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The DMPK Gene of Severely Affected Myotonic Dystrophy Patients Is Hypermethylated Proximal to the Largely Expanded CTG Repeat

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Summary

Using methylation-sensitive restriction enzymes, we characterized the methylation pattern on the 5′ side of the CTG repeat in the DMPK gene of normal individuals and of patients affected with myotonic dystrophy, showing expansions of the repetitive sequence. The gene segment analyzed corresponds to the genomic *Sac*I-*Hin*dIII fragment carrying exons 11–15. There is constitutive methylation in intron 12 at restriction sites of *Sac*II and *Hha*I, localized 1,159–1,232 bp upstream of the CTG repeat, whereas most, if not all, of the other sites of *Sac*II, *Hha*I, and *Hpa*II in this region are unmethylated, in normal individuals and most of the patients. In a number of young and severely affected patients, however, complete methylation of these restriction sites was found in the mutated allele. In most of these patients, the onset of the disease was congenital. Preliminary in vivo footprinting data gave evidence for protein-DNA contact in normal genes at an Sp1 consensus binding site upstream of the CTG repeat and for a significant reduction of this interaction in cells with a hypermethylated DMPK gene.

Introduction

Myotonic dystrophy (DM; OMIM 160900 [http://www3.ncbi.nih.gov:80/htbin-post/Omim/dispmim?160900] is an autosomal dominant multisystemic disorder that includes weakness and wasting of distal limb and face muscles and progressive myotonia (Harper 1989). The clinical manifestation is extremely variable. The most severe form is congenital, and symptoms comprise generalized muscular hypotonia, neonatal respiratory distress, feeding difficulties, bilateral facial weakness, talipes, delayed motor development, a high neonatal mortality rate, and mental retardation (Harper et al. 1992). The underlying mutations are expansions of the CTG repeat in the 3′ UTR of the DM kinase (DMPK) gene (GenBank X61378; http://www.ncbi.nlm.nih.gov/Web/Genbank/) (Aslanidis et al. 1992; Brook et al. 1992; Buxton et al. 1992; Harley et al. 1992). The repeat size shows some correlation with age at onset and with the severity of the disease (Hunter et al. 1992; Reardon et al. 1992; Tsilfidis et al. 1992), but there is no simple causal relation.

Several triplet-repeat expansions localized within a CpG island lead to hypermethylation of the corresponding gene region (Oberlé et al. 1991; Hirst et al. 1993; Knight et al. 1993; Nancarrow et al. 1994; Parrish et al. 1994; Jones et al. 1995; Schwemmle et al. 1997). In fragile X syndrome (OMIM 309550 [http://www3.ncbi.nih.gov:80/htbin-post/Omim/dispmim?309500]), hypermethylation of the CpG island of the FMR1 gene (GenBank X61378; http://www.ncbi.nlm.nih.gov/Web/Genbank) is associated with loss of transcription factor binding to the promoter region, which results in transcriptional repression (Bell et al. 1991; Oberlé et al. 1991; Pieretti et al. 1991; Verkerk et al. 1991; Sutcliffe et al. 1992; Verheij et al. 1993; Schwemmle et al. 1997). In this study, we analyzed part of the CpG island at the 3′ end of the DMPK gene for the methylation status as well as for a possible functional role of this island in transcriptional regulation.

According to previous reports, there has been no evidence of either parental imprinting playing a role in differential allelic expression of the DMPK gene (Jansen et al. 1993) or any allele-specific DNA methylation (Shaw et al. 1993). However, in contrast to the latter study, we discovered hypermethylation associated with reduced protein interaction at an Sp1 binding site localized in a gene segment upstream of largely expanded repeats with $\geqslant$1,000 CTG triplets. This modification of disease alleles was found in young, severely affected patients and may be another genetic factor causally related to earlier onset and more severe manifestation.

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Material and Methods

Cell Cultures

Cell cultures were established from dissected dura mater of a 16-wk-old DM fetus with a very large expansion (P57F), from skeletal muscle material of another, 13-wk-old, DM fetus (P70F), and from skin biopsies of normal adults, as described elsewhere (Wöhrle et al. 1995; Schwemmle et al. 1997). All cell cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM).

