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Myotonic dystrophy: evidence for a possible dominant-negative RNA mutation

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The trinucleotide expansion mutation causing myotonic dystrophy is in the 3′ untranslated region of a protein kinase gene. The molecular mechanisms by which the expanded repeat causes the clinically variable and multisystemic disease, myotonic dystrophy, are not understood. It has been particularly difficult to rationalize the dominant inheritance with the fact that the expansion mutation lies outside of the protein-encoding gene elements, and should not be translated into protein. Here we use muscle biopsies from classical adult-onset myotonic dystrophy patients to study the accumulation of transcripts from both the normal and expanded DM kinase genes in patient muscle, and compare the results to normal and myopathic controls. We found relatively small decreases of DM kinase RNA in the total RNA pool from muscle; however, these reductions were not disease specific. Analysis of poly(A)$^+$ RNA showed dramatic decreases of both the mutant and normal DM kinase RNAs, and these changes were disease-specific. Our findings are consistent with a novel molecular pathogenetic mechanism for myotonic dystrophy: both the normal and expanded DM kinase genes are transcribed in patient muscle, but the abnormal expansion-containing RNA has a dominant effect on RNA metabolism by preventing the accumulation of poly(A)$^+$ RNA. The ability of the expansion mutation to alter accumulation of poly(A)$^+$ RNA *in trans* suggests that myotonic dystrophy may be the first example of a dominant-negative mutation manifested at the RNA level.

INTRODUCTION

Myotonic dystrophy is one of the most common monogenic inherited disorders in Caucasians, and is caused by a trinucleotide repeat expansion of a gene on 19q encoding a protein kinase (1–5). Clinical expression of the disorder is extremely variable, with some patients showing only development of cataracts in middle-age, while others show severe neonatal hypotonia leading to death unless ventilatory assistance is provided. The 'classical' presentation is a progressive muscular dystrophy affecting distal muscles more than proximal, often associated with the inability to relax muscles appropriately (myotonia); this presentation is the origin of the disorder's name. The striking diversity of organ systems affected, and the marked variability in clinical symptoms seemed to be rationalized with the finding that myotonic dystrophy is one of the 'expansion disorders': a polymorphic trinucleotide repeat sequence within a gene mutates into an abnormally large size range, whereupon it becomes unstable and causes disease. Indeed, the clinical severity of myotonic dystrophy corresponds to the extent of the pathological expansion.

The effect that the pathological expansion has on the gene product of the mutant gene is clear for most of the expansion disorders, and the molecular pathology is consistent with the inheritance patterns. In Fragile X, the expanded repeat in the 5′ untranslated region of the gene causes hypermethylation of the promoter, and under-expression of the gene (6): this is consistent with a 'loss of function' in this X-linked recessive disorder. The other expansion disorders (e.g. Kennedy's disease, Huntington's disease) show expansions in the coding sequence of the protein, and the polyglutamine or polyglutamic acid tracts in the mutant protein are thought to alter the DNA binding specificity of the gene product: this is consistent with the 'change of function' seen in the mutant protein products of most dominantly inherited disorders (7). Indeed, the clinical disorder caused by a loss of function deletion of the androgen

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receptor gene (testicular feminization) is quite distinct from a repeat expansion in the same gene (Kennedy's disease; motor neuron degeneration).

The effect of the repeat expansion in myotonic dystrophy has remained an enigma, both in molecular rationale and molecular data. The repeat is located in the 3′ untranslated region of a protein kinase gene, and therefore should have no effect on the primary sequence of the protein product. However, it is dominantly inherited, which would suggest a change of function of the protein product. The effect of the expansion on tissues is also unique and apparently quite pleotropic. Many tissues are involved with distinct cellular abnormalities, and the range of tissues and abnormalities are dependent on the age of presentation. Eyes develop cataracts; muscle develops electrical hyperexcitability, degenerates, and shows striking central nucleation; there is characteristic facial dysmorphology with low-set, out-turned ears; distal and facial weakness are most prominent (most muscular dystrophies show preferential involvement of proximal musculature); the heart shows conduction defects; there is premature frontal balding; the brain is involved with frequent mental retardation.

The effect of the repeat expansion on RNA expression of the DM kinase gene is critical to distinguish between loss of function and change of function of the protein product. Results to date have been contradictory, with some studies suggesting that the expanded repeat causes a loss of function of the mutant allele (8,9), others showing decrease in expression of both the mutant and normal genes (10), and others showing increase of expression of the expanded gene (11). The variability in the results of these studies is reflected in the many different experimental systems and methods used to test for RNA levels.

In this report we use a systematic and novel approach to dissecting the question of RNA expression, using quantitative comparisons of RNA levels of normal and mutant DM kinase transcript in muscle biopsies from patients with classical adult-onset myotonic dystrophy. Our results show that the gene containing the expansion is expressed, and the levels of transcript accumulating to high levels in the total RNA pool. We show that the expansion mutation interferes with the accumulation of poly(A)$^+$ RNA of both the normal and mutant allele. Our data suggest that the pleiotropic nature of myotonic dystrophy may be caused by altered cellular RNA metabolism: a change of function at the RNA level.

