70% N_2O plus 30% O_2 and 0.2 to 2% halothane. Multiunit activity was recorded with arrays of 4 to 6 Pt-Ir electrodes with a spacing of 0.4 to 1 mm (*3*) that were placed in area 17 of either hemisphere close to the border of areas 17 and 18 [R. J. Tusa, L. A. Palmer, A. C. Rosenquist, *J. Comp. Neurol.* **177**, 213 (1978)]. In one of the experiments, in which 98 pairs of multiunit responses were recorded, the location of the electrodes in the vicinity of the border of areas 17 and 18 was verified histologically. In all recordings, the cells had receptive fields located within 4° of the vertical meridian and velocity preferences typical of area 17 neurons. For further details of recording and data processing see (*2, 3*).
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8. At 9 months of age, two cats were anesthetized with a mixture of ketamine and xylazine. After craniotomy, the corpus callosum was cut, by aspiration, as far forward as stereotaxic level A15 to eliminate all visual connections (*6*). The cats were tested several months after surgery (*5*). The use of arrays of multiple electrodes for recording from either hemisphere enabled us to monitor, as an internal control, the quality of the interactions within left and right area 17. Thus, we recorded 42 intraareal cell pairs, 33 of which displayed synchronized oscillatory responses (Fig. 3). Neuronal responsiveness was normal in the border zone despite the callosal lesion (7). The receptive fields of all cells recorded were located within 4° of the vertical meridian. In 46 of 82 interhemispheric response pairs, the receptive fields were overlapping, and in 40 pairs the orientation preferences at the two recording sites differed only by 0 to 22°. Electrode penetrations were marked by electrolytic lesions and verified in Nissl-stained sections. The callosal lesion was verified by macroscopic inspection of the perfused brain and by myelin-staining of frontal sections through the corpus callosum by the Heidenhain-Woelcke method. (M. Woelke, *J. Phychol. Neurol.* **51**, 199 (1942)].
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Fragile X Genotype Characterized by an Unstable Region of DNA

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DNA sequences have been located at the fragile X site by in situ hybridization and by the mapping of breakpoints in two somatic cell hybrids that were constructed to break at the fragile site. These hybrids were found to have breakpoints in a common 5-kilobase Eco RI restriction fragment. When this fragment was used as a probe on the chromosomal DNA of normal and fragile X genotype individuals, alterations in the mobility of the sequences detected by the probe were found only in fragile X genotype DNA. These sequences were of an increased size in all fragile X individuals and varied within families, indicating that the region was unstable. This probe provides a means with which to analyze fragile X pedigrees and is a diagnostic reagent for the fragile X genotype.

Fragile X syndrome is the most common form of familial mental retardation (*1*). It is associated with a rare, fragile site at Xq27.3 (FRAXA); this association allows for cytogenetic prenatal diagnosis and carrier detection, although incomplete penetrance of the fragile site renders these procedures inaccurate. The genetics of the syndrome are bizarre. Normal men and women can transmit the fragile X, although they do not manifest any symptom of the fragile X syndrome themselves and do not express the fragile site cytogenetically. Such transmitters or carriers can have intellectually handicapped children or grandchildren with the fragile X syndrome (*1*). The diagnosis of the fragile X genotype relies on polymorphic DNA markers that are closely linked (*2*). Neither the molecular basis for the syndrome nor the mechanism of expression of the fragile site is understood. To obtain a better understanding of the syndrome and the site, we have previously isolated a 275-kb fragment of human DNA in a yeast artificial chromosome (XTY26) that spanned the fragile site at Xq27.3 (*3*). This clone was constructed from the DNA of a fragile X–affected individual and therefore ought to contain the sequences necessary for expression of the fragile site.

To identify sequences that constitute the fragile site and to screen for DNA differences between normal and fragile X individuals in the vicinity of the fragile site, we used sequences from XTY26 as hybridization probes. We localized the fragile site by first establishing a contig of λ subclones between the two closest probes that flanked the fragile site. One of these probes (VK16, Fig. 1) was first used to isolate XTY26; VK16 has been localized proximal to the fragile site by in situ hybridization (*3*). We established the distal end of the contig by screening the λ library of XTY26 with an Alu polymerase chain reaction (PCR) product (*4*) referred to as Alu2 (Fig. 1). Subclone 91 was isolated with this probe, and it was subsequently demonstrated by in situ hybridization that the probe mapped distal to the fragile site.We used RNA probes from each end of 91 to chromosome walk away from this

Table 1. Number of individuals with each band type seen in Southern blots probed with pfxa3 (Pst I digests) in 136 fragile X individuals from 25 families and 130 unrelated controls. Males were classified as affected if they had expression of the fragile site in lymphocyte culture (*1*), mental retardation, and dysmorphic features of the fragile X syndrome (*1*). Males were classified as "transmitting" if they were phenotypically normal (no fragile site expression, no clinical features of the syndrome, and intellectually normal) and if they had either the appropriate position in the pedigree or if they had a high probability, on the basis of flanking DNA polymorphisms, of having the fragile X genotype (*9*). Female carriers were classified as affected or normal on the basis of intellectual status, regardless of fragile site expression.

