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Immunological identification of a high molecular weight protein as a candidate for the product of the Duchenne muscular dystrophy gene

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ABSTRACT **An oligopeptide was synthesized based on translation of the nucleotide sequence of the putative exon region of clone pERT87-25 from the gene for Duchenne muscular dystrophy. Immunization of rabbits with this oligopeptide induced the formation of antibodies directed against a protein present in human, rat, and rabbit skeletal muscle. This protein, which is missing in the skeletal muscle of two patients with Duchenne muscular dystrophy, has a molecular mass of ≈320–420 kDa and is clearly different from the putative Duchenne muscular dystrophy-related protein nebulin. The data suggest that this 320- to 420-kDa protein is produced by the Duchenne muscular dystrophy gene.**

In 1986, Kunkel, Monaco, and co-workers (1) identified a pair of cDNA probes, pERT87-4 and pERT87-25, lying within the gene for Duchenne muscular dystrophy (DMD). On RNA analysis these probes hybridized with skeletal muscle mRNAs of ≈14 kilobases from a variety of species (1, 2). Sequence analysis revealed that the probes contained open reading frames ≈60 base pairs lying between 5′-splice acceptor and 3′-splice donor sites. The open reading frames were also downstream from lariat consensus sequences. From these observations, the authors suggested that each probe contained translated regions coding for portions of the DMD protein.

To determine whether these cDNA probes do, in fact, contain regions coding for a specific human muscle protein, we have synthesized the oligopeptide deduced from the nucleotide sequence of the pERT87-25 cDNA probe. The synthetic peptide (87-25 oligopeptide) was then used to immunize rabbits, and the immune sera were tested by immunologic analysis for the presence of cross-reactive material in skeletal muscle.

MATERIALS AND METHODS

Materials. *p*-Methylbenzhydrylamine resin was obtained from Applied Biosystems (Foster City, CA). Keyhole limpet hemocyanin (KLH) was from Sigma. CNBr-activated Sepharose 4B was from Pharmacia. Materials for gel electrophoresis and immunoblots were from Bio-Rad.

Synthesis of the Oligopeptide. Solid-phase synthesis of the (87-25)oligopeptide amide and cysteine containing (87-25)-oligopeptide amide was done on 0.5 mmol of *p*-methylbenzhydrylamine resin at 0.48 mmol/g using an Applied Biosystems model 430A peptide synthesizer. The *Nα*-tert-Boc-protected amino acids were coupled as the symmetrical anhydrides with the exception of arginine and glutamine, which were double coupled by the dicyclohexylcarbodiimide/1-hydroxybenzotriazol method. Side-chain protection was as follows: Arg (tosyl), Asp (cyclohexyl), Cys (*p*-methylbenzyl), Glu (benzyl), Lys (2-chlorobenzyloxycarbonyl), and Ser (benzyl). Cleavage and deprotection were done using liquid hydrofluoric acid at 0°C containing 5% (wt/vol) anisole for 35 min. After removal of the hydrofluoric acid *in vacuo* at 0°C, the peptide was extracted from the resin with a small amount of 6 M urea/10% (vol/vol) acetic acid. The combined resin washes were lyophilized, and the residue was dissolved in 15% (vol/vol) acetonitrile/0.1% trifluoroacetic acid and centrifuged to remove insoluble material. The soluble supernatant was eluted on a 21.4 × 250-mm Dynamax C_{18} column at 10 ml/min by use of a 15–32% linear gradient of acetonitrile in 0.1% trifluoroacetic acid over 17 min. The major product as detected by UV at 254 nm was collected and lyophilized. The peptides were purified by reverse-phase high-performance liquid chromatography.

