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Expression of an X-linked gene from an inactive human X chromosome in mouse–human hybrid cells: Further evidence for the noninactivation of the steroid sulfatase locus in man

(X-linked ichthyosis/X-chromosome abnormalities)

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ABSTRACT **Somatic cell hybrid clones were derived from the fusion of hypoxanthine phosphoribosyltransferase (HPRT; EC 2.4.2.8)-deficient mouse cells and two different human fibroblast strains, each carrying an X chromosome–autosome translocation. One of these had an X/11 translocation [46,X,t(X;11)(p21;q13)] and the other had an X/19 translocation [46,X,t(X;19)(q22;q13)]. The structurally normal human X chromosome is the late-replicating (genetically inactive) chromosome in these two cell strains; the rearranged X chromosome is early replicating (genetically active). One primary hybrid clone carrying both the translocated X chromosome and the structurally normal X chromosome was isolated in hypoxanthine/aminopterin/thymidine medium from each of these two cell fusion experiments. These clones were then selected in medium containing 8-azaguanine to achieve the loss of the active human *HPRT* locus. Five subclones from the cell hybrid with the X/11 translocation failed to express two known human X-chromosome markers [glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) and phosphoglycerate kinase (PGK; EC 2.7.2.3)] but did express human microsomal steroid sulfatase (STS; sterol-sulfate sulfohydrolase, EC 3.1.6.2). Three of these were cytogenetically analyzed and found to contain a structurally normal human X chromosome but not the X/11 translocation. Two subclones were isolated in 8-azaguanine from the hybrid with the X/19 translocation. Cytogenetic analysis of these two clones showed the presence of a structurally normal human X chromosome; the X/19 translocation was not present. They did not express human G6PD, PGK, or HPRT but did express human STS. These results indicate that human STS is expressed from a locus on the inactive human X chromosome and support our earlier finding that the *STS* locus escapes X-inactivation in man.**

According to the Lyon hypothesis (1), one of the two X chromosomes in the somatic cells of a normal mammalian female is inactivated. A large body of experimental evidence from biochemical, genetic, and cytologic studies supports the essential premise of this theory (2, 3). The inactivation process has been thought to be irreversible and to affect the entire X chromosome. However, there is evidence that favors the existence of an area (or areas) of the human X chromosome that escapes inactivation. The somatic abnormalities associated with partial or full monosomy of the X chromosome may reflect the requirement for expression of both alleles at some X-linked loci (4); aberrations associated with extra X chromosomes may result from an excess of these same gene loci on the supernumerary X chromosomes. Furthermore, accumulated data indicate that the *Xg* locus, which specifies an X-linked erythrocyte surface antigen, escapes X-inactivation in the hematopoietic cells when on a structurally normal X chromosome (5, 6). However, since the Xg antigen can be detected only on erythrocytes, it has not been possible to use a somatic cell genetic approach to confirm the noninactivation of the *Xg* locus. It is of note that the microsomal steroid sulfatase–X-linked ichthyosis locus, which we have shown to escape X chromosome-inactivation in man (7), is linked to *Xg* with a genetic distance of 10 centimorgans (5). [Steroid sulfatase (STS) sterol-sulfate sulfohydrolase, EC 3.1.6.2.]

The finding of noninactivation of the *STS* locus was based on cloning studies of cultured fibroblasts from two females, each heterozygous for STS deficiency and glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) deficiency (7). Single-cell clones isolated from these two females were all positive for STS expression, although they belonged to two classes (+ and −) with regard to their G6PD expression. Using mouse–human somatic cell hybrids containing human X-autosome translocations, we have assigned the *STS* locus to the distal half of the short arm of the human X chromosome (8).

Since the presence of a locus on the human X chromosome that escapes inactivation is of importance in understanding the mechanism of X-inactivation, we have sought confirming evidence by using a different experimental approach. We now demonstrate that human STS is expressed from an inactive human X chromosome retained in mouse–human somatic hybrid cells providing further evidence for the noninactivation of the *STS* locus on the human X chromosome.

