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Nebulin and titin expression in Duchenne muscular dystrophy appears normal

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Monoclonal antibodies which recognize different epitopes on either titin or nebulin show normal staining patterns on frozen sections of three muscle biopsies of Duchenne muscular dystrophy (DMD). Gel electrophoresis and immunoblotting performed on two of these muscle biopsies show the normal pattern of titin and nebulin polypeptides. Since the donor of one of these biopsies has a large deletion of the 5'-region of the DMD gene, our results argue against the recent proposal that nebulin is the gene mutated in DMD.

Duchenne muscular dystrophy; Gene deletion; Nebulin; Sarcomere structure; Titin

1. INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked human disorder which occurs approximately once in every 4000 live male births (reviews [1,2]). Kunkel, Monaco and colleagues have described the DMD gene and provided DNA probes useful for carrier and prenatal diagnosis. The transcript of the DMD gene is 16 kilobases in size and therefore unusally large [1,3]. Recently Wood et al. [4] reported that the nebulin band was either absent or extremely faint in polypeptide patterns from muscle biopsies of 30 DMD patients. Thus they proposed that nebulin might be the direct product of the DMD gene [4] and that the disease could affect a structural protein of the elastic component of skeletal muscle. This component is known from the work of Maruyama [5] and Wang [6] to contain two unusually large polypeptides. The actual molecular masses of titin (1–3 MDa) and nebulin (0.5–0.8 MDa) are, however, not known exactly.

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In the course of a study on the elastic component of the sarcomer begun 2 years ago [7] we have generated sets of monoclonal antibodies specific for either titin or nebulin from chicken. Immunoelectron-microscopical data to be reported elsewhere showed that the different antibodies in each set recognize different epitopes and that these epitopes are also present in human skeletal muscle (D.F., M.O., R.N. and K.W., in preparation). Here we have used these antibodies on muscle biopsies from DMD patients and reach a conclusion distinct from that of Wood et al. [4].

2. MATERIALS AND METHODS

Biopsy I was from a 5-year-old boy. The diagnosis of DMD was based on clinical symptoms and characteristic morphological changes. Biopsy II was from a 7-year-old boy affected with DMD and was obtained from the femoral quadriceps muscle. Biopsy III was from a fetus diagnosed as affected with DMD and was obtained after abortion. Control muscle was represented by human leg striated muscle removed during surgery.

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Biopsy material, previously frozen in liquid nitrogen and then stored at −80°C, was homogenized in 5 mM Tris-HCl (pH 7.4), 5 mM EGTA, 1 mM PMSF and 1 mM iodoacetamide using a small glass-teflon homogenizer. 1 vol. of 2-times concentrated sample buffer (25 mM Tris-HCl, pH 8.4, 2.5 mM EDTA, 20% glycerol, 3.0% 2-mercaptoethanol, 10% SDS) prewarmed to 50°C was added per volume of homogenate. Samples were gently mixed, incubated at 50°C for 10 min and either immediately subjected to electrophoresis or stored frozen at −20°C. Electrophoresis was performed on linear polyacrylamide gradient gels (2–12% acrylamide; 0.5% cross-linker) in the standard buffer system but omitting a stacking gel. Immunoblotting using hybridoma supernatants was as described [7]. Indirect immunofluorescence microscopy on frozen muscle sections was performed after acetone fixation by standard procedures using the hybridoma supernatants and FITC-labeled sheep anti-mouse antibodies.

Genomic DNA was extracted from peripheral blood, lymphoblastoid cell lines or, in the case of the affected fetus (biopsy III), from liver following established procedures [8]. After digestion with restriction enzymes the samples of genomic DNA were electrophoresed on 0.8% agarose, blotted and hybridized to ^{32}P-labeled probes as in [9]. The probes used were kindly provided by Dr P. Pearson (754), R. Worton (XJ1.1), L. Kunkel (pERT), G.B. van Ommen (J-66) and J.L. Mandel (C7).