Genomic DNA Isolation and Southern Blot Analysis

Genomic DNA was extracted from white blood cells of various DM patients and normal individuals and from cultured fibroblasts, by use of standard procedures. Detailed information on the patients and on the sources of DNA samples are given in table 1. Aliquots were digested with restriction endonucleases (*Bam*HI, *Hpa*II, *Hha*I, *Hin*dIII, *Sac*I, or *Sac*II), separated on 0.8% agarose gels, blotted onto Hybond N^+ membranes, and hybridized with DNA probe pM10M6 radiolabeled by the random priming method. For analysis of DNA methylation, two types of experiments were carried out: (1) digestion of the genomic *Sac*I-*Hin*dIII fragment of the DMPK gene with methylation-sensitive enzymes *Sac*II or *Hha*I on triple digests; and (2) digestion of the genomic *Bam*HI fragment with *Hpa*II on double digests. Digests were carried out at standard conditions. Expansion sizes were measured on *Sac*I + *Hin*dIII digests.

In Vivo Footprinting and Ligation-Mediated PCR Analysis

For in vivo dimethyl sulfoxide (DMSO)–footprinting analysis, the cultured cells were washed twice with phosphate-buffered saline (PBS) and were subsequently treated with 0.1% DMSO in DMEM without serum for 8 min at room temperature. The DMSO-containing medium was then quickly removed, and the cells were washed with ice-cold PBS. DNA was then extracted by standard procedures, treated with piperidine, and finally subjected to ligation-mediated PCR (LMPCR), according to the protocols of Mueller and Wold (1989) and Pfeifer et al. (1989), which are described in detail in Schwemmle et al. (1997). The primers used for LMPCR of the lower strand were (1) 5′-GCC CCG TTG GAA GAC TGA-3′, (2) 5′-CCA CCC ACC GCC TGC CAG TTC ACA-3′, and (3) 5′-CGA GCG TGG GTC TCC GCC CAG CTC CAG T-3′.

Results

Constitutive Methylation

A schematic illustration of the gene structure, including restriction maps of the investigated gene region and the distribution of CpG dinucleotides, is presented in figure 1. On the *Sac*I-*Hin*dIII fragment, which overlaps with probe pM10M6 (Brook et al. 1992) and includes the CTG repeat, are numerous restriction sites of the methylation-sensitive enzymes *Hha*I and *Hpa*II and two sites of *Sac*II, which is also sensitive to methylation. On *Sac*I + *Hin*dIII double digests (fig. 2*a*), probe pM10M6 hybridizes to a normal fragment of ~1.78 kb. This is enlarged in mutated genes according to expansion of the CTG repeat carried on this fragment. Triple digests with *Sac*I + *Hin*dIII + *Sac*II of DNA from normal individuals resulted in *Sac*I-*Sac*II fragments of 1.55 kb, whereas the expected 1.2-kb *Sac*II-*Sac*II fragments were missing. Because there is also absence of a 1.4-kb *Sac*II-*Hin*dIII fragment, this finding indicates constitutive methylation of the upstream site of *Sac*II. Cleavage at the distal *Sac*II site is also demonstrated by *Bam*HI + *Sac*II double digests, resulting in a 1-kb fragment instead of the 1.4-kb *Bam*HI fragment carrying only the distal *Sac*II site (not shown). Triple digests, *Sac*I + *Hin*dIII + *Hha*I, gave 0.9-kb fragments, which are expected to occur if restriction sites of *Hha*I in intron 12 and exon 13 are also involved in this constitutive methylation and if most, or all, *Hha*I sites between exon 13 and the CTG repeat are unmethylated. On *Bam*HI + *Hpa*II double digests, no restriction fragments were seen, which indicates that in normal DMPK genes many, if not all, of the numerous *Hpa*II sites on the *Bam*HI fragment are unmethylated.