Classification	Normal band	Single band of increased size	Two to four bands of increased size	Multiple bands (smear)
		Males		
Affected	0	18	10	5
Transmitting	0	11	1	0
Normal	65	0	0	0
		Females		
Normal carriers	82	71	5	6
Affected	9	4	3	2
Normal	65	0	0	0

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locus, and we established the direction of the walk by hybridization back to blots of various restriction enzyme digests of XTY26. Each of the λ subclones between 91 and VK16 was mapped relative to the fragile site by in situ hybridization (*3*).

Mapping delineated the sequences that appeared to span the fragile site to about 15 kb, although the extent and boundaries of this region could not be sharply defined. Each of the λ clones that spanned the fragile site was then used as a hybridization probe to several somatic cell hybrid DNAs. Two of these, Q1X and micro 21D, had been constructed from a fragile X parent cell line (Y75-1B-M1) in such a way as to break the X chromosome at the fragile site (*5*). These hybrids have breakpoints that mapped within the same 5-kb Eco RI–restriction fragment (Figs. 1 and 2). Cell line Y75-1B-M1 demonstrated an increase in size in the common breakpoint fragment from 5 to 5.9 kb, compared with CY3, which contained an Xqter region without the fragile site (*6*). We therefore tested the hypothesis that this variation might be associated with the fragile site.

Subclone 5, which contained the 5-kb Eco RI fragment (pfxa1), was used as a probe on DNA from both normal and unrelated fragile X syndrome–affected males (Fig. 3). No variation was observed among any normal individuals, whereas every fragile X male showed an altered mobility of this sequence from the normal size of 5.0 kb up to about 7.5 kb. We localized the origin of this variability further by using a series of restriction fragments from the 5-kb Eco RI fragment as probes. Fragments a, c, and d (Fig. 1G) showed no variation between Pst I digests of normal and affected individuals (*7*). We found that the 1.0-kb Pst I fragment b (pfxa2) hybridized to repeat sequences in the human genome, whereas the 520-bp fragment e (pfxa3), derived from fragment b hybridized strongly to a single Pst I fragment that also demonstrated variations in size in unrelated fragile X–affected individuals. Some fragile X individuals had from one to four recognizable bands, varying in size from about 1.5 to 3.5 kb and decreasing in intensity as the number of bands increased. Others had multiple bands that were manifested as a smear. In those males with only a smear, PCR amplification of the 520-bp band from their genomic DNA con-

