# Unique Properties of the Inactivated X Chromosome in Mammals Revealed by Cell Hybridization

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## CONTENTS

## ABSTRACT

During early development of mammalian females, one of two X chromosomes becomes unresponsive to the transcription activators which can facilitate expression of the X-linked genes on the active counterpart in the same nucleus. Differences in the X-linked gene dosage between male and female cells is, thus, compensated by transcriptional silencing of one entire X chromosome that comprises as much as 5% of the entire genome of about 3 x $10^9$ base pairs. The inactive X chromosome shares such striking properties as late replication in the S phase, heterochromatinization in interphase nuclei, methylation of CpG islands, underacetylation of histone H4, and *Xist* (X inactivation specific transcripts) expression. The repressed state is stably maintained throughout the cell cycle, and the inactivated X chromosome is readily identified microscopically during mitosis with or without special pretreatments. In spite of extensive studies, the molecular basis of X chromosome inactivation remains enigmatic.

Replication asynchrony and heterochromatinization have been considered as the most consistent and probably essential traits that play important roles for the genetic repression. With the hope of advancing our understanding of X-inactivation, I set out the present thesis work primarily based on cell and microcell fusion methods. The first half of the thesis is devoted to examination of the relationship between the late and less familiar precocious replication shown by the inactivated X chromosome. The second half deals with the behavior of the inactive human X chromosome in the mouse genetic background with reference to gene expression, heterochromatinization, sex chromatin body formation, *Xist* expression, methylation of CpG islands, and acetylation of histone H4.

Although most inactive X chromosomes replicate late in S phase, there are occasional inactive X chromosomes characterized by an opposite behavior

replicating in early S phase especially in the extraembryonic region of the early mouse embryo and in adult lymphocytes. After extensive single cell cloning, I succeeded in obtaining two clonal cell lines, MTLB3 and MTLH8, with an inactive X chromosome replicating earlier than the active X chromosome from a cultured murine T-cell lymphoma cell line. The precociously replicating X chromosome was judged to be genetically inactive as the late replicating one on the basis of experiments determining the allele specific (1) level of X-linked genes, *Hprt*, and *Pgk-1* by Northern hybridization and G6PD enzyme activity by photometry; (2) methylation status of the 5' promoter region of *Pgk-1* and the first intron of *Hprt* gene, and (3) expression of the *Xist* (X inactive specific transcript) gene. Since the precociously replicating X chromosome was morphologically distinguishable from the synchronously replicating homolog, its genetic inactivity was further substantiated by differential heavy staining by Kanda's hot hypotonic treatment method, and the low level of histone H4 acetylation revealed by indirect immunofluorescence. When these clonal cell lines were fused with various cell lines including murine teratocarcinoma cell line OTF9-63, myoblast cell line L6TG, Chinese hamster cell line V79HP, marsupial cell line $PtK_2$, and human cervical carcinoma cell line HeLa, the replication timing of the inactive X chromosome immediately shifted from early to late S phase. It was further shown that this remarkable shift to late replication did not occur in the absence of DNA replication in the fusion partner. Taking these findings together, I concluded that (i) late replication is not a prerequisite for X-inactivation, (ii) late replication is actively maintained by a putative *trans*-acting factor, (iii) lack of the factor entails a switch from late to precocious replication as seen in the MTLB3 and MTLH8 cell lines, and (iv) the factor is activated or synthesized during S phase and is quickly inactivated or degenerated before next cell cycle.

In interphase nuclei the inactive X chromosome condenses into sex

chromatin body located specifically at the nuclear periphery. However, I found that the inactive human X chromosome failed to form a compact sex chromatin body in a CF150 human-mouse somatic cell hybrid line retaining one or more inactive human X chromosome as the only human element as reported previously by Dyer *et al.* (1989) in human-mouse somatic hybrid cell lines with several human chromosomes. Furthermore, the inactive human X chromosome did not show any sign of differential condensation during mitotic prophase, and markedly reduced reaction to antiacetylated H4 antibody. The conclusion that the human X chromosome in CF150 is inactive is based on the fact that it was indeed late replicating, the *XIST* gene on this chromosome was active, and above all, X-linked genes usually subject to X-inactivation such as *TIMP, POLA, AR, PGK1,* and *G6PD* were completely repressed. Thus, the human X chromosome is capable of maintaining the inactive state without extreme condensation culminating in the sex chromatin body formation, and underacetylation of histone H4. It was surprising to find that this X chromosome became hypoacetylated and formed a compact chromatin mass at the nuclear periphery when it was introduced into HeLa cells by microcell fusion. Probably, species-specific factor(s) such as the nuclear attachment site is essential for the full manifestations of the inactive X chromosome.

To further explore the interspecific similarities and dissimilarities in the control of X chromosome activity, I introduced the inactive human X chromosome from CF150 cells into mouse embryonal carcinoma (EC) cell lines, PSA-TG8 and OTF9-63, by microcell fusion. All hybrid cells obtained resembled parental EC cell in morphology and retained the human X chromosome(s) or its truncated derivative(s). Notwithstanding apparent reactivation of the introduced human X chromosome element shown by chromosome replication study and RT-PCR analysis, the *XIST* gene remained active continuously. Methylation status of 5'

region of the active *XIST* gene varied considerably from almost full methylation to unmethylation in these hybrids. Thus, mouse EC cells used here are capable of altering methylation status of the human *XIST* gene, but unable to repress its transcription. In addition, we failed to obtain any positive evidence for the occurrence of *de novo* X chromosome inactivation in these hybrid cells grown under the condition conducive to cell differentiation. It is likely that the human X chromosome inactivation center (Xic) including the *XIST* gene is unable to function effectively in mouse EC cells.

# GENERAL INTRODUCTION

X chromosome inactivation is a unique epigenetic event in mammals that leads to transcriptional silencing of one of two X chromosomes in female somatic cells (Lyon, 1961) to offset the X chromosome dosage difference between females (XX) and males (XY). The typical mammalian X chromosome consists of about 5% of the haploid genome, and harbors a number of genes directly proportionate to its size, whereas the Y chromosome diminished in size during evolution retains only a small number of genes primarily involved in sex determination and male fertility. An outstanding feature of X-inactivation consists essentially in the fact that two X chromosomes in the same nucleus, even if genetically identical, are rigidly distinguished, and one X chromosome is made unresponsive to the transcription machinery.

In some rodent species including mice, X-inactivation take place on at least three separate occasions during early development of female embryos apparently in association with cell differentiation that occurs at specific developmental stages in different cell lineages (Monk and Harper, 1979; Rastan, 1982; Takagi *et al.*, 1982; Sugawara *et al.*, 1985). X-inactivation may be somewhat different in detail in different cell lineages. Thus, the paternally derived X chromosome is selectively inactivated in extraembryonic trophectoderm by day 3.5 and primitive endoderm by day 4.5 after fertilization, respectively, probably due to differential imprinting on the two X chromosomes (Takagi and Sasaki, 1974; West *et al.*, 1977). Either X chromosome is inactivated at random in the epiblast cell lineage immediately prior to gastrulation (Gardner and Lyon, 1971; Rastan, 1982).

Once inactivated, the X chromosome maintains the repressed state extremely stably through successive somatic cell divisions exhibiting certain recognizable properties. The inactivated X chromosome tends to condense

throughout the cell cycle culminating in the formation of a perinuclear sex chromatin body in interphase nuclei and premature condensation at prophase. The same X chromosome at metaphase can be distinguished from the active counterpart by the Giemsa staining method involving pretreatment with heated hypotonic solution (Kanda, 1973; Kanda and Yoshida, 1979) or by *in situ* nick translation (Kerem *et al.*, 1983). Remaining prominent properties of the inactive X chromosome include DNA replication later in the S phase than other chromosomes in a cell (Lyon, 1972), methylation of CpG islands in the promoter region of most housekeeping genes (Mohandas *et al.*, 1981; Wolf and Migeon, 1982), and underacetylation of histone H4 (Jeppesen and Turner, 1993). It is conceivable that these properties manifest multiple levels of control involved in the maintenance of X-inactivation, but relationships between them and their relative importance remain unknown not to mention their exact roles.

Cytological analysis both in man and mouse has shown that a *cis*-acting major switch region, termed X chromosome inactivation center (Xic), is required for X-inactivation (Russell and Cacheiro, 1978; Therman *et al.*, 1979). Two Xics on separate chromosomes are necessary for the occurrence of X-inactivation in diploid cells. Xic has been assumed to be involved in (1) initiation of X-inactivation including the choice of the X chromosome to be inactivated, (2) promulgation of the inactive state along the entire length of the X chromosome, and (3) stable maintenance of the inactive state through cell cycle. In mouse, a genetically defined locus, X chromosome controlling element (*Xce*), mapped in the Xic region is capable of biasing the randomness of X-inactivation, hence it has long been thought to be a candidate for Xic itself (Cattanach and Papworth, 1981; Johnston and Cattanach, 1981).

Although the molecular entity of Xic remains still elusive, an important breakthrough has been made by the cloning of a novel human gene X inactivation

specific transcript (*XIST*; Brown *et al.*, 1991) and its mouse homolog *Xist* (Borsani *et al.*, 1991; Brockdorff *et al.*, 1991). The *XIST/Xist* gene is mapped within the interval of Xic region defined cytogenetically, and, as the name implies, exclusively expressed from the inactive X chromosome. Furthermore, *Xist* RNA is not translated into protein and retained in the nucleus. *Xist* is expressed prior to the onset of X-inactivation in the early female mouse embryos and it is the paternally inherited *Xist* allele that is expressed at this time in agreement with the imprinted inactivation of the paternally derived X chromosome in the extraembryonic tissues (Kay *et al.*, 1993, 1994). *Xist* expression is also increased markedly in murine embryonal carcinoma (EC) and embryonic stem (ES) cells upon induction of cell differentiation (Beard *et al.*, 1995; Tai *et al.*, 1994).

Recent gene targeting analyses (Penny *et al.*, 1996; Marahrens *et al.*, 1997) strongly supported that *Xist* is indeed essential for the initiation of X-inactivation. The X chromosome with the deleted *Xist* gene is unable to be repressed most probably due to failure of *Xist* expression. Deletion of most of the gene body invariably showed that Xist RNA is essential for X-inactivation. The fate of female heterozygous for the deletion varied greatly according to the parental derivation of the deletion (Marahrens *et al.*, 1997). Heterozygous embryos die immediately after implantation if the deletion is transmitted from father, whereas they grow normally if the deletion is transmitted from mother. Only the maternally derived X chromosome was inactivated in viable heterozygous females of the latter type. These findings are compatible with the view that Xist RNA is essential for X-inactivation, and inactivation of the maternal X chromosome is indeed prohibited in the extraembryonic structures.

There have been conflicting reports on the role of the *XIST/Xist* gene in the maintenance of X-inactivation in somatic cells. Continuous expression of *Xist* throughout the life of the animal suggests that the gene product is essential for the

maintenance of X-inactivation. The spliced and polyadenylated *XIST/Xist* transcripts are retained in the interphase nuclei and colocalized with the inactive X chromosome in interphase nuclei (Clemson *et al.*, 1996; Lee *et al.*, 1996). In accord with these observations, *XIST/Xist* expression is extinguished contingent on the reactivation of the inactive X chromosome in human-mouse somatic hybrid cells (Luo *et al.*, 1995) and mouse EC-somatic cell hybrids (Mise *et al.*, 1996). Interestingly enough, however, deletion of the Xic region including the *XIST* gene from the inactive human X chromosome does not automatically lead to its reactivation in human-mouse somatic hybrid cells (Brown and Willard, 1994) and human leukemia cells (Rack *et al.*, 1994).

In spite of extensive studies, the molecular basis of X-inactivation remains enigmatic. In the hope of advancing our knowledge, I set out to study two of most prominent properties of the inactive X chromosome, namely replication asynchrony and heterochromatinization in cultured cells. Novel murine T-cell lymphoma cell lines and a human-mouse somatic hybrid cell line subjected to cell fusion and/or microcell fusion yielded certain interesting findings.

The first chapter deals with unusual replication timing of the inactive X chromosome in novel mouse lymphoma cell lines. The inactive X chromosome usually replicates late in S phase, but there are also occasional cells in which it replicates earlier than any other chromosomes in the cell. Even newly inactivated X chromosome in trophectoderm and primitive endoderm, most probably, replicates precociously but it shifts the replication timing to the late S phase sooner or later (Takagi *et al.*, 1982; Sugawara *et al.*, 1983). It is also known that the precociously replicating X chromosome is not rare in hematopoietic tissues of adult female mice (Takagi *et al.*, 1984). Isolation of two clonal T-cell lymphoma cell lines, MTLB3 and MTLH8, with a precociously replicating, inactive X chromosome provided me with a unique opportunity to study the cause of

transition from precocious to late replication and stable maintenance of late replication in most somatic cells. It was interesting to find that the precociously replicating X chromosome from MTLB3 and MTLH8 begins to replicate late immediately after fusion with various cultured cell lines. It seems, therefore, that late replication characterizing most inactive X chromosome is actively maintained by a trans-acting factor(s) in female somatic cells, and that its lack inevitably leads to switch from late to precocious replication. My cytological observation in hybrids immediately after cell fusion showed that replication of the parental genome is necessary for the switch.

In the second chapter, I describe results of experiments examining properties and behavior of a human inactive X chromosome in the mouse genetic background. It was found that the late replicating and inactivated X chromosome in a mouse-human somatic hybrid cell was not typically heterochromatic neither forming a condensed sex chromatin body in interphase, nor showing heteropycnosis at mitotic prophase. Furthermore, this chromosome showed markedly reduced reaction to acetylated histone H4 antibody. An inevitable conclusion would be that the human X chromosome is capable of maintaining the inactive state without extreme heterochromatinization and underacetylation of histone H4. I introduced this chromosome into mouse EC cell lines, PSA-TG8 and OTF9-63, by microcell fusion, to further explore its behavior in undifferentiated conditions. Notwithstanding apparent reactivation, the *Xist* gene was not extinguished, and the 5' region of the gene varied extensively from almost complete unmethylation to heavy methylation.

Thus, the present study has yielded several unexpected findings apparently at variance with the current belief. It is tempting to postulate that one X chromosome in the adult female somatic cell maintains its inactivated state stably by synergy of several kinds of factors and modifications, some of them being

species-specific, and absence of one or two could be tolerated safely.

# I. REPLICATION ASYNCHRONY

## Correlation Between Late and Precocious Replication

# INTRODUCTION

Various lines of evidence have indicated that genomic DNA replication in animal cells occurs in a temporally ordered fashion during S phase. Thus, the mammalian chromosome consists of late replicating G-positive and early replicating R-positive bands, differing in size aligned alternately in a pattern specific to each chromosome. Reviewing the then available evidence, Holmquist (1987) arrived at a conclusion that constitutively active genes replicate early and tissue-specific genes usually replicate late, whereas the tissue-specific genes do replicate early in those cell types which express those particular gene. Although further study showed that this conclusion was an oversimplification, it is still believed that late replication is correlated with gene repression. However, we are ignorant of the underlying mechanism that controls the timing of DNA replication during S phase.

The inactivated X chromosome begins and finishes DNA replication later than autosomes and the active homolog (Lyon, 1972). In apparent contradiction to the intimate correlation between late replication and genetic inactivity, a minor fraction of the inactive X chromosome finishes DNA replication earlier than any chromosome of the cell. The precociously replicating X chromosome has been reported to appear temporarily in the extra-embryonic lineages of mouse, rat, Chinese hamster and rabbit embryos (Takagi *et al.*, 1982; Sugawara and Takagi, 1985) and in a limited proportion of bone marrow cells from the adult female mouse (Takagi *et al.*, 1984). An adequate study of the precociously replicating X chromosome has not been possible due to the lack of any known cell population with a maintaining such X chromosome stably.

We established mouse thymic lymphoma cell clones with a precociously replicating, presumably genetically inactive X chromosome. Cells of one of these clones were fused with the OTF9-63 murine embryonal carcinoma (EC) cell line

as a part of a survey to examine the ability of the EC cell line to quench the differentiated phenotype of various somatic cells upon cell fusion. To our surprise the precociously replicating X chromosome turned into a late replicating one in hybrid cells. In this chapter, we report analyses of this phenomenon together with the nature of the precociously replicating X chromosome because this might shed new light on the control mechanism of replication timing of individual chromosomes and chromosome segments. Findings obtained in this study suggest that (1) the precociously replicating X chromosome is indeed genetically repressed, (2) late replication shown by the inactive X chromosome is actively maintained in female somatic cells, and (3) the failure in the maintenance mechanism leads to precocious replication. Late DNA replication itself may not be responsible for the genetic inactivation of the mammalian X chromosome.

## MATERIALS and METHODS

### *Cell lines*

The MTL1 cell line, established from a thymic lymphoma, spontaneously occurred in an F1 female obtained from a mating between a female carrying Is(InX;7)1Ct and an AKR/Hok male, had 41 chromosomes including $X^{(7)}$ and $X^N$. MTL1 consisted of two cell populations one with a late replicating $X^N$ and the other with an apparently precociously replicating $X^N$ chromosome. MTLB3, MTLH8, and MTLD6 (shortened to B3, H8 and D6, respectively) are subclones isolated from MTL1 by limiting dilution. It may be worth mentioning that D6 is the only clone out of 104 isolated from MTL1 that has an inactive X chromosome replicating in the latter half of S phase. H8TGN and D6TGN were isolated after successive selection with 6-thioguanine and G418 following treatment of parental clones with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and infection of Moloney murine leukemia virus carrying bacterial neomycin resistance gene.

The modal cell of an HPRT-deficient and ouabain-resistant EC cell line, OTF9-63 (Rosenstraus and Levine, 1979), originated from an XY terato-carcinoma and has 42 autosomes and a single X chromosome (Figure 6a). The HPRT-deficient mouse fibroblast cell line A9, derived from the connective and fatty tissue pad of a male C3H mouse (Littelefield, 1964), has about 51 chromosomes including a single X chromosome. V79HP, an HPRT-deficient Chinese hamster cell line, and $PtK_2$, a kidney cell line from the marsupial species *Potorous tridactylis*, are characterized by a single X chromosome and have 21 and 13 autosomes, respectively. Both cell lines were derived from male animals (Ford and Yerganian, 1958; Walen, 1965). The rat myoblast cell line, L6TG.cap, established from an embryo of unknown sex (Yaffe, 1968) has a single X

chromosome and about 71 autosomes. HeLa, a human cervical carcinoma cell line, has 1-3 X chromosomes and about 68 autosomes. L6TG.cap and $PtK_2$ were obtained from the Japanese Cancer Resources Bank. All of these cell lines were grown in Eagle's minimum essential medium supplemented with 10 % fetal calf serum.

