Physical linkage of the fragile site \textit{FRA11B} and a Jacobsen syndrome chromosome deletion breakpoint in 11q23.3

Christopher Jones$^{1,*}$, Predrag Slijepcevic$^1$, Sharon Marsh$^{2,†}$, Elizabeth Baker$^3$, Wallace Y. Langdon$^4$, Robert I. Richards$^3$ and Alan Tunnacliffe$^{1,5}$

$^1$Department of Pathology and $^2$Molecular Cytogenetics Laboratory, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK, $^3$Department of Cytogenetics and Molecular Genetics, Adelaide Children's Hospital, 72 King William Road, North Adelaide, SA 5006, $^4$Department of Biochemistry, University of Western Australia, Nedlands, WA 6009, Australia and $^5$Quadrant Research Foundation, Maris Lane, Trumpington, Cambridge CB2 2SY, UK

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Autosomal fragile sites, unlike their X-linked counterparts, are not known to be associated with disease. However, one case report has highlighted a possible relationship between the inheritance of a rare folate-sensitive fragile site in band 11q23.3 (FRA11B) and the chromosome 11q23-pter deletion in Jacobsen (11q-) syndrome. The mother and brother of the reported Jacobsen syndrome child are FRA11B carriers, suggesting that in vivo breakage at the fragile site during early development could have given rise to the chromosome deletion. We have tested this hypothesis by high resolution physical mapping of FRA11B and of the deletion chromosome breakpoint in the Jacobsen syndrome patient. A detailed restriction map of 600 kb of human chromosome band 11q23.3 has been assembled which covers the \textit{PBGD, CBL2} and \textit{THY1} genes. FISH experiments with YACs and cosmids from this region have localised FRA11B to an interval of approximately 100 kb containing the 5' end of the CBL2 gene, which includes a CCG trinucleotide repeat. This class of repeat is expanded in the four cloned examples of fragile site and therefore the CBL2 repeat is a candidate for the location of FRA11B. Further, it is shown that the chromosomal deletion breakpoint of the Jacobsen syndrome child maps within the same interval as the fragile site. The breakpoint has apparently been repaired and stabilised by the de novo addition of a telomere. These data are consistent with a role for an inherited fragile site in the aetiology of a chromosome deletion syndrome.

\section*{INTRODUCTION}

Fragile sites are specific inherited chromosomal aberrations that appear as a constriction in the chromosome and render it susceptible to breakage under specific experimental conditions (1). However, although chromosomal breakage at fragile sites can be induced \textit{in vitro}, there is no direct evidence of breakage occurring \textit{in vivo} in carriers of fragile sites.

Fragile sites can be divided into two major categories, rare or common, according to their frequency within the population. Rare fragile sites are sub-classified according to the conditions under which they are expressed. The majority of rare fragile sites are expressed when cells are grown in folate acid-deficient culture medium, whereas others are induced by the addition of distamycin A, BrdU, or related compounds, to the culture medium. In contrast, common fragile sites are weakly induced by folate deficiency, more strongly by aphidicolin and most effectively by both of these conditions together (1).

The only fragile site with which a clinical phenotype has been unequivocally linked is the rare folate-sensitive fragile site at Xq27.3 (FRAXA) (2) which causes fragile X syndrome (3,4), the most common form of inherited mental retardation (5). Recently, a gene associated with FRAXA (\textit{FMR1}) has been identified (6) and shown to contain specific molecular anomalies in affected individuals (7–10). In the majority of cases, the molecular basis of fragile X syndrome has been found to be the extensive expansion of a CCG-trinucleotide repeat in the 5' untranslated region of \textit{FMR1} (6,11,12), associated methylation of an adjacent CpG island (8,9) and reduction in the transcription of \textit{FMR1} (13,14).

