The fragile X syndrome*

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INTRODUCTION

X-linked mental retardation (XLMR) accounts for an estimated 10-50% of all human mental retardation [1, 2]. It can be classified as either non-specific, characterized by a classical X-linked inheritance pattern with no other consistent clinical features, or syndromal. The discovery that X-chromosome fragility, first described by Lubs in 1969 [3], was closely associated with mental retardation in some males [4] was pivotal in the recognition of XLMR as an important disorder in man.

The fragile X syndrome (also called the Martin-Bell syndrome after the first report by Martin & Bell in 1943 [5]) is the most common single recognized form of inherited mental retardation. It is classified as an X-linked semi-dominant trait with reduced penetrance. It is associated with the appearance of a rare folate-dependent fragile site at Xq27.3, visible cytogenetically in metaphase chromosomes from lymphocytes of affected individuals which have been cultured under the appropriate conditions of folate deficiency. Unusually for an X-linked disorder, transmission may be observed through normal males (normal transmitting males, NTMs) [6-8]. Estimates of the frequency of the disorder in all human populations range from 0.3 to 1 per 1000 in males and from 0.2 to 0.6 per 1000 in females [9-11]. Fifty per cent of all XLMR may be attributable to the fragile X syndrome.

CLINICAL PHENOTYPE

The fragile X syndrome is characterized clinically by a triad of symptoms (reviewed in [12]). These consist of moderate mental retardation with an IQ typically in the range 35–50 [2], elongated facies (associated with oedema, tissue thickening and prognathism) with large everted ears, and macro-orchidism. However, this triad is seen in only approximately 60% of fragile X males. Ten per cent of affected males have normal facial features, up to 30% of affected males have no macro-orchidism and 10% present with mental retardation as the only clinical symptom. Moreover, the severity of the phenotype within kindreds may vary considerably. This variation in the degree of severity of the phenotype, which is more pronounced in heterozygote females, may mask the presence of the mutation at a much higher level in the population than has been previously estimated.

In addition to the symptoms described above, connective tissue disorders, possibly related to elastin fibre dysmorphism, have been noted. These include joint hyperextensibility and cardiac abnormalities. About 80% of adult patients show mitral valve prolapse.

Female heterozygotes for the fragile X syndrome are of two types (reviewed in [12]). The first type, daughters of NTMs, almost never show symptoms of the fragile X syndrome and do not express the fragile site cytogenetically. The second type, the grand-daughters of NTMs and sisters of affected males, show clinical manifestations in up to 35% of cases.

A considerable amount of work has been invested in the study of the psychological impairment in fragile X males and carrier females [13, 14]. In males, early symptoms are speech and language delay. Hyperactivity with short attention span, poor eye contact, a reluctance to be touched and confused speech are common. A high proportion, up to 30%, of fragile X males fulfil the required criteria for classification as autistic, an area of interest which is under continual discussion [15]. Approximately one-third of heterozygous female siblings of affected

*This review is dedicated to the memory of Isabelle Oberle, who over many years contributed so much to the research of the fragile X syndrome.

Key words: fragile X syndrome, genome mapping, microdissection, prenatal diagnosis, unstable DNA.

Abbreviations: bp, base pairs; HTF, hypopigmented tiny fragment; kb, kilobases; Mb, megabases; mRNA, messenger RNA; NTM, normal transmitting male; PCR, polymerase chain reaction; PFGE, pulsed field gel electrophoresis; SBMA, X-linked spinal and bulbar muscular atrophy; SCE, sister chromatid exchange; YAC, yeast artificial chromosome; XLMR, X-linked mental retardation.

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males are intellectually impaired. Approximately 30% of heterozygotes have been found to exhibit some signs of schizophrenic disorders, and 40% show aspects of affective disorder. Reiss et al. [16] have recently shown by nuclear magnetic resonance scanning that heterozygotes have hypoplasia of the posterior vermis, which is consistent with developmental and neuropsychiatric disorders.