### *Cell Fusion*

Cell fusion was carried out by gently stirring the mixture of parental cells in 50% polyethylene glycol (mol. wt 1500, Boehringer Mannheim) for 1 min as described by Hales (1977). Hybrid cells were selected in HAT medium containing 1.5 x $10^{-4}$ M hypoxanthine, 1.1 x $10^{-6}$ M amethopterine and 2 x $10^{-5}$ M thymidine with or without 200~800 $\mu$g/ml G418.

### *Chromosome Examination*

Chromosome studies were routinely done by an R-banding method involving continuous BrdU incorporation for 10 h including the last 1 h in the presence of Colcemid and fluorescence staining with acridine orange (RBA method). Whole chromosomes or chromosome segments replicated in the presence of BrdU showed red fluorescence, whereas those not having incorporated BrdU showed bright green fluorescence. When BrdU was present only during the latter part of S phase, an unequivocal red-green banding pattern was observed, distinguishing late and early replicating regions, respectively.

The replication behavior of the X chromosomes in B3 was evaluated further by the BrdU antibody technique (BAT)(Vogel *et al.*, 1986). Cells were exposed to 10 $\mu$g/ml BrdU for 10 min, rinsed, refed with fresh medium

containing 0.3 mM thymidine, and harvested 1~24 h after BrdU labeling. Immunostaining was carried out according to the method described by O'Keefe *et al.* (1992) with minor modifications. Chromosome slides were treated with 0.7% Triton X-100 in 0.1 M HCl for 10 min on ice, and with 50% formamide in 2 x SSC at 80°C for 10 min before immunological staining with FITC-conjugated anti-BrdU antibody (Boehringer-Mannheim). The FITC-labeled preparations were counterstained with propidium iodide (0.05 $\mu$g/ml) in 9:1 (v/v) glycerol/PBS containing 1.25% (w/v) 1,4-diazabicyclo-(2.2.2) octane (DABCO).

The precociously replicating X chromosome was also examined by the cytological method developed for the differential staining of the inactivated X chromosome (Kanda, 1973). Actively growing cells were harvested after Colcemid treatment for 1 h, incubated in 0.075 M KCl at 53°C for 5~7 min, fixed with 3:1 (v/v) methanol:acetic acid, dried on slides and stained with 2% Giemsa.

For immunological detection of acetylated histone H4 (Jeppesen *et al.*, 1992), Colcemid-treated (1 h) cells were resuspended in 0.075 M KCl at a cell density of 2~4 x $10^6$/ml. The cells were incubated for 30 min at room temperature, then placed on ice. About 50 $\mu$l of hypotonically swollen cells was added into the chamber of SC-2 cytocentrifuge (TOMY SEIKO, Tokyo) that was filled with 3 ml of 75 mM KCl. After centrifugation at 1500 rpm for 10 min, slides were transferred to a Coplin jar and immersed in KCM solution (120 mM KCl, 20 mM NaCl, 10mM Tris-HCl[pH8.0], 0.5mM EDTA, and 0.1 % [w/v] Triton X-100) for a minimum of 10 min at room temperature, before proceeding. A rabbit polyclonal antiserum against acetylated H4, a kind gift from Dr. C.D. Allis, University of Rochester, New York, was diluted 1/20 ~ 1/100 in KCM containing 10% (w/v) bovine serum albumin (BSA). Slides were incubated with 10~20 $\mu$l per slide of this dilution for 1 h at 37°C in a humidified chamber. After first antibody reaction, the slides were washed three times (for 5 min, at room temperature) with KCM

solution. Then, the slides were incubated with an FITC-conjugated anti-rabbit IgG (Cappel, Durham, NC) diluted 1/200 in KCM containing 10% (w/v) BSA, for 1 h at 37°C in a humidified and dark chamber. After second antibody reaction, the slides were washed with KCM as before, then incubated for 10 min in KCM containing 10 % formalin (v/v) and 0.1 mg/ml Hoechst 33258. After fixation and DNA fluorochrome staining, the slides were rinsed with distilled water and mounted in glycerol/PBS (9:1)(v/v) containing 1.25 % (w/v) DABCO.

***Analysis of Cell Cycle***

Chronology of X chromosome replication during S phase was analyzed by a standard method. Cells were labeled with 5 $\mu$g/ml BrdU for various periods of time before harvest. Colcemid treatment was avoided except in experiments involving fusion between B3 and OTF9-63. Approximately 100 mitoses from each period of incubation with BrdU were screened. To determine the time necessary for replication of the inactive X chromosome, B3 cells were labeled with 20$\mu$g/ml BrdU for 0.5~1.5 h followed by a 0.5 h thymidine (10 $\mu$g/ml) pulse and harvested hourly intervals up to 24 h before mitosis.

***DNA hybridization probes***

Plasmids whose inserts or whole linearized DNA were used as hybridization probes in Northern or Southern blot analysis were as follows: pHPGK-7e obtained from American Type Culture Collection, containing a 1.8 kb cDNA sequence of the human *PGK-1* gene (Michelson *et al.*, 1983); pHPT5, including a 1.3 kb cDNA of the mouse *Hprt* gene (Konecki *et al.*, 1982); pHPT $\lambda$ 13-in1, comprising a 1.8 kb genomic DNA fragment from the first intron of the mouse *Hprt* gene

(Lock *et al.*, 1986); and pBV2.1, consisting of a 2.1 kb human $\beta$-actin cDNA and pBR322.

***RNA isolation and analysis***

Total cellular RNA was prepared from various mouse tissues and cultured cells as previously described (Sambrook *et al.*, 1989). RNA samples were treated with RQ1 RNase-free DNase (Promega, Madison, WI) before extraction with phenol, precipitation with ethanol and quantification. For Northern hybridization, 10 $\mu$g of total cellular RNA was electrophoresed, blotted and hybridized as previously described (Sambrook *et al.*, 1989). A video densitometer (Vilber Lourmat, model Bio-Profile) with programs for one-Dimension analysis was used to determine the intensity of each band on X-ray films, with care being taken to be in the linear response range of the film.

1 $\mu$g of DNA-free RNA was reverse transcribed and amplified by polymerase chain reaction (RT-PCR) as previously described (Singer-Sam *et al.*, 1990c), in the presence of `Perfect match' enhancer (Stratagene, La Jolla, CA, 1 U). Primers used for the study of *Xist* expression were Xist5' (5' CTATGTCTCC-TTGTGTTGTCTA 3') and Xist3' (5' GGATGAATGCAACATGTGACCC 3'), 588-2F and 2R, 594-1F and 1R (Borsani *et al.*, 1991). Primers used for the internal marker were Bact-1a (5' CATGAAGATCCTGACCGAGCGT 3') and Bact-1b (5' ATG-ACCTGGCCGTCAGGCAGCT 3') for the amplification of mouse cytoskeltal $\beta$-actin (Tokunaga *et al.*, 1986). After PCR, a 20 $\mu$l aliquot was subjected to electrophoresis in a 2.6% (w/v) agarose gel containing 0.4 $\mu$g/ml ethidium bromide in 1 x TAE buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA).

*Measurements of enzyme activities*

Specific activities of two enzymes [X-linked glucose-6-phosphate dehydrogenase (G6PD: EC 1.1.1.49) and autosomal 6-phosphogluconate dehydrogenase (6PGD: EC 1.1.1.44)] were determined as previously described (Martin *et al.*, 1978).

*DNA isolation and analysis*

High molecular weight DNA was isolated from various mouse tissues and cultured cells by the procedures described previously (Sambrook *et al.*, 1989). 10 $\mu$g of DNA from cell lines and adult mouse tissues were digested with methylation-sensitive restriction endonucleases (*Hae*II, *Hpa*II, and *Ava*I) in 0.5 x KGB buffer (McClelland *et al.*, 1988) electrophoresed, transferred to nylon membrane and hybridized essentially as described previously (Lock *et al.*, 1986).

The *Hpa*II-PCR method was employed to study DNA methylation at an *Hpa*II site (H-7) in the CpG-rich island at 5' end of *Pgk-1* (Singer-Sam *et al.*, 1990a, b). Approximately 500 cells were suspended in proteinase K-guanidine hydrochloride lysis buffer containing $\phi$X replicative form DNA, and purified DNA was digested with *Hpa*II in combination with *Xba*I. After amplification and electrophoresis, the gel stained with ethidium bromide was directly assayed to determine the intensity of each band by the video densitometer.

# RESULTS

## *Replication timing of the precociously replicating X chromosome*

Three subclones were isolated from an MTL1 cell clone established from a murine thymic lymphoma that spontaneously occurred in a female F1 hybrid between a female carrying Cattanach's insertion, Is(In7;X)1Ct and an AKR/Hok male. B3 had 41 chromosomes including a Cattanach X chromosome [$X^{(7)}$] and a large rearranged telocentric chromosome ($X^T$) which consists of a centromeric segment of chromosome 12 and nearly the entire X chromosome (Figure 1a). Precocious replication of the X chromosome segment of the latter was concluded from its green acridine orange fluorescence after continuous incorporation of 5-bromo-deoxyuridine (BrdU) during the latter part of S phase (Figure 1a) and from the strong fluorescein isothiocyanate (FITC) fluorescence observed in cells pulse-labeled with BrdU early in S phase and stained with an FITC-conjugated anti-BrdU antibody (Figure 2a). The complementary pattern was observed when cells were pulse labeled in late S phase (Figure 2b). H8 and D6 have 40 chromosomes including $X^{(7)}$ and a morphologically normal X chromosome ($X^N$). $X^N$ replicated early in S phase in H8, whereas it replicated in the latter half of S phase in D6 (Figures 1b and 1c).

In order to characterize the precociously replicating X chromosome of B3 more fully, we tried to determine its replication timing in S phase. Acridine orange staining patterns of chromosomes from cells which continuously incorporated BrdU for various periods of time before fixation indicated that, on average, $X^T$ initiated replication 12 h before mitosis (Figure 3a) and it continued for ~1.5 h. Since the average duration of S + $G_2$ was ~14 h, the precociously replicating X chromosome replicated in the first quarter of S phase in B3 (Figure 3b), except

**Figure 1.** X chromosome differentially stained by RBA method (**a-c**) and Kanda's hot hypotonic Giemsa method (**d-f**). (**a**) MTLB3 cell with a precociously replicating $X^T$ (arrow); (**b**) MTLH8 cell with a precociously replicating $X^N$ (arrow); (**c**) MTLD6 cell with late replicating $X^N$ (arrow). The synchronously replicating $X^{(7)}$ is indicated by an arrowhead (**a-c**). In these photographs, late replicating segments appear dark and early replicating segments appear bright. A darkly staining inactive X chromosome (arrow) is evident in (**d**) an MTLB3 cell, (**e**) an MTLH8 cell and (**f**) an MTLD6 cell. The autosomal segment of $X^T$ (arrowhead) in MTLB3 is not stained darkly.

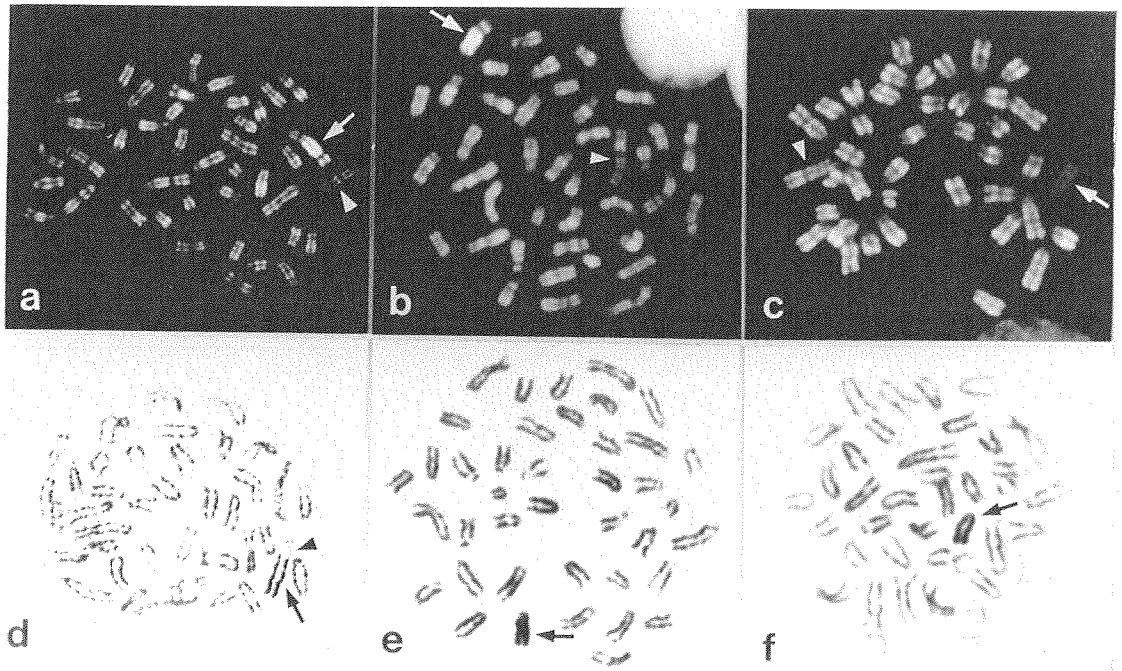

a
b
c
d
e
f

**Figure 2.** Precocious replication of $X^T$ chromosome in MTLB3 visualized by a BrdU antibody technique. (**A**) A cell labeled 23 h before harvest. The X chromosome segment of $X^T$ is heavily labeled except for band E and proximal autosomal region (arrow), whereas autosomes and $X^{(7)}$ appear labeled lightly. The pulse labeling procedure as shown in the bottom (involving centrifugation, medium change and chase with high concentration of thymidine) apparently elongated the cell cycle considerably. (**B**) A cell labeled 13 h before harvest. $X^T$ is devoid of labeling except for the proximal autosomal region and the E band (arrow). The G-band-like replication patterns of other chromosomes suggest that the cell was labeled during mid to late S phase.

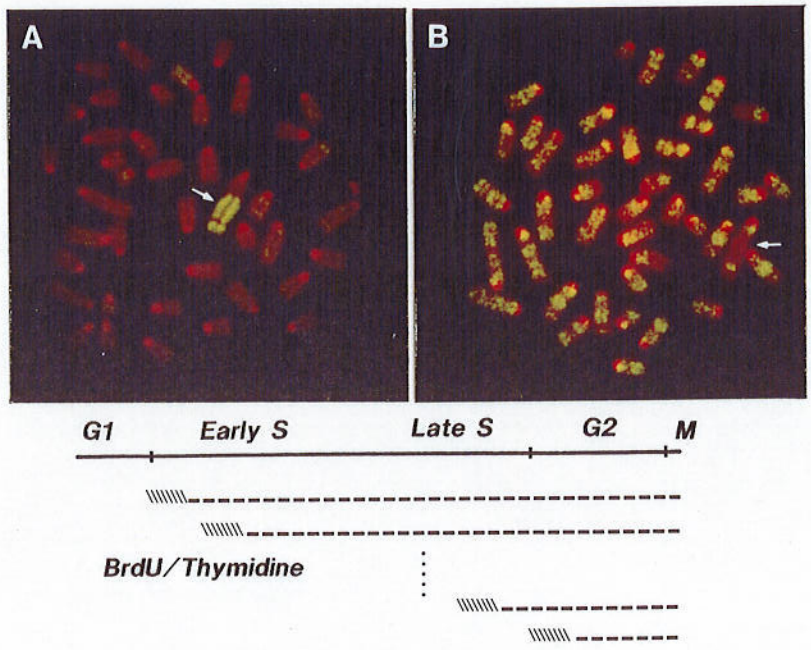
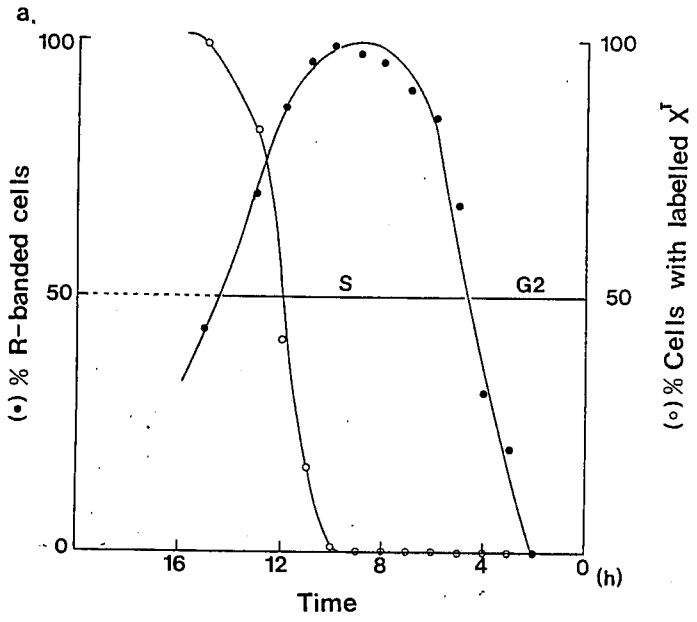
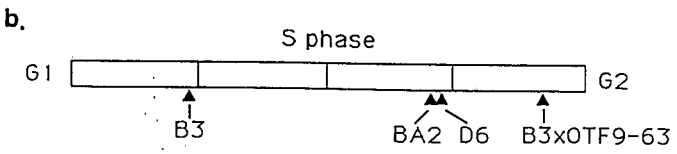

A
B
G1
Early S
Late S
G2
M
BrdU/Thymidine

**Figure 3.** (**a**) Replication time of the precociously replicating $X^T$ chromosome in MTLB3. Unsynchronized MTLB3 cells were labeled continuously with BrdU for various times before harvesting. Samples were harvested at hourly intervals and the percentage of metaphase cells showing BrdU incorporation was plotted (closed circles). Initiation of $G_2$ was determined by the time at which 50% of the cells show BrdU labeling. The duration of $S+G_2$ was defined as the time at which 50% of cells show uniform BrdU labeling. Open circles represent the percentage of cells having $X^T$ uniformly labeled with BrdU except the autosomal segment. Initiation of DNA replication by the precociously replicating X chromosome was defined as the time at which 50% of the cells showed uniform BrdU labeling along the entire length of the X chromosome segment of $X^T$. A pulse-labeling experiment showed that the whole X chromosome segment finished replication within 1.5 h. At least 100 cells were evaluated at each point. (**b**) Differing initiation time of the allocyclic X chromosome replication in MTLB3 and three related cell lines. The initiation times determined as above were normalized and are illustrated on a linear map in which S phase is divided into equal quarters. The actual lengths of S phase were 9.8 h in MTLB3, 8.5 h in MTLD6, 10.8 h in MTLB3 x OTF9 and 5.0 h in B3 x A9 (BA2).


a.
100
50
0
100
50
(•) % R-banded cells
(◦) % Cells with labelled X
S
G2
16
12
8
4
0
(h)
Time


b.
S phase
G1
G2
B3
BA2
D6
B3xOTF9-63

for the band E and the proximal autosomal region. The later replicating X chromosome of D6, on the other hand, replication at the end of the third quarter.