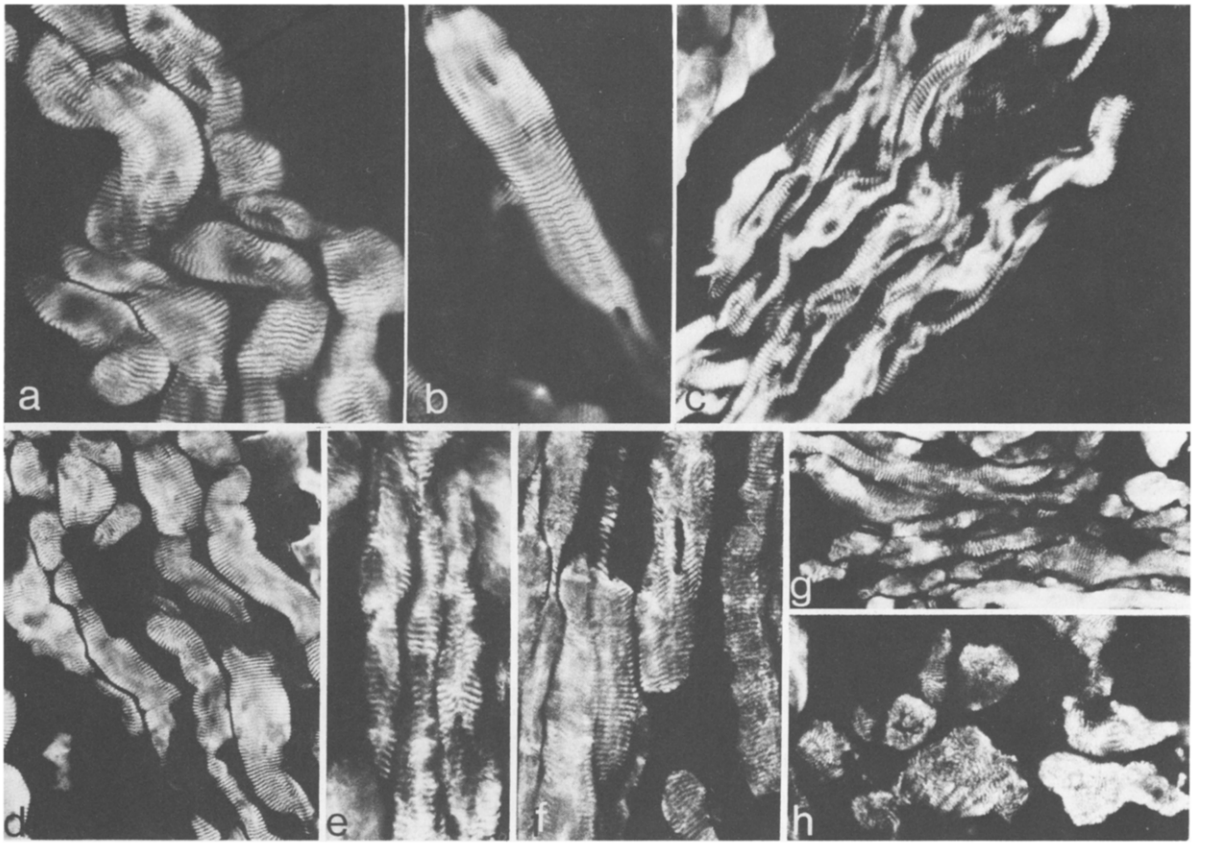

Fig.1. Immunofluorescence microscopy with nebulin and titin antibodies of control skeletal muscle and different DMD muscular dystrophy specimens. (a–c) Titin T3: (a,b) case I, (c) case III. These profiles are indistinguishable from those seen on normal human muscle (cf. [7]). (d–h) Nebulin: (d) normal human skeletal muscle, Nb6, (e) case I, Nb4, (f) case I, Nb1, (g) case III, Nb4, (h) case III, Nb1 (cross-section). Positive staining is seen in both the normal and the dystrophic samples. Magnification: (a–g) ×400, (h) ×680.

3. RESULTS

Immunofluorescence microscopy was performed with 4 titin monoclonal antibodies (T1, T3, T4, T12) and 6 nebulin monoclonal antibodies (Nb1, Nb2, Nb3, Nb4, Nb6, Nb7) on frozen sections of three DMD specimens. Although not every antibody was used on each specimen all antibodies used gave normal striated staining patterns. Examples are given in fig.1. Within the limits of the method neither the general appearance nor the intensity of labeling seemed different in DMD specimens when compared to normal human muscle.

Since some DMD patients characteristically show deletions within the DMD gene [1] we also studied the polypeptide patterns seen in total SDS extracts. For this analysis only biopsies II and III could be used as sufficient amounts of biopsy I were not available. Fig.2 shows that in both specimens titin and nebulin had apparent molecular masses equivalent to those found in control samples of normal human skeletal muscle. Moreover, the relative amounts of the two proteins seemed similar in the DMD samples and the controls. In parallel experiments gels were analyzed by immunoblotting with monoclonal antibodies to assign unambiguously the titin and nebulin reactivities. This is documented for normal human muscle and DMD biopsy III in fig.2 and again shows that titin and nebulin display apparently normal molecular masses. Significant proteolytic breakdown of nebulin is not seen, probably because iodoacetamide was included in the homogenization solution. This compound is known to inhibit thiol proteases including the intracellular Ca^{2+}-dependent proteases (calpains). Degradation of titin was very limited (fig.2) and did not give rise to the 400 kDa fragment [5] which is recognized by our titin antibodies.