RESULTS

Myotonic dystrophy patient biopsies show high levels of DM kinase RNA in total RNA pools, but a marked reduction in poly(A)$^+$ DM kinase RNA

We have recently shown quantitative multiplex fluorescence PCR (QMF–PCR) (12) to be an accurate method to measure relative levels of RNAs in small tissue samples. We used this method to quantitate the levels of DM kinase RNA relative to muscle-specific creatine kinase (CK) RNA in nine adult-onset myotonic dystrophy patient muscle samples, three myopathic disease controls, and four biopsies showing no evidence of pathology (normal controls). Relative levels of the two RNAs were tested in both total RNA and poly(A)$^+$ RNA fractions. All RNA samples were purified through CsCl cushions, and contained no DNA contamination as shown by absence of automated sequencer signals when reverse transcriptase was omitted from the RT–PCR reactions.

Total RNA (2.5 μg) and poly(A)$^+$ RNA isolated from 2.5 μg of total RNA were reverse transcribed using DM kinase (11) and CK gene-specific primers simultaneously. cDNA corresponding to 60 ng of total RNA was subjected to 20 cycle QMF–PCR with fluorescent forward primers, and the RT–PCR products analyzed on an ABI automated sequencer using GeneScan software. All samples were tested in triplicate. DM kinase PCR product was 187 bp, and CK was 224 bp, and both were located near the 3′ end of the corresponding cDNAs.

In total RNA, the levels of DM kinase RNA in myotonic dystrophy patient muscle biopsies were reduced to approximately half of the levels seen in normal controls (Fig. 1). There was inter-sample variation, with some patient biopsies showing levels which overlapped with the normal controls (Fig. 2). The myopathic controls also showed relative reductions in DM kinase RNA, with levels similar to those seen in myotonic dystrophy patients. Thus, the relatively minor reduction in DM kinase levels in myotonic dystrophy muscle was not disease-specific. A single series of experiments was done with transferrin receptor RNA as the control, and the amount of DM kinase RNA in myotonic dystrophy patient muscle showed the same two-fold reduction in total RNA pools compared with normal muscle. In addition, the raw sequencer quantitation of peak areas for CK RNA using the same amount of input cDNA were similar, and overlapped between all patient groups (data not shown).

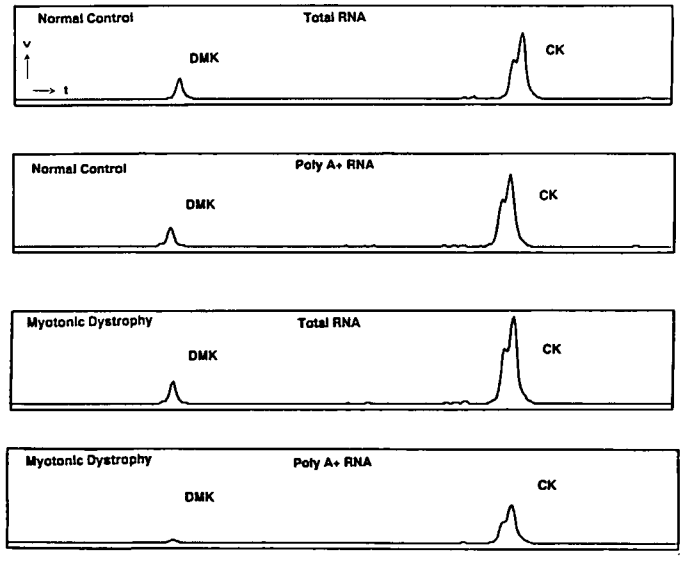

Figure 1. Quantitative multiplex fluorescent RT–PCR (QMF–PCR) of DM kinase and CK RNA in myotonic dystrophy patient muscle biopsies. Shown are automated sequencer traces of fluorescent RT–PCR products. CK and DM kinase (DMK) RNAs were simultaneously amplified from patient muscle biopsy cDNA. The areas of the peaks were used to calculate the relative levels of DM kinase RNA in each biopsy as a function of CK RNA levels. This method has been previously shown to be an accurate and reproducible means of quantitating relative RNA levels. The ratios of DM kinase and CK stay relatively constant in the normal controls, independent of whether total or poly(A)$^+$ fractions were tested. In myotonic dystrophy patient muscle, the DM kinase RNA is present at relatively high levels in the total RNA fraction, but is significantly decreased in the poly(A)$^+$ fraction.

In the poly(A)$^+$ RNA fraction of the same samples, both the normal controls and myopathic controls showed ratios of DM kinase and CK mRNAs which were similar to the ratios seen in the total RNA pools. In contrast, the myotonic dystrophy biopsies uniformly showed a marked reduction in DM kinase poly(A)$^+$ mRNA relative to CK poly(A)$^+$ mRNA (Fig. 2). This result suggested that there was a disease-specific loss in poly(A)$^+$ DM kinase mRNA in myotonic dystrophy patient muscle.