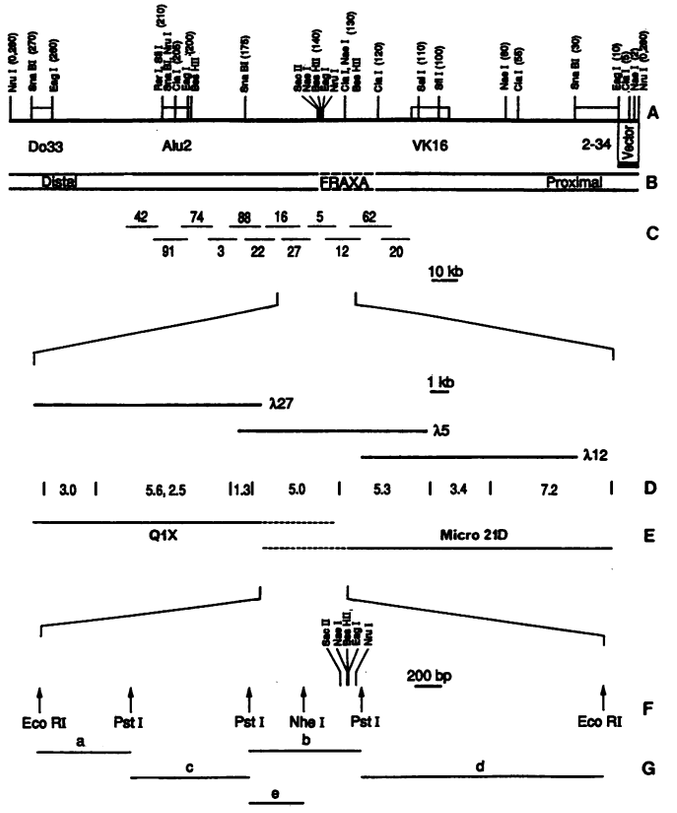

Fig. 1. Localization of the fragile X region. The diagram depicts the steps taken in localizing the sequences that compose the fragile site and the variable region. (**A**) The "rare-cutter" restriction endonuclease map of the yeast artificial chromosome XTY26 was determined by pulse-field gel electrophoresis (*3*). The locations of four DNA probes (VK16, 2-34, Do33, and Alu2) are indicated. Alu2 was generated by PCR with XTY26 DNA as a template and the Alu consensus sequence oligo (TC)65 (*4*) as a primer. Restriction site positions (in kilobases) are shown in parentheses. The localization of other probes has been reported (*3*). (**B** and **C**) A contig of subcloned DNA fragments of XTY26 was generated by construction of a partial Sau 3AI digest library in λ GEM-3 (Promega) by means of the manufacturer's protocols and packaging extracts. The library was first screened with total human DNA, then the plaque-purified array of 108 clones was probed with Alu2 and VK16. We generated RNA probes with the manufacturer's protocols and reagents from the positive clones and used the probes to walk toward and across the fragile X region. We established the direction of the walk by mapping these RNA probes back to the XTY26 restriction map. Each of the subclones was then used in a fluorescent in situ hybridization assay to localize the fragile site with respect to the contig. This localization and its approximate boundaries are shown by dashed lines. (**D** and **E**) Each of the clones that flank and span the fragile site region as defined by in situ hybridization were used as probes in Southern (DNA) blots of genomic DNAs. These results confirmed the Eco RI restriction map across this region. The location of the breakpoints in hybrids Q1X and micro 21D are indicated by dashed lines. (**F**) Restriction endonuclease map of the 5-kb Eco RI fragment pfxa1 that demonstrated instability in fragile X individuals. The CpG region is indicated by the cluster of "rare-cutter" restriction endonuclease recognition sites. (**G**) Restriction fragments used as hybridization probes to delineate the region of instability. Fragment b is designated pfxa2, and fragment e is designated pfxa3.

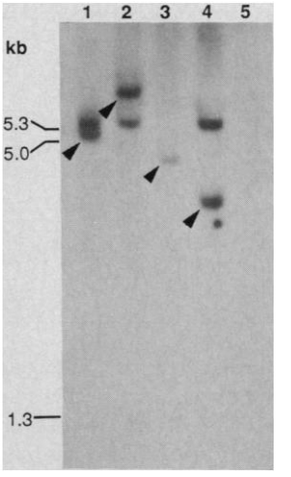

Fig. 2. Southern blot analysis of somatic hybrid cell DNAs with subclone λ5. Chromosomal DNA was isolated from the somatic hybrid cell line CY3 (*6*), which contains the Xq26-qter region intact from a normal X chromosome (lane 1); Y75-1B-M1 (lane 2); Q1X (lane 3); micro 21D (lane 4); and the mouse cell line A9, a parent line of CY3 (lane 5). The DNA was then subjected to cleavage with Eco RI. The blotted DNA was probed with random-primed λ5. The normal 5-kb Eco RI fragment (which contains the Q1X and micro 21D breakpoints and the Y75-1B-M1 instability) that demonstrated altered mobilities of Q1X, micro 21D, and Y75-1B-M1 is indicated by arrows. The 5.3- and 1.3-kb Eco RI fragments flank the unstable fragment and were present in the micro 21D and Q1X hybrids, respectively.

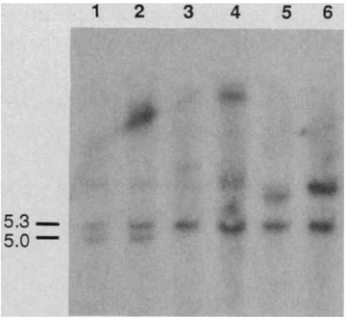

Fig. 3 Unrelated fragile X–affected males demonstrated instability of DNA sequences at the FRAXA site. DNA from a normal male (lane 1), a normal male from an affected pedigree (lane 2), and four unrelated fragile X syndrome–affected males (lanes 3 to 6) was digested with Eco RI and subjected to Southern blot analysis; subclone λ5 was used as a probe.

