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Myotonic Protein Kinase Expression in Human and Bovine Lenses

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Myotonic dystrophy (DM) is an autosomal dominant trait closely associated with CGT repeat expansions in the same locus on human chromosome 19q13.3. The expansions occur in the 3′ untranslated region of a transcription unit encoding a serine-threonine kinase (DM kinase) of a new class based upon structure and function. Lens cataracts are a prominent finding in myotonic dystrophy. DM kinase was shown to be expressed in human and bovine lenses at the RNA level and in human lenses at the protein level. Sequencing of PCR products of RNA extracted from normal human lenses demonstrated an exact match to published genomic and cDNA 3′ UTR sequences. Northern blots of bovine lens RNA showed that the transcript is similar in size to the transcript detected in other tissues that are affected in myotonic dystrophy. A polyclonal antibody (DM-2) was produced against recombinant DM protein kinase in rabbits. Development of Western blots with DM-2 showed a single reactive band of 67 kDa. Immunofluorescent studies of formalin-fixed human lens sections detected the DM kinase in the perinuclear cytoplasm of normal human lens epithelial cells and more diffusely in superficial subcapsular cortical fibers. In contrast, the same antibody labeled the nucleus most prominently in a single DM lens.

Myotonic dystrophy (DM) is inherited as an autosomal dominant trait in which characteristic cataracts of the eye lens and skeletal muscle weakness and myotonia are prominent clinical findings (1). The severity of phenotype is closely associated with an unstable expansion of a CTG repeat in the 3′ untranslated region (3′UTR) of a predicted DM protein kinase (2,3).

Although cataracts are rarely observed before age 10 even in the most severely affected congenital DM patients, virtually all DM patients exhibit specific iridescent opacities by age 50 (1,4,5). Ultrastructural studies suggest that these particles are multilaminated membranous structures derived from the plasma membrane which refract light to give the appearance of colored flecks (4-6). Protein crystalloids are occasionally detected in anterior epithelial (4-6).

As a first step toward understanding the effects of myotonic dystrophy in lens, we report here on the expression of DM protein kinase at the RNA and protein levels in human and bovine lenses. Using a specific antibody raised against DMPKH, a truncated recombinant construct containing only the catalytic and helical domains of DM kinase (7), we localized protein kinase at a cellular level by immunofluorescence microscopy in human control lenses and a DM lens.

MATERIALS AND METHODS

J.T. Baker: $MgCl_2$, KH_2PO_4, K_2HPO_4, NaCl, Kcl; Sigma: Trizma Base, EDTA, phenylmethylsulfonylfluoride (PMSF), benzoyl-L-arginine ethylester, Nα-p-tosyl-L-aryinine methylester, dithiothreitol; Fisher: glycerol, SDS, bromophenol blue, β-mercaptoethanol, glass slides; Difco: Freund's Complete and Incomplete Adjuvants; Organon Teknika Corp: fluorescein isothiocyanate-conjugated goat anti mouse IgG; Kirkegaard and Perry Labs: peroxidase-conjugated goat anti-rabbit IgG; Cinna/Biotecx: RNAzolB; Biorad: Zeta-Probe nylon filters; Boehringer-Mannheim: soybean trypsin inhibitory leupeptin, chymostatin, pepstatin; Kodak: X-omat ARX-ray film; ICN: Sequenase PCR product Sequencing Kit.

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A. Generation of rabbit polyclonal antibodies to myotonic dystrophy protein kinase. 100 μg of gel-purified DMPKH, a bacterially expressed form of DM kinase containing its catalytic and helical domains (7), in 1 ml of elution buffer was vortexed with 3 ml of Freund's Complete Adjuvant to generate an emulsion. One half of the emulsion was injected into lymph nodes of anesthetized New Zealand white female rabbits and the other half was injected intradermally at 5 sites along the back. Four and six weeks later, the rabbits were boosted with 2 ml of equal volumes of elution buffer and Incomplete Freund's Adjuvant containing 100 μg of purified DMPKH. After the second boost, rabbits were bled and boosted at two week intervals. IgG was prepared by selective precipitation with ammonium sulfate followed by DEAE-cellulose chromatography. This polyclonal antibody was designated pAB DM-2.

