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Molecular analysis of the CTG trinucleotide repeat in South African myotonic dystrophy families — implications for diagnosis and counselling

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Myotonic dystrophy is associated with an increased number of CTG repeats in the 3' untranslated region of the myotonin protein kinase gene. The recent elucidation of the molecular basis of myotonic dystrophy has, for the first time, made a specific molecular diagnosis of this condition a possibility. Ten South African families were analysed at the molecular level, using both PCR and Southern blot analyses for the detection of the trinucleotide repeat. Expansion of this repeat was found in 9 families and in 2 cases the grandparental origin, which was previously unknown, could be determined. It is now possible to counsel these families more effectively and to identify individuals, particularly women, who are at risk of passing on the DM mutation.

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Myotonic dystrophy (DM) is an autosomal dominant disorder with a worldwide prevalence of 1:8 000. The classic clinical features include myotonia, progressive muscle weakness and wasting and many associated symptoms, including cataracts, intellectual impairment and frontal balding. A hallmark of the disease is the extreme variation in severity and age of onset, both between and within families.[1] A severe, and often fatal, congenital form of DM also occurs, but only among offspring of a carrier female. The genetic basis of DM has recently been shown to be an unstable CTG repeat DNA sequence in the 3' non-coding region of the myotonin protein kinase gene, located on chromosome 19q13.3.[2-4] When amplified, this trinucleotide repeat is responsible for DNA instability and molecular pathology. A similar mechanism of trinucleotide repeat expansion has been described in fragile X mental retardation syndrome (CGG),[5,6] spinobulbar muscular atrophy (CAG)[7] and, more recently, Huntington's disease (CAG).[8] However, very little is known of the mechanisms which determine the size and stability on transmission of such expanded repeats, or the manner in which the genotype leads to the observed phenotype, which particularly in DM may be very variable in presentation. In normal individuals, the CTG repeat is highly polymorphic, with the copy number ranging from 5 to 38.[9-11] In mildly affected DM patients, the copy number is greater than 50, and has been found to be as high as 2 000 in severely affected individuals. The discovery that there is a marked tendency for an increase in CTG triplet number from one generation to the next, paralleled by earlier onset of the disease in successive generations, gave a molecular explanation for the phenomenon known as anticipation. It was initially thought that CTG expansion occurred similarly in both maternal and paternal transmission. Recent studies have, however, reported that paternal transmission of the CTG expansion may be more common in the initial expansion from the relatively asymptomatic minimally amplified allele category to the larger sizes of CTG repeats seen often in mildly affected parents of DM patients.[12] Thus, the instability of the CTG sequence appears to depend on its size as well as on the sex of the transmitting parent.[13]

Myotonic dystrophy has been described in many countries worldwide, including Japan, China, India, South Africa and Nigeria.[1,14] Very little is known about DM and its incidence in the Indian population or in people of mixed ancestry in South Africa. Also, there is no single report of the condition in South African blacks.[15,16] However, a higher incidence of DM has been described in white families in the Northern Transvaal, with a minimum prevalence rate of 14,3/100 000, higher than the 3,3 - 5,5 reported elsewhere.[1,15,16]

Subjects and methods

Ten South African families with DM were investigated, 6 of which were Afrikaans-speaking and 4 English-speaking. These families had been referred via the Genetic Counselling Clinic at the Transvaal Memorial Institute or by the Department of Human Genetics at the University of Pretoria. Genomic DNA was extracted from peripheral blood, either with a phenol chloroform extraction[17] or a simple salting out procedure.[18] Three systems were used for analysis: PCR and two Southern blotting systems. The polymerase chain reaction (PCR) with primers 101 and 102[9] was used to amplify the region containing the CTG repeat. The PCR was performed in a 25 µl reaction volume containing 100 ng of genomic DNA, 10 mmol Tris-HCl, pH 8,8, 50 mmol KCl, 1,5 mmol $MgCl_2$, 0,1% Triton-X, 200 µmol dATP, dTTP and dGTP, 2,5 µmol dCTP, 50 ng of each primer, 1,25 µCi (α-^{32}P) dCTP, and 2 U *Taq* DNA polymerase. Altogether, 35 cycles of amplification (94°C for 1 min, 66°C for 1 min, 72°C for 1 min) were carried out with a Perkin Elmer Cetus DNA Thermal Cycler. The denatured PCR products were separated on 6% polyacrylamide gels. Samples with known CTG repeat numbers were run on every gel as controls for the sizing of alleles. *Bgl*1 or *Eco*RI-digested DNA, hybridised with p5B1.4, enabled the detection of larger CTG expansions not amplifiable by PCR.[19]

