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Physical map of human Xq27-qter: Localizing the region of the fragile X mutation

(Xq28/pulsed-field gel map/telomere/X chromosome)

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ABSTRACT **We describe a physical map of the end of the long arm of the human X chromosome encompassing the region from Xq27.2 to the q telomere, inclusive of the chromosomal band Xq28. This region is of particular interest, since it contains the highest density of genes associated with genetic diseases. The map covers a total of 12 megabases (Mb) of DNA and extends from the telomere to 3 Mb beyond the most likely position of the fragile X mutation, defined by a cluster of translocation breakpoints in somatic cell hybrids. The map determines order and position of loci throughout the Xq28 region and localizes cell line breakpoints marking the fragile X region to an interval of 300–700 kilobases between 8 and 8.7 Mb proximal of the Xq telomere.**

The most distal region of the long arm of the human X chromosome, extending from the telomere to and beyond the position of the fragile X mutation (1, 2), has been implicated in a large number of human genetic diseases (3), of which only a few genes defined by known biochemical defects have as yet been cloned. Many of the genes associated with human genetic diseases that are located in this region remain to be isolated.

The high density of genes involved in human diseases in this area has stimulated an intense analysis of the region using genetic (4, 5), cytogenetic (6, 7), and pulsed-field gel mapping techniques (8–12). Questions as to the length of the region as well as the order and exact location of many of the markers have, however, remained unresolved.

Among the mutations residing in this region, the fragile X or Martin–Bell syndrome is of particular interest due to its medical importance. This syndrome accounts for the most common form of inherited mental retardation in humans and is the second leading cause of mental retardation after Down syndrome. This disease is associated with a thymidine stress-induced fragile site at Xq27.3, which appears as an unstained chromosome gap on metaphase chromosome preparations of affected patients, as well as some carriers (13, 14). Neither the molecular nature of this and other fragile sites nor the relationship between the fragile X site and the syndrome is understood. The inheritance pattern is unusual among mammalian X chromosome-linked loci and is characterized by variable expression of the phenotype in both males and females (2, 15).

In somatic cell hybrids carrying the human fragile X chromosome, it has been shown that thymidine stress induces markedly nonrandom chromosome breakage at or very near the fragile X site, which is not observed in similarly isolated somatic cell hybrids bearing a normal human X chromosome (16). By using biochemical markers flanking the fragile X site, derivative hybrids identified by marker segregation have been isolated that contain, as the sole human DNA, either human Xpter-q27.3 or Xq27.3-qter stably translocated to a rodent chromosome (17). Characterization of the hybrids by *in situ* hybridization with total human DNA showed these fragments to be the only human components, located at the ends of hamster chromosomes (data not shown). These hybrids therefore can be used to localize the position of the region of enhanced breakage associated with *FRAX* expression within a physical map.

In addition, such cell lines can now be used as essential elements in determining the physical map of the Xq28 region, since hybridization of human-specific repetitive DNA to hybrid DNAs digested with infrequent cleaving enzymes and resolved by pulsed-field gel electrophoresis identifies most restriction fragments from the region among the background of hamster DNA sequences. Both this approach and the more standard approach of hybridizing unique probes to single and double digest filters of different DNAs have been used by us to develop a long-range physical map of this portion of the human genome and to place and order more than 12 loci within the map, many of which had been positioned relative to human mutations by genetic analysis.

METHODS

Probes. Probes used in this analysis were TelBam3.4 (18), 767 (locus *DXS115*) (19), St35.239 (*DXYS64*) (20), a genomic fragment encoding factor VIII (*F8*) (21), a cDNA encoding glucose-6-phosphate dehydrogenase (*G6PD*) (22), hs7 (*CB*) (23), DX13 (*DXS15*) (24), (*GABRA3*) (25), St35.691 (*DXS305*) (26), U6.2 (*DXS304*) (27), VK21A (*DXS296*) (28), and 2.34 (*DXS477*) (6).