KLH Conjugation. The synthesized Cys(87-25)oligopeptide amide was conjugated with KLH according to the procedure of Carlsson *et al.* (3). Twelve milligrams of the Cys (87-25)oligopeptide amide was treated with 20.6 mg of KLH that had been pretreated with *N*-succinimidyl 3-(pyridyldithio)propionate. After removal of insoluble reactants, the final yield was 28.0 mg, calculated from the substitution level of the heterobifunctional linker group attached to KLH. The substitution level obtained from weight gain was 0.12 μmol/mg compared with a theoretical substitution level of 0.15 μmol/mg. Analysis of the Cys(87-25)oligopeptide amide and (87-25)oligopeptide amide was done by high-performance liquid chromatography and amino acid composition. The cysteine-containing peptide was not completely homogenous. Additional analyses by fast-atom-bombardment mass spectrometry gave evidence of an N-terminal alanine deletion and an N-terminal glutamic acid insertion in the Cys(87-25)oligopeptide used for generating the antibody. The C-terminal fragment ions seen were in agreement with the correct sequence. Specificity of the antibodies generated was subsequently verified by their recognition of the highly purified (87-25)oligopeptide from a second preparation that was used throughout the experiments reported here.

Immunization. For production of immune serum, two pathogen-free female New Zealand rabbits weighing 3 kg were immunized at multiple sites along their backs with 1 mg of (87-25)oligopeptide–KLH in complete Freund's adjuvant. The animals were boosted at 3- to 4-week intervals by intramuscular injection of 0.5 mg of the same preparation in each haunch.

Serum Fractionation. The immunoglobulin fraction of the rabbit serum was prepared by ammonium sulfate precipitation essentially according to the method of Campbell (4). For

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Abbreviations: DMD, Duchenne muscular dystrophy; KLH, keyhole limpet hemocyanin; (87-25)oligopeptide, synthetic peptide of probe pERT87–25; (87-25)protein, muscle protein against which antibody to (87-25)oligopeptide reacts.

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purification of specific antibody, (87-25)oligopeptide was covalently coupled to CNBr-activated Sepharose 4B according to the procedure of the manufacturer (Pharmacia). Two milligrams of (87-25)oligopeptide was treated with 1 g (dry weight) of CNBr-activated Sepharose 4B. After inactivation of unreacted CNBr, the mixture was placed on a 1.0-cm diameter column that had been prewashed with 50 ml of 50 mM phosphate buffer, pH 7.4. Two hundred fifty microliters of the ammonium sulfate immunoglobulin fraction, equivalent to 500 μl of undiluted immune antiserum, was added to the column and allowed to equilibrate for 30 min. The column was then extensively washed again with 50 ml of 50 mM phosphate buffer, pH 7.4, and antibody was eluted from the column with 3.5 M KSCN; 0.5-ml fractions of eluted material were collected followed by a further incubation for 2 hr at room temperature. The eluate wad dialyzed overnight with Spectra-Por tubing (μ_r cutoff = 3500) in 20 mM Tris buffer/0.5 M NaCl, pH 7.5.

Preparation of Muscle Samples. Skeletal-muscle biopsy samples were obtained with informed consent from two DMD patients and four disease control patients (one individual with scapulo-peroneal syndrome, one individual with diabetic neuropathy, and two individuals with fasciitis). Skeletal muscle was also obtained from rabbit and rat gastrocnemius muscles. Muscle tissue was either chilled on ice immediately upon removal and rapidly homogenized in cold buffer (0.5 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/2% $NaDodSO_4$, pH 7.0) or quick frozen in liquid nitrogen. To stop proteolytic degradation after homogenization, the sample was then heated for 3 min at 95°C. The sample was then mixed with an equal volume of buffer containing 100 mM Tris/2% β-mercaptoethanol/60% (vol/vol) glycerol/0.002% bromophenol blue, pH 6.8, and an aliquot was applied to polyacrylamide gel. Unused muscle tissue or homogenate could be stored at −80°C for as long as 6 months without noticeable change in the electrophoretic profile.