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Results

Direct detection of the DM mutation was possible in 9 of the 10 South African families, where the expanded CTG repeat was found. In the remaining family suspected of DM, the patient had allele sizes within the normal size range and the diagnosis of DM was refuted (see below). The parental origin of the DM mutation could be determined in all 8 families tested; 6 were maternal and 2 paternal in origin. In 1 case, the parents were not tested. In addition, the grandparental origin of the DM mutation was determined in 2 families, in both of which it was grandpaternal.

Precise determination of CTG copy number of normal (non-DM) alleles was possible with PCR analysis. Great variation in allele size was found on normal (non-DM) chromosomes (Fig. 1). All normal alleles had between 5 and 29 CTG repeats. Those DM alleles, with large CTG expansions, are not amplifiable with this method. Thus individuals with a single PCR-detected allele were confirmed on Southern blotting either to be true homozygotes or to have an expanded DM allele.

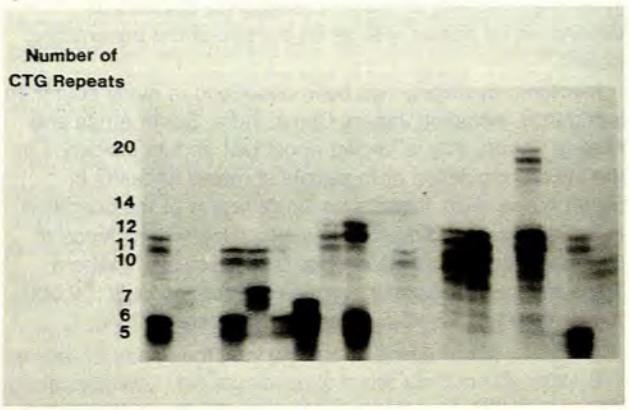

Fig. 1. A 6% polyacrylamide gel showing variability of the CTG repeat length on non-DM chromosomes. Each allele is represented by a 'doublet' on the gel. The number of CTG repeats is indicated by the number placed between the two doublet bands.

Figs 2 and 3 show how a combination of detection systems enables the inheritance of both the non-DM and DM alleles to be followed in a family, as well as determining the progressive meiotic expansion of the CTG repeat through generations.

*Bgl*1-digested DNA, probed with p5B1.4, detects a 3,4 kb *Bgl*1 fragment on non-DM chromosomes[19] (Fig. 2, I-1 and II-1). This system will detect small DM expansions given that the target fragment for the probe is small. In mildly affected individuals, the normal 3,4 kb fragment occurs together with a moderately sized fragment while in affected DM individuals a much larger fragment or a smear of fragments corresponding to the DM mutation will be seen. The *Eco*RI/p5B1.4 system, in addition to detecting the DM expansion, recognises an insertion/deletion polymorphism with normal alleles being 8,6 and 9,8 kb.[3] Studies in DM patients have revealed that the probe detects one normal allele (8,6 or 9,8 kb) and a DM-specific band of variable size in most affected individuals. The variable band is always larger than the 9,8 kb allele and sometimes appears as a diffuse smear because of somatic instability.

