Cytological mapping of the human glucose-6-phosphate dehydrogenase gene distal to the fragile-X site suggests a high rate of meiotic recombination across this site

(in situ mapping/ genetic recombination)

PAUL SZABO, MICHELE PURRELLA, MARIANO ROCCHI, NICOLETTA ARCHIDIACONO, BECKY ALHADEFF, GIORGIO FILIPPI, DANIELA TONIOLO, GIUSEPPE MARTINI, LUCIO LUZZATTO, and MARCELLO SINISCALCO

*Sloan-Kettering Institute for Cancer Research, New York, NY 10021; †Istituto di Biologia Generale, Facoltà di Medicina, Università di Catania, Italy; ‡Istituto per l’Infanzia, Trieste, Italy; §Cattedra di Genetica Medica, Università di Trieste, Italy; †Istituto Internazionale di Genetica e Biofisica, Napoli, Italy; and ‡Department of Haematology, Royal Postgraduate School, London, England

Communicated by Ernst Beutler, August 10, 1984

ABSTRACT The human gene for glucose-6-phosphate dehydrogenase (G6PD) has been subregionally mapped to band Xq28 by segregation analysis in rodent-human somatic cell hybrids [Pai, G. S., Sprinkel, J. A., Do, T. T., Mareni, C. E. & Migeon, B. R. (1980) Proc. Natl. Acad. Sci. USA 77, 2810–2813]. We have previously reported a common type of X-linked mental retardation associated with an inducible fragile site at Xq27–Xq28 that segregates in a close linkage relationship with a G6PD variant, but the latter locus has been mapped to the subtelomeric region Xq27–Xq28 [Camerino, G., Mattei, M. G., Mattei, G. F., Jaye, B. & Mandel, J. L. (1983) Nature (London) 306, 701–704]. The in situ hybridization studies reported here provide strong evidence that G6PD is located on the Xq telomeric fragment distal to the fragile site. These observations and the well-established knowledge that the genes for Deutan and Proctor colorblindness are closely linked to G6PD, but segregate independently of factor IX deficiency, suggest that the fragile site associated with this type of X-linked mental retardation occurs in a region prone to high frequency of meiotic recombination.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

The gene for the first enzyme in the hexose monophosphate pathway, glucose-6-phosphate dehydrogenase (G6PD); 6-phosphate:NADP+ oxidoreductase, EC 1.1.1.49] has been mapped to the subtelomeric region of the human X chromosome (Xq28) by cell hybrid studies (1). The gene responsible for an X-linked form of mental retardation associated with an inducible fragile site at Xq27–Xq28 (2) is probably located in the same region. The structural locus for coagulation factor IX has also been assigned recently to the subtelomeric region of the X chromosome long arm (4–8) and close genetic linkage between a TaqI restriction fragment length polymorphism (RFLP) detected by the factor IX nucleic acid probe and the fragile-X syndrome has been established (9). On the other hand, paradoxically, a solid body of data exclude measurable linkage between factor IX deficiency (hemophilia B) and at least two loci of the G6PD cluster, which includes hemophilia A, adrenal leukodystrophy, and Deutan and Proctor colorblindness (3, 10–12). In particular, at least one recombinant has occurred in the only pedigree found to segregate for both types of hemophilia (13). We have therefore taken advantage of the unique cytological features of the fragile site at Xq27–Xq28 to map precisely G6PD, by showing that a G6PD nucleic acid probe hybridizes in situ to the telomeric fragment of the X chromosome, distal to the fragile site. This finding enables us to suggest a possible solution to the paradox outlined above.