Pulsed-Field Gel Analysis. Cell lines used in this work were grown under standard conditions in Dulbecco's modified Eagle's medium or F12 medium, supplemented with 10% fetal calf serum (16). DNA in agarose blocks was prepared as described (29, 30). Pulsed-field gel electrophoresis was carried out in a commercially available contour-clamped homogeneous electric field electrophoresis apparatus (Pharmacia LKB) in 0.5× TBE (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) at a constant temperature of 12°C by using the pulse times indicated in the figure legends. The gels in Figs. 2*a* and 4 were run in a contour-clamped homogeneous electric field electrophoresis box constructed at the European Molecular Biology Laboratory workshop, at a temperature of 18°C. Chromosomes from yeast strains *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were

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Abbreviation: Mb, megabase(s).

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used as size markers in pulsed-field gel electrophoresis experiments.

Filter Transfer and Hybridization. DNA was transferred by capillary blotting under alkaline conditions to GeneScreen membranes (NEN), and filters were hybridized as described in Herrmann *et al.* (29). Probes were prepared from excised probe inserts separated on low-melting-point agarose gels after removal of the agarose by digestion with agarase and labeled by oligonucleotide priming. If appropriate, repeat sequences were blocked from hybridization by a prehybridization step with total human DNA (29).

RESULTS

Independent maps were established in two human–hamster somatic cell hybrids: the cell hybrid Q1Z, derived from a fragile X chromosome, and 578, which contains the entire (nonfragile X) human X chromosome (31). In addition, in many subregions the results have been compared with the hybrids Q1V, Q1AD, and h-g+ (analogous to Q1Z, but derived independently), Y75-1B (a hybrid containing a human X chromosome from a fragile X patient in a hamster background, used as parent to derive the hybrids Q1Z, Q1AD, and Q1V), and GM1416B (a lymphoblastoid 48,XXXX cell line) (Camden Mutant Cell Repository).

The final map consists of two segments, which as yet have not been linked physically: a distal segment, extending from the telomere to the position of the red–green pigment genes, and a proximal segment, extending from the locus *DXS15* (probe DX13) across the cluster of fragile X associated breakpoints to *DXS477* (probe 2.34), a locus mapping proximal to this mutation.

The Distal Segment: Telomere to the Red–Green Pigment Genes. The distal segment of the map defined by the probes TelBam3.4, St35.239, 767, *F8*, *G6PD*, and hs7 was established by a number of different restriction fragments (Fig. 1)

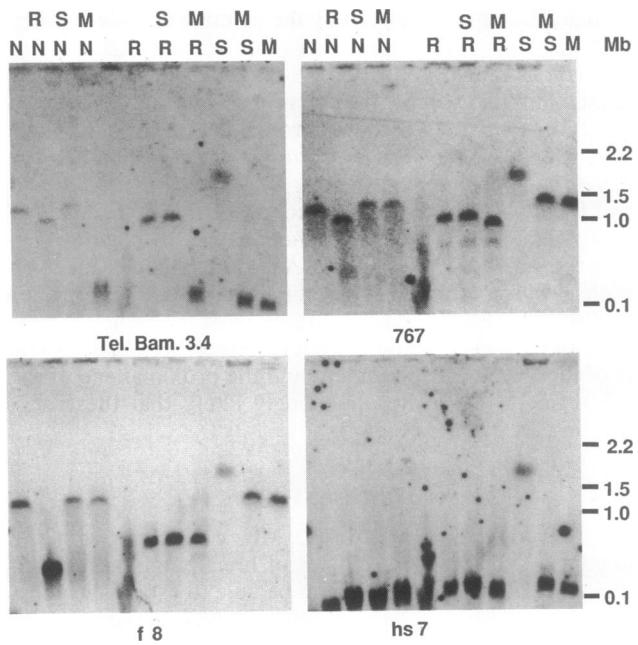

FIG. 1. Physical linkage of the markers of the distal cluster: pulsed-field gel analysis of single and double digests of Q1Z DNA with the enzymes *Not* I (N), *Mlu* I (M), *Nru* I (R), and *Spl* I (S). The same filter was hybridized with the probes TelBam3.4, 767, *F8*, and hs7. Electrophoresis was carried out at 70 V, using a pulse time increasing from 10 min to 20 min for 4 days and from 50 sec to 3 min for an additional 20 hr. Under these conditions, designed to demonstrate linkage over the high molecular weight bands, fragments in the small size range remain unresolved.

and especially an *Spl* I band detected by all probes from the segment. All data are in agreement with previous mapping information (20). The segment is oriented by the probe TelBam3.4, a telomere sequence, which crosshybridizes with the Xq telomere in some, but not all, X chromosomes. This now positions the telomere on the map and therefore defines an endpoint by which this map and subsequent X chromosome maps can be oriented.

