The EMBO Journal vol.6 no.11 pp.3277–3283, 1987

Deletions of fetal and adult muscle cDNA in Duchenne and Becker muscular dystrophy patients

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Communicated by R.Williamson

We have isolated a cDNA molecule from a human adult muscle cDNA library which is deleted in several Duchenne muscular dystrophy patients. Patient deletions have been used to map the exons across the Xp21 region of the short arm of the X chromosome. We demonstrate that a very mildly affected 61 year old patient is deleted for at least nine exons of the adult cDNA. We find no evidence for differential exon usage between adult and fetal muscle in this region of the gene. There must therefore be less essential domains of the protein structure which can be removed without complete loss of function. The sequence of 2.0 kb of the adult cDNA shows no homology to any previously described protein listed in the data banks although sequence comparison at the amino acid level suggests that the protein has a structure not dissimilar to rod structures of cytoskeletal proteins such as lamin and myosin. There are single nucleotide differences in the DNA sequence between the adult and fetal cDNAs which result in amino acid changes but none that would be predicted to change the structure of the protein dramatically.

Key words: cDNA/muscle/Duchenne/Becker

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease affecting approximately 1 in 3000 males (for review see Emery, 1987). Afflicted males are generally wheelchair-bound by the age of 12 and die in their late teens. The disease was localized to the Xp21 region by the cytogenetic assignment of the X chromosome breakpoint in an X;1 translocation in a female suffering from the disease (Lindenbaum, 1979). In X;autosome balanced translocations it is the normal X chromosome that is inactive. Thus if the breakpoint interrupts expression of the DMD gene in Xp21 the patient suffers from DMD. DNA studies confirmed this localization by segregation studies in families (Murray *et al.*, 1982; Davies *et al.*, 1983). The clinically milder disease, Becker muscular dystrophy (BMD) shows a similar segregation pattern with the DNA markers and is now known to be allelic (Kingston *et al.*, 1983, 1984).

Recently, a cDNA molecule has been reported which is deleted in DMD and BMD patients (Monaco *et al.*, 1986). This was derived by screening a fetal muscle cDNA library with conserved sequences from the pERT87 locus originally isolated by Kunkel and colleagues and shown to be deleted in 10% of DMD patients (Kunkel *et al.*, 1986). Monaco *et al.* (1986) demonstrated that the cDNA hybridized to a 16-kb mRNA in fetal muscle and that the exons were distributed over a large region of genomic DNA. Pulsed-field gel electrophoresis analyses of the extent of deletions and the physical linkage of the probes suggests that the gene is spread over a genomic region of at least 2000 kb (Burmeister and Lehrach, 1986; van Ommen *et al.*, 1986; Kenwrick *et al.*, 1987). The order of loci isolated from the region of the DMD gene and shown to be deleted in patients can be summarized as follows (Monaco and Kunkel, 1987; Ray *et al.*, 1985; Smith *et al.*, 1987):

Xpter–JBir–pERT87-41–pERT87-30–pERT87-15–pERT87-8–pERT87-1–XJ1.1–HIP25–pERT84–Xcen

Using these genomic markers, we have mapped deletions in several patients who display a variation in the severity of the disease (Davies *et al.*, 1987). This and other studies (Hart *et al.*, 1987) did not reveal differences in deletions between some mildly

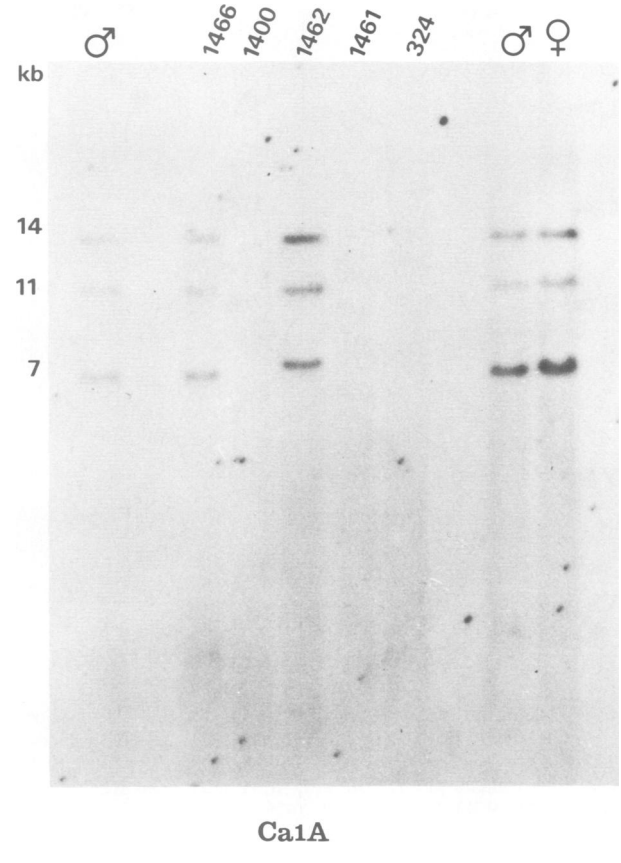


Fig. 1. Southern blot analysis of *Hind*III digests of DNA samples from DMD patients after hybridization to Ca1A. ♂, ♀, male and female genomic controls; **1466**: deletion includes pERT87-15, -8 and -1; **1400**: deletion includes JBir, pERT87, XJ1.1, HIP25 and 754; **1462**: deletion includes pERT87-30, -15, -8, -1, XJ1.1 and HIP25; **1461**: deletion includes pERT87-41, -30, -15, -8, -1, XJ1.1 and HIP25; **324**: deletion includes JBir, pERT87-41, -30, and -15.

