Am. J. Hum. Genet. 49:656–661, 1991

Isolation of a Human DNA Sequence Which Spans the Fragile X

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Summary

To identify the sequences involved in the expression of the fragile X and to characterize the molecular basis of the genetic lesion, we have constructed yeast artificial chromosomes (YACs) containing human DNA and have screened them with cloned DNA probes which map close to the fragile site at Xq27.3. We have isolated and partly characterized a YAC containing approximately 270 kb of human DNA from an X chromosome which expresses the fragile X. This sequence in a yeast artificial ring chromosome, XTY26, hybridizes to the two closest DNA markers, VK16 and Do33, which flank the fragile site. The human DNA sequence in XTY26 also spans the fragile site on chromosome in situ hybridization. When a restriction map of XTY26, derived by using infrequently cutting restriction enzymes, is compared with similar YAC maps derived from non-fragile-X patients, no large-scale differences are observed. This YAC, XTY26, may enable (*a*) the fragile site to be fully characterized at the molecular level and (*b*) the pathogenetic basis of the fragile-X syndrome to be determined.

Introduction

Fragile-X syndrome is the most common form of familial mental retardation, affecting 1 in 2,500 children (Davies 1989). A reasonably constant manifestation is the presence of a cytogenetically detectable fragile site in band q27.2 of the X chromosome, a fragile site which, if not the cause of the disorder, is closely associated with it (Sutherland and Hecht 1985). Its mode of inheritance is very unusual for an X-linked trait, with incomplete penetrance in both males and females. Recent evidence by Vincent et al. (1991) has suggested a differential DNA methylation in the FRAXA region, supporting the hypothesis of Laird (1987; Laird et al. 1990) that the fragile site and the fragile-X syndrome are due to local failure of X reactivation mediated through DNA methylation. Other hypotheses include sequence rearrangement or duplication during meiotic recombination (Pembrey et al. 1985; Ledbetter et al. 1986). In the present paper we describe the isolation of a ring yeast artificial chromosome (YAC) which contains the fragile-X region from a fragile-X syndrome patient, and we compare its restriction map with the restriction-enzyme pattern of DNA of this region from normal individuals (Vincent et al. 1991).

Received March 19, 1991; revision received May 23, 1991.

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0002-9297/91/4903-0019$02.00

Material and Methods

Construction of YAC Library

The YAC library was constructed by the procedure of Reithman et al. (1989) and was designed to rescue telomeres by complementation. The procedure was modified to employ digestion of the vector pTYAC1 with *Bam*HI and either *Eco*RI or *Cla*I, to accommodate inserts of DNA digested with either *Eco*RI or *Taq*I. The library was made from DNA from the hu-

man/hamster somatic cell hybrid X3000.11 (Nussbaum et al. 1986), which contains an X chromosome from band q24 to qter—which in turn expresses the fragile X—translocated onto a hamster chromosome. This DNA was digested with *Taq*I, ligated to pTYAC1, and transformed into yeast which were grown on selective media. The details of library construction and characterization will be described elsewhere, but, in brief, the collection includes clones with sequences from a number of locations in Xq24-Xqter.

In Situ Hybridization

The DNA probes VK16 (Suthers et al. 1990), 2–34, and Do33 (Rousseau et al. 1991) and total DNA extracted from yeast cultures containing XTY26 were fluorescently labeled (Kievits et al. 1990) and used as probes for in situ hybridization to metaphase chromosomes expressing the fragile X. The fragile X was induced in blood lymphocytes from a fragile-X male (Sutherland 1979), and metaphase spreads were prepared using standard methods. The in situ hybridization and signal detection were as described elsewhere (Callen et al. 1990; Kievits et al. 1990). Probes were nick-translated with biotin-14-dATP and hybridized in the presence of 125 × excess sonicated human placental DNA. A plasmid containing human repetitive sequences hybridizing at high stringency to the pericentromeric alphoid sequences of the X chromosome (Choo et al. 1987) was added to the hybridization solution immediately prior to hybridization, to unequivocally identify the X chromosomes (fig. 1*b–i*). Detection and amplification of the target sequences were with avidin-FITC and biotinylated goat anti-avidin. Propidium iodide was used as a counterstain. The metaphases were examined and photographed using a Leitz incident light fluorescence microscope; there was no computerized enhancement of the images.