The Proximal Segment: DX13 to the Fragile X Region. The next group of probes, DX13, *GABRA3* (the γ-aminobutyric acid receptor α subunit), and St35.691 all recognize a 2.45-megabase (Mb) *Not* I fragment (Fig. 2*A*) as well as (in Q1Z DNA) 2- and (weakly hybridizing) 2.3-Mb *Nru* I fragments, with additional fragments supporting linkage and order of probes (Fig. 2*B*). This cluster can be extended further in 578 DNA due to one of the rare methylation differences observed between Q1Z and 578 DNA. Here the differential methylation of *Nru* I sites allows the detection of an *Nru* I fragment recognized by both St35.691 and U6.2. This observation is in

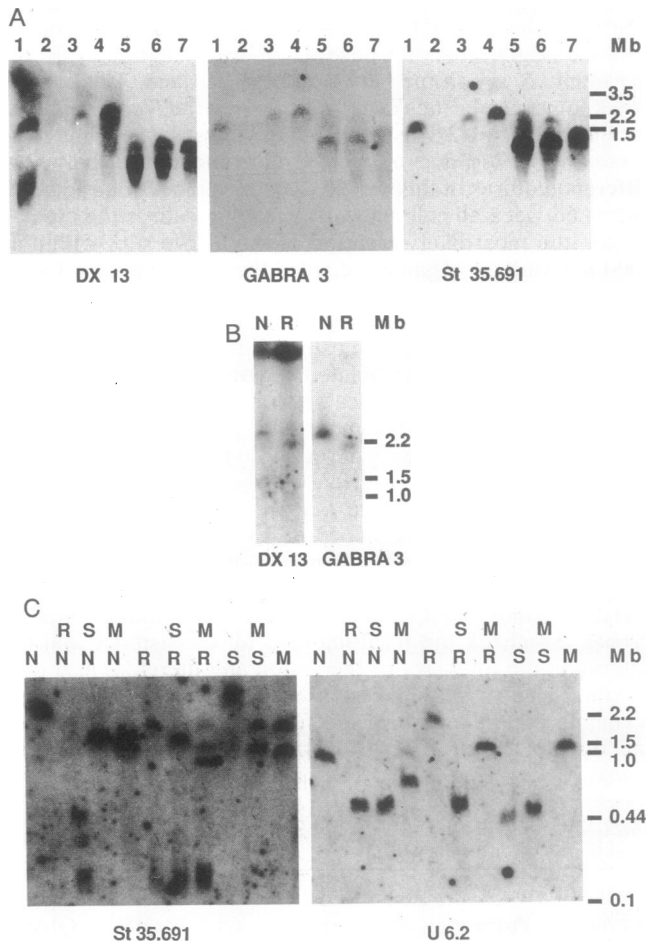

FIG. 2. Physical linkage between the markers of the proximal cluster. (*A*) Hybridization of *Not* I digests of different DNA samples to probes DX13, *GABRA3*, and St35.691 showing the common *Not* I band as well as the higher degree of methylation in cell hybrids. Electrophoresis conditions were 50 V using a pulse time of 50 min for 4 days and a pulse time of 35 min for an additional 3 days. Lane 1, 578; lane 2, hamster DNA; lane 3, Y75-1B; lane 4, Q1Z; lanes 5–7, lymphoblast lines from *FRAX* males. (*B*) Hybridization of *Not* I (N) and *Nru* I (R) digests of Q1Z DNA with probes DX13 and *GABRA3*. Electrophoresis was carried out as described in Fig. 1. (*C*) Hybridization of the probes St35.691 and U6.2 to single and double digests of 578 DNA with the enzymes *Not* I (N), *Mlu* I (M), *Nru* I (R), and *Spl* I (S). Electrophoresis was carried out at 70 V using a pulse time increasing linearly from 10 min to 20 min for 3 days and from 50 sec to 3 min for an additional 2 days.

agreement with the sizes of the double digest fragments (Fig. 2*C*). Since genetic mapping positions U6.2 proximal to DX13 (32), DX13 is located at the distal end of the cluster, with a possible gap between DX13 and the region containing the red–green pigment genes.

