Isolation of the human chromosomal band Xq28 within somatic cell hybrids by fragile X site breakage

(somatic cell genetics/fragile X syndrome/cytogenetics/human genome analysis)

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ABSTRACT The chromosomal fragile-site mapping to Xq27.3 is associated with a frequent form of mental retardation and is prone to breakage after induced deoxyribonucleotide pool perturbation. The human hypoxanthine phosphoribosyltransferase (HPRT) and glucose-6-phosphate dehydrogenase (G6PD) genes flank the fragile X chromosome site and can be used to monitor integrity of the site in human–hamster somatic cell hybrids deficient in the rodent forms of these activities. After induction of the fragile X site, negative selection for HPRT and positive enrichment for G6PD resulted in 31 independent colonies of HPRT−,G6PD+ phenotype. Southern blot analysis demonstrated the loss of all tested markers proximal to the fragile X site with retention of all tested human Xq28 loci in a majority of the hybrids. In situ hybridization with a human-specific probe demonstrated the translocation of a small amount of human DNA to rodent chromosomes in these hybrids, suggesting chromosome breakage at the fragile X site and the subsequent translocation of Xq28. Southern blot hybridization of hybrid-cell DNA, resolved by pulsed-field gel electrophoresis, for human-specific repetitive sequences revealed abundant CpG-islands within Xq28, consistent with its known gene density. The electrophoretic banding patterns of human DNA among the hybrids were remarkably consistent, suggesting that fragile X site breakage is limited to a relatively small region in Xq27–28. These somatic cell hybrids, containing Xq27.3–qter as the sole human DNA, will aid the search for DNA associated with the fragile X site and will augment the high resolution genomic analysis of Xq28, including the identification of candidate genes for genetic-disease loci mapping to this region.

We have described (5) a somatic cell-hybrid system that can monitor chromosome breakage within or near the fragile X site. Somatic cell hybrids were produced from fusions of Chinese hamster ovary cells deficient in both hypoxanthine phosphoribosyltransferase (HPRT, EC 2.4.2.8) and glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) with lymphoblasts from a male patient with fragile X syndrome. Clones were isolated that contained the human fragile X chromosome and expressed only the human forms of both HPRT and G6PD. Because HPRT and G6PD are syntenic on the human X chromosome, flanking the fragile X site at Xq26 and Xq28, respectively, positive selection for HPRT activity and histochemical staining for the loss of G6PD activity can identify hybrid cells that segregate these markers. Such segregation, after biochemical induction of the fragile X site through thymidylate stress, has been shown to result from chromosome breaks at or very near the fragile X site. Recovered HPRT+,G6PD− hybrids carried interspecific translocation chromosomes of the human X chromosome deleted of the terminal band Xq28. These chromosomes were evidence of specific chromosomal breakage at the fragile X site and also provided a heterologous breakpoint useful for mapping and cloning studies (6, 7). However, because most of the human X chromosome was retained in these hybrids (~170,000 kilobases (kb)) of DNA), their usefulness was limited for many molecular studies.

To facilitate the search for fragile X site-associated DNA, the studies reported here centered on the isolation of the reciprocal translocation event—the translocation of the human DNA of band Xq28 to a rodent chromosome, with the loss of all other human DNA. Carrying less than an estimated 10,000 kb of human DNA (based upon metaphase chromosome length, band Xq28 represents 5−6% of the X chromosome or ~0.3% of the haploid human genome), such hybrids would be amenable to molecular analysis. Moreover, the isolation of this limited region of the human genome within somatic cell hybrids will allow saturational cloning and detailed molecular analysis of Xq28. This chromosomal band is of particular interest for high-resolution genomic studies, as it is the most gene-dense region of the human genome currently recognized, containing over 24 assigned loci. [Recent map positions were obtained from the on-line data bases of Mendelian Inheritance in Man (OMIM) at the Johns Hopkins Hospital and the Howard Hughes Medical Institute's Human Gene Mapping Library (HGML) in New Haven (Yale University).] Many of these loci have alleles respon-