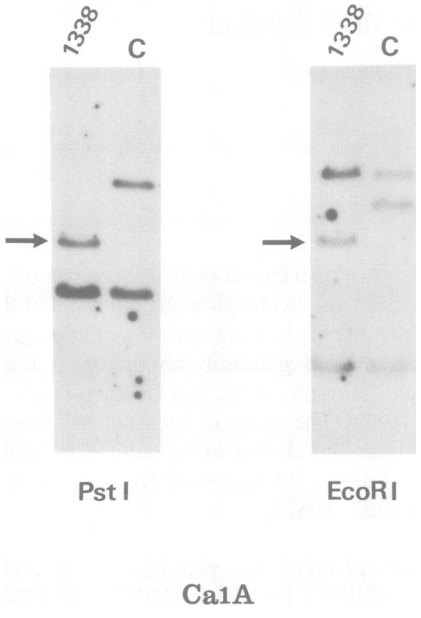


Fig. 2. Enzyme digest showing one of the deletion endpoints in the DMD patient 1338 detected by Ca1A. **C** is a normal genomic control. The arrows indicate the altered band.

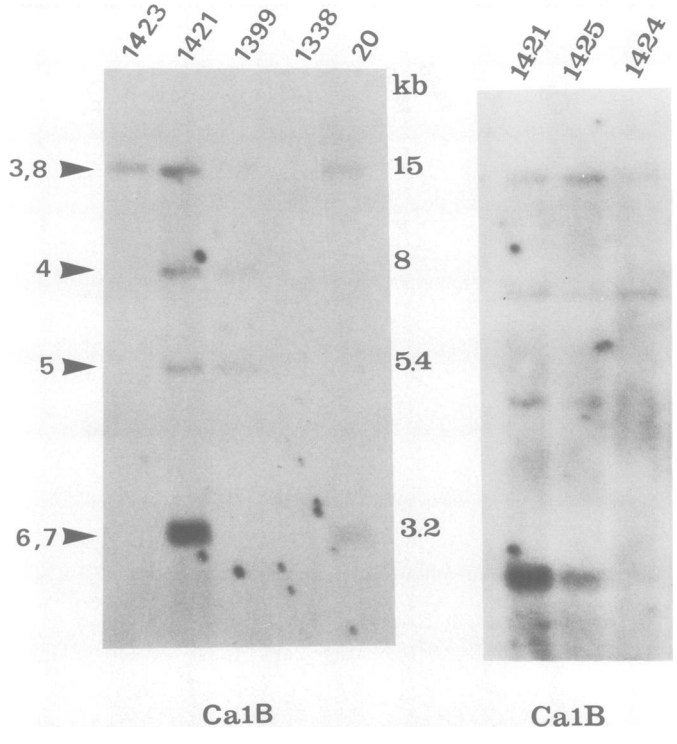


Fig. 3. *Pst*I digest of DMD patients hybridized to Ca1B. **1423**: deletion includes pERT87-15, -8, -1, XJ1.1 and HIP25; **1421**: deletion includes pERT87-1; **1399**: deletion includes pERT87-15, -8, -1, XJ1.1 and HIP25; **1338**: deletion includes pERT87-30, -15, -8, -1, XJ1.1 and HIP25; **20**: deletion includes pERT87-41, and -30; **1425**: deletion includes pERT87-8 and -1; **1424**: deletion includes pERT87-15, -8, -1, XJ1.1 and HIP25. The numbers refer to the exon numbers across Xp21 as in Figure 6.

affected patients compared with severely affected ones. Our objective in the present study was to isolate the coding sequences deleted in these cases in order to investigate a correlation, if any, between the differences in the phenotypes and the exons of the gene deleted and to determine whether differential splicing of

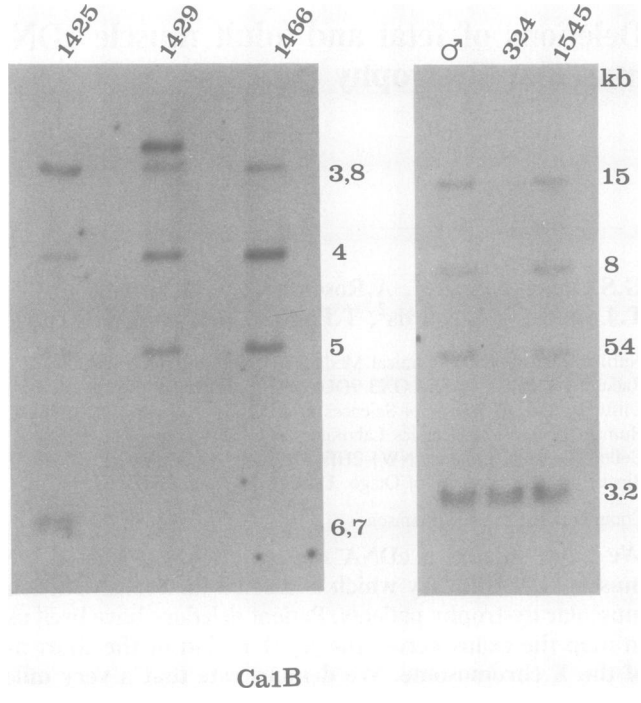


Fig. 4. *Pst*I digests of patients' DNA hybridized to Ca1B. DMD patient **1425**: deletion includes pERT87-8 and -1; DMD patient **1429**: deletion includes pERT87-15, -8 and -1; DMD patient **1466**: deletion includes pERT87-15, -8 and -1; ♂: genomic control; BMD patient **324**: deletion includes pERT87-15, -30, -41 and JBir; DMD patient **1545**: deletion includes JBir only. The numbers refer to the exon numbers across Xp21 as in Figure 6.