Immunologic Analysis. Muscle samples were electrophoresed on a 6% polyacrylamide/1% bisacrylamide slab gel (20 × 15 cm) for 4 hr at 7.5 W as described by Locker and Wild (5). Proteins were electrophoretically transferred from the polyacrylamide gels to nitrocellulose paper, which was then cut into strips for immunoblotting (6). Proteins were visualized by gold stain (Bio-Rad enhanced colloidal gold total protein detection kit). Binding of rabbit immunoglobulins was detected using goat anti-rabbit antiserum conjugated to alkaline phosphatase (Bio-Rad immunoblot assay kit). Where indicated, before detection of the protein bands serum was preadsorbed with KLH alone or with (87-25)oligopeptide as follows. Twenty milligrams of KLH or 2 mg of (87-25)oligopeptide were covalently linked to 1 g (dry weight) of CNBr-activated Sepharose 4B according to the procedure of the manufacturer (Pharmacia). Antiserum was added to the beads and shaken gently for 2 hr at 37°C and for 24 hr at 4°C.

RESULTS

The amino acid sequence deduced from the putative exon region of probe pERT87-25 is Ala-Ile-Glu-Arg-Glu-Lys-Ala-Glu-Lys-Phe-Arg-Lys-Leu-Gln-Asp-Ala-Ser-Arg-Ser-Ala-Gln-Ala-Leu. After solid-phase synthesis, the (87-25)oligopeptide with an additional N-terminal cysteine and C-terminal amide was conjugated with KLH using the heterobifunctional linker *N*-succinimidyl 3-(2-pyridyldithio)propionate. Two rabbits were immunized with the (87-25)–KLH conjugate. Fig. 1 shows the results of an immunologic analysis obtained using antiserum from one of these rabbits before and after immunization with (87-25)oligopeptide-KLH. For this analysis, human muscle proteins were resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis using a low percentage of cross linker to allow high molecular weight proteins to enter the gel.

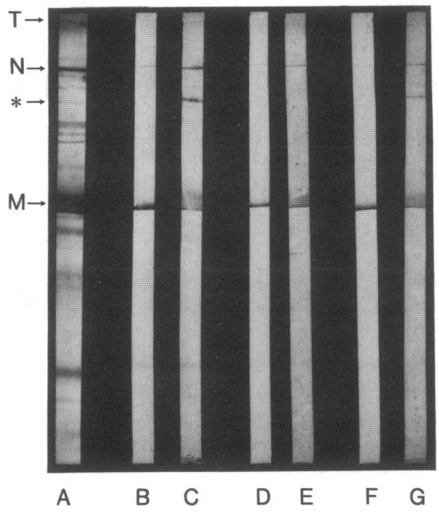

FIG. 1. Binding of rabbit antibodies to human muscle proteins. Human skeletal muscle was homogenized in $NaDodSO_4$ buffer and electrophoresed on 6% polyacrylamide slab gel with 1% bisacrylamide crosslinker to allow high molecular weight proteins to enter the gel. Protein bands were transferred to nitrocellulose by transelectrophoresis, and nitrocellulose strips were then incubated with the indicated antisera. Lane A, protein profile of human muscle protein from an individual with scapulo-peroneal syndrome visualized by gold stain. Arrows indicate the titin (T), nebulin (N), myosin (M), and (87-25)protein (*) bands. Lanes B–G, immunoblots using the immunoglobulin fraction at dilutions equivalent to a 1:200 dilution of rabbit serum. Binding of rabbit immunoglobulins was detected using goat anti-rabbit antiserum conjugated to alkaline phosphatase. Lanes: B, preimmune rabbit antiserum; C, immune rabbit antiserum; D, preimmune rabbit antiserum preadsorbed with (87-25)oligopeptide conjugated to Sepharose 4B; E, immune rabbit antiserum preadsorbed with (87-25)oligopeptide conjugated to Sepharose 4B as in D; F, preimmune rabbit antiserum preadsorbed with KLH conjugated to Sepharose 4B; G, immune rabbit serum preadsorbed with KLH conjugated to Sepharose 4B as in F.