B. Preparation of lens proteins and immunoblotting. Whole lenses were incubated in 0.01 M Tris HCl, pH 7.4, 1 mM EDTA, pH 8.0, 0.05% dithiothreitol (DTT), 1% SDS, 1 mM PMSF and 1 μg/ml each benzoyl-L-arginine ethyl ester, N^{α}-p-tosyl-L-arginine methyl ester, soybean trypsin inhibitor, leupeptin, chymostatin, and pepstatin for 6 min, the supernatant transferred to gel loading buffer (60 mM Tris-HCl, pH 6.8, 1 mM bromophenol blue, 10% glycerol, 1% SDS, and 1% β-mercaptoethanol), boiled for 2 min and 25 μg of protein loaded onto a 7.5 % SDS-PAGE. Gel electrophoresis and immunoblotting were carried out as previously described (7,8). Membranes were incubated at room temperature for 1 h with purified pAb DM-2 IgG (1.2 X 10^{-7} M), followed by incubation for 1 h with peroxidase-labeled goat anti-rabbit IgG (1:5000 dilution). Following three 10 min washes at room temperature, the membrane was immersed for 1 min in Lumiglo chemiluminescent substrate. Membranes were blotted to remove excess liquid, placed between plastic sheets, then exposed to X-ray film for various time periods.

C. Northern analysis of bovine lens RNA. Total RNA was isolated by the guanidinium thiocyanate method (9) using RNAzol B from freshly cut bovine lenses that had been immediately frozen in liquid nitrogen. Thirty μg of total RNA was separated on a 1% agarose, 6% formaldehyde gel, and transferred to nylon filters by capillary transfer (10). The blot was probed with an α-$^{32}PO_4$-labelled DM kinase cDNA (2) and incubated overnight in 0.25 M $NaPO_4$, pH 7.2, 0.25 M NaCl, 5% SDS, 10% PEG 8000, 1 mM EDTA, pH 8.0, at 60°C. The blot was washed in 1X SSC, 1% SDS twice for 5 min at room temperature, once in 0.1X SSC, 0.1% SDS for 15 min at room temperature, and once in 0.1X SSC, 0.1% SDS for 5 min at 60°C.

D. RT-PCR analysis and sequencing of normal human lens RNA. Total RNA isolated from frozen human lenses, as described above, was used for first strand cDNA synthesis using a specific reverse primer 7409 (5′ CACTTTGCG-AACCAACGATAGG 3′) following a protocol previously described (11). Forward primer 7006 (5′ GGCTCGAAG-GGTCCTTGTAGCCGG 3′) and reverse primer 7409 were used to amplify the cDNA under the following PCR conditions: an initial denaturation at 95°C for 4 min, followed by 35 cycles of 94°C for 1 min, 64°C for 45 s, 72°C for 2 min, with a final elongation of 72°C for 5 min. 25 ng of the PCR product was sequenced with either primer 7006 or 7409 using the Sequenase PCR Product Sequencing kit according to manufacturer's protocol.

E. Preparation and sectioning of formalin-fixed, paraffin-embedded human lenses. Freshly cut human lenses obtained through the Lions Eye Bank of the Baylor College of Medicine were bisected sagittally and immediately fixed in 10% formalin for 4 h, then dehydrated by successive incubations for 1 h each in 95% ethanol, 100% ethanol and 100% chloroform at room temperature. Lenses were then infused with paraffin at 52°C. Using a Leitz 1512 microtome, 5 μm sections were cut from paraffin blocks, collected on Superfrost Plus electrostatically modified glass slides, and incubated for 1 h at 60°C.

F. Immunofluorescence microscopy. Sections of normal human adult formalin-fixed, paraffin-embedded lenses were incubated with purified pAb DM-2 IgG (6 X 10^{-7} M) in PBS (0.13 M NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4), 5% goat serum, 0.1 % Tween 20 either for 1 h or overnight at room temperature. Slides were rinsed three times in PBS, pH 7.4, then incubated with an affinity purified FITC-conjugated goat anti-mouse IgG in PBS, 5% goat serum for 1 h at room temperature at 1:200 dilution. After three washes in PBS, the sections were covered by 0.1% p-phenylenediamine, 10% PBS, 90% glycerol, mounted with coverslip and stored at −20 °C in the dark.

