Korean J. Zool. 39:75-88, 1996

Control of X Chromosome Reactivation and Determination of the Ratio of Sex Chromosome to Autosome in Embryonal Carcinoma Cell-Somatic Cell Hybrids

Kwang-Ho Lee

Department of Biology, Chung-Ang University, Seoul 156-756, Korea

OTF9-63 (OTF9) and P19S1O1A1 (P19) embryonal carcinoma (EC) cells were examined for their ability to produce the reactivation of inactive X chromosomes from somatic cells. They were hybridized with various somatic cells and resulting HAT^r EC-somatic cell clones were analysed for their morphology, chromosomal replication patterns and expression profiles of X-linked and distantly located genes, *Hprt* and *Pgk-1*. The results demonstrated that ORF9 cells could reactivate the inactive X chromosome whereas P19 cells could not. In adition, EC-somatic cell hybrids tended to reduce the number of sex chromosomes in long-term culture, resulting in 1:2 ratio of sex chromosomes to autosomes. The use of EC cell hybrids provides an experimental system for studying the mechanism(s) of the X-reactivatio that is initiated and maintained from meiotic prophase of oogenesis to early embryogenesis.

KEY WORDS: Embryonal Carcinoma (EC) Cell Lines, Somatic Cell Hybridization, X Chromosome Reactivation, X Chromosome Loss, Y Chromosome Stability, Ratio of Sex Chromosome: Autosome

In early embryogenesis, one of two X chromosomes of female mammals undergoes the process of inactivation that makes all of the genes on it genetically inert. This hemizygous expression in the XX female is essential to achieve the equivalence to the XY male with respect to the dosage of X-linked gene products. The inactive condition becomes somatically heritable feature that is stably maintained within a cell lineage. The inactive state of the X chromosome is altered to express both X chromosomes when oogonia undergo the meiotic division to oocytes, and once initiated, the two active X condition is maintained to the early cleavage stage of embryo (Kratzer and Chapman, 1981).

A number of attempts have been made to understand the mechanism involved in the X-reactivation process using the somatic cell fusion technique between somatic cells and various EC cells that maintain the ability to retain the two X-active state within one cell and to differentiate into some cell lineages as observed in preimplantation embryos (Jami *et al.*, 1973; Von Kap-Herr and Mukherjee, 1977; Benham *et al.*, 1983; Takagi, 1983). The use of this somatic cell fusion system to produce the reactivation of the inactive X chromosome may provide a useful cell culture system for experimentally characterizing the elements involved in the X-reactivation process. Takagi *et al.* (1983) reported that OTF9 EC cells reactivated the previously inactive and T(X;16) 16H (designated T16H) lymphocyte-derived X chromosomes in their somatic cell hybrids. I have also reproduced the reactivation of the inactive X chromosome of the T16H lymphocyte in OTF9-T16H hybrids and have extended the previous

results to show that the X-reactivation is not limitted to the diploid lymphocytes and can be induced even in 6-thioguanine (6-TG)-resistant primary and immortalized cells (unpublished data). These results indicate that OTF9 EC cells are capable of producing the reactivation of the inactive X chromosome from somatic cells in EC cell-somatic cell hybrids.

I have examined hybrids of somatic cells with P19 EC cells to determine whether every EC cell line is equally capable of producing the X-reactivation. P19 hybrids have failed to produce the reactivation of the inactive X chromosome from somatic cells including T16H lymphocytes and 6-TG^r cultured primary and immortalized cells. Hybrids of P19 cells and T16H lymphocytes showed spontaneous differentiation into endodermal cells and late-replicating X and/or Y chromosomes. In addition, they failed to express the *Pgk-1*^{a} and *Hprt*^{a} alleles that carried on the inactive X chromosome. The T16H results were consistent with the failure to form HAT^r hybrids in the fusion experiments of P19 cells with 6-TG^r cultured cells that carry only an intact *Hprt* allele on the inactive X chromosome, reflecting that the X-reactivation did not occur in these hybrids. Moreover, P19 hybrids tended to reduce the number of X chromosomes retained in them with increasing number of passages whereas the number of Y chromosome retained in the hybrids remained unchanged.

Materials and Methods

Mice

The mouse *Hprt*$^{b\text{-}m3}$ mutation (designated BM3) was produced by Hooper (Hooper *et al.*, 1987; Thompson *et al.*, 1989). It was originally recovered as a spontaneous mutation in embryonal stem (ES) cells that were transmitted to the germline in an ES cell blastocyst injection chimera. The HPRT-deficient BM3 mice were maintained as a homozygous breeding strain in colony. A C57BL/6-*Hprt*^{a}, *Pgk-1*^{a} congenic strain (designated AT29) was developed from the transfer of *Pgk-1*^{a} from *Mus musculus* and *Hprt*^{a} from *Mus castaneus* onto a recombinant X chromosome that was subsequently transferred onto the C57BL/6 background N_{10}, F_{17} (Chapman *et al.*, 1989). The T16H translocation females were from an outbred colony of the stock that was maintained by recurrent breeding of the X;16 translocation female with a (C3H/HeHa-*Hprt*^{a}, *Pgk-1*^{a} × C57BL/6) F_1 male. The T16H females are genetically heterozygous, *Hprt*$^{a/b}$, *Pgk-1*$^{a/b}$, but phenotypically HPRT B, PGK-1B because they carry the *Hprt*^{a}, *Pgk-1*^{a} alleles on the intact X chromosome that is uniformly inactive in these females (Chapman *et al.*, 1982). *Mus spretus* (designated MS) mice were from an outbred colony that had been maintained at Roswell Park Cancer Institute since 1979. MS is *Hprt*^{a} and *Pgk-1*^{b}.

Cells

Somatic cells

Splenocytes (genotypically *Hprt*$^{a/b}$, *Pgk-1*$^{a/b}$; phenotypically HPRT B, PGK-1 B) from T16H mice were obtained from spleens of 6-7 week old mice. Splenocytes (*Hprt*^{a} *Pgk-1*^{b}/Y) from male MS mice were also obtained from spleens. Primary cells (*Hprt*$^{a/b\text{-}m3}$, *Pgk-1*$^{a/b}$) were obtained from the liver of an F_1 hybrid female (designated BM3/AT29) between BM3 stock of mice (*Hprt*$^{b\text{-}m3/b\text{-}m3}$, *Pgk-1*$^{b/b}$) and AT29 (*Hprt*$^{a/a}$, *Pgk-1*$^{a/a}$). They were exposed to 6-TG 10 μg/ml for over three weeks. The 6-TG^r primary cells were spontaneously immortalized after successive passages in low cell density. The immortalized cells (designated HOBMSL2) were grown in 6-TG in order to recover cells that carry a functionally active and BM3-derived X chromosome (that is, genotypically *Hprt*$^{a/b\text{-}m3}$, *Pgk-1*$^{a/b}$; phenotypically HPRT NULL, PGK-1 B). STO (*Hprt*^{null} *Pgk-1*^{b}) is an HPRT-deficient mouse embryo fibroblast cell line. STO cells were treated with mitomycin C for 2-4 hours and were used as a feederlayer for EC cell-somatic cell hybrids.