The Fragile X Region. In contrast to the probes located in the other regions of the map, VK21A identifies fragments differing between the different cell lines for all enzymes except *Not* I (Table 1). Since Q1Z, Q1V, Q1AD, and h-g+ were selected to break at or close to the fragile X locus, it is apparent that VK21A detects sequences extending into the randomly different parts of the hamster genome that are adjacent to the human chromosome fragments. Fragments hybridizing to VK21A in 578 DNA are therefore likely to span the fragile X region. This is verified by the identification of shared fragments between VK21A and 2.34, the closest probe proximal to the Q1Z breakpoint (Fig. 3). Since 2.34 is absent in the Xq27.3-qter hybrids Q1Z, Q1V, Q1AD, and h-g+, but hybridizes to DNA from the reciprocal translocation hybrids, such as micro21D (Xpter-q27.3) (6, 16), the breakpoints in all hybrids must be located between VK21A and 2.34. Consistent with the notion that these breakpoints are associated with the fragile site is the positioning, by linkage analysis, of the fragile X syndrome between these markers. The hybrid breakpoints can be localized further, since the *Not* I fragment recognized by VK21A is not changed (the absence of the *Not* I site in Q1Z is due to methylation and differs between different cultures of this hybrid), whereas the *Nru* I fragment differs between all cell lines. This result localizes the breakpoints, the most likely position of the fragile site, within a 700-kilobase (kb) segment between the *Not* I and *Nru* I site indicated by a box in Fig. 4.

In contrast to the situation in the majority of the cell lines, in which the human DNA must have translocated next to a CpG-poor or highly methylated region of the hamster genome, leading to the appearance of very large junction fragments, the junction fragments observed in h-g+ are fairly short. From the fragments observed in this cell line, we can conclude that the breakpoint in this hybrid lies within a region of at most 300 kb (hatched box in Fig. 4), at a maximal distance of 700 kb proximal of VK21A. This is in reasonable agreement with the genetic distance of 2 centimorgans established for this locus and the fragile X mutation (1, 7).

Hybridizations of Cell Line Digests with Human Repeat Probes. To check the resulting map for consistency and to determine the total size of the region and therefore also the maximal size of possible gaps between the different map segments, we have hybridized filters from gels with single and double digests of Q1Z DNA with radioactive human repeat sequences (33, 34). The banding pattern obtained by hybridization to human repeats was compared with the hybridization pattern from unique probes (Fig. 5*B*). The results shown schematically in Fig. 5*A* demonstrate that, with the exception of the 700-kb *Not* I fragment postulated to be located between St35.169 and U6.2, all larger fragments detected by human repeat probes are also recognized by one or more unique probes. Though the existence of additional (larger) comigrating bands or bands showing little or no hybridization cannot be ruled out, this is unlikely given the close agreement between the results with different restriction enzymes, as well as the agreement between the size of the map and the predicted size of this region (<12 Mb) based upon metaphase length (17). We would however expect the presence of very small (<100 kb) *Not* I, *Mlu* I, and *Nru* I bands in the region between the end of the most distal cluster (hs7) and DX13, a region especially rich in CpG islands, which are either not resolved by the electrophoresis or carry no or too few repeat sequences to be detected by this approach.