RESULTS

A. Myotonic Dystrophy Protein Kinase RNA Is Expressed in Human and Bovine Lenses

To determine whether a myotonic dystrophy protein kinase transcript is expressed in lens at the RNA level, total RNA was extracted from pairs of lenses from post-mortem normal adult human subjects and from frozen bovine lenses. Since the availability of human lenses for RNA extraction was limited, DM kinase transcription was evaluated using RT-PCR analysis. Using oligonucleotide primers flanking the CTG repeat region in the 3′ untranslated region of DM protein kinase, PCR products of the predicted size (194 base pairs) were detected (Figure 1A). To verify that the PCR bands were genuine products of the DM locus, PCR products were sequenced and the sequence was identical to the published DM protein kinase cDNA sequence (Figure 1B) (2,3,12-14).

Total RNA from bovine lenses were transferred to Immobilon-NC and probed with α-$^{32}PO_4$-

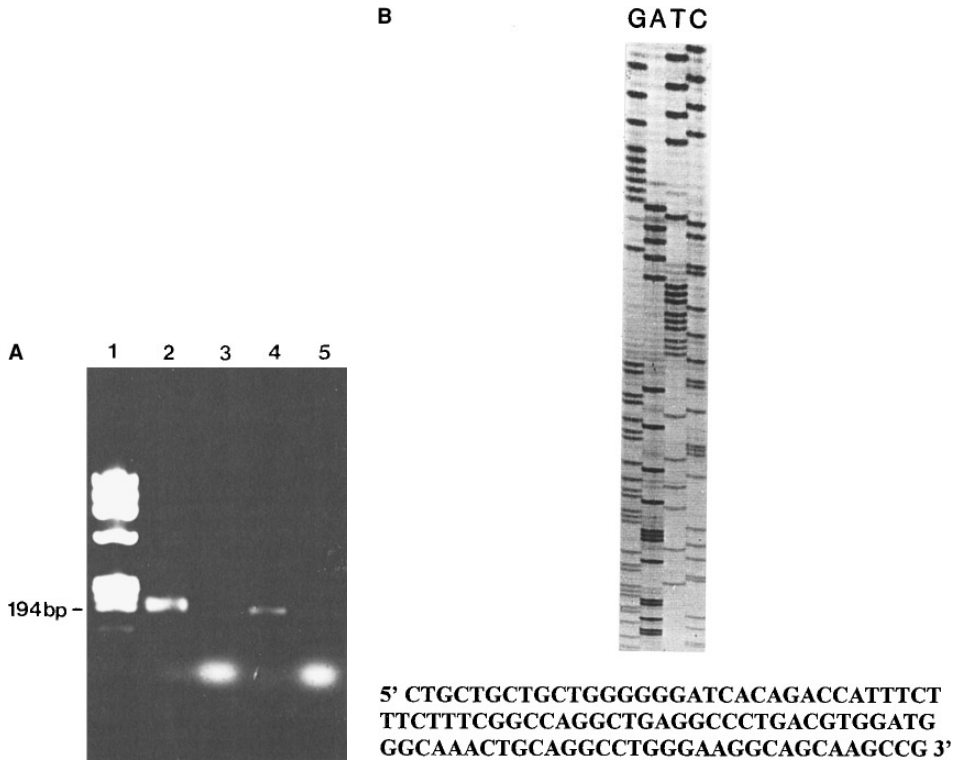

FIG. 1. Expression of the myotonic dystrophy protein kinase mRNA in lens. (**A**) RT-PCR products of total RNA from a pair of post-mortem human lenses from each of two normal (non-DM) subjects. Lane 1, ΦX174-HaeIII molecular weight standards (Life Technologies), lanes 2 and 4, RT-PCR products of RNA from individuals 1066 and 1119, respectively, lanes 3 and 5 are the same as 2 and 4 with the omission of reverse transcriptase to control for genomic DNA contamination. (**B**) Sequence of RT-PCR product from individual 1066 using the forward primer 7006. The sequence is shown at the bottom.

labelled DM kinase cDNA (2). A single band of 3.1 kb was detected (Figure 2), consistent with DM kinase transcript sizes reported previously (2,3).

