Triplet Repeat Mutations in Human Disease

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Triplet repeats are the sites of mutation in three human heritable disorders, spinal and bulbar muscular atrophy (SBMA), fragile X syndrome, and myotonic dystrophy (DM). These repeats are GC-rich and highly polymorphic in the normal population. Fragile X syndrome and DM are examples of diseases in which premutation alleles cause little or no disease in the individual, but give rise to significantly amplified repeats in affected progeny. This newly identified mechanism of mutation has, so far, been identified in two of the most common heritable disorders, fragile X syndrome and DM, and one rare disease, SBMA.

Reports of the genetic alterations in SBMA, also known as Kennedy disease (*1*), fragile X syndrome (*2–4*), and DM (*5–7*) over the past 7 months have identified a new class of human mutation, triplet repeat amplification. There is sufficient information at clinical and molecular levels regarding these diseases to provide investigators with simple approaches to searching for this mutational mechanism in other genes and diseases (*8*). This article will summarize the clinical and genetic features of the first three diseases in which simple sequence amplification mutations were identified, speculate on the mechanism of their amplification, discuss the affects on gene expression, and outline new medical genetic issues raised by the ability to identify premutation sequences.

Clinical and Genetic Features

Spinal and bulbar muscular atrophy (SBMA), or Kennedy disease, is an X-linked recessive genetic disorder characterized by progressive muscular weakness of upper and lower extremities that starts in adults and is secondary to neural degeneration (*9*). Affected males have reduced fertility and excessive development of the male mammary glands (gynecomastia); female carriers have few or no symptoms. The mapping of SBMA to Xq11–12, the region of the gene encoding the androgen receptor (*AR*), led to the study of *AR*'s highly polymorphic CAG repeat. This genetic analysis not only revealed highly significant linkage between *AR* and SBMA (a lod score of >13 at 0 cM) but identified the putative molecular defect within *AR* at the CAG repeat sequence (*1*). This same mutation has been observed in all SBMA patients. A highly polymorphic triplet repeat, CAG, located in exon 1 and encoding a polyglutamine stretch, is amplified to approximately twice normal size. Point mutations and deletions in *AR* were already well known as being responsible for androgen-resistant genetic defects in humans and mice (*10–12*).

Fragile X syndrome is an X-linked recessive disorder with incomplete penetrance; it is characterized by moderate to severe mental retardation, large head, long face, large ears, and large testicles (macroorchidism) (*13*). It is one of the most common forms of mental retardation, with an estimated incidence of 1 in 1250 males and corresponding 1 in 2500 females (heterozygotes) (*14, 15*). Some males, referred to as normal transmitting males (NTMs), are clinically normal but are inferred to carry the genetic defect by a position in pedigrees rendering them obligatory carriers. One-third of female carriers have evidence of mental impairment, but they rarely show severe retardation and have no other distinguishing clinical features (*16, 17*). The cytogenetic identification of a constriction in Xq was made in such a family in 1969 (*18*). Cytogenetic detection of fragile X reached clinical utility with the discovery that the fragile site at Xq27 could be induced by peripheral leukocyte cell culture in special media (*19*). The fragile X site is defined as a folate-sensitive site since its expression cytogenetically can be induced by media deficient in folate or containing the folate inhibitor methotrexate. Inhibitors of DNA replication, (such as fluorodeoxyuridine or high concentrates of thymidine) are also effective in fragile site induction. Cytogenetic diagnosis with peripheral leukocytes, particularly in carrier females, has lacked absolute accuracy (never >30%). Fragile site induction is even less reliable in amniocytes and chorionic villus cells used in prenatal diagnosis (*20*).

Genetic linkage studies localized the fragile X gene to Xq27 by conventional analysis of restriction fragment length polymorphisms in informative pedigrees (*21*). The physical position of the gene was further delineated by the development of somatic cell hybrids whose human fragile X chromosome was induced to break in the fragile X region (*22*). It was this genetic information, linkage probes, and cell hybrids that made possible the positional cloning of the fragile X region (*2*), and discovery of a highly polymorphic CGG repeat within a gene, *FMR-1* (*4*). This discovery permitted the correlation of the clinical genetic feature of anticipation (referred to as the Sherman paradox for fragile X syndrome) to the repeat amplification in the DNA. Anticipation is defined as the appearance of increasing disease severity or earlier onset in successive generations with a heritable disorder. This correlation will be discussed more fully in the following sections.