MATERIAL AND METHODS

Metaphase Chromosome Spreads. Metaphase cells with a fragile site were prepared from a short-term culture of peripheral blood lymphocytes in 2'-deoxy-5-fluorouridine medium according to the protocol described by Glover (2). The donor of the blood samples was a male with the fragile X syndrome who had been found to exhibit the fragile site at Xq27–Xq28 in 68% of the metaphases (3). Moreover, in this patient, like in other published studies (14, 15), the incidence of C-group chromosomes, other than the X, with a fragile site at the tip of their long arm was found to be <4%. Well-spread metaphase cells were prepared from this patient on slides precoated with 5× Denhardt’s solution (1× Denhardt’s solution = 0.02% bovine serum albumin/0.02% Ficoll/0.2% polyvinylpyrrolidone) at 65°C overnight (16). Some of the slides were stained with acetic orcein prior to their exposure to the labeled probe so that the metaphase cells with a clearly identifiable fragile site at Xq27–Xq28 could be earmarked by recording their coordinates. Slides with metaphase cells derived from a normal 46(XY) female were used as test slides to optimize the autoradiographic exposure time.

Radiolabeling of the Probe. The molecular probe pGD3 (17) used in this study contains a 3-kilobase genomic nonrepeated DNA sequence homologous to the original cDNA recombinant clone pGD6405 and inserted into the PstI site of pBR322 (18). The plasmid DNA was labeled by nicktranslation (19) with [32P]-labeled dCTP (Amersham) to a specific activity of 7 × 106 dpm/μg. After spin dialysis through Sepharose 6 B-Cl and ethanol precipitation, the labeled DNA was dissolved at a concentration of ~40 ng/ml in the hybridization buffer [50% formamide/2× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate)/25 mM phosphate buffer, pH 6.8/2× Denhardt’s solution/DNA (10 μg/ml) from Escherichia coli] containing 10% dextran sulfate.

Abbreviations: RFLP, restriction fragment length polymorphism; G6PD, glucose-6-phosphate dehydrogenase.
To whom reprint requests should be addressed.
In Situ Hybridization. The labeled probe was hybridized in situ according to the protocol of Gerhard et al. (16) except that a more stringent washing was applied and the slides were exposed to the emulsion for a relatively shorter period of time to limit the spreading of the autoradiographic signal beyond the site of the molecular hybridization (20). Thirty microliters of probe solution (i.e., ~1.2 ng or 8.4 x 10^6 cpm) was added to each slide and covered with a coverslip in a humid chamber saturated with the hybridization buffer. After incubation at 42°C for 16 hr, the slides were washed 4 times in 2x NaCl/Cit at 25°C, twice in 2x NaCl/Cit at 64°C, and twice in 0.1x NaCl/Cit at 45°C. Exposure to the photographic emulsion (Kodak, NTB) was for 5 days at 4°C for the slides with the patient's metaphase cells and for 2 days for the test slides containing the control normal metaphase cells. The statistical analysis of silver grain distribution was initially done on randomly selected well-spread metaphase cells that were karyotyped on the basis of chromosome length and arm ratio. Confirmation of the karyotype—with particular reference to the X chromosome—was obtained by successive destaining and restaining of the slides with the Wright's stain, which allows identification of the X chromosome by its banding pattern (21). The control metaphase cells from the normal female were exposed to the same molecular probe and treated in the same manner as the patient's metaphase cells, except that they were banded (21) soon after the hybridization so that the analysis of the silver grain distribution could be carried out on clearly identified X chromosomes.