GENETICS

Undoubtedly, transmission of the fragile X syndrome through apparently normal males was first reported by Camerino et al. [6] using polymorphic DNA markers to study the segregation of the X-chromosome in fragile X families. Following this, in a large collaborative study, Sherman et al. [7, 8] analysed a total of 206 families segregating for the fragile X syndrome. In addition to male transmission, it was apparent that there was an imbalance of penetrance of the phenotype in the different generations of kindreds segregating for the disorder. For example, there were fewer than expected affected male siblings of NTMs. The mothers and daughters of NTMs were almost never affected, in contrast to the sisters of affected males, who showed 35% penetrance. The overall penetrance in males was estimated at 79%. The likelihood of developing mental impairment is dependent upon an individual's position in the pedigree. As the mutation progresses through generations, the risk of mental impairment increases. These observations are not consistent with classical X-linkage, and are collectively known as the 'Sherman paradox' [17].

THE FRAGILE SITE AT Xq27.3

Fragile sites are detected microscopically as non-staining gaps in one or both chromatids in metaphase chromosomes after induction by suitable biochemical agents (reviewed in [18]). The fragile site at Xq27.3 (FRAXA) is induced under culture conditions of folate deficiency, excess thymidine or in the presence of several chemical additives which disrupt folate metabolism (reviewed in [19]). The appearance of the fragile X site is variable, presenting in several different forms. It usually appears as a gap in the chromatid or as a triradial figure owing to breakage and non-disjunction at mitosis. It can also be visualized as a terminal deletion of Xq28 owing to loss of theacentric fragment, which may be observed elsewhere in the metaphase chromosome spread.

The detection of a fragile site at Xq27.3 in mentally retarded male patients is regarded as diagnostic of the fragile X syndrome. However, there are possible cases in which the fragile X site expression and the fragile X syndrome phenotype are dissociated with a sibship [20]. A family segregating for the fragile site, but without mental retardation [21], has also been reported. Recent molecular advances have provided evidence for a non-fragile X Martin–Bell syndrome phenotype (see below).

Affected females generally express the fragile site, but unaffected obligate carrier females (not daughters of NTMs) express the fragile site in less than 50% of cases. The assessment of cytogenetic fragile X expression is not therefore a totally accurate means for detecting carrier females and is incapable of detecting carrier males.

MODELS FOR THE FRAGILE X SYNDROME

A number of hypotheses have been proposed to explain the unusual genetics and progression in penetrance of the fragile X syndrome. Most of these predict the existence of the mutation in two forms which differ in their penetrance. The first form, premutation, is non-phenotypic, but is capable of progression to the full mutation upon passage through a female oogenesis. The exact nature of this second step in the progression toward the full phenotype has been the subject of many hypotheses. It has been suggested that abnormal recombination [22] or the amplification of repetitive sequences by unequal cross-over events [23] is the mechanism. The theory of Laird [24] suggested that the premutation acts to block local reactivation of a previously inactivated X-chromosome, thus leaving the chromosome ‘imprinted’ and presumably with one or more genes in the region silenced. As we shall describe later, none of these hypotheses has been shown to be absolutely correct, although mutations found at the fragile X gene share common factors with these theories.

DNA STUDIES AND THE FRAGILE X SYNDROME

The human X-chromosome comprises approximately 150 000 kilobases (kb) of linear DNA. Although many regions of the chromosome have been extensively mapped by both physical and genetic means, the area around the fragile X site remained refractory for many years. Until recently, only a few DNA markers had been isolated from this region and this was held to be indicative of the unusual or unstable DNA elements present at the fragile X site itself. When new DNA markers became available, two approaches towards the construction of a linear map across the region containing the fragile X mutation were used. Linkage analysis, that is the study of polymorphic marker segregation with respect to the fragile X mutation, allowed markers to be mapped with respect to the disease gene itself. This was complemented by the analysis of these genetic markers and additional markers with respect to their physical localization on the chromosome relative to each other and to the site of cytogenetic fragility.
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Fig. 1. Physical maps across the fragile X locus. (a) The linear map across 5 Mb of the fragile X region was established using the markers as described in the text. Sites for the restriction enzymes SacII (S), BssHII (B), MluI (M) and NolI (N) are shown. Clusters of these sites represent HTF islands (see the text). The HTF island hypermethylated in fragile X affected individuals is marked with an asterisk. (b) The 600 kb region immediately flanking the fragile X site is enlarged to show the location of the closest DNA markers to each other relative to the hypermethylated HTF island. (c) The 5 kb region flanking the FMR1 HTF island, containing the unstable CGG trinucleotide repeat, is further enlarged. The enzyme sites and the location of DNA probes which are used in Southern blot molecular diagnosis of fragile X syndrome (described in [29, 52, 55-59]) are shown.