Myotonic dystrophy (DM) is an autosomal-dominant disease characterized by myotonia, cardiac arrhythmias, cataracts, male balding, male infertility (hypogonadism), and other associated endocrinopathies (*23*). The rare congenital form of DM is associated with profound newborn hypotonia and mental retardation (*24*). Such children are invariably the offspring of affected mothers, never affected fathers (*25*). These mothers frequently have mild to subclinical features of DM. It is this feature together with clinical documentation of progressively increasing disease severity through generations in adults that defined anticipation as a feature of DM. Through an international working group sponsored by the Muscular Dystrophy Association, the DM gene was mapped by genetic linkage to 19q13.2–13.3, and a consensus genetic and physical map developed (*26–30*). Evidence of an unstable sequence in the region associated with DM was provided in three of these reports (*28–30*). We used a sequence scanning method based on synthetic oligonucleotides containing all GC-rich triplets to identify a highly polymorphic GCT repeat as the DM mutation (*6*), while others used positional cloning strategies (*5, 7*). This triplet repeat, GCT, undergoes progressive expansion in DM families as will be discussed in the following sections. The gene containing the repeat has been designated myotonin protein kinase (*MT-PK*).

Molecular Features of Triplet Expansion

The localizations of triplet repeats within *AR*, *FMR-1*, and *MT-PK* transcripts are illustrated in Fig. 1. *AR* has a highly poly-

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morphic CAG repeat encoding polyglutamine and a second smaller GGN repeat encoding polyglycine. This second site also appears polymorphic but has been less well studied (*31*). Triplet repeats associated with diseases can occur at different positions in genes; the CGG repeat of *FMR-1* is thought to be 5′ to the initiation AUG and the GCT repeat of *MT-PK* lies in the 3′ untranslated portion of the mRNA (*6*). The DNA sequences encoding the *AR* polyglutamine tract and the 3′ repeat of *MT-PK* (GCT) are identical, although on opposing DNA strands with respect to gene transcription. Furthermore, the 5′ repeat of *FMR-1* (CGG) is similar to the polyglycine repeat tract (GGN) in *AR*. These correlations should not be interpreted as being the limits of sequence composition for unstable repeats. We have previously reported a wide spectrum of polymorphic triplet, tetramer, and pentamer repeats (*32, 33*).

The polymorphic variation of the CAG repeat in *AR* is illustrated for four ethnic groups in Fig. 2. There is unusual ethnic variability (for example, compare the peaks for blacks and Caucasians) and allele distribution (>95% of the people sampled are heterozygotes). The population genetics of these alleles was previously analyzed in detail (*33*). SBMA has been reported in multiple ethnic groups and, where measured, the size of the repeat ranged from 40 to 52 units. This is well beyond the normal range but within a doubling of allele sizes found in the normal population (*1*). There was no recorded incidence of a large expansion of the repeat as has been observed for fragile X and DM. Small changes in the repeat number have been observed, and indicate that the CAG disease-associated allele is unstable from generation to generation. Thus, the narrow repeat range for disease association indicates that there could have been a single ancestral derivative, rather than multiple independent mutations involving a doubling of different normal alleles (Fig. 3).

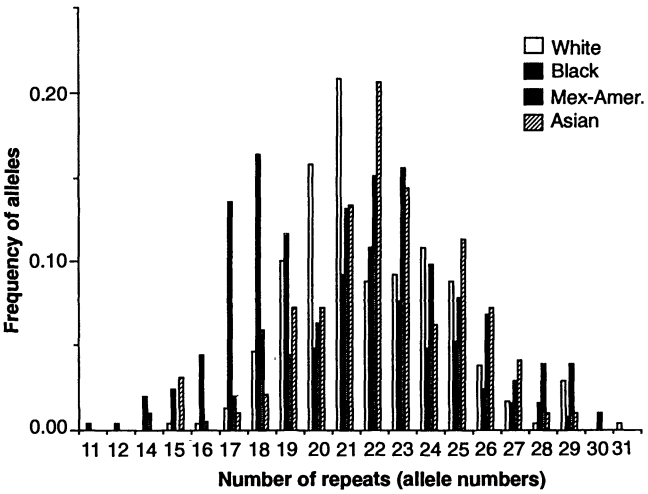

Fig. 2. Allele frequencies of four population groups for the AGC polymorphic repeat in *AR*. [Adapted from (*33*)]

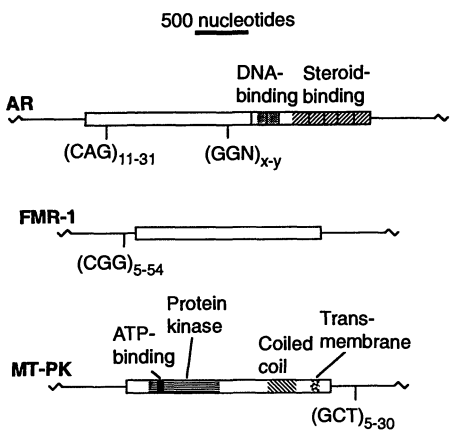

Fig. 1. Schematic of three mRNA molecules containing polymorphic triplet repeats. The range of allele numbers seen in normal individuals is shown. The extent of polymorphism of the GGN repeat in *AR* is not fully explored. Open bars indicate coding regions divided into exons by vertical lines where known; some functional domains are indicated. AR, androgen receptor; MT-PK, myotonin protein kinase; FMR-1, fragile X gene.