MATERIALS AND METHODS

Isolation of Cell Hybrids. Two independent mouse–human somatic cell hybrid lines containing an inactive human X chromosome were used in the studies reported here. One hybrid was derived from the fusion of mouse line A9 (GM 0346, Institute for Medical Research, Camden, NJ), which is deficient in hypoxanthine phosphoribosyltransferase (HPRT; EC 2.4.2.8), with human fibroblasts containing an apparently balanced X/11 translocation [46,X,t(X;11)(p21;q13)] (GM 1695). The structurally normal human X chromosome is late-replicating and genetically inactive in these cells (9), as is the case in the majority of balanced X chromosome–autosome translocations (10). Fusion of human fibroblasts with A9 cells and isolation of primary hybrid clones followed described procedures (8). Hybrids were isolated in hypoxanthine/aminopterin/thymidine (HAT) medium (11), which selects for the retention of the human X chromosome carrying the active locus for HPRT. One primary clone (37-26) was found to have the translocated X

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Abbreviations: STS, microsomal steroid sulfatase (sterol-sulfate sulfohydrolase, EC 3.1.6.2); HPRT, hypoxanthine phosphoribosyltransferase; G6PD, glucose-6-phosphate dehydrogenase; PGK, phosphoglycerate kinase; HAT, hypoxanthine/aminopterin/thymidine.

(expressing the *HPRT* locus) and the structurally normal inactive human X chromosome. This clone was allowed to grow in normal growth medium for a few generations, after which it was selected in medium containing 8-azaguanine (3 μ/ml) to achieve the loss of the chromosome carrying the active *HPRT* locus. Secondary clones were isolated from clone 37-26 and characterized.

The second hybrid clone was derived from the fusion of mouse line A9 and human fibroblasts containing an apparently balanced X/19 translocation. [46,X,t(X;19)(q22;q13)] (GM 89). Again, the structurally normal human X chromosome is late replicating in these cells. The details of the production and isolation of hybrids with the inactive X chromosome from this experiment have been published (12).

Mouse line A9 was also fused with leukocytes from a normal human male volunteer. [Earlier a total of five independent hybrid clones from the fusion of A9 cells and leukocytes from two individuals deficient in STS activity had been isolated (8).] Fusion of A9 cells with human leukocytes and isolation of hybrids were carried out as described (13). In addition, four independent hybrid lines were isolated from the fusion of A9 cells and human fibroblasts from an individual lacking STS activity.

Enzyme Assay and Electrophoresis. STS activity in somatic cell hybrids was measured by using tritiated cholesterol sulfate as substrate (14). Protein was determined by the method of Lowry *et al.* (15), and the specific activity of cholesterol sulfatase was calculated in pmol/mg of protein per hr. The enzyme assay uses substrate concentrations substantially below the K_m of the enzyme. This is done to achieve maximum sensitivity with a high percentage of conversion of substrate to product; activity as low as 0.02 pmol/mg of protein per hr can be detected. However, under such conditions, linearity with protein concentration cannot always be achieved. Different culture dishes of necessity were utilized for biochemical and cytogenetic analyses, although always at the same passage level. Because the assay is not always completely linear, apparent discrepancies between the percentages of cells containing an active *STS* gene and quantitative enzyme activity occasionally were noted among the hybrid clones analyzed.

STS expression in mouse–human hybrids also was evaluated by agarose/acrylamide gel electrophoresis. The details of the electrophoretic technique have been reported (8). By using this electrophoretic assay, it is possible to distinguish between mouse and human STS (Fig. 1).

Expression of three established markers on the long arm of the human X chromosome was evaluated by electrophoretic procedures; these markers were phosphoglycerate kinase (PGK; EC 2.7.2.3) (17), (G6PD) (18), and HPRT (12).

