Am J Hum Genet 34:811–817, 1982

Brief Communication

Genetic Evidence for the Inactivation of a Human Autosomal Locus Attached to an Inactive X Chromosome

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SUMMARY

Mouse-human cell hybrid clones retaining an inactive translocated chromosome involving the human X and 13 were isolated. Esterase D, a marker on the segment of chromosome 13 translocated to the X, was not expressed in these clones. These results provide genetic evidence for the spreading of inactivation into the autosomal segment in an inactive human X-autosome translocation.

INTRODUCTION

In somatic cells of chromosomally normal mammalian females, one of the two X chromosomes is inactivated [1]. The inactive X chromosome is heterochromatic and replicates its DNA late in the S phase of the cell cycle [2, 3]. Females who are balanced carriers of reciprocal X-autosome translocations have a structurally normal X chromosome and two re-arranged chromosomes each consisting of contiguous X chromosomal and autosomal segments. In such individuals, the structurally normal X is usually inactivated [4]. Occasionally, however, a translocated X is observed to be late replicating [4]. Studies on mouse X-autosome translocations have provided cytogenetic and biochemical evidence for the spreading of X inacti-

Received January 29, 1982.

This study was supported in part by grants HD-05616, HD-04612, HD-12178, and HD-15193 from the U.S. Public Health Service, and grant I-639 from the March of Dimes Birth Defects Foundation. T. M. and L. J. S. are recipients of Research Career Development Awards from the National Institute of Child Health and Human Development.

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vation into the autosomal segment [5, 6]. In human X-autosome translocations, spreading of inactivation into the autosome has been inferred from the late replication of the autosomal segment and from the clinical phenotype of the translocation carrier. However, biochemical confirmation of such spreading has not been available. We now present evidence for the inactivation of a human autosomal locus for the enzyme esterase D (ESD) attached to an inactive X chromosome using somatic cell genetic techniques.

MATERIALS AND METHODS

Isolation of Hybrid Clones

An apparently balanced de novo translocation between the X chromosome and chromosome 13 [46,X,t(X;13) (p22;q12)] was identified in a patient with bilateral retinoblastoma and failure to thrive [7, 8]. Chromosome replication studies in the lymphocytes showed the structurally normal X to be late replicating. However, similar studies on fibroblasts showed the der(X) chromosome (Xqter→Xp22::13q12→13qter) (fig. 1) to be late replicating in each of 104 cells examined [8]. The entire der(X) appeared to be late-labeling, suggesting that the autosomal portion is also inactivated. Eight independent somatic cell hybrid clones were isolated in hypoxanthine aminopterin and thymidine (HAT) medium [9, 10] from the fusion of these fibroblasts and a hypoxanthine guanine phosphoribosyltransferase (HPRT)-deficient mouse cell line (A9; GM 0346, Institute of Medical Research, Camden, N.J.). Detailed cytogenetic and biochemical characteristics of these clones have been reported [11].

Enzyme Analysis

Expression of the following autosomal enzyme markers were evaluated in the hybrid clones by electrophoretic techniques described previously: esterase D (ESD) [12], phosphoglucomutase-2 (PGM2) [13], lactate dehydrogenase A (LDHA) [13], nucleoside phosphorylase (NP) [13], peptidase A (PEP A) [13], glucose phosphate isomerase (GPI) [13], adenosine deaminase (ADA) [13], and superoxide dismutase soluble (SOD1) [13]. In addition, the following X-linked markers were examined by enzyme electrophoresis: glucose-6-phosphate dehydrogenase (G6PD) [14] and phosphoglycerate kinase (PGK) [15]. HPRT, also an X-linked enzyme marker, was examined by isoelectric focusing [16, 17] followed by fluorography [18].

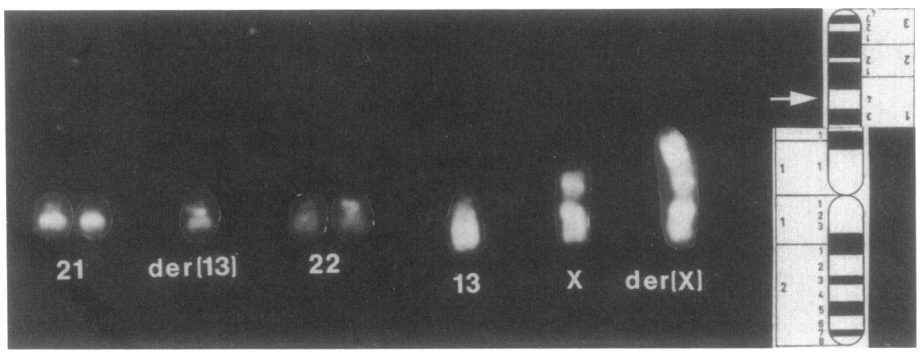

FIG. 1.—Partial Q-banded karyotype of the X/13 translocation carrier. An idiogram of the der(X) chromosome is shown with *an arrow pointing* to band 13q14, to which the locus for human ESD has been assigned.