The distribution of CGG repeat polymorphisms in *FMR-1* has been measured by amplification through the polymerase chain reaction (PCR) followed by polyacrylamide gel analysis (*4*) or by agarose gel sizing of restriction fragments containing the CGG repeat and then DNA hybridization (Southern) analysis (*2, 34, 35*). In contrast to the situation with *AR*, we found no significant variation among ethnic groups at *FMR-1*; fragile X syndrome has been reported in a broad spectrum of ethnic groups. We have reported that the range of CGG repeats is 6 to 54 in the normal population with a peak at 29 (*4*). These measurements are in general agreement with those made by several groups.

It was the study of fragile X families that revealed two types of instability associated with the CGG repeat (Fig. 3). A selected pedigree (Fig. 4A) illustrates these types. Unstable alleles are observed in a normal transmitting male (NTM) and his asymptomatic daughter. Such alleles have been referred to as premutation alleles. The NTM's allele (82 repeats) is slightly altered in his daughters (83 and 90 repeats). Two of the females with premutation alleles (80 and 83 repeats) have male children with fragile X syndrome, whose repeat sequence expanded remarkably in one generation resulting in disease.

It was the study of numerous families of this type that permitted a correlation of the phenomenon of anticipation and the molecular events of CGG amplification. These studies indicate that NTMs carry numbers of CGG repeats outside the range of normal (from 52 to 200 repeats) and below those found in affected males (>200). Males transmit the repeat to their progeny with small changes in the repeat number. It is also well documented that somatic mosaicism occurs indicating mitotic instability of premutation alleles (*4*). Females who carry similar premutation alleles are prone to bear progeny (male or female) with large expansions of the repeat region (250 to 4000 repeats). Thus, large CGG amplification associated with fragile X syndrome appears to be a predominantly female meiotic event, with somatic instability found in many patients. Mentally retarded males with fragile X syndrome rarely have children, which therefore limits opportunities to study transmission of large repeats via male meiosis. There is the possibility of ascertainment bias in this circumstance since reproducing normal female heterozygotes are frequently observed to transmit large alleles while mentally retarded males rarely have offspring. There is good agreement among several investigative groups that CGG repeats above 50 have significant instability. Our studies indicate a rapidly increasing risk for disease in progeny over the repeat range between 60 (17%) and 90 (100%) repeats. Thus, fragile X syndrome is the first human disorder in which premutation DNA sequences in the parent predict substantial disease risk to progeny.

The distribution of normal GCT repeat alleles in *MT-PK* mRNA is compared to that of the CAG repeat alleles in *AR* in Fig. 5. While both are highly polymorphic, the distribution of the alleles differs at the two loci suggesting site, rather than triplet sequence specificity of polymorphic variation. PCR- and Southern-based methods of allele measurement have been used to observe generation-to-generation amplification of the GCT triplet repeat, which is in keeping with the clinical feature of anticipation for

DM. The reported experience is limited at this time, but patients with few or no symptoms of DM are reported to have repeats in the 50 to 100 range (Fig. 3). Such high-risk alleles can expand via both male and female meiosis.

A selected pedigree (Fig. 4B) illustrates some of these features. The grandmother of the congenital myotonic child is asymptomatic while her daughter has clinical features of DM. The instability of the GCT repeat is illustrated by the grandmother's DM allele of 75 repeats giving rise to the symptom-associated allele of 105 repeats in the daughter and the smaller, symptom-free allele of 72 repeats in her son. We detected no mosaicism in the grandmother or daughter in peripheral blood cells. The congenital myotonic and retarded grandson inherited a 25-fold enlarged GCT repeat from his mother. Although our studies indicate a larger average size of amplification for congenital myopathic children (7.3 kb) than their parents (3.6 kb), caution must be exercised at this time in relating size to a prediction of clinical severity. For example, we have observed a smaller allele in one congenital myopathic child than that of his affected mother. More extensive clinical and molecular comparisons will be needed before genotype should be used to predict disease severity.