In the DNA of all DM patients examined, an expansion of the CTG repeat was detected on *Sac*I + *Hin*dIII digests, giving the normal band at $\geqslant$1.78-kb fragments corresponding to the various expansions (fig. 2*a* and 2*c*). After cleavage with *Sac*I + *Hin*dIII + *Sac*II, the normal signals are still seen, whereas, in many patients, the expansions could no longer be detected clearly (exceptions are described below, in the Hypermethylation of Disease Alleles in Severely Affected Patients subsection). This finding confirms that the restriction site of *Sac*II, 42 bp upstream of the repeat, is unmethylated. Cleavage of this site results in a restriction fragment with the repeat flanked by single-copy sequences of 42 bp on the 5′ and 126 bp on the 3′ side. The expanded repeats usually could not be visualized in this situation, because these expansions already appeared as broad smears when carried on much larger genomic fragments. On *Sac*I + *Hin*dIII + *Hha*I digests, again, only the normal 0.9-kb bands were detected, which shows that the repeat is not carried on such a fragment and that sites of *Hha*I adjacent to the unmethylated *Sac*II site are also unmethy-

Table 1

DM Patients, by Methylation Status

Methylation Status and Patient	Age at Examination (years)	Expansion Size (kb)	Parental Origin of Mutation	Disease Status
Hypermethylation:				
P57F	Prenatal	5.5	Maternal	(CMD)
4211	<1	3.6	Maternal	CMD
3842	<1	3.55	Maternal	CMD
3692	<1	3.5	Maternal	CMD
1496	?	3.5	Maternal	CMD
4106	24	3.4	Maternal	CMD
3320	12	3.4	Maternal	(CMD)
4009	8	3.3	Maternal	CMD
3319	17	3.2	Maternal	(CMD)
2931	20	3.1	...	?
Normal (constitutive):				
3103	45	4.2[a]	...	DM
3954	48	3.5[a]	...	?
3953	62	3.4[a]	...	DM
3252	40	3.1[a]	Maternal	DM
4148	38	3.0[a]	...	?
1778	53	2.8[a]	...	DM
4267	19	2.6[a]	Maternal	DM
0076	31	2.4[a]	...	?
1578	33	2.3[a]	...	DM
2416	26	1.8[a]	...	DM
2146	36	1.8[a]	Maternal	DM
1264	36	1.6[a]	Maternal	DM
1265	30	1.5[a]	Maternal	DM
1958	42	1.5[a]	...	DM
0055	26	1.5[a]	...	?
2621	33	1.4[a]		DM
2608	20	1.3[a]	Paternal	DM
1497	61	0.7[a]	...	DM
1413	58	0.6[a]	...	DM
P70F	Prenatal	0.6	Paternal	?

NOTE.—P57F and P70F are cell cultures established from tissues of DM fetuses (see Material and Methods). All the other numbers refer to patients whose DNA was isolated from white blood cells. CMD = congenital DM; (CMD) = onset probably congenital; and DM = disease onset not congenital. Ellipses dots (...) indicate missing data. A question mark (?) indicates that disease status is unknown.

[a] Smear of expansions.

lated (fig. 2*a*, 2*b*). A similar result was obtained on *Bam*HI + *Hpa*II digests (not shown).

Hypermethylation of Disease Alleles in Severely Affected Patients

We identified a number of DM patients whose DNAs gave a very different pattern. When additionally cleaved with methylation-sensitive enzymes *Sac*II, *Hha*I, or *Hpa*II, the sizes of expanded fragments remained unchanged (fig. 2*b* and 2*c*). There was one exceptional case (P57F) showing partial methylation of the disease allele on *Hpa*II but complete methylation on *Hha*I digests. Although there are many restriction sites (fig. 1), the genomic *Sac*II-*Hin*dIII fragment carrying the expanded CTG repeat is resistant to the methylation-sensitive enzymes. The mutated genes of these particular patients, therefore, in addition to the constitutive methylation described above, show specific hypermethylation of a large gene segment containing numerous CpG dinucleotides (fig. 1). This hypermethylation of disease alleles was found in both fibroblasts and leukocytes and was present in an affected fetus (cell line P57F) as well as in adult tissues.