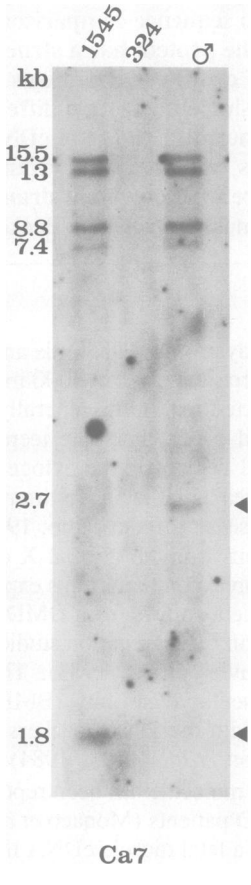


Fig. 5. *Pst*I digests of BMD patient 324 (deleted JBir, pERT87-41, -30 and -15) and DMD patient 1545 (delected JBir). ♂: genomic control. Hybridization to Ca7. The arrows indicate the polymorphic band also detected by Ca1A.

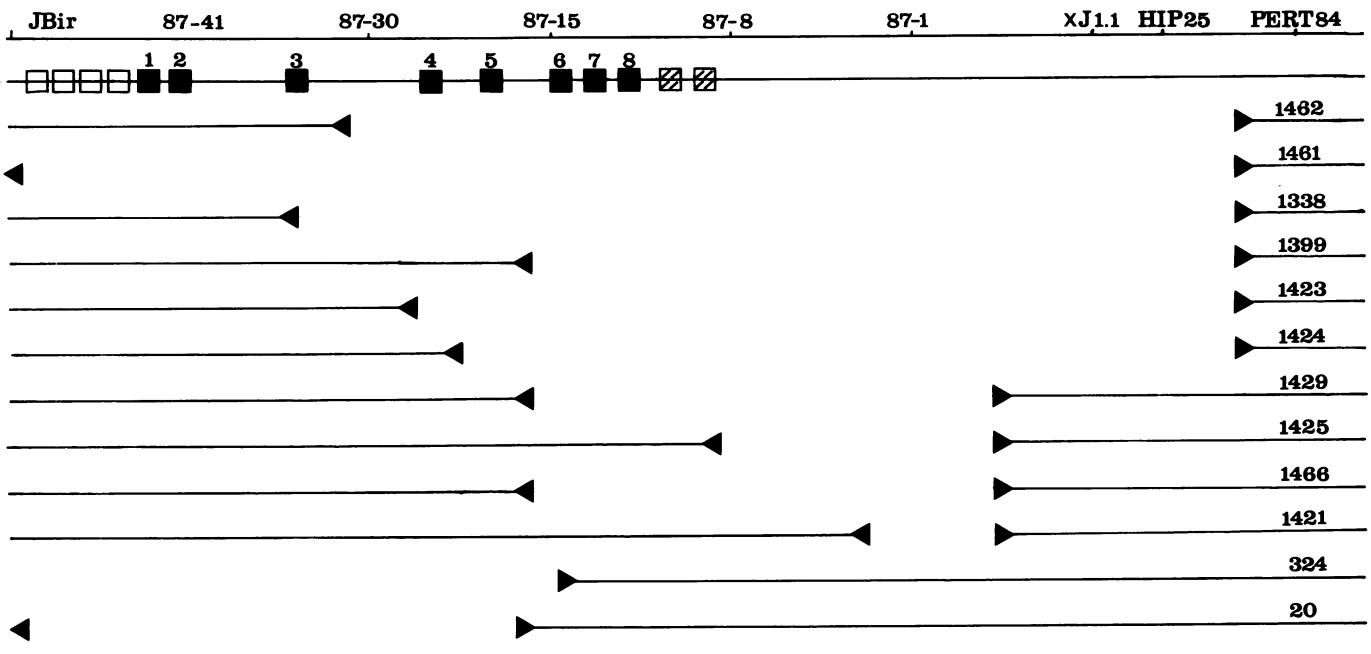


Fig. 6. Summary of the mapping of exons in the adult muscle cDNA. ■ adult exons in Ca1; □ additional adult exons in Ca7; ▨ additional fetal exons in Cf16 (not to scale).

the gene in fetal and adult muscle could explain the clinical observations.