The protein profile as visualized by gold staining is shown in lane A. Bands indicated as myosin and nebulin were identified by comigration with the authentic proteins, and identification of the doublet designated as titin is based on previously published R_f values under similar electrophoretic conditions (5). The cross-reactivity of the immunoglobulin fraction of preimmune antiserum from this rabbit with human muscle protein is shown in lane B. Two clearly discernible antibody bands directed against nebulin and myosin as well as several other, faintly visible, anti-muscle antibody bands are present in the preimmune serum. These results, indicating that the naive rabbits had endogenous immunity to muscle proteins, are consistent with previous reports showing the presence of antibodies to autoantigens in the preimmune sera of several species including humans and rabbits (7, 8). Such autoantibodies are often found to be directed against cytoskeletal proteins including actin and tubulin (7, 9). Because the structures of cytoskeletal proteins are often highly conserved among species and are also often closely homologous to muscle contractile proteins, the presence in rabbit preimmune serum of antibodies cross-reacting with human skeletal muscle proteins is not surprising. To make sure that the presence of antibody binding to a number of human skeletal muscle proteins in rabbit preimmune serum was not the result of nonspecific binding of immunoglobulins to these proteins, nitrocellulose transblots were pretreated with goat antiserum to saturate nonspecific immunoglobulin binding sites. No differences between the pretreated and the control immunoblots could be detected (data not shown).

Fig. 1, lane C shows the pattern of antibodies to human muscle protein present in the serum of this rabbit after

immunization with (87-25)oligopeptide-KLH. In addition to the previously detected bands, a new band cross-reacting with a high molecular weight protein migrating between nebulin and myosin is now present. Because of the paucity of molecular weight markers with consequent uncertainties in the measurement of molecular weights in this region of the gel, we have chosen to temporarily designate the protein recognized by the new antibody as the (87-25)protein. This protein migrates one-fourth of the distance between nebulin and myosin and has an estimated molecular mass between 320 and 420 kDa. In addition to the appearance of the new band, there is also an increase in the intensity of the anti-nebulin band and a smearing of the anti-myosin band in the immune serum.

To determine whether the changes detected in the immune serum are specifically due to antibody directed against (87-25)oligopeptide, antiserum was preadsorbed with (87-25)oligopeptide covalently bound to Sepharose 4B (lanes D and E). The banding pattern of the immunoblot using preimmune serum (lane D) is not different from the untreated perimmune serum of lane B. In immune serum, however, preadsorption with (87-25)oligopeptide–Sepharose 4B results in almost complete removal of the anti-(87-25)oligopeptide antibody. There is also a marked reduction in the intensity of the antinebulin band relative to that present in lane C. Because the bands present in immune serum could have been produced in response to epitopes on KLH rather than on (87-25)oligopeptide, preimmune antiserum (lane F) and immune antiserum (lane G) were preadsorbed with KLH–Sepharose 4B. This preadsorption did not remove the anti-(87-25)oligopeptide antibody from the immune serum of lane G, supporting the conclusion from lanes D and E that immunization has induced the production of antibody specifically directed against (87-25)oligopeptide. The observation that the anti-nebulin band in lanes E and G are of equal intensity indicates that the reduction in these bands relative to lane C is independent of the presence of (87-25)oligopeptide and is therefore a nonspecific effect related to Sepharose 4B exposure. Results with the preimmune and immune antisera of a second, similarily immunized rabbit were identical to the results with the rabbit antiserum shown in Fig. 1 except that the autoantibody banding pattern in the preimmune serum was slightly different (data not shown).

These results, suggesting that antibodies produced in response to the (87-25)oligopeptide cross-react with a specific protein of human skeletal muscle, were extended to a second human muscle sample and to skeletal muscle from two other species (Fig. 2). *I*, *II*, and *III* show the cross-reactivity of the immunoglobulin fraction of rabbit serum with skeletal muscle proteins from a patient with fasciitis, from a rat, and from a rabbit, respectively. Antibody specifically induced by immunization against (87-25)oligopeptide-KLH cross-reacts with an (87-25)protein of skeletal muscle in all of the species tested (lanes C), and preadsorption of the antiserum with (87-25)oligopeptide–Sepharose 4B reverses this effect (lanes D).