Cytogenetic Analysis

Standard air-dried preparations from hybrid clones were Q-banded [19], and cytogenetic analysis was done on photographs of well-spread banded metaphases.

Re-activation of Human HPRT

Hybrid cells containing the inactive der(X) chromosome were treated with 5-azacytidine (2.5 μM) for 24 hrs [17]. Following a recovery period of 72 hrs, the cells were plated in HAT medium. Fourteen HAT-resistant colonies were isolated 3 weeks later.

RESULTS

Of eight independent primary hybrid clones isolated in HAT, seven retained a structurally normal X chromosome and expressed human G6PD, a human X-linked marker. One clone (25-8) retained the der(X) chromosome with no other human X chromosome material and was also positive for human G6PD. Thus, there appears to be a small population of cells in the fibroblasts of the patient in which the der(X) is active, although late-replication studies failed to demonstrate their presence.

The gene locus for the human enzyme ESD has been assigned to human chromosome 13 [20, 21]. Regional localization studies have shown that *ESD* is in band q14 of chromosome 13 [22]. Expression of ESD was evaluated in the mouse-human somatic cell hybrid clones by starch gel electrophoresis (fig. 2). The hybrid clone (25-8) containing the active der(X) chromosome, but not a normal 13, was positive for the expression of human ESD. Another clone containing the reciprocal product of the translocation, der(13), [13pter→13q12::Xp22→pter], but not a der(X), was negative for human ESD. These results are consistent with the assignment of

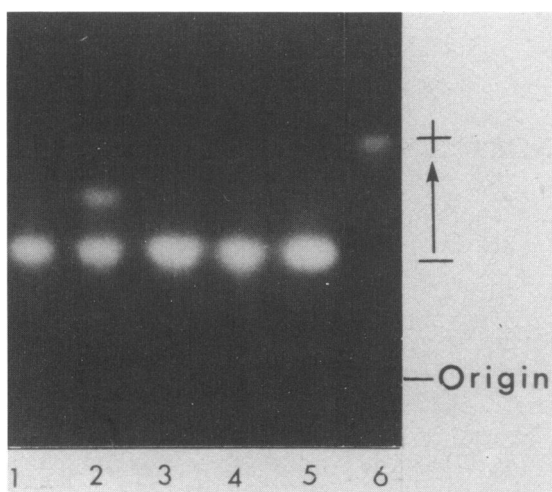

FIG. 2.—ESD electrophoresis. *Channel 1*, mouse parental cell line (A9); *channel 2*, mouse-human hybrid clone positive for the expression of human ESD; *channels 3–5*, mouse-human hybrid clones negative for the expression of human ESD; *channel 6*, human fibroblasts.

ESD to band 13q14 and demonstrate that the *ESD* locus is on the der(X) chromosome.

One of the seven hybrid clones (25-2) containing the structurally normal active X chromosome was found to contain the der(X) chromosome in 13% of the cells analyzed. This clone was placed in medium containing 8-azaguanine to select for cells that had lost the human chromosome carrying the active HPRT locus. From this experiment, a clone (25-2R) was isolated that continued to retain the der(X) chromosome in 10% of analyzed cells and was negative for the expression of human G6PD and ESD. Serial dilution experiments using lysates of cell hybrids with and without an active human X showed that 3% of cells with an active X chromosome is sufficient for our electrophoretic technique to detect the expression of human G6PD. Similar studies for ESD showed that 13% of the cells must have a human chromosome 13 for human ESD to be detected by enzyme electrophoresis. Therefore, the lack of expression of human G6PD in 25-2R shows that the der(X) is indeed inactive. However, the lack of expression of ESD could be due to the low percentage of hybrid cells carrying the der(X) chromosome.

To obtain hybrid clones containing the der(X) chromosome in a high proportion of cells, we re-activated the HPRT locus on the inactive der(X) chromosome and selected for cells that could grow in HAT medium. This approach is based on our recent experiments that indicate that gene loci on the inactive X chromosome can be re-activated by treating cells with low concentrations (1–10 μM) of 5-azacytidine (5-azaC) [17]. Clone 25-2R was therefore treated with 5-azaC. Three weeks later, 14 independent HAT-resistant colonies were isolated. Clone 25-2R contained human chromosomes 3, 4, 5, 11, 14, 15, 18, 19, 20, and 21 in addition to der(X). The 5-azaC-induced HAT-resistant clones isolated from 25-2R were evaluated for the expression of the following markers known to be on the der(X) chromosome: ESD, G6PD, HPRT, and PGK. In addition, they were also assessed for the expression of seven other autosomal enzyme markers known to be on the human autosomes retained in 25-2R. These enzyme markers were: phosphoglucomutase-2, lactate dehydrogenase-A, nucleoside phosphorylase, peptidase-A, glucose phosphate isomerase, adenosine deaminase, and superoxide dismutase (soluble). The gene loci for these enzymes are on chromosomes 4, 11, 14, 18, 19, 20, and 21, respectively.