EC cells

HPRT-deficient and ouabain-resistant OTF9 (*Hprt*^{null} *Pgk-1*^{b}) and P19 (*Hprt*^{null} *Pgk-1*^{b}/ Y) cells showed a typical morphology of EC cells in culture without feederlayers and were used as fusion counterpartners with various somatic cells.

EC cell-somatic cell hybrids

HOTXF9 was a mixture of several HAT^r clones between female T16H splenocytes and OTF9 cells. HOTXF9 cells were maintained undifferentiated in a prolonged culture period both with and without feederlayers. HOTXP19 was a mixture of several HAT^r clones derived from the fusion of female T16H splenocytes with P19 cells. HOSF9 was a mixture of several HAT^r clones obtained by the fusion of OTF9 cells with male MS splenocytes.

Culture conditions

All cultures were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with non-essential amino acids and 10% fetal bovine serum. EC cells and EC cell-somatic cell hybrids were subcultured with 4 day-interval. Four days after passage, initial cell number of 5×10^6 was expanded to $0.8\text{-}1 \times 10^8$ cells/100 mm petridish.

Somatic cell hybridization

Hybridization was accomplished using the method described by Hales (1977) with minor modifications. For fibroblast-EC cell hybridization, 1×10^6 cells per each parental cell were fused. For lymphocyte-EC cell hybridization, 5×10^7 splenocytes from T16H or MS were fused with 5×10^6 EC cells.

Fluorescence in situ hybridization (FISH)

Chromosome preparation

Replication-R banding pattern was produced by exposing the cells to bromodeoxyuridine (BrdU; 150 μg/ml) for 6.5-10 hrs to cover the duration from late S phase to metaphase. During final 30 min of the BrdU treatment, colcemid (0.1-1 μg) was added to cultures. Conventional air-dry method was used for chromosome preparation. Slides were kept at −20°C until used.

DNA probes and biotin-labelling

DXSmh141 that had been previously localized to X chromosome at A3 band (Disteche *et al.*, 1989) was used for the direct identification of X chromosomes in EC and EC cell hybrids. It has an 1.2 kb mouse insert in pSP64 and was labelled by nick translation using enzymatic incorporation of biotin-11-dUTP. Unincorporated nucleotides were separated from the probe DNA by sephadex G50 spin columns (Pharmacia) equilibrated with 50 mM Tris HCl/1mM EDTA/0.1% SDS. The Y-painting probe obtained from flow-sorted MS Y chromosomes by arbitrarily primed polymerase chain reaction (AP-PCR) was used for the identification of Y chromosomes in EC and EC cell hybrids (Weier *et al.*, 1994). It was labelled by second round of PCR in the reaction mixture containing biotin-11-dUTP and was used without purification.

In situ hybridization and probe detection

The biotin-labelled DXSmh141 (10-20ng) was ethanol-precipitated with 10 μg of salmon sperm DNA and 40 μg of tRNA and then dissolved in 20 μl of hybridization mixture (50% (v/v) deionized formamide/ 2xSSC/10% dextran sulfate). The probe was then denatured at 75°C for 10 min and placed on ice. PCR-amplified and -labelled Y-painting probe was ethanol-precipitated with 2 μg mouse Cot1 DNA and 1 μg salmon sperm DNA. The probe mixture was preannealed at 37°C for 15 min after denaturation at 75°C for 5 min. Hybridization and detection of probes on R-banded chromosomes were accomplished by the method described by Lee *et al.* (1994).

Biochemical assay of HPRT and PGK-1

Isoelectrofocusing and isotopic identification of HPRT activity were previously descrived (Chapman *et al.*, 1983). PGK-1 electrophoretic forms were separated on cellulose acetate gels (Titan III, Helena Laboratories) and identified by the procedure described in Chapman *et al.* (1983).

Results

Establishment of EC cell-somatic cell hybrids

P19 EC cells that do not require STO feederlayers in culture were fused with T16H splenocytes and subject to HAT selection. Two weeks after HAT selection, HAT^r colonies (that is, HOTXP19) were obtained at an average of

211/10^6 P19 cells. HOTXP19 cells resembled P19 parental EC cells in morphology at early stages of hybrid cell growth with HAT selection, but they gradually changed into endoderm-like cells in morphology (Fig. 1).

The change was clearly detected about 1-2 weeks after the fusion although it is unclear whether the morphological change had been initiated immediately after the fusion and continued during successive selection. This finding may indicate that HOTXP19 cells are too unstable to maintain EC cell-like features even at the early stage after the hybrid formation. HOTXP19 cells were successfully maintained on the STO feederlayer for prolonged culture period although part of hybrid stem cells showed spontaneous differentiation. Retinoic acid (RA)-differentiated P19 cells have been known to give rise to skeletal and cardiac muscle, neurons and glial cells (Pruitt and Natoli, 1992). However, no evidence of the differentiation into those embryonic lineages was observed in HOTXP19 cells for prolonged culture period after the fusion. By contrast, HAT^r colonies from the fusion of OTF9 EC cells with T16H splenocytes were obtained at an average of 32/10^6 OTF9 cells. They showed no sign of differentiation for the prolonged culture period after the fusion.

The somatic cell fusion of P19 cells with BM3/AT29- derived 6-TG^r cultured primary and immortalized cells (that is, HOBMSL2) failed to form HAT^r colonies. This result may indicate that the failure of the formation of HAT^r colonies in these fusion experiments reflects that both an obligatory reactivation of the X chromosome and derepression of the inactive *Hprt* gene, which are required to recover HAT^r colonies, did not occur in the hybrids since only a functional $Hprt^a$ allele is located on the inactive and AT29-derived X chromosome of the 6-TG^r BM3/AT29 cells. On the contrary, OTF9-BM3/AT29 hybrids were successfully obtained at an average of 4.5 colonies/10^6 OTF9 cells. The hybrids could be maintained in culture for a prolonged culture period without any sign of differentiation.

In addition, OTF9 cells were hybridized with splenocytes from male MS mice and the resulting HAT^r clones (that is, HOSF9) were recovered at an average 82 colonies/10^6 OTF9 cells. HOSF9 cells could be maintained in culture for a long period (more than 3 months) without showing any spontaneous differentiation into endodermal lineages.

