A study of DNA methylation in myotonic dystrophy

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Abstract

We have examined the hypothesis that the severe congenital form of myotonic dystrophy is caused by genomic imprinting at the level of differential DNA methylation of maternal and paternal alleles. Probes encompassing the 5′, central, and 3′ regions of the myotonic dystrophy protein kinase gene were used on blots of blood DNA from congenital and adult onset patients, digested with combinations of methylation sensitive and insensitive restriction enzymes. We observed similar patterns of methylation in each of the different classes of patient, and found no methylation differences between paternally and maternally derived alleles. Within the limitations of the experiment, our results provide no evidence for a role for genomic imprinting in congenital myotonic dystrophy and suggest that the explanation for this form of the disease will be found elsewhere.

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Myotonic dystrophy (DM) is one of the commonest inherited neuromuscular disorders, and shows autosomal dominant inheritance with highly variable expression and a wide range of symptoms. In DM families, it is commonly observed that the severity of the symptoms increases with each successive generation, and the age at onset decreases similarly. This phenomenon is termed anticipation. A particularly severe congenital form of the disease may occur in affected infants born to affected mothers (but never when the father is the affected parent). For a review of the disease in general, see Harper.[1]

We and others have recently identified the mutation causing DM and the gene that is affected. Linkage studies had mapped the locus to 19q13.3[2–4] and linkage disequilibrium analysis predicted that most cases would have a common mutational origin.[5] Extensive analysis using probes derived from the DM candidate region identified a DNA fragment that expanded in size in DM families, and showed that this expansion was exclusively associated with the DM phenotype.[6–8] Further investigation showed that the expansion was the result of an unstable tandemly repeated CTG sequence, located in the 3′ untranslated region of a protein kinase family gene.[9–10] Progressive expansion of this sequence in generations of a DM family was correlated with increasing disease severity and earlier age at onset, and hence provided a biological basis for anticipation.[6 7 11] An analogous mechanism had already been described in the fragile X syndrome, in this case an unstable CCG repeat sequence in the 5′ untranslated region of a gene designated FMR-1.[12]

Patients with congenital myotonic dystrophy (CDM) have DNA expansions of between 1·5 and 6 kb.[11 13] Although this is larger on average than the expansions seen in non-congenital, adult onset or minimal forms of DM, there is considerable overlap between the size ranges of DNA expansion in the various disease classes.[11 13] Therefore DNA expansion alone is not sufficient to explain the congenital form of DM, nor why it is exclusively maternally transmitted.

Genomic imprinting is a process whereby the maternal and paternal genetic contributions to the offspring are expressed differently, and has been studied extensively in the mouse.[14] By using transgenic animals it was shown that imprinting effects are strongly correlated with DNA methylation.[15] It has been suggested[16] that imprinting might account for the fact that the congenital form of DM is exclusively maternally transmitted. If the paternally derived normal allele of the DM gene were inactivated by methylation, and the mother's allele was mutated, then the offspring might be expected to show a particularly severe form of the disease. Before the discovery of the mutation in fragile X syndrome, it had been shown that DNA methylation at a specific site in Xq27.3 was associated with clinically affected subjects[17] and it is now known that this site is an HTF island at the 5′ end of the FMR-1 gene.[18] The island is hypermethylated in males affected with the fragile X syndrome and on the fragile X chromosome of female carriers. In general the degree of methylation is positively correlated with the degree of length expansion of the fragile X CCG repeat sequence. It is not known whether methylation directly affects the expression of the FMR-1 gene, or whether it is merely a consequence of the mutation.[19]

In order to investigate whether genomic imprinting at the level of DNA methylation might have a role in the congenital form of myotonic dystrophy, we used sequences from within and around the DM protein kinase gene to probe Southern blots of DNA digested with methylation sensitive restriction enzymes, in combination with enzymes detecting DNA polymorphisms that allow the parental origin of the alleles to be distinguished. If there were differences in methylation between the maternally and paternally derived alleles in DM patients, these should be detectable using such an approach.

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Materials and methods

Myotonic dystrophy families were selected from the large collection studied in this Department over the years. Patients were classified as either congenital (symptoms, including hypotonia and respiratory problems, present at birth) or adult onset (symptoms first noted during the third or later decade of life). Congenital cases were exclusively maternally transmitted, whereas examples of both maternally and paternally transmitted adult onset cases were used in the study.

DNA analysis was done using conventional agarose gel electrophoresis, Southern blotting, and hybridisation with ^{32}P labelled probes. All DNA samples were obtained from peripheral blood. No cell lines were used.

