

# 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)

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**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 segregates in a close linkage relationship with a G6PD variant, but the relative position of G6PD with respect to the fragile site has not yet been established. This fragile-X syndrome has been shown to be closely linked also to a *Taq* I restriction fragment length polymorphism detected by a cDNA probe for factor IX, and the latter locus has been mapped to the subtelomeric region Xq26-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 Protan 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 human gene that specifies the first enzyme in the hexose monophosphate pathway [glucose-6-phosphate dehydrogenase (G6PD); D-glucose-6-phosphate:NADP<sup>+</sup> 1-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, as suggested by a measurable genetic linkage between this fragile-X syndrome and G6PD deficiency (3). 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 *Taq* I 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 Protan colorblindness (3, 10-12). In particu-

lar, at least one recombinant has occurred in the only pedigree found to segregate for both types of hemophilia (13). We have 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/0.02% 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(XX) 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 *Pst* I site of pBR322 (18). The plasmid DNA was labeled by nick-translation (19) with <sup>125</sup>I-labeled dCTP (Amersham) to a specific activity of 7 × 10<sup>7</sup> 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.

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Abbreviations: RFLP, restriction fragment length polymorphism; G6PD, glucose-6-phosphate dehydrogenase.

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**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.,  $\approx 1.2$  ng or  $8.4 \times 10^4$  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  $2\times$  NaCl/Cit at 25°C, twice in  $2\times$  NaCl/Cit at 64°C, and twice in  $0.1\times$  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 orcein, 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  $\approx 5\%$  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 random-

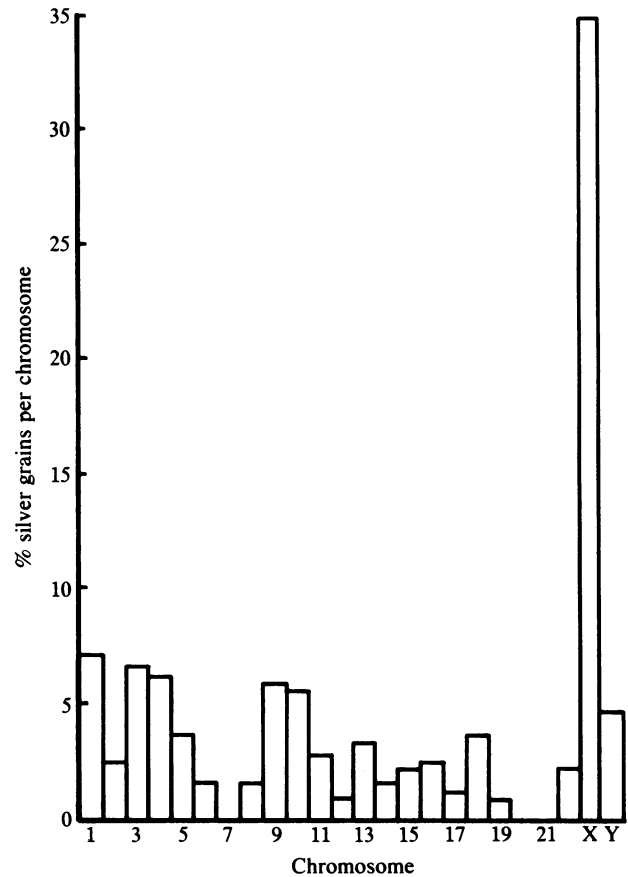


FIG. 1. Distribution of autoradiographic grains over metaphase chromosomes after *in situ* hybridization with the  $^{125}\text{I}$ -labeled G6PD probe. Data are presented as percentage of total silver grains associated with each individual chromosome. About 90% of the grains on the X chromosome were found to be at the tip of Xq. In six of the seven metaphases with a recognizable fragile site, the signal was always distal to the breakage point at Xq27.3. The seventh metaphase with fragile site was unlabeled.

ly—i.e.,  $6/7 = 0.86$ . By applying the Poissonian formula  $[\binom{n}{x} p^x (1-p)^{n-x}]$ , 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  $[\binom{7}{6} (0.0125)^6 (0.9875)] = 1.2 \times 10^{-12}$ .

