Overexpression of Myotonic Dystrophy Kinase in BC₃H₁ Cells Induces the Skeletal Muscle Phenotype*

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Myotonic muscular dystrophy is an autosomal dominant defect that produces muscle wasting, myotonia, and cardiac conduction abnormalities. The myotonic dystrophy locus codes for a putative serine-threonine protein kinase of unknown function. We report that overexpression of human myotonic dystrophy protein kinase induces the expression of skeletal muscle-specific genes in undifferentiated BC₃H₁ muscle cells. BC₃H₁ clones expressing myotonic dystrophy kinase appear equivalent to differentiated cells with respect to expression of myogenin, retinoblastoma tumor suppressor gene, M creatine kinase, β-tropomyosin, and vimentin. In addition, differential display analysis demonstrates that the pattern of gene expression exhibited by myotonic dystrophy kinase-expressing cells is essentially identical to that of differentiated BC₃H₁ muscle cells. These observations suggest that myotonic dystrophy kinase may function in the myogenic pathway.

Myotonic muscular dystrophy (DM) is an autosomal dominant myopathy of variable onset, producing pleiotropic effects including skeletal muscle weakness and wasting, cardiac conduction abnormalities, frontal baldness, and cataracts (1, 2). Severity of the disease correlates with expansion of an unstable CTG trinucleotide repeat within the 3′-untranslated region (UTR) of the gene for DM kinase (DMK) (3–7). The triplet repeats expand in subsequent generations, producing the genetic phenomenon of anticipation; progeny of affected individuals generally display earlier onset and greater severity of symptoms (8, 9).

The product of the DM locus has been identified as a putative serine-threonine kinase that shares homology with protein kinases A and C. The full-length cDNA codes for a protein product with an apparent molecular mass of 71 kDa (10, 11). The 71-kDa gene product appears to be post-translationally modified to produce a second DMK form with an apparent molecular mass of 84 kDa. Structural predictions from the primary sequence suggest that DMK consists of an amino-terminal kinase domain, an α-helical coiled-coil domain, and a carboxyl-terminal transmembrane domain (12). The catalytic activity of DM kinase has been verified (13), but in vivo substrates have yet to be identified. In skeletal muscle, DMK appears to be localized to the neuromuscular junction, while in cardiac muscle the kinase is present in the intercalated disc (10, 11). DMK mRNA is present in a variety of tissue types, including muscle, brain, and eye. Quantitation of DMK mRNA concentrations from alleles with expanded triplet repeats in affected individuals has proven difficult, leading to conflicting reports as to expression of mutant alleles (14–16).

The disease state of DM is likely to be related to the aberrant expression of DMK and the subsequent failure of the kinase to act upon its proper substrates or ectopic interaction with other substrates. In an effort to establish whether DMK operates within a known signal transduction pathway, we constructed stably transfected BC₃H₁ muscle cell lines that constitutively overexpress human DMK. DMK-expressing and untransfected cells were cultured in the presence of 20% fetal calf serum; differentiated BC₃H₁ cells were produced by mitogen withdrawal. Total cellular RNA was isolated and subjected to mRNA differential display analysis, a polymerase chain reaction (PCR)-based technique useful in the identification of differentially expressed genes (17). The pattern of gene expression was substantially changed in DMK-expressing clones, and the new pattern of expression was essentially identical to that of differentiated BC₃H₁ cells. Two differentially expressed gene products, consistent with a skeletal muscle phenotype, were cloned by differential display, sequenced, and used as Northern blot probes. Skeletal muscle-specific β-tropomyosin mRNA expression was increased, and vimentin mRNA expression was decreased in DMK-expressing clones. Northern blot analysis of other genes implicated in myogenesis showed that myogenin mRNA was induced in both DMK-expressing and differentiated BC₃H₁ cells. Retinoblastoma tumor suppressor (Rb) mRNA was present in untransfected cells but absent from DMK-expressing and differentiated cells. Creatine kinase activity assays demonstrated that DMK-expressing clones up-regulate expression of the M creatine kinase isoform. Thus, despite the presence of mitogens, overexpression of DMK in BC₃H₁ cells induces the skeletal muscle phenotype.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—A coding-only DMK cDNA construct was derived from a full-length (flDMK) cDNA (kindly supplied by R. G. Korenuk) by PCR (Perkin-Elmer) using primers that annealed to the first methionine and termination codons. Primers were designed such that EcoRI restriction sites would be added to the termini of the amplified product: forward primer (5′-GGCGGAAATTCTAGGAGCGCGG- GCGG-3′); reverse primer (5′-CAGGGAATTCTAGGAGCGCGG- CGCG-3′). A 1889-base pair PCR product was amplified and ligated into the EcoRI site of the eukaryotic expression vector pcDNA3 (Invitrogen). This clone, designated pcDNA3/coDMK was sequenced (Sequenase 2.0, United States Biochemical Corp.) to verify integrity of the cDNA sequence. 3′-UTR sequences were subcloned from the full-length
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RESULTS AND DISCUSSION