All 14 of the 5-azaC-induced HAT-resistant clones expressed human HPRT (fig. 3). Human G6PD was expressed in one clone, while all clones were negative for human PGK. The concomitant re-activation of G6PD was also observed in our earlier studies with 5-azaC [17]. None of these clones expressed human ESD, while clone 25-8, containing the der(X) in the active form (used as a control), consistently expressed human ESD. Detailed cytogenetic analysis on at least 30 photographed Q-banded metaphases were done on each of five of these clones. On each of the remaining nine clones, 10 Q-banded cells were analyzed directly under the microscope. The der(X) chromosome was present in every analyzed cell in all 14 clones. The expression of the seven autosomal enzyme markers was concordant with the presence of the relevant human chromosome in the five hybrid clones analyzed in detail.

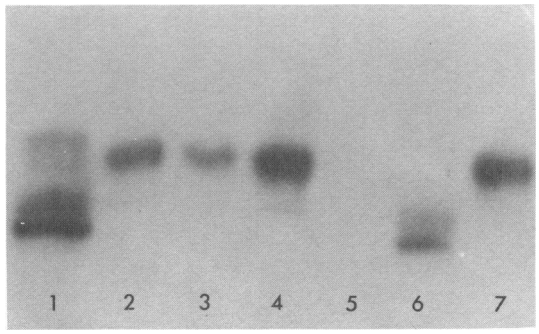

FIG. 3.—Isoelectric focusing of HPRT. *Channel 1*, mixture of mouse cells and human fibroblasts; *channels 2–4*, mouse-human hybrid clones expressing human HPRT; *channel 5*, clone 25-2R which has no HPRT; *channel 6*, mouse cells; *channel 7*, human fibroblasts.

DISCUSSION

The results indicate that the *ESD* locus is inactivated when it is a part of an inactive der(X) chromosome. It is unlikely that the 5-azaC treatment itself rendered the *ESD* gene nonfunctional in these clones, as the expression of seven other constitutive enzyme markers was not affected by 5-azaC. The use of a somatic cell hybrid system in which the inactive X can be selectively retained while permitting segregation of the active X and normal chromosome 13 has allowed us to obtain definitive evidence for the inactivation of a human autosomal locus attached to an inactive X chromosome. One report [23] presented evidence for the inactivation of the human superoxide dismutase (soluble) locus in an X/21 translocation carrier, based on quantitation of the enzyme. However, gene-dosage data need to be interpreted with caution.

Deletion of band 13q14 has been associated with a predisposition to the development of retinoblastoma [24, 25]. Based on the fact that the entire der(X) is late-labeling, it had been proposed that the patient carrying the X/13 translocation (who had retinoblastoma) was effectively monosomic for 13q14 in the majority of her cells resulting from the spreading of inactivation into the corresponding autosomal region [8]. Our finding that *ESD* (which has also been assigned to 13q14) is indeed inactive on the inactivated der(X) chromosome supports this hypothesis.

Investigation of mouse X-autosome translocations employing specific autosomal gene markers has shown that the spreading of the inactivation into the adjacent autosome is variable [5]. This variability is illustrated in studies of Cattanach translocation in the mouse. The attached autosomal segment replicated its DNA late in only half of the metaphases examined. Measurements of the levels of mitochondrial malic enzyme (whose locus is on the relevant autosomal segment) were consistent with variable spreading of inactivation into the autosome [6]. When inactivation does spread into the autosome, it is thought to exert a gradient influence originating in the X-chromosome material, such that autosomal genes farther removed from the point of attachment of the X may be unaffected while

autosomal genes closer to the breakpoint are inactivated [5]. The somatic cell genetic approach we have outlined should be useful in future studies to assess the spreading of inactivation in human X-autosome translocations. With increasing knowledge of the human gene map, such studies should enhance our understanding of human X inactivation.

ACKNOWLEDGMENTS

We gratefully acknowledge the expert technical assistance of Merry B. Passage, Maryellen C. Sparkes, and Roberta Weiss, and the assistance of Kathleen Nelson in preparation of the manuscript.

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