Isolation of new DNA markers

Several DNA sources enriched for the X-chromosome region carrying the fragile X site were used in the isolation of new DNA markers. These sources included somatic cell hybrid DNA or DNA isolated directly from Xq27 by chromosome microdissection. Markers which were developed across this region are shown in Fig. 1. The markers VK21, VK16 and VK23 were isolated on the basis of being human in origin from a hybrid cell line carrying a fragment of the human X-chromosome [25]. Another such hybrid was the DNA used as a source for a selective technique for isolating gene-associated sequences in Xq24–q28 [26] and this led to the isolation of the probe 2-34. Rousseau et al. [27] used inter-Alu polymerase chain reaction (PCR) [28] to generate probe Do33 from a reduced irradiation hybrid containing only the fragile X region. A highly polymorphic dinucleotide repeat, DXS548 [29], was isolated from the human yeast artificial chromosome (YAC) RS46, which was made from another hybrid cell line [30].

Microdissection, that is the direct physical extraction of DNA from a specific area of a metaphase chromosome, achieves a great enrichment of DNA sequences in a defined chromosomal area [31, 32]. Microdissection of the fragile X region [33] resulted in the saturation of the region with more than 40 new DNA markers [34]. Although none of these markers was used for genetic studies, the markers M759, M125 and M749 were used to develop the physical map of the area [35] and to aid the saturation cloning of the fragile site region [36].

Genetic linkage studies

The first report of linkage of a DNA marker to the fragile X syndrome was a study of the segregation of the factor IX gene (F9) [6]. This and other studies confirmed the localization of the mutation and the fragile site to the same region of the X chromosome. In the progress to isolate candidate gene sequences for the fragile X mutation, many new closely linked polymorphic markers were isolated (see Fig. 1). The first of these new markers, RN1 (DXS369) [37, 38] and U6.2 (DXS304) [39, 40], were found to flank the mutation, although still to be some distance away. Thereafter the markers VK21 and VK23 [41] increased the resolution of this map, with VK21 becoming the closest marker to the disease locus. The marker 2-34 was found to map between these markers, but its localization with respect to the fragile X mutation was dependent upon the assumed carrier status of several females with very low levels of fragile X site expression [27]. It was clear, however, that this marker was very close to the disease gene. These data and more detailed multipoint mapping allowed the localization of the fragile X mutation to be established very precisely [42]. These studies also demonstrated that no increase in recombination was present in the fragile X region [43], as had been suggested previously [44].
The map across the fragile X region

Concurrent with the development of the genetic map, many markers were mapped physically using the techniques of somatic cell hybrid localization and pulsed field gel electrophoresis (PFGE). Using hybrids, studies consistently ordered markers across the region, and placed VK23, M759, 2-34, M749, Do33, R546, VK16 and VK21 all in close proximity to the fragile site and the mutation [27, 29, 34, 45].

Also in this region were a series of somatic cell hybrids, studies consistently ordered markers across the region, and placed VK23, M759, 2-34, M749, Do33, R546, VK16 and VK21 all in close proximity to the fragile site [46, 47]. Such data further pinpointed the region for a further analysis by both PFGE and more extensive cloning in YACs [48].

The analysis of genomic DNA by PFGE relies upon the relative infrequency of digestable restriction enzyme sites due to the methylation of 98% of human genomic DNA. Areas in which non-methylated sites for these enzymes cluster (HTF or CpG islands) are frequently associated with expressed genes [49]. As these sites are relatively infrequent in the human genome, the fragments generated tend to be very large, usually in the range 100–1000 kb. Fragments of this size can only be resolved with the use of PFGE. Such an analysis was carried out in the fragile X region and revealed two important findings [35]. First, the 6 Mb map physically linked markers genetically proximal and distal to the fragile X mutation, thereby localizing the mutation to a defined region of DNA of known physical size. Secondly, four HTF islands were identified within this region, two of which were candidates for genes mutated in the fragile X syndrome [35, 50, 51].

Thus the combined approaches of genetic and physical mapping led to the creation of a map across the fragile X region (see Fig. 1). A further analysis of this map was to lead to the identification of the first DNA changes in fragile X individuals, an observation which led to the isolation of the fragile X site itself and to the eventual isolation of the fragile X gene.