Association of Hypermethylation with Large Repeat Expansion and Severe Phenotype

In the DM patients who exhibited hypermethylation of the disease allele (table 1), repeat expansions were 3.0–5.5 kb and appeared in most cases as sharp bands on Southern blots. These patients were young and se-

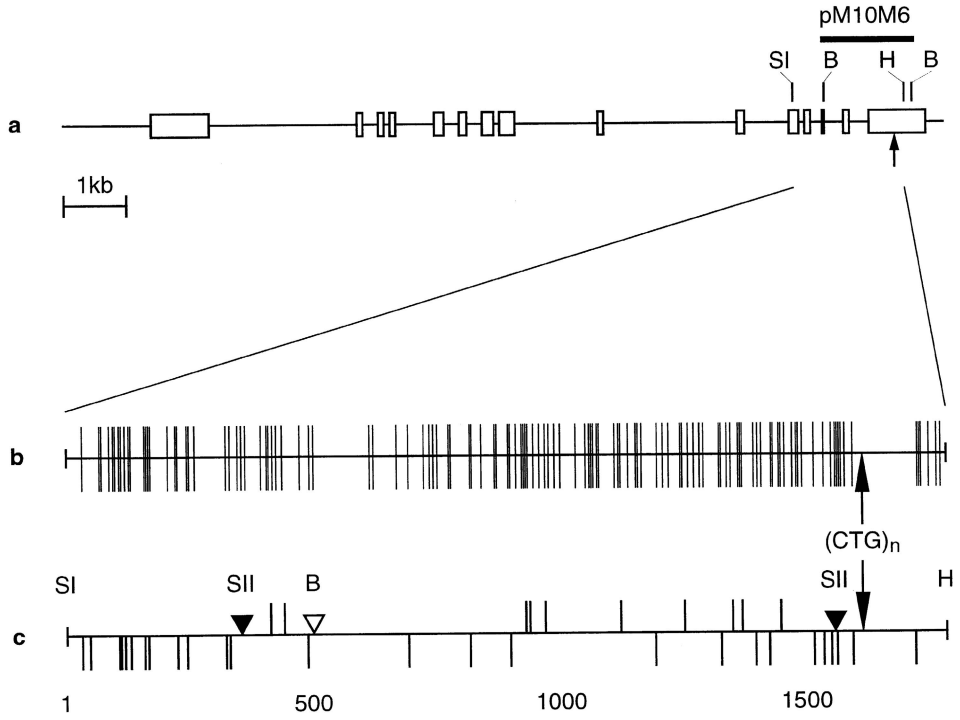

Figure 1 Schematic presentation of the DMPK gene structure and of the genomic region investigated (nt 1–1775). *a,* Exon-intron boundaries. *b,* CpG dinucleotide distribution. *c,* Sites of restriction enzymes in 5′ region of the CTG repeat. B = *Bam*HI; H = *Hin*dIII; SI = *Sac*I; SII = *Sac*II: ⊥ = *Hha*I; and ⊤ = *Hpa*II. In this scheme, the the first nucleotide corresponds to nt 12634 of the published sequence (Mahadevan et al. 1993).

verely affected, most of them having congenital or very early onset of the disease. One case of hypermethylation was identified prenatally in a 16-wk-old fetus who was the donor of the cell line P57F and was suspected to develop congenital myotonic dystrophy because of a maternally inherited expansion. In contrast, the DM patients showing no specific methylation of the disease allele were less severely affected, had older ages, and had later onsets of disease. Their expansions were 0.6–4.2 kb, with those extending into the size range of the hypermethylated alleles appearing as broad smears. These patterns suggest that the patients originally received smaller expansions and that their smears of larger expansions resulted from continual instability of the repeat in somatic tissues (Monckton et al. 1995; Wöhrle et al. 1995).

