A YAC contig across the fragile X site defines the region of fragility

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Received March 26, 1991; Revised and Accepted May 8, 1991

ABSTRACT

The fragile X syndrome is a common cause of mental retardation and is associated with a fragile site at Xq27.3 (FRAXA). Recently, evidence has been presented for the role of methylation and genomic imprinting in the expression of the disease. We have identified a site of methylation in patients by long range restriction mapping of the region. In this paper we present a YAC contig of this area, localise the CpG sequences which are methylated, and show by *in situ* hybridisation that the site of fragility lies within this region.

INTRODUCTION

The fragile X, or Martin Bell, syndrome is the commonest genetic cause of mental retardation after Down's syndrome with a frequency of 1 in 1,500 males (1, 2, 3). The disease is associated with the expression of a rare fragile site at Xq27.3 in lymphocytes grown under conditions of thymidine stress (4, 5). The syndrome displays an unusual segregation pattern since about one third of carrier females are mentally retarded and approximately 20% of males carrying the mutation have a normal phenotype (6, 7). Most hypotheses explain these observations by suggesting that the generation of the disease phenotype involves a two step process. Recently, we (8) and others (9) have presented evidence for the role of methylation and genomic imprinting in the expression of the disease consistent with Laird's hypothesis (10). However, the exact relationship between the degree of methylation and the phenotype is not clear. We have analysed fragile X positive, mentally retarded males who appear not to be methylated and a fragile X positive, phenotypically normal male who is methylated in this region (8). Using the markers M749 and M759, which were isolated by microdissection of the fragile X region (11), we constructed a long range restriction map across the fragile site and identified the site methylated in fragile X patients (8). Using STSs derived from these two microclones we have now constructed a yeast artificial chromosome (YAC) contig over the region involved in methylation imprinting. Using YAC and cosmid clones we show by *in situ* hybridisation that the contig crosses the fragile site.

MATERIALS AND METHODS

Microclone STSs

The complete sequence of the microclones M749 (231bp) and M759 (109bp) was generated using standard dideoxy chain termination sequencing protocols with Sequenase (USB) as recommended by the manufacturers. STS-M749 (749F, 5'-CAGTTTTCATTTCTCTGTCTTCAA, 749R, 5'-AATGTG-GCAAGGAAGCCAGCA) and STS-M759 (759F, 5'-GAA-GGGCCAGCAAATTGC, 759R, 5'-CTGTAGACTACTTGA-TGTGA) were used to screen pools of DNA made from various genomic YAC libraries by PCR (see text). Amplifications were carried out for 35 cycles of 94°C denaturation, annealing temperatures of 53°C (M759) and 61°C (M749) and extensions at 72°C for 2 minutes in standard buffer (50mM KCl, 10mM Tris.HCl pH8.4) containing 1.5mM $MgCl_2$.

DNA analysis and probe preparation

High molecular weight yeast DNA was prepared in low melting point agarose using standard protocols. For restriction mapping, yeast DNA in agarose was digested with restriction enzymes and

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size fractionated by CHEF pulsed field gel electrophoresis using the LKB Pulsaphor system. Typically, gels were run at 180 volts for 20 hours at a switching time ranging from 80 seconds to 15 seconds, to resolve DNA fragments in the range 1000Kb−10Kb. The DNA was depurinated for 30 minutes in 0.25M HCl, denatured, neutralised and transferred onto Hybond-N (Amersham Int PLC) according to the manufacturers instructions. Hybridisations to pYAC4 vector arms and internal probes were carried out under conditions as previously described (8, 11).

pYAC4 vector arm probes were prepared directly from pYAC4 DNA by PCR. The left arm probe (330bp) is made by amplification with the primer pairs YACLR1 (5'-GTGTGGTCGCCATGATCGCG) and YACLP (5'-ATGCGGTAGTTTATCACAGTTAA). The right arm probe (265bp) is made by amplification with the primer pairs YACRP (5'-GATCATCGTCGCGCTCCAGCGAAAGC) and YACRR3 (5'-CTCGCCACTTCGGGCTCA). After 30 cycles of PCR (standard buffer conditions containing 1.5mM $MgCl_2$, annealing temperature 55°C), vector arm probes were gel purified in low melting point agarose for radiolabelling. pYAC4 vector arm probes, total inter-alu PCR product and G9−4 were radiolabelled by random priming and probes 291L, 291R, 141R, G9L, M759, M749 by PCR labelling as described previously (11).