IDENTIFICATION OF DNA ALTERATIONS IN FRAGILE X INDIVIDUALS

Hypermethylation of an HTF island on the fragile X-chromosome

The process of X-inactivation involves the silencing of gene expression from one of the X-chromosomes in females to allow for equal gene dosage between male and females. This silencing is reflected in the methylation of most HTF islands on the inactive X-chromosome. On the single male X-chromosome these HTF islands are usually unmethylated. An analysis of the HTF islands in the candidate region for the fragile X mutation, that is between VK23 and VK21, led to the discovery that one of these islands was abnormally methylated on the fragile X-chromosome [35, 50]. This abnormal methylation is only found in individuals carrying the full mutation. The implication of this observation is that the gene which is normally expressed in this region is transcriptionally silenced. This was the first DNA alteration found in fragile X individuals and raised much interest, particularly as genomic imprinting by methylation had been proposed as the mutation mechanism several years previously [24].

Amplification of DNA at the FMR-1 HTF island

Isolation of the DNA covering the hypermethylated HTF island was aided by the use of YAC clones corresponding to markers in the region. The markers M759, Do33, 2-34, DXS548 and VK16 were all within a 500 kb area around the hypermethylated HTF island and were used to isolate YAC clones covering this critical region [29, 36, 52–54]. In situ hybridization with these YAC clones to induced fragile X-chromosomes showed that the site of chromosomal fragility was confined to this region [36, 53]. Further in situ hybridization with DNA markers immediately flanking the HTF island further localized the site of chromosome fragility to this region.

Analysis of the region immediately adjacent to the hypermethylated HTF island in fragile X individuals revealed that, in addition to abnormal methylation, an unusual amplification of DNA was present on the fragile X-chromosome [29, 55–58]. The molecular basis of this amplification appears to be an expansion of a CGG trinucleotide repeat. A gene which is transcribed from the HTF island was isolated shortly afterwards and was termed FMR-1 (for fragile X mental retardation) [29]. Interestingly, the CGG repeat appears in the messenger RNA (mRNA) for this gene, suggesting the possibility that it encodes a polyarginine stretch within the N-terminal portion of the FMR-1 protein. However, it has not been shown that this polyarginine peptide region is present in the FMR-1 protein. Furthermore, most of the breakpoints in the hybrids broken at the fragile site have recently been shown to occur in close proximity to each other immediately adjacent to the FMR-1 HTF island [29]. Thus most of the molecular evidence suggests that the region is both the primary source of the fragile X mutation and the region of chromosomal fragility.

Genotypic profiles of the fragile X-chromosome

The number of CGG repeats on the normal X-chromosome is polymorphic between individuals with the number varying up to a maximum of 50 copies (see Fig. 2). On normal X-chromosomes these are stably inherited in families [59, 60]. On the fragile X-chromosome, the CGG array can exist in
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**Fig. 2. Variation of the fragile X trinucleotide array in the normal population.** DNA samples from 17 non-fragile X males was analysed by PstI digestion and probed with the DNA probe OxO.55 [56]. As can be seen from Fig. 1, this fragment is approximately 1 kb in size, but varies in length owing to alterations in the repeat array length. The position of a control size marker at 1.02 kb is shown alongside.

two states, dependent upon the copy number, which mirror the pre- and full mutation. By analysing the size of a DNA fragment extending across the FMR-1 HTF island, each of these genotype states has a characteristic profile when analysed on a Southern blot. This type of analysis is illustrated in a fragile X family shown in Fig. 3. Two normal transmitting grandfathers (1 and 6) have amplifications (represented as $\Delta$) of 170 base pairs (bp) and 180 bp. Their mutations are passed on to their daughters (2 and 7), each of which has a doublet corresponding to the mutant and normal X-chromosomes. In the third generation, the grandpaternal alleles expand to full mutations. The mutant alleles in individuals 4, 5 and 9 have increased in size to over 1 kb. In the case of individual 8, the mutation is small in size (600 bp), but nevertheless results in a fully penetrant individual. Thus by assaying the DNA genotype at the FMR-1 locus we can predict the phenotype of an individual with a high degree of accuracy.