The results of a detailed analysis carried out in the family of the aborted DMD fetus indicated the presence in the mother, in an affected son and in the same fetus of an extensive deletion of the DMD gene. This deletion does not include the locus DXS84 recognized by probe 754, and includes the region of the gene revealed by probes XJ1.1, pERT 87.1, 87.8, 87.15 but not that revealed by J.66. According to present knowledge [1] this deletion covers therefore at least 400 kb of the 5′-portion of the gene. Partial data on the analysis regarding the pERT region are shown in fig.3. The last two

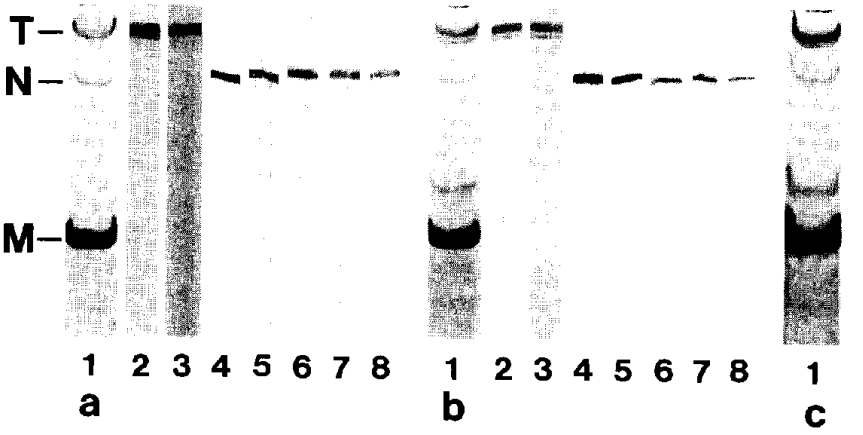

Fig.2. Gel electrophoretic and immunoblot analysis of normal (a) and different DMD (b,c) human muscle samples. Lanes 1 show Coomassie blue-stained gel patterns of muscle samples. Lanes 2–8 (in a,b) show corresponding immunoblots of the muscle samples with monoclonal antibodies directed against titin (lanes 2,3) or nebulin (lanes 4–8). Titin antibodies were T3 (lanes 2) and T10 (lanes 3). Nebulin antibodies were Nb1 (lane 4), Nb2 (lane 5), Nb3 (lane 6), Nb4 (lane 7) and Nb6 (lane 8). Degradation of titin is minimal (see section 3). Note intactness of the nebulin polypeptide in the DMD samples. Lanes b1 and c1 give results on DMD cases III and II, respectively. The positions of titin (T), nebulin (N) and myosin heavy chain (M) are marked on the left. Only the upper relevant part of gels and blots are shown. An unidentified polypeptide above myosin was only weakly present in normal muscle (a).

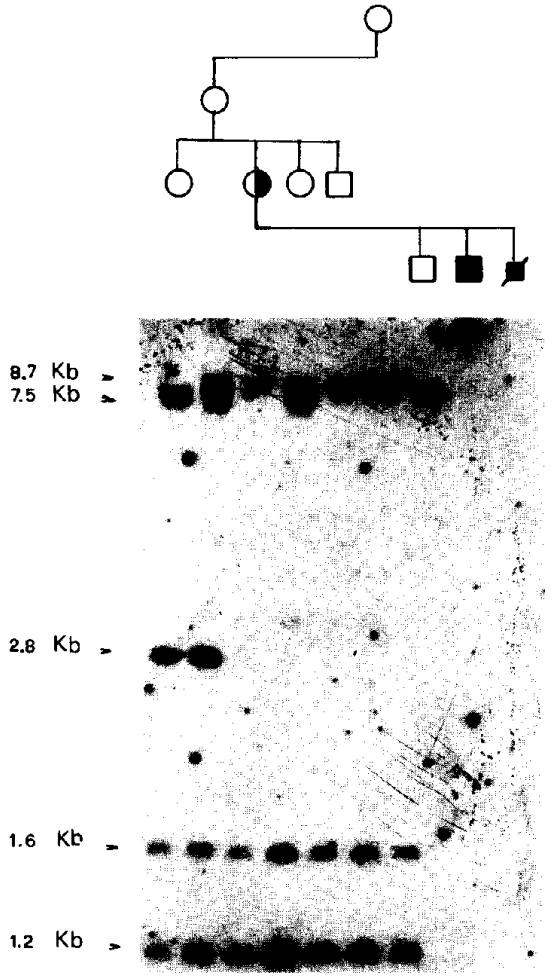

Fig.3. Results of Southern blotting after digestion with *Xmn*I and electrophoresis of genomic DNA from the individual members of the family showing a deletion of the DMD gene (biopsy III). The radiolabeled probes pERT 87.1 and 15 hybridized respectively to the following sets of fragments: 8.7 and 7.5 kb alleles revealed by pERT 87.1 at the top, and 2.8 kb (common fragment) plus 1.6 and 1.2 kb alleles revealed by pERT 87.15 at the bottom. Each lane corresponds to the genomic DNA of the individuals indicated at the top of the figure as follows: (○) normal female; (◑) carrier female (by CPK test); (□) normal male; (■) affected male; (◩) aborted affected fetus.

individuals on the right of this figure (affected living son and affected aborted fetus) do not show any signal with the pERT probes, while their mother is a carrier as indicated by her altered creatine phosphokinase (CPK) test.

