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Duchenne Muscular Dystrophy Gene Expression in Normal and Diseased Human Muscle

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A probe for the 5′ end of the Duchenne muscular dystrophy (DMD) gene was used to study expression of the gene in normal human muscle, myogenic cell cultures, and muscle from patients with DMD. Expression was found in RNA from normal fetal muscle, adult cardiac and skeletal muscle, and cultured muscle after myoblast fusion. In DMD muscle, expression of this portion of the gene was also revealed by in situ RNA hybridization, particularly in regenerating muscle fibers.

DUCHENNE MUSCULAR DYSTROPHY (DMD) is a severe hereditary degenerative disease of muscle with an incidence of about 1 in 3500 live male births. Cytogenetic and linkage studies have localized the genetic defect to the middle of the short arm of the X chromosome (*1*), and two approaches have led to the identification of a segment of DNA deleted in many patients with the disease (*2*). Portions of this DNA segment have been used to isolate complementary DNA (cDNA) from a 14-kb transcript specifically expressed in fetal and adult skeletal muscle (*3–5*). The protein encoded by this transcript has recently been identified and given the name dystrophin (*6*). We sought to determine whether expression of the dystrophin gene is detectable in muscle from DMD patients. We found that at least a part of the gene was expressed in every patient studied.

The probe used in this study was a 1.4-kb subclone of a 2.0-kb cDNA isolated with genomic DNA from the region of an X;21 translocation that produced symptoms of DMD in a female patient (*4*). The portion of the transcript identified by this probe is at the 5′ end of the gene (*4, 5*). The cDNA was subcloned into a Bluescript vector (Stratagene), which allowed production of labeled strand-specific RNA probes. Strand polarity was identified by sequence analysis; experiments done with antisense probes included controls done with probes of the opposite polarity. Sense and antisense probes had specific activities that differed by less than 20%, and equal counts of each probe were used.

The probe identified a messenger RNA (mRNA) transcript of about 14 kb in Northern blots of total RNA from human fetal muscle and adult cardiac and skeletal muscle (Fig. 1A). Detection of this signal depended on isolation of high-quality (nondegraded) RNA. RNA isolated from human muscle cells grown in monolayer culture also showed a 14-kb transcript after 23 days of growth from primary explants. The signal was noted only after the appearance of myotubes in the culture plates.

Muscle specimens were taken from patients with typical clinical manifestations of DMD. Each patient had the usual hereditary pattern, including distribution of weakness, elevation of serum creatine kinase, and muscle biopsy findings that are characteristic of this disease. None of the patients studied had deletions of genomic DNA on Southern blot with the cDNA probe we were using. Total cellular RNA isolated from the muscle

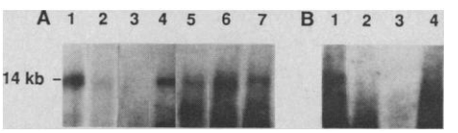

Fig. 1. Northern blot analysis of human muscle RNA with a probe for the DMD gene. (**A**) Samples from normal muscle; lane 1, quadriceps muscle from a 29-year-old male, lane 2, skeletal muscle from an 18-week fetus; lane 3, tissue culture 17 days; lane 4, tissue culture 23 days, lane 5, tissue culture 43 days; lane 6, cardiac muscle (atrium); lane 7, cardiac muscle (ventricle). (**B**) Quadriceps muscle samples from patients with DMD, aged 4 to 10 years: a transcript is seen in lane 1 but not in lanes 2 to 4. Total RNA was isolated from human muscle by means of a guanidine isothiocyanate/cesium chloride gradient technique (*9*). RNA was isolated from tissue culture preparations with a guanidine hydrochloride precipitation procedure (*10*). The RNA was separated in a 0.8% agarose-formaldehyde gel and transferred onto Biotrans nylon membranes (ICN) after a water rinse to remove excess formaldehyde (*9*). The RNA was not alkali-treated before transfer. Antisense probes were labeled with [^{32}P]UTP by means of T3 polymerase (Stratagene). The blots were hybridized in 5× standard saline citrate (SSC) (1× SSC is 0.15*M* NaCl, 0.015*M* sodium citrate), 50% formamide, and 10% dextran sulfate at 52°C and washed in 0.1× SSC, 0.1% SDS at 65°C (*11*). The mRNA size is based on comparison with a standard RNA ladder (BRL).