RESULTS

In preliminary trials, we observed that the molecular hybridization signal obtained in metaphase cells that had been previously photographed was unsatisfactory, presumably because of excessive photosensitivity of 2'-deoxy-5-fluorouridine-substituted DNA. Twenty well-spread metaphase cells were analyzed for grain distribution. Ten of these had been prestained with acetic orcin, but none of them was directly photographed prior to the hybridization experiment. Karyotypes were prepared according to standard arm ratio classification and the percentage of silver grains per chromosome was calculated (Fig. 1). This analysis was carried out to determine the average background noise per metaphase for each chromosome. Chromosome banding was not carried out at this stage, because it makes the identification of the fragile site more difficult (14, 15). As expected, the percentage distribution of grains was found to be approximately proportional to the size of the individual autosomes. By contrast, the percentage of grains on the X chromosome was 7 times higher than the ~5% expected by chance (Fig. 1). Of the twenty X chromosomes analyzed, one had the fragile site but no grains. Thirteen were labeled at the tip of their long arm but they did not show a fragile site. Six had both the fragile site and an autoradiographic signal, and the latter was clearly distal to the fragile site (Fig. 2 a and b). This was confirmed also in one metaphase cell photographed prior to hybridization (Fig. 2 c and d). The classification of these X chromosomes was confirmed at the end of the analysis by successive cycles of destaining and restaining with Wright's stain (21). In total, 99 grains were found on individual chromosomes in the 20 metaphase cells scored—i.e., an average of 5 grains per total genome. An estimate (approximated in excess) of the size of the telomeric fragment distal to the fragile site is 0.0025 of the total genome, based on the assumption that the X chromosome is 5% of the human haploid genome and that the fragment in question is 1/20th of the human X chromosome. Thus, the number of grains expected on this fragment by chance alone is (0.0025)5 = 0.0125. We observed, instead, 6 labeled fragments of 7 scored randomly—i.e., 6/7 = 0.86. By applying the Poissonian formula \([\exp(1-p)]\), where \(n\) is the number of X chromosomes with fragile site, \(x\) is the number of X chromosomes with fragile sites with the silver grain on the fragment distal to the fragile site, \(p = 0.0125\), and \(1 - p = 0.9875\), it follows that the probability of our finding being due to chance alone is \([1/(0.0125)(0.9875)] = 1.2 x 10^{-12}\).

Fifty-one banded normal metaphase cells from the test slides were analyzed for silver grain distribution. Since the exposure time of these slides was only 2 days, the labeling frequency was found to be correspondingly lower. However, all the labeled X chromosomes in this experiment (35 of 99 banded X chromosomes) had silver grains at the tip of their long arm. These results indicate that the G6PD gene is located distal to the fragile site and that such a conclusion agrees with the localization of G6PD obtained on the normal X chromosome.

DISCUSSION

The precise location of the X-linked fragile site has now been established by prophase banding (22) and by electron microscopy (23) to be Xq27.3. Formal genetic analysis (Table 1) had previously shown close linkage of G6PD to hemophilia A (25), Deutan and Protan color blindness (12, 28), and adrenal leukodystrophy (27). Since we have now mapped G6PD distal to the fragile site, we infer that all these genes,
to which we shall refer for convenience as the G6PD cluster, are distal to Xq27.3.

Genetic variants at both the G6PD (3) and the factor IX (9) loci have been found to segregate in a close linkage relationship with the fragile-X syndrome (Table 1, x–xii). However, factor IX deficiency (hemophilia B) segregates independently from Deutan (12) and Protan (11) color blindness and the occurrence of recombination between the two types of hemophilias has been reported (13). In addition, we have recently found a Sardinian family with at least one recombiant between hemophilia B and G6PD itself. Thus, we have a paradoxical situation whereby the G6PD cluster and factor IX are each closely linked to the fragile-X syndrome but not to each other. Indeed, within the G6PD cluster there are only 5/411 recombinants (Table 1, i–vi), whereas between the G6PD cluster and factor IX there are 16/37 (Table 1, vii–ix). This paradox can be illustrated in a simple diagram, as follows:

Thus, the genetic distance between the factor IX locus and the G6PD cluster appears to be nonmeasurable when derived from pedigrees segregating for factor IX deficiency and a mutant allele at one of the loci in the G6PD cluster, whereas it would seem close if derived by adding the numbers of
Table 1. Segregation data between pairs of X-linked genes flanking the fragile site at Xq27.3

<table>
<thead>
<tr>
<th>Loci compared</th>
<th>Recombinants observed</th>
<th>Total scorable sibs</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Deutan/G6PD</td>
<td>3</td>
<td>238</td>
<td>24</td>
</tr>
<tr>
<td>(ii) Protan/G6PD</td>
<td>1</td>
<td>51</td>
<td>24</td>
</tr>
<tr>
<td>(iii) HA/G6PD</td>
<td>0</td>
<td>58</td>
<td>25</td>
</tr>
<tr>
<td>(iv) HA/Deutan</td>
<td>1</td>
<td>39</td>
<td>11, 12, 25</td>
</tr>
<tr>
<td>(v) HA/Protan</td>
<td>0</td>
<td>7</td>
<td>26</td>
</tr>
<tr>
<td>(vi) ALD/G6PD</td>
<td>0</td>
<td>18</td>
<td>27</td>
</tr>
<tr>
<td>(vii) HA/F-IX (HB)</td>
<td>1</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>(viii) F-IX (HB)/Deutan</td>
<td>12*</td>
<td>27</td>
<td>12</td>
</tr>
<tr>
<td>(ix) F-IX(HB)/Protan</td>
<td>3*</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>(x) FS/F-IX(Taq I)</td>
<td>0</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>(xi) FS/G6PD</td>
<td>2</td>
<td>24</td>
<td>3, †</td>
</tr>
<tr>
<td>(xii) FS/Protan</td>
<td>0</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