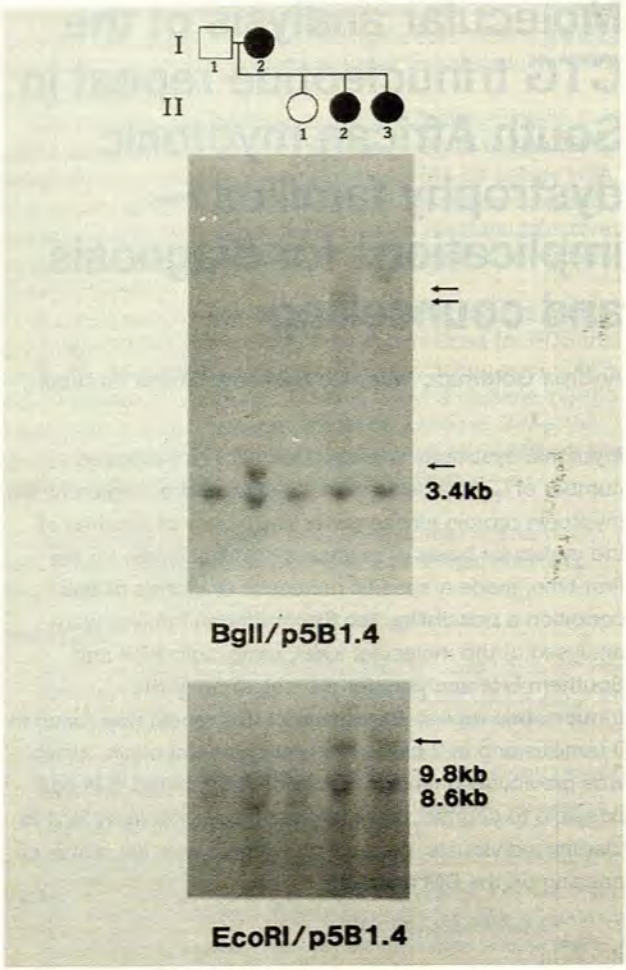

Fig. 2. Southern blot analysis of a DM family illustrating the inheritance and progressive meiotic expansion of the CTG repeat in the myotonin protein kinase gene. DNA was digested with *Bgl*1 and *Eco*RI as indicated and hybridised with p5B1.4. The father (I-1) has two normal alleles detectable with both PCR (11 and 29 repeats) and Southern blotting (8,6 and 9,8 kb alleles). The mildly affected mother (I-2) has one normal allele (13 repeats) as well as a larger myotonic dystrophy-specific fragment. II-1 has received two normal alleles (13/29). The two severely affected daughters (II-2 and II-3) each received one normal allele (29 repeats) from their father and a DM-specific fragment from their mother. Mitotic instability, seen as a smear of DNA fragments on Southern blots (II-2 and II-3 on the *Bgl*1 blot), has been observed in affected individuals. The fragment size is seen to correlate well with the increase in severity of symptoms in the next generation.

Discussion

The identification of an unstable CTG repeat sequence, in the 3' untranslated region of the myotonin gene, as the basis for the mutation in DM has, for the first time, enabled direct molecular diagnosis in this condition. The implication of this finding is of immediate practical relevance.

It was not known whether the aetiology of DM in South Africa was the same as elsewhere and, therefore, the main aim of this study was to test for the CTG expansion as the DM-causing mutation in South African DM families. The range of CTG repeats found in the South African families was similar to that found in other populations. On the non-DM chromosomes, all normal alleles had between 5 and 29

