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14. We have evidence that, after selective deactivation of the SII hand representation, the adjacent SII representations of the foot and trunk expand into the vacated territory.
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# The Fragile X Site in Somatic Cell Hybrids: An Approach for Molecular Cloning of Fragile Sites

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**Fragile X syndrome is a common form of mental retardation associated with a fragile site on the human X chromosome. Although fragility at this site is usually evident as a nonstaining chromatid gap, it remains unclear whether or not actual chromosomal breakage occurs. By means of somatic cell hybrids containing either a normal human X or a fragile X chromosome and utilizing two genes that flank the fragile site as markers of chromosome integrity, segregation of these markers was shown to be more frequent if they encompass the fragile site under appropriate culture conditions. Hybrid cells that reveal marker segregation were found to contain rearranged X chromosomes involving the region at or near the fragile site, thus demonstrating true chromosomal breakage within this area. Two independent translocation chromosomes were identified involving a rodent chromosome joined to the human X at the location of the fragile site. DNA analysis of closely linked, flanking loci was consistent with the position of the breakpoint being at or very near the fragile X site. Fragility at the translocation junctions was observed in both hybrids, but at significantly lower frequencies than that seen in the intact X of the parental hybrid. This observation suggests that the human portion of the junctional DNA may contain part of a repeated fragility sequence. Since the translocation junctions join heterologous DNA, the molecular cloning of the fragile X sequence should now be possible.**

FRAGILE SITES ARE SPECIFIC CHROMOSOMAL loci, inherited in a Mendelian co-dominant manner, where chromatid gaps and/or breaks occur after specific biochemical induction. The molecular mechanism by which such sites exhibit fragility is unknown, although various genetic analyses have concluded that it is due to some, as yet unidentified, DNA sequence or sequences residing at the observed chromosomal site (*1*). Of the several human fragile sites currently recognized, the X chromosome site mapping to the vicinity of band Xq27.3 has been the most intensely studied as it is associated with mental retardation (*2*). This condition, referred to as fragile X–linked mental retardation or fragile X syndrome, is inherited in an X-linked semidominant fashion and is the single most common form of inherited mental deficiency in humans with a prevalence of greater than 1 per 2000 newborn males. It is an unusual X-linked disorder in that approximately 30% of carrier females express the phenotype of mental retardation and there is a relatively high frequency of nonpenetrant carrier males (*3*).

Chromosome breakage at the fragile X site, as well as at the autosomal sites, has been observed in individual metaphase spreads; questions have been raised as to whether chromosome breakage also occurs at fragile sites in the intact cell since physical shearing during cytogenetic preparation may influence chromatid stability at these sites (*4*). Furthermore, genetic evidence for breakage at fragile sites, such as their involvement in chromosome deletions or rearrangements, is not observed in appreciable frequencies. Although a correlation has been suggested between fragile site loci and the breakpoints of nonrandom chromosomal rearrangements in certain forms of cancer (*5*), significance of this association remains unclear, particularly in the absence of relevant data on fragile site breakage. We therefore wished to determine if there is a predilection for chromosome breakage at the fragile X site within the dividing cell and, if so, to determine if such breakage leads to useful rearrangements that could be utilized for subsequent molecular cloning experiments.

A strategy for using interspecific somatic cell hybrids to detect fragile site breakage was developed through the following rationale. First, we and others have shown that the fragile X site is cytogenetically expressed in hybrid cells and results in a cell system more easily manipulated for genetic analysis (*6*). Second, since there is evidence that the de novo loss of Xq28 (the region distal to the fragile site) in early embryogenesis leads to preferential X inactivation (*7*), it is possible that later somatic loss of this region on the fragile X chromosome results in mitotic failure and, hence, the absence of cytogenetic observations. A similar loss in a somatic cell hybrid would presumably be complemented by rodent loci and result in no significant effect on cell viability. Third, the experimental design could employ two X-linked loci which flank the fragile X site, those for hypoxanthine guanine phosphoribosyl transferase (HGPRT), and glucose-6-phosphate dehydrogenase (G6PD). By using rodent cells deficient in both activities fused with human cells, hybrids are isolated that express only the human forms of these enzymes. Since the HGPRT locus is proximal to the fragile X site relative to the centromere at Xq26–Xq27.2 (*8*), breakage in the vicinity of the fragile X site should result in an acentric fragment containing the G6PD locus at Xq28 (*8*) which would be mitotically unstable. It should then be possible to follow segregation of these two enzymes by placing positive selective pressure upon cells to maintain HGPRT activity followed by histochemical staining for G6PD activity.