Results

Four probes were used for this study, allowing visualisation of three contiguous regions of the DM protein kinase gene and the segment of DNA downstream from its 3′ end. These DNA regions are defined by sites for the enzymes *Eco*RI and *Eco*RV (fig 1). Region 1 (probe p59A) includes the 5′ end and exons 1 to 4 of the DM protein kinase gene, and the 3′ end of a neighbouring gene (cDNA59, Shaw *et al*, submitted).[20] Region 2 (probe B2.7) includes the central portion of the DM gene and the site of a 1 kb DNA insertion giving rise to an RFLP that enables the alleles to be distinguished.[21] Region 3 (probe pM10M6) includes exons at the 3′ end of the DM gene, the 3′ untranslated region, and the unstable CTG repeat sequence. The expansion of this sequence enables the allele bearing the DM mutation to be distinguished as a restriction fragment of increased length. The probe pM10M7 maps 3′ to the end of the gene and allows detection of the next *Eco*RI–*Eco*RV fragment in this direction.

Samples of DNA were selected from patients in each of the following clinical categories: 10 cases of congenital myotonic dystrophy (CDM); 16 cases of adult onset, paternally transmitted (AOP); and five cases of adult onset, maternally transmitted (AOM). DNA was digested with *Eco*RI + *Eco*RV, with *Eco*RI + *Eco*RV + *Msp*I (methylation insensitive), and with *Eco*RI + *Eco*RV + *Hpa*II (methylation sensitive). *Msp*I and *Hpa*II both recognise the sequence CCGG.

Region 1, which includes the 5′ end of the DM gene and 3′ end of the neighbouring 59 gene, was digested with *Hpa*II regardless of parental origin (fig 2A). The *Eco*RI–*Eco*RV fragment was absent when digests included *Hpa*II or *Msp*I, indicating that there are unmethylated CCGG sites on both chromosomes. The same result was obtained with samples from CDM, AOP, and AOM patients. In region 2, containing the central part of the DM gene, there was no digestion of either allele with *Hpa*II, although there was with *Msp*I (fig 2B). This indicates that the CCGG sequences within this region are methylated on both chromosomes. Again there were no differences between the three classes of DM patient. In region 3, containing the 3′ end of the gene and the CTG repeat, there was complete digestion of the *Eco*RI–*Eco*RV fragment with *Hpa*II or *Msp*I, showing that both alleles contain unmethylated sites (fig 2C). The fourth probe (M10M7), that maps distal to the 3′ end of the gene, hybridised to a larger *Eco*RI–*Eco*RV fragment (8 kb) that was completely digested with *Hpa*II or *Msp*I, again indicating the presence of unmethylated sites on both alleles (results not shown).

With probe pM10M6 the expansion of the CTG repeat characteristic of the DM mutation could usually be visualised, and an example of a congenital patient with an expansion smear of 4 to 5 kb is shown in fig 2C. The range of expansion sizes seen in the various classes of patient were described fully in our previous paper.[11]

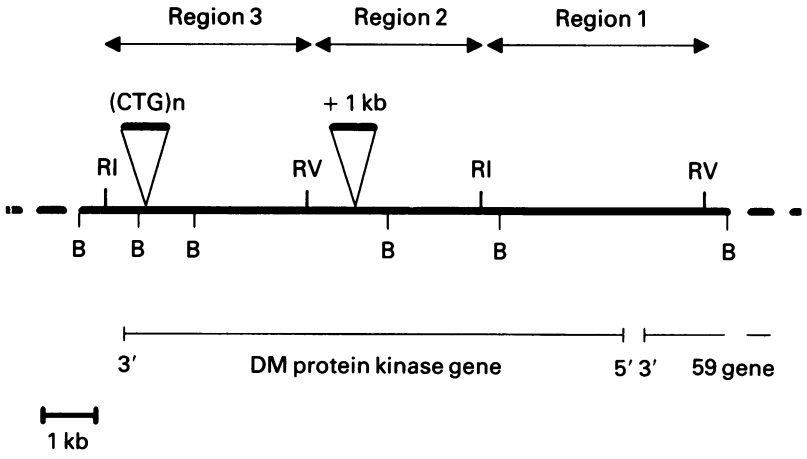

Figure 1 Restriction map of the genomic locus encompassing the myotonic dystrophy protein kinase gene and part of the neighbouring 59 gene. The intervals in which the exons of these genes are located are shown. The three regions of DNA detected by the probes used in the study are indicated, as are the positions of the CTG unstable repeat sequence and the 1 kb insertion giving rise to an RFLP. Sites for restriction enzymes: RI, EcoRI; RV, EcoRV; B, BamHI.

Discussion

Our results show that in the samples tested, there were no detectable differences in methylation status of the DM protein kinase gene between the paternally and maternally derived alleles. This observation was true for all the clinical categories of patients studied (congenital or adult onset with transmission from either parent). Thus we have found no evidence to support the hypothesis that genomic imprinting at the level of DNA methylation plays a role in the congenital form of myotonic dystrophy.

