20

Fragile Sites, Trinucleotide Repeat Expansion, and the Fragile X Syndrome

Fragile sites are chromosomal regions that show breaks when cells are exposed to certain drugs or grown in media with a deficiency of folate. The more than 80 *common fragile sites* can be induced in anyone, while *rare fragile sites* are seen in only a small proportion of individuals and are inherited in a Mendelian fashion. Fragile sites appear as unstained or stretched regions in the chromosomes (Figs. 20.1 and 22.2). Aphidicolin, an inhibitor of DNA polymerase, induces common fragile sites in a small percentage of cells, while camptothecin, an inhibitor of topoisomerase I, enhances the percentage of cells showing them. Lack of folic acid or thymidine in the culture medium induces folate-sensitive rare fragile sites, such as FRAXA and FRAXE on the X chromosome, while BrdU induces other rare fragile sites, such as FRA10B and FRA16B on chromosomes 10 and 16, respectively. Distamycin A, a peptide that binds in the minor groove of AT-rich DNA, induces some fragile sites, including FRA16B. Most rare fragile

O. J. Miller et al., *Human Chromosomes*

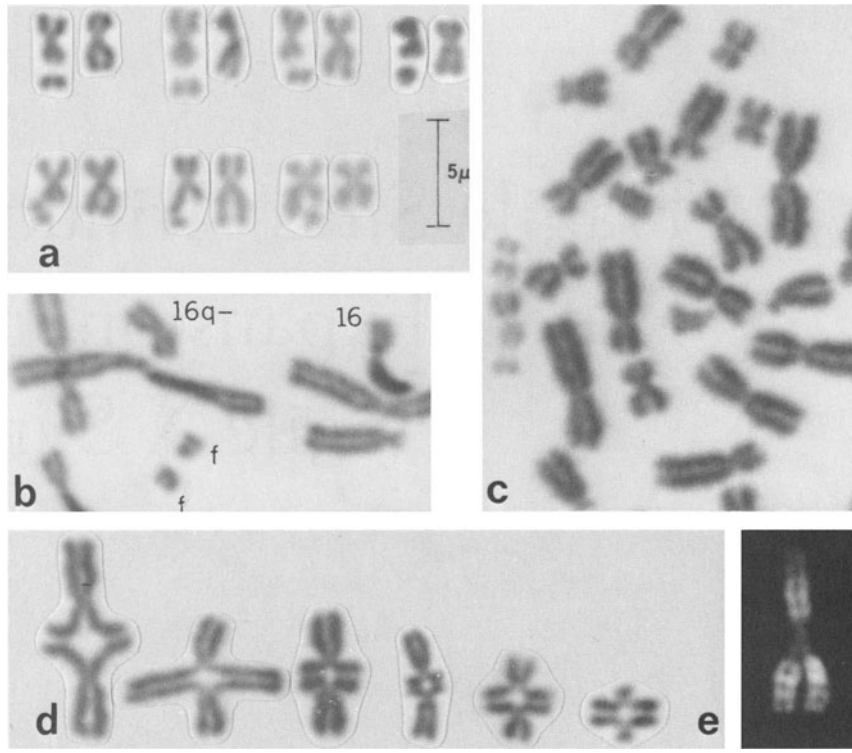

Figure 20.1. (a) Chromosome 16 with a fragile site, and its normal homologue, from several cells. (b) Break at the fragile site and the replicated fragment. (c) Allocyclic C-group chromosome with banding. (d) Mitotic chiasmata of different chromosome pairs. (e) Triradial chromosome 1 resulting from partial endoreduplication.

sites, even in the homozygous condition, have no phenotypic effects. The exceptions are FRAXA, the fragile site at Xq27.3, which is responsible for the fragile X mental retardation syndrome, and the nearby FRAXE at Xq28, which is sometimes associated with mild mental retardation.