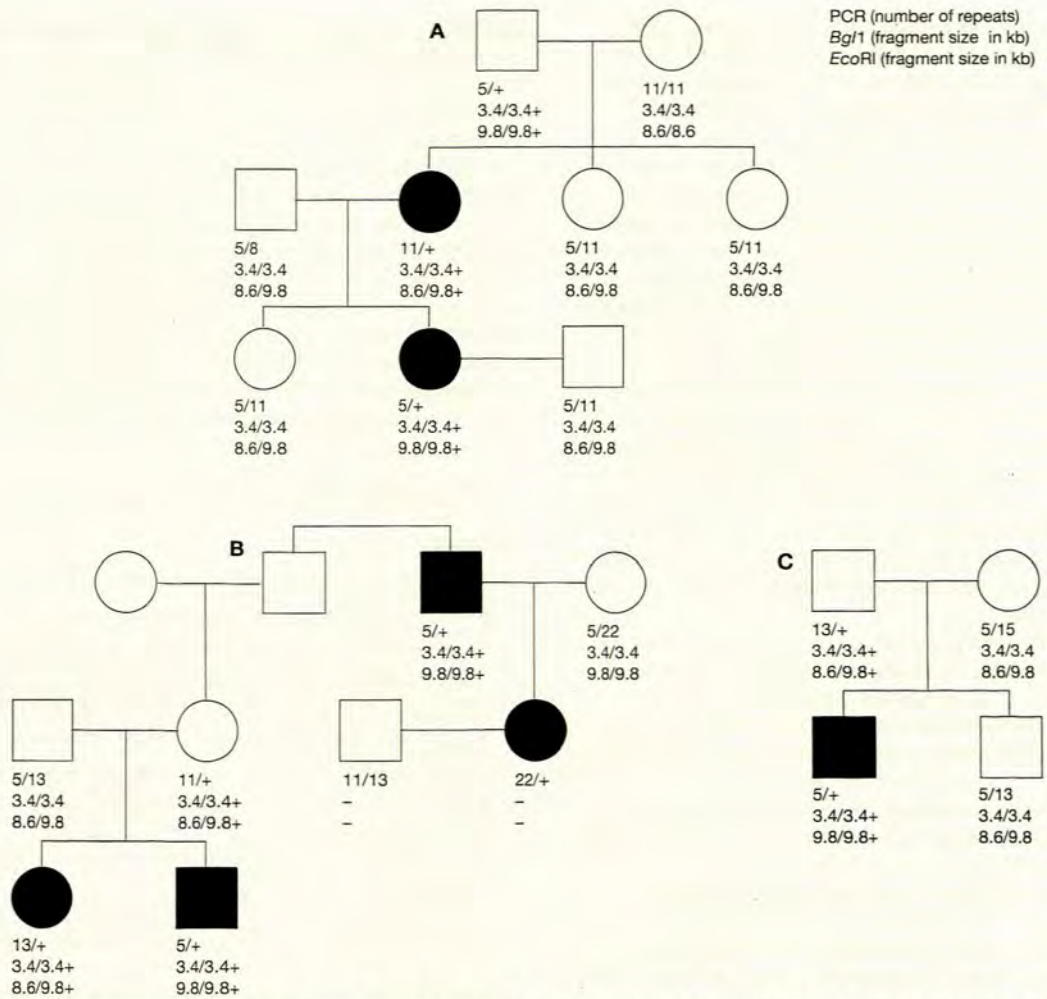

Fig. 3. Three DM pedigrees showing the PCR results and Southern blotting systems, *Bgl*1 and *Eco*RI, where possible. For PCR results, the '+' denotes repeats greater than the normal range. In the *Bgl*1 system, 3,4kb represents a fragment of normal size, while 3,4+ represents an expanded fragment. For the *Eco*RI system, 8,6 kb and 9,8 kb fragments are normal-sized. The 9,8+ represents an expanded fragment. Pedigree 3A shows how the triplet repeat expansion (the DM mutation) is inherited through three generations. Pedigree 3B illustrates the importance of identifying the parent or grandparent that has transmitted the CTG expansion. In this case, other 'at-risk' family members may be counselled and tested. Pedigree 3C illustrates that many carrier parents are mildly affected or clinically unaffected and are only identified once they have had an affected child.

CTG repeats, where the normal range has been reported as 5 - 37 repeats.[9-11,20] On DM chromosomes, the principal mutation causing DM (the CTG expansion) could be detected directly by Southern blotting analysis (using the probe p5B1.4) together with PCR analysis.

The CTG trinucleotide repeat expansion was seen in 9 of the 10 families investigated. Affected DM individuals with large alleles at the DM locus often had a smear of DNA fragments on Southern blots (Fig. 2), reflecting mitotic instability,[11] whereas smaller alleles could be seen as distinct fragments. It is likely that the *Eco*RI/p5B1.4 Southern blotting system will efficiently detect almost all clinically significant cases of DM (i.e. large expansions) as was the case in this study. However, in minimally affected DM patients, the DNA expansion may be difficult to distinguish from the normal 9,8 kb allele since the minimum expansion size seen with *Eco*RI is 0,2 kb.[19] In such circumstances, it can be detected more easily with *Bgl*1; an example of this is shown in Fig. 2, Individual I-2, where the mother's small expansion can be seen with *Bgl*1 but not with *Eco*RI.