In Vivo Footprinting Studies

In the fetal cell line (P57F) and in fibroblasts from normal adult individuals, the in vivo protein–DNA interaction was studied in a segment of 120 bp (nt 1490–1610, according to the numbering system of fig. 1), located within the hypermethylated region of the mutated gene (fig. 3). In normal cells (fig. 3, lane 2), we identified a footprint at an Sp1 consensus binding site (5′-GGC GGG-3′). In the P57F cells, carrying a normal gene and a hypermethylated disease allele, the G residues of this binding site were clearly visible, indicating missing of protein binding on either one or both alleles (fig. 3, lane 1).

Discussion

Our findings address the long-standing question why the CTG-repeat expansion in the 3′ untranslated sequence of the DMPK gene can result in an autosomal dominant and variable phenotype (Wieringa 1994). Several hypotheses have been put forward. The precise level of DMPK may be critical to normal cellular function, and repeat expansion may give rise to haploinsufficiency (Fu et al. 1993). The mutant allele may alter chromatin structure and affect transcriptional activity of both the DMPK and other closely linked genes on chromosome 19 (Boucher et al. 1995; Jansen et al. 1995; Otten and Tapscott 1995; Wang and Griffith 1995). This conclusion implies the existence of a locus control region (Wieringa 1994). The expansion of the repeat may be a dom-

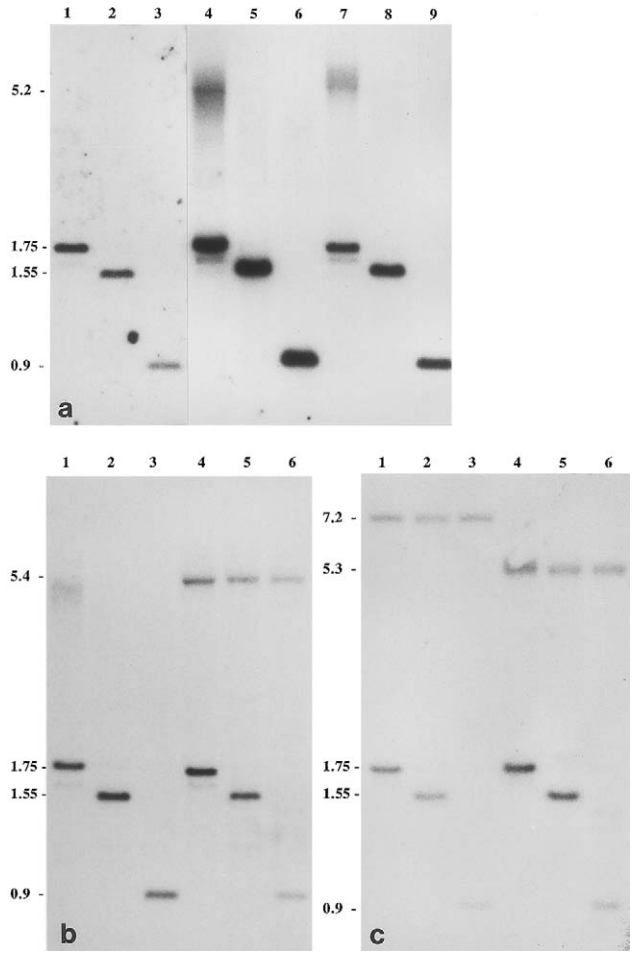

Figure 2 Southern blot analysis of DNAs from a normal individual (*a,* lanes 1–3) from DM patients with early- or late-adult onset (*a,* lanes 4–9; *b,* lanes 1–3), and from DM patients with congenital onset (*b,* lanes 4–6; *c,* lanes 1–6). The DNAs were digested with *Sac*I + *Hin*dIII (*a,* lanes 1, 4, and 7; *b,* lane 4; *c,* lanes 1 and 4), with *Sac*I + *Hin*dIII + *Sac*II (*a,* lanes 2, 5, and 8), or with *Sac*I + *Hin*dIII + *Hha*I (*a,* lanes 3, 6, and 9; *b,* lane 6; *c,* lanes 3 and 6) and were hybridized to probe pM10M6. The patients' identifiers (see table 1) are as follows: 3953 (*a,* 4–6), 3954 (*a,* 7–9), 4148 (*b,* 1–3), 4211 (*b,* 4–6), P57F (*c,* 1–3), and 1496 (*c,* 4–6). With the exception of P57F, all the DNA samples were isolated from white blood cells. P57F is a cell line from a fetus suspected to have congenital DM.