Common Fragile Sites: Methods of Induction

The common fragile sites are induced by drugs that inhibit DNA replication or drugs that attack DNAase I hypersensitive sites, which are associated

with transcriptionally active genes. The common fragile sites are important because they are hotspots for chromosome breakage and perhaps for integration of foreign DNA. Chromosomes have a tendency to break at fragile sites, and this can lead to the formation of deletions and translocations (Glover and Stein, 1988). For example, breakage at the fragile site at 11q23.3, FRA11B, can lead to del(11)(q23.3–qter) and the Jacobsen 11q– deletion syndrome (Jones et al., 1995). FRA8E at 8q24.11 is near deletion breakpoints in the Langer-Giedion syndrome (Hill et al., 1997). The common fragile sites appear to correspond to chromosomal breakpoints associated with cancer (Yunis, 1984). Sister chromatid exchanges may be increased at these sites (Glover and Stein, 1987), which may act as branchpoints in triradial chromosomes (Kuhn and Therman, 1982).

The most common fragile site is FRA3B, in 3p14.2. Breaks at this site occur at the highest frequency when DNA replication is blocked by folate deficiency or by aphidicolin, which inhibits two DNA polymerases. The site contains no trinucleotide or other repeat motifs, but FISH analysis shows it is late replicating (LeBeau et al., 1998). Molecular analysis of the replication timing of each allele of 21 marker loci in 3p14.2 has confirmed this and shown that the allele on the homologue that shows more breaks in 3p14.2 is later replicating than the allele on the other homologue (Fig. 20.2). Exposure to aphidicolin delays replication of FRA3B still more, suggesting that the inducible fragility of this site is due to failure to replicate all the DNA in the region (LeBeau et al., 1998; Wang et al., 1999). FRA3B is often a site of spontaneous breakage, with loss of alleles distal to the breakpoint. This can be detected for loci that are heterozygous in an individual by looking for loss of heterozygosity (LOH) in selected cell populations. This is seen in over 60% of renal and other carcinomas and presumably contributes to the malignancy (Shridhar et al., 1997). Most of the very common rearrangements and mutations involving 3p14.2 are probably triggered by carcinogens in cigarette smoke that act on the FRA3B fragile site (Sozzi et al., 1997). FRA3B extends over a considerable distance in 3p14.2 and includes a spontaneous integration site for herpes virus type 16 (HPV16). This site is commonly deleted in uterine cervical cancers, which are usually HPV16-associated. It may be that HPV16 integration leads to specific breakage at this site, with loss of distal alleles and tumor formation (Wilke et al., 1997).

The insertion of telomeric TTAGGG repeats into an interstitial location can produce a novel fragile site (Chapter 14). The rare insertion of a different kind of tandem repeat, for example, an rRNA gene cluster (Fig. 20.3), can also lead