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of six patients was studied by Northern blot. A faint transcript of roughly normal size was detected in one patient and possibly a second; no discrete signal of any size was seen in the other samples, even under low-stringency hybridization conditions (Fig. 1B). Under low stringency and without ribonuclease digestion of the nonhybridized probe, there was substantial nonspecific binding to ribosomal RNA, which could have obscured a faint signal from a transcript less than half the normal size. This nonspecific binding to smaller RNA species was seen with both sense and antisense probes. The quality of RNA in these preparations was assured by normal hybridization with a control probe for myosin heavy chain. Additional evidence that the RNA was not degraded came from

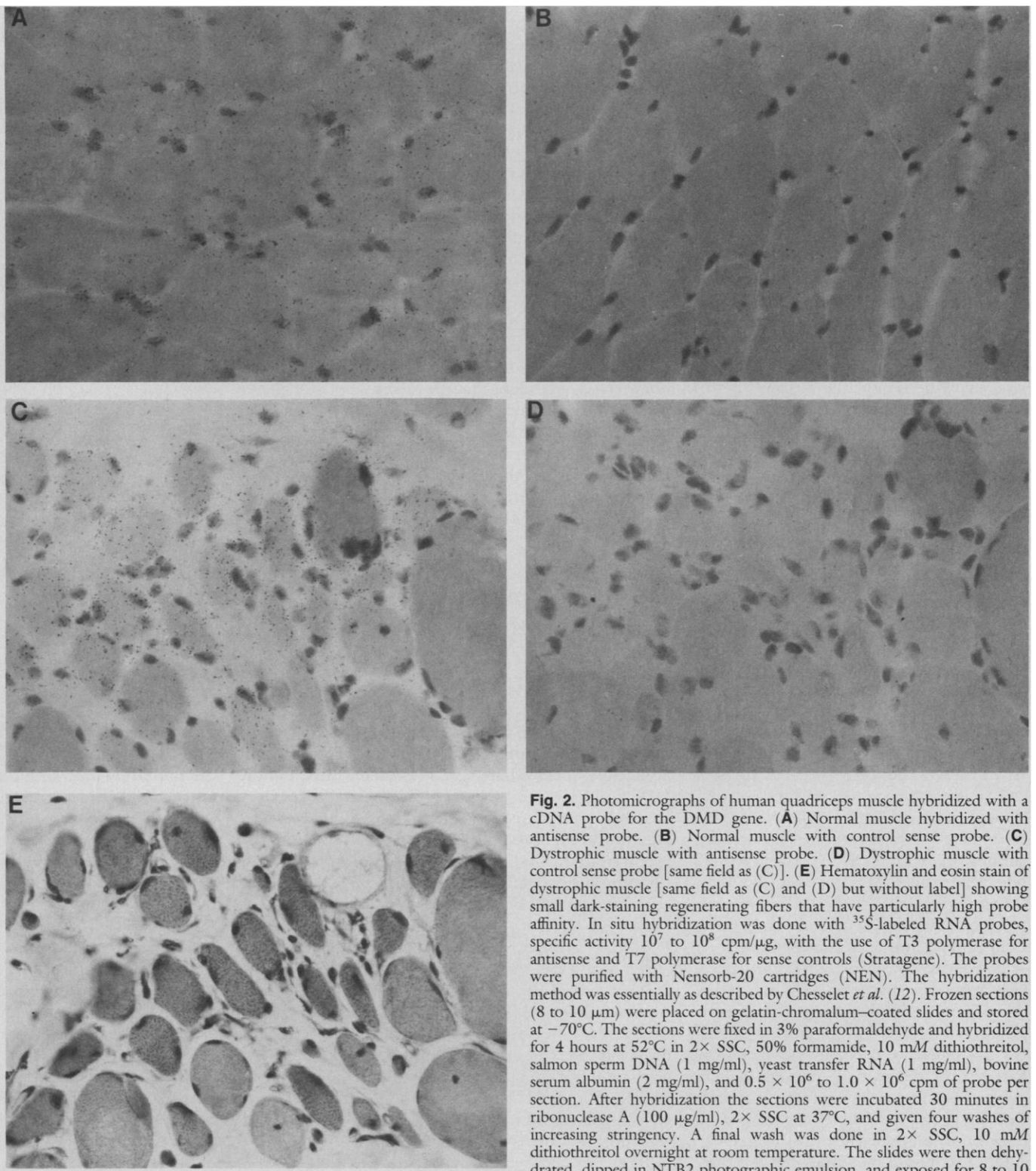

Fig. 2. Photomicrographs of human quadriceps muscle hybridized with a cDNA probe for the DMD gene. (**A**) Normal muscle hybridized with antisense probe. (**B**) Normal muscle with control sense probe. (**C**) Dystrophic muscle with antisense probe. (**D**) Dystrophic muscle with control sense probe [same field as (C)]. (**E**) Hematoxylin and eosin stain of dystrophic muscle [same field as (C) and (D) but without label] showing small dark-staining regenerating fibers that have particularly high probe affinity. In situ hybridization was done with ^{35}S-labeled RNA probes, specific activity 10^7 to 10^8 cpm/μg, with the use of T3 polymerase for antisense and T7 polymerase for sense controls (Stratagene). The probes were purified with Nensorb-20 cartridges (NEN). The hybridization method was essentially as described by Chesselet *et al.