The first attempt to produce a stable DMK-overexpressing cell line was unsuccessful. Stably transfected clones were produced by transfecting BC3H1 cells with a plasmid construct containing the full-length DMK cDNA, including the 5'- and 3'-UTR, DMK mRNA and protein expression in stable full-length DMK (flDMK) and coding-only DMK (coDMK) BC3H1 clones. A Northern analysis. Total RNA samples from cell lines were isolated, electrophoresed on a 2% agarose-formaldehyde gel (20 μg loaded/lane), transferred to a nylon membrane, and hybridized with a DMK cDNA probe. Positions of 28 and 18 S ribosomal RNAs are as noted in figure. Lane 1, untransfected BC3H1; lane 2, coDMK clone 1; lane 3, DMK clone 10; lane 4, flDMK clone 8; lane 5, flDMK clone 10; lane 6, DMK clone 11 B. Western analysis. Protein samples from cell lines were isolated, resolved on a SDS-polyacrylamide gel (20 μg loaded/lane), transferred to a nylon membrane, and incubated with an anti-DMK primary antibody. Position of the 69-kDa standard is as noted in figure. Lane 7, untransfected BC3H1 control; lane 8, DMK 3'-UTR clone; lane 9, coDMK clone 1; lane 10, flDMK clone 7; lane 11, flDMK clone 8; lane 12, flDMK clone 10; lane 13, flDMK clone 11.
fected BC3H1 cells.

In order to identify changes in gene expression associated with DMK or DMK 3'-UTR overexpression, we performed differential display analysis of the stable coDMK and 3'-UTR cell lines. The patterns of gene expression in the 3'-UTR clone and the untransfected control cells were nearly identical (Fig. 2, panels A–C, lanes 2, 3, 5, 6, 8, and 9). In comparison, cells expressing DMK exhibited a markedly different pattern of mRNA expression (Fig. 2, panels A–C, lanes 1, 4, and 7). Expression of numerous mRNAs were affected, apparently representing both up- and down-regulation of several genes. We initially reamplified and subcloned nine differentially expressed cDNA products. The products were both sequenced and used to probe Northern blots to verify that differential display correlated with differential expression. Six of the products were unidentifiable, either showing no significant similarity to sequences in the GenBank™ database, or matching the 3' end of a partially sequenced but uncharacterized cDNA. One product represented mitochondrial sequences and was not examined further.

One product, designated DM114.10, hybridized to an mRNA unique to DMK-expressing cells (Figs. 2C and 3A, lanes 1–3). DM114.10 also hybridized to an mRNA expressed at high concentrations in differentiated BC3H1 cells (data not shown). This product was identified as a differentiation-dependent form of b-tropomyosin unique to skeletal muscle. Rodents have been shown to produce two different tissue-specific b-tropomyosin messages (27). Fibroblasts and smooth muscle produce a 1.1-kilobase mRNA transcript; skeletal muscle transcripts are larger (1.2 kilobases) and possess a different 3' end produced by alternative splicing and usage of a distal polyadenylation site. Our DM114.10 product is limited to those 3' sequences unique to the skeletal muscle mRNA.

The clone designated DM114.07 was identified as vimentin and recognized an mRNA present in untransfected BC3H1 cells that became diminished in DMK-expressing cells (Figs. 2B and 3B, lanes 4–6) as well as differentiated BC3H1 cells (data not shown). Studies in rodents and humans have established that vimentin expression is high in satellite cells and regenerating muscle, but undetectable in mature skeletal muscle (28–31).

Because of the differential expression of b-tropomyosin and vimentin, we began to suspect that cells expressing DMK were initiating a portion of a skeletal muscle differentiation program. Although the cognate messages could not be identified, the expression patterns of two of the differential display products, DM114.02 and DM114.03, were consistent with a skeletal muscle phenotype. DM114.02 recognized a transcript present in cells expressing DMK that was absent from untransfected cells (data not shown) and had high similarity to a human cDNA isolated from skeletal muscle (GenBank accession no. Z28822). DM114.03 hybridized to a transcript in untransfected cells that was absent in the DMK clone (data not shown) and shared similarity with a human cDNA from cardiac muscle (GenBank™ accession no. Z33441).