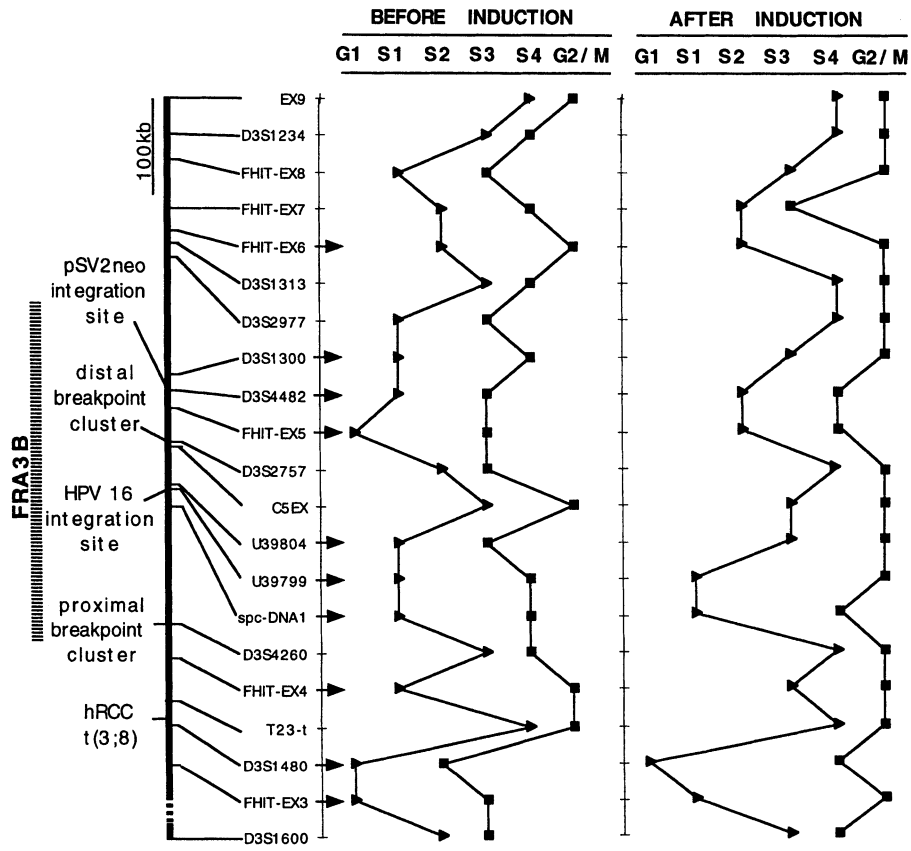

Figure 20.2. The time of replication of the two alleles at each of 21 marker loci encompassing the FRA3B fragile site and most of the *FHIT* gene (exons 3–9), before and after induction with aphidicolin. Arrows indicate loci with maximum allelic differences in replication timing. Breaks and gaps occurred preferentially on the chromosome with the later-replicating allele (reproduced from Wang et al., Hum Mol Genet 8:431–437, 1999, with permission of Oxford University Press).

to chromosome instability. An interesting feature of this is the formation of micronuclei containing these genes in individuals who have an insertion of an rRNA gene cluster into the middle of the long arm of chromosome 7 (Guttenbach et al., 1998).