4. DISCUSSION

Our results argue that nebulin and titin are readily detected in DMD specimens and the two DMD muscle biopsies studied by SDS gel analysis show no obvious differences when compared to normal human striated muscle. The further analysis of patient III and of his family using DNA probes is particularly important in that it showed that the 5′-portion of the DMD gene was deleted. Thus, it seems unlikely that either nebulin or titin represents the protein coded for by the DMD gene. After our results were completed we learned from Dr Maruyama that he and his colleagues had studied 2 preclinical DMD cases as well as 3 symptomatic DMD cases by gel electrophoresis and immunoblotting using polyclonal antibodies to titin and nebulin [10]. While they noted some proteolytic degradation they could ascertain that nebulin was present in all cases. These authors did not report, however, any indication regarding the structure of the DMD gene in the patients they studied [10].

The two studies, which complement each other, yield results which differ from those of Wood et al. who reported that in 30 DMD patients the nebulin was "absent or extremely faint" [4]. Currently it is difficult to account for the difference. While Maruyama's laboratory favours differential in situ proteolysis there is an additional possibility. Both we and Maruyama's group processed frozen biopsy material directly for SDS gel electrophoresis while Wood et al. seem to have included an additional step involving the preparation of "myofibrillar-sediments" [4]. Thus currently it cannot be excluded that proteolysis of DMD samples could have occurred at this stage and Wang [6] has already alluded to the difficulties in documenting nebulin in gel electrophoresis.

The final identification of the protein coded for by the DMD gene should come from the isolation and sequence of the DMD gene. While it may still take some time to obtain limited amino acid sequence data on nebulin, and to search for the presence of such sequences in the product predicted by the DNA sequence of the DMD gene, it seems unlikely on the basis of the present data that nebulin represents the DMD gene product. An additional consideration contributes to exclude the latter hypothesis. Titin and nebulin are major

myofibrillar proteins accounting for some 10% of the sarcomeric proteins [6] and therefore should arise from abundant mRNA species. The transcript corresponding to the DMD gene is instead scarcely represented in muscle cells [11], which is again in disagreement with the 'nebulin hypothesis'.

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REFERENCES

[1] Monaco, A.P. and Kunkel, L.M. (1987) Trends Genet. 3, 33–37.
[2] Robertson, M. (1987) Nature 327, 372–373.
[3] Monaco, A.P., Neve, R.L., Colletti-Feener, C., Bertelson, C.J., Kurnit, D.M. and Kunkel, L.M. (1986) Nature 323, 646–650.
[4] Wood, D.S., Zeviani, M., Prelle, A., Bonilla, E., Salviati, G., Miranda, A.F., DiMauro, S. and Rowland, L.P. (1987) N. Engl. J. Med. 316, 107–108.
[5] Maruyama, K. (1986) Int. Rev. Cytol. 104, 81–114.
[6] Wang, K. (1985) in: Cell and Muscle Motility (Shay, J.W. ed) vol. 6, pp. 315–369, Plenum, New York.
[7] Hill, C. and Weber, K. (1986) J. Cell Biol. 102, 1099–1109.
[8] Kunkel, L.M., Smith, K.D., Boyer, S.H., Borgaonkar, D.S., Wachtel, S.S., Miller, D.J., Breg, W.R., Jones, H.W. and Rary, J.M. (1977) Proc. Natl. Acad. Sci. USA 74, 1245–1249.
[9] Roncuzzi, L., Fadda, S., Mochi, M., Prosperi, L., Sangiorgi, S., Santamaria, R., Sbarra, D., Besana, D., Morandi, L., Rocchi, M. and Romeo, G. (1985) Am. J. Hum. Genet. 37, 407–417.
[10] Sugita, H., Nonaka, I., Itoh, Y., Askura, A., Hu, D.H., Kimura, S. and Maruyama, K. (1987) Proc. Jap. Acad. 63, Ser. B. 107–110.
[11] Monaco, A.P., Neve, R., Colletti-Feener, C., Bertelson, C.J., Kurnit, D.N. and Kunkel, L.M. (1986) Nature 323, 646–650.