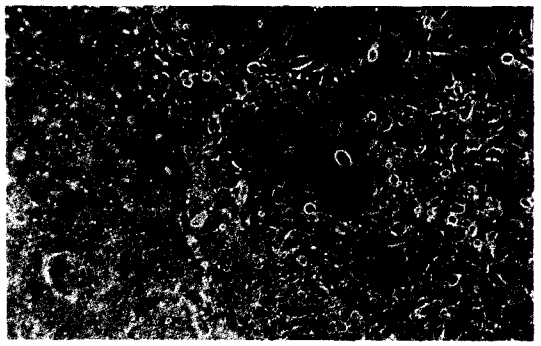

Fig. 1. Spontaneous differentiation of HOTXP19 cells at early stage after the somatic cell fusion. HOTXP19 cells clearly showed spontaneous differentiation into endoderm-like cells that outgrow the colonies from stem cells two weeks after the HAT selection. (a) and (b) show HOTXP19 stem cells and outgrown endoderm-like cells, respectively.

Basic cytogenetic data

I analyzed the karyotypes of EC cells, somatic cells and their somatic cell hybrids using the conventional Q-banding technique (Table 1). OTF9 had 38-43 chromosomes containing a metacentric chromosome that was easily discriminated from other chromosomes and additional two or three minute chromosomal elements. It had XO sex chromosome constitution. P19 had a modal range of 63-68 chromosomes. It had two X chromosomes in 93% of cells examined and one to three Y chromosomes in 88%. HOBMSL2 showed a modal range of 41-43 chromosomes with trisomy 19 in every cell analyzed. HOTXF9 had 70-82 chromosomes, indicating that the fusion ratio of OTF9:T16H splenocyte was 1:1. The fusion ratio was also determined by counting the number of the OTF9-specific marker chromosome retained in the hybrids. They showed the loss of only a few chromosomes. HOSF9 had 70-94 chromosomes, indicating that the fusion ratio of OTF9:MS-derived lymphocyte is 1:1 or 2:1. In case of 2:1,

Table 1. Karyotypes of paretal cells and of their hybrids.

Cells	No. of metaphases examined	Modal range of chromosome (EC cell:somatic cell)[a]	Sex chromosomes observed (%)[b]
OTF9	100	38-43	XO (98) XX (2)
P19	100	63-68	XO (1) XY (6) XX (11) XXY (61) XXYY (15) XXYYY (6)
HOBMSL2	100	41-43	XO (1) XX (99)
HOSF9[c]	100	70-94 (1:1, 2:1)	XO (3) XY (4) XX (70) XXY (23)
HOTXF9[d]	72	70-82 (1:1)	XX (22) XXX (78)
HOTXP19[e]	100	71-87 (1:1)	XX (3) XXY (17) XXYY (2) XXX (5) XXXY (19) XXXYY (4) XXXYYY (2) XXXYYYY (1) XXXX (9) XXXXY (29) XXXXYY (6) XXXXYYY (1) XXXXYYYY (1) XXXXXYYYYY (1)

a: The fusion ratio of EC cell:somatic cell. b: 100 × (No. of cells with the indicated sex chromosome constitution/No. of total cells examined). c: Cells examined at 12th passage after HAT^r colony formation. d: Cells examined at 5th passage. e: Cells examined at 1st passage.

the total chromosome number of 116-126 would be expected as a sum of chromosomes of two OTF9 cells and one MS-derived lymphocyte. However, the finding that HOSF9 cells ranged 70-94 in their chromosome number may indicate that hexaploid HOSF9 cells are unstable in culture and tend to lose their chromosomes. HOTXP19 had a modal range of 71-87 chromosomes, indicating that most of the hybrid cells derived from the 1:1 fusion of P19 cell:T16H splenocyte. They also showed the extensive loss of chromosomes with increasing number of passages as did HOSF9 cells, indicating that HOTXP19 cells are very unstable in culture. All EC cell-somatic cell hybrids examined tended to be near-tetraploid in their chromosome number irrespective of the ratio of EC cell:somatic cell.

Replication patterns of X and Y chromosomes in EC cell hybrids

Replication patterns of X and Y chromosomes were examined to ascertain whether the previously inactive X and Y chromosomes are early-replicating or replicating asynchronously in HOTXP19 cells. Replication-R banding patterns were obtained by UV irradiation to BrdU-incorporated chromosomes that were subsequently stained with fluorescence dyes such as acridine orange (AO) (Takagi *et al.*, 1982; Lee *et al.*, 1990). Since the whole inactive X and Y chromosomes replicate late in somatic cells and XY EC cells (McBurney and Rogers, 1982), the incorporation of BrdU to chromosomes during late S phase results in damage to the DNA of the UV-irradiated chromosomes with a marked reduction in DNA fluorescence staining along the entire chromosome length whereas autosomes and active X chromosomes show a typical R-banding pattern. The dull AO staining of the inactive X and Y chromosomes allows their discrimination from autosomes and active X chromosomes.

At various passages after HAT^r colony formation, HOTXP19 cells showed a late-replicating X chromosome in 15-24% of cells examined (Table 2). The result indicates that one of X chromosomes from either P19 or T16H cells remained inactivated in 15-24% of HOTXP19 cells but remaining 76-85% of the hybrids had no late-replicating X chromosome. The finding that 76-85% of HOTXP19 cells had no late-replicating X chromosome demonstrates that a previously late-replicating X chromosome of the T16H lymphocyte has been lost or changed in its replication pattern to early without expression of $Hprt^a$ and $Pgk\text{-}1^a$ alleles. I could not find HOTXP19 cells that have more than two late-replicating X chromosomes within one hybrid cell. In addition, Y chromosomes were all late-replicating in 78% (a sum of 67% of Y^l and 11% of X^l+Y^l at 8th passage) - 86% (a sum of 65% of Y^l and 21% of X^l+Y^l at 5th passage) of HOTXP19 cells examined (Table 2).

HOTXF9 cells showed no late-replicating X chromosomes in all cells examined (Data not shown). HOSF9 cells had a MS splenocyte-derived Y chromosome in 27% of cells examined (Table 1). All Y chromosomes retained in the hybrids were late-replicating. The Y chromosomes were clearly identified by dull fluorescence staining along the entire chromosome length whereas X chromosomes derived from OTF9 cells and MS male splenocytes were synchronously replicating with autosomes.

The results from HOTXP19 and HOSF9 cells demonstrate that replication patterns of Y chromosomes in EC cell hybrids can not be changed from late to early. Thus, it can be easily assumed that the process of reactivation of whole chromosome may occur only in the previously inactive X chromosome, but not in Y chromosome, with respect to at least the chromosomal replication pattern.