To further assess the differentiation state of DMK-expressing cells, we examined expression of two genes known to be implicated in myogenic development: myogenin (32) and retinoblastoma (Rb) (33, 34). A myogenin cDNA probe detected high levels of myogenin mRNA induced in both DMK-expressing and differentiated cells (Fig. 4, A and B). Rb mRNA expression was affected in a reciprocal fashion; it was abundant in
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untransfected cells but decreased to undetectable levels in DMK-expressing and differentiated BC3H1 cells (Fig. 4, C and D).

Creatine kinase (CK) isoforms serve as markers of skeletal muscle differentiation (35–37). Undifferentiated myogenic cells predominantly express the B isofrom (Fig. 5, lane 1). Following myogenesis, M creatine kinase becomes the major isofrom. We performed CK activity assays on DMK-expressing clones and observed that with respect to CK activity, DMK-expressing cells appeared to be equivalent to differentiated cells; M creatine kinase activity was strongly increased (Fig. 5, lanes 2 and 3).

Finally, we performed differential display on RNA from untransfected/undifferentiated, differentiated, and DMK-expressing cells. The patterns of mRNA expression shown by DMK-expressing and differentiated cells were nearly identical (Fig. 2D, lanes 11 and 12), and both were substantially different from the pattern of mRNA expression detected in untransfected/undifferentiated cells (Fig. 2D, lane 10). This observation suggests that many of the changes in gene expression associated with DMK overexpression are similar to those induced during differentiation in BC3H1 cells.

We have not determined whether the apparent myogenic effect of DMK overexpression is a specific one, or if it is simply a result of the nonspecific activity of an ectopically expressed protein kinase. However, overexpression of protein kinase A or C, the two kinases that share the greatest homology with DMK, actually inhibit muscle-specific transcription (38, 39). Although no protein kinase has yet been conclusively implicated in the control of myogenesis, it is likely that kinase cascades are involved in the establishment and maintenance of the differentiated state in muscle (40). It has been speculated that DMK may influence muscle development through an effect upon myogenic gene products (41). Recent observations by Meissel et al. suggest that myogenin can serve as an in vitro substrate for DMK. Ultimately, however, any formal model will rely upon identification of the actual in vivo substrate. It is interesting to note that DMK-expressing cells maintain a differentiated phenotype while actively dividing in high mitogen conditions, suggesting that cell cycle arrest is not a prerequisite for differentiation in these cells.