Lymphoblasts, established from a clinically normal male or a retarded male with confirmed fragile X syndrome, were fused with Chinese hamster ovary cells deficient in both HGPRT and G6PD activities (*9*). Somatic cell hybrids expressing HGPRT were selected in medium containing hypoxanthine and azaserine [HAS medium; (*10*)] and determined to be expressing the human isomeric form of G6PD by electrophoresis. Two clones were selected for further analysis: Y75-1B, in which cytogenetic analysis revealed the presence of an intact fragile X chromosome as well as a human chromosome 13; and Y130-3A, which contained an intact X chromosome from a normal male and two unidentified human autosomes.

Colonies from either hybrid, grown in medium containing HAS, were consistently $G6PD^+$ (>90%) on the basis of in situ histochemical staining, which results in blue positive colonies and yellow negative colonies (*11*). Back-selection of the hybrids in

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**Table 1.** Frequency of somatic cell hybrid colonies staining negative for G6PD activity. Hybrids containing either a normal human X chromosome (Y130-3A) or a fragile X chromosome (Y75-1B) were isolated from fusions between lymphoblastoid cell line GM130-3A or MGL75 and a Chinese hamster ovary cell line (YH21) that is deficient in HGPRT and G6PD activities (*9*). Somatic cell fusion and cell culture protocols were performed as described (*6*). Cells ($1 \times 10^4$) were inoculated into either medium F12 or Dulbecco's modified Eagle's medium (DMEM), both supplemented with 10% fetal bovine serum, 100 $\mu M$ hypoxanthine, and 10 $\mu M$ azaserine to maintain positive selection on colonies expressing HGPRT activity. After cell attachment, thymidine stress was placed upon the cells by adding either $5 \times 10^{-8} M$ fluorodeoxyuridine (FUdR; in supplemented DMEM medium) or 1 m$M$ thymidine (in supplemented F12 medium). The stress was removed 96 hours later by changing the medium with either supplemented DMEM or F12 and the cells were allowed to recover for 24 hours. The cells were then trypsinized, counted, and used to inoculate 100-mm culture dishes at a density of 500 cells per dish. Approximately 10 days later the resulting colonies were rinsed with phosphate-buffered saline and histochemically stained in situ for G6PD activity as described (*11*). After counterstaining with metanil yellow, colonies were scored for G6PD activity. Colonies were scored $G6PD^+$ if they contained less than 10% negatively staining cells microscopically while $G6PD^-$ colonies were scored as such if they contained less than an estimated 10% positive cells. The remainder, containing mixed or sectored colonies, were not included in the count but did not constitute greater than 5% of the total colonies in any experiment.

| Cell line | Thymidine stress | $G6PD^-$ colonies (%)* |
|---|---|---|
| Y75-1B (fragile X) | No | 6 (2–9) |
| Y75-1B (fragile X) | Yes | 19 (6–32) |
| Y130-3A (normal X) | No | 4 (1–7) |
| Y130-3A (normal X) | Yes | 6 (2–14) |

*Data represent the means of 15 independent experiments resulting in approximately $2 \times 10^4$ total colonies per group. Numbers in parentheses show the range among the experiments.

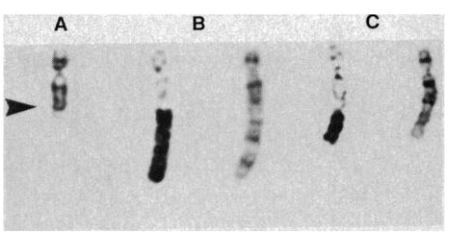


**Fig. 1.** Cytogenetic analysis of fragile X–containing somatic cell hybrid and two $HGPRT^+/G6PD^-$ subclones. (**A**) GTG-banding of the human X chromosome from hybrid Y75-1B showing expression of the fragile X site at Xq27 (arrowhead); (**B**) G-11 staining (left) and GTG-banding (right) of translocation chromosome from hybrid subclone Y75-1B-3C; (**C**) G-11 staining (left) and GTG-banding (right) of translocation chromosome from hybrid subclone Y75-1B-28. G-11 staining differentiates human (light) from rodent (dark) chromosomal material. GTG-banding and G-11 staining as described (*8*).