When the increases in allele size via male meiosis and female meiosis were examined, substantial increases occurred in both. Thus, DM GCT triplet repeat amplification differs from fragile X, which is limited to female meiosis.

It has been possible to demonstrate in vitro via PCR amplification that segments of DNA containing CA repeats can participate in strand-switching during amplification (36). Repeats in the range of 50 to 100 are prone to small allele changes for all three loci. Some have referred to this type of small allele variation as polymerase slippage. Large segment amplification of genes such as multiple drug resistance (37), dihydrofolate reductase (38), and adenosine deaminase (39) in cell culture and in cells of patients with cancer has been extensively studied. This type of amplification results in amplifications of several thousand times from single copy sequences. Episomal amplification of these sequences is an intermediate event before the re-entry of amplified sequences into nonhomologous chromosome sites. It is doubtful if these gene amplification mechanisms relate to specific triplet amplification such as observed for fragile X and DM, since immediate flanking sequences are not amplified and amplification of the triplets occurs at the authentic gene locus.

It is difficult to envision recombination events which can, in a single generation, expand triplet repeats 10- to 40-fold. New explanations must be sought for simple sequence amplification. One model that has appeal relates to the difficulty of replication of GC-rich sequences. Inequality of rate of DNA synthesis may lead to multiple incomplete single strands of complimentary, triplet, re-initiated sequences. Strand switching during replication between multiple incomplete strands (leading and lagging) might then occur. This model predicts that longer alleles would be more prone to incomplete ends.

Circularization of single-stranded DNA and resultant self-copying is unlikely given the nucleotide sequence of the CGG and GCT repeats. Another noteworthy feature of DNA size and structure is that the DNA associated with each nucleosome is about 150 to 200 bp, which roughly coincides with the size of the unstable triplet repeat sequences. Is it possible that expansion of a GC-rich triplet repeat beyond ~50 interferes with the packaging and phasing of the nucleosome structure? In vitro and in vivo models should be capable of testing these mechanisms.

The premutation alleles could arise continually or might represent ancestral rare mutations which themselves have become polymorphic. The rarity of SBMA, linkage

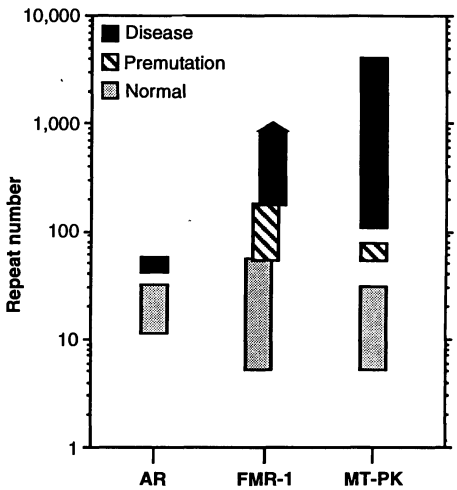

Fig. 3. Variation of polymorphic triplet repeat number in three heritable diseases. Bars corresponding to the *FMR-1* repeat are offset to demonstrate overlap among the three classes. The average alleles for *AR* and *MT-PK* are given in Figs. 2 and 5. The average alleles for *FMR-1* are repeated elsewhere (*35*). The numbers of triplet repeats in the *AR*, *FMR-1*, and *MT-PK* are displayed. The range of repeat numbers is shown for normal individuals, those carrying premutations, and those affected with the associated clinical disorder (spinal and bulbar muscular dystrophy, fragile X syndrome, and myotonic dystrophy, respectively). The value of 1000 repeats as the upper limit of *FMR-1* repeat amplification is a lowest estimate; the precise number may actually be higher. A logarithmic scale has been used for convenient display of the information.