Based on the results of the repeat hybridizations, we expect the gap between the proximal end of the distal segment and the distal end of the proximal segment to be small or nonexistent, since almost all *Spl* I, *Not* I, *Mlu* I, and *Nru* I fragments in Q1Z can be accounted for by the maps of the subsegments. The results of the repeat hybridization also verify the map of the U6.2–VK21A region, which is based on the observation of multiple matching sites from both probes and especially on the exact positioning of the *Not* I, *Nru* I, *Mlu* I, and *Spl* I sites. The perfect match of sites at the distal end of the VK21A map segment and the proximal end of the U6.2 segment makes it convincingly likely that these segments in fact overlap (Fig. 4).

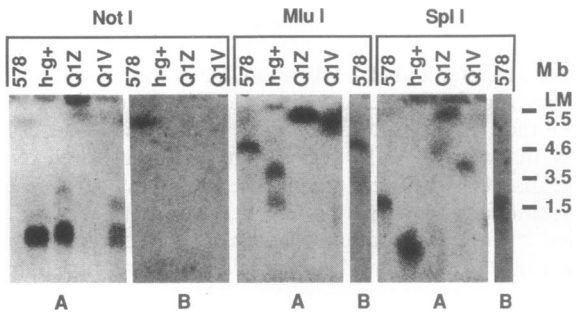

FIG. 3. Localization of the cell line breakpoints associated with the fragile X region. Hybridization of probes VK21A (A) and 2.34 (B) to *Not* I, *Mlu* I, and *Spl* I digests of DNA from the cell lines 578, h-g+, Q1Z, and Q1V. The lanes corresponding to the hybridization of h-g+, Q1Z, and Q1V DNA with probe 2.34 show no hybridization for all enzymes. These lanes are therefore only shown once. Electrophoresis conditions were as described in Fig. 2*A*. LM, limiting mobility.

Table 1. Comparison of fragments crossing the cell line breakpoints in different cell lines

Enzyme	Probe	Cell line 578	h-g+	Q1Z	Q1V	Q1AD
Not I	VK21A	1.1	1.1	>5	1.1	1.1
	2.34	>5	2		1.8	
Mlu I	VK21A	4.05	1.8	>5	5	>5
	2.34	4.05	3.5			
Spl I	VK21A	2	ND	4.5	3.5	ND
	2.34	2				
Nru I	VK21A	2.2	1.75	4	3	ND
	2.34	1.25			4.8	
		2.15				
		2.6				

Fragment sizes are given (in Mb). Fragments indicated as >5 were not resolved under conditions separating the three chromosomes of *Schizosaccharomyces pombe* (3.5 Mb, 4.6 Mb, and 5.5 Mb). ND, not determined.

DISCUSSION

This study defines a map of 12 Mb, extending from the telomere of Xq through a cluster of breakpoints associated with the fragile X site (Fig. 4). To be able to establish such a large map in a region rich in CpG islands, we have specifically selected somatic cell hybrids in which this region is heavily methylated. In addition, three of the four enzymes used (*Spl* I, *Mlu* I, and *Nru* I) have A and T in their recognition sequence and are therefore found less often in CpG islands.

The order of the loci *F8*, *G6PD*, *CB*, and *GABRA3* postulated by the physical mapping results described here agrees well with the results of genetic mapping experiments in mouse, in which the order *GABRA3/RCP-GDX* (close to *G6PD*)/*F8C*-telomere has been found (25).