Individuals carrying a pre-mutation chromosome and exhibiting a non-penetrant phenotype (e.g. NTMs and their daughters) have a copy number of repeats which is higher than the 50 copies found in the normal population but which is below 150 copies. The FMR-1 HTF island in these individuals is non-methylated. Although the number of repeats is somatically stable within the individual, it is highly unstable upon transmission from generation to generation. Indeed, it is estimated to mutate with a frequency of 1 (that is in 100% of meioses) with 93% of the changes resulting in an increase in the size of the array [60]. With the increase in size of the array, the genotype progresses over a threshold level into that found in the full mutation range.

Affected individuals carry a repeat array usually greater than 150 copies, the FMR-1 HTF island is hypermethylated and the chromosome exhibits cytogenetic fragility. The mutant allele in these individuals frequently exhibits somatic variation, resulting in either multiple fragments or a heterogeneous smear of fragments on Southern blot analysis.

The analysis of fragile X heterozygote females is more complicated. The daughters of NTMs, who are unaffected and cytogenetically negative for fragile X expression, have a mutant allele within the non-penetrant pre-mutation size range. Several patterns for the mutant allele are found in other heterozygotes. Those females with defined fragments of increased size in the non-penetrant range are phenotypically normal [59, 61]. Females where the mutation is visualized as a smear of fragments fall into the classification of both unaffected and affected. The smear of fragments above the normal fragment is sometimes difficult to resolve as it appears extremely diffuse, but can be compacted by use of an alternative digest (BglII [56, 58, 59]). Although there are general trends in the relationship of carrier female phenotype to genotype, this area still remains to be clarified. There are several factors which may contribute to this lack of certainty. First, in many cases, the clinical assessment of phenotype in females is incomplete. Secondly, the expression of the carrier phenotype may depend upon the proportion of cells in which the mutant chromosome is the ‘active’ chromosome, and the distribution of these cells within the body. Hopefully more detailed psychometric assessment will be established in order to study in further detail the genotype-phenotype relationship.

Molecular diagnosis of fragile X syndrome

For diagnostic purposes, molecular changes at the FMR-1 HTF island can be used as a direct test for the prediction of clinical phenotype [62–64]. Assays of genotype can be carried out using a series of DNA digestions, the aim of which is to assess CGG amplification and methylation of the FMR-1 HTF island. The accuracy of tests based upon the direct amplification of the CGG array by PCR has been limited to pre-mutation genotypes as amplification of the full mutation is difficult [60]. Recently, however, a protocol has been established to analyse the full mutation by PCR [65].

In the first trimester, prenatal diagnosis using DNA from chorion villus samples can only be based upon analysing the CGG array amplification at the FMR-1 locus. This is because methylation patterns
Fig. 3. Typical Southern blot genotype profile in a three-generation fragile X family. (a) Pedigree of a fragile X family. The numbers for each individual correspond to the lanes on the Southern blot in (b) below. \( \Delta \) = size of DNA amplification present at the fragile X locus. (b) DNA from individuals was digested with HindIII, the resultant DNA fragments size separated by gel electrophoresis and the DNA transferred on to a membrane. Amplifications are visualized after hybridization with the DNA probe Ox1.9. Lane numbers correspond to the numbers of individuals in the pedigree in (a). The control sample (lane 10) contains the wild-type fragment size of 5.2kb (see Fig. 1). A control hybridization to the DNA probe V21 is shown below.

DNA alterations in a fragile X negative Martin-Bell male

More recently, DNA analysis of a cytogenetically fragile X negative male presenting with a typical Martin-Bell phenotype revealed a deletion of genetic material in the FMR-1 region [66]. Using DNA probes across the region it is estimated that less than 250kb of DNA in the FMR-1 region is deleted. The deletion removes the FMR-1 HTF island and the several exons of the FMR-1 gene, including the CGG trinucleotide repeat. This provides further evidence that the FMR-1 region is the only critical region for the development of the fragile X phenotype and fragile X expression. The identification of such individuals highlights the need for a systematic analysis of all fragile X negative mentally impaired individuals for changes in this region.