This study does, however, have some limitations that make it impossible entirely to exclude the involvement of methylation. First, the approach taken did not directly assay methylation at individual CCGG sequences, and it is possible that there are more subtle differences that would have been missed, such as the presence of one differentially methylated CCGG surrounded by others that were completely unmethylated and hence digested with *Hpa*II. Second, the samples of DNA assayed

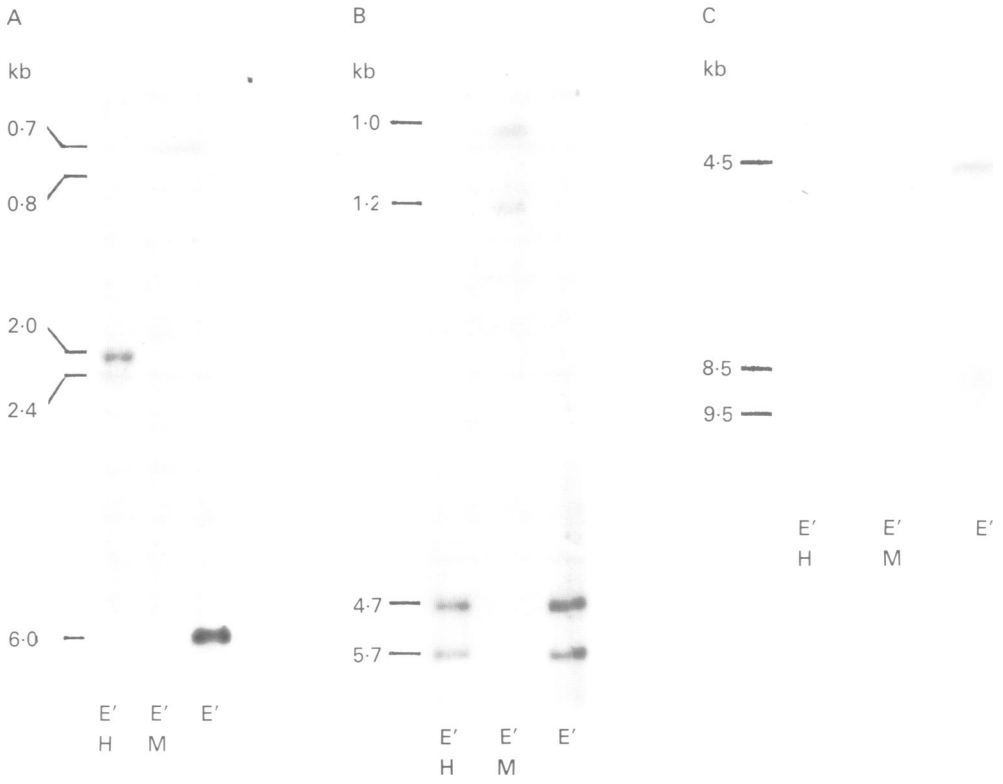

Figure 2 Southern blots of DNA from myotonic dystrophy patients, hybridised with (A) region 1 probe (p59A), (B) region 2 probe (pB2.7), (C) region 3 probe (pM10M6). The DNA in each track was digested with EcoRI+EcoRV (E′); with EcoRI+EcoRV+MspI (E′M); or with EcoRI+EcoRV+HpaII (E′H). In panel C is shown DNA from a congenital patient with a CTG expansion smear of 4 to 5 kb. In panel B the two alleles of the insertion polymorphism (4·7 and 5·7 kb) can be seen.

were taken from blood cells of relatively mature persons. In the case of congenital myotonic dystrophy patients, the samples would have been taken at a time when the patient was no longer affected with this form of the disease. It is possible that methylation may play a role at a stage of development, or in a particular tissue, that was not accessible to this study. For example, the development of the congenital form might be influenced by genomic imprinting in utero, with subsequent loss of the imprint after birth. For obvious practical reasons it would be hard to investigate this hypothesis extensively, although investigation of the DNA of congenitally affected infants who unfortunately died in the perinatal period, or of chorionic villus DNA of pregnancies predicted to end in a congenitally affected child, could be tried. In fragile X it has been shown that the characteristic methylation of the FMR-1 gene HTF island is present in fetal tissue but not in chorionic villus.[22]

A second gene (designated 59) has been found to lie very close to the myotonic dystrophy protein kinase locus, and its equivalent in the mouse was characterised recently.[20] In the present study we used a probe that identifies the region containing the 5′ end of the kinase gene and the 3′ end of the 59 gene, which have been shown by DNA sequencing to be less than 500 bp apart (Shaw *et al*, submitted). A role for the 59 gene in the pathology of myotonic dystrophy has yet to be established, but if this gene is important in the disease it will be of interest to study the methylation of its 5′ regions in the same way as has been done in the present study.

There is still no satisfactory explanation for the congenital form of myotonic dystrophy. It appears likely that the mother's degree of severity (which is related to the size of DNA expansion) plays a part, since generally speaking only mothers with overt muscle symptoms are at risk for congenitally affected offspring.[23] Thus there appears to be an interaction between the genotypes of the mother and offspring, which might be mediated, for example, by a transplacental factor or by the physiological state of the uterus. We are presently investigating the precise relationship between the extent of DNA expansion in the DM alleles of parents and offspring, the expression of the protein kinase and 59 genes, and the development of the congenital form of the disease.

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