Isolation of YAC end fragments

End fragments from YAC clones were isolated by the method of inverse PCR (12). 100ngm of total yeast DNA was digested to completion with restriction enzymes TaqI, RsaI, HaeIII, HincII, Sau3A and AccI. After precipitation, the resulting fragments were circularised using 2 units of DNA ligase (Promega) at 16°C for 16 hours in a volume of 50μl (a final DNA concentration of 2mg/ml). After heat inactivation of the ligase, PCR was carried out on 10ngm of circularised template using inverse primer pairs for 34 cycles of 94°C, 1 minute; 55−58°C, 1 minute and 72°C, 4 minutes under standard conditions. YAC left end fragments were isolated from the TaqI, RsaI, Sau3A and HaeIII circularisations with the primer YACLF (5'-AAGTACTCTCGGTAGCCAAG) paired with either YACLR1, 5'-GTGTGGTCGCCATGATCGCG (TaqI and HaeIII), YACLR2 (5'-ACTGGCGATGCTGTCGGAAT) (RsaI) or YACLR3 (5'-TCAGAGTGAAATTTATACTAAAGAA) (Sau3A). Right end fragments were isolated from HincII, AccI and HaeIII circularisations with the primer YACRF (5'-AGTCGAACGCCCGATCTCAA) paired with either YACRR1 (5'-GGAGTCGCATAAGGGAGAGC) (HincII and AccI) or YACRR2 (5'-TTCAAGCTCTACGCCGGAC) (HaeIII). Individual fragments were gel purified and cloned into pUC18.

Isolation of Cosmid clones

Cosmid G9LB (ICRFc104D08104) was identified by hybridisation screening of a gridded flow sorted×chromosome library (ICRF Reference Library (13)).

Inter-Alu PCR

Inter-Alu PCR (14) was carried using a single alu consensus primer, 938 (5'-CCACTGCACTCCAGCCTGGG). 27 cycles of 94°C, 1 minute; 50°C, 2 minutes; 72°C, 2 minutes were carried out on 50ngm of total yeast DNA in standard buffer conditions containing 1.5mM $MgCl_2$. The resulting fragments were either gel purified to isolate individual products or precipitated to provide a total Alu-PCR probe for hybrid analysis.

In situ hybridisation

The fragile site was induced in culture and chromosome spreads prepared by standard techniques. Whole yeast DNA for YAC XY291 and DXZ1 were labelled with biotin using a BRL BioNick labelling system. Whole yeast DNA for YAC G9, whole cosmid DNA for G9LB and DXZ1 were labelled with digoxigenin (BCL) by nick translation. Hybridisation and detection procedures were as described by Kievits et al (15). DNA concentrations in the hybridisation mix were 1.5ng/μl for DXZ1, 8ngm/μl for the cosmid G9LB, 30ng/μl for the YACs and 750ng/μl for the competitor total human DNA. This mixture was denatured and preannealed for 2h at 37°C. Slides were hybridised with 10μl of hybridisation mix under an 18×18 mm cover slip overnight (cosmids) or four days (YACs) at 42°C, and then washed down to 0.1×SSC at 65°C without formamide. Biotinylated probes were detected by alternate layers of fluorescein conjungated avidin (DN, Vector) and biotinylated anti-avidin antibody (Vector) both at dilutions 5μg/ml in 4×SSC with 5% non-fat dried milk. Slides were washed in 4×SSC with 0.05% Tween 20. Digoxigenin-labelled probes were detected by layers of mouse anti-digoxigenin antibody (Sigma, 1.5μl/ml), fluorescein conjugated rabbit anti-mouse antibody (Sigma, 1μl/ml) and fluorescein-conjugated goat anti-rabbit antibody (Sigma, 0.5μl/ml). Antibodies were diluted in AB buffer (0.1M Tris-HCl/ 0.15M NaCl) with 0.5% non-fat dried milk and slides were washed in AB buffer with 0.05% Tween-20. All slides were finally rinsed in PBS and mounted in antifade (1 part 1% p-phenylenediamine dihydrochloride to 9 parts glycerol) with 1μg/ml propidium iodide. A confocal laser microscope (Biorad MRC 600) was used for the analysis. Images were collected in the dual mode channel and then merged.