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REFERENCES
1. Steinert, H. (1909) Dtsch. Z. Nervenhkl. 37, 38
2. Harper, P. S. (1989) in The Metabolic Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D.) 6th Ed. Vol. 2, pp. 2899–2902, McGraw-Hill, New York
3. Aslanidis, C., Jansen, G., Amemiya, C., Shutter, G., Mahadevan, M., Tsilfidis, C., Chen, C., Alleman, J., Wormskaap, N. G. M., Voolis, M., Buxton, J., Johnson, K., Smeets, H. J. M., Lennon, G. G., Carrano, A. V., Korneluk, R. G., Wieringa, B., and de Jong, P. J. (1992) Nature 355, 548–551
4. Buxton, J., Sheehan, P., Davies, J. J., Jones, C., Van Tongeren, T., Aslanidis, C., de Jong, P., Jansen, G., Avnery, M., Riley, B., Williamson, R., and Johnson, K. (1992) Nature 355, 547–548
5. Fu, Y.-H., Pizzuti, A., Fenwick, R. G., King, J., Rajanayakan, S., Dunne, P. W., Dubel, J., Nasser, G. A., Ashizawa, T., de Jong, P., Wieringa, B., Korneluk, R. G., Perryman, M. B., Epstein, H. F., and Caskey, C. T. (1992) Science 255, 1256–1258
6. Harper, P. S., Brook, J. D., Rundle, S. A., Crow, S., Reardon, W., Buckler, A. J., Harper, P. S., Houseman, D. E., and Shaw, D. J. (1992) Nature 355, 545–546
7. Mahadevan, M., Tsilfidis, C., Sabouri, L., Shutter, G., Amemiya, C., Jansen, G., Neville, C., Naranj, M., Barcelo, J., O’Hoy, K., Leblond, S., Earle, Macdonald, J., de Jong, P. J., Wieringa, B., and Korneluk, R. G. (1992) Science 255, 1253–1255
8. Ashizawa, T., Dunne, C. J., Debel, J., Pizzuti, A., Perryman, M. B., Epstein, H. F., Boenink, E., and Hejtmancik, J. F. (1992) Neurology 42, 1871–1877
9. Ashizawa, T., Debel, J. R., Dunne, C. J., Fu, Y. H., Pizzuti, A., Caskey, C. T., Boenink, E., Perryman, M. B., Epstein, H. F., and Hejtmancik, J. F. (1992) Neurology 42, 1877–1883
10. Whiting, E. L., Warin, J. D., Tamai, K., Somerville, M. J., Hinke, M., Staines, W. A., Ikeda, J., and Korneluk, R. G. (1995) Hum. Mol. Genet. 4, 1063–1072
11. Maeda, M., Taft, C. S., Bush, E. W., Holder, B., Bailey, W. M., Neville, H., Perryman, M. B., and Bies, R. D. (1995) J. Biol. Chem. 270, 20246–20249
12. Perryman, M. B., Friedman, D. L., Fu, Y. H., and Caskey, C. T. (1993) Trends Cardiovasc. Med. 3, 82–84
13. Dunne, P. W., Walsh, E. T., and Epstein, H. F. (1994) Biochemistry 33, 10899–10904
14. Fu, Y.-H., Friedman, D. L., Richards, S., Pearman, J. A., Gibson, R. A., Pizzuti, A., Ashizawa, T., Perryman, M. B., Scarlato, G., Fenwick, R. G., and Caskey, C. T. (1993) Science 260, 235–238
15. Sabouri, L. A., Mahadevan, M. S., Naranj, M., Lee, D. S. C., Surh, L. C., and Korneluk, R. G. (1993) Nature Genet. 4, 233–238
16. Carango, P., Noble, E. J., Marks, H. G., and Funanage, V. L. (1993) Genomics 19, 340–348
17. Liang P., and Pardee, A. B. (1992) Science 257, 967–971
18. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
19. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. **215**, 403–410
20. Pulley, P. R. (1989) Clin. Chem. **35**, 1452–1455
21. Mountford, P. S., Brandon, M. R., and Adams, T. E. (1992) J. Neuroendocrinol. **4**, 656–658
22. Rastinejad, F., and Blau, H. M. (1993) Cell **72**, 903–917
23. Jackson, R. J. (1993) Cell **74**, 9–14
24. Grossman, M. E., Lindzey, J., Kumar, V. M., and Tindall, D. J. (1994) Mol. Endocrinol. **8**, 448–455
25. Ostareck-Lederer, A., Ostareck, D. H., Standart, N., and Thiele, B. J. (1994) EMBO J. **13**, 1476–1481
26. Olson, E. N., Caldwell, K. L., Gordon, J. I., and Glaser, L. (1983) J. Biol. Chem. **258**, 2644–2652
27. Wang, Y.-C., and Rubenstein, P. A. (1992) J. Biol. Chem. **267**, 2728–2736
28. Bennett, G. S., Fellini, S. A., Toyama, Y., and Holtzer, H. (1979) J. Cell Biol. **82**, 577–584
29. Gallanti, A., Prelle, A., Moggio, M., Ciscato, P., Checcarelli, N., Sciacco, M., Comini, A., and Scarlato, G. (1992) Acta Neuropathol. **85**, 88–92
30. Sarnat, H. B. (1992) Neurology **42**, 1616–1624
31. Vater, R. V., Cullen, M. J., and Harris, J. B. (1994) Histochem. J. **26**, 916–928
32. Edmondson, D. G., and Olson, E. N. (1993) J. Biol. Chem. **268**, 755–758
33. Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. (1993) Cell **72**, 309–324
34. Halevy, O., Novitch, B. G., Spiro, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., Lassar, A. B. (1995) Science **267**, 1018–1021
35. Morris, G. E., Cooke, A., and Cole, R. J. (1972) Exp. Cell Res. **74**, 582–585
36. Turner, D. C., Maier, V., and Eppenberger, H. M. (1974) Dev. Biol. **37**, 63–89
37. Perriard, J.-C., Perriard, E. R., and Eppenberger, H. M. (1978) J. Biol. Chem. **253**, 6529–6535
38. Li, L., Heller-Harrison, R., Czech, M., and Olson, E. N. (1992) Mol. Cell. Biol. **12**, 4478–4495
39. Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M. P., and Olson, E. N. (1992) Cell **71**, 1181–1194
40. Castellani, L., Reedy, M. C., Gauzi, M. C., Provenzano, C., Alena, S., and Falzone, G. (1990) J. Cell Biol. **130**, 871–885
41. Shaw, D. J., McCurrach, M., Rundle, S. A., Harley, H. G., Crow, S. R., Shhn, R., Thriron, J. P., Hamshere, M. G., Buckler, A. J., Harper, P. S., Housman, D. E., and Brook, J. D. (1993) Genomics **18**, 673–679