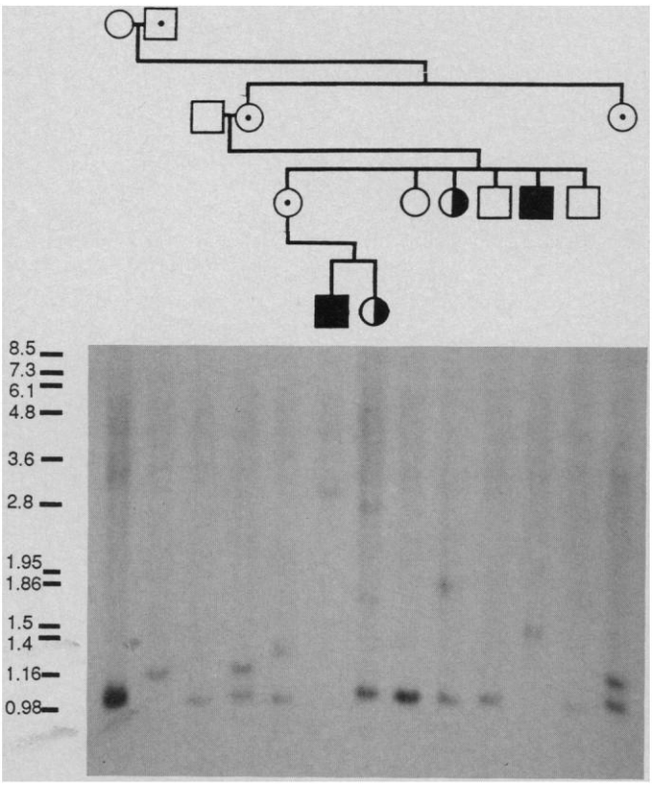

Fig. 4. Instability of FRAXA sequences in a fragile X syndrome pedigree. DNA from members of the fragile X syndrome pedigree in-dicated was digested with Pst I and subjected to Southern blot analysis (the corresponding lane is found under each symbol); pfxa3 was used as a probe. We performed the hybridization at 65°C, followed by washing in 0.1× SSC (0.015 M sodium chloride and 0.0015 M sodium citrate) at 70°C for 30 min because the T_m (denaturation temperature of 50% of this probe) was 77°C. Pedigree symbols: normal carrier males (dot in a square) or normal carrier females (dot in a circle) do not express the fragile X; half shaded circle, normal female that does express the fragile X; shaded square, retarded fragile X syndrome male that expresses the fragile X; open circle, normal female; and open square, normal male. Normal individuals in the third generation had a <2% chance of carrying the fragile X, as determined by flanking DNA polymorphisms (*9*).

firmed that this sequence was present, was always of the same size in fragile X and normal genotypes, and had not been deleted from any fragile X genomes (7). The number of fragile X genotype and normal DNA samples analyzed and the patterns of hybridization seen in the samples are summarized in Table 1.

The nature of this variable sequence was further investigated in fragile X syndrome pedigrees; a sample pedigree is shown in Fig. 4. This analysis demonstrated segregation of the variable sequence with the fragile X genotype, with altered mobilities observed in nonpenetrant transmitting males and in carrier females as well as in affected males. The alteration in mobility varied within families: a single band was observed and usually increased in size from generation to generation when the genotype was transmitted by females, but not when it was transmitted by males. The size of the band was usually larger in affected individuals than in normal carriers; however, there was no obvious relation between band size and phenotype. The increase in band size suggests amplification or insertion. The lack of a single hybridizing band in some fragile X genotypes may reflect somatic heterogeneity because the probe sequence is known to be present. These properties suggest that the sequences inserted into or amplified from the 1-kb Pst I fragment are unstable in fragile X individuals. The molecular basis for the instability is not clear because of difficulties in performing the sequence analysis; however, the observation of repeat sequences (7) in the unstable region suggests that the instability of the fragile X might be due to variations in the length or number of these repeats. The restriction map of XTY26 that was derived from a fragile X individual did not appear to differ from the restriction map of normal human DNA in the region of instability. This may be due to an undetected small difference in the size of the 1.0-kb Pst I fragment or to a deletion of the amplified region during cloning.

The 1-kb Pst fragment is GC-rich and is refractory to PCR analysis (7). A high GC content is reflected in the existence of a region that contains recognition sites for several CpG-dependent restriction enzymes. Three of these recognition sites are subject to variations in methylation status that segregate with the fragile X syndrome phenotype, but not the genotype (*8*). The observation of unstable sequences at the fragile site locus that segregate with genotype, regardless of fragile site expression or phenotype, suggests that the degree of size increase in these sequences might modulate fragile X expression and the associated syndrome. The immediate proximity of the unstable sequences to a CpG island suggests interference with either the expression of a gene or the function of its product as a molecular basis for the disease's phenotype.

The isolated 520-bp segment (pfxa3) of the 1-kb Pst I fragment is a diagnostic reagent for detection of the fragile X genotype. This segment can detect all fragile X males by the altered mobility of a 1-kb Pst I band or by the band's apparent absence; it will, however, reliably detect only those fragile X females in which there is a band or bands of altered size, because for those females where the abnormal band is a smear, the pattern appears to be similar to that of normal females. The primary diagnosis of fragile X could move soon from the cytogeneticist to the molecular geneticist. Further experience with the probe in fragile X families could conceivably lead to a means of fragile X phenotype prediction, as well as genotype identification.

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