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,

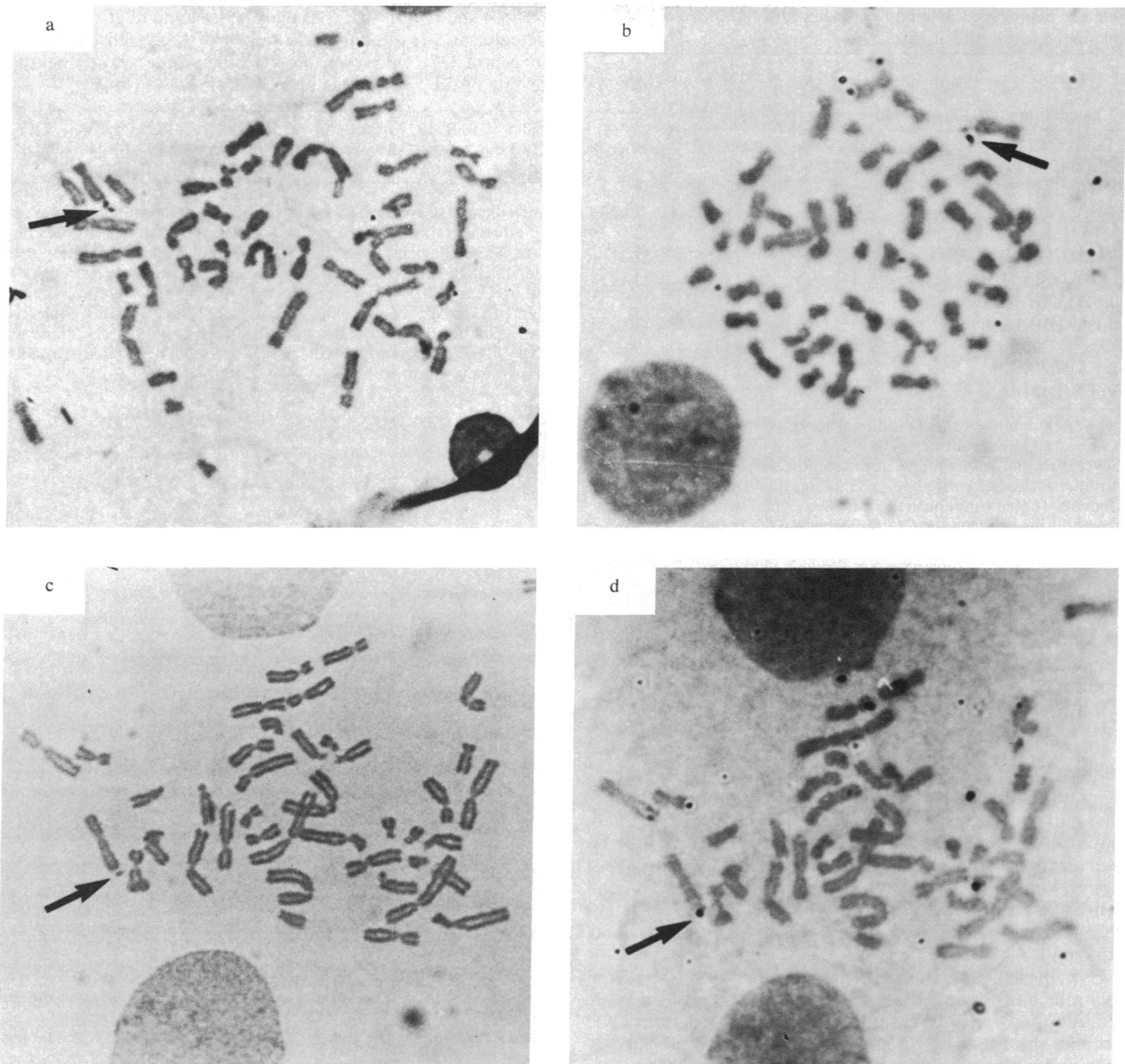
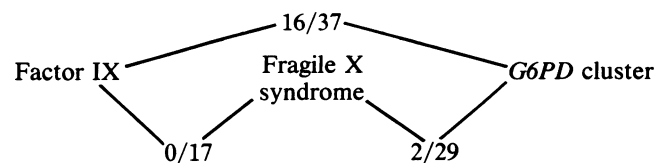


FIG. 2. Localization of the *G6PD* locus to the chromosomal region distal to the fragile site at Xq27.3 in metaphase cells from a patient with fragile-X syndrome. (a and b) Two typical metaphases showing the autoradiographic signal on the X chromosome fragment distal to Xq27.3. (c and d) A third metaphase photographed before and after hybridization to the probe. Metaphases were photographed by focusing on the silver grains.

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 recombinant 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

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

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 ( $\chi^2 = 17.5$ ;  $df = 1$ ;  $P < 0.0001$ ).

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 breakage 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<sup>h</sup>. 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.

<sup>h</sup>Sherman, S. L., Morton, N. E., Jacobs, P. A. & Turner, G., Thirty-Fourth Annual Meeting of the American Society for Human Genetics, October 30, 1983, Norfolk, VA, p. 207A (abstr.).

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