Figure 3 In vivo footprint analysis showing the Sp1 consensus binding site at position 13149–13156 in the published genomic sequence (Mahadevan et al. 1993). Lane 1, Fibroblasts of a DM fetus (P57F) with hypermethylated disease allele. Lane 2, Fibroblasts of a normal control individual. G = visible G residue; (G) = protected G residue.

inant gain-of-function mutation exerted at the RNA level (Wang et al. 1995; Timchenko et al. 1996). Repeat expansion may lead to reduction of poly(A)+ mRNAs (Carango et al. 1993; Fu et al. 1993; Hofmann-Radvanyi et al. 1993; Pizzuti et al. 1993; Wang et al. 1995) affecting DMPK gene expression at the posttranscriptional level (Krahe et al. 1995). Transcripts from the mutant allele have been found, by DMPK mRNA–specific in situ hybridization analysis, to accumulate within intranuclear foci (Taneja et al. 1995). Subcellular fractionation of RNA and the separated analysis of DMPK transcripts from each allele revealed that trancripts from expanded alleles were completely retained within the nucleus (Davis et al. 1997; Hamshere et al. 1997). Nuclear retention of DMPK transcripts occurs above a critical threshold of 80–400 CTGs but does not effect the cytoplasmic levels of transcripts from other genes at the DM locus (Hamshere et al. 1997). Because there is no simple causal relation between repeat size and phenotype, which is documented by several examples of patients with similar repeat sizes but different forms of the disease, many of the proposed factors, as well as other mechanisms hitherto unknown, may contribute to the pathophysiology of DM.

We have detected disease allele–specific DNA methylation in a segment proximal to the largely expanded CTG repeat of severely affected patients, most if not all

of them having congenital DM. This finding was substantiated by in vivo footprinting analysis detecting binding of transcription factor Sp1. The Sp1 footprints are present on unmethylated alleles, but most of them are probably absent from the methylated gene sequence. Sp1 binding is known to be methylation insensitive (Harrington et al. 1988) but to be involved in the regulation of methylation at CpG islands (Brandeis et al. 1994).

Our results seem to be in contrast to a previous study of DNA methylation in DM by Shaw et al. (1993). On the 4.5-kb *Eco*RV-*Eco*RI fragment encompassing the 1.8-kb *Sac*I-*Hin*dIII fragment that we investigated, they did not find any methylation at the *Msp*I-*Hpa*II sites in any DM patient. Ten patients with symptoms present at birth, including hypotonia and respiratory problems, were analyzed; however, the report of Shaw et al. did not include information on either the ages of patients at the time the blood samples were drawn or the sizes of expansions and the patterns the expansions formed on Southern analysis. The latter were shown from only one congenital case, who presented an expansion of ~3.5 kb, forming a heterogeneous smear as broad as 1 kb. In our study, we never detected any disease-specific methylation with a smear. All the methylated alleles we described formed sharp bands of expansions >3 kb in size that are much more typical of young patients with congenital DM.

Another difference between the two methylation studies is that Shaw et al. investigated only the *Msp*I-*Hpa*II sites, whereas we studied the sites of different enzymes (*Sac*II, *Hha*I). *Hpa*II sites were also studied in two cases, both showing complete methylation of *Sac*II and *Hha*I sites on the disease alleles. One was a blood DNA sample from a congenitally affected patient (1496) who also showed complete methylation of the *Hpa*II sites on the *Bam*HI fragment that includes probe M10M6. The other DNA sample was from cultured cells of a DM fetus (P57F) and showed only partial methylation of at least one of the *Hpa*II sites on the *Bam*HI fragment. When our findings and the results reported by Shaw et al. are taken together, the following suggestion can be made: hypermethylation of disease alleles may show variable spreading toward the CTG repeat from a hypothetical methylation center that could be located near the proximal, constitutively methylated *Sac*II site (fig. 1). Therefore, the most distant *Hpa*II sites at the 5′ end of the CTG repeat could only occasionally be involved in (possibly unstable) methylation, in contrast to the distal *Sac*II site and the sites of *Hha*I, which are closer to the hypothetical center and more distant from the CTG repeat. Variable spreading and a possible age-dependent instability of methylation at the edge of the methylated region might explain why Shaw et al. never detected an expanded *Eco*RV-*Eco*RI fragment that was resistant to cleavage with *Hpa*II but we always found the expanded *Sac*I-*Hin*dIII fragment of congenitally affected patients unchanged on *Sac*II and *Hha*I digests.