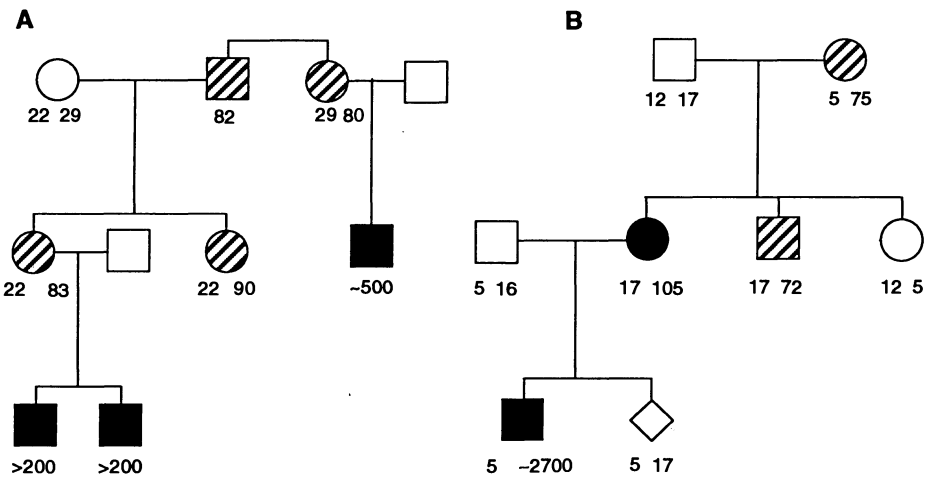

Fig. 4. Transmission and expansion demonstrates the instability of premutational alleles for fragile X syndrome and myotonic dystrophy. Open symbols, normal (phenotypically and genotypically); closed symbols, affected; hatched symbols, phenotypically normal individuals who are heterozygous for a premutation allele. Numbers of repeats for each allele are shown below the symbols. (**A**) Pedigree for a portion of a family affected by fragile X syndrome. Filled symbols indicate affected individuals and hatched symbols identify individuals who, by family history, carry the genetic defect but have normal phenotypes. Indicated genotypes represent the number of CGG repeats in *FMR-1* as determined by PCR amplification, which detects values to about 200 repeats with a range for normal individuals of 11 to 50, or by DNA hybridization analysis (*4*). The results show unstable transmission of the premutational allele with gross enlargements to a fully mutant allele during transmissions from mother to son. (**B**) Pedigree of a family affected by myotonic dystrophy. Indicated genotypes represent the number of GCT repeats in *MT-PK* as determined by PCR amplification, which detects values to about 150 repeats with a range for normal individuals of 5 to 30, or by DNA hybridization analysis (*6*). The diamond indicates a fetus that was prenatally tested.

disequilibrium between linked markers and DM (*7, 26, 28*), and lack of observed new mutation events (normal → premutation) suggest but do not prove that premutation alleles are ancient.

Finally, we have emphasized the expansion of repeats since this event leads to disease. It should be remembered that reduction in size of repeat is well documented for fragile X syndrome (*35*). This reduction also occurs for *MT-PK* alleles but the extent has not been determined accurately.

Pathophysiology

The role of androgen receptor mutations in androgen insensitivity syndromes was well established prior to the discovery of the SBMA mutation (*10*). Androgen receptors are concentrated in spinal and bulbar motor neurons, thus providing some correlation to the progressive motor weakness associated with SBMA but not with other androgen insensitivity syndromes (*40*). It is noteworthy that Reifenstein's syndrome, another androgen receptor defect, has associated loss of the sense of smell (anosmia) (*44*). Thus two neural cell types must be affected by mutations in the androgen receptor. On the basis of this experience, *AR* should be considered a candidate gene for other neurologic disorders mapping to this region of the X chromosome. Since amplification of the triplet repeat in SBMA occurs in the coding region of a multifunctional protein, perhaps essential, there may be biologic restriction on observing large amplification in patients. It is interesting to speculate that large amplifications could lead to loss of *AR* function, as has been reported for testicular feminization.

The role of FMR-1 in the pathophysiology of fragile X syndrome is inferred since the triplet expansion is within the gene and *FMR-1* mRNA is undetectable in affected males (*42*). Although it is possible that the repeat sequence simply perturbs hnRNA processing, another mechanism for the lack of *FMR-1* is more likely. Several groups identified a CpG island, later found to be ~200 bp from the 5′ transcription site of *FMR-1*, which was methylated in fragile X males and normal females carrying one inactive X chromosome (*43, 44*). The correlation of methylation and lack of *FMR-1* mRNA detection in affected males was quite good. This raised the possibility that methylation of a promoter region rather than defects in mRNA processing was the mechanism of mRNA reduction or absence.

A recent prenatal diagnostic case was instructive in this regard. Chorionic villus cells of an affected male fetus, contained *FMR-1* mRNA that was detected by reverse-transcription PCR; Bss HII methylation was not observed. However, when fetal cells were studied, *FMR-1* mRNA was reduced and the Bss HII site was methylated. We conclude that methylation in the region of the CGG amplification may be the critical event in *FMR-1* mRNA expression.

Such a mechanism necessarily involves one or more DNA methylases and points toward an association of DNA conformation change at the repeat site that allows regional recognition by the methylase. Methylation has significant effects on DNA structure, chromatin organization, and expression in diverse eukaryotes, and is also important in prokaryotes. Methylation modification is used as an in vivo analytic method to distinguish B and non-B DNA conformations in prokaryotes (*45*).