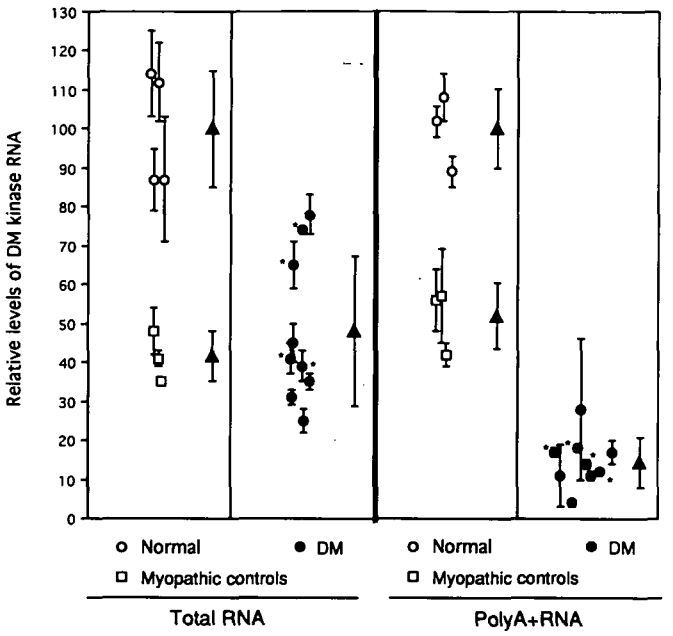

Figure 2. Myotonic dystrophy patient muscle shows normal accumulation of DM kinase in the total RNA pool, but decreased accumulation in the poly(A)$^+$ mRNA pool. Shown are the average and standard error for triplicate measurements of DM kinase RNA as a function of CK RNA levels in muscle biopsies from four normal control muscle biopsies (open circles), three myopathic control muscle biopsies (open squares), and nine myotonic dystrophy patient muscle biopsies (closed circles). The average and standard error for all samples within each patient group is also shown (triangles). Those data points marked with an asterisk indicate the myotonic dystrophy patients used for the allele-specific DM kinase RNA quantitations (Tables 2 and 3). All values from total RNA are normalized to the average ratio seen in normal muscle controls. Likewise, poly(A)$^+$ RNA values are normalized to the average value seen in normal muscle biopsies. DM = myotonic dystrophy patients.

Quantitation of normal and expanded transcripts shows expression of the mutant allele in patient muscle

To determine the contribution of the normal and mutant DM kinase RNAs in the QMF–PCR results, 27 myotonic dystrophy patients and 18 unrelated disease controls were tested for heterozygosity of a *Bpm*I polymorphism (11) in exon 11 of the DM kinase coding sequence. Thirty patients were studied using DNA isolated from frozen muscle biopsy, and 15 from peripheral blood DNA. Six muscle biopsy DNA samples were found to be heterozygous (four DM, two normal controls). Blood samples were obtained from the parents of three of the four DM patients to determine the phase between the polymorphism and the mutant repeat expansion. In each case, the presence of the enzyme site (allele A) was on the same DM kinase gene as the CTG expansion mutation (Fig. 3). Parents of the fourth heterozygous patient were not available for analysis; however, all of the remaining 26 DM patients showed linkage of the DM allele with allele A of the *Bpm*I site. Given linkage disequilibrium studies and the high frequency of the *Bpm*I site, it is likely that the fourth patient showed the same phase as the other three patients (>96% chance). All four patients showed a clinical phenotype and CTG expansions consistent with classical adult-onset myotonic dystrophy (Table 1).

Fluorescent RT–PCR was done with DM kinase primers flanking the *Bpm*I polymorphism. Omission of reverse transcriptase eliminated all fluorescent signal, indicating that all signal was specific for RNA. Formation of heteroduplexes between the two *Bpm*I alleles after RT–PCR and prior to restriction enzyme digestion could be a source of error in quantitation; the heteroduplexes will not cut with the *Bpm*I enzyme. The formation of heteroduplexes is dependent on PCR product concentration; the amount of undigested PCR product should increase relative to the digested product with increasing cycle number. We used cDNA synthesized from total RNA from patient A II-1, and performed triplicate RT–PCR experiments for 20, 22, 24, and 26 cycles of PCR. We observed the expected increase in signal intensity with increasing cycle number, and the 24 and 26 cycle reactions required a 1/10 dilution to remain in the linear detection range of the automated sequencer. The ratio of peak area (*Bpm*I digested vs. undigested PCR product; $n = 3$) were as follows: 20 cycles = 0.86 ± 0.11; 22 cycles = 0.82 ± 0.09; 24 cycles = 0.79 ± 0.08; 26 cycles = 0.89 ± 0.06. These data show no change in the ratio of digested/undigested PCR product with increasing cycle number, indicating that hetero-

Table 1. Clinical and molecular genetic presentation of four DM patients with *Bpm*I polymorphism

Patient	Age of biopsy	Age of onset	Clinical severity					(CTG) repeats
			Muscle	Endocrine	Brain	Heart	Eye	
A II-1	33	14	Myotonia, marked distal atrophy and hypotonia	Testicular atrophy	Moderate mental impairment	Normal	Bilateral cataract	1485
B II-1	30	24	Myotonia, marked distal atrophy and hypotonia	Testicular atrophy	Normal	Mitral valve prolapse	Lens opacities	1140
B II-2	27	27	Myotonia and marked distal atrophy	Not evaluated	Normal	Mitral valve prolapse	Normal	850
C	65	30	Myotonia, marked distal atrophy and hypotonia	Testicular atrophy	Mild mental impairment	Conduction defect	Macular dystrophy	795

duplexes are not a significant source of error. Twenty cycle PCR was chosen for all subsequent experiments.