***Genetic inactivity of the precociously replicating X chromosome***

There was no simple and unequivocal way to ascertain the activity state of the precociously replicating X chromosome. Hence, we set out to collect various circumstantial evidence to show that it is, in fact, the genetically inactivated chromosome as the late replicating one. Attributes of the precociously replicating X chromosome we examined included (1) dark Giemsa staining after hot hypotonic treatment of cells before fixation (Kanda, 1973), (2) expression of the *Xist* gene (Brown *et al.*, 1991), (3) transcriptional activity of X-linked *Hprt* and *Pgk-1* genes, (4) the activity level of an X-linked G6PD enzyme, (5) methylation of several restriction endonuclease sites present in the intron 1 of *Hprt* gene (Lock *et al.*, 1986) and a HpaII site (H-7) in the promoter region of *Pgk-1* gene (Singer-Sam *et al.*, 1989, 1990a, b), and (6) the lack of histone H4 acetylation (Jeppesen and Turner, 1993).

Figure 1 panel d-f show metaphase plates from B3, H8 and D6 stained according to Kanda's hot hypotonic Giemsa method. A single darkly stained X chromosome or X chromosomal part was observed in more than half of the metaphase cells examined in each clone. Thus, the precociously replicating X chromosome in B3 and H8 and the late replicating one in D6 were considered equally heterochromatic.

The *Xist* gene located within the Xic, is expressed exclusively from the inactivated X chromosome (Borsani *et al.*, 1991; Brockdorff *et al.*, 1991). Consequently, *Xist* expression was studied in RNA samples from eight different cell lines including B3, H8 and thymus specimens by an RT-PCR assay (Figure

4a). There was a complete lack of amplification in all the samples without reverse transcriptase, ruling out contamination of the sample with genomic DNA, and the presence of $\beta$-actin products certified that amplification occurred normally in samples containing reverse transcriptase. The PCR product of 433 bp amplified from the *Xist* transcript was detected in B3 and H8 as well as in D6 and XX thymocytes, suggesting that these cells had an inactive X chromosome from which *Xist* was expressed.

Transcription levels of the *Hprt* and *Pgk-1* genes assessed by Northern hybridization were virtually equal in B3, H8 and D6 cell lines and in thymocytes from XX, X0 and XY mice after normalization by $\beta$-actin signals (Table 1). This was consistent with the expectation that compensation of the X-linked gene dosage occurred in B3 and H8 as in D6 and normal female somatic cells. Genetic repression of genes on the precociously replicating X chromosome was further demonstrated by the nearly identical level of G6PD activity in B3 and normal X0 thymocytes (Table 1). Although the actual specific activities varied, two additional experiments showed similar results (data not shown).

The first intron of the mouse *Hprt* gene contains a cluster of restriction endonuclease sites that are completely unmethylated when carried on the active X chromosome and are methylated to variable extent when carried on the inactive one (Lock *et al.*, 1986; Figure 5c). Hence, we examined the methylation states of this region in somatic cells and cell lines including B3 and H8 (Figure 5). DNA from male thymocytes digested with *Taq*I and *Hae*II produced a 4.1 kb band, suggesting that the H1.1 and H1.2 sites on the active X chromosome were completely unmethylated. DNA from female thymocytes gave rise to two female-specific bands at 4.4 and 5.3 kb in addition to a 4.1 kb band. As illustrated in Figure 5c, a 5.3 kb fragment should be detected when both the H1.1 and H1.2 sites are methylated and thus cannot be cleaved by *Hae*II. The 4.4 kb fragment

**Figure 4.** (**a**) *Xist* expression revealed by RT-PCR. Reverse-transcribed total RNA samples were amplified with two sets of primers, one for *Xist* (433 bp) and the other for $\beta$-actin (177 bp). Plus and minus signs indicate the presence or absence of reverse transcriptase in the reaction mixture containing 1 $\mu$ g total RNA. It should be noted that the signal intensity of each samples is not necessarily proportional to the level of gene expression, since the number of amplification cycles is large (35 cycles). Lanes: 1, XX thymocyte; 2, XO thymocyte; 3, XY thymocyte; 4, MTLB3; 5, MTLH8; 6, MTLD6; 7, B3 x A9 (BA2); 8, A9. (**b**) Methylation of the H-7 *Hpa*II site in the promoter region of the *Pgk-1* gene assessed by *Hpa*II-PCR. Purified DNA was digested with (+) or without (-) *Hpa*II in combination with *Xba*I. After heat inactivation of HpaII, samples were assayed by PCR in the presence of 200 molecules of internal standard. The internal standard gave a 133 bp PCR product (**I**), while the genomic template gave rise to a 170 bp PCR product (**G**) in the presence of the same primer set. The level of methylation estimated from G/I ratio is given in parenthesis. Lane 1, XX thymocyte (75.7±3.9%); 2, XO thymocyte (19.7± 4.9%); 3, XY thymocyte (17.8±8.0%); 4, MTLB3 (65.4±5.8%); 5, MTLH8 (76.6±7.2%); 6, MTLD6 (76.5±11.4%); 7, BA2 (83.2±5.3%); 8, A9 (21.6± 0.5%). *Hae*III digested $\phi$ X-DNA was used for size calibration.

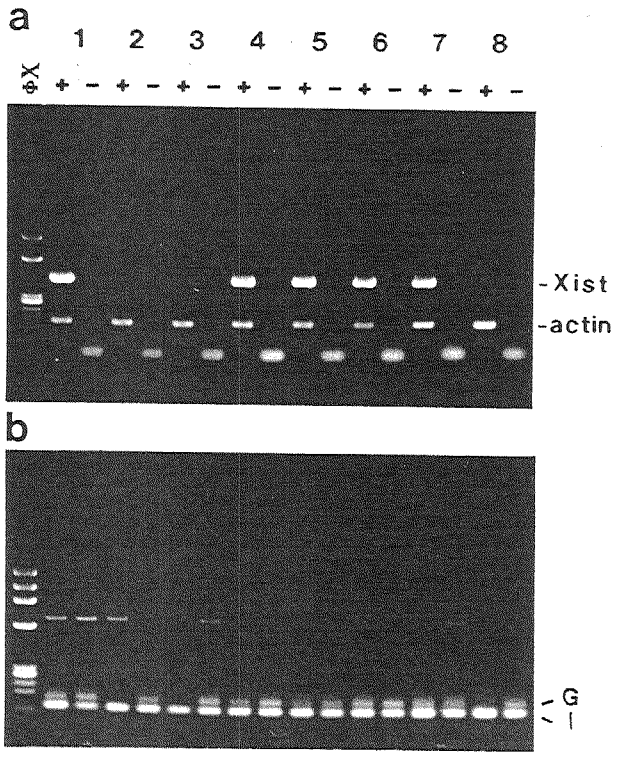
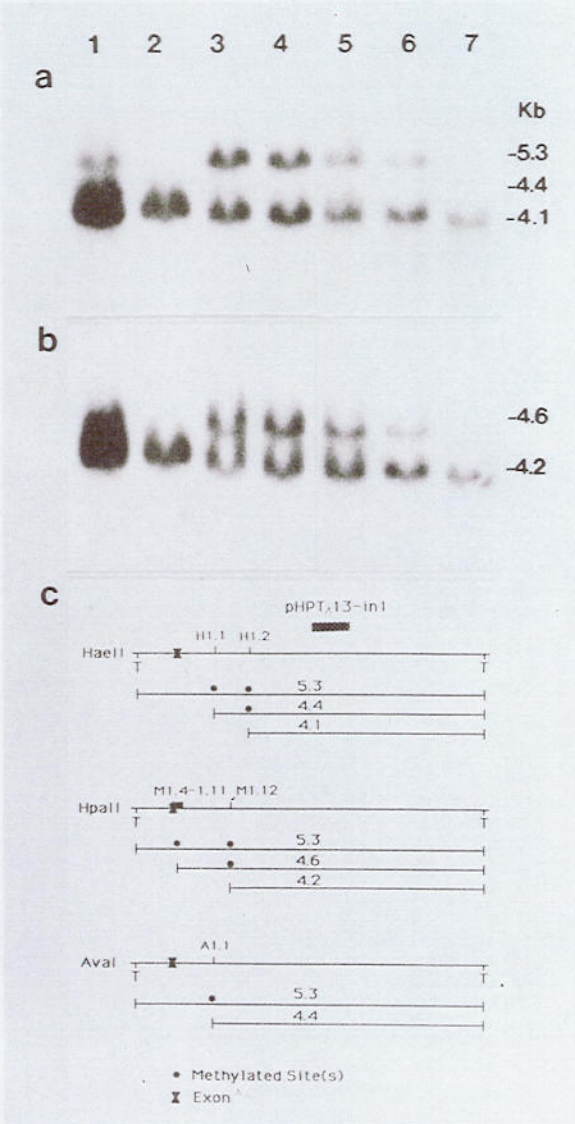

a
1 2 3 4 5 6 7 8
ΦX + − + − + − + − + − + − + − + −
-Xist
-actin
b
G
I

Table 1. Non-expression of these X-linked genes, *Hprt*, *Pgk-1*, and G6PD on the precociously replicating X chromosome revealed by mRNA levels and enzyme activities

| Cells | Ratio of specific activities | | |
|---|---|---|---|
| | *Hprt*/ β -actin | *Pgk-1*/ β -actin | G6PD/6PGD |
| XX thymocyte | 0.62 ± 0.01 | 0.54 ± 0.02 | - |
| X0 thymocyte | 0.73 ± 0.03 | 0.66 ± 0.01 | 2.71 ± 0.38 |
| XY thymocyte | 0.72 ± 0.03 | 0.67 ± 0.04 | - |
| MTLB3 | 0.70 ± 0.04 | 0.60 ± 0.01 | 3.02 ± 0.10 |
| MTLH8 | 0.63 ± 0.01 | 0.63 ± 0.01 | - |
| MTLD6 | 0.53 ± 0.02 | 0.62 ± 0.02 | - |

**Figure 5.** Methylation state of intron 1 of the *Hprt* gene as revealed by Southern blot analysis. Genomic DNA digested with *Hae*II (**a**) or *Hpa*II (**b**) in combination with *Taq*I was hybridized with an intron 1 probe, pHPT λ 13-in1. Lane 1, female thymocyte; 2, male thymocyte; 3, MTLB3; 4, MTLH8; 5, MTLD6; 6, B3 x A9 (BA2); 7, A9. The low intensity of 5.3 and 4.6kb bands in the BA2 sample in comparison with MTL cells may be due to aneuploidy of the hybrid cells. (**c**) Partial map of the mouse *Hprt* gene showing the location of restriction sites in and near the first exon and intron cited from Lock *et al.* (1987). Each of three maps shows the restriction fragments detected in Southern blots of female genomic DNA digested with *Hae*II, *Hpa*II or *Ava*I in combination with *Taq*I (T) and hybridized to the pHPT λ 13-in1 probe.


1 2 3 4 5 6 7
a
Kb
-5.3
-4.4
-4.1
b
-4.6
-4.2
c
pHPTA13-in1
HaeII
HI.1 HI.2
T T
5.3
4.4
4.1
HpaII
MI.4-I.11 MI.12
T T
5.3
4.6
4.2
AvaI
A1.1
T T
5.3
4.4
• Methylated Site(s)
Exon

will be detected when only the H1.2 site is methylated.

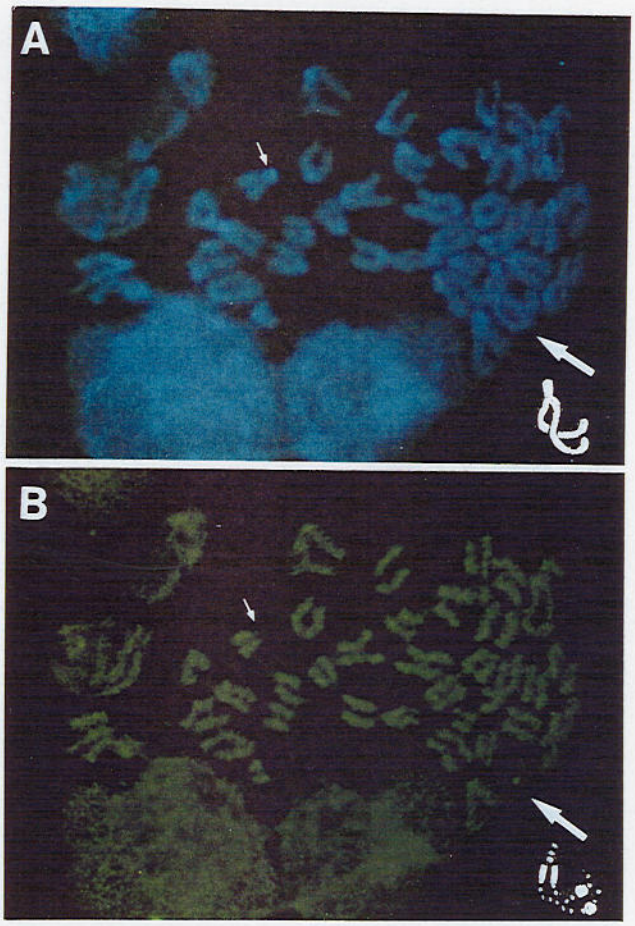
As shown in Figure 5a, 5.3, 4.4 and 4.1 kb fragments were detected in DNA samples from B3, H8 and D6, although the intensity of a 4.4 kb band in these cell lines was low in comparison with that in female thymocytes. This finding suggested that (1) the H1.1 and H1.2 sites on the precociously replicating X chromosome were methylated to the same extent as the late replicating one and (2) the H1.1 site of these cell lines was methylated more heavily than that of female thymocytes *in vivo*. Similarly, a female-specific 4.6 kb fragment was detected in samples from B3, H8, D6 and female thymocytes digested with *Hpa*II, implying that the M1.12 site of the precociously replicating X chromosome and the late replicating one was not cleaved due to methylation. A female-specific 5.3 kb fragment was also detected in samples from B3, H8 and D6 cells digested with *Ava*I and *Taq*I (data not shown).

By means of *Hpa*II-PCR, we studied DNA methylation of the H-7 *Hpa*II site in the promoter region of the *Pgk-1* gene, the methylation status of which is found to be correlated well with transcriptional silence (Singer-Sam *et al.*, 1989). DNA samples from B3, H8, D6 and female thymocytes yielded a 170 bp amplification product (G) whether or not *Hpa*II digestion was performed prior to the PCR reaction, indicating that the H-7 site on the precociously replicating X chromosome was methylated like that on the late replicating one (Figure 4b). The validity of this judgment was based on the detection of the control 133 bp PCR product (I) derived from the exogenously added internal standard in all lanes, and on the complete absence of the 170 bp amplification product from DNA samples of male and X0 thymocytes digested with *Hpa*II before PCR. The origin of the 700 bp band that appeared in every lane remains unknown. The G/I ratios suggested that the levels of methylation of three T-cell lymphoma cell lines were not statistically different from that of XX thymocytes (see legend to Figure 4b).

Jeppesen and Turner (1993) used antibodies specific to acetylated forms of histone H4 to show by immunofluorescence that the mammalian inactive X chromosome is deficient in acetylated H4. By means of an antibody specific to acetylated histone H4 derived from *Tetrahymena* macronucleus (Lin *et al.*, 1989), we evaluated the acetylation status of the precociously replicating X chromosome. In agreement with previous observation (Jeppesen and Turner, 1993), all chromosomes of D6 cells except for the late replicating inactive X chromosome were labeled with this antibody (data not shown). As shown in Figure 6, a single chromosome, except for a proximal block and a distal band, was scarcely labeled with the antibody in B3 cells. This labeling pattern is compatible with early replicating regions of the translocated X chromosome of B3 cell (see Figure 2). Thus, the precociously replicating X chromosome is most probably deficient in acetylated H4, as well as the late replicating one.

These findings unanimously showed that the precociously replicating X chromosome is not distinguished from the late replicating one, except for the remarkable differences in the timing of their replication.

**Figure 6.** Distribution of acetylated histone H4 in metaphase chromosome of the MTLB3 cell. A metaphase spread prepared from MTLB3 cells with fluorescently stained by Hoechst 33258 is shown in (A), and the indirect FITC labeling of the same cell using a rabbit antibody specific to acetylated histone H4 is shown in (B). The underacetylated pericentric heterochromatin regions (small arrow) and $X^T$ chromosome (large arrow) are indicated. On the $X^T$ chromosome, an autosomal region and band E remain hypoacetylated as illustrated.


A
B

***Time course of shift in replication timing of the precociously replicating X chromosome from B3 upon fusion with OTF9-63.***

As mentioned earlier, we found that the precociously replicating X chromosome from B3 turned into a late replicating one when OTF9-63 (Figure 7a) was fused with B3 (Figures 3b and 7b). To examine the time course of this change, we carried out further cell fusion experiments. As shown in Table 2, the replication timing of $X^T$ remained unchanged for the initial 10 h after cell fusion in almost all hybrid cells. Late replicating $X^T$ was found in ~8 % of hybrid cells harvested 2 h thereafter. It was evident that these cells were at the first mitotic metaphase after cell fusion in view of the chromosome banding pattern observed and BrdU labeling schedule employed. The frequency of cells having a late replicating $X^T$ increased to 19.7% and 63.9%, 15 and 24 h after cell fusion, respectively. It reached 95.2% 48 h after cell fusion and remained at the same level for another 24h. This shift in the chronology of X chromosome replication was limited to the X chromosomal region of $X^T$. It may be worth mentioning that $X^T$ did not show replication asynchrony in a considerable proportion of hybrid cells. Although our previous study (Takagi *et al.*, 1983) suggested that reactivation may not explain these synchronously replicating X chromosomes, those increasing gradually after day 4 probably reflect reactivation.

