commitment and cellular differentiation. Thus, I suggest that different EC cell lines represent embryonic cells arrested at different stages of X chromosome inactivation.

Differentiation does not Induce Reinactivation

The induction of differentiation of C86S1A1AZA clones resulted in the reduction of HGPRT specific activity by approximately 50%, while other X encoded enzymes did not change or had increased specific activities. The majority of the cells retained lower, but significant, levels of ^{3}H-hypoxanthine incorporation and did not contain a late replicating X chromosome. Since the differentiated C86S1A1AZA cells expressed similar HGPRT specific activities and ^{3}H-hypoxanthine incorporation at day 5 and day 14 after RA addition, this result is probably not due to a slow turnover rate of the HGPRT mRNA or protein after inactivation.

These data suggest some form of gene dosage regulation at the reactivated hgprt locus which occurs in the absence of late replication or transcriptioanl inactivation. The persistence of two isocyclically replicating X chromosomes is consistent with this interpretation. Alternatively, the reduction in HGPRT activities may be a differentiation phenomenon, possibly due to the decreased numbers and rates of cells undergoing DNA replication.

The activities of PGK, G6PD and alpha-GAL before and after differentiation suggest that these genes do not undergo inactivation or they are already transcriptionally inactive. My measurements of specific activities for these enzymes in C86S1A1AZA clones support the later explanation.

Thus, C86S1A1AZA clones may be equivalent of C100 cells (McBurney and Adamson, 1976) but in the former cells the hgprt locus is active and other loci are transcriptionally repressed while in the later the alpha gal locus is active.

The apparent absence of reinactivation of the $hgprt^+$ and the induction of late replication of this X chromosome in C86S1A1AZA cells after differentiation, is in contrast to the induction of transcriptional inactivation and late replication in other EC cell lines possessing two Xa (McBurney and Strutt, 1980; Takagi and Martin, 1984). This may suggest that the initiation of X inactivation is a sequential event involving the methylation of the X chromosome DNA and the second mechanism of inactivation which requires that the Xi is methylated. Demethylation of the Xi DNA by 5-AC in C86S1A1AZA cells may make this X incapable of being recognized by the second inactivation system.

Germ Cells or Embryonic Cells

My results are entirely consistent with the interpretation that EC cells are equivalent to germ cells at various stages of X reactivation, prior to their entry into meiosis. According to this interpretation, C100AG1 and C86S1A1 cells represent germ cells just prior to expression

of both X chromosomes and DNA demethylation allows the expression of genes on the Xi. C145FA12 and C145FAG11 are more primitive germ cells whose Xi are maintained by X inactivation mechanisms that are independent of DNA methylation and they are thus analogous to somatic cells.

Takagi et al. (1984) have been able to reactivate the inactive pgk-1 locus and induce the early replication of the Xi in mouse thymocytes, by fusing them with an X0 EC cell line. These results implicate some kind of trans acting "reactivation factor" that is present in EC cells and may be the same mechanism which reactivates the Xi during germ cell maturation. The reactivation of the Xi by fusion with some EC cell lines (Takagi et al., 1984) but not with others (Graves and Young, 1982) suggests that this reactivation factor is present or absent in different EC cell lines, depending upon their state of X chromosome differentiation. However, these reactivation factors may also be present in early embryo cells and may be involved in mechanisms which reactivate the heteropycnotic X^p in embryos shortly after fertilization.

Conclusions

I have shown that DNA methylation is a primary mechanism in mediating X ohromosome inactivation. The treatment of some female EC cell lines with the DNA demethylating agent 5-AC induces the high frequency reactivation of genes on the Xi and alterations in the replication patterns of the Xi. The ability to reactivate Xi genes was directly dependent upon the undifferentiated state of the cell. My data supports a

model where the inactive state of the X in undifferentiated cells is maintained by DNA methylation. An unknown, second mechanism replaces, or is present in addition to, DNA methylation and maintains the inactive state of the X chromosome in differentiated cells.