Restriction Map of XTY26

The map of the yeast artificial ring-chromosome, XTY26, was derived by pulsed-field gel electrophoresis of single and double digests of the restriction enzymes shown and by hybridization of the blotted DNA

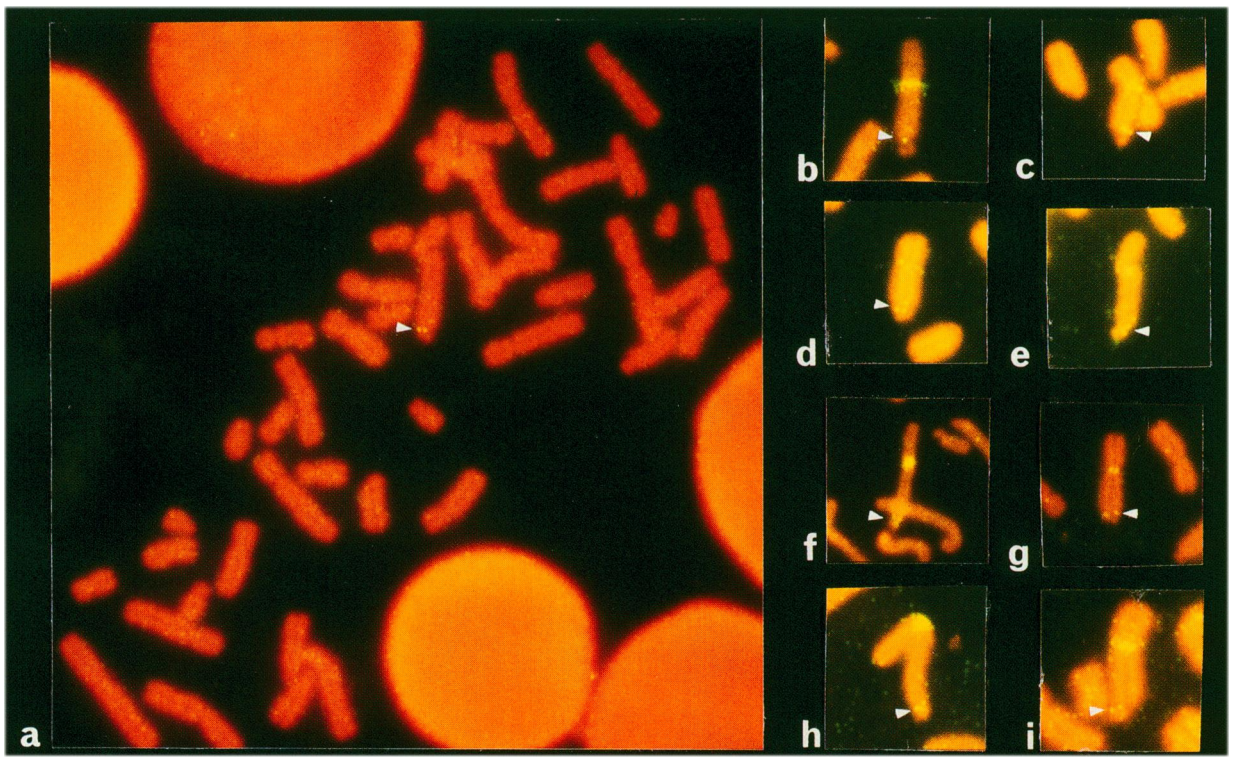

Figure 1 Partial metaphases showing in situ hybridization results for various probes to fragile-X chromosome. *a*, Metaphase showing VK16 (whole lambda clone) proximal to fragile site and general level of background. *b* and *c*, VK16 showing signal proximal to fragile site. *d–g*, XTY26 showing signal proximal, central, and distal to the fragile site. *h* and *i*, Do33 showing distal signal.

fragments with probes derived from pUC19 (vector), 2–34 (*DXS463*), Do33 (*DXS465*), and two subclones (A3 and B3) of VK16 (*DXS293*) (fig. 2). Restriction enzymes were used according to the suppliers' protocols. The yeast clone XTY26 was encapsulated in agarose beads as described elsewhere (Overhauser and Radic 1989). The DNA samples were usually digested for 12–16 h.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis of *Sfi*I digests of both XTY26 and normal human DNA was undertaken. DNA was isolated from the lymphocytes of a normal human female and from yeast containing XTY26 and was cleaved with the restriction enzyme *Sfi*I and electrophoresed to resolve fragments in the 50–300-kb size range. The DNA was transferred to a nylon membrane (Hybond N^+) and hybridized with ^{32}P-labeled VK16A3 (*DXS293*) probe.

Results and Discussion

There are a number of DNA probes closely linked to the fragile X (Suthers et al. 1991); but the fragile site itself has not been cloned, and nothing is known of its molecular nature. In order to identify the sequences involved in the expression of the fragile site and to characterize the molecular basis of the genetic lesion, we have constructed YACs containing human DNA and screened them with cloned DNA probes which map close to the fragile site at Xq27.3 (Suthers et al. 1990; Rousseau et al. 1991). One of these constructs, XTY26, was isolated by hybridization with a unique subclone (VK16B3) of the *DXS293* locus which is one of the DNA probes closest to the fragile site. It was not known whether *DXS293* was proximal or distal to the fragile site. The method of construction of the telomere complementation YAC library, involving telomere rescue, selects for clones which acquire or no longer need an additional telomere. A few clones contain sequences that hybridize to the human telomere repeat probe $(ttaggg)_n$; others contain segments from nontelomeric regions; yet others are circular chromosomes, maintained as such in yeast (Hieter et al. 1985), which also satisfy the selection. Insertion of human DNA sequences into the *Cla*I site was verified by sequence analysis of a subclone of XTY26, a subclone containing the vector-insert junction fragment (data not shown). The telomere complementation YAC library, as well as the library with human inserts in pYAC4 (Abidi et al. 1990), were screened with the pVK16B3 probe (*DXS293*) as described. Only one clone, XTY26, from the telomere library was found to be positive.