* (*12*). Frozen sections (8 to 10 μm) were placed on gelatin-chromalum–coated slides and stored at −70°C. The sections were fixed in 3% paraformaldehyde and hybridized for 4 hours at 52°C in 2× SSC, 50% formamide, 10 m*M* dithiothreitol, salmon sperm DNA (1 mg/ml), yeast transfer RNA (1 mg/ml), bovine serum albumin (2 mg/ml), and 0.5×10^6 to 1.0×10^6 cpm of probe per section. After hybridization the sections were incubated 30 minutes in ribonuclease A (100 μg/ml), 2× SSC at 37°C, and given four washes of increasing stringency. A final wash was done in 2× SSC, 10 m*M* dithiothreitol overnight at room temperature. The slides were then dehydrated, dipped in NTB2 photographic emulsion, and exposed for 8 to 10 days before development with Kodak D19. The slides were counterstained with hematoxylin and eosin after development of the emulsion. Final magnification, ×300.

the intensity of the 28*S* ribosomal RNA band in ethidium-stained test gels and methylene blue–stained filters after transfer, and in vitro translation of high molecular weight protein from the RNA in two samples.

Because of difficulty visualizing the 14-kb dystrophin message in disease muscle, even under conditions that produced much nonspecific binding, we used the alternative technique of in situ RNA hybridization, which proved to be both more sensitive and more specific. In situ hybridization studies of sections of normal human skeletal muscle showed binding of the probe to the mRNA sequence within muscle fibers. The signal intensity was consistently greatest in and around muscle fiber nuclei (Fig. 2A). This binding pattern is similar in distribution but less intense than we have found with a probe for creatine kinase. With the DMD gene probe there was no apparent variation in the labeling pattern by muscle fiber type, and sections of cardiac muscle showed a pattern similar to that of skeletal muscle although the intensity of the signal was less. There was very little nonspecific binding by this technique: control sections hybridized with a probe of reverse polarity showed signal intensity about 1/20 of that seen in the experimental sections, without an increase in signal around muscle fiber nuclei (Fig. 2B).