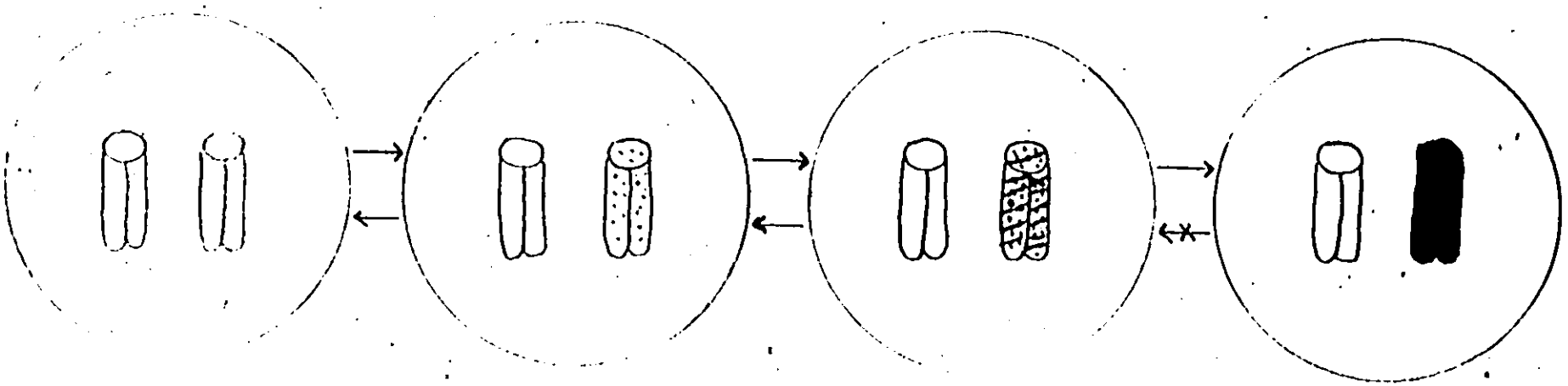
I also suggest that different female EC cell lines represent embryonic cells arrested at different stages of X chromosome inactivation. This is supported by the different stabilities of the inactive state in different EC cell lines and the different responses to 5-AC. The EC cell lines used in this study can be classified in a hierarchy of stages of inactivation (Figure 27). My results are entirely consistent with the interpretation that different EC cell lines are germ cells at different stages of X chromosome reactivation.

Regardless of the origin of of EC cells, they provide a system for the study of X chromosome inactivation <u>in vitro</u>. The availability of different EC cell lines, arrested at different stages X inactivation provides the potential for elucidating the molecular mechanism for X inactivation and will further our understanding the control of differential gene expression during development.

FIGURE TWENTY-SEVEN

X chromosome differentiation in lines of EC cell.?

X CHROMOSOME DIFFERENTIATION



-both X chromosomes transcriptionally active
-X chromosomes replicate in synchrony in early "S" phase
-e.g. P10

-Methylation of X chromosome DNA sequences leads to transcriptional suppression
-reversable by demethylation
-X chromosomes replicate in synchrony early in "S" phase
- e.g. C100

-transcriptional supression results in chromatin structural changes leading to heteochromatinization and late replication
-initially reversable by demethylation
-e.g. C8651A1

-Additional mechanisms replace or are present in addition to DNA methylation to maintain X inactivation
-not reversable by demethylation
- e.g. C145F and somatic cells