B. Polyclonal IgG to Recombinant DM Protein Kinase Recognizes a 67 kDa Protein in Human Lens

We used an immunochemical approach in order to determine whether DM protein kinase was expressed at the protein level in bovine and human lenses. A polyclonal antibody (pAb DM-2) was raised against the bacterially expressed truncated DM protein kinase (DMPKH) which contains the catalytic and α-helical zipper domains of the predicted full-length gene product (7). The antibody reacts primarily with the carboxyl terminal portion of the recombinant protein which contains the helical region since it reacted only weakly with the kinase domain alone. Whole adult bovine and post-mortem normal adult human lenses were separated by SDS-PAGE, transferred to Immobilon-NC membranes and reacted with pAb DM-2. Figure 3 shows that a single broad band of 67 kDa reacted with the antibody in human lens. The same size band was detected in both human cardiac and skeletal muscle reacted with DM-2 (P.W. Dunne, L. Ma and H.F. Epstein, unpublished experiments). The antibody did not react with mouse or rat lenses, and therefore may be specific to the human DM kinase α-helical zipper domain. The antibody reacted with both the bacterially expressed DMPKH (71 kDa) in lane

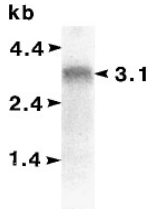

FIG. 2. Northern transfer of total RNA from bovine lens probed with ^{32}P-ATP-labeled DMPK cDNA[2].

1 and the predicted full-length construct expressed in BC3H1 cells (72 kDa) (8). The basis for the lower M_R of the lens protein is not known. Two potential explanations, each with an experimental basis, are 1) RNA spliceoforms that do not express the most carboxyl terminal putative transmembrane domain have been detected (2,3,13,14). The encoded protein would be similar to our DMPKH construct. 2) The phosphorylation patterns of DM kinase appear to be complex. The M_R of the protein is sensitive to its state of phosphorylation (7,8) as is the case with certain other protein kinases (17). The several immunoreactive bands of the bacterially expressed DMPKH are most likely due to phosphorylation differences (8), and all of these bands coordinately increase in mobility with further genetic truncation to the catalytic domain alone (7). The breadth of the main bands possibly result from the creation of microheterogeneous species due to phosphorylation at multiple different sites which is compatible with predictions of potential phosphorylation sites in the kinase itself (2,3).

C. Anti-DM Protein Kinase Antibodies Label Cytoplasm in Lens Epithelial Cells and Subcapsular Cortical Fibers

Formalin-fixed, paraffin-embedded sagittal sections of post-mortem normal adult human lenses were reacted with pAb DM-2. The antibody displayed cytoplasmic labeling with increased intensity in the perinuclear region in anterior epithelial cells and a more uniform cytoplasmic staining in anterior and posterior subcapsular cortical fibers whereas the cell nuclei appeared unlabelled (Figure 4 A, B). The depth of fiber staining varied, being most shallow at the posterior pole and having the greatest depth in the equatorial region. The mature anucleate nuclear fibers were unlabelled. Goat anti-rabbit secondary antibody alone or with preimmune rabbit serum did not label similar lens sections. Recombinant DMPKH protein was capable of blocking all labeling of lens sections with pAb DM-2.

D. pAb DM-2 Labels Epithelial Cell Nucleus in DM Patient Lens

To determine whether the expansion mutation affects the localization of DM protein kinase in the lens, formalin-fixed sections from both normal human lenses and a lens from a DM

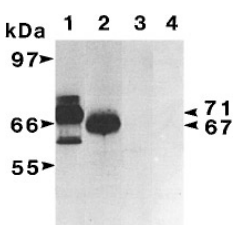

FIG. 3. Immunoblot of control human lens protein reacted with pAb DM-2. Lane 1, 0.1 μg of recombinant DMPKH as control. Lens proteins reacted with pAb DM-2 (lane 2), with rabbit preimmune serum (lane 3), or with secondary antibody alone (lane 4). The lanes are all from the same slab SDS-PAGE but have been rearranged for direct comparison.