It is unclear at this time if other genes in the region are also affected in their expression. One must keep open the possibility that fragile X syndrome is the consequence of more than the lack of *FMR-1* expression. Complex phenotypes are well established for contiguous deletion syndromes in which more than one gene is affected (*46*). It is too early to exclude this possibility for fragile X syndrome. Continued search for patients with the clinical fragile X phenotype but lacking CGG amplification and identification of a deleterious mutation in *FMR-1* could clarify this uncertainty.

FMR-1 is expressed in a variety of tissues, with highest expression in brain and testis. Macroorchidism and head enlargement are features of fragile X syndrome but the mechanisms by which these are induced is unknown. The predicted protein sequence of *FMR-1* has given little hint of its function, with the exception of a putative nuclear localizing sequence. Thus, although the gene is cloned and the mutational mechanism established, our knowledge of the cause of mental retardation is lacking. Since the gene is expressed in many tissues of humans and found in mouse and *Caenorhabditis elegans* (*2*), the function appears to have been conserved during evolution. This disease therefore provides an excellent challenge for cell biologic study.

The role of myotonin protein kinase in myotonic dystrophy is in the early stages of study. The predicted protein sequence of *MT-PK* indicates a set of domains that are only partially represented in Fig. 1. Given the ATP-binding site and catalytic domain for protein kinase, these functions can be predicted with certainty. Other interesting domains correspond to muscle structural proteins and transmembrane domains. The kinase is present in many tissues but is particularly highly expressed in skeletal and cardiac muscle. Its features would imply that *MT-PK* is a highly specialized signal transduction protein. Myotonic dystrophy

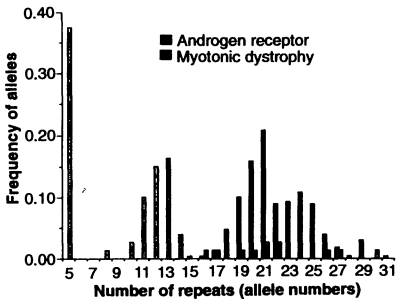

Fig. 5. Allele frequencies of Caucasians for the *MT-PK* and *AR* trinucleotide repeats.

cells have previously been reported to have abnormalities in protein phosphorylation (*47, 48*). Several studies have related the pathophysiology to ion channel activities. Chloride, potassium, and sodium channels have all been variously associated with the pathogenesis of myotonia in myotonic syndromes such as myotonia congenita (Thomsen's disease), paramyotonia congenita, and hyperkalemic paralysis periodica (*49–51*). These would appear to be excellent candidate proteins for the *MT-PK* ligand (or ligands). The availability of these clones and their protein products should facilitate the search.

It is not possible at this time to define precisely the mechanism by which *MT-PK* mutations lead to DM in adults or newborns given the very recent discovery of repeat amplification. We presume, but it has not been proven at this time, that expression of *MT-PK* is reduced as an associated feature of the GCT triplet expansion. There is no documentation of methylation events associated with the DNA sequence differences of mutant and normal *MT-PK*. As the disorder is inherited in an autosomal dominant fashion and the mutation does not arise in the coding sequence it would be unlikely that the dominant phenotype is due to interference of a multisubunit protein as observed for collagen disorders (*52*). Protein kinases can consist of nonidentical subunits; an alteration in the level of one subunit could affect overall function as occurs in hemoglobin α tetramers in β-thalassemia. Finally, it could be a simple reduction in protein level as found for the autosomal dominant disorder of the low density lipoprotein receptor associated with coronary artery disease (*53*).

The remarkable difference in severity of congenital myopathy and adult disease deserves special comment. Congenital myopathy is associated with maternal rather than paternal inheritance of the *MT-PK* mutant allele. Parental imprinting should be considered as the basis of the difference. Could the paternally derived *MT-PK* gene be expressed at lower levels than maternal *MT-PK* gene? If so, then inheritance of a ma-

ternal disease allele would have greater disease impact. These many possibilities can be resolved given the availability of the isolated gene, the gene sequence, protein probes, and informative patients.

Molecular Diagnosis

The only method of SBMA diagnosis presently available is PCR amplification of the CAG repeat and allele quantitation by sequencing gel analysis. Detection of the alleles can be achieved by generating radioactive or fluorescent reaction products (*32*).

The diagnosis of fragile X syndrome has traditionally been cytogenetic. Now that the CGG repeat has been documented as the fragile site, DNA-based methods should replace the cytogenetics. Southern methods, although labor-intensive, have the capacity to detect large CGG expansions found in fragile X patients and female carriers. We utilize Eco RI restriction cleavage ± Bss HII and the probe pE 5.1. Such an analysis will yield a 5.1-kb Eco RI fragment that is cleaved by Bss HII to 2.4- and 2.7-kb fragments. The 2.7-kb Eco RI–Bss HII fragment contains the CGG repeat. Patients with fragile X syndrome will have a single expanded fragment under both digestion conditions because the Bss HII site is methylated (resistant to cleavage) and the CGG repeat is expanded. NTMs occasionally have somatic heterogeneity, are not methylated, and usually exhibit a band above 2.7 kb (50 to 200 bp). The 2.4-kb band is unchanged in all NTMs and serves as an internal control.