In situ RNA hybridization study was done on sections of biopsied muscle from ten DMD patients. In each case signal was clearly detected (Fig. 2C), although it varied more than in normal muscle. The signal was reduced in fibers with relatively normal appearance, and clusters of regenerating muscle fibers [those with basophilia and small size (see Fig. 2E)] showed particularly high signal intensity. In regenerating fibers the signal was again most intense in the vicinity of the nuclei. The control sections (hybridized with a probe of reverse polarity) showed substantially less signal than the experimental sections, and the characteristic labeling over regenerating fibers and around muscle fiber nuclei was not seen (Fig. 2D).

Our finding of a 14-kb DMD gene transcript of relatively low abundance in RNA from normal muscle is consistent with results reported by others (*3, 4*). The expression found in myogenic cells in culture differs from the initial report (*3*) that the gene is not expressed in cultured myoblasts, but this difference is readily explainable by the state of differentiation of the culture preparations in our experiments. It appears that the gene is normally transcribed in muscle cells only after myoblast fusion (*7*).

Diseased muscle showed evidence of transcription of at least the 5′ end of the gene by in situ hybridization, although full-length mRNA was not seen on Northern blot in most cases. This finding could have been due to greater sensitivity of the in situ hybridization technique. It is also likely that the message seen in diseased muscle by in situ hybridization is not seen on Northern blot because it is truncated or rapidly degraded. This interpretation is consistent with recent findings that 50% or more of DMD patients have deletions of part of the gene, frequently in the central portion (*5, 8*). In situ hybridization studies with another cDNA probe from the central part of the gene (*5*) show a loss of signal (nonspecific background binding only) in muscle from two patients with known deletions in this region. Our results indicate that in some patients transcription of the full-length message does not occur. On the basis of these findings we would predict that the complete gene product is markedly reduced or absent in patients with the disease.

Our finding of signal for the 5′ end of the transcript indicates that none of the DMD patients sampled had a true null mutation, however. Regenerating muscle fibers appeared to have greater signal intensity than normal, perhaps indicating a compensatory increase in transcription. If the reading frame is preserved, these patients might be expected to have protein corresponding to at least this portion of the message. The disease phenotype likely depends on the site of the defect within the gene and whether translation of the message is affected.

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Human Immunodeficiency Virus May Encode a Novel Protein on the Genomic DNA Plus Strand

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The genome of the human immunodeficiency virus (HIV) is known to contain eight open reading frames (ORFs) on the minus strand of the double-stranded DNA replicative intermediate. Data presented here indicate that the DNA plus strand of HIV contains a previously unidentified ORF in a region complementary to the envelope gene sequence. This ORF could encode a protein of approximately 190 amino acid residues with a relative molecular mass of 20 kilodaltons if translation began from the first initiation codon. The predicted protein is highly hydrophobic and thus could be membrane associated. It is possible, therefore, that the HIV genome encodes a protein on antisense messenger RNA.

THE HUMAN IMMUNODEFICIENCY virus (HIV) is the primary etiologic agent of the acquired immunodeficiency syndrome (AIDS). Like other retroviruses, HIV replicates by a mechanism involving reverse transcription of RNA and generation of a double-stranded DNA intermediate. Virion RNA of plus strand polarity, transcribed from minus strand DNA, contains the three open reading frames (ORFs) that encode the viral capsid, envelope (*env*), and polymerase proteins, as well as five additional proteins not commonly found in other retroviruses: tat, sor, trs/art, R, and 3′-orf (*1*). Computer analysis of HIV type 1 (HIV-1), presented here, indicates the presence of a ninth ORF with a novel location: the plus strand of the virus DNA genome.

Computer analysis (*2*) of the plus strand DNA sequence of HIV reveals a long ORF located in the region of the genome complementary to the *env* gene sequence of the

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