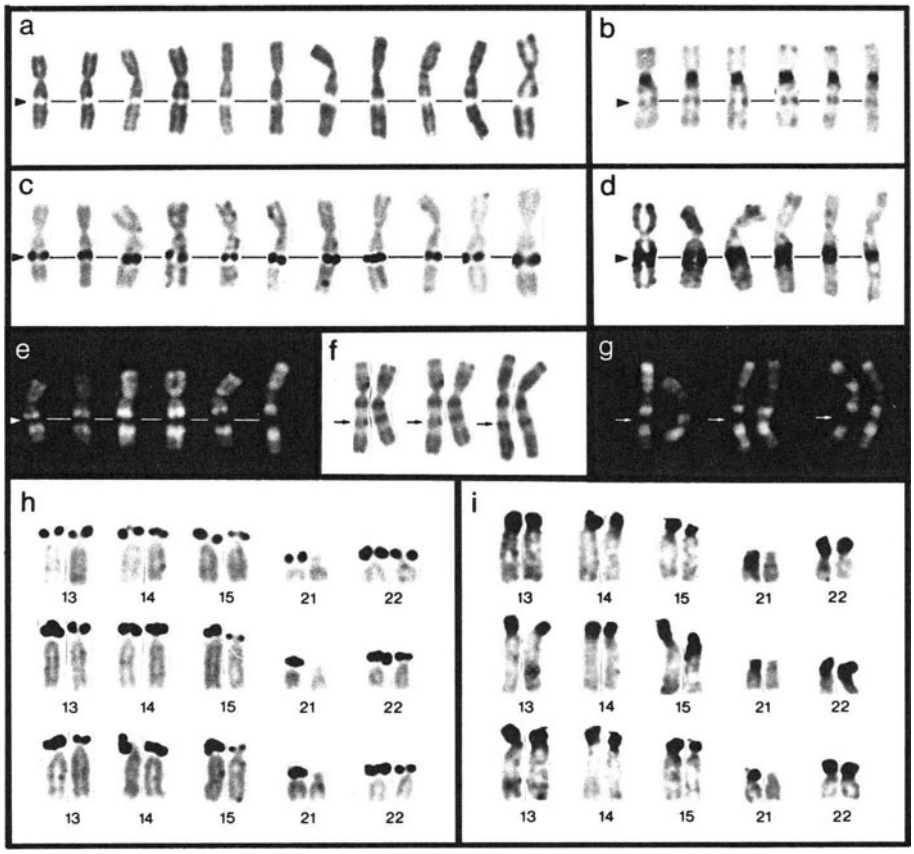

Figure 20.3. Insertion of an NOR into an interstitial site (indicated by arrowheads) on 7q. The altered chromosome after (a) Giemsa staining, (b) C-banding, (c) silver staining, (d) in situ hybridization with (GACA)n, and (e) Q-banding. Both chromosomes 7 from three cells after (f) R-banding and (g) Q-banding. The acrocentric chromosomes from three cells after (h) silver staining and (i) in situ hybridization with (GACA)n (Guttenbach et al., 1998, reproduced with permission of S. Karger AG, Basel).

Heritable (Rare) Fragile Sites

Rarely, more than one heritable fragile site is seen in a family (Romain et al., 1986) Many of the heritable fragile sites, including FRAXA and FRAXE, are made visible by inducing a folate deficiency. All these folate-sensitive sites are due to the pres-

ence of expanded repeats of the CCG trinucleotide (reviewed in Yu et al., 1997). Heritable fragile sites may undergo spontaneous breakage. One breakpoint in a ring X chromosome that arose in the sister of a male with the fragile X syndrome was close to the CCG repeat in the gene (Mornet et al., 1993). FRA16B is a rare fragile site at 16q22.1. It is an expansion, with up to 75 repeats, of a 33-bp AT-rich minisatellite repeat (Yu et al., 1997). This may explain its sensitivity to distamycin A, which binds preferentially to AT-rich DNA. It is also inducible by daunomycin A or bromodeoxyuridine (BrdU). FRA10B contains repeats of a 42-bp unit; it is not inducible by daunomycin A (Hewett et al., 1998).

FRAXA, the Fragile X Syndrome, and the *FMR1* Gene

The presence of a fragile site at Xq27.3 is associated with particular, though somewhat variable, clinical findings, called the *fragile X syndrome*. Minor features include slightly increased head size, prominent ears and jaw, hyperextensible joints, and cardiac mitral valve prolapse. A useful diagnostic feature is macroorchidism (large testes), present in most of the adult males. The most important features are neurological. Mental retardation is present in about 90% of the males, with IQs sometimes as low as the 20–60 range. The incidence of the fragile X syndrome is about 1 in 3000 males, based on molecular analysis of the size of the CCG repeat (Morton et al., 1997). Many heterozygous (carrier) females are also affected, even though the disorder is much less severe in females than in males (Nussbaum and Ledbetter, 1995). The frequency of the FRAXA fragile site in females, who have two X chromosomes, is twice that in males, who have only one, and about half of women with FRAXA are affected (Rousseau et al., 1994).

Individuals with the fragile X syndrome do not express the *FMR1* gene that is adjacent to the FRAXA site (Pieretti et al., 1991). Similarly, individuals with the FRAXE fragile site do not express the adjacent *FMR2* gene (Gecz et al., 1996). The *FMR1* gene in normal individuals is replicated late in S, according to some studies (Hansen et al., 1997), although FISH replication studies suggest much earlier replication (Torchia et al., 1994; Yashaya et al., 1998). Nevertheless, all agree that the mutant allele in patients with fragile X syndrome replicates much later, in what is normally G2. The zone of delayed replication in band Xq27 can extend more than 400 kb 5′ of *FMR1* and, depending on the extent of the CCG expansion, can involve one, two, or three replicon-sized domains (Hansen et al., 1997).