Despite the lack of an obvious clinical phenotype in carriers of other fragile sites, there is some statistical evidence that the incidence of nine autosomal fragile sites is higher in mentally retarded individuals than in the general population (15). In addition, there are case reports suggesting links between fragile sites and other phenotypes such as breast cancer (16), multiple spontaneous abortions (17,18) and autosomal deletion syndromes (19). Since FRA16A is the only autosomal fragile site to have

*To whom correspondence should be addressed
†Present address: Sanger Centre, Hinxton Hall, Hinxton, Cambridge CB10 1RQ, UK
been defined to date (20), the precise relationship between disease and fragile site expression has yet to be resolved.

Jacobsen syndrome (or \(11q-\) syndrome) is the clinical presentation of the loss of part of the long arm of chromosome 11, typically from band \(11q23-qter\), and is recognised by specific dysmorphic features and severe mental retardation (21,22). Voulaire et al. (19) reported a case in which a child who had a deletion del(11)(q23) of one chromosome 11 homologue, and who had signs of Jacobsen syndrome, was born to a mother who expressed \(FRA11B\). A second child in this family was also reported to express the fragile site. \(FRA11B\) has previously been shown to map to band 11q23.3 (23), hence it is possible that the chromosomal deletion in this Jacobsen patient was due to inheritance of the mother's \(FRA11B\) chromosome and its subsequent breakage at an early stage in development. If this were the case, autosomal fragile sites may have more important clinical implications than previously believed. To test this hypothesis, we are attempting to characterise both \(FRA11B\) and the deletion breakpoint in the \(11q-\) case reported by Voulaire et al. (19). In this study we have mapped the location of both \(FRA11B\) and the \(11q-\) breakpoint to a region of 11q23.3 of approximately 100 kb and we have identified a likely candidate for the molecular basis of fragility at 11q23.3.

### RESULTS

Using standard banding techniques, \(FRA11B\) has previously been assigned to chromosome band 11q23.3 (23). However, the precise location of the fragile site within 11q23.3 has not been deduced. To date, three folate-sensitive fragile sites have been characterised at the molecular level (\(FRAXA, FRAXE,\) and \(FRA164\)) and have all been found to be due to the expansion of CCG-trinucleotide repeats (6,11,12,20,24). The proto-oncogene \(CBL2\) maps to 11q23.3 (25,26) between the \(PBGD\) and \(THY1\) genes (26) and has a CCG-repeat in its 5' untranslated region (27), which is therefore a candidate for the location of \(FRA11B\). YACs containing the 3' end of the \(CBL2\) gene (\(y323E1\)) and the \(PBGD\) gene (\(y15E5\)) were used in preliminary FISH experiments with \(FRA11B\)-expressing cells and were shown to flank the fragile site (see Table 1). Therefore, we decided to restriction map these clones in order to position them with respect to a genomic map of the interval (26) and hence to more accurately position \(FRA11B\).

**Restriction map of YACs containing the \(PBGD, CBL2\) and \(THY1\) genes**

The YACs containing the \(CBL2\) gene (\(y323E1\)) and the \(PBGD\) gene (\(y15E5\)) were mapped with rare-cutting restriction enzymes using conventional pulsed field gel electrophoresis (PFGE) and Southern blotting technology. Details of probes used in hybridisation experiments are given in Materials and Methods. Hybridisation of \(y15E5\) with a YAC-specific probe identifies a single band of approximately 125 kb. Digestion of \(y15E5\) with \(SalI\) yields a 60 kb fragment that is recognised by both the YAC-left probe and the \(PBGD\) probe (Fig. 1A). However, the \(PBGD\),...
A

![Diagram of mapping information](image)

B

![Diagram of mapping information](image)

Figure 2. Summary of the mapping information interpreted from restriction enzyme and Southern analysis of cosmids and YACs, and FISH analysis of FRA11B and Jacobsen (11q−) chromosomes. (A) Restriction map of YACs yl5E5 and y323El, showing the relative positions of FRA11B and the PBGD, CBL2 and THY1 genes and (dark shaded boxes; arrows denote the known orientation of genes), probes mentioned in the text (lightly shaded boxes) and cosmids from this region. (B) The long range restriction map of this region as previously reported by Tunnacliffe and McGuire (26). Restriction sites mapped in this study are aligned with those identified by Tunnacliffe and McGuire as described in the text. N, NotI; Nr, Nrul; S, Sail; (S), possible additional Sail sites; c and y prefixes denote cosmid and YAC respectively; Circled R and L denote right and left YAC arms respectively.