RESULTS

Isolation of YACs

Primers for M749 and M759 were used to screen genomic YAC libraries made from a 48, XXXX lymphoblastoid cell line (16, 17) a 46, XY lymphocyte sample (18) and a 46, XY lymphoblastoid cell line (19). No positive PCR pools were obtained with M749 but several positive YACs were identified using the M759 primers. A comparison of the rare cutter restriction maps of the M759 YACs shows that they overlap and is consistent with the absence of any gross rearrangement or co-ligation events in the cloning procedure. The map of the YACs, which covers a megabase of DNA, is presented relative to the genomic map of the region in figure 1. Comparison of the YAC and genomic maps shows that most of the restriction sites containing CpG dinucleotides, except for those in the cluster between VK21 (DXS296) and M749 (DXS533) and between M749 and M759 (DXS532) are methylated in genomic DNA and therefore do not cut.

M749 region

M749 identified a YAC (XY291) in a grid of YACs constructed from a library made from a somatic cell hybrid containing Xq24-qter (20). These YACS had previously been localised to Xq27/q28 by *in situ* hybridisation (21). End fragments from the insert of this and other YACs were isolated by inverse PCR on whole yeast DNA (see Materials and Methods). Probe 291L, which maps in the same region as M749, hybridises to a 600kb genomic BssHII fragment and detects the hypermethylation of

this site in fragile X positive, mentally retarded patients (8). The cluster of NotI, SacII, MluI and SalI immediately distal to 291L coincides with the CpG island between M749 and VK21. Inter-Alu PCR products from XY291 confirm that the 400kb up to the central BssHII site is of Xq27 origin and is contiguous with M749. The proximal end of XY291 (291R) was found to hybridise to DNA which is of rodent origin (data not shown). This appears to make up the proximal 200kb of the YAC. XY291