The four heterozygous DM patients and two heterozygous normal controls were tested for relative levels of RNA corresponding to the two alleles by fluorescent RT−PCR. The same amount of total RNA (2.5 μg), and poly $(A)^+$ RNA isolated from 2.5 μg total RNA, were reverse transcribed using a DM kinase gene-specific primer. cDNA corresponding to 60 ng of total RNA was subjected to 20 cycle PCR, the PCR products digested with *Bpm*I, and the digested products analyzed on an ABI automated sequencer using Genescan software. All cDNA samples were tested in triplicate RT−PCR experiments, and data presented using peak areas and standard deviation.

A normal control homozygous for the *Bpm*I site showed complete digestion, with a fluorescent peak at 136 bases (Fig. 4, top panel). There was a slightly larger secondary peak which probably represents partial filling in of the 5′ overhang of the *Bpm*I site by *Taq* polymerase. These peaks were considered additive in determination of peak areas. The two heterozygous normal controls showed two major peaks of similar height in RT−PCR of total RNA (allele A = *Bpm*I digested; allele B = undigested; Fig. 4, normal control #1). In these two normal muscle biopsies studied, allele A was found in total RNA at a higher level (130–174%) compared to allele B (100%). Parallel analysis of the four heterozygous DM patients showed the mutant allele (allele A) to be expressed at relatively high levels (56–86% of normal allele B)(Table 2). These data indicate that the DM kinase gene containing the expansion is expressed, and the RNA accumulates in patient muscle.

Analysis of poly$(A)^+$ RNA from the same samples showed similar levels of the two alleles in the normal controls. To the contrary, the myotonic dystrophy patients showed a reduction in the amount of the mutant allele to 20–25% of the normal allele (Table 2).

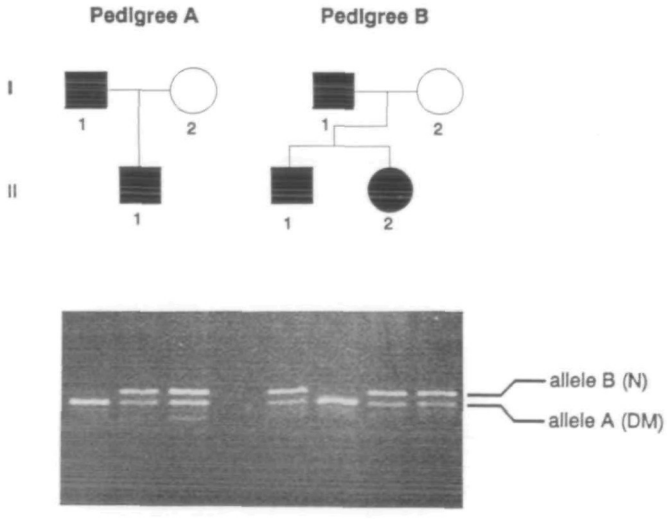

Figure 3. Determination of phase between *Bpm*I polymorphism and CTG expansion mutation. Shown are the pedigrees corresponding to three of the four DM patients used in this study (pedigree A, II-1; pedigree B, II-1, II-2). Each patient shows linkage of the disease with allele A, consistent with the presence of the *Bpm*I enzyme site being in phase with the CTG expansion mutation.

Both normal and mutant DM kinase RNAs show a disease-specific decrease in poly$(A)^+$ RNA accumulation

The results of the QMR−PCR and the allele-specific *Bpm*I RT−PCR data were correlated for the four myotonic dystrophy patients and two normal controls (Table 2). The QMF−PCR data provided the levels of the DM kinase RNAs (mutant + normal alleles) relative to CK RNA. The *Bpm*I experiments gave the relative levels of mutant and normal DM kinase RNA. Thus, we can derive the levels of the mutant and normal DM kinase RNAs relative to CK RNA by combining the two sets of data (Table 3).