The centromeric boundary of sequences recognised by the vCBL probe is identified in the long range restriction map (26) by NotI and Nrul sites between PBGD and CBL2 (Fig. 2A). The telomeric boundary of sequences recognised by the vCBL probe in the long range restriction map is identified by Sail and NotI sites between CBL2 and THY1 (Fig. 2B). These sites are in the same orientation and of very similar spacing to the sites that mark the telomeric boundary of the vCBL probe in y323E1 (Fig. 2A). The long range restriction map estimates the boundaries of vCBL to be 250 kb apart (Fig. 2B), so
alignment of the two physical maps allows us to estimate the physical distance between the ends of y15E5 and y323E1 as approximately 100 kb (Fig. 2A). Therefore according to the preliminary FISH results using these YACs, FRA11B must lie in this 100 kb interval. This was confirmed in FISH experiments using cosmids corresponding to the approximate boundaries of this interval.

Isolation of cosmids containing PBGD and CBL2 sequences

Initial attempts to clone the region between y15E5 and y323E1 in YACs were unsuccessful, so in order to link y15E5 and y323E1 to form one continuous contig and also to identify the position of the trinucleotide repeat of the CBL2 gene, we attempted to clone this region in cosmids. We used probes made from a previously described PBGD cosmid, cP1 (P2 and P7HR) (26) and the CBL2 gene (CBL2-5', vCBL and CBL-RI) to isolate several cosmids from the ICRF chromosome 11 gridded cosmid library (28). These cosmids were aligned with each other and with YACs y15E5 and y323E1 by restriction and Southern analyses (data not shown; Fig. 2A). Only one cosmid, cA0353, hybridises to the CBL2-5' probe (data not shown; Fig. 2A) and the presence of the CCG-trinucleotide repeat was confirmed by sequence analysis of part of this cosmid (data not shown). cA0353 was found neither to overlap with the small cosmid contigs identified, nor with the YACs y15E5 or y323E1 (data not shown; Fig. 2A). Therefore this cosmid is predicted to lie in the gap between these two YACs and CBL2 must be transcribed in a telomeric direction (Fig. 2A). Knowing the direction of the CBL2 gene has allowed us to orientate cosmids that span this region (Fig. 2A) and to estimate that the CBL2 gene covers over 100 kb.

Cosmids containing the PBGD and CBL2 genes flank the fragile site FRA11B

To localise the position of the fragile site FRA11B, we performed FISH on a lymphoblastoid cell line (JE) derived from an individual that expresses the fragile site, using cosmids cC0617 and either cA0734 or cE0182 as probes. The region between these cosmids must contain the CCG-trinucleotide repeat of the CBL2 gene, a candidate for the molecular basis of fragility at 11q23.3.

Expression of the fragile site was induced by growing cells in medium deficient in folic acid. Biotin-labelled cosmid cC0617 (red fluorescence) and digoxigenin-labelled cosmid cA0734 (green fluorescence) were co-hybridised to metaphase spreads prepared from the fragile site-induced cells and detected using standard procedures (see Materials and Methods). Figure 3A shows part of a DAPI-stained metaphase spread with the normal 11 (blue arrow) and FRA11B chromosome (red arrow) labelled. Figure 3B shows the same image as Figure 3A, but with the
Figure 4. Fluorescence in situ hybridisation of the Jacobsen (11q-) cell line C262, with cosmid probes. (A) DAPI stained metaphase spread showing normal chromosome 11 (blue arrow) and Jacobsen (11q-) chromosome (red arrow). (B) The same metaphase spread as in (A) but with the hybridisation signals of cC0617 (red fluorescence) and cA0734 (green fluorescence).