THE FRAGILE X MUTATION

Progression of the fragile X mutation and the Sherman paradox

In the pre-mutation range, the CGG repeat array is genetically highly unstable, with a tendency toward increasing in size from generation to generation. This progression toward an array of a fully penetrant mutation, provides a molecular explanation for the Sherman paradox described above (see Fig. 4). A detailed analysis by PCR across the CGG array demonstrated that in successive generations descendant from a pre-mutation, the array will be increased in size. Thus descendant generations will have a higher probability of progression toward a fully penetrant mutation [60].

Most of the studies of the fragile X region have been carried out using DNA prepared from peripheral blood lymphocytes. Analysis of tissues from a fragile X positive fetus showed that the molecular changes described above are also present in every tissue examined throughout the fetus [67]. Hypermethylation of the FMR-1 HTF island was also observed in all these tissues, although the patterns of genotype fragment length variation and methylation differed between different tissues.

on the X-chromosome are not consistently maintained in extra-embryonic material [63]. Both in amniocentesis (where the sampled cells derive from the fetal genito-urinary tract) and postnatal diagnoses, the combined analyses of amplification and methylation are readily performed. Although the amplification test is accurate, it is important to perform both tests. This is illustrated by the case of an affected male with the amplification genotype of a NTM who expressed the fragile X site at 30%. Analysis of his methylation status revealed the FMR-1 HTF island to be totally methylated, indicating an affected status [59].

Although this case illustrates the need to perform both assays, it also highlights the critical role that methylation plays in the development of the fragile X phenotype. The amplification in this case is in the pre-mutation range, a state which is usually non-phenotypic, but the FMR-1 gene is hypermethylated and therefore probably not expressed. This indicates that the causative factor in the development of the fragile X phenotype is not the amplification, but the loss of FMR-1 expression possibly due to methylation of the HTF island.
The role of brain and also to be expressed in peripheral blood tissues have shown it to be highly expressed in the lymphocytes. Expression of the gene in lymphocytes has been utilized in a study of transcription in lymphoblastoid cell lines established from fragile X individuals carrying the pre- and full mutation [68]. This study showed that the gene is transcribed in non-penetrant individuals, but is not expressed in amplification and/or FMR-1 HTF island methylation [29]. Thus the isolation [68].

We await to see how the expression patterns of the FMR-1 gene in cell and tissue functions of a novel gene, perhaps the first in a new family. Several affected individuals who do express the gene are mosaic for CGG amplification and/or FMR-1 HTF island methylation [68].

A comparison of the predicted protein composition and structure with other known proteins identifies only weak similarities [29]. Thus the isolation of the FMR-1 gene represents the identification of a novel gene, perhaps the first in a new family. We await to see how the expression patterns of FMR-1 and its role in cell and tissue functions relate both to mental impairment and to the dysmorphic features with which sufferers of the fragile X syndrome present.

Possible mechanisms of mutation

The molecular alterations at the fragile X locus have identified a novel genetic entity, that of hereditary unstable DNA [69]. Although the mechanisms underlying such instability are as yet unknown, it is possible to speculate about them in the light of studies on the progression of the mutation.

The basic mechanism of mutation appears to be the amplification or addition of genetic material consisting of a CGG repeat array immediately adjacent to the FMR-1 HTF island. Whilst the origin of this amplified DNA is unknown, analyses of amplified DNA in other regions suggest that it could arise as either an artefact of unscheduled DNA replication or by unequal pre-mitotic sister chromatid exchange (SCE) recombination [70].

On the basis of observations made regarding the behaviour of the mutation in families, it must function within several constraints. Within the pre-mutation range, amplifications can occur without the opportunity for meiotic X interchange, as demonstrated by the increase in pre-mutation size on passing from an NTM to his daughter [58]. In this situation, where only one X-chromosome is present, the most likely source of genetic exchange that between sister chromatids (SCE).

Amplification of the CGG array does not appear to involve a single recombination event. First, genetic studies with flanking polymorphic markers within 10 kb of the CGG array indicate that no meiotic recombination cross-over events are occurring within the array as the alteration in array size occurs [71]. Although one cannot rule out that double cross-over events are occurring, this would have to happen over a very small region of DNA (less than 10 kb) and in every case of amplification within a family. Secondly, the size of the final amplification products is inconsistent with a straightforward unequal cross-over event. These observations are similar in nature to those of the generation of new alleles at several minisatellite loci, amplifications in size which do not involve much flanking marker segregation [72]. Here, however, the amplifications are much smaller. One would expect an equal number of increased as decreased sized alleles, whereas most (95%) of the changes in allele length result in increased size fragments. Additionally, in several cases the final amplification product exceeds that which is possible by combining the mutant and normal allele lengths (which would occur at unequal meiotic cross-over), or by combining mutant chromosome lengths (as would occur in SCE) in completely unequal exchange reactions.