Asynchrony between chromosomes from B3 and OTF9-63 in hybrid cells was not rare during the first day of cell fusion. Hybrid cells harvested up to 24 h after cell fusion could be assigned to four different classes according to the presence or absence of BrdU incorporation into chromosomes from two parental cells: in class I, both B3 and OTF9-63 chromosomes incorporated BrdU and hence displayed replication bands (Figure 7b); in class II, only B3 chromosomes displayed replication bands (Figure 7c); in class III, only OTF9-63 chromosomes

displayed replication bands; in class IV, chromosomes from neither B3 nor OTF9-63 displayed replication bands. As shown in Table 3, late replicating $X^T$ was found only in hybrid cells belonging to class I, whereas $X^T$ remained precociously replicating in most class II hybrid cells. Replication timing of $X^T$ could not be determined in class III and class IV cells due to the lack of BrdU incorporation into B3 chromosomes. This finding strongly indicated that replication of the OTF9-63 genome is essential for the change in the chronology of $X^T$ replication induced by cell hybridization.

***Behavior of $X^T$ chromosome after fusion of B3 with various cell lines***

To examine the universality of what had happened in cell hybrids between B3 and OTF9-63, we fused B3 cells with seven different cell lines derived from potoroo (*Potorous tridactylis*), Chinese hamster, rat, mouse and man (Table 4). The $X^T$ chromosome was found to be late replicating in the majority of hybrids between thioguanine- and neomycin-resistant (TGN) clone of D6 and B3 (Figure 7d) and B3 x A9 hybrid cells, whereas $X^T$ from B3 and $X^N$ from H8 remained precociously replicating in B3 x H8TGN hybrids (Figure 7e). Both of the $X^N$s replicated late in S phase in H8 x D6TGN hybrid cells (Figure 7f). Genetic inactivity of the late replicating $X^T$ in B3 x A9 hybrids (Figure 3b) was inferred from its dark Giemsa staining (data not shown), positive *Xist* gene expression (Figure 4a), and methylation of several restriction sites in intron 1 of *Hprt* gene (Figure 5) and the H-7 site of the promoter region of the *Pgk-1* gene (Figure 4b).

In experiments involving other cell lines, the pattern of X chromosome replication was examined soon after cell fusion, because stable hybrid clones could not be isolated (Table 4). Although the numbers of cells observed were small, hybrid cells having a late replicating $X^T$ were found in every combination.

Thus, it is evident that the ability to switch the replication timing of the inactive X chromosome has been conserved in mammals from marsupial to human.

**Figure 7.** X chromosome replication patterns of OTF9-63 and four hybrid cells. **(a)** OTF9-63 metaphase cell with a synchronously replicating $X^N$ chromosome (small arrow), a large metacentric chromosome (small arrowhead) and four minute marker elements. **(b)** B3 x OTF9-63 hybrid metaphase cell 48 h after cell fusion. Note that B3 and OTF9-63 chromosomes replicate synchronously. The X chromosome segment of $X^T$ replicated late (large arrow), whereas $X^N$ derived from OTF9-63 (small arrow) and $X^{(7)}$ from B3 (arrowhead) replicated synchronously with autosomes. **(c)** B3 x OTF9-63 hybrid metaphase cell 24 h after cell fusion. $X^T$ (large arrow) remained unchanged. Note that the OTF9-63 genome, which did not incorporate BrdU, is characterized by the metacentric (small arrowhead) and four minute markers. **(d)** B3 x D6TGN hybrid metaphase cell having late replicating $X^T$ (large arrow) and $X^N$ (small arrow). **(e)** B3 x H8TGN hybrid metaphase cell retaining precociously replicating $X^T$ (large arrow) and $X^N$ (small arrow). **(f)** H8 x D6TGN hybrid metaphase cell with two late replicating $X^N$ chromosomes (small arrows).

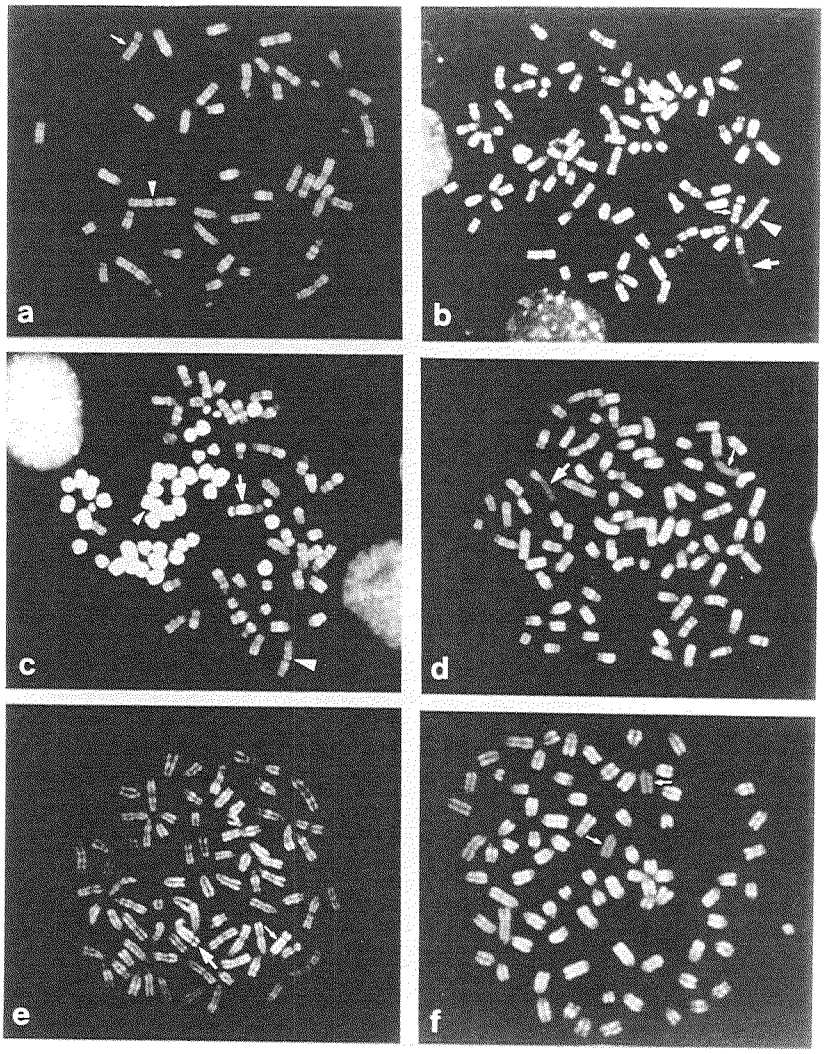

a
b
c
d
e
f

Table 2. Changes in replication timing of the inactive X chromosome from MTLB3 after fusion with OTF9-63

| Days after fusion | No. Of cells examined | No. of cells with* | | |
|---|---|---|---|---|
| | | $PRX^T$ | $SRX^T$ | $LRX^T$ |
| 0.4 (10h) | 57 | 53 (93.0)** | 4 ( 7.0) | 0 ( 0) |
| 0.5 (12h) | 139 | 93 (66.9) | 35 (25.2) | 11 ( 7.9) |
| 0.6 (15h) | 66 | 34 (51.5) | 19 (28.8) | 13 (19.7) |
| 1 | 97 | 21 (21.6) | 14 (14.4) | 62 (63.9) |
| 2 | 62 | 1 ( 1.6) | 2 ( 3.2) | 59 (95.2) |
| 3 | 71 | 2 ( 2.8) | 0 ( 0) | 69 (97.2) |
| 4 | 116 | 0 ( 0) | 22 (19.0) | 94 (81.0) |
| 5 | 95 | 0 ( 0) | 36 (37.9) | 59 (62.1) |
| 6 | 27 | 0 ( 0) | 16 (59.3) | 11 (40.7) |
| 7 | 63 | 0 ( 0) | 38 (60.3) | 25 (39.7) |
| 10 | 42 | 0 ( 0) | 30 (71.4) | 12 (28.6) |
| 11 | 34 | 0 ( 0) | 30 (88.2) | 4 (11.8) |
| 13 | 27 | 0 ( 0) | 23 (85.2) | 4 (14.8) |
| 14 | 38 | 0 ( 0) | 33 (86.8) | 5 (13.2) |

*$PRX^T$, precociously replicating $X^T$; $SRX^T$, $X^T$ replicating synchronously with autosomes; $LRX^T$, late replicating $X^T$. **percentage given in parenthesis.

Table 3. Necessity of OTF9-63 chromosome replication for the switch from early to late replication displayed by the inactive XT chromosome of MTLB3 in MTLB3 x OTF9-63 cell hybrids

| BrdU incorporation into | | Hours after | No. of cells | No. of cells with | | |
|---|---|---|---|---|---|---|
| MTLB3 | OTF9-63 | cell fusion | counted | $PRX^T$ | $SRX^T$ | $LRX^T$ |
| + | + | 10 | 41 | 38 | 3 | 0 |
| | | 12 | 120 | 84 | 25 | 11 |
| | | 15 | 57 | 28 | 16 | 13 |
| | | 24 | 76 | 6 | 8 | 62 |
| + | - | 10 | 16 | 15 | 1 | 0 |
| | | 12 | 19 | 9 | 10 | 0 |
| | | 15 | 9 | 6 | 3 | 0 |
| | | 24 | 21 | 15 | 6 | 0 |

Abbreviation are as in Table 2.

Table 4. X chromosome replication patterns in various hybrid cells

| Cells | Hours after fusion | No.cells examined | No. of cells with late replicating | | | | No. of cells with precociously replicating | | | | No. of cells without allocyclicX chromosome |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $X^TX^N$ | $X^NX^N$ | $X^T$ | $X^N$ | $X^TX^N$ | $X^NX^N$ | $X^T$ | $X^N$ | |
| B3 x A9 | >240 | 118 | - | - | 107 | - | - | - | 6 | - | 5 |
| B3 x D6TGN | >240 | 100 | 81 | - | 1 | 1 | 7 | - | 4 | 2 | 4 |
| H8 x D6TGN | >240 | 100 | - | 87 | - | 1 | - | 4 | - | 0 | 8 |
| B3 x H8TGN | >240 | 100 | 0 | - | 0 | 0 | 94 | - | 0 | 5 | 1 |
| B3 x $PtK_2$ | 12 | 36 | - | - | 20 | - | - | - | 12 | - | 4 |
| B3 x V79HP | 10 | 64 | - | - | 20 | - | - | - | 36 | - | 8 |
| | 13 | 20 | - | - | 10 | - | - | - | 8 | - | 2 |
| B3 x L6TG | 28 | 50 | - | - | 26 | - | - | - | 18 | - | 6 |
| B3 x HeLa | 27 | 28 | - | - | 4 | - | - | - | 22 | - | 2 |

# DISCUSSION

Late DNA replication and heteropycnosis are two of the most prominent properties of the genetically inactivated X chromosome (reviewed by Lyon, 1972). A second type of heterochromatic X chromosome that replicates early in S phase was previously reported in mice and in some laboratory animals (Takagi, 1974, 1978; Takagi *et al.*, 1982; Sugawara *et al.*, 1985). Although DNA replication asynchrony and heteropycnosis predict that the precociously replicating X chromosome is genetically inactive, the lack of a stable cell line having such a chromosome has hindered proof of this. The isolation of B3 and H8 cell clones thus provided us with a unique opportunity for the detailed characterization of this behaviorally interesting chromosome and for understanding the mechanism of the temporal control of chromosomal DNA replication. The present study showed that the precociously replicating X chromosome shared, as has been assumed so far, all properties with the genetically inactivated, late replicating X chromosome except replication early in S phase. Thus, late replication may not be directly responsible for the initiation of inactivation, but may possibly be involved in its maintenance. It is interesting that band E is consistently late replicating even in the precociously replicating X chromosome (see Figures 1a, 1b and 2).

Cell fusion sometimes induces noteworthy alteration in the chromosomal replication pattern. Thus, the late replicating X chromosome of the female mouse lymphocyte becomes synchronously replicating and genetically active when fused with the murine teratocarcinoma stem cell (Takagi *et al.*, 1983). In a similar hybrid cell line, Selig *et al.* (1988) found that the timing of replication of the centromeric heterochromatin of lymphocytes shifted from late to middle S phase. Complete reactivation of the inactive X chromosome was also induced in cell hybrids between female human chorionic villus cells and mouse A9 cells (Migeon

*et al.*, 1986). The last example would be band E of mouse chromosome 15, which is early replicating in normal T cells, but late replicating in T cell lymphoma (Somssich *et al.*, 1982). In any tetraploid hybrid between a lymphoma cell and another mouse cell, the bands E of all chromosome 15 are either early or late replicating. Hybrid cells with late replicating 15Es are tumorigenic, but those with early replicating 15Es are non-tumorigenic (Somssich *et al.*, 1984).

Despite the findings mentioned above, it was surprising to find that the precociously replicating X chromosome began to replicate late upon fusion with OTF9-63 teratocarcinoma stem cells. This striking switch in the time of replication suggests the existence of a potent mechanism to keep the inactivated X chromosome late or early replicating. In the trophectoderm and primitive endoderm cell lineages of mouse embryo, precocious replication precedes late replication (Takagi *et al.*, 1982). Taking these results together, it is tempting to postulate that early replication of the inactive X chromosome in B3 and H8 was due to lack of a cellular factor that renders the chromosome late replicating, and that this factor is supplied by the fusion partner or produced in hybrid cells by activation of a hitherto dormant gene by cell fusion, thus turning the precociously replicating X chromosome into a late replicating one. Selig *et al.* (1988) postulated a similar *trans*-acting cellular factor that controls the replication timing of the centromeric satellite sequences. The putative X chromosome-related cellular factor seems to be amply expressed, hence the inactive X chromosome is late replicating in most female somatic cells *in vivo* and *in vitro*. Occasional occurrence of the precociously replicating X chromosome in adult bone marrow cells, thymocytes, splenocytes and certain cancer cells may imply that it is depleted spontaneously from certain type of cells (Takagi *et al.*, 1984, 1986).

During S phase of the cell cycle, the genome of higher eucaryotes replicates in a bipartite manner separated by a well-demarcated or a somewhat indistinct

pause (Holmquist *et al.*, 1982). High-resolution G-banding of human chromosomes reveals ~2,000 separate bands averaging 1300 kb each (Yunis, 1981). Each band is a cluster of 12~100 DNA replication units (replicons) comprising 50~300 kb, and all replicons in a cluster synchronously initiate and terminate DNA synthesis (Hand, 1978). Replication of the eucaryotic chromosome is directed by the *cis*-acting replication origin. Although not much is known about replication origins in mammals, it is believed that there are more origins than seem necessary and that different origins initiate replication at different times in S phase (reviewed by Fangman and Brewer, 1992). The late replication of the inactive X chromosome and the isocyclic replication of the active one in the same cell of a female mammal seem to suggest either that the time of activation of an origin is not an inherent property of the origin, or that different replication origins are used for the replication of this chromosome according to the state of chromatin structure (Holmquist, 1987).

The late replicating X chromosome appeared for the first time 12 h after cell fusion, which implies that the *trans*-acting factors supplied by OTF9-63 or activated by cell fusion reacted quickly with the $X^T$ chromosome to make this shift possible. Since the average duration of $S+G_2$ was 14 h, replication of $X^T$, but not that of other chromosomes including the active X chromosome, must have been inhibited at the beginning of the first S phase after cell fusion. The simplest explanation would be that this putative factor suppresses those replication origins of $X^T$ that are to be activated at the beginning of S phase and leaves those origins to be activated at the end of the S phase to replicate the entire inactive X chromosome. It may further be speculated that this remarkable activity is present in the cell only during S phase, because the replication of OTF9-63 genome seems necessary for the switch from precocious to late replication. An intriguing question to be asked here is, why does the inactive X chromosome have to

replicate early in S phase in the absence of the factor?

Several lines of evidence suggest that changes in DNA methylation may be directly or indirectly involved in controlling replication timing. Chronology of satellite DNA replication altered dramatically in murine RAG fibroblasts treated with 5-azacytidine (5azaC)(Selig *et al.*, 1988). Similar observations were made with the constitutively heterochromatic X chromosome arms in *Microtus agrestis*: 5azaC treatment caused striking shift in their replication time from late to early S (Jablonka *et al.*, 1987). Furthermore, Paterno *et al.* (1985) demonstrated that 5azaC treatment converted the typical inactive X chromosome of C86S1A1 teratocarcinoma cell into genetically active and synchronously replicating one. The quick change in the replication timing found in the inactive X chromosome of fused cell, however, may not be consistent with massive change in the level of DNA methylation. Although the number of sites examined was small, we could not find any methylation difference between the late and precociously replicating X chromosome.

The present study showed that seven unrelated cell lines of dissimilar character were capable of turning the precociously replicating inactive X chromosome contributed by B3 into late replicating one. These cell lines were different in species origin, sexes, morphology in culture and chromosome constitution. Thus, the ability to switch replication timing of the inactive X chromosome is not correlated with the female sex or the XX sex chromosome constitution. It may be reasonable to assume that such a ubiquitous factor has functions other than simply confining replication of the inactive X chromosome to a short period in late S phase. A preliminary examination of autosomal replication patterns of cells having a late replicating X chromosome and those of B3, however, failed to reveal any consistent difference. This study was difficult because the replication pattern of each chromosome could potentially be altered by the

putative modifying factor and hence we had no unequivocal reference chromosome for comparison. A more refined approach may, however, disclose a particular genomic DNA fraction that becomes late replicating together with the change found in the inactive X chromosome.

# II. HETEROCHROMATINIZATION

## Behavior of an Inactive Human X Chromosome in the Mouse Genetic Background

# INTRODUCTION

The inactive human X chromosome introduced into cultured mouse heteroploid cells remains continuously inactive under appropriate culture conditions. This may indicate that the human X chromosome can be brought under control of the mouse X-inactivation machinery, in spite of the fact that the human and the mouse *Xist* genes share only limited nucleotide sequence similarity (Brockdorff *et al.*, 1992; Brown *et al.*, 1992). Alternatively, it may be that the human X chromosome is independent of the murine rule and the inactive state is maintained autonomously. Introduction of an inactive human X chromosome into mouse embryonal carcinoma (EC) cells is a potentially useful approach to determine if the human Xic can be regulated in mouse cells.