To evaluate the status of the (87-25)protein in DMD, muscle biopsies were obtained from two DMD patients and two patients with other diseases for control data. The samples were quick-frozen in liquid nitrogen immediately after biopsy, and muscle protein was prepared for immunoblotting as before. The (87-25)protein band, which was clearly visible in both controls (Fig. 3*I*, lanes A and C), was undetectable in both DMD patients (Fig. 3*I*, B and D). Total protein profiles as revealed by gold stain confirmed the loss of the (87-25)protein band in the two DMD patients (Fig. 3*II*). However, several other changes were also apparent in the patient protein profiles. In one DMD patient (*II* lane B), high-molecular weight bands above the nebulin band were markedly reduced or absent. In both DMD patients, a reduction in bands adjacent to the (87-25)protein was seen. In addition, two new bands above myosin and two new bands below myosin appeared in the DMD patient protein profiles.

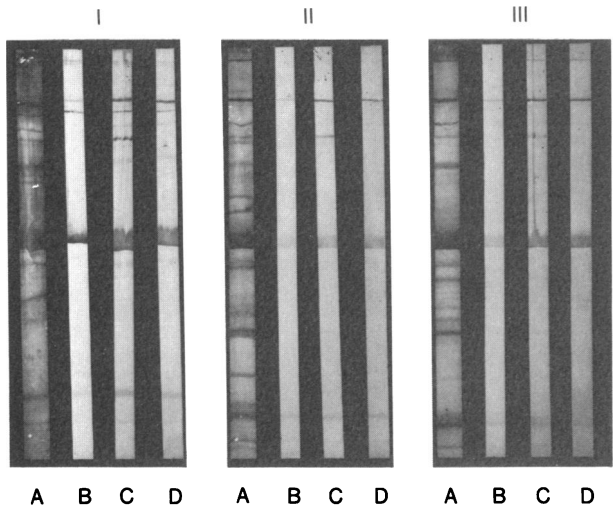

FIG. 2. Species cross-reactivity of anti-(87-25)oligopeptide antiserum. Nitrocellulose transblots were prepared and analyzed as in Fig. 1. (*I*) Human skeletal muscle from an individual with fasciitis. (*II*) Rat gastrocnemius muscle. (*III*) Rabbit gastrocnemius muscle. Lanes: A, protein profiles visualized by gold stain; B, immunoblot of preimmune serum preadsorbed with KLH–Sepharose 4B; C, immunoblot of immune serum preadsorbed with KLH–Sepharose 4B; D, immunoblot of immune serum preadsorbed with (87-25)oligopeptide–Sepharose 4B.

The specificity of immunized rabbit antibody directed against the (87-25)protein was further examined in competition experiments. Fig. 4*I* shows various concentrations of rabbit anti-serum incubated with electrophoretically separated human muscle proteins in the presence or absence of excess (87-25)oligopeptide. Without competing oligopeptide, the anti-(87-25)protein band is clearly visible at anti-serum dilutions of 1:200 (*I* lane B) and 1:100 (*I* lane D). With (87-25)oligopeptide, this band is absent (*I* lanes C and E) even at the higher antiserum concentration. At serum dilutions of 1:500 the anti-(87-25)protein band is only faintly visible (data