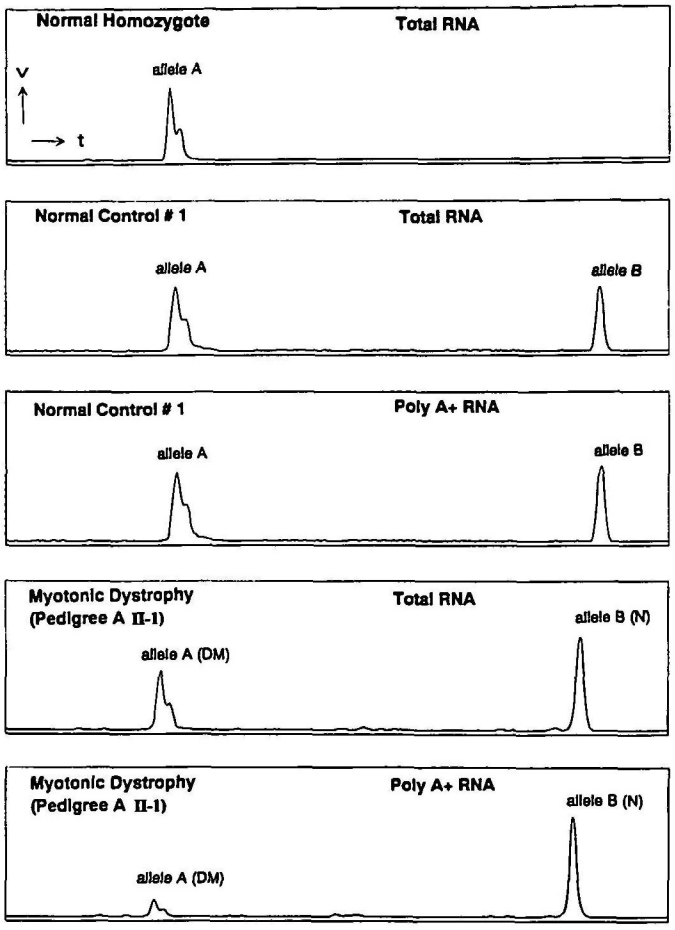

Figure 4. RNA from the mutant DM allele is expressed, but shows decreased polyadenylation. Shown are automated sequencer traces (ABI 373A) from fluorescent 20 cycle RT−PCR experiments. Total RNA and poly$(A)^+$ RNA from each muscle biopsy was reverse transcribed using a gene-specific primer downstream of the *Bpm*I polymorphic site in the DM kinase coding sequence. Single-stranded cDNA was amplified by PCR for 20 cycles with the forward primer covalently labeled with fluorescein. Fluorescent RT−PCR products were digested with *Bpm*I, and electrophoresed on automated sequencer gels to resolve the two alleles. The top panel shows RT−PCR product from a normal control muscle, and shows complete digestion of the PCR product by *Bpm*I (allele A). The next two panels show RT−PCR products from heterozygous control muscle. Both allele A and allele B show similar levels of RNA expression in both total RNA and poly$(A)^+$ RNA. The lower two panels show RT−PCR products from a myotonic dystrophy patient (pedigree A, II-1). Allele A is in phase with the DM mutation, and shows decreased expression relative to the normal allele (allele B) in both total and poly$(A)^+$ RNA. Expression is more dramatically decreased in the poly$(A)^+$ RNA.

In the two heterozygous normal controls, each of the two DM kinase genes in each biopsy showed a slight decrease in poly(A)$^+$ RNA levels relative to CK RNA (11–15%; Table 2). This result shows that the levels of each DM kinase gene in normal muscle maintains a constant ratio relative to CK RNA both in total RNA and poly(A)$^+$ RNA.

In the myotonic dystrophy muscle samples, solving for the levels of the normal and mutant DM kinase RNAs relative to CK RNA showed that, in total RNA, the normal allele accumulated to levels equal to those seen in the control muscles. Accumulation of the DM kinase RNA containing the expansion mutation in total RNA was about half that of the normal gene, but this value still overlapped with those seen in the disease controls. In the poly(A)$^+$ fraction, the expanded mRNA showed dramatic reductions of accumulation; relative to CK RNA, 86% of the mutant DM kinase RNA in the total RNA pool was lost upon isolation of poly(A)$^+$ RNA. Importantly, the normal DM kinase RNA also showed dramatic reductions in accumulation in the poly(A)$^+$ fraction; about 70% of the RNA corresponding to the normal DM kinase gene was not shifted to the poly(A)$^+$ fraction. This was in marked contrast to the normal controls, where only 10% of the DM kinase alleles were not shifted to the poly(A)$^+$ compartment (Table 3).

DISCUSSION

Previous studies of DM kinase gene expression in myotonic dystrophy have provided discordant results. Some studies have suggested that the expanded repeat precludes gene expression, leading to a loss of function of the protein product. This scenario suggests that myotonic dystrophy may represent a 'haploinsufficiency' syndrome, where gene expression from the normal DM kinase gene present in each patient is not sufficient to preclude clinical symptoms. There are a number of observations which seem to argue against this model. First, monogenic haploinsufficiency syndromes are quite rare. The primary examples are inherited cancers, but these result in somatic loss of function, and therefore represent a pathogenetic mechanism quite distinct from myotonic dystrophy. A few other examples exist (familial hypercholestrolemia, acute intermittent porphyria); however, these disorders are not 'congenital' *per se*, instead reflecting insufficient biochemical reserve during the patient's interaction with the environment. Myotonic dystrophy is a congenital defect.

Second, it is conceptually difficult to synthesize the haploinsufficiency model with the clinical variability associated with varying trinucleotide repeat size. For example, a pathological repeat expansion of 500 repeats in a 'classical' adult-onset myotonic dystrophy patient presumably causes loss of expression of the mutant DM kinase gene. An expansion of 3000 repeats in a congenital myotonic dystrophy baby presumably similarly causes loss of function of the mutant allele. If we assume that the adult-onset patient shows some residual expression of the mutant allele (5%), then his total kinase protein (mutant plus normal genes) should be about 55% of normal. If we assume that the congenital patient shows complete absence of expression of the mutant allele, then his total kinase protein should be about 50% of normal. It is difficult to visualize how very minor changes in DM kinase protein (55% vs. 50%) could cause such dramatically different symptoms in a wide variety of tissues.