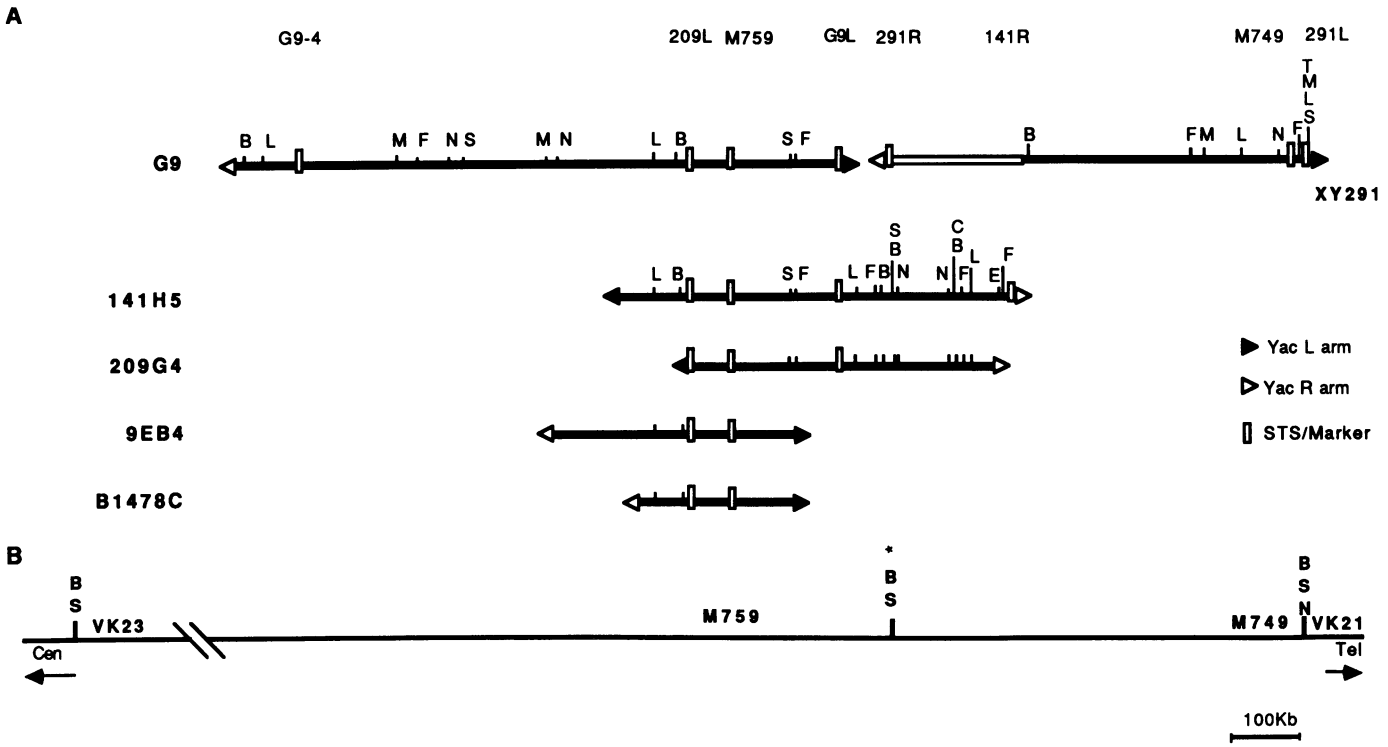

Figure 1. (A) Mapping of a YACs in the fragile X region. YACs for M759; G9 (950Kb), 9EB4 (350Kb), B1478C (200Kb), 141H5 (570Kb), 209G4 (470Kb) and M749; XY291 (620Kb) were aligned by a comparison of restriction maps generated with the enzymes M=MluI, T=NotI, B=BssHII, S=SacII, F=SfiI, N=NruI, C=ClaI, E=EagI and L=SalI. In the case of EagI and ClaI, only partial mapping on the 141H5 and 209G4 YACs is shown. Boxes indicate the positions of the markers and STSs used to nucleate and map the region. M749 and M759 are microdissection clones. YAC end fragments were isolated by inverse PCR following digestion with TaqI (G9L, 209L), HaeIII (141R), RsaI (291L) and HincII (291R) using conditions described in Materials and Methods. G9-4 is an inter-alu PCR product isolated as described in Materials and Methods. Open triangles represent pYAC4 vector right arms and closed triangles pYAC4 vector left arms. For XY291 the open line represents non-contiguous DNA (see text) **(B)** The YAC maps areshown above the genomic map of the region taken from reference 8 with the markers used for this analysis; VK23 (DXS297), VK21 (DXS296), M749 (DXS533) and M759 (DXS532). The hypermethylated HTF island is marked with an asterisk.