medium containing 6-thioguanine caused virtually all colonies (99%) to revert to the $G6PD^-$ phenotype of the rodent parent. Since this back-selection favors colonies that have lost HGPRT activity, and therefore the human X chromosome, these data establish syntenic behavior of the two enzyme activities in our experimental system.

The hybrids were cultured in HAS medium under thymidine stress for a period of several days, allowed to recover, and replated in HAS medium to form colonies. As shown in Table 1, histochemical staining of these colonies showed a significantly greater number of $G6PD^-$ colonies in the fragile X–bearing hybrid, Y75-1B, than the normal X hybrid, Y130-3A. Over the course of several such experiments, the absolute numbers varied considerably for reasons not yet understood. However, in all experiments the fragile X hybrid had at least a twofold greater number of $G6PD^-$ colonies. In both hybrids, up to 9% of the colonies were $G6PD^-$ in the absence of thymidine stress, presumably due to the inherent instability of human chromosomes in a rodent background. We interpret these data as evidence (i) for segregation of the genes for HGPRT and G6PD due to chromosome breakage and (ii) that segregation is more frequent if these loci encompass the fragile X site. Sectored colonies that were one-quarter negative and three-quarters positive for G6PD were occasionally observed, which also suggests the occurrence of a stable genetic event such as chromosome breakage.

Random colonies from either Y75-1B or Y130-3A were isolated after culturing under conditions of thymidine stress, as described in Table 1. Cells were cultured in HAS medium in duplicate wells; after growth to subconfluence in HAS medium, one well of each duplicate was histochemically stained for G6PD activity. Four $G6PD^-$ colonies from 56 Y75-1B and two $G6PD^-$ colonies from Y130-3A were identified from 49 isolations. All colonies maintained their phenotype after expansion and rescreening. Cytogenetic analysis by G-11 staining (*12*), which results in dark magenta rodent chromosomes and light blue human chromosomes, was performed on these subclones to determine the structure of the human X chromosomes. Two Y75-1B subclones as well as the two Y130-1B subclones showed rearrangements of the human X chromosome not interpretable by GTG-banding. However, two other Y75-1B subclones, Y75-1B-3C and Y75-1B-28, were found to carry a translocation between the centric human X and varying amounts of rodent chromosomal material, with the junction near the end of the long arm of the X (Fig. 1). Subsequent GTG-banding on both subclones confirmed the human breakpoint in the vicinity of the Xq27–28 interband region; human chromosome 13 was also maintained in these subclones. On the basis