In all patients who were found to have a hypermethylated disease allele, the expanded *Sac*I-*Hin*dIII fragments formed a sharp band on Southern analysis and the expansion sizes were >3 kb. Only the latter condition was met by some older patients showing smears of expansions and only the constititive methylation on both alleles. In contrast to the younger patients, whose expansion sizes may still be similar to that of the originally inherited progenitor allele, the smears in the older patients probably resulted from an ongoing mitotic instability of the repeat (Wöhrle et al. 1995), leading to slow but continual further expansion throughout the patients' lives, associated with an increase of size heterogeneity (Monckton et al. 1995; Wong et al. 1995; Zatz et al. 1995). The overlap of repeat size in the two groups of DM patients separated in table 1 is probably explained by age-related unstable expansion, since hypermethylation of expanded disease alleles may only occur in an early developmental stage.

Because hypermethylation is associated with a large size of the CTG repeat, an important question addresses the origin of such a methylation. Hypermethylation of adjacent CpG islands has also been found to occur with large expansions of CGG and CCG repeats (Oberlé et al. 1991; Knight et al. 1993; Nancarrow et al. 1994; Parrish et al. 1994; Jones et al. 1995; Schwemmle et al. 1997) and may be a consequence of expansion of these repeats (Smith et al. 1994; Malter et al. 1997). A detailed discussion of this interesting point is included in another article (S. Schwemmle, unpublished data). In brief, largely expanded triplet repeats form secondary structures interfering with chromatin structure and, in particular, with protein-DNA contacts by which CpG-island methylation is regulated. For example, large expansion of the CTG repeat probably eliminates an adjacent nuclease-hypersensitive site of a putative transcriptional enhancer of the DM locus-associated homeodomain protein (DMAHP) gene (Otten and Tapscott 1995; Klesert et al. 1997). The 5′ end of the DMAHP gene, previously identified by Boucher et al. (1995), is only ~1 kb downstream of the CTG repeat of DMPK. Repeat expansion converting the region surrounding the repeat to a more condensed chromatin structure may lead to a reduced accessibility of transcription factors and thereby to reduced expression in *cis* of DMAHP, as was recently found to be the case in DM patients with expansions of $\geqslant$900 triplets (Klesert et al. 1997; Thornton et al. 1997). Such a conclusion must, however, remain speculative, because there are other published data that demonstrate no effect of CTG-repeat expansion on the level of expression of the 59 and DMAHP genes (Hamshere et al. 1997).

Instead of this significant, as yet unresolved discrep-

ancy between the published data on DMAHP expression, there are two lines of evidence that the hypermethylation of disease alleles, described in our study, may have a functional significance in modifying the phenotypic manifestation of the DM mutation by affecting local gene expression. (1) Hypermethylation is not only associated with repeat expansion to $\geqslant$1,000 CTG triplets but also with earlier onset and more severe manifestation of the disease. (2) The hypermethylated 3′ region of the DMPK gene is part of a large CpG island with many CpGs being unmethylated in normal individuals and does, according to our footprinting results, contain at least one site of protein-DNA interaction, indicating its possible functional involvement in transcriptional regulation.

Repeat expansion probably also affects transcription factor binding to the putative enhancer of the downstream gene of DMAHP (Klesert et al. 1997). Therefore, the large CpG island extending on both sides of the CTG repeat could be a region controlling the chromatin structure and thereby the expression of genes at the DM-chromosome locus.

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