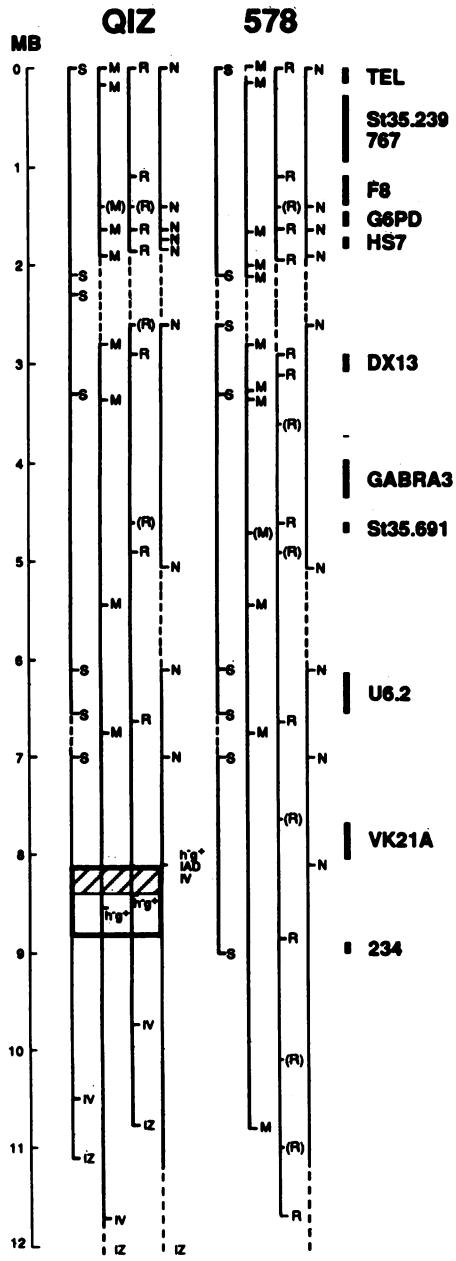

FIG. 4. Map of Xq27-Xq28. Weakly cut *Nru* I and *Mlu* I sites are indicated by parentheses. S, *Spl* I; M, *Mlu* I; N, *Not* I; R, *Nru* I; IV and IZ indicate restriction sites that are different in the corresponding cell lines. A table of fragment sizes and a detailed description of the map construction will be made available on request.