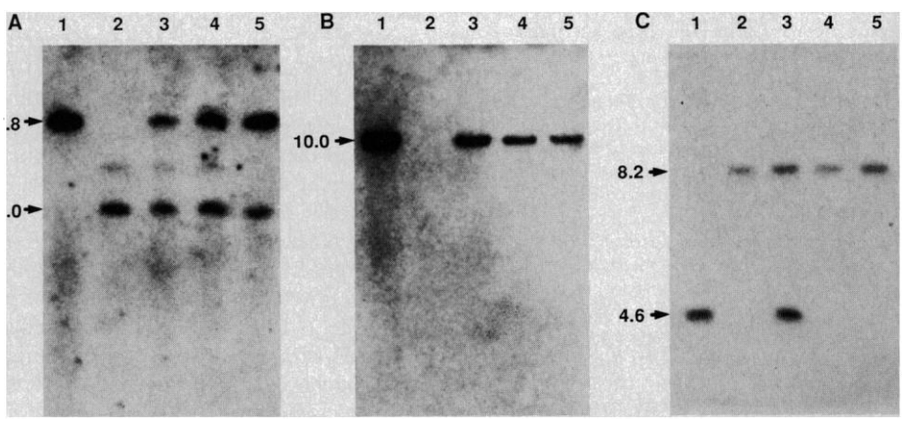


**Fig. 2.** Southern blot analysis of genomic DNA from parental cell lines, derived somatic cell hybrid, and two $HGPRT^+/G6PD^-$ subclones. Lane 1, human parent MGL75; lane 2, rodent parent YH21; lane 3, somatic cell hybrid with intact human X Y75-1B; lane 4, translocation hybrid Y75-1B-3C; lane 5, translocation hybrid Y75-1B-28. (**A**) Pvu II–digested genomic DNA hybridized with a human factor IX probe detecting a human 7.8-kb band and a 4.0-kb rodent band. (**B**) Eco RI–digested genomic DNA hybridized with the human probe CX55.7 detecting a 10.0-kb human band. (**C**) Pvu II–digested genomic DNA hybridized with a human factor VIII probe detecting a 8.2-kb rodent band and a 4.6-kb human band. Genomic DNA (10 $\mu$g) was digested according to manufacturer's recommendations, separated on a 0.7% agarose gel, stained with ethidium bromide, and photographed with ultraviolet illumination. Gels were soaked for 10 minutes in 0.25$M$ HCl prior to capillary transfer to Zeta-bind nylon membrane with 0.4$M$ NaOH. Filters were prehybridized overnight at 65°C in a hybridization solution containing 7% SDS, 1% bovine serum albumin, 10% PEG-8000 (w/v), and 250 $\mu$g of sonicated, denatured salmon sperm DNA in 0.25$M$ NaCl, 1 m$M$ EDTA, and 0.25$M$ sodium phosphate buffer. DNA fragments were labeled with $^{32}$P-dCTP by means of random primers, denatured, and added to the hybridization buffer. Hybridization was carried out for 20 hours at 65°C. The filters were washed twice in 2× SSC at 65°C and then in 0.5× SSC at 65°C for 30 minutes. Filters were subjected to autoradiography as described (*13*).

of the resolution afforded by cytogenetic analysis, it appears that a chromosomal break occurred in two independent clones between the loci for HGPRT and G6PD with loss of the distal band of the human X, which rejoined to different rodent chromosome arms.

Southern blotting of genomic DNA isolated from the two translocation hybrids, as well as from the original hybrid and the parental lines, was performed to determine how close the translocation breakpoint was to the fragile X site. Restriction endonuclease digests were separated on agarose gels and the DNA fragments were blotted onto nylon filters (*13*). The filters were hybridized with radiolabeled fragments from the human factor IX gene and the human factor VIII gene, which flank the fragile site at locations 12 centimorgans (cM) proximal and 5 cM distal, respectively (*14*). An additional anonymous DNA probe (CX55.7) was used which maps closer to the proximal side of the fragile site than factor IX at 5 cM (*15*). A 7.8-kb band, representing the human factor IX sequence, was observed in both translocation hybrids, suggesting that the breakpoint is distal to this gene (Fig. 2). The probe CX55.7, which is homologous to a sequence distal to factor IX but still proximal to the fragile site, detects a 10.0-kb band still retained in both translocation hybrids. Analysis with the factor VIII probe, however, reveals absence of the 4.6-kb human band in both translocation hybrids although the rodent 8.2-kb band is observed, indicating the presence of hybridizable DNA in those lanes. These data suggest that the human X chromosome breakpoint in both independent $HGPRT^+$, $G6PD^-$ subclones of the fragile X–bearing hybrid are within a 10-cM region (inclusive of the fragile X site). Since segregation of G6PD from HGPRT could result from any chromosome break within the estimated 40 cM that separate these loci (*16*), the observation that at least half of such segregants have breakpoints within a specific 10-cM region suggests the presence of nonrandom chromosome rearrangement.