Results and discussion

Our initial aim was to characterize the deletions in patients whose proximal breakpoints lay within the pERT87 locus. In particular, we wished to discover which, if any, exons were deleted in a Becker patient who is the mildest case so far reported, despite having a very large genomic deletion, beginning within pERT87 and extending towards the telomere (patient 324 in Davies *et al.*, 1987). From the exon map of Monaco *et al.* (1986) it was known that this deletion includes at least 3 exons of the fetal mRNA. It is possible, however, that there is differential exon usage in adult and fetal mRNAs. It was thus desirable to obtain cDNA clones from adult skeletal muscle to test this hypothesis. Therefore an oligonucleotide was synthesized from the published sequence of the conserved region of the pERT87 (DXS164) locus (Monaco *et al.*, 1986) and used to screen a human adult muscle cDNA library in λgt11. A 2.0-kb cDNA clone, Ca1, was isolated which consisted of two 1.0 kb *Eco*RI fragments. The localization of the sequence within Xp21 is demonstrated by hybridization to DNA from DMD patients possessing deletions of Xp21 (see Figures 1,2,3 and 4). The cDNA detected a large mRNA in muscle consistent with that observed in fetal muscle by Monaco *et al.* (1986).

The *Eco*RI fragments of Ca1 were used independently to order the exons across the pERT87 locus by Southern blotting to DNA from patients with previously described deletions (Davies *et al.*, 1987). An example is shown in Figure 1 where the more telomeric *Eco*RI cDNA probe (Ca1A) was hybridized to various patient DNAs after digestion with *Hin*dIII. The deletions as defined previously are given below the blot. A normal individual gives three clear bands under these conditions. The deletion in patient 1461 begins between pERT84 and HIP25 and ends between pERT87-41 and JBir. This patient is deleted for all the bands. The deletion in patient 1462 begins between pERT84 and HIP25 and ends between pERT87-30 and pERT87-41. This patient is not deleted for any of the bands. Ca1A does not possess a *Hin*dIII site and thus the bands must represent individual exons. This maps the exons homologous to Ca1A distal to pERT87-30. Pa-

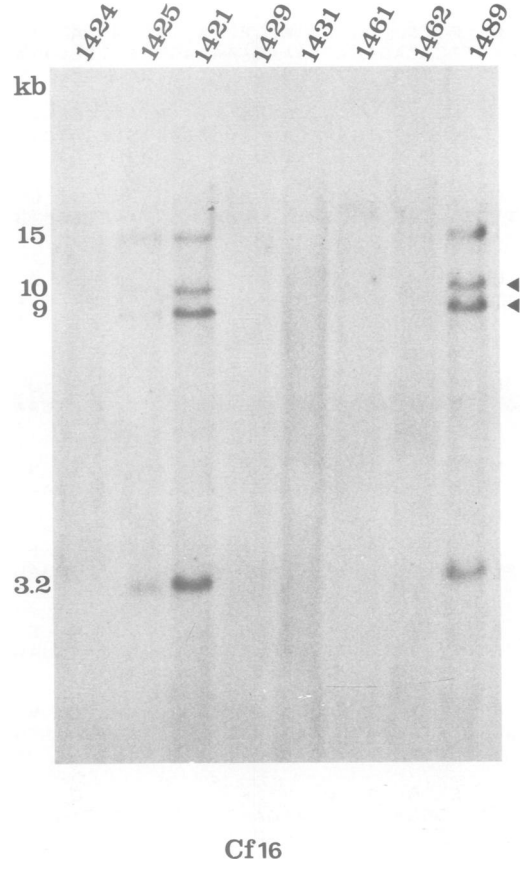
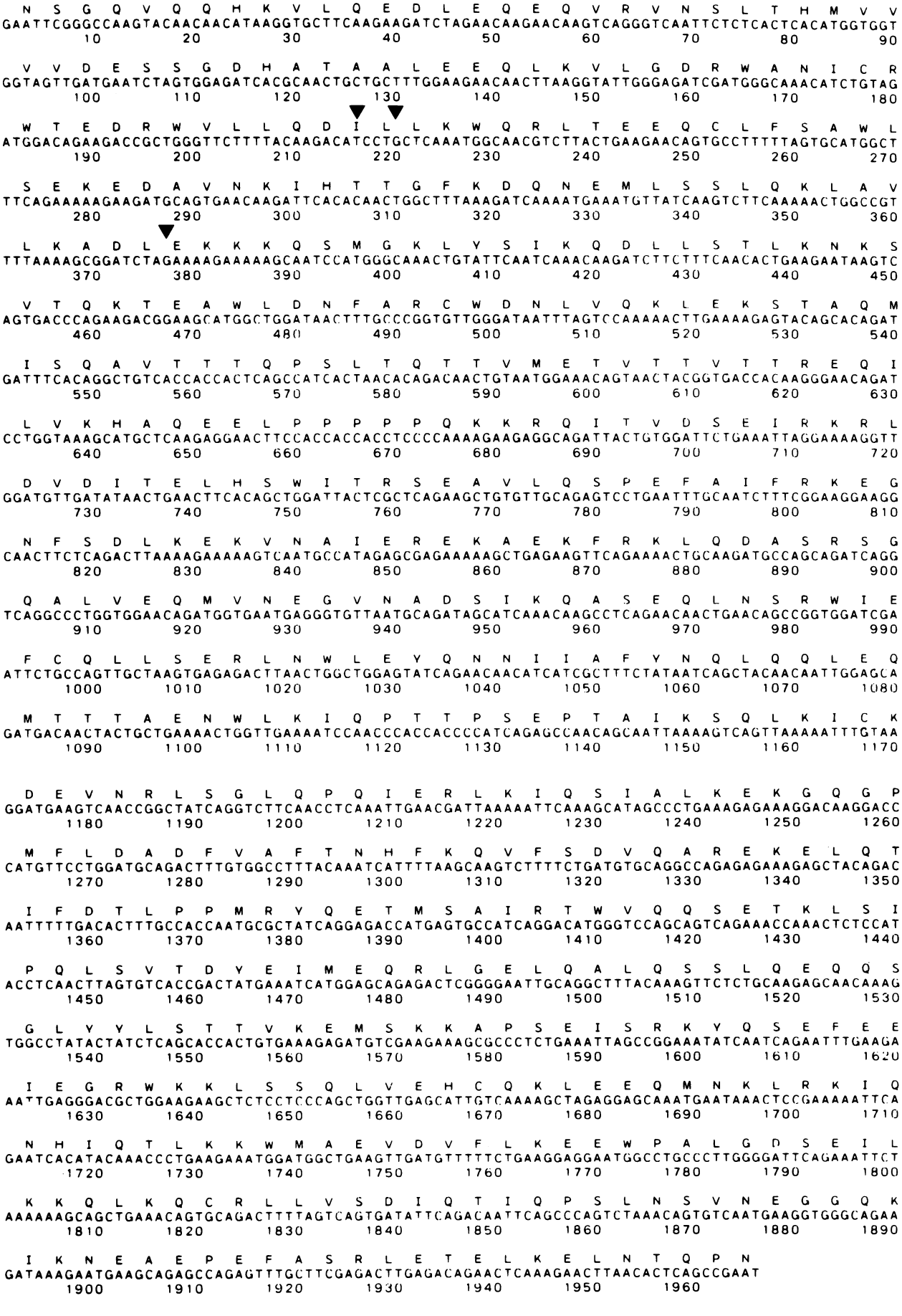