Figure 5. Fluorescence in situ hybridisation of the Jacobsen (11q-) cell line C262, with a telomeric probe. (A) DAPI stained metaphase spread showing normal chromosome 11 (blue arrow) and Jacobsen (11q-) chromosome (red arrow). (B) The same metaphase spread as in (A) but with the hybridisation signal of the telomeric probe (yellow fluorescence).

Fluorescent labels of the hybridised probes superimposed. Both cC0617 (red) and cA0734 (green) hybridise to the normal chromosome 11 at band q23.3 (blue arrow) as had been predicted by the mapping information described above. Following hybridisation with cC0617 (red), a clear signal is seen on the FRA11B chromosome centromeric to the fragile site (red arrow), whereas hybridisation with cA0734 (green) produces a clear signal telomeric to the fragile site (green arrow). The result using cA0734 is confirmed by FISH with the overlapping clone, cE0182, and the results of FISH with this and cC0617 are shown in Table 1. This localises the fragile site FRA11B to the 100 kb region between these two cosmids (Fig. 2), which includes the CCG-trinucleotide repeat at the 5' end of the CBL2 gene and confirms the preliminary FISH experiments with YAC probes. Since expansion of similar repeats has been shown to be the cause of fragility at other chromosomal loci, the CBL2 repeat would now appear to be a strong candidate for the location of FRA11B. FISH experiments with the cosmid cA0353, which lies...
within this interval and contains the CCG-trinucleotide repeat, were unsuccessful (data not shown). Preliminary mapping information suggests that this cosmid consists mainly of high copy repeat sequences that may be difficult to suppress (data not shown).

The breakpoint of an 11q- chromosome maps to the FRA11B region

Voullaire et al. (19) reported a case in which a child with apparent Jacobsen syndrome and a deletion del(11)(q23) was born to a mother who expressed FRA11B. To investigate whether the breakpoint on the 11q- chromosome of this child coincides with the position of the fragile site FRA11B, we performed FISH upon metaphase spreads from a fibroblast cell line (C262) derived from this patient, with cosmids flanking FRA11B.

Digoxigenin-labelled cosmid cCO617 (red fluorescence) and biotin-labelled cosmid cA0734 (green fluorescence) were co-hybridised to metaphase spreads prepared from the C262 cell line and detected using standard procedures (see Materials and Methods). Figure 4A shows part of a DAPI-stained metaphase spread of C262 with the normal 11 (blue arrow) and the 11q- chromosome (red arrow) labelled. Figure 4B shows the same image, but with the fluorescent labels of the hybridised probes superimposed. Both cCO617 (red) and cA0734 (green) gave clear signals on the long arm of the normal chromosome 11 (blue arrow). In contrast only cCO617 (red), the more centromeric cosmid, gave a signal on the 11q- chromosome (red arrow). This demonstrates that the breakpoint of the 11q- chromosome is between these two cosmids and is therefore localised to the same 100 kb region containing the fragile site FRA11B.

The truncated q-arm of the 11q- chromosome contains a telomere

A previous report has analysed the breakpoint of a terminal truncation of the p-arm of chromosome 16 and demonstrated that a tandem repeat of the telomeric sequence (TTAGGG)n was added directly to the site of the break (29). The truncated chromosome was stably inherited, demonstrating that telomeric truncation of the p-arm of chromosome 16 and demonstrated that telomere.

The truncated q-arm of the 11q- chromosome contains a telomere

A digoxigenin-labelled telomere (TTAGGG)n probe (yellow fluorescence) was hybridised to metaphase spreads prepared from the C262 cell line and detected using standard procedures (see Materials and Methods). Figure 5A shows a DAPI-stained metaphase spread of C262 with the normal 11 (blue arrow) and the 11q- chromosome (red arrow) labelled. Figure 5B shows the same image, but with the fluorescent label of the hybridised probe superimposed. The telomere probe (yellow) gives clear signals at the ends of most chromosomes, including the long arm of both the normal 11 (blue arrow) and the 11q- chromosomes (red arrow; Fig. 5B). Of 15 metaphase spreads from C262 that were analysed, all demonstrated a specific signal with the telomere probe at the deleted long arm of chromosome 11. This demonstrates that the 11q- chromosome has been stabilised by the addition of a telomere or telomere-like sequences.