HA, hemophilia A; ALD, adrenal leukodystrophy; F-IX, factor IX; FS, fragile-X syndrome; HB, hemophilia B.

*Several recombinants found in the same sibships.

†Additional Studies carried out in collaboration with the Greenwood Genetic Center (unpublished) have brought now the number of scorable sibs between the G6PD cluster and the fragile-X syndrome to a total of 29 with only 2 recombinants. The new maximum likelihood estimate of recombination is 7% with a lod score of 5.57 and a 90% upper fiducial limit of 16%.

recombinants (2 of 46) observed in the two separate comparisons: factor IX(Taq I)/fragile-X syndrome and fragile-X syndrome/G6PD cluster. The difference in these two estimates of the genetic distance between factor IX and the G6PD cluster is highly significant ($x^2 = 17.5; df = 1; P < 0.0001$).

We can think of three ways to explain this paradox: (i) the Taq I-RFLP and the hemophilia B mutations may occur at separate unlinked X chromosome loci; (ii) the fragile site mutation occurs at or near DNA sequences highly prone to chromosomal breaks and/or rearrangements in meiotic as well as mitotic divisions; (iii) the chromosomal region overlapping with the location of the fragile site may be hypercondensed so that its length in DNA base pairs may be considerably greater than that of chromosomal regions distal and proximal to it.

Hypothesis i is unlikely for two reasons. First, the factor IX probe has been successfully used to detect molecular alterations associated with some hemophilia B mutations (29). Second, the Taq I-RFLP and hemophilia B cosegregate according to expectation in all informative pedigrees thus far examined (30).

Hypothesis ii is in keeping with previous suggestions that the chance of meiotic recombination between loci of the subtelomeric region of the X chromosome long arm may not necessarily be proportional to their physical distance in DNA base pairs (31), and it is supported by the unusually high incidence of fresh mutations reported for the fragile-X syndrome (3). According to this hypothesis, loci at the opposite side of Xq27.3 would recombine freely with respect to each other, whereas those on either side would segregate proportionately to physical distances in base pairs. To explain why in families with the fragile-X syndrome both the factor IX Taq I-RFLP and the G6PD cluster behave as closely linked to the fragile site mutant, we must also postulate that the mutation responsible for the disease suppresses the normally high rate of recombination characteristic of the region.

Hypothesis iii is complementary rather than alternative to ii in that it attributes the high rate of recombination between genes at the opposite sides of the fragile site to a higher DNA density per chromosome unit length in this region.

The availability of nucleic acid probes for both factor IX (33–35) and factor VIII (36) and the large number of RFLPs detected in the subtelomeric region of the X chromosome long arm (37) will soon allow further direct experimental testing of the hypotheses proposed.

Dedicated to Bernard L. Horecker for his 70th birthday as a token of esteem, gratitude, and appreciation. We thank Drs. R. E. Stevenson, M. C. Phelan, and R. A. Saul for allowing us to mention the unpublished data referred to in Table 1. These studies were supported by Grants NIH-HD 16782, NIH-CA-08748, and March of Dimes NF 1-882 to M.S.; Controllo Crescita Neoplastica and Basi Molecolari Malattie Ereditarie (Consiglio Nazionale della Ricerca, Italy) to G.F., D.T., and G.M.; Medical Research Council (Great Britain) Programme Grant to L.L.

27. Giannelli, G., Choo, K. H., Winship, P. R., Rizza, C. R., An-