Fig. 7. *Pst*I digests of patient DNA samples after hybridization with Cf16. Deletions in patients 1424, 1425, 1421, 1429, 1461 and 1462 are described in Figures 1, 3 and 4. Patient **1431** is deleted for pERT87-41, -30, -15, -8, -1, XJ1.1 and HIP25. Patient **1489** is deleted for XJ1.1 and HIP25. The arrows indicate the new bands detected by Cf16, not detected by Ca1B.


N S G Q V Q Q H K V L Q E D L E Q E Q V R V N S L T H M V V
GAATTCGGGCCAAGTACAACAACATAAGGTGCTTCAAGAAGATCTAGAACAAGAACAAGTCAGGGTCAATTCTCTCACTCACATGGTGGT
10 20 30 40 50 60 70 80 90
V V D E S S G D H A T A A L E E Q L K V L G D R W A N I C R
GGTAGTTGATGAATCTAGTGGAGATCACGCAACTGCTGCTTTGGAAGAACAACTTAAGGTATTGGGAGATCGATGGGCAAACATCTGTAG
100 110 120 130 140 150 160 170 180
W T E D R W V L L Q D I L L K W Q R L T E E Q C L F S A W L
ATGGACAGAAGACCGCTGGGTTCTTTTACAAGACATCCTGCTCAAATGGCAACGTCTTACTGAAGAACAGTGCCTTTTTAGTGCATGGCT
190 200 210 220 230 240 250 260 270
S E K E D A V N K I H T T G F K D Q N E M L S S L Q K L A V
TTCAGAAAAAGAAGATGCAGTGAACAAGATTCACACAACTGGCTTTAAAGATCAAAATGAAATGTTATCAAGTCTTCAAAAACTGGCCGT
280 290 300 310 320 330 340 350 360
L K A D L E K K K Q S M G K L Y S I K Q D L L S T L K N K S
TTTAAAAGCGGATCTAGAAAAGAAAAAGCAATCCATGGGCAAACTGTATTCAATCAAACAAGATCTTCTTTCAACACTGAAGAATAAGTC
370 380 390 400 410 420 430 440 450
V T Q K T E A W L D N F A R C W D N L V Q K L E K S T A Q M
AGTGACCCAGAAGACGGAAGCATGGCTGGATAACTTTGCCCGGTGTTGGGATAATTTAGTCCAAAAACTTGAAAAGAGTACAGCACAGAT
460 470 480 490 500 510 520 530 540
I S Q A V T T T Q P S L T Q T T V M E T V T T V T T R E Q I
GATTTCACAGGCTGTCACCACCACTCAGCCATCACTAACACAGACAACTGTAATGGAAACAGTAACTACGGTGACCACAAGGGAACAGAT
550 560 570 580 590 600 610 620 630
L V K H A Q E E L P P P P P Q K K R Q I T V D S E I R K R L
CCTGGTAAAGCATGCTCAAGAGGAACTTCCACCACCACCTCCCCAAAAGAAGAGGCAGATTACTGTGGATTCTGAAATTAGGAAAAGGTT
640 650 660 670 680 690 700 710 720
D V D I T E L H S W I T R S E A V L Q S P E F A I F R K E G
GGATGTTGATATAACTGAACTTCACAGCTGGATTACTCGCTCAGAAGCTGTGTTGCAGAGTCCTGAATTTGCAATCTTTCGGAAGGAAGG
730 740 750 760 770 780 790 800 810
N F S D L K E K V N A I E R E K A E K F R K L Q D A S R S G
CAACTTCTCAGACTTAAAAGAAAAAGTCAATGCCATAGAGCGAGAAAAAGCTGAGAAGTTCAGAAAACTGCAAGATGCCAGCAGATCAGG
820 830 840 850 860 870 880 890 900
Q A L V E Q M V N E G V N A D S I K Q A S E Q L N S R W I E