It has been shown that the inactivated X chromosome from a female mouse lymphocyte is activated after fusion with a mouse EC cell such as OTF9-63, PSA1 and LT-1, and essentially random *de novo* X-inactivation may occur in such hybrid cells when they are allowed to differentiate *in vitro* (Takagi, 1993). Rat Xic is functional in the mouse EC cells, as *de novo* X-inactivation occurs in near-tetraploid hybrid cell formed by a mouse PSA1(X0) cell and a male rat lymphocyte (Takagi, 1983). It appears that the hybrid cell was able to count both the mouse and the rat X chromosome in a nucleus and choose an X chromosome for inactivation. It has not been possible to apply the EC hybrid approach to an inactive human X chromosome, because no viable hybrids suitable for study have ever been isolated following hybridization of human lymphocytes with mouse EC cells. In the present study we adopted an alternative approach making use of microcell fusion to introduce a single inactive human X chromosome from a mouse-human somatic cell hybrid into a mouse EC cells.

Studies in monochromosome EC hybrids successfully isolated showed that

the human X chromosome shifted its replication timing from late to middle in the S phase and human X-linked genes which had been repressed in the donor human-mouse monochromosome hybrid cell (CF150) were derepressed in the new environment. The human *XIST* gene, on the other hand, remained transcriptionally active in spite of apparent reactivation of the X chromosome bearing it. Methylation status of certain CpG sites in the 5' region of the *XIST* gene was modified *de novo* in some of these hybrid clones. These and other findings suggest that the human *XIST* gene has diverged from its mouse homolog such that it is unable to respond to certain murine signals. In agreement with the finding made by Brown and Willard (1994), results obtained in this study seem to indicate that the inactive human X chromosome remains inactive in mouse fibroblasts simply because this chromatin state, once established, is maintained autonomously in most somatic cells irrespective of the activity state of the *XIST* gene. The present findings imply that heterochromatinization is not an attribute indispensable for maintenance of inactivation of the X chromosome, and support the view that the *Xist* gene is not directly involved in activation of the inactive X chromosome in EC-hybrid cells.

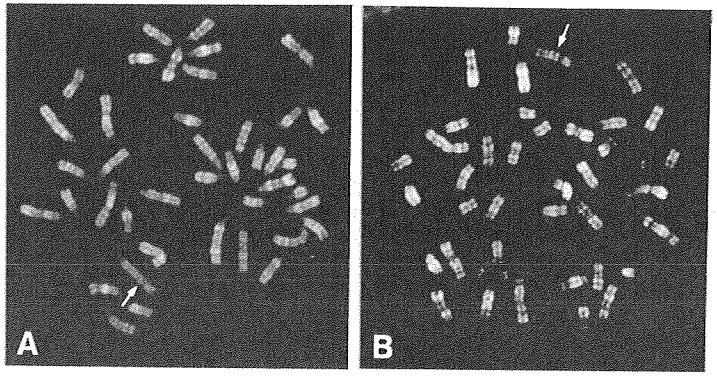
# MATERIALS and METHODS

## *Cell line*

PSA-TG8 is a 6-thioguanine (6-TG) resistant subclone of a mouse pluripotential teratocarcinoma cell line PSA1 (Martin *et al.*, 1978). The modal cell had 41 chromosome with a morphologically normal X chromosome (Figure 8A). OTF9-63 is an ouabain and 6-TG resistant, pseudonullipotent subline (Rosenstraus and Levine, 1979) of mouse F9 EC cell line. Modal cells had 43 chromosomes including a morphologically normal X chromosome and 4~5 minute elements (Fig. 8B). A human female and a male lymphoblastoid cell lines from normal donors were used for DNA and RNA isolation.

As microcell donor, we used two clones of mouse A9 cell line containing the human X chromosome as the only human element. CF150 cell line, a kind gift of Dr. T.K. Mohandas, Dartmouth-Hitchcock Medical Center, New Hampshire, U.S.A., had 1~6 morphologically normal, late replicating X chromosomes (Fig 11A). The *HPRT* gene on this X chromosome had undergone spontaneous reactivation before isolation of CF150 (Ellison *et al.*, 1992). A9(7149)-5, which is a kind gift of Dr. Mitsuo Oshimura, Tottori University, had 1~3 structurally normal, synchronously replicating active human X chromosome. HPRT-deficient HeLaTG contains one or more active X chromosome and its derivative(s), and human fibroblast cell IMR90 derived from a normal donor were obtained from the Japanese Cancer Resources Bank. Neomycin- resistant A9neo was isolated from A9 cells transfected with bacterial neomycin resistant gene. HHIX5 is a monochromosome hybrid of HeLaTG cell line comprising one or more human inactive X chromosome(s) from CF150 cells. The introduced human inactive X chromosome replicates late in S phase (data not shown).

**Figure 8.** R-banded metaphase chromosomes of (A) PSA-TG8 and (B) OTF9-63 used as recipient EC cell lines. Both cell lines have a single morphologically normal and synchronously replicating X chromosome (arrow).


A
B

*Cytogenetic examination*

Chromosome studies were done by a replication banding method involving 5-bromodeoxyuridine (BrdU) labeling in the late S phase and fluorescence staining with acridine orange. For fluorescence *in situ* hybridization (FISH), biotin-labeled HeLa DNA or FITC-conjugated human X chromosome-specific probe (Cambio, Cambridge, MA) was used. FITC signals were amplified by the serial treatment with anti-FITC goat IgG and FITC conjugated anti-goat IgG as previously described (Yoshida *et al.*, 1996). Chromosome preparation by cytocentrifuge and immunological detection of acetylated H4 were performed as described in the forgoing chapter.

***Microcell-mediated chromosome transfer.***

Chromosome transfer was performed as previously described by Koi *et al.* (1989) with slight modifications. Microcell donor cell lines grown in 25 $cm^2$ tissue culture flask were treated for 48 hours with Colcemid (0.05 $\mu$g/ml) for micronucleation. Microcells were obtained by centrifugation of monolayer cells in serum-free medium containing Cytochalasin B (10 $\mu$g/ml) at 10,000g for 60 min at 37°C. Microcell preparations serially filtered through polycarbonate filters with the size of 8, 5, and 3 $\mu$m were centrifuged, and mixed with recipient cells in serum-free medium containing phytohemagglutinin (5~10 $\mu$ g/ml). After fusion with polyethylene glycol (M.W. 1500), about 1.5 x $10^6$ cells were plated onto each 100 mm dish, and HAT selection was initiated 24 to 48 hours later. In experiments involving PSA-TG8, the selection medium contained leukemia inhibitory factor (LIF) and $\beta$-mercaptoethanol (BME) to prevent cell differentiation.

### *Cell fusion*

Cell fusion was carried out with the aid of polyethylene glycol (M.W. 1500) according to the method described in the preceding chapter. Hybrids were selected in HAT medium containing G418 (800$\mu$g/ml).

### *Probe*

A 5 kb DNA fragment was amplified from the first exon of human *XIST* with `Expanded Long PCR System' (Boehringer Mannheim). The nucleotide sequences of primers tagged with *Kpn*I or *Hin*dIII sites were: XI-F, AAGTCGACTCTCT CGGGGCTGGAAGCTTCCTGACT; XI-R3, AAGTCGACAGGTACCGACTC TGGGTTTCCAGCATCCCTTTCTATA. PCR products digested with *Kpn*I and *Hin*dIII were cloned into pBluescriptII SK(+) and used for Northern hybridization. This 5 kb *Hin*dIII/*Kpn*I fragment was further digested with *Bam*HI and a 1.1 kb *Bam*HI-*Hin*dIII fragment was cloned into pBluescriptII SK(+) for Southern hybridization. pR97E1 plasmid containing a 1.8 kb mouse *Xist* gene (Sado *et al.*, 1996) was also used for Southern hybridization. A 345 bp PCR product of mouse glycelaldehyde-3-phosphate dehydrogenase gene (*G3pdh*) was used as probe directly. Nucleotide sequences of primers used were: AGGTCGGTGTGAACGG ATTTGG and GAGATGATGACCCGTTTGGCTC. This probe was used for the human as well as for the mouse because of the high sequence similarity between these two species.

*RNA isolation and analysis*

Total cellular RNA was isolated from various cell lines by acid guanidium phenol chloroform method (Chomczynski and Sacchi, 1987), and digested with RQ1 RNase-free DNase (Promega, Madison, WI) before Northern blotting and reverse transcriptase-polymerase chain reaction (RT-PCR). Northern blotting was carried out according to the conventional method (Sambrook *et al.*, 1989) with 0.8% (w/v) agarose gel containing 0.66 M formaldehyde. Blots were hybridized with $^{32}$P-labeled cDNA probe at 42°C for 48 hours and washed once in 1 x SSPE, 0.1% (w/v) SDS at 65°C for 15 min, and twice in 0.1 x SSPE, 0.1% (w/v) SDS at room temperature for 60 min. Fujix BAS2000 image analyzer was used to quantitate radioactivity. RT-PCR was carried out as described in the first chapter. Nucleotide sequences of primers used for RT-PCR are shown in Table 5. Complete lack of amplification was substantiated in all samples in the absence of reverse transcription.

Table 5. RT-PCR assay conditions and nucleotide sequence of primers used

| Gene | Nucleotide sequence of primer set | Bases spanned | Cycles of PCR | Annealing condition |
|---|---|---|---|---|
| *TIMP* | gtcatcagggccaagttcgtg/agggagccacgaaactgc | 183-446 | 30 | 60℃, 1min |
| *POLA* | agggggaagatttagaagcc/actgccatactgaaatacat | 4925-5281 | 40 | 54℃, 1min |
| *AR* | aggaaagcgacttcaccgca/gagctccatagtgacaccca | 1896-2141 | 30 | 60℃, 1min |
| *MIC2* | acccagtgctggggatgact/tctccatgtccacctcccct | 315-679 | 35 | 54℃, 1min |
| *XIST* | tttcttactctctctctcggggct/tatagaaaagagagtggaagag | 43-365 | 30 | 60℃, 2min |
| | gagggaacatatgcagaggt/ttgacatccttctccgagaa | 10901-11390 | 30 | 58℃, 2min |
| | tttatgcagacacaaggaat/ttccaaacagcaaagactca | 112-260 | 30 | 58℃, 2min |
| *PGK1* | ttcctgctccgcccctaagt/gggagagaggtcggtgattc | 182-513 | 35 | 60℃, 1min |
| *HPRT* | taaaccacagcactattgag/ttgatgtgaaaattgactgc | 996-1178 | 30 | 54℃, 1min |
| *G6PD* | cctcacaagctctgagccct/atttgaggaatgtagctggg | 2004-2175 | 35 | 58℃, 1min |

*DNA isolation and analysis*

High molecular weight DNA was isolated from cultured cells as described previously (Sambrook *et al.*, 1989). A number of X-linked markers were examined by PCR or Southern blot hybridization in each hybrid cells to determine the extent of deletions, if any, in the human X chromosome. Primers for simple sequence repeat were purchased from Research Genetics (Huntsville, AL). Nucleotide sequences of other primers were: DXS56, GTGGGATTACTGTATT ATTTCAGA and ATACTCAGTAGCCACTACTC; DXS227, TATCAGAACAT GGTCCAGTT and CATGGGTGCCTGGTTTGTTT; *MIC2*, TATCTGTCCTGC CGCCTTCG and CGACCAGAACACCCAGCAG. *AR*, AGGAAAGCGACTTC ACCGCA and TGCCAGGGACCATGTTTTGC. Genomic DNA was digested with a large excess of *Eco*RV or *Eco*RI. After purification and quantification, 5 $\mu$ g of DNA was electrophoresed and blotted as described previously (Sambrook *et al.*, 1989). Blots were hybridized with a $^{32}$P-labeled 1.1 kb human *XIST* cDNA at 42°C for 24~48 hours. After hybridization, blots were washed in 1 x SSPE, 0.1% (w/v) SDS at 65°C for 15 min, and in 0.1 x SSPE, 0.1% (w/v) SDS at 65°C for 15 min twice.

To determine the methylation status of *XIST/Xist* alleles, 5 $\mu$g of the *Eco*RV or *Eco*RI digested DNA was further digested with one of methylation sensitive restriction enzymes, *Ava*I, *Sac*II, *Cfo*I, *Hpa*II and *Mlu*I or methylation insensitive *Msp*I. After the second digestion, all samples were precipitated with ethanol. Southern blot hybridization was done as described above.

# RESULTS

## *Activity of the human X chromosome in parental hybrid cell lines used for microcell fusion*

Human X chromosomes in CF150 cells replicated late in S phase (Figure 11A). In keeping with this cytogenetic observation, no expression of five genes which were known to be subject to X-inactivation, *TIMP, POLA, AR, PGK1,* and *G6PD*, was detected by RT-PCR. Three genes, *XIST, MIC2* in the pseudoautosomal region and *HPRT* were transcribed in this cell line (Table 8). The *HPRT* gene on this X chromosome was spontaneously reactivated before isolation of CF150 (Ellison *et al.*, 1992). Thus, the human X chromosome retained by CF150 was judged as the one genetically inactivated in consistent with previous data (Ellison *et al.*, 1992). We could not, however, find in CF150 cells any perinuclear-localized sex chromatin body at interphase (Figure 9), and chromosomes markedly understained with an anti-acetylated histone H4 antibody at metaphase (Figure 10), two of important traits characterizing the inactive X chromosome. These data suggest that the human X chromosome retains its inactive state without sex chromatin body formation and hypoacetylation of histone H4. The same human X chromosome was hypoacetylated and formed SCB, when it was transferred into HeLaTG cells (Figures 9, and 10). It is probable that certain human specific factor(s) is involved in the manifestation of these characters.

A cytogenetic study in A9(7149)-5 showed that the human X chromosome replicated synchronously with other chromosomes, and all human X-linked genes examined so far except *XIST* were expressed. The human X chromosome in this hybrid cell line was apparently the one conformed to the genetically active X chromosome.

**Figure 9.** Unsuccessful formation of sex chromatin body in interphase nuclei of CF150. Fixed monolayer cells grown on the slide glass stained with 4 % Giemsa (a, c, and e) prior to chromosome painting. The same cells after painting of the human X chromosome domains (b, d, and f). (a) The typical sex chromatin body is evident in 46,XX IMR90 cells, and (b) colocalization of the sex chromatin body with one of two painted human X-domains is observed in all nuclei (n=34). Remaining diffused signals define the active human X chromosomes. (c) No obvious sex chromatin bodies were observed in any nuclei of CF150 cells (n = 63), whereas (d) human X-domains in CF 150 cells are as condensed as the inactive X chromosome in IMR90 nuclei. (e, f) About 10 % of HHIX5 cells observed (n = 50) has sex chromatin bodies (arrow), suggesting the human inactive X chromosome introduced from CF150 into HeLaTG reforms sex chromatin body. HeLaTG has no sex chromatin bodies (n = 30; data not shown).

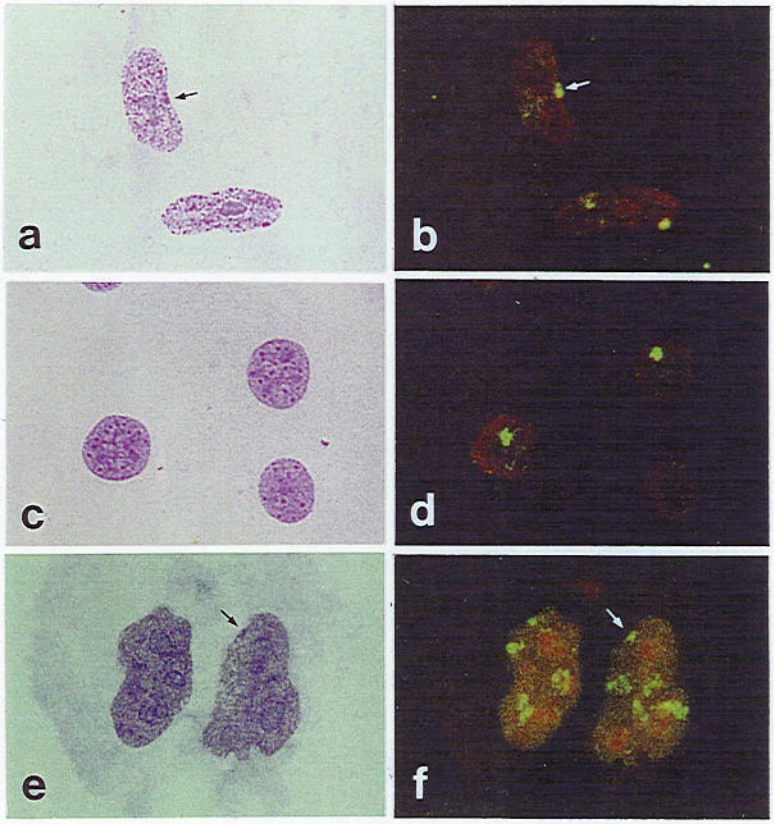
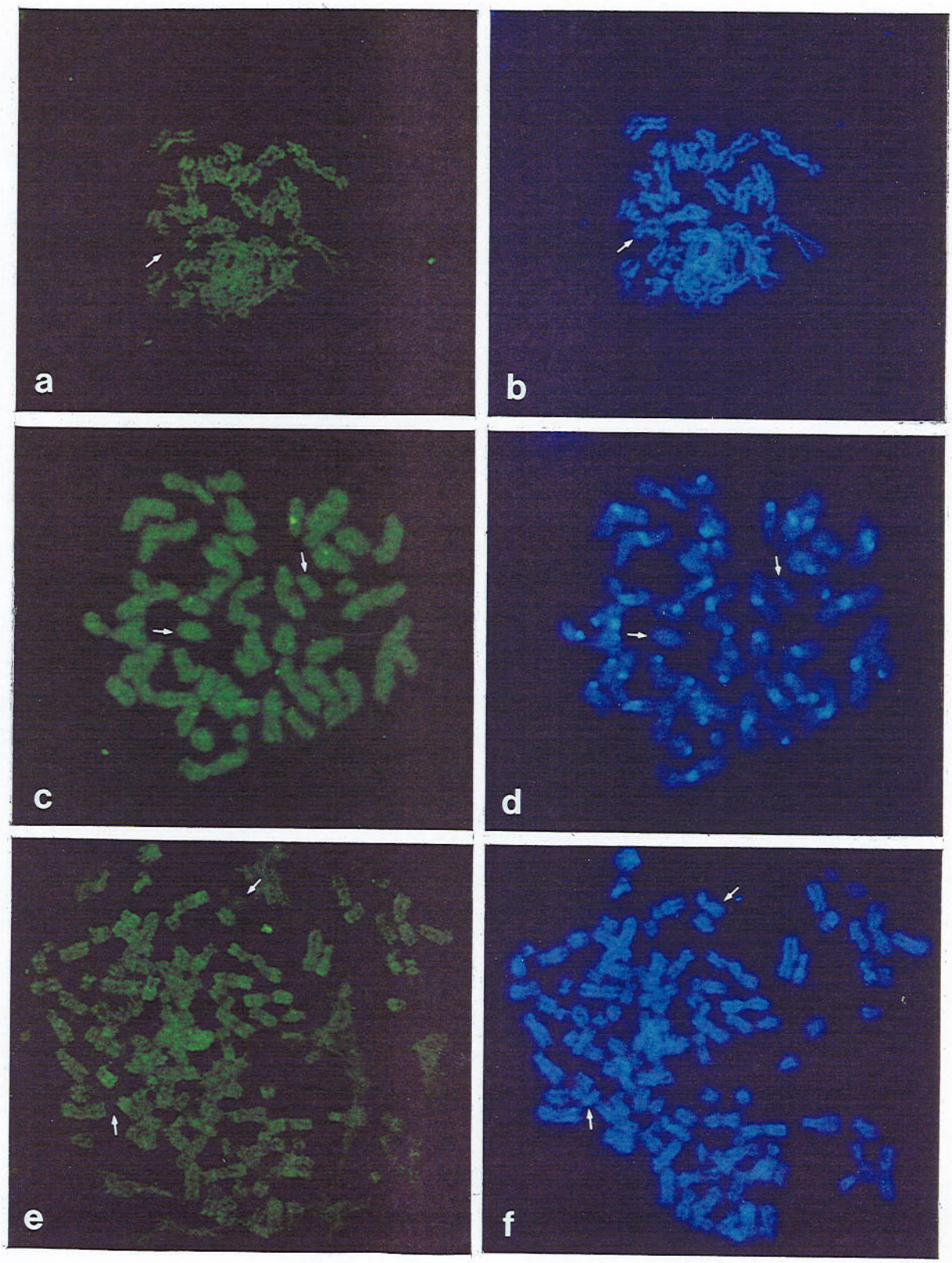

a
b
c
d
e
f

**Figure 10.** Acetylation of the human inactive X chromosome in CF150 cell. A metaphase spread prepared from various cell lines with fluorescently stained by Hoechst 33258 is shown in (b, d, f), and the indirect FITC labeling of the same cells using a rabbit antibody specific to acetylated histone H4 is shown in (a, c, e). The inactive X chromosome (arrow) in a human fibroblast IMR90 cell is apparently underacetylated (a, b). The human inactive X chromosomes (arrows) can be easily distinguished from mouse chromosomes by Hoechst 33258 staining in a metaphase from CF150, because all human chromosomes have an only small pericentric C-band, whereas most mouse chromosomes have a prominent C-band (d). The human X chromosomes (arrows) are not underacetylated in CF150 (c; n = 25). One or more underacetylated chromosomes are represent in HHIX5 cell (e, f; n=48). HeLaTG cell has no such chromosomes (n = 35; data not shown).