Fragile sites are heritable chromosomal loci that form cytologically evident gaps and/or breaks after specific biochemical induction (1, 2). These loci occur throughout the human genome and are found at variable population frequencies, from very rare to constitutive. The molecular nature of fragile sites and their biological consequences are poorly understood. The rare fragile-site mapping to Xq27.3, referred to as the fragile X site, is the only fragile site associated with a distinct phenotype, being found among patients and carriers of Martin–Bell syndrome (also known as the fragile X syndrome). This chromosome X-linked condition is responsible for the most frequent form of inherited mental retardation in humans, occurring at frequencies of >1 per 2000 newborns (3). It is unusual among mammalian X-linked loci because the gene is penetrant in ~30% of female carriers and there is a relatively high frequency (~20%) of nonpenetrant carrier males (4). The fragile X site itself is either the basis of this syndrome or is tightly linked to the gene(s) responsible.

Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; G6PD, glucose-6-phosphate dehydrogenase; HAS, hypoxanthine/ azaserine medium.

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sible for significant genetic diseases of which little is understood at the molecular level, such as those for Emery–Dreifuss muscular dystrophy, adrenoleukodystrophy, incontinentia pigmenti, nephrogenic diabetes insipidus, and an inherited form of manic–depressive illness. Thus, the prospects of identifying and isolating candidate genes for these diseases, by reverse genetic approaches, should also be enhanced by the availability of Xq28–only somatic cell hybrids.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Cells were grown, unless otherwise noted, in F-12 medium/6% fetal bovine serum in a humidified atmosphere of 5% CO2 at 37°C. Hybrid Y75-1B-M1 carries the human fragile X as the sole human chromosome and was isolated by the microcell transfer of hybrid Y75-1B (described in ref. 5) to the Chinese hamster ovary line Y21 (deficient in rodent HPRT and G6PD), as described by Warren et al. (8). The hybrids were maintained in the above medium with the addition of 100 μM hypoxanthine/1 μM azaserine (HAS medium) to select for retention of the human X chromosome. A human fragile X chromosome-only hybrid (frax 3200-3, ref. 9) was also used to assess diameide sensitivity relative to G6PD activity because this hybrid contained human and rodent G6PD activities and had a total activity 1.9 times that of hybrid Y75-1B-M1.

Selection for HPRT-G6PD* Hybrid Cells. Ten culture dishes (100 mm), containing HAS medium, were each inoculated with 7 × 105 Y75-1B-M1 cells and allowed to attach. The fragile X site was induced to break by changing to HAS medium/2 mM thymidine. Twenty hours later, caffeine was added to a final concentration of 2 mM, and the cells were further incubated for 6 hr. The medium was then removed, the cells rinsed twice with prewarmed phosphate-buffered saline, and nonselective F-12 medium/6% fetal bovine serum was added. Twenty-four hours later, the cells were subcultured as follows: the medium was removed, and the cells were rinsed with phosphate-buffered saline and trypsinized. The trypsinized cells were used to inoculate sets of 10 culture dishes (100 mm) at 1 × 10⁴ cells per dish. Another set of dishes was inoculated with 200 cells per dish for subsequent G6PD histochemical staining. Every 5 days, the sets of 10 dishes were subcultured as above, and the colonies of the low-density dishes were stained for G6PD activity.

To select for a cell deficient in HPRT activity, the cells were subcultured as above, except 10 μM 6-thioguanine was added to the medium. A set of dishes, containing 6-thioguanine medium, was inoculated at 5 × 10³ cells per dish in addition to dishes containing nonselective medium inoculated at 200 cells per dish. The latter groups of dishes were used, once colonies developed, to assess the frequency of G6PD* colonies by histochemical staining. To enrich for G6PD* colonies after selection against HPRT, the cells were subcultured as described immediately above, except that 40 μM diameide was added to the 6-thioguanine medium. The amount of diameide used was determined from sensitivity studies, relative to G6PD activities, by measuring colony-forming ability in the presence of increasing diameide concentrations, relative to control dishes, of cells with <1%, 100%, and ~200% G6PD activity. (Y75-1B-M1 and frax 3200-3, respectively). Once the frequency of G6PD* cells was appreciable, random colonies were picked and used to inoculate 2 wells of 36-well plates. After sufficient growth, 1 of the 2 wells was histochemically stained for G6PD activity. Histochemical staining for G6PD activity was done as described (10).