The Fragile X Phenotype Reflects the Number of CCG Repeats

Analysis of families segregating for the fragile X syndrome yielded striking results, called the *Sherman paradox*. The risk of an abnormal phenotype depends on the position in the pedigree; affected individuals receive the mutant allele from the mother, not the father; and the disorder tends to occur earlier in each generation and its severity to increase (*anticipation*). The reason for this became clear only after the discovery that males with the fragile X syndrome have a greatly increased number of repeats of the CCG trinucleotide (CGG in the other strand). Normally, individuals have 6–50 repeats, while affected males have 200–2000. Individuals with an intermediate number, roughly 50–200, are almost always phenotypically normal despite the increase in copy number (Fu et al., 1991). Meiotic expansion of the number of copies is rare in individuals with 6–50 copies but relatively common in individuals with 50–200 copies. The intermediate expansion has therefore been called a *premutation* and is a necessary step for the further expansion of the repeats that produces clinical disease. The CCG repeat array is usually interrupted after every 9 or 10 CCGs by an AGG, and this stabilizes the array. When the length of uninterrupted CCGs (CGGs in the other strand) reaches 34–38, the instability threshold is passed and further expansion occurs very readily (Eichler et al., 1994).

Mechanism of Expansion of Trinucleotide and Other Repeats

One mechanism of expansion of trinucleotide repeats may be strand slippage secondary to mispairing during replication. Other mechanisms have also been proposed (Sinden, 1999). All of there involve the formation of alternative DNA structures within the triplet repeats. The alternative structures found in CAG, CGG, or CTG triplet repeats inhibit processing of Okazaki fragments by flap endonuclease 1 (FEN-1), leading to site-specific expansions (Spiro et al., 1999). Strand slippage has been established for variations in size of other types of microsatellites. Microsatellites are tandem repeats of a DNA region only 1–6 bp long: mono-, di-, tri-, tetra- penta-, and hexanucleotide repeats. They are very abundant throughout the genome and highly variable in length. These features,

and their ease of scoring using polymerase chain reaction (PCR) and gel electrophoresis, have made microsatellites the most widely used genetic markers (Chapter 29). Strand slippage usually activates a mismatch repair system, and a deficiency in mismatch repair is associated with high rates of size variation in microsatellites. This size variation occurs only in the perfect repeat (no other sequences interspersed) part of the microsatellite, not in the region of imperfect repeats (Eichler et al., 1994). The same is true of the trinucleotide repeat class of microsatellites, as in the fragile X syndrome; alleles with imperfect repeats are more stable than alleles with perfect repeats.

Why should there be an instability threshold at 34–38 uninterrupted CCGs? One explanation is that the Okazaki fragments that are so central to replication of the lagging strand of DNA (Chapter 3) play an important role in the dynamic mutation of short repeats. Thus, Okazaki fragments that are anchored to a unique DNA sequence flanking short repeats permit, at most, minor expansions, whereas Okazaki fragments that fall entirely within large repeats can undergo major expansions. The threshold for dynamic mutation corresponds to the approximate length of an Okazaki fragment (Sutherland et al., 1998).

Expansion of CCG Premutation Only in Maternal Meiosis

A striking feature in fragile X families is that females with a premutation can have sons and daughters carrying the full mutation, or *expansion*, but males with a premutation never do. That is, the trinucleotide expansion appears to take place in the female germline, not in the male germline. A surprising finding is that four men with the full mutation of the *FMR1* gene in somatic tissues had a much lower premutational level of CCG expansion in sperm. This could mean that the germline has maintained only this lower number of repeats and that the full mutation arises postzygotically, as a developmental somatic mutation (Reyniers et al., 1993). If so, this must occur very early in embryogenesis, because somatic variability in CCG repeat length is established early and is maintained unchanged in clones established from 13- and 21-week-old fetuses (Wohrle et al., 1993). It seems more likely that the male germline is acting like any other specialized tissue in establishing its own level of expansion. This provides a simpler explanation of the pedigree data.