Control of the expression of two X-linked and previously inactive genes, *Hprt* and *Pgk-1*, in EC cell hybrids

Analyses of *Hprt* and *Pgk-1* allelic expression provide a functional assessment of X chromosome gene expression. I found that OTF9 cells expressed $Hprt^a$ and $Pgk\text{-}1^a$ alleles that carried on the inactive X chromosome in both T16H

Table 2. Replication patterns of X and Y chromosomes in HOTXP19 cells at various number of passages.

No. of passages	No. of metaphases with[a]				Total cells examined
	X^l	Y^l	X^l+Y^l	N[b]	
1	2	69	13	16	100
5	3	65	21	11	100
8	11	67	11	11	100

a: X^l; late-replicating X chromosome, Y^l; late-replicating Y chromosome. b: N; Number of metaphases without X^l and Y^l.

lymphocytes (Fig. 2 and 3) and 6-TG^r cultured primary and immortalized BM3/AT29 cells (unpublished data), indicating that they can reactivate the previously inactive X chromosome following the somatic cell hybridization. In order to ascertain whether P19 cells can also produce the X-reactivation, I examined HPRT and PGK-1 activities in HOTXP19 cells.

HOTXP19 cells failed to show a heteropolymeric pattern for the *Hprt*$^{a/b}$ genozyme (Fig. 2) and the heterozygous expression for *Pgk-1*$^{a/b}$ alleles (Fig. 3), suggesting that P19 did not reactivate the intact and inactive X chromosomes of T16H lymphocytes. That is, the resulting HAT^r hybrids could survive under HAT selection by the expression of *Hprt*^{b} allele on the active and translocated X chromosome without the reactivation of the intact and inactive X chromosome carrying an *Hprt*^{a} allele. The fusion of HPRT-deficient P19 cells and T16H splenocytes (HPRT B) resulted merely in the expression of *Hprt*^{b} allele whereas HOTXF9 and HOSF9, two different OTF9 hybrids, produced the heteropolymeric patterns for HPRT although the mobility of their HPRT subunits was different each other (Fig. 2). HOSF9 cells produced three visible subunits for the *Hprt*$^{a/null}$ alleles instead of five for the *Hprt*$^{a/b}$ alleles of HOTXF9 cells. The occurrence of the heteropolymeric pattern of *Hprt*$^{a/null}$ alleles in HOSF9 cells may indicate that the OTF9 *Hprt*^{null} alleles produce enzymatically inactive HPRT subunits that can combine with those of MS HPRT A to form the heteropolymeric pattern. HOSF9 cells showed the complete absence of 3rd and 5th subunits from the first HPRT A subunits of HPRT A/B of HOTXF9 cells.

HOTXP19 cells did not express the *Pgk-1*^{a} allele that carried on the inactive X chromosome whereas HOTXF9 cells showed the heterozygous expression for *Pgk-1*$^{a/b}$ alleles (Fig. 3). This result provides additional evidence of the failure of the X-reactivation in HOTXP19 cells.

The T16H results were consistent with the failure to form HAT^r colonies in P19 hybrids with 6-TG^r BM3/AT29-derived cultured primary and

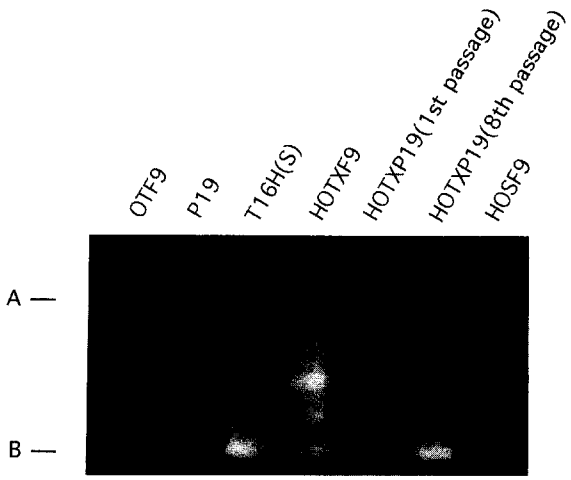

Fig. 2. Isoelectric focusing pattern of HPRT enzyme. OTF9 and P19 cells showed no HPRT activity as expected. T16H splenocytes showed a uniform expression of the *Hprt*^{b} allele, reflecting that the C3H/HeHa-derived X chromosome was fuctionally inactivated at least at the *Hprt* locus. HOTXF9 cells showed the heteropolymeric pattern of HPRT A/B, indicating that both *Hprt*^{a} and *Hprt*^{b} alleles are expressed within the same cell. On the contrary, HOTXP19 cells failed to show the heteropolymeric pattern. The uniform expression of only *Hprt*^{b} allele in HOTXP19 cells was observed irrespective of the number of passages. HOSF9 cells showed the heteropolymeric pattern although the pattern is different from HPRT A/B. They had three subunits, showing no 3rd and 5th (that is, corresponding to B subunits) subunits from A subunits instead of five subunits of HPRT A/B in HOTXF9 cells. (S); Splenocyte.

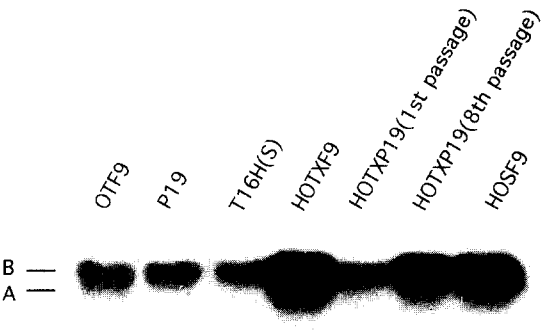

Fig. 3. PGK-1 isozyme patterns. OTF9 and P19 cells showed PGK-1 B activity. T16H splenocytes showed a uniform expression of the *Pgk-1*^{b} allele, reflecting that the C3H/HeHa-derived X chromosome was fuctionally inactivated at the *Pgk-1* locus. HOTXF9 cells showed the heterozygous expression of *Pgk-1*$^{a/b}$ alleles whereas HOTXP19 cells resulted only in the expression of the *Pgk-1*^{b} allele. PGK-1B expression in HOTXP19 cells was detected both at 1st and 8th passages. HOSF9 cells also showed PGK-1 B activity. (S); Splenocyte.

immortalized cells. HAT^r colonies were not recovered in several repeated somatic cell fusions and this may result from the fact that the only functional *Hprt^a* allele that carried on the inactive X of the BM3/AT29 cells can not be expressed without the reactivation of the entire inactive X chromosome or at least derepression of the inactive *Hprt^a* allele.