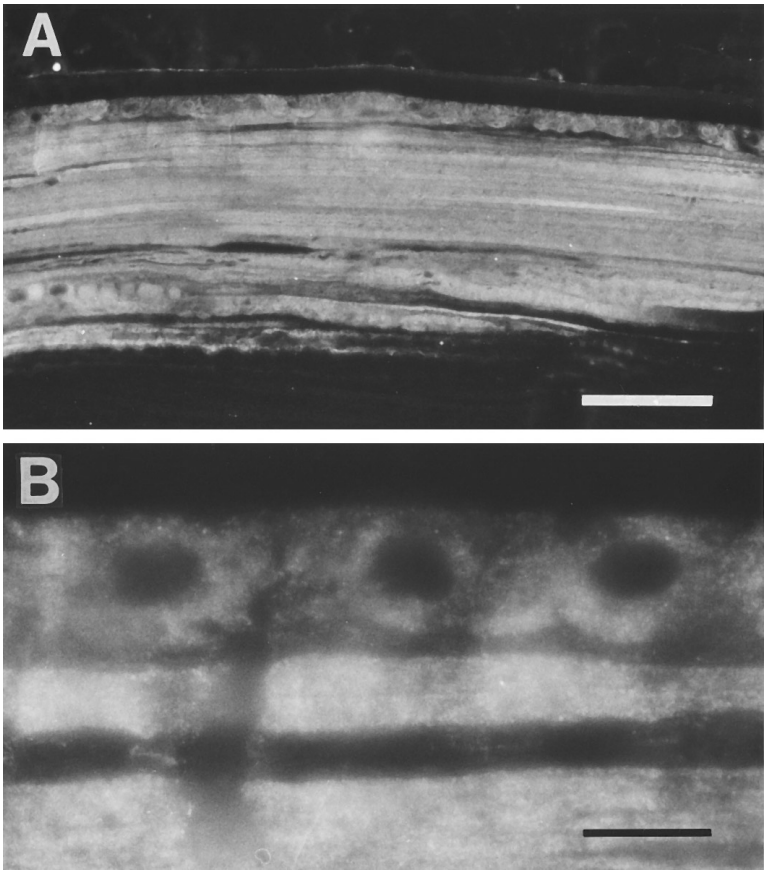

FIG. 4. Polyclonal antibody DM-2 reacts with perinuclear antigens in human lens epithelial cells and subcapsular cortical fibers. Panels (**A**) and (**B**) are midsagittal sections of a formalin-fixed, paraffin-embedded post-mortem normal adult human lens labeled with pAb DM-2. (**A**) shows the intense labeling of the superficial cortical fibers and the less intense labeling of perinuclear structures in epithelial cells. (**B**) is a more detailed view of the epithelial cells and the underlying cortical fibers (Bar, 50 μm in (**A**); 10 μm in (**B**).

patient with cataracts were reacted with pAb DM-2 and visualized by indirect immunofluorescence. Figure 5B shows that a significantly different distribution of DM kinase is detected with the DM lens compared to normal controls (Figure 5A). In the DM lens, the reaction of the DM-2 antibody is primarily detected inside the cell nucleus in contrast to the dominant cytoplasmic distribution in normal lens. The two images were photographed under similar conditions using the automatic photometer of the Zeiss Photomicroscope III system.

DISCUSSION

We have shown for the first time that the myotonic dystrophy locus is expressed at both the mRNA and protein levels in human and bovine lenses. Sequencing of products of RNA extracted from normal human lenses demonstrated their identity with published genomic and cDNA 3′ UTR sequences. Northern blots of bovine lens RNA showed that the transcript is similar in size to the transcript detected in other tissues that are affected in myotonic dystrophy (3). Immunofluorescent studies of formalin-fixed human lens sections showed that the DM kinase appears to be localized to the cytoplasm in normal human lens epithelial cells and superficial subcapsular cortical fibers. The antibody did not label the nucleus of either control