CHAPTER FIVE

A MODEL FOR X CHROMOSOME INACTIVATION

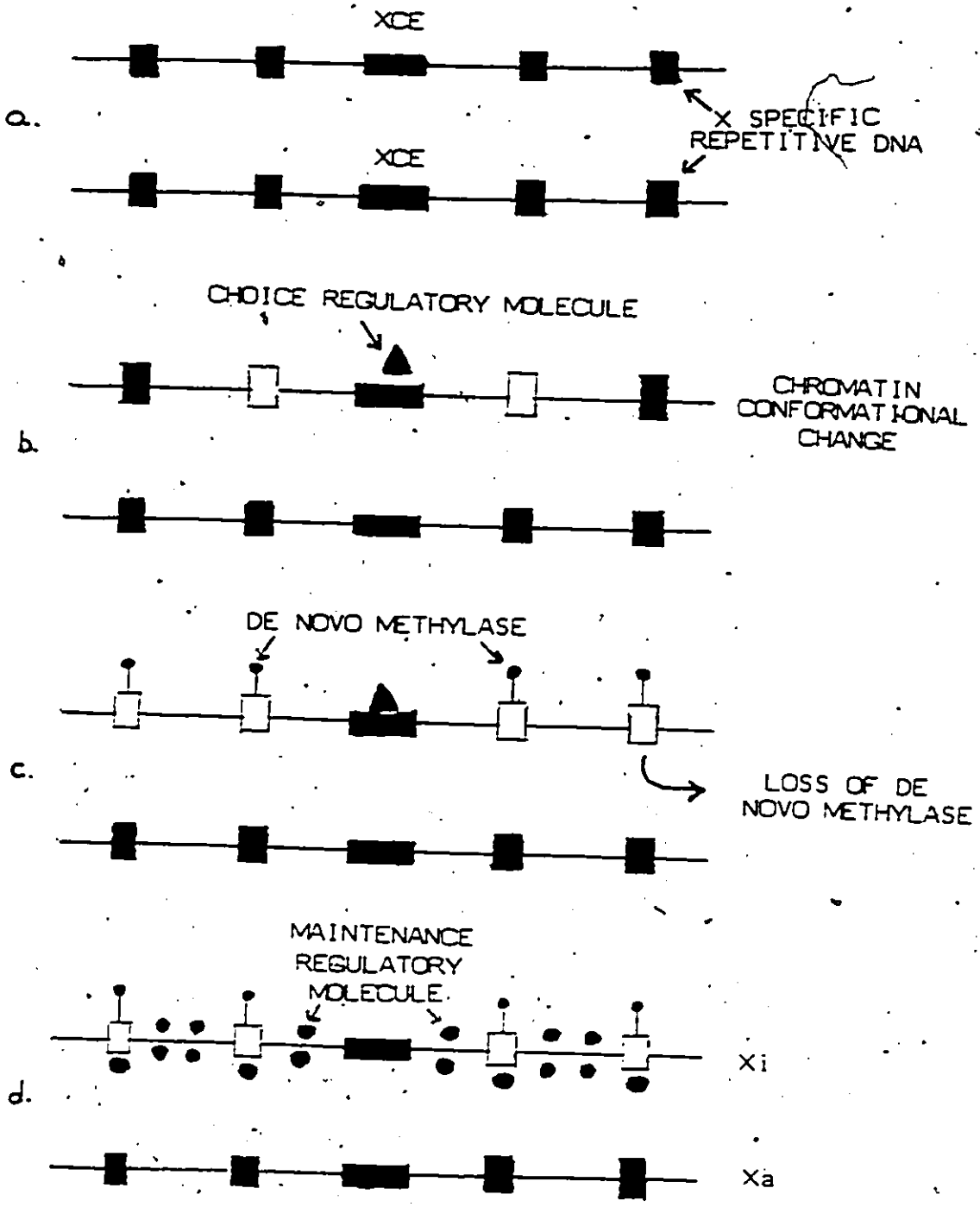
The primary objective of this project was to investigate some of the mechanisms which are responsible for controlling X chromosome inactivation. I have attempted, here, to construct a possible model for X inactivation (Figure 28) which incorporates the results from this study and what is known about X inactivation in other systems.

a) initially both X chromosomes are transcriptionally active. The X chromosomes contain DNA sequences which are important for the control and initiation of X inactivation (i.e. Xce locus). The X chromosome can contain a single or multiple sites for the control of inactivation. The X chromosome may also contains blocks of interspersed repetitive DNA sequences which are unique to the X chromosome. Repetitive sequences unique to the centromeric region of the human X chromosome have been described (Willard et al., 1983; Yang et al., 1983).

b) cells synthesize from autosomal genes, a limited number of "choice molecules" which bind specifically to the Xce locus. The characteristics of choice and X chromosome counting are difficult to study and my results do not address this issue directly: In a female cell, one X is active for each diploid set of autosomes. this observation is best explained by an activator model, where one X binds a specific molecule which keeps it active and all other X chromosomes are accosted by the inactivation mechanism. The

FIGURE TWENTY-EIGHT

A possible model for X chromosome inactivation. The details of model are described in the text.