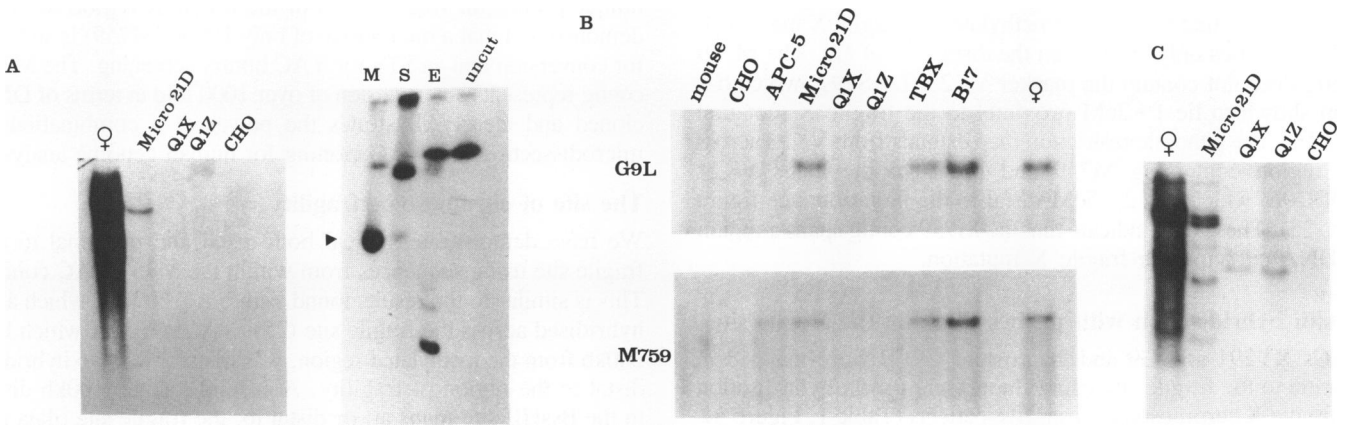

Figure 2A. Hybridisation of inter-Alu PCR product G9-4 in the presence of cold competitor human DNA to a somatic cell hybrid panel and fragments of the G9 YAC resolved by CHEF electrophoresis. Somatic cell hybrid panels contain 5μg hybrid DNAs digested with EcoRI. The hybrid Micro21D contains Xpter-q27.3 and Q1X and Q1Z contain Xq27.3-qter (11). M=Mlu I; S=SacII; E=Eag I. The arrow shows the position of the 240kb Mlu I proximal end fragment of the YAC. **2B.** Localisation of the distal end of the YAC G9 defined by the probe G9L relative to the microclone M759. 5μg hybrid DNAs were digested with Taq I and are as in figure 2A with the addition of TBX (Xpter-qter), B17 (Xq27-qter) and APC-5 which contains a small region of Xq27 (11). **2C.** Hybridisation of total inter-Alu PCR products from 141H5. Total inter-Alu PCR products (see Materials and Methods) were precipitated and used as a hybridisation probe to a panel of somatic cell hybrids as described in figure 2A.

does not, therefore, extend to the hypermethylated BssHII site, but represents a contiguous block of DNA lying only 200kb distal to the hypermethylated region.

M759 region

The contig nucleated at M759 contains 5 YACs and covers 1.25Mb of DNA (figure 1A). Verification of the proximal end of the contig was performed by mapping inter-Alu PCR products from G9 in somatic cell hybrids and against the restriction fragments of the YAC itself, an example of which is shown in figure 2A. Alu-PCR product G9-4, which identifies the most proximal 240kb MluI fragment in G9, maps to the same hybrid interval as M759 (11). These data demonstrate that sequences in G9 map to Xq27.

Orientation of M759 YAC contig

A probe isolated from the left end of G9, G9L, has been shown to detect hypermethylation of the BssHII site in fragile X males from the proximal side (8). A comparison of the hybridisation of G9L and M759 to somatic cell hybrids containing breakpoints in the region is shown in figure 2B. G9L is deleted from hybrid APC-5, whereas M759 is present. This allows us to orientate G9 with respect the centromere and telomere. As the breakpoint in APC-5 lies distal to M759 (11), G9 must extend proximal away from the hypermethylated site. This conclusion was confirmed when inter-Alu PCR products from 141H5 were found to identify bands in somatic cell hybrids carrying either distal or proximal portions of the X chromosome constructed after induction of breakage of a fragile X chromosome (22; Figure 2C). The distal end of YAC 141H5 (141R) also maps in this interval (data not shown). The YAC contig derived from M759 therefore crosses these breakpoints and extends into the same somatic hybrid interval as M749 (11).

As we have established that M759 and M749 are on opposite sides of the hypermethylated BssHII and SacII sites (8), and 141H5 crosses into the M749 hybrid interval, we would predict the presence of such a restriction site cluster in 141H5. An examination of the restriction maps of 209G4 and 141H5 indicates the presence of coincident SacII and BssHII sites, 170kb from the right end of 141H5. These sites must therefore represent the HTF island found to be hypermethylated in fragile X males (8). This island lies only 80kb from the distal end of G9. The M759 contig does not contain the marker VK23 (DXS297) which has been shown to lie 1−2cM proximal to the fragile X mutation (22, 23). The genomic restriction map extends from VK23 across the region containing M759 and M749 to the locus VK21 (DXS296) which lies 2−5cM distal to the mutation (see figure 1; 8, 23). These data indicate that the M759 contig spans a region of DNA carrying the fragile X mutation.