Thus, HPRT and PGK-1 data clearly indicate that the previously inactive X chromosome carrying *Hprt^a* and *Pgk-1^a* alleles in HOTXP19 cells might be lost at early stage after the fusion or remained inactivated while other X chromosomes from P19 cells and T16H lymphocytes were functionally active.

Loss of X chromosomes in EC cell hybrids

I found that HOTXF9 cells show a clear tendency to reduce the number of active X chromosomes (Table 3) that parallels the loss of one X chromosome from diploid EC cells (McBurney and Strutt, 1980) and the reduction to a single active X chromosome in ES cell lines (Robertson *et al.*, 1983). That is, the loss of X chromosomes continued to have the two X chromosomes in most of HOTXF9 cells while the number of total chromosomes remained stable as near-tetraploid (unpublished data). HOTXF9 cells retained three X chromosomes in 78% of cells examined at 5th passage (Table 3). The proportion has decreased up to 15% at 20th passage. On the contrary, the proportion of cells with two X chromosomes incresed 22% at 5th passage to 83% at 20th passage. These results were consistent in HOTXP19 cells that undergo the loss of X chromosomes. FISH analyses of DXSmh141 on replication-R banded chromosomes demonstrated that HOTXP19 cells retained four X chromosomes in 46% of metaphases examined at 1st passage after the HAT^r colony formation and in 3% at 8th passage. Moreover, the number of HOTXP19 cells that retained only one X chromosomes increased 0% at 1st passage to 10% at 8th passage. These results indicate that HOTXP19 cells also undergo the loss of X chromosomes as do HOTXF9 cells. Thus, the loss of X chromosome may be the EC-cell specific feature and may occur irrespective of the different genetic nature of EC cells in their sex, origin and ability to produce the X-reactivation, reflecting that this phenomenon may be controlled by other factor(s) than those that are reponsible for the X-reactivation.

Stability of late-replicating Y chromosomes in EC cell hybrids

Both P19 and OTF9 cells derived from male embryonic cells that originated as teratocarcinoma cell lines in embryos transplanted to ectopic sites (McBurney and Rogers, 1982; Artzt *et al.*, 1973). FISH analyses using Y-painting probe, however, showed that OTF9 cells have no Y chromosome content whereas P19 cells showed 1-3 Y chromosomes in 88% of the metaphases examined (Table 4, Fig. 4). The stability of the Y chromosome content in OTF9 and P19 EC cells also extends to their hybrids with somatic cells.

HOSF9 cells, in case of 1:1 fusion of OTF9:

Table 3. The number of X chromosomes retained in HOTXP19 and HOTXF9 cells at various passages.

Cells	No. of passages	Percentage of metaphases with[a]						Total cells examined
		1X	2X	3X	4X	5X	6X	
HOTXP19	1	–	22	31	46	1	–	100
	5	7	21	45	23	2	2	100
	8	10	52	33	3	2	–	100
HOTXF9	5	–	22	78	–	–	–	72
	12	–	41	59	–	–	–	69
	20	2	83	15	–	–	–	80

a: The number of X chromosomes was determined by counting the hybridization signals of the X chromosome-specific probe, DXSmh141.

Table 4. The number of Y chromosomes retained in various cell lines.

Cells	No. of metaphases with					Total cells examined
	0	1Y	2Y	3Y	>4Y	
MS (XY) (splenocytes)	–	100	–	–	–	100
MS (XX) (splenocytes)	100	–	–	–	–	100
OTF9	100	–	–	–	–	100
P19	12	67	15	6	–	100
HOSF9[a]	73	27	–	–	–	100
HOTXP19[b]	17	65	12	3	3	100
HOTXP19[c]	16	64	16	4	–	100

a: Cells examined at 12th passage after HAT^r colony formation. b and c: Cells examined at 1st and 8th passages after HAT^r colony formation, respectively. The number of Y chromosomes was determined by counting the hybridization signals of the Y-painting probe.

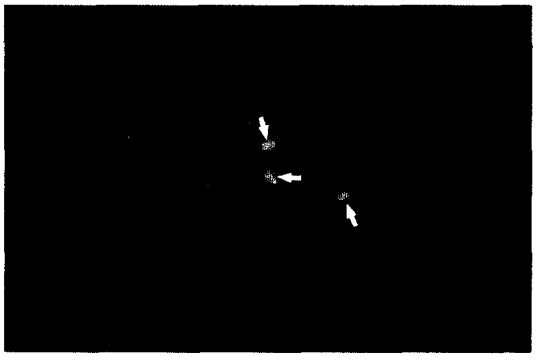

Fig. 4. A metaphase showing three Y chromosomes in P19 cells. Three small chromosomes were hybridized with Y-painting probe, indicating that the cell has three Y chromosomes. Arrows indicate Y chromosomes.

splenocytes from a male MS mouse, readily retained two X chromosomes but have lost the splenocyte-derived Y chromosome in 73% of cells examined at 12th passage (Table 4). By contrast, P19 cells and HOTXP19 cells (at 1st passage) had more than one P19-derived Y chromosome in 88% and 83% of cells examined, respectively (Table 4). HOTXP19 cells (at 8th passage) showed almost the same proportion (that is, 84%) as that observed at 1st passage, indicating that HOTXP19 cells did not lose Y chromosomes with increasing number of passages. Thus, difference in the stability of Y chromosome between HOSF9 and HOTXP19 cells is obvious, reflecting that P19-derived Y chromosomes would be different from OTF9- and somatic cell-derived ones in some roles in EC stem cells and EC cell-somatic cell hybrids.

Discussion

The result that OTF9 could reactivate the inactive X chromosome of T16H somatic cells whereas P19 could not may suggest that there is difference in the ability to reactivate the inactive X chromosome between OTF9 and P19 EC cell lines. There is difference in the origin between OTF9 and P19 EC cell lines although it is not clear whether the difference could result in the success or the failure to show the EC cell characteristics in OTF9 and P19 hybrids. That is, P19 cells were established from the primary tumor obtained by *in vivo* transplantation of embryos whereas OTF9 cells were from *in vitro* culture of teratoma-derived embryoid bodies. The difference between OTF9 and P19 in their origin, in the degree of differentiation and in the ability to reactivate the inactive X chromosomes provides an experimental system to determine the factor(s) that is involved in the reactivation of X chromosomes, the maintenance of the X-active condition and the differentiation.