TCAGGCCCTGGTGGAACAGATGGTGAATGAGGGTGTTAATGCAGATAGCATCAAACAAGCCTCAGAACAACTGAACAGCCGGTGGATCGA
910 920 930 940 950 960 970 980 990
F C Q L L S E R L N W L E Y Q N N I I A F Y N Q L Q Q L E Q
ATTCTGCCAGTTGCTAAGTGAGAGACTTAACTGGCTGGAGTATCAGAACAACATCATCGCTTTCTATAATCAGCTACAACAATTGGAGCA
1000 1010 1020 1030 1040 1050 1060 1070 1080
M T T T A E N W L K I Q P T T P S E P T A I K S Q L K I C K
GATGACAACTACTGCTGAAAACTGGTTGAAAATCCAACCCACCACCCCATCAGAGCCAACAGCAATTAAAAGTCAGTTAAAAATTTGTAA
1090 1100 1110 1120 1130 1140 1150 1160 1170
D E V N R L S G L Q P Q I E R L K I Q S I A L K E K G Q G P
GGATGAAGTCAACCGGCTATCAGGTCTTCAACCTCAAATTGAACGATTAAAAATTCAAAGCATAGCCCTGAAAGAGAAAGGACAAGGACC
1180 1190 1200 1210 1220 1230 1240 1250 1260
M F L D A D F V A F T N H F K Q V F S D V Q A R E K E L Q T
CATGTTCCTGGATGCAGACTTTGTGGCCTTTACAAATCATTTTAAGCAAGTCTTTTCTGATGTGCAGGCCAGAGAGAAAGAGCTACAGAC
1270 1280 1290 1300 1310 1320 1330 1340 1350
I F D T L P P M R Y Q E T M S A I R T W V Q Q S E T K L S I
AATTTTTGACACTTTGCCACCAATGCGCTATCAGGAGACCATGAGTGCCATCAGGACATGGGTCCAGCAGTCAGAAACCAAACTCTCCAT
1360 1370 1380 1390 1400 1410 1420 1430 1440
P Q L S V T D Y E I M E Q R L G E L Q A L Q S S L Q E Q Q S
ACCTCAACTTAGTGTCACCGACTATGAAATCATGGAGCAGAGACTCGGGGAATTGCAGGCTTTACAAAGTTCTCTGCAAGAGCAACAAAG
1450 1460 1470 1480 1490 1500 1510 1520 1530
G L Y Y L S T T V K E M S K K A P S E I S R K Y Q S E F E E
TGGCCTATACTATCTCAGCACCACTGTGAAAGAGATGTCGAAGAAAGCGCCCTCTGAAATTAGCCGGAAATATCAATCAGAATTTGAAGA
1540 1550 1560 1570 1580 1590 1600 1610 1620
I E G R W K K L S S Q L V E H C Q K L E E Q M N K L R K I Q
AATTGAGGGACGCTGGAAGAAGCTCTCCTCCCAGCTGGTTGAGCATTGTCAAAAGCTAGAGGAGCAAATGAATAAACTCCGAAAAATTCA
1630 1640 1650 1660 1670 1680 1690 1700 1710
N H I Q T L K K W M A E V D V F L K E E W P A L G D S E I L
GAATCACATACAAACCCTGAAGAAATGGATGGCTGAAGTTGATGTTTTTCTGAAGGAGGAATGGCCTGCCCTTGGGGATTCAGAAATTCT
1720 1730 1740 1750 1760 1770 1780 1790 1800
K K Q L K Q C R L L V S D I Q T I Q P S L N S V N E G G Q K
AAAAAAGCAGCTGAAACAGTGCAGACTTTTAGTCAGTGATATTCAGACAATTCAGCCCAGTCTAAACAGTGTCAATGAAGGTGGGCAGAA
1810 1820 1830 1840 1850 1860 1870 1880 1890
I K N E A E P E F A S R L E T E L K E L N T Q P N
GATAAAGAATGAAGCAGAGCCAGAGTTTGCTTCGAGACTTGAGACAGAACTCAAAGAACTTAACACTCAGCCGAAT
1900 1910 1920 1930 1940 1950 1960