In Situ Hybridization. Metaphase cells were prepared by using routine methods and subjected to fluorescent in situ hybridization by using biotinylated human DNA as probe. The procedure was essentially as described by Pinkel et al. (11) using human genomic DNA sonicated to <1 kb in size and biotinylated by nick translation with biotin-dUTP. Selected hybrids were also analyzed by using G-11 staining, as described (12), to differentiate between rodent and human chromosomal material.

DNA Preparation and Southern Analysis. Genomic DNA was isolated according to a modification of that described by Miller et al. (13). Ten to fifteen micrograms of DNA was digested with restriction endonucleases as recommended by the supplier and subjected to agarose gel electrophoresis and Southern analysis, as described (14, 15). Cloned DNA probes were provided by the following investigators: C. T. Caskey ( Baylor University, Waco, TX) (pHPT), D. Drayna (Genentech) (p52A, DX13), R. Nussbaum (University of Pennsylvania, Philadelphia) (4D8), J. L. Mandel (Institut National de la Santé et de la Recherche Médicale, Strasbourg) (S14.1), J. Nathans (Johns Hopkins University, Baltimore) (hs7), J. Gitschier (University of California, San Francisco) (FBC), D. Toniolo (Consiglio Nazionale delle Ricerche) (pTV-3A), and P. Pearson (Johns Hopkins University) (ccxs 7.7). Pulsed-Field Gel Electrophoresis. Cells were harvested, and agarose plugs were prepared as described (16). Restriction endonuclease digestion was accomplished by equilibrating the plugs for 30 min in the supplier’s suggested buffer before the addition of enzyme at 100 units per ml in fresh buffer. Digestion occurred overnight at 37°C. Pulsed-field gel electrophoresis was carried out, as described (16), on a Bio-Rad CHEF-DR II system using λ DNA ladders (FMC) and Saccharomyces cerevisiae chromosomes (Bio-Rad) as size markers. Electrophoresis was done through 1% agarose (J. T. Baker) in 0.5× TBE buffer (1× TBE) is 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.6) at 230 V at 11.5°C with pulse times of 30 sec for 18 hr followed by 50-sec pulses for an additional 9 hr.

Gels were then stained with ethidium bromide, destained in 0.5× TBE buffer, and photographed over UV illumination. The gels were then treated with 0.25 M HCl (20 min), denatured in 1.5 M NaCl/0.5 M NaOH (30 min), and renatured in 1 M Tris-HCl, pH 7.0/1.5 M NaCl (twice for 30 min). DNA was transferred onto Zeta-Probe membrane (Bio-Rad), and hybridized (16) with oligo-labeled sonicated total human DNA as probe. Filters were washed to very high stringency of 0.05× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) for 3 hr at 65°C. Under these conditions, no hybridization to rodent chromosomal DNA was detected (data not shown). Single-copy probe filters were washed to a final stringency wash of 0.2× SSC for 1 hr at 65°C. Autoradiography was carried out at −80°C overnight to 3–4 days.

RESULTS AND DISCUSSION

Isolation of HPRT-G6PD* Somatic Cell Hybrids. From previous work on human fragile X chromosome-bearing somatic cell hybrids (5), it was shown that specific breakage at or near the fragile X site, induced by thymidine stress, can lead to rejoining of the broken human chromosome ends to rodent chromosomes. By using the flanking loci of HPRT and G6PD as markers of breakage, a strategy was developed to result in (i) breakage at or near the fragile X site, causing human Xq28 to translocate to a rodent chromosome in a small number of cells; (ii) segregation of the intact and deleted centric human X out of the hybrid by subsequent selection with 6-thioguanine to ensure the loss of HPRT activity; and (ii) exposure to the oxidant diameide to enrich the population for presence of G6PD activity. Histochemical staining for G6PD activity is used throughout the protocol to monitor the presence and frequency of Xq28 within the hybrids as well as to identify HPRT-G6PD* cells for further analysis.