Cytogenetic Analysis. Biochemical and cytogenetic analyses were done on hybrid cells at the same passage level. Cytogenetic analysis was done with the aid of Q-banding (Fig. 2) as described (13). Chromosome content of a clone was determined from photographs of 25–30 well-banded metaphase preparations.

RESULTS

Expression of STS in A9–Human Cell Hybrids. Repeated analyses of the mouse parental line, A9, showed it to be deficient in STS activity (<0.02 pmol/mg of protein per hr). A9–human fibroblast hybrids retaining a structurally normal and genetically active human X chromosome or the distal half of the short arm of an active human X chromosome express STS activity (8). The presence of STS activity in these mouse–human hybrid clones was concordant with the retention of only the human X chromosome and not with any other human chromosome. Two independent hybrid clones were isolated from the fusion of A9 and leukocytes from a normal male. Both of these expressed STS activity and human G6PD activity and had a cytogenetically demonstrable normal human X chromosome. However, the five independent hybrids derived earlier from the fusion of A9 and human leukocytes from STS-deficient individuals did not have any detectable STS activity, although all of these expressed human G6PD activity (8). Four independent mouse–human hybrid clones isolated from the fusion of A9 and fibroblasts from an STS-deficient individual also failed to express STS activity, whereas they all expressed human G6PD activity. In addition, the STS expressed by hybrids migrated to the same position on agarose/acrylamide gel electrophoresis as did the enzyme from human fibroblasts (8), (Fig. 1). From these experiments, we

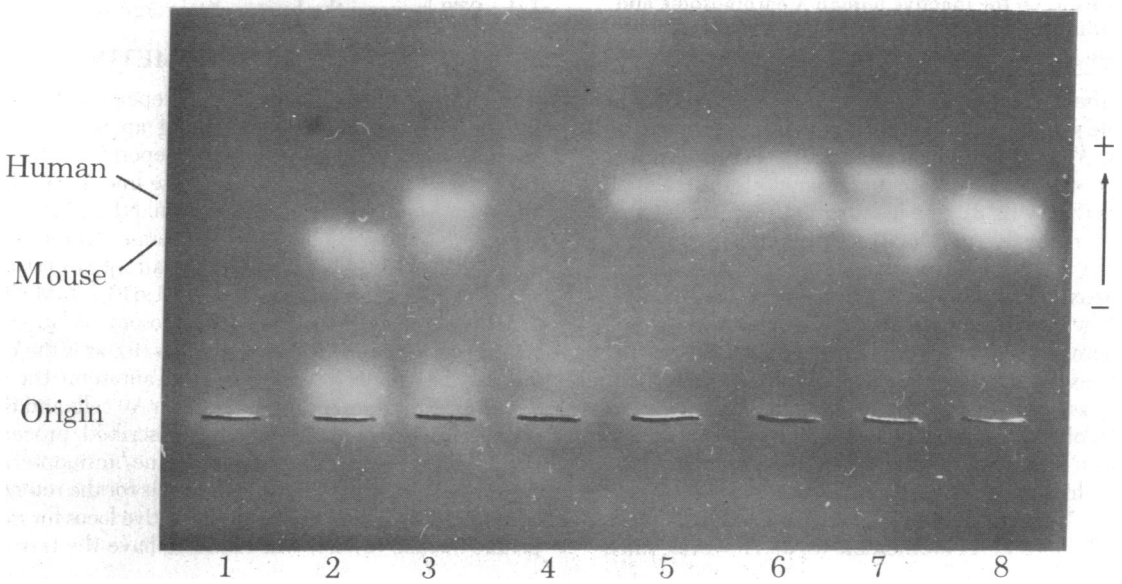

FIG. 1. Agarose/acrylamide electrophoretic pattern for STS. Vertical arrow indicates direction of migration. The lanes contained the following samples: 1, mouse A9 cells; 2, mouse hepatoma cells (16) that express STS activity; 3, mixture of hepatoma cells and human fibroblasts; 4, A9–human hybrid with no STS activity; 5, A9–human hybrid expressing human STS activity; 6, human fibroblasts; 7, mixture of human fibroblasts and mouse liver (from strain SJL/J mice, The Jackson Laboratory); 8, mouse liver.