PCR offers the opportunity for rapid detection of alleles in the normal range (6 to 54), premutation alleles associated with NTM and female carriers (52 to 200), and different alleles in cell lines from an individual (mosaicism) found in NTMs. We have found that PCR amplification requires special conditions including 10% DMSO and addition of 75% 7-deaza-dGTP. It has been reported that high molecular weight CGG repeats of several kilobases can be amplified with 100% 7-deaza-dGTP and detected with CGG synthetic oligonucleotide probes (*54*). We find it quite simple to make a fragile X diagnosis associated with a large triplet amplification by the inability to conduct PCR amplification of the CGG repeat (null). The amplification is internally controlled by coamplification of the *AR* CAG repeat. Furthermore, using primers that amplify the Bss HII site, we can directly detect the presence of methylation by nuclear DNA Bss HII cleavage prior to amplification.

A typical fragile X male will therefore have amplification elements that are: AR^+, fragile X^- (too large for amplification), and Bss HII–protected$^+$. This simple PCR approach is rapid and inexpensive. It remains to be resolved whether DNA hybridization or PCR will provide accuracy at lowest cost. The detection of female carriers is now achieved by family linkage analysis, Southern and PCR analysis. There is little doubt that DNA-based methods already supercede cytogenetic methods for female carrier detection and prenatal diagnosis. It remains to be determined if *FMR-1* mRNA or protein quantitation will replace or augment current methods. It is our opinion that no single DNA method can be recommended presently, given the high risk of serious disease in progeny and early experience with these DNA methods. Genetic centers should strive for family studies rather than single patient submissions. This approach will lead to highest accuracy and minimize error.

The diagnosis of DM has been greatly improved by development of DNA probes for the GCT repeat. No longer is it necessary for patients to undergo muscle biopsy, muscle enzyme study, and electromyography as the first diagnostic procedures for DM. Given the availability of DNA methods, they should be used first as is now accepted for clinical evaluation of Duchenne muscular dystrophy (*55*). The repeat expansion can be quantitated by DNA hybridization analysis after Bam HI digestion, which yields a fragment of 1.4 kb containing the GCT repeat. As the complete sequence of the region is known, other restriction endonucleases can be rationally chosen. For example, endonucleases Nco I, Eco RI, and Hind III yield fragments in the range of 7 to 10 kb. These and many other endonucleases will be satisfactory for the identification of large GCT expansions found in affected individuals as the size of DM GCT expansion ranges from 90 to 4000 bp in our current experience. However, just as with fragile X NTMs, some patients possess a premutation sequence that is between 50 and 78 repeats. PCR is a simpler and more rapid approach to detection of normal and premutation alleles with repeats up to 120.

Presently, the optimal conditions for PCR amplification of large alleles are not finalized. The detection of carriers is now achieved by family linkage analysis with closely linked dinucleotide repeats, Southern, or PCR detection of normal premutation and disease-associated GCT alleles. It is likely that improvements in the DNA-based diagnosis of DM will rapidly evolve. Furthermore, the usefulness of quantitating *MT-PK* mRNA has not yet been evaluated.

New Considerations

The discovery of the mutational mechanism of triplet repeat amplification for these three disorders is expected to lead to discovering many other disease-related genes. An oligonucleotide scanning method proved to be useful in identifying the DM mutation (6). A similar approach can be used to scan cloned loci of diseases with features of anticipation (for example, Huntington disease and olivopontocerebellar ataxia) or somatic events (such as neoplasia and cystic disease of the kidney). A search of GenBank has revealed more than 100 human loci with AGC/GTC repeats larger than 5 copies. Scanning of cDNA libraries via oligonucleotide hybridization has identified a significant number (>40) of independent cDNAs containing these sequences. Thus, triplet repeats are common in the human genome. It is yet to be determined what the importance of such sequences will be for other inherited and somatically acquired genetic diseases.