In situ hybridisation with probes flanking the fragile site

YACs XY291 and G9 and the cosmid G9LB were mapped in relation to the fragile site using fluorescent *in situ* hybridisation on fragile X chromosomes from five patients (Table 1, Figure 3). Two of these are brothers and thus represent a single FRAXA locus. XY291 appeared to hybridise only to the distal side of the fragile site in all cells examined from the four patients. FITC signal from G9 was located on the constriction and to different sides of the fragile site in different cells from each of the four patients. In two particular cases, signal was seen both sides of the induced fragile site constriction (see Table 1 legend). Much more discrete hybridisation signal was obtained with the cosmid G9LB and in all three individuals tested, the signal was observed either proximal or at the fragile site (Figure 3k and l).

Table 1. Localisation of FITC hybridisation signal for YACs XY291 and G9 and cosmid G9LB in relation to the fragile site in fragile X patients

Probe	Patient	Number of fragile X chromosomes exhibiting signal		
		Proximal	Mid	Distal
XY291	1	–	–	7
	2	–	–	5
	3	–	–	6
	4	–	–	5
G9	1	3	5*	3
	2	3	3	2
	3	4**	3	4
	4	2	1	1
G9LB	1	13	7	–
	3	6	5	–
	5	6	4	–

Patients 3 and 4 are brothers and thus carry the same X chromosome.
* Two of these X chromosomes exhibited discrete signal both proximal and distal to the fragile site constriction.
** An additional cell was noted with signal right at the long arm telomere of the X chromosome. In this one case the acentric fragment distal to the fragile site is presumed to have been lost.

DISCUSSION

YAC contig

The YAC contig presented here is consistent with genomic mapping experiments that we have reported previously (8). There is only one HTF island in over 3Mb of DNA in the fragile X region and this island is methylated in a large number of fragile X patients. There is an additional cluster of CpG cutting restriction enzyme sites approximately 100kb distal to this island but some of these sites are uncleaved in genomic DNA. Our map is in agreement with that recently reported by Heitz et al (25) for the YAC 141H5, but extends a further 500kb proximal.

Microdissection has allowed us to generate a large number of unique probes for Xq27 (11). For the fragile X region we have demonstrated that a microclone of only 109bp (M759) is suitable for conversion into an STS for YAC library screening. The M759 contig represents an expansion of over 1000 fold in terms of DNA cloned and clearly illustrates the power of a combination of microdissection and YAC cloning for human genome analysis.

The site of chromosome fragility

We have demonstrated signal both distal and proximal to the fragile site using sequences from within the M759 YAC contig. This is similar to the results found with YAC 209G4, which also hybridised across the fragile site (25). YAC XY291, which lies 200kb from the methylated region, was found always to hybridise distal to the region of fragility. A cosmid located 100kb distal to the BssHII site maps at, or distal to, the fragile site (data not shown) which suggests that chromosomal fragility occurs only in DNA proximal to XY291. In order to refine the area of fragility, we hybridised a cosmid corresponding to the distal end of YAC G9. This cosmid produced signal only proximal to, or at, the fragile site. These observations suggest that the region of chromosomal fragility is confined to a few hundred kb surrounding the HTF island in the M759 contig presented here.