We next wished to determine whether or not the fragile site remained on the translocations. We performed cytogenetic examination of metaphase spreads prepared from the subclones cultured under conditions of thymidine stress with or without 2 m*M* caffeine treatment prior to harvest. Caffeine was recently shown to enhance cytogenetic expression of the fragile X site in somatic cell hybrids (*17*). Both translocation chromosomes revealed fragility near the vicinity of the junction between the human and rodent chromosomes. As shown in Fig. 3, the translocation in subclone Y75-1B-3C breaks clearly between the light and dark staining regions of the junction at the bottom of band Xq27 (as observed with either G-11 staining or GTG-banding). This finding suggests retention of the fragile site sequence on the translocation chromosomes. However, the fragility frequencies (Table 2) are markedly different from that of the original hybrid and the parental line MGL75. The original hybrid, Y75-1B, containing the intact fragile X chromosome, expresses the fragile site in 12% of metaphases scored as compared with 23% expression in the parental human lymphoid line. This observation is consistent with data obtained from various laboratories, including our own, where fragile X expression is often, but not consistently, lower than that observed in the parental cells (*6*). Both translocation subclones exhibited fragility at the translocation junction, but at considerably lower frequencies. The difference was especially evident when caffeine was added prior to cell harvest, the original hybrid exhibited the fragile X site in 52% of the examined metaphases compared to the translocation hybrids Y75-1B-3C and Y75-1B-28, which exhibited fragility in only 17% and 26% of the metaphase, respectively. Thus, while the translocation junctions were still fragile, they were consistently less fragile than in the intact X chromosome.

Although there are many possible explanations for reduced fragility in the translocation hybrids, our data are consistent with a model proposed by Ledbetter *et al.* (*18*) who suggested that the fragile X site is a reiterated DNA sequence of variable length, the longest length being found in fully penetrant males and the shortest in phenotypically normal individuals who would carry a common fragile site at Xq27. According to this model, transmitting or nonpenetrant males would have an intermediate amount of fragile sequence repeats. Fragility in this region of the X has been shown to support this model in that normal, transmitting, and affected male X chromosomes (in somatic cell hybrids) show increasing frequencies of fragility. Thus, in the studies reported here, if chromosome breakage in the original somatic cell hybrid occurred within this postulated repeat sequence, and some portion remained to rejoin with the rodent chromosome, fragility at the translocation junction would be retained but at reduced frequencies.

Therefore, it is possible that the immediate human portion of the translocation junctions in the two hybrids just described contains part of the fragile X sequence itself. Since these junctions join heterologous DNA, it should now be possible to isolate an insert from a cosmid library of genomic DNA that contains both human as well as hamster DNA by probing for species-specific repeated sequences. The human portion

**Table 2.** Chromosome fragility at the human fragile X site in the parental human lymphoblastoid cell line (MGL75), the derived somatic cell hybrid (Y75-1B) that contains the intact fragile X chromosome, and two independent $HGPRT^+/G6PD^-$ subclones (Y75-1B-3C and Y75-1B-28) each containing a human fragile X–rodent translocation chromosome. Fragility was induced in the lymphoid cells by culturing in medium RPMI 1640 supplemented with 15% fetal bovine serum and $1 \times 10^{-7}$ *M* FUdR for 24 hours prior to harvest. Hybrid cell lines were cultured in medium F12 supplemented with 10% fetal bovine serum, 100 μ*M* hypoxanthine, and 10 μ*M* azaserine. Thymidine stress was induced by the addition of 2 m*M* thymidine to the medium for 24 hours. Caffeine enhancement was achieved by the addition of 2 m*M* caffeine to the medium 2 hours prior to harvest. Chromosomes of the cell hybrids were stained with G-11 for analysis while the human cell line was plain-stained with Giemsa. In selected experiments, fragile X–positive metaphases were GTG-banded for confirmation. Data were obtained from at least two independent experiments. Numbers in parentheses indicate total metaphases showing fragile X expression over total metaphases examined, N.T., not tested.

| Cell line | Percent expression after thymidine stress | |
|---|---|---|
| | Without caffeine | With caffeine |
| Parental | | |
| MGL75 | 23 (23/100) | N.T. |
| Hybrid | | |
| Y75-1B | 12 (24/200) | 52 (104/200) |
| Translocation hybrids | | |
| Y75-1B-3C | 5 (3/115) | 17 (40/230) |
| Y75-1B-28 | 8 (19/238) | 26 (34/130) |