The discovery of highly mutable sequences in DM and fragile X syndrome raises new issues for medical genetics. In these diseases, premutation sequences do not cause disease in the individual bearing them but forecast a risk of disease for their progeny above the general population. If carrier testing for Tay-Sachs disease, sickle cell anemia, β-thalassemia, and cystic fibrosis (56) are utilized for informed family planning, is it not reasonable to consider DM and fragile X syndrome? The difference in screening for DM and fragile X syndrome is, however, identification of an unstable allele and its associated risk potential rather than inheritance of a well-characterized fixed allele. Operationally, the screening of a high-risk pregnancy for fragile X and DM is not significantly different from screening pregnancies for aneuploidy for mothers over the age of 35 years. We need to determine accurately the disease risk for premutation alleles and develop educational methods related to these new concepts for families and professionals. Such information on these diseases and development of effective educational approaches will provide the medical geneticist the first opportunity to deal with mutation-induced disease in an anticipating manner and to provide options to families with risk of disease in progeny. Discussions can assist in the socially and scientifically responsive application of this technology and concept.

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Malignant Hyperthermia

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In humans genetically predisposed to malignant hyperthermia, anesthesia can induce skeletal muscle rigidity, hypermetabolism, and high fever, which, if not immediately reversed, can lead to tissue damage or death. The corresponding condition in swine leads to stress-induced deaths and devalued meat products. Abnormalities in the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum (the ryanodine receptor) have been implicated in the cause of both the porcine and human syndromes by physiological and biochemical studies and genetic linkage analysis. In swine, a single founder mutation in the ryanodine receptor gene (*RYR*1) can account for all cases of malignant hyperthermia in all breeds, but a series of different *RYR*1 mutations are likely to be uncovered in human families with MH. Moreover, lack of linkage between malignant hyperthermia and *RYR*1 in some families indicates a heterogenous genetic basis for the human syndrome.

Although anesthesia provides little cause for concern for most humans, exposure to a combination of potent inhalational anesthetics and depolarizing skeletal muscle relaxants presents a hazard to those genetically predisposed to malignant hyperthermia (MH) (*1*). The commonly used combination of halothane and succinylcholine can trigger skeletal muscle rigidity, accompanied by hypermetabolism, high fever, and cellular ion imbalances in susceptible individuals. If therapy is not initiated immediately, the patient may die within minutes from ventricular fibrillation, within hours from pulmonary edema or coagulopathy, or within days from neurological damage or obstructive renal failure. The practice of monitoring for the early symptoms of an MH episode and responding to such symptoms by terminating the anesthetic process and infusing the clinical antidote, dantrolene, has lowered the death rate for such episodes from over 80% to less than 7% in recent years. Neurological or kidney damage, however, still contributes to the morbidity resulting from MH episodes.

MH also occurs in domestic animals such as swine and therefore has worldwide economic consequences (*2*). Swine are seldom exposed to anesthesia, but animals homozygous for the abnormality respond to stress in the same way that heterozygous humans respond to anesthetics—with muscle rigidity, hypermetabolism, and high fever. The stress-induced death of such animals (porcine stress syndrome or PSS) is but one aspect of economic loss due to the syndrome. An equally serious problem is that the same reaction can be triggered when a hog experiences acute stress prior to slaughter, resulting in pale, soft, exudative (PSE) pork in large segments of the carcasses of susceptible animals.

An abnormality in the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum [the ryanodine receptor (*3*)] may account for the disorder because, in skeletal muscle, both contraction and metabolism are regulated by the concentration of intracellular Ca^{2+} (*1*, *2*). The deduced amino acid sequences derived from sequences of cDNAs encoding the Ca^{2+} release channels from an MH and a normal pig differ at a single amino acid; in the MH pig Cys is substituted for Arg^{615} (*4*). This mutation was linked to MH with a lod score (the logarithm of the odds that genetic linkage exists) of 102 for a recombination fraction of 0.0 in studies of 338 informative meioses (*5*). This discovery has made it possible to develop a diagnostic test for normal, heterozygous (carrier), and MH-susceptible animals in all breeds of swine. The availability of a diagnostic test for the mutation provides breeders with the opportunity to eliminate the MH gene from their herds, thereby eliminating the major cause of stress-induced death and PSE. On the other hand, the MH gene may contribute to leanness and heavy muscling in swine (*6*) and breeders might find it advantageous to retain the gene and benefit from possible uniform gains in dressed carcass weight if heterozygous market animals can be produced economically.

Linkage between polymorphisms in and near the skeletal muscle ryanodine receptor (*RYR*1) gene and MH has also been established in studies of inheritance in human families (*7*, *8*). Moreover, substitution of Cys for Arg^{614}, corresponding to the porcine MH mutation, was found in 1 of 35 human families studied, and this mutation also cosegregated with MH (*9*). The combination of a very high lod score for linkage between the porcine *RYR*1 mutation and MH, with the existence of the corresponding mutation in humans, indicates that this mutation in *RYR*1 is the cause of at least

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