XCE
a.
X SPECIFIC REPETITIVE DNA
XCE
CHOICE REGULATORY MOLECULE
b.
CHROMATIN CONFORMATIONAL CHANGE
DE NOVO METHYLASE
c.
LOSS OF DE NOVO METHYLASE
MAINTENANCE REGULATORY MOLECULE
d.
Xi
Xa

binding of of "choice molecules to the Xce site may involve, sequence specific binding proteins, a sequence specific de novo methylase or an integration of a site specific DNA insertion element. In addition, once one X is affected by the choice molecule there must be a rapid inactivation of other choice molecules to prevent binding to the second X chromosome. This "fast" inactivation step is difficult to comprehend especially since it probably involves autosomal genes. Several suggestions have included cooperative binding proteins, a limited number of choice molecules and an inducer-repressor titration system (Gartler and Riggs, 1984)

The binding of the "choice molecule" to the Xce induces a conformational change in the DNA or chromatin structure which is amplified and propagated along the X chromosome by the DNA repetitive sequences. Repetitive DNA elements have been shown to prpogate chromatin condensation signals in Drosophila (Tartof et al., 1984). Alternatively, this conformational change may involve a sequence specific methylase which methylates these sites and alters DNA secondary structure and chromatin structure by altering the binding of chromosomal proteins.

c) The conformational changes at repetitive sequences signal them to be methylated by a specific de novo methylase which stabilizes the conformational change and mediates transcriptional inactivation. The results from my study and from others have shown that demethylation of the DNA can

reactivate expression of Xi genes and alter the chromatin replication patterns of inactive X chromosome. Stable reactivation of specific genes may be due to the escape of local regions on the X from remethylation and reinactivation. There is also evidence that a de novo methylase exists in embryonic cells (Jahner et al., 1982)) and in EC cells (Stewart et al, 1982) at the time when X inactivation is known to occur.

d) further differentiation of the cell induces the synthesis of a second "regulatory" molecule which binds to the transcriptionally inactive X chromosome. This differentiation also suppresses the synthesis of the site specific de novo methylase. The binding of this molecule mediates additional changes in chromatin structure which result in stable and permanent inactivation and which are not dependent upon DNA methylation. This differentiation may also involve the loss of "reactivation factors" which may be present in the undifferentiated cells.

According to this model, different EC cell lines are at different stages in this inactivation scheme. P10 cells have two Xa. C100 cells have inactivated their X chromosome by methylation of their X sequences. C86S1A1 cells are further differentiated and have altered chromatin structure, as demonstrated by the presence of a late replicating X, but the inactivation is still mediated by DNA methylation. C145F cells have a fully inactivated X mediated by the second

inactivation mechanism which is independent of DNA methylation.

This model is not unique to X chromosome inactivation and may have an application in the control of other transcriptional regulation mechanisms. For example, it may provide a model for differential gene expression where cells synthesize regulatory molecules which bind to unique sequences at, or around, tissue specific genes. This binding induces a conformational change in the chromatin and signals this region to be demethylated to allow transcriptional expression.

Future Directions

There are a number of experiments for continuing the studies of this project and to test the above model.

These studies should include the monitoring of progressive changes in DNA methylation patterns at, and around, X encoded genes during the differentiation of P10 EC cells. This should also be done in the EC cell lines which differ in the inactivation status of their X chromosomes in an attempt to correlate changes in the methylation patterns with the state of inactivation of the X chromosome. By monitoring DNAase 1 sensitivities of X encoded genes in these cell lines before and after differentiation, it should be possible to determine whether or not chromatin conformational changes precede methylation or vice versa.