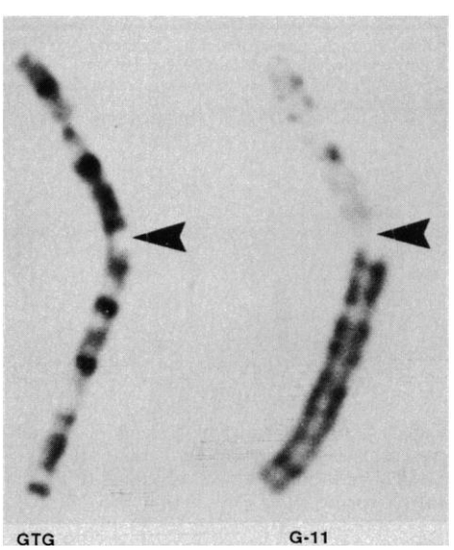


**Fig. 3.** Chromosome fragility at the translocation junction of hybrid subclone Y75-1B-3C (as described in Table 2). (Left) GTG-banding and (right) G-11 staining. Arrowhead indicates a single chromatid break, used for graphic clarity although most gaps involved both chromatids typical of fragile sites.

of such a cosmid insert should be a portion of the fragile site. (Microcell transfer of the translocations to fresh rodent backgrounds or isolation of the translocations through chromosome sorting should significantly enhance the ability to detect the translocation junction.) Although it remains possible that the break actually occurred distal to the fragile site, the human portion of the translocation junction would still be closer than 5 cM from the site. Indeed, the genetic distance of this region may be inflated because of unusually frequent recombination (*19*) such that a nearby DNA fragment may be physically closer to the fragile site than the distance of 5 cM suggests. This physical distance would approach the resolution of pulsed field and field inversion gel electrophoresis as well as "jumping" libraries (*20*). Furthermore, such nearby fragments, if not of the fragile site itself, would be immediately useful for linkage analysis in families segregating the fragile X chromosome (*21*).

Several conclusions can be drawn from the data presented. First, it is shown that a fragile site can influence specific chromosome breakage within the dividing cell. Second, this fragile site-mediated breakage can lead to nonrandom chromosome rearrangement involving the fragile site. This finding is of relevance concerning the postulated relationship between certain autosomal fragile sites and the breakpoints of nonrandom chromosome translocations seen in leukemias and lymphomas (*5*). Third, the observation of reduced chromosome fragility at the translocation junctions lends support for the model of the fragile X site as a reiterated DNA sequence. Lastly, the method of identifying cell hybrids that carry chromosome rearrangements of a human fragile site and rodent DNA suggests a means for molecular cloning of fragile site sequences, and may be used for selected autosomal fragile sites where appropriate markers exist.


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# Smooth Muscle–Mediated Connective Tissue Remodeling in Pulmonary Hypertension


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**Abnormal accumulation of connective tissue in blood vessels contributes to alterations in vascular physiology associated with disease states such as hypertension and atherosclerosis. Elastin synthesis was studied in blood vessels from newborn calves with severe pulmonary hypertension induced by alveolar hypoxia in order to investigate the cellular stimuli that elicit changes in pulmonary arterial connective tissue production. A two- to fourfold increase in elastin production was observed in pulmonary artery tissue and medial smooth muscle cells from hypertensive calves. This stimulation of elastin production was accompanied by a corresponding increase in elastin messenger RNA consistent with regulation at the transcriptional level. Conditioned serum harvested from cultures of pulmonary artery smooth muscle cells isolated from hypertensive animals contained one or more low molecular weight elastogenic factors that stimulated the production of elastin in both fibroblasts and smooth muscle cells and altered the chemotactic responsiveness of fibroblasts to elastin peptides. These results suggest that connective tissue changes in the pulmonary vasculature in response to pulmonary hypertension are orchestrated by the medial smooth muscle cell through the generation of specific differentiation factors that alter both the secretory phenotype and responsive properties of surrounding cells.**


PERSISTENT PULMONARY HYPERTENsion in infancy leads to severe lung vascular changes that include marked hypertrophy, extension of vascular smooth muscle to small vessels, adventitial thickening, and increased connective tissue deposition in both the media and adventitia (*1, 2*). In animal models of pulmonary hypertension, increased collagen deposition appears to be important in causing altered mechanical properties of hypertensive vessels (*3, 4*), but other connective tissue components also


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