Third, knock-out mice with a complete lack of DM kinase activity show none of the phenotypic features of human patients with myotonic dystrophy (D.Housman, personal communication). Indeed, DM kinase knock-out mice show no abnormality of any kind. While animal models of human disease often show phenotypic differences compared with the human disease, this observation would seem to argue against a loss of function of the DM kinase as the molecular basis for myotonic dystrophy.

Finally, some reports suggest that transcription is increased rather than decreased from the expanded gene (11). These authors have legitimately pointed out that an increase in expression from the mutant gene is more consistent with a dominant inheritance pattern (change/gain of function). It is, however, difficult to compare the results of the different DM kinase expression studies, as there have been substantial variability in patient tissues and experimental methods employed. For example, most studies have used tissues from autopsies from neonatal congenital myotonic dystrophy

Table 2. The DM kinase RNA from allele containing the expansion mutation (allele A) shows reduced accumulation in DM patient's muscle biopsies

Individual	Total RNA allele A% relative to B	Poly (A)$^+$ RNA allele A% relative to B
Normal #1	174 ± 18 %	162 ± 31%
Normal #2	135 ± 12%	129 ± 8%
DM A II-1	86 ± 10%	24 ± 1%
DM B II-1	62 ± 5%	22 ± 1%
DM B II-2	84 ± 7%	25 ± 1%
DM C	56 ± 6	20 ± 2%

Table 3. Decreased levels of DM kinase RNA from both normal allele (allele B) and mutant allele (allele A) in DM patient's muscle biopsies

Individual	Allele A (mutant allele)			Allele B (normal allele)		
	Total RNA	Poly(A)$^+$ RNA	% of loss in poly(A)$^+$RNA	Total RNA	Poly(A)$^+$ RNA	% of loss in poly(A)$^+$ RNA
Normal #1	1371 ± 252	1158 ± 225	14.6 ± 10.3	787 ± 124	703 ± 106	11.6 ± 14.3
Normal #2	1287 ± 63	1110 ± 19	13.7 ± 2.9	929 ± 81	817 ± 46	11.7 ± 5.5
DM A II-1	845 ± 22	94 ± 32	88.3 ± 3.5	989 ± 17	260 ± 22	73.7 ± 2.5
DM B II-1	344 ± 47	46 ± 6.6	86 ± 3.5	530 ± 35	176 ± 20	67 ± 2.6
DM B II-2	476 ± 39	56 ± 16	87 ± 4	558 ± 53	159 ± 28	71 ± 3
DM C	357 ± 22	38 ± 4	89.6 ± 1.5	617 ± 56	204 ± 9	66.3 ± 4.5

Levels DM kinase from both alleles are nomalized with CK RNA from the muscle biopsies: (DMK/CK)×10 000.
Allele loss (%) in poly(A)$^+$RNA: [allele A (total) − allele A (polyA$^+$)/allele A total]×100.

patients; this neonatal disease is rare, and is clinically quite different from the classical adult-onset disease.

We have recently developed two RNA quantitation protocols using automated sequencers. One, QMF–PCR, accurately measures the relative levels of two or more mRNA species. The second takes advantage of polymorphic restriction enzyme sites to quantitate RNA levels from the two alleles of the same gene; we recently used this assay to show that clinical severity in dominantly inherited periodic paralysis (change of function of sodium channel) may be correlated with the relative levels of mutant and normal allele transcription (13). In this study, we used QMF–PCR to quantitate the levels of DM kinase RNA relative to CK RNA. We studied a series of muscle biopsies from classical adult-onset myotonic dystrophy patients, and compared the results with both normal and myopathic control biopsies.

We found a reduction in the level of DM kinase RNA in the total RNA fraction relative to CK RNA. Importantly, we found that the reductions in DM kinase RNA in the total fraction were not disease specific; myopathic controls showed similar relative decreases. CK protein levels are known to change with physiological state of the muscle, or during degeneration/regeneration in myopathies.

Isolation of poly(A)$^+$ RNA from the same biopsies showed dramatic reductions in DM kinase poly(A)$^+$ RNA compared with both normal and myopathic controls. The disease-specific decrease in the accumulation of poly(A)$^+$ DM kinase RNA to approximately 10–20% of levels seen in normal and normal myopathic muscle biopsies were greater than could be explained by loss of function of mutant transcript alone.

The QMF–PCR experiments did not differentiate between the normal and mutant DM kinase RNAs. To quantitate the contribution of both the normal and expanded DM kinase RNAs to the total DM kinase RNA measurements, we identified four myotonic dystrophy patients and two normal controls who were heterozygous for a polymorphism in the coding sequence of the DM kinase gene. Twenty cycle RT–PCR using sequence-specific primers flanking this site allowed precise quantitation of the relative levels of the mutant and normal DM kinase transcripts in each patient. Combining the results of this analysis with the QMF–PCR data for these six biopsies permitted quantitations of each RNA relative to CK RNA.