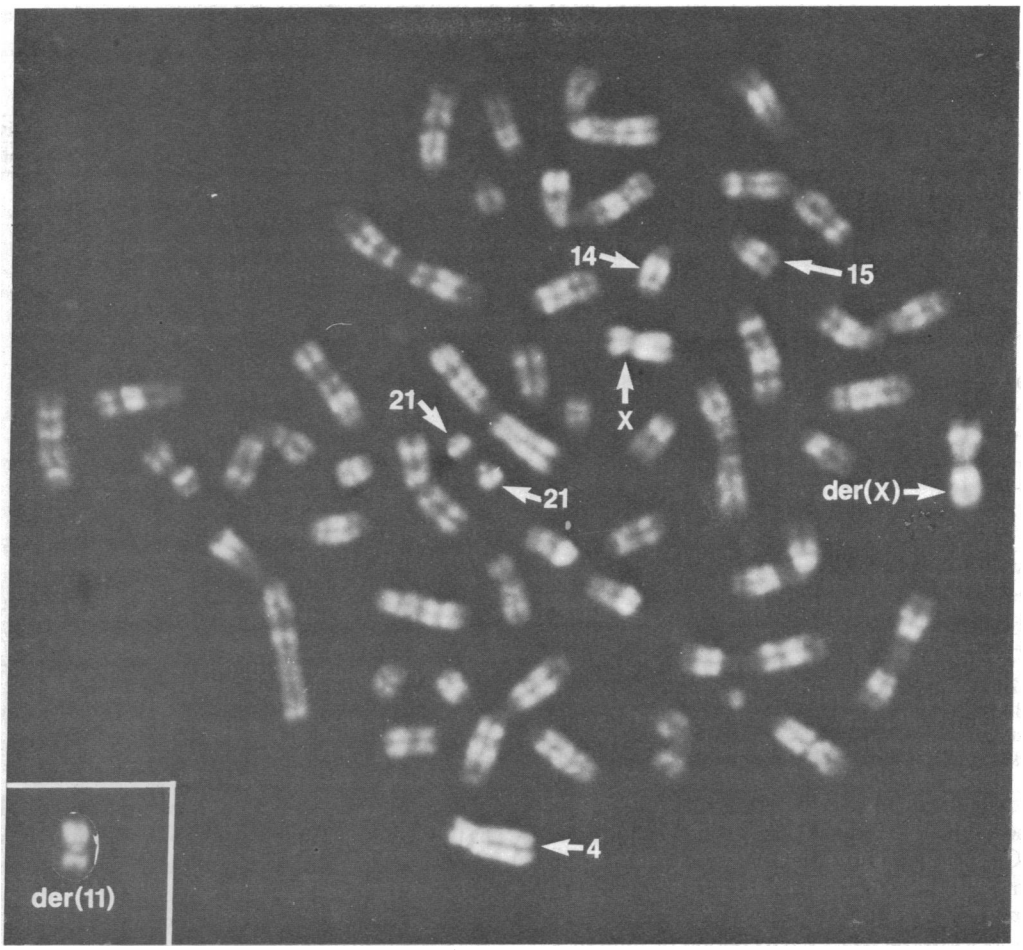

FIG. 2. A Q-banded metaphase spread from clone 37-26. The human chromosomes are identified by arrows. Note the presence of a structurally normal X chromosome and the der(X). (*Inset*) Reciprocal product der(11), cut out from the metaphase photograph of another hybrid clone. This chromosome is clearly distinguishable from mouse chromosomes and other human chromosomes.

conclude that the STS activity detected in A9–human hybrids represents human STS activity.