In this regard, the identification and characterization

of *de novo* methylases should be done. My results would predict that there are different methylases in different lines of EC cells, depending upon their state of inactivation.

Identification of DNA repetitive elements unique to the X chromosome should be isolated. It would be interesting to see if they are similar to the human repetitive elements described by Yang *et al* (1982). A homology between these sequences may indicate they are important in X inactivation.

It would be useful to investigate the methylation patterns of *hgprt* genes in different reactivated clones of C86S1A1 and C100AG1 to determine if there are critical sites of methylation that allow expression of the gene. Identification of such sites may be found in regulatory regions and may be found in similar regions in other X encoded genes. These common regulatory regions may be unique to the X chromosome and important in the regulation of X inactivation.

It would be useful to be able to identify and isolate the Xce^c locus from P10 cells in order to investigate the influence of this sequence on inactivation. Since the product of the Xce is not known, it maybe difficult to identify. However, it is tightly linked to the *pgk-1* locus, less than 0.5 centimorgans away. The *pgk-1* gene could serve as a starting point for a "chromosomal walk" into the region of the Xce gene. The Xce may be identified by cloning the

sequence into an expression vector containing the entire pgk-1a gene and transfecting it into X0 P10 cells containing only the pgk-1b gene. The expression of the pgk-1 genes before and after differentiation could be monitored to determine if the Xce had any influence on pgk-1 expression. Once isolated, the Xce sequence could be used to isolate specific DNA binding proteins which may be important in inactivation, to identify sites which may be affected by site specific DNA methylases or modifications of the DNA structure itself by identifying insertion sequences.

Other transcriptional regulatory mechanism which exist in embryonic cells need to be characterized. One interesting investigation would involve the study the expression of the ornithine carbamyl transferase gene (oct). This gene is coded for by the X chromosome, however, it is only expressed in the tissues of the fetal liver (Deler et al, 1984). It would be valuable to test whether or not oct expression can be induced in C86S1A1 cells by 5-AC and if the expression involves the Xa or Xi. This experiment should test whether or not reactivation of the Xi is complete or whether transcription can be regulated by other, tissue specific mechanisms in addition to X inactivation.

DNA mediated transformation experiments involving the hgprt gene in the different EC cell used in my study may help to elucidate that involvement of DNA modifications in the maintenance of X inactivation. I have performed some

preliminary transformation experiments with C86S1A1 XX cells and the data suggest that the hgprt gene from the Xi may function normally. The hgprt transformation experiments should include all EC lines used in this study which have different Xi characteristics and their differentiated progeny. these experiments should reveal if DNA modifications occur, when they occur, which are permanent and which are transitory and which sites are critical for maintaining inactivation. In this regard, other DNA modifications (i.e. ADP ribosylation) should also be investigated.

All of these studies will be important in understanding the molecular mechanisms controlling X inactivation and will further our understanding of differential gene expression during embryonic development.

Appendix 1

Specific Activities of Some X Encoded and Autosomal Enzymes in XX and XO P10 Cells

Enzyme	Cell Line P1042 (XX)	P1042111 (XX)	P1042112 (XX)	P1041S1 (XO)	P1041S7 (XO)	P1042S9 (XO)
G6PD	36±6	25±3	28±4	25±4	18±5	28±5
HGPRT	2.3±.4	1.3±.1	1.3±.1	1.0±.1	1.0±.1	0.9±.1
alpha-GAL	.5±.2	.4±.1	.4±.1	.3±.1	.3±.0	.3±.1
PGK	990±350	820±240	860±190	600±190	590±270	620±150
6PGD	17±4	16±3	15±5	16±3	15±6	17±2
APRT	.9±.2	.8±.1	.9±.1	.7±.1	.8±.1	.8±0
beta-GAL	.4±.1	.3±.1	.2±.1	.3±.1	.3±.1	.3±.1

Specific activities are expressed in nmoles per min per mg protein.

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