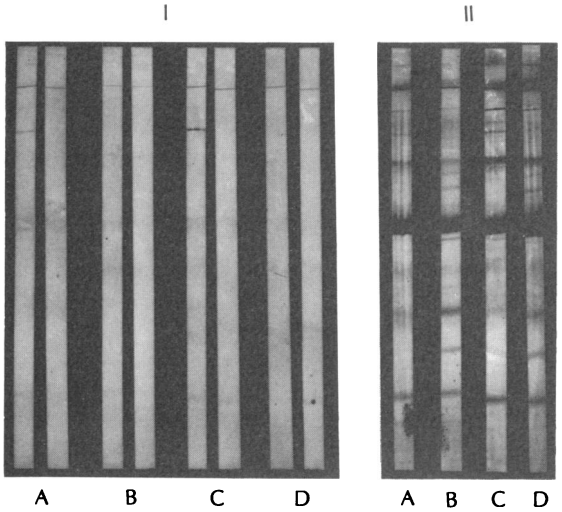

FIG. 3. Cross-reactivity of anti-(87-25)oligopeptide antiserum with DMD muscle protein. Nitrocellulose transblots were prepared and analyzed as in Fig. 1. (*I*) Immunoblots of the binding of immune serum to protein from two control patients (lanes A and C) and two DMD patients (lanes B and D). The second immunoblot in each pair was done in the presence of (87-25)oligopeptide. (*II*) Gold stain protein profiles of the same patients. (In this experiment, antiserum from rabbit 2 was used instead of antiserum from rabbit 1 used in the previous experiments.)

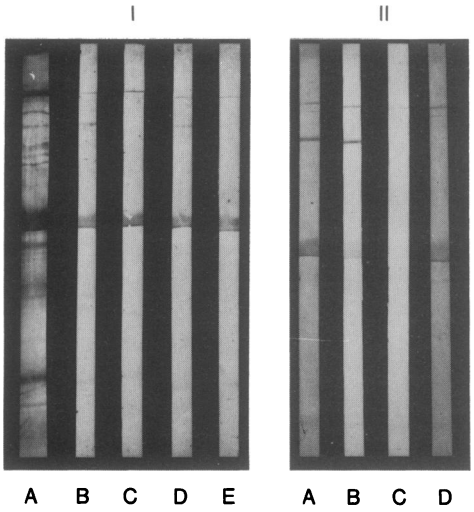

FIG. 4. Competition of (87-25)oligopeptide with (87-25) muscle protein for binding of rabbit antibodies and partial purification of the anti-(87-25) antibody. Nitrocellulose transblots of human skeletal muscle protein were prepared as in Fig. 1. (*I*) Effect of addition of (87-25)oligopeptide at 0.1 mg/ml on binding of rabbit antibodies to human skeletal muscle. Lanes: A, protein profile of skeletal muscle obtained as in Fig. 1; B and D, antibody binding pattern obtained with 1:200 and 1:100 dilutions of immune antiserum, respectively. Lanes C and E, the same conditions as for lanes B and D except (87-25)oligopeptide at 0.1 mg/ml was present during incubation of the nitrocellulose transblots with rabbit antisera. (*II*) Elution of rabbit antiserum from an (87-25)oligopeptide–Sepharose 4B affinity column [2 mg of (87-25)oligopeptide reacted with 1 g (dry weight) of CNBr-activated Sepharose 4B]. Lanes: A, binding pattern of immune antiserum (1:200) before affinity-column purification; B, binding pattern of affinity-purified rabbit antibodies eluted between 4.8 and 6.4 ml of 3.5 M KSCN; C, the same conditions as for lane B except (87-25)oligopeptide at 0.1 mg/ml was present during incubation of the nitrocellulose transblot with affinity-purified antibodies; D, the same conditions as for lane A except that excess myosin was present during incubation of the nitrocellulose transblot with rabbit immune antiserum.

not shown), whereas in preimmune serum the anti-(87-25)band is not detectable (see Fig. 1, lane B).