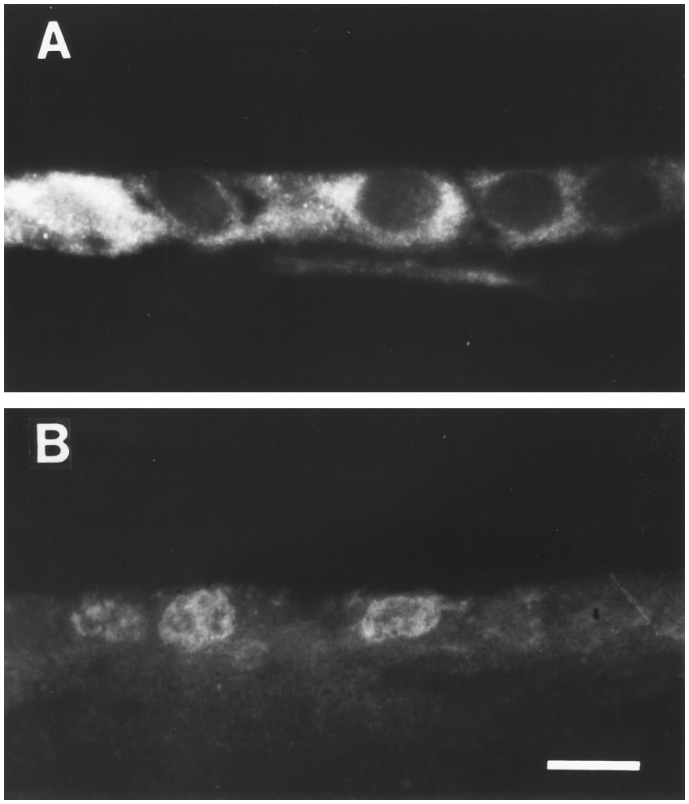

FIG. 5. Immunofluorescence of normal and DM lenses. Normal human lens reacted with pAb DM-2 (**A**). (**B**) is DM lens 84D reacted with pAb DM-2 (Bar, 10 μm).

or DM lenses. In contrast, the same antibody primarily labeled epithelial cell nuclei in the single DM lens available.

The cytoplasmic staining of normal human and rat lens epithelial cells does not exclude more specific associations with cytoplasmic structures such as the endoplasmic reticulum and Golgi complex. Our preliminary results with the human B3 human lens cell line suggest such an association (Dunne, P.W., Andley, U.P. and Epstein, H.F., unpublished experiments). Immunolocalization of DM kinase to specialized membranes in human skeletal muscle, the sarcoplasmic reticulum and transverse tubules of the triadic junction, has been reported by several groups. Fractionation studies of rat heart (P.W. Dunne, L. Ma, and H.F. Epstein, unpublished experiments) are consistent with compartmentation of the DM kinase to the cytosol and to membrane fractions enriched in sarcoplasmic reticulum. We showed that DM kinase localized to the triadic region in rodent and human skeletal muscle using a monoclonal antibody to DMPKH (8). Salvatori et al. (1994) using an antibody to a different isoform of the DM kinase reported localization of DM protein kinase to SR-containing fractions using antisera to a DM protein kinase peptide (16). Timchenko et al. (1995) report that their preparation of full-length DM protein kinase specifically phosphorylates the β-subunit of the voltage sensor channel which is a component of the transverse tubules (17). van der Ven et al. (1993) reported primary reactivity of antipeptide antibodies to subsarcolemmal structures in normal and DM muscle, but they also found reactivity to intracellular sites in muscle fibers (18). The localization of a specific protein kinase or phosphatase to multiple sites within cells has been reported

previously (19-21). Complete biochemical analysis of the isoforms of DM kinase and their localization at the ultrastructural level should permit a more definitive understanding of their intracellular associations in muscle and in the lens.

The striking difference between the nuclear labeling of DM kinase in the anterior epithelium of a DM lens and the cytoplasmic reaction in normal lenses, although tentative until further confirmation, is consistent with alteration of the localization of the DM kinase as a gain of function effect at the protein level of the autosomal dominant DM mutation. In support of this interpretation, we recently reported an altered immunolocalization of DM kinase from triad regions to peripheral sarcoplasmic masses in severely affected DM skeletal muscle (8).

Our findings that DM kinase is expressed in the lens at the level of RNA and protein support the possibility that altered expression of the DM locus in lenses from myotonic dystrophy patients could be responsible for the characteristic cataract of myotonic dystrophy. One plausible model would propose an alteration in regulation of normal lens fiber maturation. During lens maturation, lens fibers normally lose all their membranous organelles (22-24). The abnormal presence of these organelles in mature fibers are associated with cataracts in humans (25) and mice (26). Electron microscopy of early cataracts in DM reveals whorls of multilaminate membranes (4,5). Since DM kinase may play a regulatory role in one or more signal transduction pathways (P.W. Dunne, L. Ma and H.F. Epstein, unpublished experiments), it may regulate organelle loss during fiber cell maturation. Failure to degrade light-refracting organelles in mature fibers could produce the iridescent particles seen by biomicroscopy and the multilaminate whorls of membrane described by electron microscopy. Transgenic studies of the lens provide further evidence that alteration of the pathways controlling lens development/differentiation can lead to cataract formation (27-31).

Additional lenses from affected individuals must be examined in order to confirm the provocative but solitary observation of a change in subcellular distribution of DM protein kinase associated with this disorder. More complete delineation of the pathways regulated by DM kinase in the lens and its potential association with specific organelles is necessary in order to provide an experimental basis for understanding the alterations of the lens in myotonic dystrophy.

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