OTF9 hybrids with lymphocytes of normal female rats showed the EC cell-like morphology at

early stage after the fusion, but gradually changed to the endoderm-like cells in their morphology (Okuyama *et al.*, 1986). Also, hybrids derived from the fusion between PSA-6TG1 (PSA-1) EC cells and lymphocytes from normal male rats showed the spontaneous differentiation into the endodermal cells (Takagi, 1983). The former hybrids showed that the rat X chromosome would undergo the reactivation and re-inactivation at the early stage after the fusion and the latter resulted in *de novo* inactivation of the X chromosome of PSA-1 EC cells. The fact that OTF9 and PSA-1 EC cell lines that were capable of producing the reactivation of the inactive X chromosome from T16H lymphocytes (Takagi *et al.*, 1983) and 6-TG^r cultured BM3/AT29 somatic cells (unpublished data) failed in the reactivation of the previously inactive X chromosome from XX rat lymphocytes and showed de novo inactivation of a PSA-1-derived X chromosome may indicate that the failure of X-reactivation in those hybrids results from the different genetic nature of somatic cells used as fusion counterpartners. In contrast, OTF9 cell-XY mouse lymphocyte hybrids (that is, HOSF9) as well as OTF9 cell-XX mouse somatic cell hybrids (that is, HOTXF9) showed neither spontaneous differentiation into the extra-embryonic lineage nor *de novo* inactivation of OTF9-derived X chromosome. Taken together, the ability of mouse EC cells to reactivate the inactive X chromosome may be limited to the mouse-mouse hybrids, indicating that mouse-derived factor(s) that is responsible for the maintenance of the state of X-reactivation can not play its role on the rat background.

I, however, demonstrated in this study that HOTXP19 cells (that is, mouse-mouse hybrids) also showed the spontaneous differentiation into the endoderm-like cells at early stage after the fusion and failed in the reactivation of the inactive X chromosome from T16H lymphocytes and BM3/AT29 somatic cells. The failure of the X-reactivation even in mouse-mouse hybrids as well as in some mouse-rat hybrids may indicate that every EC cell line can not produce the EC cell characteristics in mouse-mouse hybrids and the spontaneous differentiation occurs in concordance with the failure of the X-reactivation. These results may suggest that the intrinsic genetic nature of EC cells rather than that of somatic cells is crucial for the reactivation of the inactive X chromosome in mouse-mouse hybrids.

Hybrids and karyobrids of C86S1A1 EC cells with whole 3T3 fibroblasts and karyoplasts of 3T3 fibroblasts, respectively, failed to show the EC cell-like morphology and the differentiation into a varied spectrum of cell lineages (McBurney and Strutt, 1979). On the contrary, their cybrids resembled C86S1A1 EC parental cells, resulting in the failure to provide evidence for the presence of 3T3 fibroblast-derived regulators involved in the expression of gene(s) that can make the cybrids resemble 3T3 fibroblasts. In contrast, Iwakura *et al.* (1985) demonstrated that cybrids derived from the fusion of mouse EC (PCC4) cells and cytoplasts of rat myoblastic cells (L_6TG CAP^r) were differentiated at a high proportion. They suggested that a cytoplasmic factor(s) from the rat somatic cells may regulate the expression of gene (s) that induces the differentiation of mouse teratocarcinoma stem cells in the cybrids. The discrepancy between above two results on whether there is a cytoplasmic factor(s) to regulate expression of the gene(s) may be solved by considering the previous reports including the present paper (Okuyama *et al.*, 1986; Takagi, 1983). Mouse EC cell-mouse somatic cell hybrids, when somatic cells that are diploid or near-diploid were fused with EC cells, resembled their EC cell parents, but mouse EC cell-rat diploid lymphocyte hybrids resulted in the differentiation to endoderm-like cells. The results may imply that the differentiation of mouse EC cell-rat cytoplast fusion cybrids is due to that the factor(s) of mouse EC cell parents can not properly regulate the expression of the mouse EC cell-derived gene(s) involved in the maintenance of undifferentiated condition on the rat cytoplasmic background rather than rat somatic cell-derived cytoplasmic factor(s) induces the differentiation of the cybrids. In addition, the fact that the C86S1A1 EC cell hybrids and karyobrids failed to show EC cell-like features whereas the cybrids resembled EC cell parents may indicate that the EC cell-derived factor(s) involved in the maintenance of EC cell-like features is too diluted to exert its action both

in the hybrids and karyobrids that can be expected to contain 103-110 chromosomes as a sum of chromosomes of a 3T3 fibroblast (62-69) and a C86S1A1 EC cell (41), but the factor(s) can regulate the gene expression in the cybrids containing EC cell-derived 41 chromosomes only. This speculation was consistent with the result obtained by Benham *et al.* (1983). They demonstrated that most EC cell (PCC4) hybrids containing a single or a few human chromosomes on the PCC4 background showed the EC cell phenotype, indicating that the PCC4-derived factor(s) can regulate expression of gene(s) responsible for the ability to retain the EC cell characteristics in the interspecific hybrids containing almost all of genetic factors from EC cells and a few from human cells. That is, the ability of EC cells to maintain EC cell-like features in EC cell-somatic cell hybrids may be species-specific and may depend largely upon the genomic size of somatic cells as well as the intrinsic genetic nature of EC cells.

It could not be excluded the possibility that hypotetraploid (63-68 chromosomes) P19 EC cells used in this study had already lost their ability to maintain the EC cell phenotypes such as the ability to differentiate into a varied spectrum of cell lineages and, as a consequence, they resulted in the failure to show the EC cell-like features following the somatic cell fusion with somatic cells. McBurney (1976), however, described that tetraploid EC cells with a modal chromosome number of 78-79 were also pluripotential in spite of chromosomal instability, indicating that their factor(s) involved in the maintenance of pluripotency still exerts its action. In addition, the exactly same P19 cells as those used in this study were differentiated into endodermal, mesodermal and neuro-ectodermal cell lineages under a variety of induction conditions (Pruitt and Natoli, 1992). These results may indicate that the hypotetraploid P19 EC cells are pluripotential and the failure in producing the EC cell phenotypes in HOTXP19 cells is due to the other factor(s) than the numerical aberration of P19 EC cells although the possibility that P19 cells had been doubled in their chromosome number but had lost 12-17 chromosomes including the chromosome(s) carrying the factor(s), resulting in dilution of dosage of the factor(s) still remains.