DISCUSSION

The significance of the co-localisation of FRA11B and an 11q- breakpoint to the same 100 kb region of band 11q23.3 is enhanced by examination of the family of the Jacobsen patient. The patient's mother is FRA11B positive and has apparently transmitted the FRA11B chromosome to her second child (19). The patient was apparently mosaic for del(11)(q23) with three out of 465 lymphocytes having a normal karyotype. It is therefore tempting to speculate that the affected child also inherited the FRA11B chromosome and that this suffered a deletion at the fragile site at an early stage of development. Haplotyping of this family would allow us to determine that the deleted chromosome was the FRA11B-expressing maternal homologue, but insufficient family members are currently available for this analysis to be performed.

Penny et al. (30) have located the breakpoints of 18 other Jacobsen patients and show that eight of these are clustered between D11S1341 and D11S924, which is consistent with the map position of the Jacobsen case studied here. Hence it seems likely that the localisation of a Jacobsen syndrome chromosome deletion breakpoint to the FRA11B region of 11q23.3 as described in this study may not be a unique event. Cytogenetic analysis of the parents of the Jacobsen cases described by Penny et al. (30) may reveal other individuals expressing FRA11B. In addition, molecular analysis following characterisation of the fragile site will allow the identification of individuals who are FRA11B carriers, but are otherwise cytogenetically normal. Carriers of FRA1A and FRA1B are found to have a premutation of the FMR1 p(CCG)n repeat that does not cause expression of the fragile site in that individual, which predisposes to further expansion in future generations (11,31).

Although many chromosomes expressing fragile sites are known to break under specific cell culture conditions (hence their name), there is currently no direct evidence that chromosome breakage occurs in vivo in other fragile site carriers. For example, chromosome deletion syndromes associated with the inheritance of fragile sites on the X chromosome have not been described. Such deletions may be lethal however, and it is likely that partial aneuploidy is only viable for certain regions of the genome. Therefore we might predict, based on our data, that a subset of fragile sites will have associated chromosomal deletion syndromes. In addition, a pathological role of fragile sites might also be invoked in cases of early pregnancy loss resulting from chromosomal aberrations that are, in contrast, not sustainable. Molecular cloning of FRA11B and Jacobsen syndrome breakpoints will allow us to further test the possible link between fragile sites and chromosome breakage. Should this link be demonstrated, it would be necessary to re-evaluate the role of fragile sites in the aetiology of chromosomal abnormalities.

MATERIALS AND METHODS

Probes

vCBL is a 400 bp EcoRI–PstI fragment from the vCBL cdNA (32). CBL-5′ is a 175 bp BamHI–Sall fragment from a pUC clone of part of the human CBL2 cdNA (pUC-hCBL) (27) and contains the first 171 bp of the CBL2 cdNA, which includes the CCG-trinucleotide repeat. CBL-RI is a 2.3 kb EcoRI fragment isolated from pUC-hCBL and contains CBL2 sequences from the internal EcoRI site at position 578 bp to the stop codon (27). PBGD is an 800 bp BamHI–EcoRI fragment from the porphobilinogen deaminase (PBGD) cdNA. P2 is a 700 bp XhoI–NcoI fragment subcloned from a cosmid, pP1, isolated using the PBGD probe (26). P7HR is a 800 bp HindIII–EcoRI fragment also subcloned from
cF1 (26). THY1 is a 1 kb BamHI—PstI fragment isolated from the THY1 cDNA (33). Probes specific for the left and right arms of pYAC4 were generated from vector DNA PCR using appropriate primers (34); for the right end, YACRP 5′-ATCATGCTTCGGTCCAGCG and YACRR3 5′-CTCGCAACTTTGGCGTCA, which generates a 265 bp fragment; for the left end, YACLR1 5′-GTTGTTGTCGATGATCAGG and YACLP 5′-ATGCTGATTTTATCAGTTAA, which generates a 330 bp fragment. All of the above probe fragments were separated by electrophoresis on low-melting point agarose gels and excited using the Wizard DNA clean-up system (Promega). All of the above probes were radio-labelled with [α-32P]dCTP (Amersham) using a previously described oligo-labelling protocol (35). The telomere probe is a multimer of the telomeric repeat sequence (TTAGGG)n. generated by PCR (36).