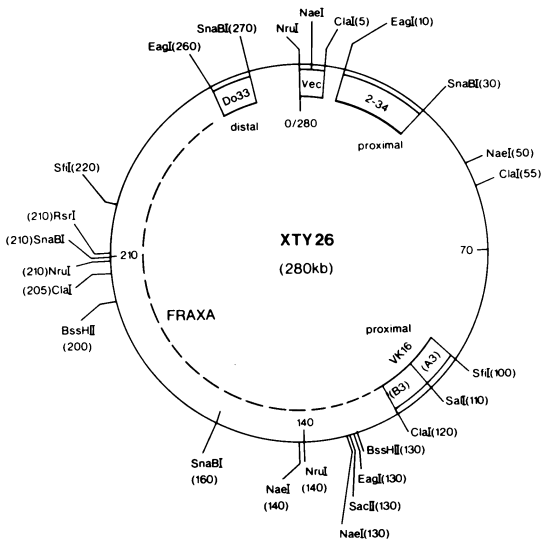

Figure 2 Restriction map of XTY26. Complete map sites in human insert DNA are shown only for *Sal*I, *Sfi*I, *Nru*I, *Rsr*I, and *Sac*II. Each of the other enzymes may have one or more additional sites. Multiple additional *Nae*I sites within the vector sequences are not shown.

Because of the close proximity of XTY26 to the fragile site, we investigated by means of in situ hybridization the possibility that the human DNA sequences it contained might span the fragile site. The results in table 1 demonstrate that the majority of the fluorescent signal is distal to the fragile site, whereas the probe (VK16) used to isolate the YAC was proximal to it on in situ hybridization (fig. 1*b*).

The integrity of the human DNA in XTY26 was established by hybridization to additional flanking DNA probes known to map close to the fragile site. Both Do33 (*DXS465*) and 2–34 (*DXS463*) (Rousseau et al. 1991) were found to be present in XTY26, and their maps for the restriction enzymes *Bam*HI, *Hin*dIII, and *Taq*I were identical in both XTY26 and human chromosomal DNA (data not shown). Linkage mapping has shown that Do33 and 2–34 are distal and proximal, respectively (Rousseau et al. 1991). Subsequently Do33 was shown to map distal to the fragile site (fig. 1*h* and *i*)— and 2–34 was shown to map proximal to it—by fluorescence in situ hybridization.

Restriction mapping of XTY26 by using infre-

Table 1

Location of Signal for Various Probes, in Relation to Fragile Site at Xq27.3

	Position of Signal (in relation to fragile site)			
Probe	Proximal	Central	Distal	Proximal and Distal
XTY26....	11	10	39	8
VK16.....	10	2	0	0
2-34.....	9	3	0	0
Do33	0	0	10	0

Note.—Sequential metaphase spreads from two fragile-X males were examined until at least 10 X chromosomes expressing the fragile site and exhibiting signal from probe hybridization had been scored. The position of the signal was scored as proximal, central (i.e., overlying the gap in the chromosome), or distal to the fragile site. Sensitivity and specificity was such that 35%–90% of all metaphases (depending on the probe) had yellow fluorescent dots on the end of at least one chromatid of the X chromosome, with virtually no background signal (fig. 1).

quently cutting enzymes revealed that this construct was indeed a ring (fig. 2). The evidence for this comes from several restriction digests which are only compatible with a circular map. The first is *Sal*I, which cuts XTY26 only once yet maps within *DXS293,* which in turn, according to other digests (e.g., *Nae*I), maps toward the middle of the human DNA sequence. Compared with undigested DNA, the *Sal*I digest gives only a minimal alteration in the size of XTY26, a finding which presumably reflects the difference between the mobility of the circular DNA and that of linear DNAs. Second, *DXS293* mapped onto the same *Nru*I fragment (140 kb) as did 2–34 but onto a different *Sfi*I fragment (*DXS293,* 120 kb; 2–34, 160 kb). The two *Sfi*I fragments equaled the total length of XTY26. This is only possible in a circular map which has two *Sfi*I sites (rather than in a linear map with one *Sfi*I site). In addition, 2–34 mapped to within 60 kb of one end of the human DNA insert on a *Nae*I digest and to a 50-kb *Cla*I fragment, yet the vector sequences which map to the same 60-kb *Nae*I fragment are found on an 80-kb *Cla*I fragment. The *Cla*I sites at map positions 5 kb, 55 kb, and 205 kb indicate the origin of these fragments.