I observed that the HOTXF9 cells showed a clear tendency to reduce the number of active X chromosomes (Table 3) and this phenomenon was also found in diploid female EC and ES cells that show the reduction to a single active X chromosome (McBurney and Strutt, 1980; Tai *et al.*, 1994). These results were consistent in HOTXP19 cells that undergo the loss of X chromosome. These findings may suggest that EC cell hybrids with an elevated dosage of active X chromosomes are unstable in culture and undergo a reduction in the number of active X chromosomes. As a consequence, the cells with a reduced number of active X chromosomes become dominant in cell population. As shown in Tables 1, 3 and 4, HOSF9 cells that have two X chromosomes in 93% of cells examined tended to lose the Y chromosome of somatic cells whereas HOTXP19 cells that have Y chromosomes of P19 EC cells in 83% tended to lose the X chromosome, resulting in the marked increase in proportion of HOSF9 and HOTXP19 cells with XX and XY sex chromosome constitution, respectively. That is, HOTXP19 cells that retain EC cell-derived Y chromosomes need only one fuctionally active X chromosome in order to achieve the 1:2 ratio of sex chromosome: autosome. With the same reason, HOSF9 cells that retain two functionally active X chromosomes do not necessarily need a Y chromosome. These findings were consistent in HOTXF9 cells that had XX sex chromosome constitution in 83% of cells examined at 20th passage. HOTXF9 cells that had three X chromosomes including two from the T16H and one from OTF9 have also lost one X chromosome to achieve the 1:2 ratio of sex chromosome:autosome. Moreover, Mittwoch (1992) demonstrated that Y-chromosomal genes are essential for spermatogenesis and spermiogenesis, but do not function effectively in the presence of more than one X chromosome, reflecting that there is quantitative relationship between X and Y chromosomes in the expression of the genes. Steinmann-Zwicky (1993) described that there is difference in the sex determination between germ and somatic cells of *Drosophila*,

and the X chromosome:autosome ratio in *Drosophila* produces a primary signal for the sex determination. Taken together, it seems that the loss of sex chromosomes in EC and EC cell hybrids is essential to maintain the stability in culture and they retain the ability to recognize the ratio of sex chromosome: autosome.

Recently, several genes including *Sry, Hya, Spy, Ube 1y* and *Zfy* that carried on the Y chromosome have been identified impling that the Y chromosome has other male-associated fuctions in addition to its testis-determining function (Burgoyne *et al.*, 1986; Gubbay *et al.*, 1990; Koopman *et al.*, 1989; McLaren and Burgoyne, 1983; Mitchell *et al.*, 1991). Burgoyne (1993) demonstrated that Y chromosomes have some effect on mouse blastocyst cell number through the expression of a gene(s) that speeds up the rate of cell division and Burgoyne *et al.* (1992) also suggested that a Y-chromosomal "spermiogenesis" gene that carried on the long arm of the Y chromosome is responsible for the fertility by participating in the normal development of the sperm head. Furthermore, the activity of some Y-chromosomal genes is different in germ and somatic cells (Steinmann-Zwicky, 1993), and genes such as serologically detected male antigen (SDMA) and *Zfy-1* can be expressed in preimplantation embryos and embryo-derived stem cells, respectively (Koopman *et al.*, 1989). Thus, these results may imply that the Y chromosome exerts its action both in EC cells and EC cell hybrids as does it in germ cells and preimplantation embryos, and its action can be cotrolled in connection with the X chromosome although each of X and Y chromosome can independently play its major role. Although it is unclear whether the difference in the presence of the Y chromosome alters the ability to retain EC cell-like morphology and to reactivate the inactive X chromosome in EC cell hybrids, the fact that HOTXP19 cells containing P19-derived Y chromosome(s) failed to retain the EC cell characteristics including the reactivation of the previously inactive X chromosome and the EC cell-like morphology whereas HOSF9 cells that have lost the somatic cell-derived Y chromosome showed the EC cell characteristics suggests that the EC cell-derived Y chromosome may play some roles in the production of the X-reactivation and in the maintenance of EC cell-like morphology in EC cell-somatic cell hybrids.

Acknowledgements

This paper was supported by SPECIAL FUND for UNIVERSITY RESEARCH INSTITUTE, Korea Research Foundation.

References

Artzt, K., P. Dubois, D. Bennett, H. Condamine, C. Babinet, and F. Jacob, 1973. Surface antigens common to mouse cleavage embryos and primitive teratocarcinoma cells in culture. *Proc. Nat. Acad. Sci. USA* **70:** 2988-2992.

Benham, F.J., M.V. Wiles, and P.N. Goodfellow, 1983. Differentiation *in vitro* of human-mouse teratocarcinoma hybrids. *Mol. Cell. Biol.* **3:** 2259-2270.

Burgoyne, P.S., 1993. A Y-chromosomal effect on blastocyst cell number in mice. *Development* **117:** 341-345.

Burgoyne, P.S., E.R. Levy, and A. McLaren, 1986. Spermatogenic failure in mice lacking H-Y antigen. *Nature* **320:** 170-172.

Burgoyne, P.S., S.K. Mahadevaiah, M.J. Sutcliffe, and S. J. Palmer, 1992. Fertility in mice requires X-Y pairing and a Y-chromosomal "spermiogenesis" gene mapping to the long arm. *Cell* **71:** 391-398.

Chapman, V.M., P.G. Kratzer, and B.A. Quarantillo, 1983. Electrophoretic variation for X chromosome-linked hypoxanthine phosphoribosyl transferase (HPRT) in wild-derived mice. *Genetics* **103:** 785-795.

Chapman, V.M., P.G. Kratzer, L.D. Siracusa, B.A. Quarantillo, R. Evans, and R.M. Liskay, 1982. Evidence for DNA modification in the maintenance of X-chromosome inactivation of adult mouse tissues. *Proc. Natl. Acad. Sci. USA* **79:** 5357-5361.

Chapman, V.M., D.R. Miller, D. Armstrong, and C.T. Caskey, 1989. Recovery of induced mutations for X chromosome-linked muscular dystrophy in mice. *Proc. Natl. Acad. Sci. USA* **86:** 1292-1296.

Disteche, C.M., G.K. McConnell, S.G. Grant, D.A. Stephenson, V.M. Chapman, S. Gandy, and D.A. Adler, 1989. Comparison of the physical and recombination maps of the mouse X chromosome.

Genomics **5:** 177-184.

Gubbay, J., J. Collignon, P. Koopman, B. Capel, A. Economou, A. Munsterberg, N. Vivian, P. Goodfellow, and R. Lovell-Badge, 1990. A member of a novel family of embryonically expressed genes mapping to the sex-determining region of the mouse Y chromosome. *Nature* **346:** 245-250.

Hales, A., 1977. A procedure for the fusion of cells in suspension by means of polyethylene glycol. *Somat. Cell Genet.* **3:** 227-230.

Hooper, M., K. Hardy, A. Handyside, S. Hunter, and M. Monk, 1987. HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature* **326:** 292-295.