We found that the expanded trinucleotide dramatically reduced accumulation of both the normal and mutant DM kinase transcripts in the poly(A)$^+$ mRNA compartment. The normal transcript was present at levels similar to those seen in normal muscle; however, the level of the same transcript in the poly(A)$^+$ fraction was decreased by over 66%, and this alteration was disease-specific. The mutant transcript showed mild reductions in the total RNA fraction (~60% of normal), but showed even greater reductions in the poly(A)$^+$ fraction; over 86% of the expanded RNA did not move to the poly(A)$^+$ pool compared with normal controls.

There are a number of interpretations of our results. It is possible that the assay method we used (relative levels of DM kinase vs. CK RNAs by quantitative fluorescent RT–PCR), is subject to some unknown artefact which is biasing the results. We found similar results in total RNA pools with DM kinase vs. transferrin; however, other RNAs should be tested as controls to verify our results in both total and poly(A)$^+$ RNA pools. Unfortunately, alternative experimental procedures such as Northern blotting, or RNAse protection, are difficult to perform with limited muscle biopsy specimens. Furthermore, these techniques are generally unable to accurately quantitate and distinguish between the mutant and normal alleles.

If we assume that the results we obtained accurately reflect the steady-state levels of RNAs in patient muscle *in vivo*, our results suggest that the expansion mutation has only a minor effect on transcription and accumulation of DM kinase hnRNA containing the expansion mutation. Instead, the mutation dramatically alters the ability of the mutant RNA to be processed into poly(A)$^+$ mRNA. More importantly, the same expansion-containing hnRNA seems to affect the accumulation of the normal DM kinase mRNA from the normal allele *in trans*.

This interpretation suggests a 'dominant-negative RNA mutation' model, which is consistent with the dominant inheritance pattern observed in myotonic dystrophy. The expansion mutation is transcribed into hnRNA which then alters the RNA metabolism in myofibers, inhibiting the accumulation of poly(A)$^+$ DM kinase mRNA from both the normal and abnormal gene. If the mutant RNA can affect alterations in the accumulation of the normal DM kinase mRNA, then it is possible that it can alter the accumulations of other species of mRNA sharing similar regulatory elements. This model could explain the tissue- and development-specific features of myotonic dystrophy; each tissue has populations of stage-specific RNAs, which could be affected differently by the expanded DM kinase RNA. The lack of a clinical phenotype in the homozygous mouse knock-out if the DM kinase gene is also consistent with this model; the mutant gene must be expressed in order to produce the clinical disease.

A 'dominant-negative RNA mutation' would be a novel mechanism for generating human disease, and considerable additional study is required to validate this hypothesis. If this model is correct, then the DM kinase RNAs should probably bind specific RNA regulatory proteins, and these must be identified and characterized. Furthermore, *in vitro* transfection studies should confirm the effect of the expansion mutation on RNA metabolism both *in cis* and *in trans*.

MATERIALS AND METHODS

Muscle biopsies

Muscle biopsies flash-frozen in isopentane cooled in liquid nitrogen were studied from 16 patients. Nine biopsies were from patients with classical adult-onset myotonic dystrophy, and all had DNA-confirmed expansions of the trinucleotide repeat (range 83–1550 repeats). Hematoxylin and eosin (H&E) staining was done for all biopsies. Cryosections of each biopsy were stained for embryonic fetal myosin heavy chain (MHCemb) (14,15) (diluted 1:1000) to test for the presence of regenerating myofibers. Five of the nine biopsies showed very mild histopathology with no evidence of degeneration of myofibers by H&E, and no regenerating fibers by MHCemb immunostaining. Four of the nine DM biopsies showed more extensive histopathology, with a high prevalence of central nuclei, focal fibrofatty replacement of the muscle, myofiber splitting. Two of the biopsies showed isolated degenerating myofibers, and two showed MHCemb-positive regenerating fibers (1–2% of total fibers; >1000 fibers studied). The four *Bpm*I heterozygotes used for allele-specific RNA quantitation included three patients with 'mild' histopathology, and one patient with 'more extensive' histopathology.

Three biopsies (myopathic controls) were from patients referred for dystrophin protein testing, but all had normal dystrophin. One patient was a 15 year old male with a 3 year history of progressive muscle weakness, elevated serum CK (3950–7377), and myopathic electromyograph and muscle biopsy. This patient's biopsy showed an active dystrophic process with myofiber

splitting, central nuclei, and mild endomysial and perimysial fibrosis, and approximately 1% regenerating myofibers by MHCemb staining. A second myopathic control was a 9 year old male with a history of myoglobinurea and proximal muscle weakness, elevated serum CK (2000–3000), with a myopathic muscle biopsy. This patient's muscle showed no evidence of active degeneration or regeneration, with mild perimysial fibrosis. The third myopathic control was a 23 year old male with a history of myoglobinurea and distal muscle wasting, elevated serum CK (1073–11 530), and myopathic muscle biopsy. This patient's muscle showed an active dystrophic process, with central nuclei, endo and perimysial connective tissue proliferation, fiber splitting. Approximately 20% of myofibers stained positive for MHCemb antibodies.