YAC isolation
YACs y15E5 and y33E1 were isolated from the CEPH MkI library by PCR screening of primary pools in Cambridge, and secondary screening by CEPH in Paris and final colony purification in Cambridge. y15E5 was isolated using a PBGD STS and y33E1 using a THY1 STS (37).

Cosmid isolation and purification
Nylon membranes containing DNA from the ICRF chromosome 11-specific Lawrist 4 cosmid library no. 107 (La/FS11, ICRF Reference library) (28) were screened by hybridisation with the single copy probes CBL-RI, vCBL, CBL-2′-S, P2, P7 and PBGD (see above for details). DNA from positive clones was prepared from bacterial grown in Trypticase broth supplemented with kanamycin at 25 μg/ml, using a standard alkaline lysis protocol (38) followed by a multiple precipitation procedure. Briefly, following precipitation of the bacterial lysate with isopropanol, excess protein was removed by precipitation with 2.5 M ammonium acetate (DNA precipitated with 2.5 vol ethanol) and RNA was removed by treatment with 0.2 mg/ml RNase (DNA precipitated with 7.5% PG/1.5 M NaCl). Following treatment with 1 mg/ml protease K, DNA was cleaned by extraction with phenol/chloroform and precipitated with ethanol.

Pulsed field gel electrophoresis and Southern analysis
High molecular weight YAC DNA was prepared in low-melting point-agarose plugs using a previously described method (39). Restriction digestions of YAC-plug DNA were performed with at least 100 U of enzyme in a 250 μl volume overnight. DNA was electrophoresed on 1% agarose gels using the CHEF DR-H apparatus (Bio-Rad) at 200 V in 0.5 XTBE for 20 h, with the switch time linearly ramped from 1 to 30 s. Size markers were a bacteriophage λ oligomeric ladder and bacteriophage X HindIII digest (New England Biolabs). DNA was blotted from pulsed field gels onto Zeta-probe GT nylon membranes (Bio-Rad) by alkaline capillary transfer using standard techniques (38). Pre-hybridisation (with 0.5 mg/ml sonicated salmon sperm DNA) and hybridisation with specific probe were performed in a standard dextran sulphate; 0.5 mM Tris-HCl pH 7.6; 0.1 mM EDTA; 0.1 μg/μl sonicated salmon sperm DNA). Metaphase spreads were denatured in 70% formamide/2×SSC at 70°C for 2 min and preannealed probe was hybridised in a moist chamber at 42°C overnight. Detection of probes hybridised to the C622 (11q-) cell line was achieved using either FITC-conjugated avidin (Vector Laboratories) for biotinylated probes; or Texas red-conjugated anti-digoxigenin (Sigma) for digoxigenin labelled probes. Detection of probes hybridised to the JE (FRA11B) cell line was achieved using either Texas red-conjugated avidin (Sigma) for biotinylated probes; or anti-digoxigenin/anti-mouse FITC antibodies (Sigma) for digoxigenin labelled probes. Slides were mounted in antiadhesive solution (AFL; Citifluor) containing DAPI (4,6-diamino-2-phenylindole; 10 μg/ml). Staining and hybridisation signals were analysed using a computer-controlled Nikon epifluorescence microscope equipped with a cooled charge-coupled device (CCD) camera. Computer images were merged and Q-banding (generated by DAPI staining) converted to G-banding, using IP Lab Spectrum software (Digital Scientific).

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