Iwakura, Y., M. Nozaki, M. Asano, M.C. Yoshida, Y. Tsukada, N. Hibi, A. Ochiai, E. Tahara, M. Tosu, and T. Sekiguchi, 1985. Pleiotropic phenotypic expression in cybrids derived from mouse teratocarcinoma cells fused with rat myoblast cytoplasts. *Cell* **43:** 777-791.

Jami, J., C.F. Failly, and E. Ritz, 1973. Lack of expression of differentiation in mouse teratoma-fibroblast somatic cell hybrids. *Exp. Cell Res.* **76:** 191-199.

Koopman, P., J. Gubbay, J. Collignon, and R. Lovell-Badge, 1989. *Zfy* gene expression patterns are not compatible with a primary role in mouse sex determination. *Nature* **342:** 940-942.

Kratzer, P.G. and V.M. Chapman, 1981. X chromosome reactivation in oocytes of *Mus caroli*. *Proc. Natl. Acad. Sci. USA* **78:** 3093-3097.

Lee, K.-H., B. Keitz, M. Taira, and V.M. Chapman, 1994. Linkage of phosphoribosylpyrophosphate synthetases 1 and 2, *Prps1* and *Prps2*, on the mouse X chromosome. *Mamm. Genome* **5:** 612-615.

Lee, K.-H., Y. Matsuda, and V.M. Chapman, 1990. Simultaneous Visualization of Nonisotopic in Situ Hybridization on Thymidine-synchronized and Banded Chromosomes from Various Mouse Cells, 4th International Workshop on Mouse Genome Mapping, Bethesda, Maryland.

McBurney, M.W., 1976. Clonal lines of teratocarcinoma cells *in vitro*: Differentiation and cytogenetic characteristics. *J. Cell. Physiol.* **89:** 441-456.

McBurney, M.W. and B.J. Rogers, 1982. Isolation of male embryonal carcinoma cells and their chromosome replication patterns. *Dev. Biol.* **89:** 503-508.

McBurney, M.W. and B. Strutt, 1979. Fusion of embryonal carcinoma cells to fibroblast cells cytoplasts and karyoplasts. *Exp. Cell Res.* **124:** 171-180.

McBurney, M.W. and B.J. Strutt, 1980. Genetic activity of X chromosomes in pluripotent female teratocarcinoma cells and their differentiated progeny. *Cell* **21:** 357-364.

McLaren, A. and P.S. Burgoyne, 1983. Daughterless *XSxr/YSxr* mice. *Genet. Res.* **42:** 345-349.

Mitchell, M.J., D.R. Woods, P.K. Tucker, J.S. Opp, and C. Bishop, 1991. Homology of a candidate spermatogenic gene from the mouse Y chromosome to the ubiquitin- activating enzyme E1. *Nature* **354:** 483-486.

Mittwoch, U., 1992. Sex determination and sex reversal: genotype, phenotype, dogma and semantics. *Hum. Genet.* **89:** 467-479.

Okuyama, K., N. Takagi, and M. Sasaki, 1986. Sequential X-chromosome reactivation and inactivation in cell hybrids between murine embryonal carcinoma cells and female rat thymocytes. *Exp. Cell Res.* **164:** 323-334.

Pruitt, S.C. and T.A. Natoli, 1992. Inhibition of differentiation by leukemia inhibitory factor distinguishes two induction pathways in P19 embryonal carcinoma cells. *Differentiation* **50:** 57-65.

Robertson, E.J., M.J. Evans, and M.H. Kaufman, 1983. X-chromosome instability in pluripotential stem cell lines derived from parthenogenetic embryos. *J. Embryol. Exp. Morphol.* **74:** 297-309.

Steinmann-Zwicky, M., 1993. Sex determination in *Drosophila: sis-b*, a major numerator element of the X:A ratio in the soma, does not contribute to the X:A ratio in the germ line. *Development* **117:** 763-767.

Tai, H.H., J. Gordon, and M.W. McBurney, 1994. *Xist* is expressed in female embryonal carcinoma cells with two active X chromosomes. *Somat. Cell Mol. Genet.* **20:** 171-182.

Takagi, N., 1983. *De novo* X-chromosome inactivation in somatic hybrid cells between the XO mouse embryonal carcinoma cell and XY rat lymphocyte. *Exp. Cell Res.* **145:** 397-404.

Takagi, N., O. Sugawara, and M. Sasaki, 1982. Regional and temporal changes in the pattern of X-chromosome replication during the early post-implantation development of the female mouse. *Chromosoma* **85:** 275-286.

Takagi, N., M.A. Yoshida, O. Sugawara, and M. Sasaki, 1983. Reversal of X-inactivation in female mouse somatic cells hybridized with murine teratocarcinoma stem cells *in vitro*. *Cell* **34:** 1053-1062.

Thompson, S., A.R. Clarke, A.M. Pow, M.L. Hooper, and D.W. Melton, 1989. Germ line transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. *Cell* **56:** 313-321.

Von Kap-Herr, C. and B.B. Mukherjee, 1977. Stability

of inactive X-chromosome in mouse embryoid body-mule cell and transformed mouse cell-mule cell heterokaryons. *Exp. Cell Res.* **104:** 369-376.

Weier, H.-U., D. Polikoff, J.J. Fawcett, K.M. Greulich, K.-H. Lee, S. Cram, V.M. Chapman, and J.W. Gray, 1994. Generation of five high-complexity painting probe libraries from flow-sorted mouse chromosomes. *Genomics* **21:** 641-644.

(Accepted November 21, 1995)

배종양 세포와 체세포 간의 융합 세포에서 X 염색체 재활성화의 조절과 성염색체에 대한 상염색체 비율의 결정

이광호(중앙대학교 이과대학 생물학과)

OTF9-63(OTF9)과 P19S1O1A1(P19) 배종양 세포들의 체세포에 존재하는 불활성 X 염색체의 재활성화 유발 능력을 조사하였다. 배종양 세포와 체세포들의 융합에 의해 얻어진 HAT^r 클론들의 형태, 염색체 복제 양상을 비롯하여 X 염색체에 존재하나 그 위치는 상당히 먼 유전자들인 *Hprt*와 *Pgk-1*의 발현 양상을 분석한 결과, OTF9 세포는 불활성 X 염색체를 재활성화 시킬 수 있는데 반해 P19 세포는 불가능한 것으로 나타났다. 또한, 모든 융합세포는 장기간 배양되었을 때 성염색체 수가 감소하였으며, 결국 1:2의 성염색체:상염색체의 비율을 나타내었다. 배종양 세포-체세포 융합세포의 이용은 초기 배발생 과정에서 시작되어 난자형성 과정의 감수분열 전기까지 유지되는 X 염색체의 재활성화 기작을 연구하기 위한 실험 방법을 제공한다.