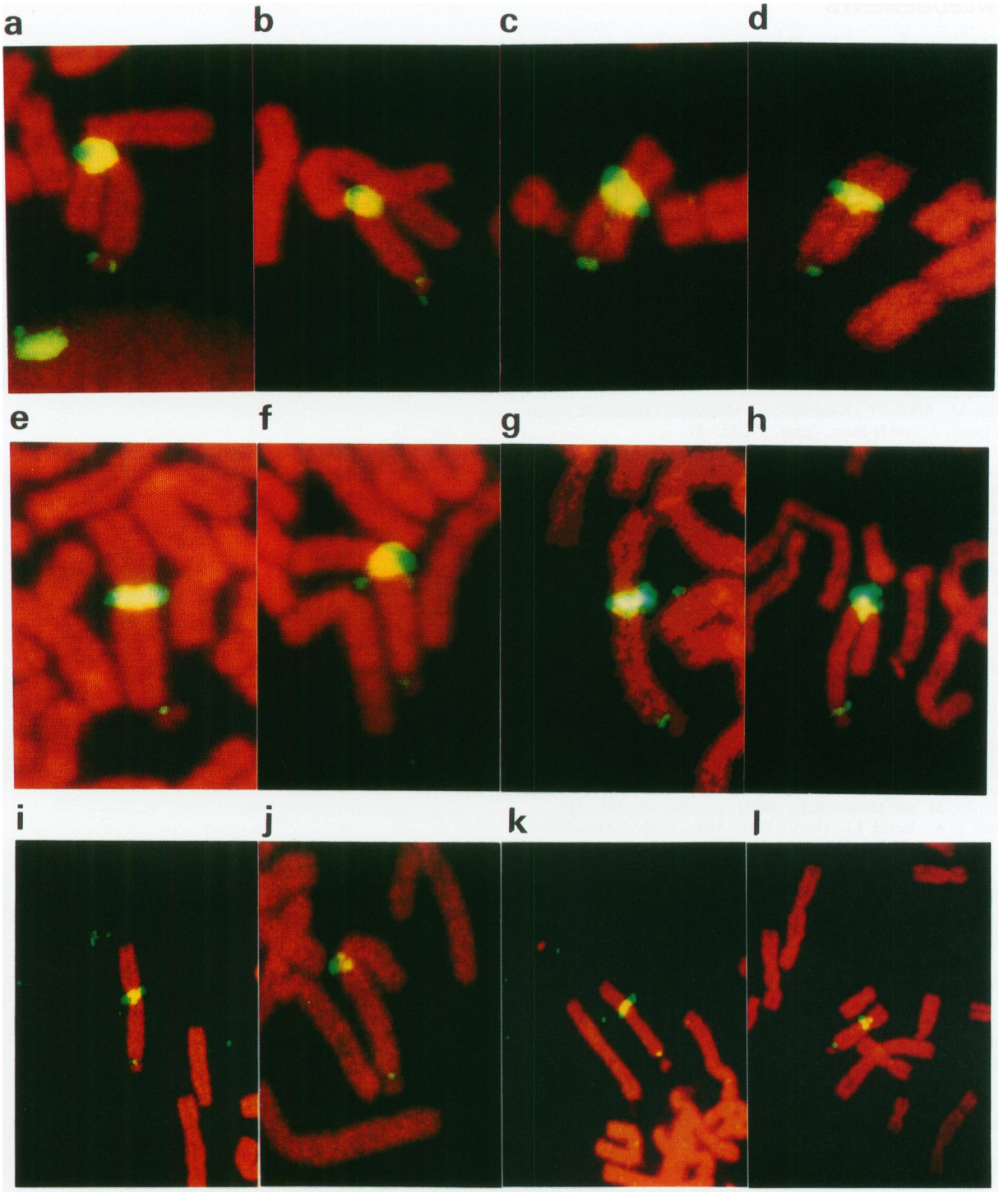

Figure 3. *In situ* hybridisation of YACs XY291, G9 and cosmid G9LB to chromosome preparations from five patients with X-linked mental retardation. An alphoid centromere probe specific for the X chromosome (DXZ1, Oncor) was also hybridised to confirm the identity of chromosome examined. (a–d) Hybridisation of YAC XY291 distal to the fragile site in all four patients examined. (e–j) Hybridisation of YAC G9: (e and f) distal to the fragile site; (g and h) proximal to the fragile site; (i and j) on top of the fragile site; (k and l) Hybridisation of cosmid G9LB proximal to the fragile site.

Since the chromosome breakpoints which have been induced in a fragile X chromosome (22) cluster in the region of the CpG island in the centre of our YAC contig, this appears to be a critical region for the expression of the fragile site. However, the relationship between the methylation of this CpG island and the clinical phenotype remains unclear as a normal, fragile X positive male shows almost total methylation at this site and 2/21 fragile X positive, mentally retarded males are not methylated at this site (8). The demonstration that chromosomal fragility may extend further proximal may mean that genes not associated with this CpG island within this contig may be involved in the pathology of this disorder.

ACKNOWLEDGEMENTS

We thank Dr Breuning and J.G.Dauwerse for help in establishing non-isotopic detection systems, Mrs M.Fitchett for supplying patient samples, Dr A.Monaco for assistance with the YAC library, Dr D.Nizetic and Dr C.Douglas for cosmid clone isolation, Dr S.Warren for hybrids and H.Blaber for help with manuscript preparation. The project was funded by the Medical Research Council (as a Programme and as part of the UK Human Genome Mapping Project) and Action Research for the Crippled Child.

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