Analysis of Hybrids with the X/11 Translocation. Five independent primary hybrid clones were isolated in HAT medium from the fusion of A9 and human cells containing the X/11 translocation (Table 1). As expected, all of these retained the der(X), (11qter→11q13::Xp21→Xqter), that carries the active *HPRT* locus. However, in the presence of the der(X) alone (clones 37-6 and 37-16), the hybrids did not express STS, although they expressed human G6PD (Table 1). Clones 37-15 and 37-20 retained both products of the translocation and were positive for STS expression. The STS expressed in these hybrids migrated to the same position as did the enzyme from human fibroblasts (Fig. 1). The expression of STS was not concordant with any of the human autosomes in these hybrids. Thus, these results confirm the assignment of the gene locus for *STS* to the distal half (p21 → pter) of the short arm of the human X chromosome.

Clone 37-26 was particularly interesting as it retained the der(X) and the structurally normal human X chromosome (presumably inactive, based on late-labeling studies) and was positive for STS activity, human G6PD, and PGK. Because the der(X) lacks the *STS* locus, the structurally normal X chromosome must have been responsible for the expression of STS in this hybrid clone. Clone 37-26 was selected in 8-azaguanine. Five independent secondary clones were analyzed, all of which expressed human STS activity but failed to express human G6PD or PGK activities. Cytogenetic analyses of three of these hybrid clones showed the presence of a structurally normal X chromosome but not the der(X) (Table 1). The lack of expression of human G6PD, PGK, and HPRT (the latter by inference, because these subclones were selected in 8-azaguanine) show that the structurally normal X chromosome retained in these secondary clones is inactive. Yet, they expressed human STS activity, indicating that the *STS* locus escapes inactivation in man.

One of the five secondary clones (37-26R-A) had the inactive X chromosome in a low proportion of analyzed cells (2:30) (Table 1). Consequently, subcloning this clone should result in tertiary clones, the majority of which would be deficient in STS activity. Three of the tertiary clones isolated from 37-26R-A were analyzed and found to be deficient in STS activity (Table 1).

Analysis of Hybrids with the X/19 Translocation. One primary clone (GM 89 A99C) from this fusion experiment was found to retain the translocated chromosomes der(X), Xpter→Xq22::19q13→19qter, and der(19), 19pter→19q13::Xq22→Xqter, in addition to a structurally normal human X chromosome (presumably inactive, based on late-labeling studies). This primary clone was selected in medium with 8-azaguanine, and three subclones so isolated were analyzed. The results are summarized in Table 2. Two of these clones (GM89 A99C-1 and GM89 A99C-9) retained the normal X chromosome alone, but failed to express human G6PD, PGK, and HPRT activities, showing that this X chromosome is inactive. However, these two clones were positive for human STS ac-

Table 1. Cytological and biochemical analyses of cell hybrids derived from the fusion of mouse line A9 and human cells carrying an X/11 translocation

Clone	Cytological analysis: Metaphases analyzed	Metaphases with human activity: X	der(X)*	der(11)†	Biochemical analysis: STS activity,‡ pmol/mg of protein per hr	Human activity§: G6PD	PGK
Primary clones isolated in HAT medium:							
37-6	36	—	30	—	<0.02	+	NA
37-15	26	—	20	21	0.20	+	NA
37-16	35	—	27	—	<0.02	+	NA
37-20	31	5	27	4	0.57	+	NA
37-26	33	32	32	—	2.36	+	+
Secondary clones isolated in medium containing 8-azaguanine from clone 37-26:							
37-26-B		NA			0.65	−	−
37-26-C		NA			0.46	−	−
37-26-D	25	24	—	—	0.62	−	NA
37-26-E	18	14	—	—	0.88	−	−
37-26-A	36	2	—	—	0.36	−	−
Tertiary clones isolated in normal growth medium from clone 37-26-A:							
37-26-A-3	NA				<0.02	NA	
37-26-A-5					<0.02		
37-26-A-6					<0.02		