The four normal controls had no evidence of myopathic histopathology. Two were surgical specimens obtained during heart surgery. One was diagnosed with hyperparathyroidism. After surgery for removal of the parathyroid gland, muscle weakness was noted. Muscle biopsy showed normal histology. The last patient is a 19 year old female who reported pain in the quadriceps when running. Neurological exam and laboratory studies were normal. Muscle biopsy showed no abnormalities.

Isolation of DNA from peripheral blood and muscle biopsy

Blood was collected in EDTA tubes, and processed for PCR as previously described (16). DNA was purified from frozen muscle biopsies as follows. Approximately 40 cryostat sections, 4 μm thick, were cut for each biopsy. Muscle sections were kept frozen until solubilized in lysis buffer (16). Proteinase K digestion in PCR buffer was done at 60° for 2 h. Proteinase K was inactivated with phenol-chloroform and chloroform extraction. DNA was concentrated using Amicon Microcon microconcentrators.

Identification of *Bpm*I heterozygotes

PCR primers for detection of the *Bpm*I polymorphism were as previously described (11). For amplification of RNA (RT–PCR) primers DMK-1 and DMK-2 were used. For amplification of DNA, primers DMK-2 and DMK-3 were used (11). Digested PCR products were resolved on 2% agarose gels.

Purification of muscle biopsy RNA and synthesis of cDNA

Muscle biopsies were preexisting specimens used for diagnostic purposes. All biopsies had been frozen in isopentane cooled in liquid nitrogen soon after excision, and stored at −80°C. Fragments of the biopsies (150 mg) were homogenized with Brinkman Polytron homogenizer in isothiocyanate buffer (17), and RNA pelleted through a CsCl cushion. Samples were rinsed with cold 70% ethanol, and resuspended in TE and 1% SDS. RNA integrity was verified by agarose gel electrophoresis, and concentration determined by OD260.

cDNA was produced from both total RNA and poly(A)$^+$ RNA. Poly(A)$^+$ RNA was purified from 2.5 μg of total RNA from each biopsy using Fasttrack mRNA isolation kits (Invitrogen). Total RNA (2.5 μg), or all poly(A)$^+$ RNA isolated from 2.5 μg of total RNA, was input into the cDNA synthesis. Each cDNA synthesis contained 200 ng of sequence-specific primer (DMK-1 and/or CK-1), 5.0 units of AMV reverse transcriptase (BRL), and dNTPs and buffers as per manufacturers' instructions. cDNA synthesis was done at 42°C for 1 h. Each cDNA synthesis was boiled for 2 min to inactivate reverse transcriptase and denature the RNA/DNA hybrid cDNA.

Quantitation of DM kinase RNA

The same DMK-1 and DMK-2 primers11, located 1014–1201 bp in the cDNA sequence, were used for QMF–PCR quantitation of DM kinase RNA relative to CK, and for the allele-specific *Bpm*I quantitation of normal and mutant RNAs, and amplify a 187 bp fragment. For the CK RNA, primers were designed against region 997–1221 bp of the human CK cDNA sequence18, and amplify a 224 bp fragment. Primer sequences were: CK1 (5′ CTTCTGGGCGGGGATCATGTC 3′); CK2 (5′ AGTTCGAGGAGATCCTCACCCGC 3′). The transferrin receptor primers were as previously described (8). Primers DMK-2, CK-2, and the reverse transferrin receptor primer were synthesized with an amino-link (ABI) residue at the 5′ end, and covalently bound to carboxyl-fluorescein (Molecular Probes) as previously described (14). PCR reactions for both QMF–PCR and allele-specific *Bpm*I quantitations contained primers (100 ng each) and cDNA corresponding to 60 ng of total RNA, and were amplified for 10 cycles at 94°C 1 min, 55°C 1 min, 72°C 1 min, and then an additional 10 cycles at 94°C 1 min, 60°C 1 min, and 72°C 1 min (total of 20 cycles). For QMF–PCR of DM kinase RNA relative to CK or transferrin receptor RNAs, portions of PCR reactions (25 μl) were mixed with an equal volume of sequencing gel loading buffer, denatured, and aliquotes electrophoresed on an ABI 373A automated sequencer using matrix files specific for fluorescein.

For allele-specific *Bpm*I detection of normal and mutant DM kinase RNA, portions of the RT–PCR reactions (10.5 μl) were digested for 1 h at 37°C with 3.0 units of *Bpm*I using conditions suggested by the manufacturer. Equal volumes of the restriction enzyme digestion and sequencing gel loading buffer were mixed, denatured at 94°C for 2 min, and then placed on ice. Samples (5 μl) were loaded on automated sequencer gels. The amount of digested PCR products loaded in each lane corresponds to amplification from 4 ng of total RNA input. Automated sequencer gels were run for 6 h at 30 W using Genescan software (ABI). Lane assignments were done manually from the image files, and the areas of the peaks corresponding to fluorescent peaks quantitated by the Genescan software using PMT voltages. All experiments were done in triplicate, and results presented as mean and standard error.

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