Mechanism of Inactivation of the *FMR1* Gene

The CCG trinucleotide repeat is close to a CpG island near the 5′ end of the *FMR1* gene. Like other CpG islands, this one is normally unmethylated despite the large number of potential sites of methylation. This is also true of premutations. However, the situation is different when the repeat is fully expanded: The CpG island becomes hypermethylated, and the *FMR1* gene is silenced. CCG trinucleotide repeat amplification and hypermethylation of an adjacent CpG island are also characteristic of the FRAXE mental retardation syndrome (Knight et al., 1993; Barnicoat et al., 1997).

The *FMR1* protein product, FMRP, associates with polyribosomes and fosters mRNA translation into proteins. In the absence of FMRP, or in the presence of an abnormal FMRP, translation is impaired (Feng et al., 1997). The importance of FMRP for understanding the fragile X syndrome may be that it is produced near synapses in response to neurotransmitter activation and may thus be important for the development of synaptic connections (Weiler et al., 1997).

Other Trinucleotide Expansion Disorders

A growing number of diseases have been associated with trinucleotide expansions (Table 20.1), and at least some of them also show anticipation. This has been most clearly established for myotonic dystrophy, which is associated with expansion of a CTG repeat. Here, the length variations from one generation to the next are most likely to occur in early embryonic mitotic divisions in both somatic and germline cells. As in the fragile X syndrome, there is a bias against very high expansion levels in sperm (Jansen et al., 1994). The CTG expansion occurs in the 3′ region of the protein kinase gene and alters the adjacent chromatin structure, with loss of the DNAase-hypersensitive sites so important for transcriptional activity (Otter and Tapscott, 1995). There is slightly preferential segregation of the larger allele into gametes in female meiosis (segregation distortion of 56.5 : 43.5 instead of 50 : 50) but none in male meiosis (Chakraborty et al., 1996). Segregation distortion is even more marked (73 : 23 in favor of the larger allele) in spinocerebellar ataxia 3 and Haw River syndromes, but in both

Table 20.1. Diseases Due to Expansions of Trinucleotide Repeats

Disease	Fragile site or gene	Repeat unit	Number of repeats		
			Normal	Premutation	Disease
Fragile X syndrome	*FRAXA/FMR1*	CGG	6–52	59–230	230–2000*
FRAXE syndrome	*FRAXE*	CCG	4–39	31–61?	200–900*
Jacobson syndrome	*FRA11B*	CGG	11	80	100–1000*
Kennedy syndrome	*SMBA*	CAG	14–32	?	40–55†
Myotonic dystrophy	*DM*	CTG	5–37	50–80	80–3000‡
Huntington disease	*HD*	CAG	10–34	36–39	40–121†
Spinocerebellar ataxia 1	*SCA1*	CAG	6–39	None	40–81†
Spinocerebellar ataxia 2	*SCA2*	CAG	14–31	None	34–59†
Spinocerebellar ataxia 3	*SCA3*	CAG	13–44	?	60–84†
Spinocerebellar ataxia 6	*SCA6*	CAG	4–18	?	21–28†
Spinocerebellar ataxia 7	*SCA7*	CAG	7–17	?	38–130†
Haw River syndrome	*HRS/DRPLA*	CAG	7–25	?	49–75†
Friedreich ataxia	*FRDA*	GAA	6–29	34–40?	200–900

*Expansion silences the gene by promoter methylation

†Expansion produces a polyglutamine expansion in protein product, precipitates in neurons

‡Expansion 3′ to coding region, alters gene expression or RNA processing

Source: Adapted from Sinden, 1999, copyright 1999, used by permission, the University of Chicago Press

these disorders it occurs in male meiosis and not in female meiosis (Ikeuchi et al., 1996).

Huntington disease (HD) is another trinucleotide expansion disorder. The HD gene, *IT15*, maps to 4p16.3 and contains an unstable CAG trinucleotide repeat expansion (Huntington's Disease Collaborative Research Group, 1993). Leeflang et al., (1999) determined the size of the (CAG)n repeat in more than 3500 individual sperm from males in the large Venezuelan HD pedigree, whose number of repeats varied (n = 37–72). The size of the repeat had changed in 82% of the sperm overall and in 98% of the sperm from men with n > 50. This high frequency of dynamic mutation strongly suggests that the change in repeat length occurs in the abundant mitotic divisions in the germline rather than in the final meiotic division. For more details about dynamic mutation and some of the resultant neurological diseases, see Wells and Warren (1997).