Key to this strategy was the ability to significantly enrich the population for G6PD* cells among an excess G6PD
population. As shown earlier by Rosenstrauss and Chasin (17), cells deficient in G6PD are markedly sensitive to diamide as their capacity to counter oxidative damage is compromised by the deficiency of NADPH, a product of G6PD metabolism. To determine the optimal diamide concentration for enrichment, survival studies were performed with Chinese hamster ovary cells deficient in G6PD and somatic cell hybrids bearing a human X chromosome (and therefore an expressed G6PD gene) within a G6PD− or a G6PD+ hamster cell (Y75-1B-M1 and frax 3200-3, respectively). Spectrophotometric assays for total G6PD activity demonstrated for cell lines Y21, Y75-1B-M1, and frax 3200-3 specific activities of 0.001 ± 0.004 unit/mg of protein, 0.043 ± 0.005 unit/mg of protein, and 0.099 ± 0.006 unit/mg of protein, respectively. As shown in Fig. 1, G6PD activity and diamide sensitivity were inversely correlated. Cell line Y21, deficient in G6PD activity, is markedly more sensitive to diamide exposure than cell lines with such activity. This correlation between diamide sensitivity and G6PD activity is further supported by the observation that frax 3200-3 cell line is >2-fold more resistant to diamide than Y75-1B-M1 cell line. At diamide concentrations >35 μM a >500-fold sensitivity differential is apparent between Y21 and Y75-1B-M1 cells, allowing <0.1% of the G6PD-deficient cells to survive. For the experiments described below, 40 μM diamide was chosen for selective enrichment of G6PD+ cells among a population of deficient cells.

Selections were carried out by using hybrid Y75-1B-M1, which contains the human fragile X chromosome as the only human DNA against a Chinese hamster background. Under selective growth in medium containing hypoxanthine/azaserine (HAS) medium, this hybrid was stable and expressed only the human isomeric forms of HPRT and G6PD; reversion of the hamster mutations at these loci was <1 × 10−6. Results of a typical experiment are shown in Table 1. After treatment known to induce both cytogenetic expression and chromosome breakage of the fragile X site (3), the cells were removed from HAS medium and propagated serially under nonselective conditions. By the ninth passage, ~28% of the colonies were deficient in histochemical G6PD activity. This loss of G6PD activity reflects segregation of the human X chromosome out of the hybrid cell line in the absence of positive selection for the syntenic HPRT gene. Of the G6PD+ cells, most would consist of hybrids that had not yet lost the human X chromosome and maintain HPRT activity, whereas a relatively small pool of cells would be HPRT−,G6PD− due to X chromosome breakage and translocation. Selection against this HPRT+,G6PD+ cell majority was accomplished with 6-thioguanine, which forms a toxic product when metabolized by HPRT and results in resistant HPRT-deficient cells; of these, 1−2% stained positive for G6PD activity. To facilitate the clonal isolation of these cells, enrichment for G6PD+ cells by treatment with 40 μM diamide was performed. After diamide treatment, there was a 50-fold enrichment for G6PD+ cells, resulting in a cell population of which ~90% were HPRT−,G6PD+. From seven such experiments (of serial passages and selections of individual experimental groups), 31 independent HPRT−,G6PD+ hybrids were isolated for study.

Characterization of HPRT−,G6PD+ Somatic Cell Hybrids. Initial cytogenetic screening by G-11 analysis, which differentiates human from rodent chromosome material (12), showed that 17 of the 31 hybrids had small amounts of human material as the only apparent human DNA within the hybrids. The remaining hybrids had more complex rearrangements resulting in retention of much greater than expected amounts of human DNA, some in multiple locations, and were not studied further. For further evaluation of human DNA in the apparent QX28 hybrids, fluorescent in situ hybridization was done by using biotinylated human DNA as probe. Most hybrids (14/17) showed only a small amount of fluorescent human DNA, consistent in size with QX28 and translocated to centric rodent chromosomes (Fig. 2). Although different rodent chromosomes were involved in many of the translocations, each independent cell line showed the same translocation in 50 metaphase cells examined. Slot-blot densitometric analysis of hybrid DNA with labeled human DNA probe indicated ~0.4% of the hybrid genome to be human, corresponding to <12 megabases (data not shown).