To purify the anti-(87-25) antibody by affinity chromatography, immune serum was applied to an (87-25)oligopeptide–Sepharose 4B column which was then extensively washed with phosphate buffer to remove nonspecifically bound antibodies, as judged by return of OD_{280} to basal levels. Bound antibody was then eluted from the column with 3.5 M KSCN. The results are shown in Fig. 4*II*, lane B shows the antibody-binding pattern in one fraction of material eluted from the affinity column. Relative to the untreated immune serum of *II* lane A, there is a substantial enrichment of the anti-(87-25)protein antibody in the affinity-purified material. Competition with excess (87-25)oligopeptide for binding of the affinity-purified material (*II* lane C) completely removes both the anti-(87-25)protein band and the small amount of remaining anti-myosin band, and the anti-nebulin band is also decreased. A possible rationale for the effective competition of (87-25)oligopeptide with the affinity-purified antibodies directed against myosin was found in the results of a search for amino acid-sequence similarities between the (87-25)oligopeptide and other proteins. The search revealed a run of six amino acids in the middle of the (87-25)oligopeptide (Lys-Phe-Arg-Lys-Leu-Gln) with complete similarity to amino acids 218–233 of rat skeletal muscle myosin, whereas other regions of similarity were also found to occur in several other myosins. This limited similarity between the (87-25)protein and myosin was further substantiated by the ability of excess myosin to effectively compete with antiserum for binding to the (87-25)protein (compare Fig. 4*II* lanes A and D). Although the data indicate that myosin and, perhaps, nebulin share some sequence similarity with the (87-25)protein, these are clearly different proteins as evidenced by the differences in molecular weight and amino acid sequence and by the failure of the anti-nebulin and anti-myosin antibodies present in the preimmune serum to react at all with the (87-25)protein (see Fig. 1, lane B).

DISCUSSION

Wood *et al.* (10) have proposed that the high molecular weight protein nebulin may be a product of the DMD gene. This suggestion was based on their observations that nebulin is markedly decreased in a number of DMD patients and the observation of others that nebulin appears to be functionally involved in muscle contraction and elasticity (11). However, other recent evidence has cast some doubt on the role of this protein in DMD because nebulin is apparently absent from cardiac tissue, whereas the DMD gene transcript has been detected in cardiac muscle (as reported by M. Robertson in a review of the proceedings of a meeting held at Versailles, April 8–10, 1987; see ref. 12). In our studies, the antibody induced by immunization with the (87-25)oligopeptide recognizes a protein that, based on its electrophoretic migration, clearly differs from nebulin. Although the possibility exists that the (87-25)oligopeptide could be derived from nebulin as a result of limited proteolysis, this is unlikely because the time between obtaining the muscle samples and tissue homogenization in $NaDodSO_4$ buffer was usually <15 min and often <5 min, conditions consistent with only minimal autoproteolysis. On the other hand, because autoantibodies to nebulin in the preimmune sera could be masking the detection of a small increase in anti-nebulin activity, we cannot state unequivocably that antibodies induced by the (87-25)oligopeptide do not recognize nebulin.

The loss of the (87-25)protein bands seen in muscle samples from two DMD patients agrees with the conclusion that the (87-25)protein is the DMD gene product. It would, however, be premature to conclude from this small sample that absence of the (87-25)protein will be found in all DMD cases. The strength of the conclusions from the patient data is also diminished by the protein profile data, which showed the loss of several other bands besides the (87-25)protein and the gain of four additional bands. The presence of different bands could be explained by partial degradation of higher molecular weight proteins in DMD tissue. Alternatively, these different proteins could result from attempts by DMD cells to restore muscle tissue or from invasion of the DMD muscle by nonmuscle cells. It is noteworthy that the DMD patient whose high molecular weight proteins (above the nebulin band) were well preserved was also unusual in showing delayed loss of motor functions.

These results show that a region of the pERT87-25 cDNA probe codes for a specific human skeletal muscle protein. This protein is well conserved in evolution because cross-reacting proteins of nearly identical molecular weight are present in rat and rabbit skeletal muscle. This conservation is not unexpected because the method used by Monaco *et al.* (1) in selecting this probe relied, in part, on the mRNA being well conserved among species.

While this paper was being reviewed, two articles by Hoffman, Kunkel, and co-workers appeared (13, 14) identifying the protein product of the DMD gene by the use of a different immunological approach from our technique. From the data presented in these papers, the protein that they identified and named dystrophin and the (87-25) protein that we identified appear to be the same. We therefore suggest that the (87-25)protein be referred to as dystrophin in future work.

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