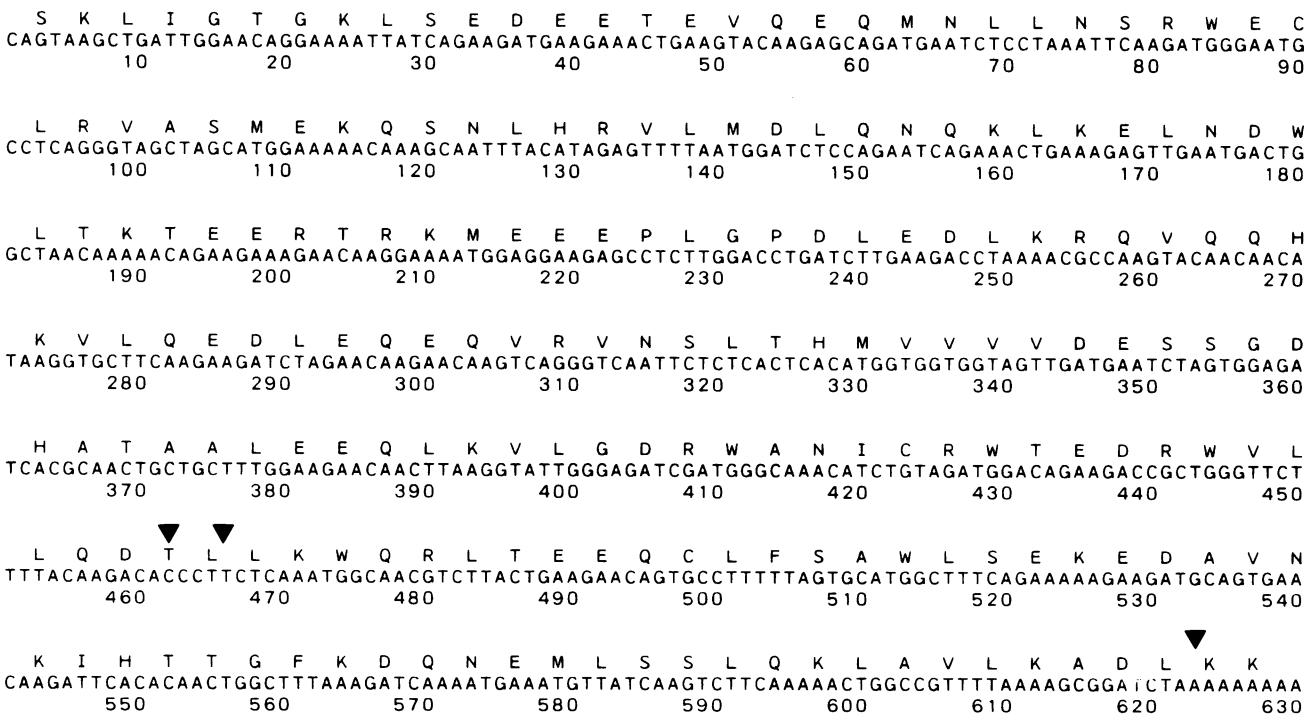


Fig. 8. Sequence of the adult muscle cDNA clone, Ca1 (**A**) and the fetal cDNA clone Cf16 (**B**). Arrows indicate the single base differences.

tient 1462 does however show a band which is slightly larger than the normal 7-kb band. This same changed band is detected by the Ca1B fragment suggesting that the *Eco*RI site in the 2.0-kb cDNA clone lies in this exon.

Our main patient of interest, with the exceptionally mild phenotype, is patient 324. He is deleted for all three exons of Ca1A. DMD Patient 1400 has a large deletion of the locus that is cytogenetically detectable and has been reported previously as patient SS (Wilcox *et al.*, 1986).

The blot in Figure 1 was performed with *Hin*dIII digested DNA so that we could compare our clone with that of Monaco *et al.* (1986) isolated from fetal muscle. No bands higher than 8.0 kb were detected by these workers indicating that our clone detects more telomeric sequences from the pERT87 locus or that the exons for the adult muscle mRNA are different from the fetal muscle ones.

We had the opportunity of precisely localizing one of the exons because Ca1A identified a changed band in patient 1338 known to possess a deletion endpoint between pERT87-30 and pERT87-41. As shown in Figure 2, his breakpoint is detected with both *Eco*RI and *Pst*I. This maps this exon telomeric from pERT87-30. Since the breakpoint is not detected by hybridization to pERT87-41, the exon must lie between pERT87-41 and pERT87-30. Patient 1338 has all the other bands detected by Ca1A which must therefore lie distal to this exon. Patient 1338 is deleted from pERT87-30 to a position centromeric from pERT87-1.

Figure 3A shows the more refined mapping of the cDNA fragment Ca1B. A *Pst*I digest was used since the individual bands observed must correspond to independent exons of the gene. This is because neither of the cDNA fragments has a *Pst*I site. The largest *Pst*I band must be a doublet because a lighter hybridizing band is present in both patient 1339 who is deleted for pERT87-15 toward the centromere and in patient 20 who is deleted from between pERT87-30 and pERT87-15 towards the telomere. This is confirmed by the presence of this band in patient 324 who is deleted from between pERT87-8 and -15 towards the telomere (Figure 4). The exons can be ordered relative to each other by their progressive deletion in patients with larger deletions in this region (see Figures 3A,B and 4). They are numbered in the figure according to their positions in Xp21 (see Figure 6). Exon 3 is detected by both Ca1A and Ca1B as both hybridize to the same altered band in patient 1462 (Figure 1). The exons of Ca1B do not appear to extend proximal to pERT87-8 because they are present in patient 1425 who is deleted for pERT87-8 and pERT87-1. The alternative explanation is that the exons map proximal to pERT87-1 which is not consistent with the map published by Monaco *et al.* (1986) which placed exons in the region of pERT87-8 and -1. Exons 4 and 5 are ordered by their presence in patients 1429 and 1466 who are deleted for 87-15 and by the presence of exon 4 and not exon 5 in patient 1424 whose deletion breakpoint lies between 87-30 and 87-15. They were localized proximal to pERT87-30 because of their absence in patient 1423 who is not deleted at the pERT87-30 locus (Figure 3A). Exons 6 and 7 map proximal to pERT87-15 because they are present in patient 324, whose deletion extends telomeric from and includes pERT87-15, and absent in patient 1424 who is also deleted at the pERT87-15 locus but whose deletion extends towards the centromere. Patient 1429 consistently showed a high molecular weight altered band with this probe and hybridization to the largest band corresponding to exon 3. The altered band probably corresponds to a deletion breakpoint which alters the size of the band detected by exon 6, 7 or 8.