a
b
c
d
e
f

### *Introduction of the human inactive X chromosome into mouse EC cells*

We tried to transfer the inactive human X chromosome to reactivating-competent, near-diploid mouse EC cells by microcell fusion to study its behavior in undifferentiated mouse cells. We succeeded in isolating 11 (PXI) and 9 (OXI) HAT-resistant clones in experiments using PSA-TG8 and OTF9-63 as recipients, respectively. All of these hybrid clones were indistinguishable from parental EC cells in morphology. PXI clones tended to differentiate into epithelial-like cell and required LIF to maintain their undifferentiated state, whereas OXI clones remained undifferentiated in the absence of such reagent. Another feature common to both types of clones was that they acidified culture medium much more quickly than parental EC cell lines.

The modal chromosome number varied from 41 to 43 in PXI clones, whereas it was 41 in PSA-TG8, suggesting successful transfer of one or two chromosomes from CF150 cell. Replication banding and FISH studies showed that all PXI clones contained an apparently intact human X chromosome or its truncated derivative (Figures 11 and 12). However, each clone was by no means homogeneous (Table 6). In PXI-1, for example, out of 100 cells examined 74 had one, and one had two apparently intact human X chromosome, but one cell had an X chromosome segment inserted into a mouse chromosome. We could not demonstrate positive cytogenetic evidence for the presence of human X chromosome in the remaining 24 cells. A truncated human X chromosome was found in more than half of PXI-3 cells. No X chromosomal segment was detected with certainty in remaining cells. Such cells showing no hybridization signal ranged from 24% to 52% in PXI clones. We presumed that these cells retained a small X chromosomal region encompassing the *HPRT* gene, but the present FISH technique was not sensitive enough to demonstrate it.

Table 6. Human X chromosomes in PXI, OXI, and PXA hybrids revealed by FISH

| Clone | Modal chr. numbers | No. cells observed | Numbers of cell having following no. of apparently intact human X chromosome | | | deleted human X chr. | human X recombined with mouse chr. | no human X chr. |
|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | ≧3 | | | |
| CF150 | 58 | 100 | 44 | 33 | 13 | 5 | 1 | 4 |
| A9(7149)-5 | 53 | 100 | 74 | 1 | 2 | 12 | 1 | 1 |
| PXI-1 | 42 | 100 | 74 | 1 | 0 | 0 | 1 | 24 |
| PXI-2 | 41 | 50 | 18 | 2 | 0 | 12 | 1 | 17 |
| PXI-3 | 42 | 75 | 0 | 0 | 0 | 48 | 0 | 27 |
| PXI-4 | 42 | 50 | 23 | 0 | 0 | 1 | 0 | 26 |
| PXI-5 | 41 | 84 | 6 | 0 | 0 | 38 | 0 | 40 |
| PXI-6 | 41 | 50 | 0 | 0 | 0 | 25 | 0 | 25 |
| PXI-7 | 43 | 101 | 48 | 1 | 0 | 6 | 0 | 46 |
| PXI-8 | 42 | 143 | 0 | 0 | 0 | 122 | 0 | 21 |
| PXI-9 | 42 | 142 | 31 | 37 | 0 | 5 | 5 | 64 |
| PXI-10 | 41 | 200 | 140 | 2 | 0 | 1 | 0 | 57 |
| PXI-11 | 42 | 109 | 57 | 1 | 0 | 0 | 0 | 51 |
| OXI-1 | 42 | 74 | 1 | 0 | 0 | 42 | 11 | 20 |
| OXI-2 | 42 | 112 | 0 | 0 | 0 | 0 | 106 | 6 |
| OXI-3 | 42 | 59 | 2 | 0 | 0 | 11 | 40 | 6 |
| OXI-4 | 46 | 56 | 26 | 7 | 1 | 2 | 0 | 20 |
| OXI-5 | 42 | 183 | 1 | 0 | 0 | 131 | 0 | 51 |
| OXI-6 | 43 | 66 | 2 | 0 | 0 | 10 | 40 | 14 |
| OXI-7 | 43 | 24 | 0 | 0 | 0 | 12 | 7 | 5 |
| OXI-8 | 42 | 59 | 0 | 0 | 0 | 21 | 13 | 25 |
| OXI-9 | 43 | 64 | 0 | 0 | 0 | 5 | 39 | 20 |
| PXA-2 | 42 | 100 | 28 | 0 | 0 | 14 | 9 | 49 |

The modal chromosome number was 43 in OTF9-63, and it was 42~43 in all OXI clones except OXI-4 which had 46 chromosomes (Table 6). In contrast to PXI clones, the human X chromosome was apparently morphologically intact in only a small proportion of cells and deletion or truncation was frequent in OXI clones. Cellular differences between the two recipient EC cell lines could be responsible for the differential stability of the introduced human X chromosome.

***Reactivation of the human inactive X chromosome introduced into mouse EC cells***

In PXI and OXI clones, all human X chromosomes or their derivatives replicated synchronously with other mouse chromosomes (Figures 11B, 11C and 11D), which suggested that the human inactive X chromosome transferred from CF150 was reactivated in mouse EC cells after microcell fusion. To prove reactivation, expression of several X-linked genes was analyzed in PXI or OXI clones by RT-PCR. We also conducted genomic PCR to distinguish between transcriptional silencing and physical loss of genes (Figure 13 and Table 7).

The human *HPRT* transcripts were detected in all PXI and OXI clones as expected because these clones were selected in HAT medium. Three genes, *POLA, AR,* and *G6PD*, normally subject to X-inactivation and *MIC2* gene which is known to escape inactivation were expressed in all clones that retained them (Figure 14 and Table 8). Results were less consistent in the *TIMP* gene localized on the proximal short arm: RT-PCR products were not recovered in 9 clones, but transcriptional silencing was likely only in 4 OXI clones. Apparent loss of *POLA* and *AR* flanking *TIMP* strongly indicated that the *TIMP* gene had been deleted together with *POLA* and *AR* in remaining 5 clones. We could not detect *PGK1*

**Figure 11.** Switch from late to synchronous replication of the transferred X chromosome from CF150 to mouse EC cells. (A) Two late replicating human X chromosomes (arrows) in a CF150 cell. (B) An apparently normal human X chromosome replicating synchronously (arrow) in a PXI-1 hybrid cell. (C) A deleted human X chromosome replicating synchronously (arrow) in a PXI-3 hybrid cell. (D) A morphologically intact human X chromosome replicates synchronously (arrow) in an OXI-4 hybrid cell.

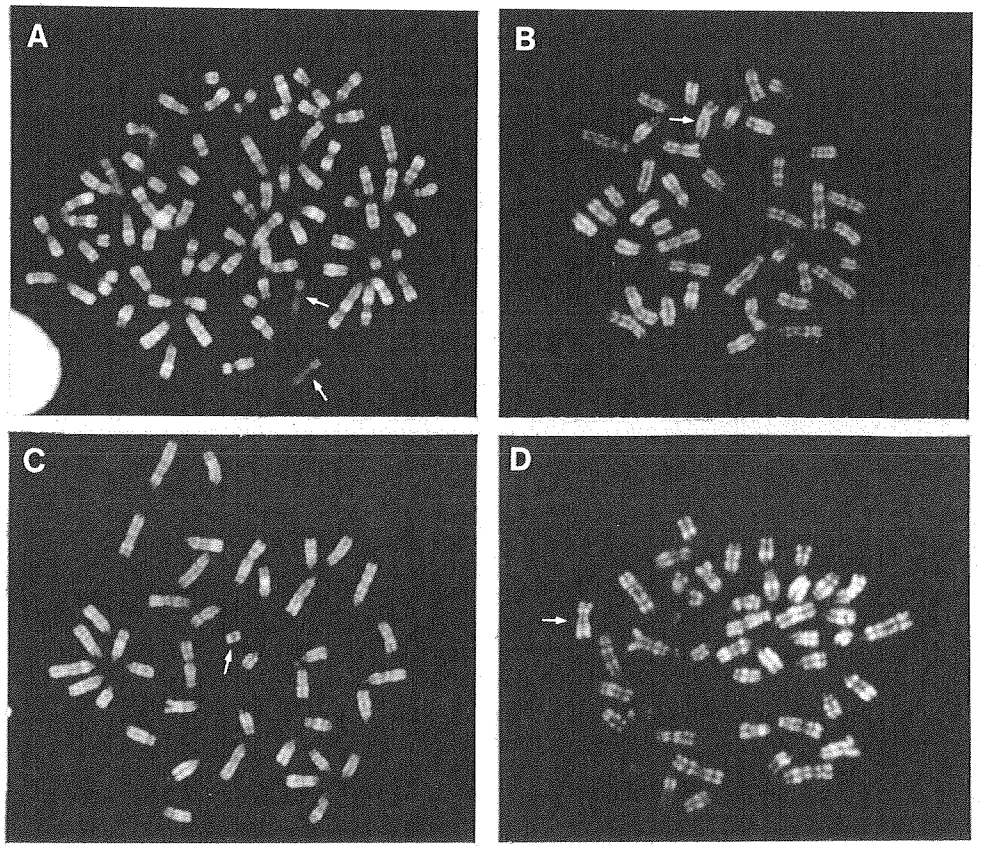
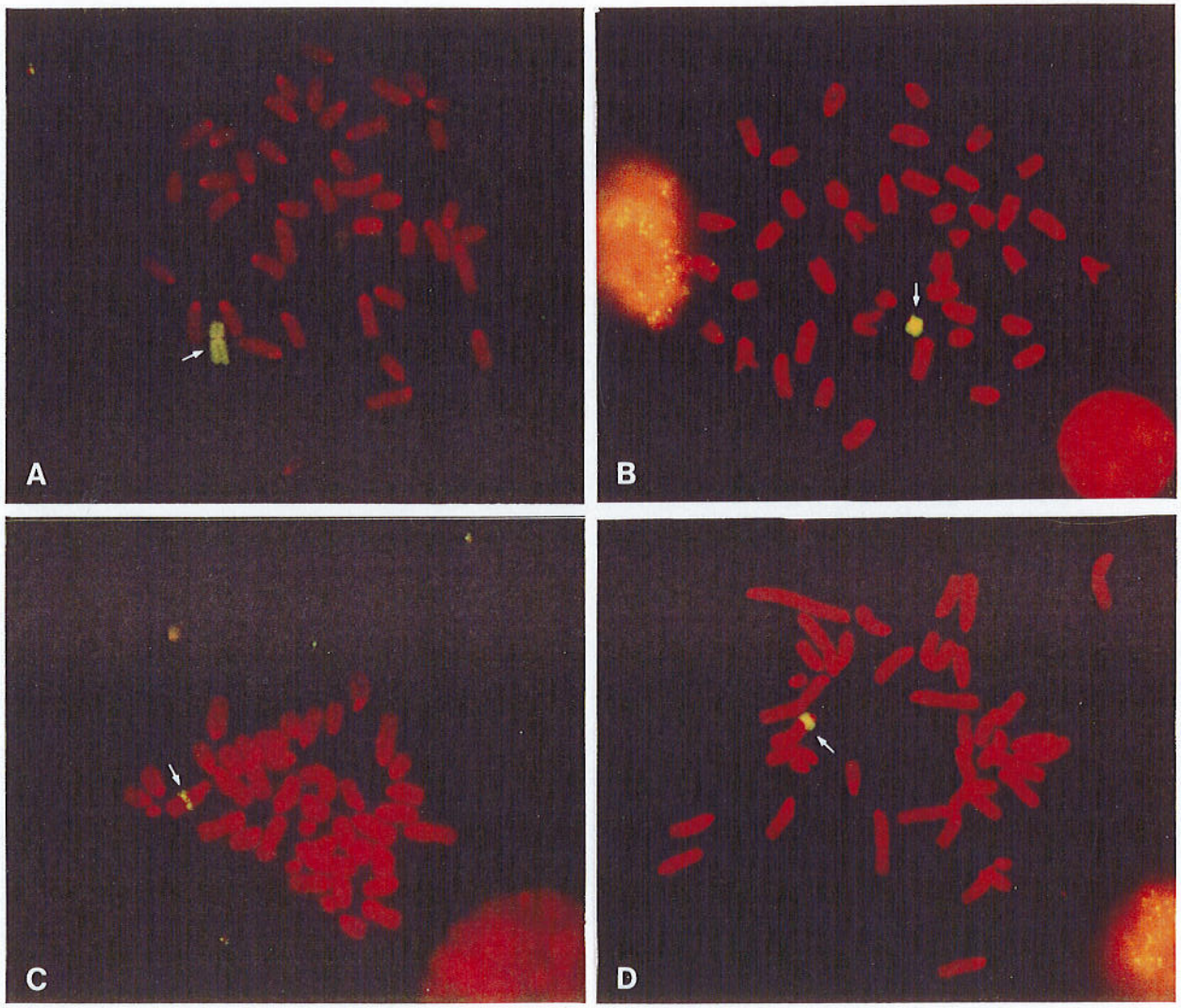

A
B
C
D

**Figure 12.** Human X chromosome and its derivative in (A) PXI-1, (B) PXI-3, (C) OXI-3, and (D) OXI-8 hybrid cells revealed by *in situ* hybridization with biotin-labeled total human DNA (arrows). The hybridization signal was visualized by anti-biotin goat IgG and FITC-conjugated anti-goat IgG.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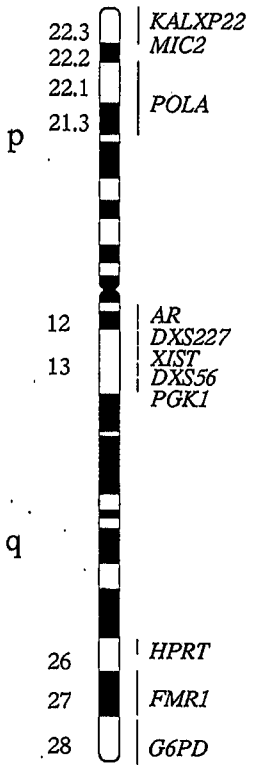



**Figure 13.** Distribution of human X chromosome markers examined in PCR analysis.

**Figure 14.** Transcriptional activity of human X-linked genes in PXI and OXI clones revealed by RT-PCR. The *MIC2* primers amplified a 365 bp human-specific product in all RNA samples from cells with human X chromosomes, as the pseudoautosomal *MIC2* at Xp22.3 is expressed from both the active and inactive X chromosomes. *POLA* at Xp22.1-p21.3 and *AR* at Xq11-12 are subject to X-inactivation. Amplification of 357 and 246 bp products showed that these genes were reactivated in PXI and OXI clones. *XIST* at Xq13 usually expressed from the inactive X chromosome was persistently transcribed in PXI and OXI clones whose human X chromosome had apparently been reactivated. Loss of genes by chromosomal deletion rather than changes in gene activity explains the lack of amplification in certain hybrid clones (cf. Table 6).