To determine the X chromosome breakpoints among the 17 hybrids, Southern blot analysis was performed with a panel of 10 probes that hybridize to loci flanking the fragile X site. As shown in Fig. 3, the majority (13/17), or almost half of the original 31 isolates, gave results consistent with chromosome breakage at or near the fragile X site with loss of the five proximal loci and retention of five distal Xq28 loci. Four other hybrids had breaks elsewhere between HPRT and G6PD, apparently not involving the fragile X site. However, these hybrids, some of which retained the fragile X site region, will be useful in fine mapping in the vicinity of the fragile X site as well as in strategies to isolate fragile X site-associated sequences.

Table 1. Frequency of G6PD+ colonies during the three-phased selection strategy to isolate HPRT−,G6PD+ somatic cell hybrids

<table>
<thead>
<tr>
<th>Passage number</th>
<th>HAS Nonselective</th>
<th>6-TG</th>
<th>6-TG + diamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&gt;99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>98.5 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>95.6 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>92.9 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>90.3 ± 1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>85.2 ± 1.9</td>
<td>1.5 ± 0.2*</td>
<td>83.0 ± 2.0*</td>
</tr>
<tr>
<td>9</td>
<td>72.1 ± 4.0</td>
<td>1.9 ± 0.2*</td>
<td>89.1 ± 1.5*</td>
</tr>
</tbody>
</table>

6-TG, 6-thioguanine.

*aPassage number n + 1.

*bPassage number n + 2.

![Fig. 1. Clonal survival of cells with different G6PD activities after treatment with the oxidant diamide. Cell lines: λ, Y21 (G6PD−); α, Y75-1B-M1 (G6PD+); and β, frax 3200-3 (approximately twice the G6PD activity of Y75-1B-M1). All survival is expressed relative to untreated controls, and each point represents the mean of triplicate cultures.](image)
ments occurred within Xq28. Hybridization with probes hs7, Stl4.1, and F8C showed band sizes identical to those seen in normal human DNA. For example, as shown in Fig. 4, hybridization with a portion of the human factor 8 gene (probe F8C) results in a 160-kb band for Sac II-digested DNA from normal human cells and three of the Xq28 hybrids. The rodent band (>700 kb) was clearly distinguishable from the human locus. Another, perhaps more sensitive, assessment of Xq28 integrity is the hybridization of radiolabeled human DNA to filters of pulsed-field-separated Xq28 hybrid DNA. The results of such an experiment are shown in Fig. 5. A remarkably consistent pattern of bands was observed, using either Sac II, Sal I, or Sfi I, suggesting that the human breakpoint in these hybrids is nonrandom. Because the selection protocol used could result in breaks anywhere between the possible 30,000–40,000 kb that separate the genes for HPRT and G6PD, these data suggest that breakage is confined to a much smaller region, perhaps <500 kb, which is likely to contain the fragile X site.

The pattern of human bands observed indicates an abundance of CpG-islands within Xq28. CpG-islands are small CpG-rich, undermethylated regions found 5' to vertebrate genes that contain sites for rare-cutting restriction enzymes (18). Because 75% of Sac II sites reside within CpG-islands,
the frequent and relatively small bands of the Sac II digests of Fig. 5 indicate extensive CpG-islands, particularly in comparison with Sal I and Sfi I digests, which do not occur preferentially within CpG-islands. This finding suggests that the extraordinary number of genetic-disease loci within Xq28 is not due to a possible mapping bias alone (two historically important genes for linkage analysis, red/green color pigment and G6PD, map to Xq28) but may, indeed, represent an unusually gene-dense region.

In summary, a selective strategy has been devised to recover the human chromosomal band Xq28 within somatic cell hybrids, through fragile-X-site breakage. Analysis of such hybrids has shown that fragile-X-site breakage is non-random and limited to a relatively small region of Xq27–28. These hybrids, identified with breakpoints at or above the fragile X site, should prove useful for the isolation of fragile-X-site DNA sequences. Pulsed-field gel studies of these hybrids also have demonstrated the presence of abundant CpG-islands within Xq28, consistent with its extraordinary gene density. Finally, the availability of these somatic cell hybrids, containing Xq7.3–qter as the sole human DNA, will significantly accelerate the high-resolution genomic analysis of Xq28, including identification of candidate genes for the plethora of genetic-disease loci mapping to this region.

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