Most of the restriction endonucleases used to generate the pulsed-field gel map of XTY26 contain CpG dinucleotides in their recognition sequences. While this contributes to their underrepresentation in the genome and therefore to their utility in long-range restriction mapping, the methylation of mammalian DNA at these sites rendered a direct comparison between the XTY26 map and human chromosomal DNA all but useless. A fortunate exception was *Sfi*I, which does not contain CpG and generates a 120-kb *Sfi*I fragment from XTY26, which in turn contains *DXS293* and most of the DNA between this locus and Do33 (approximately 150 kb). The same 120-kb *Sfi*I fragment was detected in human lymphocyte DNA from a normal individual (fig. 3), confirming the integrity of at least a portion of the human DNA sequence in XTY26. The integrity of the human insert is further supported by evidence from restriction maps of YACs in this area (Vincent et al. 1991) that show the probes 2–34 and Do33 to be approximately 210 kb apart. We had previously estimated, on the basis of our pulsed-field gels, that these probes are 230–260 kb apart in XTY26. This discrepancy is probably due to different pulsed-field gel electrophoresis conditions and not to a rearrangement of either YAC. Vincent et al. (1991) have suggested that methylation affects

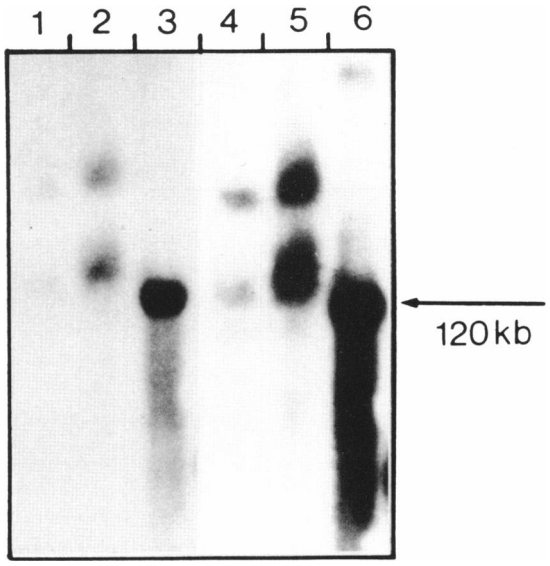

Figure 3 *Sfi* digests of XTY26 and normal human DNA probed with VK16A3. Lanes 1 and 4, Approximately 5 μg human DNA. Lanes 2 and 5, Approximately 25 μg human DNA. Lanes 3 and 6, Approximately 1 μg total yeast DNA including XTY26. Lanes 1–3 show results after 16 h autoradiography, and lanes 4–6 show results for the same filter after exposure for 72 h. The slight differences in mobility of the 120-kb fragment (e.g., between lanes 2 and 4) occur because of the different amounts of DNA loaded in these lanes, as demonstrated by ethidium bromide staining (not shown). The additional approximately 200-kb band in lanes 1, 2, 4, and 5, as well as the 280-kb band in lane 6, are presumably due to incomplete digestion.

cleavage of the restriction-endonuclease *Bss*HII and *Sac*II sites at or near the FRAXA region in affected lymphoblastoid cell lines or leukocyte DNA samples of fragile-X patients. The location of these sites is conserved in fragile-X (XTY26) and normal (Heitz et al. 1991) DNAs. The conservation of these sites suggests that the observed differences in the mobility of *Bss*HII and *Sac*II restriction fragments from this region (*a*) are not due to large amplification or rearrangement in fragile-X affected individuals and (*b*) support a role for methylation. The clustering of CpG-containing restriction-endonuclease recognition sequences at position 130 kb (fig. 1) is indicative of the promoter region of a gene and suggests that the observed methylation differences may effect expression of such a gene.

The screening of the human insert in XTY26 for AC repeat microsatellite sequence (Weber and May 1989) should provide additional highly polymorphic DNA markers for carrier detection and prenatal diagnosis of the fragile X (Richards et al. 1991). Identification of the DNA sequences for fragile-X expression should now be possible, and the identification of transcribed sequences may allow the gene(s) responsible for the fragile-X syndrome to be characterized. The CpG-rich region delineates a logical position at which to commence the search for such a gene.

Acknowledgments

We thank Fatima Abidi for preliminary characterization of the telomere library, and we thank Sandy Johnson for screening the YAC libraries. This work was supported by grants from the National Health and Medical Research Council of Australia and from The Adelaide Children's Hospital Research Foundation.

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