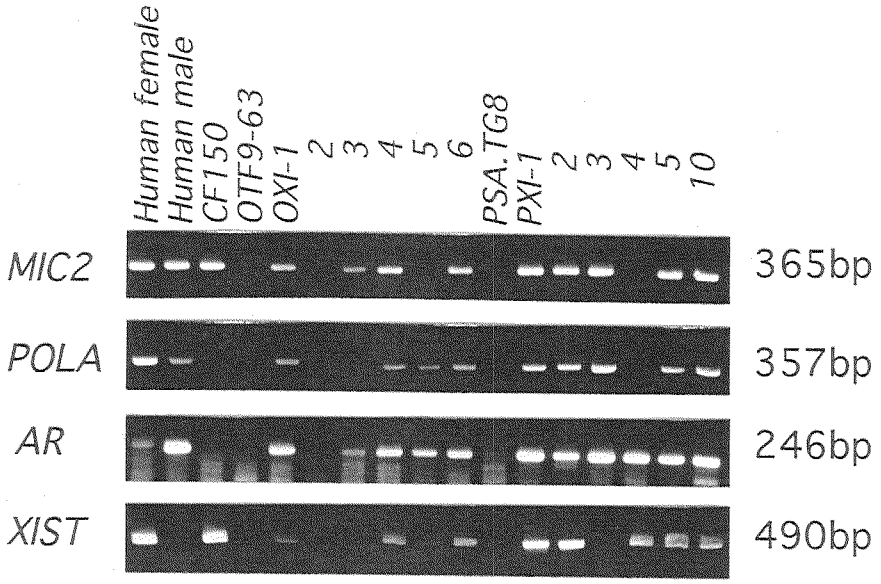

Human female
Human male
CF150
OTF9-63
OXI-1
2
3
4
5
6
PSA.TG8
PXI-1
2
3
4
5
10
MIC2
365bp
POLA
357bp
AR
246bp
XIST
490bp

Table 7. Composition of the human X chromosome in PXI, OXI, and PXA hybrids determined by genomic PCR and Southern hybridization (*)

| | PXI | | | | | | | | | | | OXI | | | | | | | | | CF150 | A9(7149)-5 | PXA-2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | | | |
| *MIC2* | + | + | + | - | + | - | + | - | + | + | + | + | - | + | + | - | + | - | - | - | + | + | - |
| *KALXP22* | + | + | + | - | + | - | + | + | + | + | + | + | - | + | + | + | + | + | + | - | + | + | - |
| *POLA* | + | + | + | - | + | - | + | + | + | + | + | + | - | + | + | + | + | - | + | - | + | + | - |
| *AR* | + | + | + | + | + | - | + | + | + | + | + | + | - | + | + | + | + | - | + | - | + | + | + |
| DXS227 | + | + | + | + | + | - | + | + | + | + | + | + | - | + | + | + | + | - | + | - | + | + | + |
| *XIST*[a] | + | + | + | + | + | - | - | - | + | + | + | + | - | - | - | - | - | - | - | - | + | + | + |
| *XIST* | | | | | | | | | | | | | | | | | | | | | | | |
| 5' ex1 | + | + | + | + | + | - | + | + | + | + | + | + | - | + | + | + | + | - | + | - | + | + | + |
| 3' ex1 | + | + | + | + | + | - | + | + | + | + | + | + | - | + | + | + | + | - | + | - | + | + | + |
| 3' ex8 | + | + | + | + | + | - | + | + | + | + | + | + | - | + | + | + | + | - | + | - | + | + | + |
| DXS56 | + | + | + | + | + | - | + | + | + | + | + | + | - | + | + | + | + | - | + | - | + | + | + |
| *PGK1* | + | + | + | + | + | - | + | + | + | + | + | - | - | - | + | + | + | - | + | - | + | + | + |
| *HPRT* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *FMR1* | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | - | + | + | - | + | + | + |
| *G6PD* | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + |

[a]Determined by Southern hybridization

Table 8. Expression of human X-linked genes in PXI, OXI, and PXA hybrids

| | PXI | | | | | | | | | | | OXI | | | | | | | | | CF150 | A9(7149)-5 | PXA-2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | | | |
| *MIC2* | + | + | + | (-) | + | (-) | + | (-) | + | + | + | + | (-) | + | + | (-) | + | (-) | (-) | (-) | + | + | (-) |
| *POLA* | + | + | + | (-) | + | (-) | + | + | + | + | + | + | (-) | + | + | + | + | (-) | + | (-) | - | + | (-) |
| *TIMP*[a] | + | + | + | (-) | + | (-) | + | + | + | + | + | + | (-) | - | + | (-) | - | (-) | - | (-) | - | + | + |
| *AR* | + | + | + | + | + | (-) | + | + | + | + | + | + | (-) | + | + | + | + | (-) | + | (-) | - | + | + |
| *XIST* | + | + | - | + | + | (-) | + | + | + | + | + | + | (-) | (-) | + | + | + | (-) | (-) | (-) | + | - | - |
| *PGK1* | + | + | + | - | + | (-) | + | + | + | + | + | (-) | (-) | (-) | + | + | + | (-) | - | (-) | - | + | + |
| *HPRT* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *G6PD* | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | - | + | + |

Note. +, active; -, inactive; (-), deleted. [a]Genomic PCR was unsuccessful

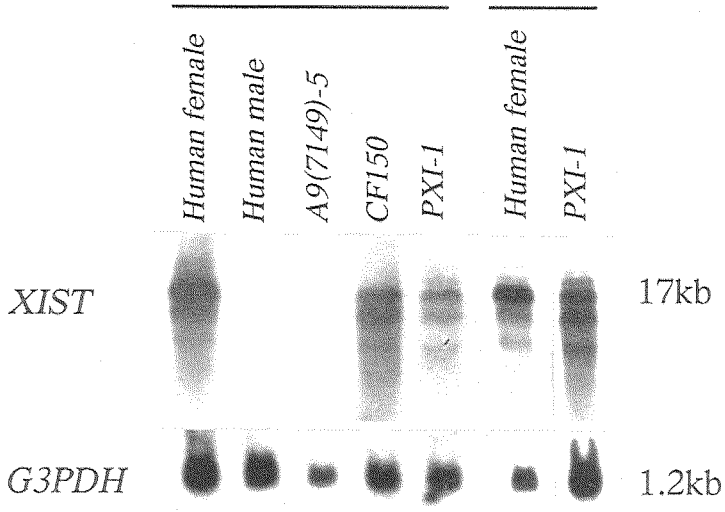
transcription in PXI-4 and OXI-8. The present cytogenetic and RT-PCR data, though not unanimous, were consistent with the view that the human inactive X chromosome transferred to PSA1 and OTF9-63 mouse EC cell lines is activated.

***Persistent XIST expression from the reactivated human X chromosome***

Unexpectedly, our RT-PCR study showed that the human *XIST* gene, though carried by an activated X chromosome, did not stop transcription in 13 out of 16 PXI and OXI clones (Figure 14 and Table 8). We conducted Northern blot hybridization to examine the possibility that RT-PCR picked up *XIST* transcripts limited to a minority of hybrid cells whose human X chromosome had not been activated. Figure 15 showed three clear *XIST* mRNA bands in a female lymphoblastoid cell line, CF150, and PXI-1. No *XIST* signal was detected in a male lymphoblastoid cell line and A9(7149)-5 hybrid cell. The level of *XIST* expression in PXI-1 normalized by the activity of *G3pdh* was 50% that in CF150, and 29~46% that in the female lymphoblastoid cell line. Since the average copy number of active *XIST* per cell was 0.8 in PXI-1 and 1.6 in CF150 (Table 6), the level of *XIST* expression seemed roughly identical in these cell lines. By the same token, the level of *XIST* expression in PXI-1 was estimated at 36~57% of that in female lymphoblastoid cell line.

In spite of chromosomal reactivation, two mouse EC cell lines used in this study failed to turn off the *XIST* gene possibly with the exception of PXI-3, OXI-3 and OXI-8. Southern hybridization and PCR analysis showed that the *XIST* gene and its neighboring markers (*AR*, DXS56, and DXS227) were apparently present in these three exceptional clones. In OXI-3 and OXI-8 clones, only genomic PCR revealed the existence of the *XIST* gene. Similar results were also obtained in *HPRT* and *PGK1* loci (data not shown), suggesting that small fraction of cells

**Figure 15.** Expression of the human *XIST* gene in A9(7149)-5, CF150 and PXI-1 cells. The *XIST* probe detect discrete messages of at least three different sizes in human female lymphoblastoid, CF150 and PXI-1 cells, but none in human male lymphoblastoid and A9(7149)-5 cells. The same filter was reprobed with a mouse *G3pdh* probe to show that RNA specimens were intact and to normalize the level of *XIST* expression. The relative level of the *XIST* expression thus obtained were 3.54 for a female lymphoblastoid cell line, 2.08 for CF150 and 1.04 for PXI-1. Independent experiment showed that the values were 0.89 for the female lymphoblastoid cell line and 0.41 for PXI-1.


Human female
Human male
A9(7149)-5
CF150
PXI-1
Human female
PXI-1
XIST
G3PDH
17kb
1.2kb

retained the human X chromosome(s) or its derivative(s) in these clones (cf. Table 6). It is likely that the *XIST* gene is repressed in these cells.

It would be possible that the human *XIST* gene is turned on in the milieu of the mouse EC cell lines. This possibility was examined by introduction of a human active X chromosome from A9(7149)-5 hybrid cell into PSA-TG8. PXA-2 clone thus isolated was identical to PSA-TG8 in morphology, and about 40% of cells retained a synchronously replicating human X chromosome deleted at the distal segment of short arm (Table 7). No sign of *XIST* activity was detected in the clone, whereas every human X-linked gene retained was transcriptionally active (Table 8).

***Methylation status of the 5' region of XIST gene in PXI and OXI clones***

A cluster of CpG sites in the 5' region of the *XIST/Xist* gene is fully methylated on the active X chromosome, whereas it is almost completely unmethylated on the inactive X chromosome (Hendrich *et al.*, 1993; Norris *et al.*, 1994). These CpG sites are partially methylated in undifferentiated mouse EC and ES cells in which *Xist* is not transcribed (Sado *et al.*, 1996). It would be of special interest to see methylation status of the human and the mouse allele of this gene (Figures 16A and 17A) in PXI and OXI clones. Analysis of the mouse allele was done according to the method we employed earlier (Mise *et al.*, 1996). With the exception of minor differences at *Cfo*I sites, the methylation pattern of the mouse *Xist* allele in all PXI and OXI clones closely resembled that reported in PSA1 and OTF9-63 (Figure 16B). It has been reported (Hendrich *et al.*, 1993) that two *Sac*II sites, one *Hha*I (*Cfo*I) site and one *Ava*I site in the 5' region of the human *XIST* gene studied here are differentially methylated on the active and unmethylated on the inactive X chromosome (Figure 17A). Southern blots of genomic DNA

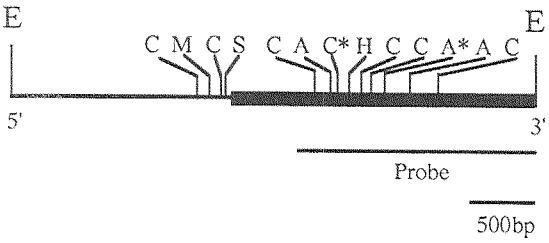
digested with *Eco*RV in combination with one of three methylation-sensitive restriction enzymes were hybridized with the probe shown in Figure 17A. In the case of double digestion with *Eco*RV and *Sac*II, full methylation of *Sac*II site gives rise to 1.3 kb band, whereas unmethylation gives rise to 1.2 kb band. As shown in Figure 17B, only the 1.3 kb band appeared in a human male lymphoblastoid cell line, A9(7149)-5, and PXA-2 clone, and only the 1.2 kb band appeared in CF150. Both 1.2 and 1.3 kb bands most probably corresponding to the active and the inactive *XIST* allele were visualized in a female lymphoblastoid cell line. Methylation status of this site varied considerably in PXI and OXI clones. Only the 1.2 kb band was detected in PXI-1, PXI-2, and PXI-11 as in CF150. The 1.3 kb band was heavier than the 1.2 kb band in PXI-3 and PXI-5, whereas the reverse was true in PXI-4 and PXI-9. These results suggest that *de novo* methylation occurred at this site to a varying degree. Demethylation was not detected in PXA-2 clone. Similar results were obtained for the *Ava*I and the *Hha*I site (data not shown).

Correlation between CpG methylation and expression of *XIST* remained elusive in PXI and OXI clones. Almost full methylation in the 5' region of *XIST* was compatible with expression in PXI-5, whereas similarly methylated *XIST* was not expressed in PXI-3. The use of RT-PCR might have complicated the situation. It is possible that *XIST* expression detected in certain monochromosome hybrid clones did not represent the whole cell population but only a small proportion of cells having unmethylated *XIST*. We indeed detected *XIST* expression in PXI-7 and PXI-8 clones by 30 cycles of RT-PCR, but the presence of the *XIST* gene itself was verified only by genomic PCR (30 cycles) not by Southern hybridization. An alternative possibility would be that *XIST* expression is independent of methylation status in undifferentiated cells. Beard *et al.* (1995) showed that the hypomethylated *Xist* gene expresses at a low level in undifferentiated male

embryonic stem (ES) cells deficient in DNA methyltransferase, but it is highly expressed not only in their differentiated derivatives *in vitro* but in male embryo *in vivo*. It is possible that PXI-5 was slightly more differentiated than PXI-3, and that *XIST* expression restricted to PXI-5, in spite of similar methylation pattern in these clones.

**Figure 16.** Methylation status of the 5' end of the mouse *Xist* gene as revealed by Southern blot analysis. (A) Partial map of *Xist* gene showing methylation sensitive enzyme sites cited from Mise *et al.* (1996). The thick solid line indicates the 5' end of the first exon. E, *Eco*RI; A, *Ava*I; C, *Cfo*I (=*Hha*I); H, *Hpa*II; M, *Mlu*I; S, *Sac*II. Asterisks indicate the presence of two restriction sites recognized by one enzyme. The relative position of probe is indicated under the restriction map. (B) Methylation status of *Xist* in PSA-TG8 and PXI-1. Following *Eco*RI digestion, genomic DNA was further digested with one of five methylation sensitive restriction enzymes. Partial methylation was evident at every analyzable CpG site except the *Mlu*I site. Molecular sizes (kb) are marked on the left.

(A)


E
E
C M C S C A C* H C C A* A C
5'
3'
Probe
500bp

(B)

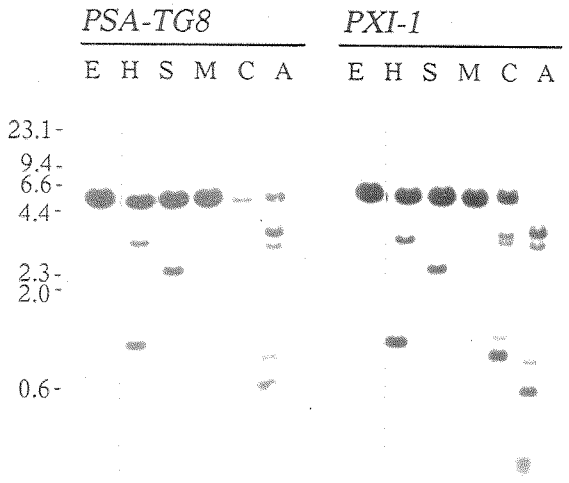
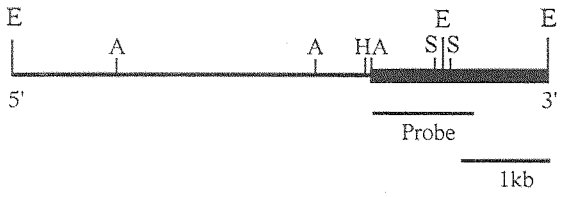
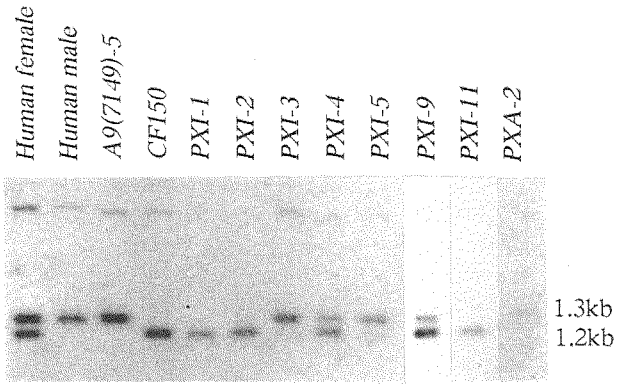

PSA-TG8
PXI-1
E H S M C A
E H S M C A
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
0.6-

**Figure 17.** Methylation status of the 5' end of the human *XIST* gene as revealed by Southern blot analysis. (A) Partial map of *XIST* gene showing the location of methylation sensitive enzyme site cited from Hendrich *et al.* (1993). The thick solid bar indicates the 5' end of the first exon. E, *Eco*RV; A, *Ava*I; H, *Hha*I; S, *Sac*II. The relative position of the probe used in this study is indicated under the restriction map. According to Hendrich *et al.* (1993), differential methylation was found at the *Hha*I site, the 3' *Ava*I site and two *Sac*II sites. (B) Methylation status of *XIST* in female and male lymphoblastoid cells, CF150 and PXI cells. Genomic DNA digested with *Sac*II in combination with *Eco*RV produces 1.3 or 1.2 kb bands corresponding unmethylated or methylated *Sac*II sites.

(A)


E
A
A
HA
E
S
S
E
5'
3'
Probe
1kb

(B)


Human female
Human male
A9(7149)-5
CF150
PXI-1
PXI-2
PXI-3
PXI-4
PXI-5
PXI-9
PXI-11
PXA-2
1.3kb
1.2kb

### *Mouse genome is unable to modify the activity of the human XIST gene*

Undifferentiated state of PXI and OXI clones might have been responsible for *XIST* expression from the human active X chromosome. To explore this possibility, we hybridized PXI-1 with mouse A9 fibroblast. Six fibroblast-like PXI-1 x A9 hybrids clones (P1A), products of 1:1 fusion, had a synchronously replicating human X chromosome and continued to express all human X-linked genes examined here including *XIST*, although repression of *PGK1* was detected frequently (Table 9). Thus, expression of the *XIST* gene was not correlated with undifferentiated state of PXI clones. *XIST* expression was never detected in 6 fibroblastic P3A clones of hybrid between PXI-3 and A9 as in the parental PXI-3. Remaining X-linked genes examined were expressed uniformly in these clones. These findings suggest that the mouse genome is not capable of controlling the activity of the human *XIST* gene.

### *XIST expression is not modified in HeLa cells of human origin*

To examine the potency of human genome to modify *XIST* expression, we transferred the human X chromosome from P1A-5 and P3A-3 hybrid clones to HeLaTG cells. The *XIST* gene was active in P1A-5, but was completely inactive in P3A-3. Ten times more monochromosome hybrids were obtained from microcell fusion involving HeLaTG cells than mouse EC cells as recipients. Since these monochromosome hybrids morphologically indistinguishable from HeLaTG cells had endogenous active X chromosomes, activity of X-linked genes on the introduced X chromosome was not determined. *XIST* mRNA was detected easily in all hybrid clones which received an X chromosome from P1A-5, whereas it was below the level of detection in hybrid clones which received an X chromosome

from P3A-3 (data not shown). Taken together, we may conclude that the activity state of *XIST* was stably maintained in the milieu of the human and mouse somatic cell once it had been established during embryonic development.