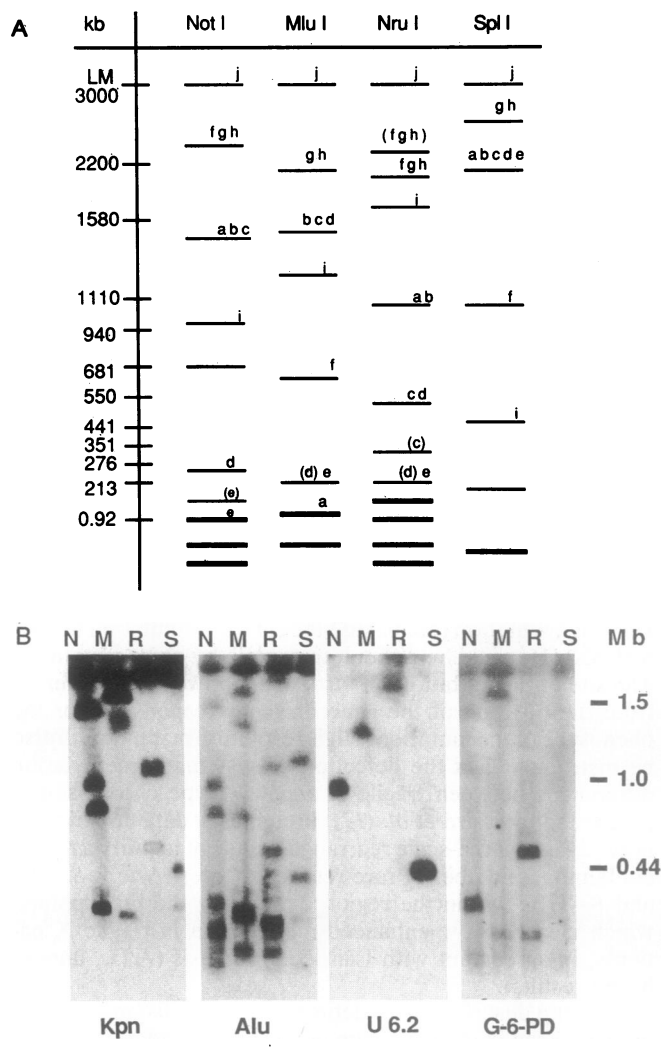

FIG. 5. Hybridization of human repeat sequences and unique probes to digests of Q1Z DNA. (*Alu* and *Kpn* hybridizations correspond to neighboring lanes, causing a slight difference in migration of corresponding bands). (*A*) Schematic representation of the results of hybridization of human repeat probe (*Alu* repeat) to filters from gels containing *Not* I, *Mlu* I, *Nru* I, and *Spl* I digests of Q1Z DNA. Bands hybridizing to unique probes are indicated by letters (a, TelBam3.4; b, St35.239; c, *F8*; d, *G6PD*; e, hs7; f, DX13; g, *GABRA3*; h, St35.691; i, U6.2; j, VK21). Parentheses indicate partial cleavage products. Dark bands indicate potentially unresolved bands. LM, limiting mobility. The diagram is compiled from pulsed-field gel runs covering different separation ranges. (*B*) Autoradiogram of hybridization of *Not* I (N), *Mlu* I (M), *Nru* I (R), and *Spl* I (S) digests of Q1Z DNA to human repeat sequences [*Kpn* I (34) and *Alu* I (35) and unique probes (probes U6.2 and *G6PD*)].

This orientation does disagree with two separate observations, both of which can, however, have alternative explanations. One of these, based on distance measurements in interphase nuclei (35), could be caused by the specific folding of DNA close to the telomere, already postulated to explain contradictory orders from the analysis of metaphase hybridization results and the analysis of distances in interphase nuclei.

Similarly the orientation of the telomeric segment (TelBam3.4 to the red–green pigment genes) postulated here is opposite to that proposed by Kenwrick and Gitschier (12), which was based on analysis of DNA of a patient carrying a large deletion in this region. This could either be caused by accidental comigration of fragments or be due to the occurrence of further rearrangements (e.g., a large inversion) during formation of the deleted chromosome.

This orientation now positions the X–Y homology region (locus *DXYS64*) close to the telomere. Since TelBam3.4 is found in both X and Y chromosomes (19), this could define a continuous X–Y homology region extending to the telomere of the long arm of the X chromosome, in analogy to the pseudoautosomal region on the short arm of the X chromosome.

Although most mapping results in the various cell lines were found to be similar, variation was detected in the breakpoint region, the region of the red–green color pigment genes (due to variation in tandem gene repeats) (23) and in the methylation patterns of a few sites distributed throughout the map. Analysis of GM1416B (48,XXXX) however showed a lower and variable degree of methylation in this cell line, which unmasks a number of sites methylated in the hybrid lines (data not shown).

This low degree of variation allowed the straightforward identification of the position of the cell line breakpoints defining the *FRAX* region. Although physical mapping in general is unable to localize mutations unless associated with major changes in the DNA like translocations, deletions, or insertions, we took advantage of the expected fragility of this position in the genome to induce chromosome breaks, which mark the fragile site, and therefore to localize it within the physical map. The clustering of breakpoints in an area of only 300–700 kb strengthens the evidence that the breakpoint region and the cytogenetically defined fragile site are closely linked. The cytogenetically defined fragile site in turn has to be related to the position and nature of the genetically defined fragile X mutation. The position of the mutation could (but does not have to) coincide with the position of the fragile site (36). The physical mapping information described here now opens the way for a high-resolution comparison of the area surrounding the fragile site for differences between fragile X and wild-type chromosomes and will allow the localization of smaller changes like insertions, deletions, or expansion of minisatellite sequences expected to be responsible for the mutation. Such an analysis seems especially hopeful since the very high mutation rate postulated (one new mutation in 3000 meiosis) (1) would be difficult to explain by point mutations. The site of the mutation, in turn, could however be different from the position of the gene or genes responsible for the phenotype of the mutation. High-resolution mapping will also be instrumental in the detection of systematic methylation differences between fragile X and wild-type chromosomes postulated by Laird *et al.* (37). Preliminary data do however argue against large-scale rearrangements but identify a region containing a number of rare restriction sites (*Nae* I, *Sac* II, and *Bss*HII) within the region defined by the breakpoints, which clearly show enhanced methylation in fragile X patients, in agreement with Laird's hypothesis (A.D., unpublished results).

The map described here defines order and distances of the markers used in the genetic mapping and will therefore be of considerable importance in improving the genetic localization of mutations within the map. It will help to assign mutations to molecularly defined intervals and will be instrumental in the design of experiments to clone the corresponding genes and especially the gene for the fragile X mutation.

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