References

Barnicoat AJ, Wang Q, Turk J, et al. (1997) Clinical, cytogenetic, and molecular analysis of three families with FRAXE. J Med Genet 34:13–17

Chakraborty R, Stivers DN, Deka R, et al. (1996) Segregation distortion of the CTG repeats at the myotonic dystrophy locus. Am J Hum Genet 59:109–118

Eichler EE, Holden JJA, Popovich BW, et al. (1994) Length of uninterrupted CGG repeats determines instability in the FMR1 gene. Nat Genet 8:88–94

Feng Y, Absher D, Eberhart D, et al. (1997) FMRP associates with polyribosomes as an mRNP, and the 1304N mutation of severe fragile X syndrome abolishes the association. Mol Cell 1:109–118

Fu Y-H, Kuhl DPA, Pizzutti A, et al. (1991) Variation in the CCG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 67:1047–1058

Gecz J, Gedeon AK, Sutherland GR, et al. (1996) Identification of the gene *FMR2*, associated with FRAXE mental retardation. Nat Genet 13: 105–108

Glover TW, Stein CK (1987) Induction of sister chromatid exchanges at common fragile sites. Am J Hum Genet 41:882–890

Glover TW, Stein CK (1988) Chromosome breakage and recombination at fragile sites. Am J Hum Genet 43:265–273

Guttenbach M, Nassar N, Feichtinger W, et al. (1998) An interstitial nucleolus organizer region in the long arm of human chromosome 7: cytogenetic characterization and familial segregation. Cytogenet Cell Genet 80: 104–112

Hansen RS, Canfield TK, Fjeld AD, et al. (1997) A variable domain of delayed replication of FRAXA fragile X chromosomes: X-inactivation-like spread of late replication. Proc Natl Acad Sci USA 94:4587–4592

Hewett DR, Handt O, Hobson L, et al. (1998) *FRA10B* structure reveals common elements in repeat expansion and chromosome fragile site genesis. Mol Cell 1:773–781

Hill A, Harada Y, Takahashi E, et al. (1997) Assignment of fragile site 8E (FRA8E) to human chromosome band 8q24.11 adjacent to the hereditary multiple exostoses gene and two overlapping Langer-Giedion syndrome deletion endpoints. Cytogenet Cell Genet 78:56–57

Huntington's Disease Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable in Huntington's disease chromosomes. Cell 72:971–983

Ikeuchi T, Igarashi S, Takiyama Y, et al. (1996) Non-Mendelian transmission of dentato-pallidoluysian atrophy and Machado-Joseph disease. The maternal allele is preferentially transmitted in male meiosis. Am J Hum Genet 58:730–733

Jansen G, Willems P, Coerwinkel M, et al. (1994) Gonosomal mosaicism in myotonic dystrophy patients: involvement of mitotic events in (CTG)n repeat variation and selection against extreme expansion in sperm. Am J Hum Genet 54:575–585

Jones C, Penny L, Maltina T, et al. (1995) A fragile site within the proto-oncogene *CBL2* is associated with a chromosome deletion syndrome. Nature 376:145–149

Knight SJ, Flannery AV, Hirst MC, et al. (1993) Trinucleotide repeat amplification and hypermethylation of a CpG island in FRAXE mental retardation. Cell 74:127–134

Kuhn EM, Therman E (1982) Origin of symmetrical triradial chromosomes in human cells. Chromosoma 86:673–681

LeBeau MML, Rassool FV, Neilly MG, et al. (1998) Replication of a common fragile site, FRA3B, occurs late in the S phase and is delayed further upon induction: implications for the mechanism of fragile site induction. Hum Mol Genet 7:755–761