***Human Xic does not function in mouse EC cells***

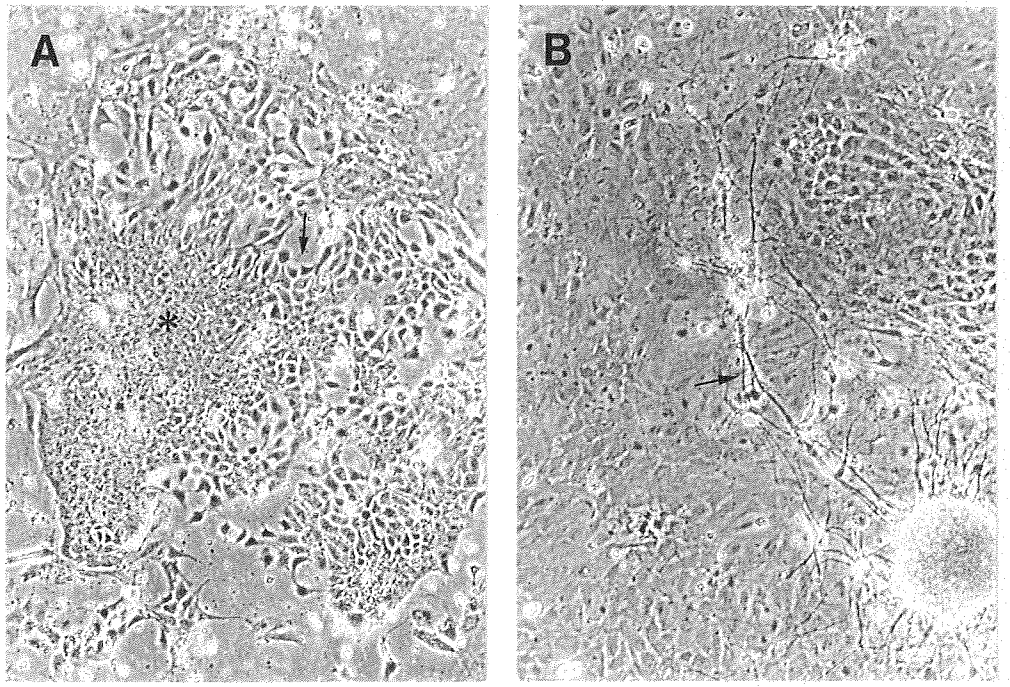
Finally, we examined cytogenetically whether or not human Xic is functional in mouse cells. In practice, we looked for the occurrence of late replicating X chromosome in PXI clones differentiating with the aid of retinoic acid (RA). After RA treatment, cell populations containing flat epithelial cells and neuron-like cells to a considerable extent were obtained in every independent experiments (Figure 18). Replication study failed, however, to demonstrate any positive evidence for the occurrence of X-inactivation about 200 suitably labeled PXI cells which retained a human X chromosome(s). No late replicating X chromosome was found in OXI clones, either. Thus, we conclude that human Xic is not functional in mouse EC cells.

Table 9. Expression of human X-linked genes in PXI x A9neo hybrids

| | P1A | | | | | | P3A | | | | | | A9neo |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | |
| *MIC2* | + | + | + | + | (-) | + | + | + | + | + | + | + | - |
| *POLA* | + | + | + | + | + | + | + | + | + | + | + | + | - |
| *TIMP* | + | + | + | + | + | + | + | + | + | + | + | + | - |
| *AR* | + | + | + | + | + | + | + | + | + | + | + | + | - |
| *XIST* | + | + | + | + | + | + | - | - | - | - | - | - | - |
| *PGK1* | - | - | - | + | - | - | + | + | + | + | + | + | - |
| *HPRT* | + | + | + | + | + | + | + | + | + | + | + | + | - |
| *G6PD* | + | + | + | + | + | + | + | + | + | + | + | + | - |

Note. +, active; -, inactive; (-), deleted.

**Figure 18.** Differentiation of PXI-1 cells induced by retinoic acid treatment. (A) Flat epithelial-like cells (arrow) emerged in the peripheral region of an apparently undifferentiated PXI-1 colony (*). (B) Neuron-like cells (arrow) appearing on the lawn of flat epithelial-like cells.


A
B

# DISCUSSION

The established mouse cell line is capable of maintaining the inactive state of the human X chromosome as a solitary human element as shown in the case of CF150 cell line used here, although the inactivation state is not completely maintained in these cell lines. The same chromosome was reactivated in mouse EC cell line, PSA-TG8 and OTF9-63, when it was transferred by microcell fusion. These observations suggest that the activity state of the human X chromosome is under the control of mouse genome, and, in particular, species specific regulatory factors except those coded by the inactive X chromosome are not required for the process of reactivation. If so, these mouse EC cells may be able to reactivate the inactive X chromosome derived from any other mammalian species introduced individually.

Although reactivation found in PXI and OXI clones appeared complete on the basis of switch from late to synchronous replication, and re-expression of certain X-linked genes silenced in CF150, it is apparently incomplete because the *XIST* gene continued to be expressed from the human X chromosome at a level comparable to that in CF150. Transcription of the *XIST* gene on the "reactivated" X chromosome could not be suppressed in HeLa human tumor cell line as well as A9 mouse fibroblast cell line. Complete reactivation of the inactive X chromosomes so far known *in vivo* and *in vitro* accompanies silencing the *XIST/Xist* gene. Thus, *Xist* is expressed in mouse oogonia which have an inactive X chromosome, but it is repressed in oocytes in which X-inactivation is reversed (McCarrey and Dilworth, 1992). Luo *et al.* (1995) noted complete or nearly complete suppression of *XIST* transcription in hybrid cells between human female chorionic villus cells and the mouse A9 cells. The *Xist* gene is never expressed in EC cell-mouse female lymphocyte hybrids in which the inactive X chromosome

was activated (Mise *et al.*, 1996). In this last case, complete reactivation was substantiated by the occurrence of nearly random X inactivation, irrespective of the activity state of each X chromosome immediately before cell fusion, when hybrid cells were allowed to differentiate *in vitro*. X chromosome reactivation without turning off the *XIST* gene is, therefore, unusual and merits further consideration. It is of special interest that the *PGK1* gene is repressed in PXI-4, OXI-8 and most P1A clones expressing *XIST*, in spite of apparent reactivation of entire human X chromosome. An intriguing possibility would be suppressive effect of *XIST* RNA limited to the immediate vicinity of the *XIST* locus in these clones.

*XIST* expression in PXI and OXI clones in which the human X chromosome was reactivated together with the previous report by Brown and Willard (1994) seem to support the view that the *XIST* gene on the human inactive X chromosome is not essential for the maintenance of the inactive state in the mouse genetic background. This may be compatible with a considerable sequence diversity of the *Xist* gene between mouse and man. Notwithstanding general similarities in gene structure, the sequence divergence even in the most conserved 5' region among different species is extensive being greater than the average coding regions but less than that of intronic or untranscribed regions (Hendrich *et al.*, 1993). In fact, Southern blots probed with the 5'-most human *XIST* probe revealed only an extremely faint band in mice. A detailed *in situ* hybridization study showed, however, close association between the heterochromatic X chromosome and *XIST* RNA for the maintenance of X inactivation (Clemson *et al.*, 1996; Lee *et al.*, 1996).

These apparently contradictory observations may be explained at least partly by qualitative differences between the inactive human X chromosome in the normal human female fibroblasts and that in rodent-human somatic hybrids as

advocated by Gartler *et al.* (1992). Dyer *et al.* (1989) showed that the inactive X chromosome in rodent-human hybrid cell is often central in location and disperse in structure, whereas it is compact and peripherally located in normal human female fibroblasts. We found, in addition, that CF150 cells never had an heterochromatic X chromosome and the apparent SCB in interphase nuclei that are common in human female somatic cells. Thus, it is likely that X-inactivation is possible without heterochromatinization. Gartler *et al.* (1985) proposed that the inactive X chromosome has a double repressor system, heterochromatinization and DNA methylation in somatic cells, whereas it has only heterochromatinization in extraembryonic cells. In normal cells, demethylation of individual genes on the X is not sufficient for bringing about reactivation because repression by heterochromatinization override activation. In hybrid cells, on the other hand, demethylation brings about reactivation because the step of heterochromatinization or sex chromatin formation has already been reversed. It is likely that the human inactive X chromosome remain inactive in rodent-human hybrid cells because the repressed chromatin structure is autonomously maintained through mitosis mainly, but may not be exclusively, by the conservation of DNA methylation pattern.

This study clearly showed that the mouse EC genome is capable of modifying the methylation status in the promoter region of human *XIST* gene with all its sequence diversity, but is unable to repress the gene activity. In other words, persistent *XIST* expression did not impede activation of the inactive X chromosome. It seems likely that the failure in suppressing *XIST* transcription was due to absence of human specific regulatory proteins in our monochromosome hybrids. There are several lines of evidence to show that *XIST/Xist* expression alone is not sufficient for the initiation of X chromosome inactivation. Tai *et al.* (1994), for example, detected *XIST* expression at the level of 10~20% that of

female somatic cells in P10 EC cell line having two active X chromosomes. Furthermore, Kay *et al.* (1994) found that *Xist* expression is initiated in androgenetic mouse embryos at the 4 cell stage as in normal female embryos, and terminated by the blastocyst stage. They concluded that continued *Xist* expression in early mouse embryos, hence initiation of X inactivation, is regulated by a novel imprinted maternally expressed gene.

Detailed mechanisms involved in the reactivation of the exogenous inactive human X chromosome in mouse EC cells remain unknown. In mouse EC-rat female lymphocyte hybrids, reactivation of the inactive rat X chromosome temporarily coincides with extinction of the lymphocyte phenotype, and *de novo* inactivation occurs almost simultaneously with the appearance of endodermal cell types characterizing early mouse embryos (Okuyama *et al.*, 1986). Undifferentiated nuclear conditions to which the inactive X chromosome was exposed seem conducive to reactivation. Available evidence, though circumstantial, is consistent with demethylation as a main underlying mechanism. EC and ES cells are hypomethylated like epiblast cells from blastocysts to early postimplantation embryos. It was shown that the *Hprt* gene in mouse EC hybrid cells was uniformly hypomethylated (N. Mise, unpublished data), although both *de novo* methylation and demethylation were evident in such endogenous imprinted genes as *H19*, *Igf2*, and *Xist* (M. Tada, unpublished data).

Our failure in inducing X-inactivation in differentiating PXI clones may be explained in various ways: (1) human Xic failed to respond to inactivation signals from the mouse genome; (2) X-inactivation did not initiate from the outset because human Xic was not recognized as such by the hybrid cell; (3) the problem was not diversified Xic but cell differentiation was not extensive enough for the occurrence of X-inactivation. The low sequence homology between human and mouse *Xist* genes and the inability of the mouse EC cells to turn off the human

*XIST* seem to favor the first two explanations, though it would be difficult to rule out the third possibility. Further study is needed to distinguish between the two remaining possibilities.

## FINAL REMARKS

Thirty years after the discovery of Lyon's hypothesis, the isolation of the *XIST/Xist* gene has made an epoch in the molecular study of X-inactivation. Although knockout and transgene studies suggest that this gene is essential for the initiation of X-inactivation (Penny *et al.*, 1996; Marahrens *et al.*, 1997; Herzing *et al.*, 1997; Lee and Jaenisch, 1997), we have not obtained any definitive answers to two major problems, (1) the molecular basis of X-inactivation, and (2) roles of *Xist* in the maintenance of inactivation.

### *Role of DNA methylation in X-inactivation*

DNA methylation and acetylation of histone H4 are only molecular parameters extensively studied in association with X-inactivation. It has been shown that the methylation patterns are clearly different in the promoter region of most X-linked genes according to their activity state in accord with the hypothesis advanced by Riggs (1978). The promoter region is hypermethylated on the inactive X chromosome, whereas it is hypomethylated on the active X chromosome. However, controversy has still continued as to the significance of DNA methylation in the initiation and maintenance of X-inactivation. It has long been believed that methylation is secondary to X-inactivation, because X chromosome is inactivated without methylation in cells in which imprinted inactivation prevails, *e.g.*, marsupial species and murine tissues such as trophectoderm and visceral endoderm, and methylation in the first intron of *Hprt* gene occurs about 3 days after inactivation (Lock *et al.*, 1987). Singer-Sam *et al.* (1990b) showed, however, that the methylation occurs much earlier, 6 days after fertilization, in the *Pgk-1* gene.

DNA methylation may be functional at, at least, two different levels, the major switch of the chromosome activity and at individual gene level influencing transcription activity. It is of prime importance to distinguish between these two situations for further discussion. DNA methylation is critical for X-inactivation in the sense that the *Xist* gene activity depends on the methylation status at the promoter region (Norris *et al.*, 1994; Sado *et al.*, 1996; Mise *et al.*, 1996). Panning and Jaenisch (1996) obtained evidence indicating the occurrence of X-inactivation in differentiating DNA methyltransferase deficient ES cells and homozygous embryos derived thereof. It seems probable that methylation at the individual gene level, except *Xist*, is a lock-in mechanism insuring transcriptional suppression. Further studies are necessary before we understand the role of DNA methylation.

***Histone H4 acetylation and sex chromatin body formation***

The histone H4 in hyperactive X chromosome of male *Drosophila* is excessively acetylated (Turner *et al.*, 1992), whereas it is hypoacetylated in human inactive X chromosome in normal female fibroblasts and lymphocytes (Jeppesen and Turner, 1993). It is surprising to find that the inactive X chromosome in CF150 was exempt from underacetylation and sex chromatin body formation, and this chromosome became hypoacetylated and formed a compact chromatin mass at the nuclear periphery when it was introduced into HeLa cell by microcell fusion. Thus, the human X chromosome is capable of maintaining the inactive state without extreme condensation and underacetylation of histone H4. Although confirmation is needed, it is probable that underacetylation is the consequence rather than the cause of X-inactivation.

This observation also suggests that a certain human specific factor(s) is

lacking in CF150 cells, which prevented the inactive X chromosome from sex chromatin body formation and/or underacetylation of histone H4. In CF150, the maintenance of human inactive X chromosome depend entirely on the mouse machinery most probably including histone acetylase and deacetylase. Hence, acetylation of this chromosome may be interpreted in three different ways: (i) mouse deacetylase can not act on human inactive X chromosome; (ii) mouse acetylase overacetylate the human inactive X chromosome; (iii) failure of sex chromatin body formation allows accession of histone acetylase to the human inactive X chromosome. First two possibilities seem unlikely, because the inactive X chromosome in the CF150 cells showed R-band-like distribution of acetylated histone H4 indicating that mouse enzymes functioned normally on the human chromosome. Since the half-life of the acetyl group on histone is less than 10 min, functional imbalance of two enzymes would result in extreme hyperacetylation or hypoacetylation. Culturing cells in the presence of sodium butylate to inhibit deacetylation, Jeppesen and Turner (1993) showed that underacetylation of the inactive X chromosome is a result of reduced acetyltransferase activity. Thus, the third possibility seems most likely. The present findings imply the presence of a species-specific factor(s) essential for the formation of a compact sex chromatin body in female somatic cells. Although no definitive image of this factor is envisaged, it may be fruitful to examine possible manifestations of the properties characterizing the inactive X chromosome.

Another difference between the mouse and man emerged from the present microcell fusion experiment involving mouse EC cell lines. Although the mouse EC cell genome was capable of reactivating the human inactive X chromosome of CF150 introduced by microcell fusion, the human *XIST* gene continued to express at relatively high levels. Methylation status of the 5' region of this gene varied considerably from almost full methylation to unmethylation in these hybrids. The

inactive X chromosome from the female somatic cells is reactivated after cell fusion with OTF9-63 or PSA1. In this case, the methylation status turned from full methylation to mosaic methylation in the promoter region of the *Xist* gene. In addition, we failed to obtain any positive evidence for the occurrence of *de novo* X-inactivation in these hybrid cells grown under conditions conducive to cell differentiation. It is likely that the human X chromosome inactivation center including the *XIST* gene is unable to function properly in mouse EC cells.

***XIST expression in initiation and maintenance of X chromosome inactivation***

Fluorescence *in situ* hybridization studies showed that XIST/Xist RNA apparently coats the inactive X chromosome, and is released from the chromosome during mitosis (Clemson *et al.*, 1996; Lee *et al.*, 1996). It is proposed, therefore, that Xist RNA is a structural element of chromosome territory in interphase and involved in chromatin packaging. Although close association between the inactive X chromosome and Xist RNA, and ubiquitous expression of *Xist* in adult female cells are indicative of a role in the maintenance, Brown and Willard (1994) unequivocally showed that the inactive human X chromosome remains inactive in spite of the loss of Xic region including *XIST* in mouse-human somatic hybrid cells. Our present study and unpublished works on radiation hybrids (Sekiguchi *et al.* unpublished) fully supported this finding. It is possible that Xist RNA is necessary *in vivo* to stabilize the inactive state preventing mutation. One should recall the fact that demethylation by 5-azacytidine may be capable of reactivating house keeping genes such as *Hprt*, *G6pd*, and *Pgk-1* on the inactivated X chromosome efficiently in somatic hybrid cells, but not in parental non-hybrid cells.

Gene targeting studied of *Xist* in ES cells has demonstrated that this gene is

required for X-inactivation to occur *in cis* (Penny *et al.*, 1996; Marahrens *et al.*, 1997). Recent transgenic studies limited the Xic to 450kb of sequence including the *Xist* gene (Lee *et al.*, 1996; Lee and Jaenisch, 1997). More definitive data were obtained by Herzing *et al.* (1997). They introduced a cosmid construct containing the *Xist* gene onto an autosome in ES cells. This ectopic copy of *Xist* gene is sufficient by itself for inactivation *in cis* and Xist RNA becomes localized around the autosome into which the gene is integrated. In some cells having the ectopic *Xist* gene, activation of endogenous *Xist* gene is observed. These data strongly suggested that the *Xist* gene itself has properties of Xic, such as counting of the number of X chromosome in a cell.

In summary, the late replicating and inactivated X chromosome in a mouse-human somatic hybrid cell is not typically heterochromatic neither forming a condensed sex chromatin body in interphase, nor showing heteropycnosis at mitotic prophase. Furthermore, this chromosome shows markedly reduced reaction to antiacetylated histone H4 antibody. It is tempting to postulate that inactivated state of one X chromosome in the adult female somatic cell is maintained stably by synergy of several kinds of factors including those having species-specificity, and deficiency of one or more factors or traits does not endanger the inactive state. However, apparently we have not identified every factors involved in X-inactivation, and deciphered relationships among known factors not to mention their exact roles. Thus, we have to admit that we are still far away from understanding the fascinating phenomenon of X-inactivation.

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