* Translocated chromosome: 11qter → 11q13::Xp21 → Xqter.
† Translocated chromosome: 11pter → 11q13::Xp21 → Xpter.
‡ Enzyme activity <0.02 indicates STS deficiency.
§ +, presence of the human chromosome or enzyme marker; −, absence; NA, not analyzed.

tivity. The third subclone (GM 89 A99C-7) retained the der(X) containing the short arm of X and was positive for STS and PGK but negative for G6PD and HPRT. The expression of STS in this clone is consistent with the assignment of *STS* to the distal half of Xp and the expression of PGK is in agreement with the assignment of this locus to the q12 → q13 region of human X chromosome (19).

DISCUSSION

The results from the X/11 translocation experiment show that the gene locus for STS can be assigned to the p21 → pter region of human X chromosome. This is in agreement with studies (8) that showed that the expression of STS in A9–human hybrids is concordant with the distal half of Xp only and not with any other human chromosome. Evaluation of human X markers in secondary clones retaining an inactive X chromosone (which were derived from two independent primary hybrid clones) show that, although these clones failed to express human PGK, G6PD, and HPRT, they expressed human STS. These results taken together with results of our cloning studies (7) show that the *STS* locus, indeed, escapes X chromosome inactivation in man.

Inactivation of one X chromosome in a normal human female is thought to achieve dosage compensation between males and females for X chromosome loci. However, individuals with a 45,X0 karyotype (Turner syndrome) show somatic abnormalities and are infertile. A hypothesis to account for the somatic abnormalities is that two active X chromosomes are required in the early stages of embryonic development before X chromosome inactivation takes place. An alternate hypothesis is that inactivation does not affect the whole X chromosome (20). The noninactivation of the *STS* locus supports the latter hypothesis. Since the mechanism of X chromosome inactivation allows *STS* to remain active, it is possible that there are other loci that escape inactivation as well. Thus, the somatic anomalies associated with the Turner syndrome and other numerical and structural changes in the X chromosome constitution of man may be due to true genetic imbalance at certain of these loci that escape inactivation.

Although several theories have been proposed to explain X chromosome inactivation, experimental evidence is lacking in support of any of these mechanisms. These hypotheses can be broadly classified into those that involve binding to DNA of

Table 2. Cytological and biochemical analyses of primary clone GM89A99C and its subclones isolated in medium with 8-azaguanine

Clone	Cytological analysis*: Metaphases analyzed	Metaphases with human activity: X	der(X)†	der(19)‡	Biochemical analysis*: STS activity, pmol/mg of protein per hr	Human activity: G6PD	PGK	HPRT
GM89A99C	15	+	+	+	0.31	+	+	+
Subclones:								
GM89A99C-1	25	17	−	−	0.15	−	−	−
GM89A99C-7	33	−	27	−	0.67	−	+	−
GM89A99C-9	33	23	−	−	0.38	−	−	−

* +, Presence of the human chromosome or enzyme marker; −, absence.
† Translocated chromosome: Xpter → Xq22::19q13 → 19qter.
‡ Translocated chromosome: 19pter → 19q13::Xq22 → Xqter.

specific proteins (21, 22) and those that involve DNA modification (23, 24). The maintenance of the inactive state of the X chromosome in the mouse–human hybrids in the absence of the majority of human autosomes and the active human X chromosome, tends to support a DNA-modification mechanism. In any case, future models for X chromosome inactivation will have to account for noninactivation of at least a part of the human X chromosome.

Note Added in Proof. Since submission of this manuscript, Müller *et al.* (25) have reported using Chinese hamster–human hybrids with X chromosome–autosome translocations to confirm the assignment of steroid sulfatase to the short arm of the X chromsosome. In the course of these experiments, they observed one hybrid which contained the long arm of the human X chromosome as well as the structurally normal, presumably inactive X. This line did express steroid sulfatase activity.

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