Leeflang EP, Tavare S, Marjoram P, et al. (1999) Analysis of germline mutation spectra at the Huntington's disease locus supports a mitotic mutation mechanism. Hum Mol Genet 8:173–183

Mornet E, Boggs A, Deluchat C, et al. (1993) Molecular analysis of a ring chromosome X in a family with fragile X syndrome. Hum Genet 92:373–378

Morton NE, Burdey S, Webb TP, et al. (1997) Fragile X syndrome is less common than previously estimated. J Med Genet 34:1–5

Nussbaum RL, Ledbetter DH (1995) The fragile X syndrome. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 795–810

Otter AD, Tapscott SJ (1995) Triplet repeat expansion in myotonic dystrophy alters the adjacent chromatin structure. Proc Natl Acad Sci USA 92: 5465–5469

Pieretti M, Zhang FP, Fu YH, et al. (1991) Absence of expression of the FMR-1 gene in fragile X syndrome. Cell 66:817–822

Reyniers E, Vits L, De Boulle K (1993) The full mutation in the FMR-1 gene of male fragile X patients is absent in their sperm. Nat Genet 4:143–146

Romain DR, Columbano-Green LM, Smythe RH, et al. (1986) Studies on three rare fragile sites: 2ql3, 12ql3, and 17pl2 segregating in one family. Hum Genet 73:164–170

Rousseau F, Heitz D, Tarleton J, et al. (1994) A multicenter study on genotype-phenotype correlations in the fragile X syndrome, using direct diagnosis with probe StB12.3: the first 2253 cases. Am J Hum Genet 55:225–237

Shridhar V, Wang L, Rosati R, et al. (1997) Frequent breakpoint in the region surrounding FRA3B in sporadic renal cell carcinoma. Oncogene 14: 1269–1277

Sinden RR (1999) Human genetics '99. Trinucleotide repeats: biological implications of the DNA structures associated with disease-causing triplet repeats. Am J Hum Genet 64:346–353

Sozzi G, Sard L, DeGregorio L, et al. (1997) Association between cigarette smoking and FHIT gene alterations in lung cancer. Cancer Res 57: 2121–2123

Spiro C, Pelletier R, Rolfsmeier ML, et al. (1999) Inhibition of FEN-1 processing by DNA secondary structure at trinucleotide repeats. Mol Cell 4: 1079–1085

Sutherland GR, Baker E, Richards RL (1998) Fragile sites still breaking. Trends Genet 14:501–505

Torchia BS, Call LM, Migeon BR (1994) DNA replication analysis of FMR1, XIST, and factor 8C loci by FISH shows nontranscribed X-linked genes replicate late. Am J Hum Genet 55:96–104

Wang L, Darling J, Zhang J-S, et al. (1999) Allele-specific late replication and fragility of the most active common fragile site, FRA3B. Hum Mol Genet 8:431–437

Weiler IJ, Irwin SA, Klintsova AY, et al. (1997) Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. Proc Natl Acad Sci USA 94:5395–5400

Wells RD, Warren ST (eds) (1997) Genetic instabilities and hereditary neurological diseases. Academic, San Diego

Wilke CM, Hall BK, Hoge A, et al. (1997) FRA3B extends over a broad range and contains a spontaneous HPV16 integration site: direct evidence for the coincidence of viral integration sites and fragile sites. Hum Mol Genet 5:187–195

Wohrle D, Hennig I, Vogel W, et al. (1993) Mitotic stability of fragile X mutations in differentiated cells indicates early post-conceptional trinucleotide repeat expansion. Nat Genet 4:140–142

Yashaya J, Shalgi R, Shohae M, et al. (1998) Replication timing of the various FMR1 alleles detected by FISH: inferences regarding their transcriptional status. Hum Genet 102:6–14

Yu S, Mangelsdorf M, Hewett D, et al. (1997) Human chromosomal fragile site FRA16B is an amplified AT-rich minisatellite repeat. Cell 88:367–374

Yunis JJ, Sorenq AL (1984) Constitutive fragile sites and cancer. Science 226: 1199–1204