A *Bgl*1 RFLP, not described previously, has been found in the southern African black population.[21] If this RFLP is present in populations where DM does occur, it may interfere with interpretation of results with the *Bgl*1/p5B1.4 system and lead to the erroneous assumption of DM in unaffected individuals.[21] This polymorphism has, however, not been found in the white South African families tested in this study.

One of the 10 South African DM families did not appear to have a trinucleotide repeat expansion. This result may be explained either by misdiagnosis or by the occurrence of a mutation (other than the CTG expansion) elsewhere in the myotonin protein kinase gene. In this case, there was a problem with the clinical diagnosis. A repeat muscle biopsy and second clinical opinion concluded that the disorder was not DM. This example illustrates the usefulness of DNA testing for confirmation of a clinical diagnosis.

In most families the size of the DM-specific unstable fragment is passed onto affected offspring unchanged or increased and both maternal and paternal transmission has

been reported. Recent studies have, however, suggested that this may be an oversimplification. Numerous DM families have been reported in which transmission of the affected (DM) chromosome 19 is accompanied by a decrease in length of the triplet repeat. In 4 families, reduction led to fragment sizes within the range of the normal population.[22-26] At present these patients are asymptomatic and have no signs of DM, but as the subjects get older, it will be of great interest to see if clinical features develop. All four reversions to the normal range have occurred on transmission from affected men. This suggests that there is a trend towards CTG length reduction on transmission from men with repeat sizes in excess of 2 kb.[24,25,27,28] These cases of reversion of the length of triplet repeats to normal size in DM families appear to be uncommon, but have serious consequences for prenatal diagnosis. No example of reversion was found in the present study. The finding of more maternal transmission (6 cases) than paternal transmission (2 cases) may be a function of small sample size as well as ascertainment bias, since the more severe cases, that are more likely to be referred, will be congenital DM.

Hunter *et al.*[12] made the preliminary observation that paternal transmission may be more common in the initial expansion of the CTG repeat from the relatively asymptomatic, minimally amplified allele category to the larger sizes of CTG repeat visible on Southern blot analysis. Lavedan *et al.*[28] also reported a significant excess of minimally affected grandfathers who had transmitted DM. Ascertainment bias cannot be rejected since (*i*) women with severely affected DM children will not appear as grandmothers of affected cases, and (*ii*) affected men appear to have more children than affected women.

The chief application of the identification of the DM mutation as an unstable CTG repeat, is the confirmation of the clinical diagnosis of DM. Molecular studies will be particularly valuable when evaluating an isolated proband with equivocal signs of DM, or when evaluating the apparently normal parents of a classically affected person. In Fig. 2, the mother (I-2) is so mildly affected that the diagnosis of DM was only made after the identification of the condition in her two affected daughters. This is a common occurrence in DM families. In cases where both parents are clinically unaffected, DNA analysis is especially helpful in determining the parental or grandparental origin of the DM mutation to enable the detection of 'at-risk' family members. In addition, identification of DM individuals enables presymptomatic testing as well as assessment of their risks of having severely affected children[29] and the possibility of offering prenatal diagnosis.

Studies of a large number of DM patients have shown a definite correlation between the extent of amplification and the age of onset and severity of DM.[12] However, the ranges are broad and there is significant overlap between, and variability within, categories of allele size. At times this is clinically highly relevant.[12,30] The only clear distinction that can be made is between congenital DM (CTG copy numbers: > 500) and the minimal form of the disease (copy number: 50 to 100).

Most siblings with similar allele sizes show comparable ages of onset, and those with larger alleles show earlier onset.[12] Thus knowledge of parental or sibling copy number and clinical history within families can be of value in predicting age of onset or disease severity more accurately in an individual or fetus whose CTG copy number is available.[24] In the case of a patient with no other family members available, the size of the expanded allele can be used to estimate the most likely clinical phenotype.

In conclusion, molecular analysis of DM patients is an accurate and reliable means of identifying the mutation and confirming the clinical diagnosis. Since DM is an exceptionally variable disorder, if DNA analysis is to be used for the prediction of DM phenotype, particularly in prenatal diagnosis, it is essential that the relationship between different mutations, allele sizes, expansions and the phenotype be clearly defined.[30,31] With the current state of knowledge, however, molecular analysis generally cannot provide a precise identification of disease severity and age of onset.

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