Whilst methylation clearly plays a role in the generation of the full fragile X clinical phenotype, does it play a role in the progression of the DNA mutation? The observation that alterations occur in the pre-mutation range with a mutation rate of 1 [60] suggest that X-inactivation (which occurs on 50% of chromosomes) is not responsible for the mutation generation. It cannot be ruled out, however, that some type of developmentally associated imprinting mechanism, acting via methylation, may be imposed upon the paternal (pre-mutation) X-chromosome.

Amplifications of simple sequences at other disease loci

Amplifications of trinucleotide repeats have also recently been identified at several other loci. In a
The triplets are arranged in the order coding/non-coding strands. Affected alleles are also subject to methylation of the FMR-1 HTF island.

<table>
<thead>
<tr>
<th>Disease gene</th>
<th>Trinucleotide repeat</th>
<th>Copy number</th>
<th>Position in gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragile X syndrome</td>
<td>CGG/CGG</td>
<td>5-50</td>
<td>5' end of FMR-1 mRNA</td>
</tr>
<tr>
<td>SBMA</td>
<td>CAG/CTG</td>
<td>17-26</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>(Kennedy's disease)</td>
<td></td>
<td>40-52</td>
<td>protein</td>
</tr>
<tr>
<td>Myotonic dystrophy</td>
<td>CTG/CAG</td>
<td>5-27</td>
<td>3' UTR of mRNa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50-1600</td>
<td>related to cyclic AMP-dependent protein kinase</td>
</tr>
<tr>
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<td>5, 11-15*d</td>
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</tbody>
</table>

*The triplets are arranged in the order coding/non-coding strands.

*Pre-mutation allele size; the precise boundary with the full mutation is unclear.

*Affected alleles are also subject to methylation of the FMR-1 HTF island.

*60% of individuals fall into one of these two repeat length groupings.

A CTG repeat, complementary to the CAG repeat found in SBMA, has recently been identified in the gene responsible for myotonic dystrophy. The repeat is found in the untranslated region of a gene encoding a protein kinase polypeptide which maps in the area of chromosome 19 known to contain the mutation by genetic linkage analysis. Between 5 and 27 copies of this repeat are found within the gene of normal individuals and amplifications of between 50 and 1600 copies have been identified in diseased individuals. The increase in size of the amplification found in SBMA-affected individuals is dramatic.

In contrast to the changes in the SBMA gene, the amplifications of simple trinucleotide elements in the fragile X and myotonic dystrophy genes is dramatic. This is particularly intriguing in the case of the myotonic dystrophy-associated repeat as it is the exact DNA complement to the SBMA triplet. One would expect, therefore, that these repeats would be subject to the same mutation mechanisms unless other constraints are operating, such as coding potential. Unlike the fragile X and myotonic dystrophy proteins, the SBMA CAG repeat is known to be translated as part of a protein. The limited amplification found in SBMA-affected individuals might arise because of restraints upon protein structure and function, the complete loss of which may be detrimental to the cell. As the fragile X CGG repeat is also capable of being modified by methylation, this still leaves open a role for methylation.

**SUMMARY**

An amplification of a highly unstable DNA element has been identified at the fragile X locus in Xq27.3. This sequence appears to be both the source of the primary mutation causing the fragile X syndrome, apparently having its causative effect through the methylation of the FMR-1 HTF island and the region of cytogenetic fragility. The direct analysis of the genotype of carrier and affected individuals can be used as a direct diagnosis tool which will improve both the accuracy and speed of diagnosis.

The identification of hereditary unstable DNA in a disease with such a wide level of non-penetration and variable phenotype may give clues as to the basis of non-penetration in other human genetic disorders.

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**REFERENCES**


A molecular analysis of the fragile X syndrome


72. Wolff, K., Plaetke, R., Jeffreys, A.J. & White, R. Unequal crossing over between homologous chromosomes is not the major mechanism involved in the generation of new alleles at VNTR loci. Genomics 1989; 5, 382-4.