Further information on the ordering of exons homologous to Ca1A was obtained by walking in the cDNA library. A sequence Ca7 was isolated which only overlapped with the 2.7-kb band of Ca1A (exon 1) in a *Pst*I digest (Figure 5) and extends 1.0 kb towards the telomere. The overlap was confirmed since both clones detected a *Pst*I polymorphism in this band, the other allele being 1.8 kb. Therefore this exon must be the most telomeric exon of Ca1A. The blot also shows our mildly affected patient,

324, who is missing all four new exons detected by Ca7. Thus he is deleted for at least nine exons of coding sequence.

To compare the exons detected by fetal and adult cDNAs, we have used Ca1B to isolate fetal cDNA clones. Clones were identified which gave the same Southern blot pattern as the adult cDNA, Ca1B. However, one clone, Cf16, detected two extra exons which are also present in patient 1425 who is deleted for pERT87-8 and pERT87-1 and patient 1421 who is deleted for pERT87-1. Thus these exons must lie proximal to pERT87-1 or distal to pERT87-8 (see Figure 7). We have recently found that these two extra exons are deleted in a patient who is deleted for pERT87-15 and -8 but not deleted for pERT87-1 (T.J.Smith *et al.*, in preparation). These exons must therefore lie between pERT87-15 and -8. If the sequence reads as a continuous open reading frame then a total of five exons map between pERT87-15 and -8. This exon map differs from that of Monaco *et al.* (1986) who only reported three exons in this region. These authors examined genomic DNA clones and identified restriction fragments hybridizing to their cDNA probe. It is possible that some of these contained more than one exon. We sequenced Cf16 and showed it to overlap partially with the sequence of Ca1B and extend the open reading frame (Figure 8B). No evidence for differential splicing between the adult and fetal muscle mRNAs was found in this region of the gene sequence although three single nucleotide sequence differences were observed. Two of these resulted in amino acid changes that are not predicted to substantially alter the protein structure. The third change did not alter the protein sequence.

The sequence of the adult muscle cDNA clone, Ca1, is presented in Figure 8A. An uninterrupted open reading frame is evident orientating the 5′−3′ direction Xcen−Xpter. The DNA sequence shows no exact homology to any other known sequence in the data bank. Interestingly, the sequence matches structural proteins such as myosins, lamin and keratin. Taking the sequence of Ca1A alone, the sequence matches the rod portion of lamin A (Fisher *et al.*, 1986; RDF score 7.3). The whole sequence matched residues 1−417 of rat cardiac heavy chain myosin (total 430 residues; Mahdavi *et al.*, 1982; RDF score 5.1).

Conclusion

We have isolated a cDNA sequence from 3.3 kb of the DMD gene which overlaps with a previously described fetal muscle cDNA clone (Monaco *et al.*, 1986) but which also covers a more telomeric region . We have mapped exons of the part of the DMD gene in the region Xp21 by taking advantage of previously characterized deletions. The mildest BMD patient (324) showed no neurological symptoms until his thirties and at the age of 61 years, although he is having difficulties in walking, he is still able to drive a car. We have shown that this patient is deleted for at least nine exons which are normally present in adult mRNA. Therefore, the explanation for the mildness of his symptoms cannot be that the exons found in the region of his deletions in fetal cDNA (Monaco *et al.*, 1986) are normally spliced out in adult muscle. A more likely explanation would be that his deletion has removed protein domains which are relatively inessential for overall function.

Nebulin, a component of the sarcomere, has been suggested as a possible candidate gene for the basic defect in DMD because of its absence in some DMD and BMD patients (Wood *et al.*, 1987). The sequencing data presented here are consistent with a structural muscle protein but its precise function remains to be determined. We are currently expressing the cDNA molecules in order to develop antibodies for immunochemical studies.

Materials and methods

cDNA libraries

cDNA libraries from human adult muscle (Lloyd *et al.*, 1985) and fetal muscle (S.M.Forrest *et al.*, in preparation) in λgt11 were screened with end-labelled oligonucleotide (20-mer) made from the published sequence (Monaco *et al.*, 1986).

Deletion analysis

Patient DNA was extracted from whole blood or EBV-transformed cell lines (Davies *et al.*, 1987). Gels were run in 0.8% agarose and blotted onto Hybond (Amersham) according to Southern (1975). Probes were labelled by random priming (Feinberg and Vogelstein, 1983).

Sequencing

Sequencing of the fetal cDNA was carried out using the standard M13 protocol. The sequence analysis for the adult cDNA was performed for both strands using the supercoil plasmid sequencing method of Chen and Seeburg (1985) using purified 20-mer oligonucleotide primers according to Sanchez-Pescador and Urdea (1984). The primers were synthesized on a 380B Applied Biosystems DNA synthesizer with a stepwise yield generally higher than 98%. The sequence was used to search the National Biomedical Research Foundation Database, December 1986, using FASTP and RDF (Lipman and Pearson, 1985).

Acknowledgements

We are most grateful to Mike Jennings for assistance in the computer sequence analysis. We would also like to thank Sue Kenwrick and Mark Patterson for helpful discussions, Professor Bobrow (London), Dr Read and Dr Bundey (Birmingham), Dr Muller (FRG), Dr Spiegler and Dr Herrmann (GDR) for clinical samples, and Rachel Kitt for typing of the manuscript. We are also indebted to Dr Gottfried Herrmann for oligonucleotide synthesis and Heidi Billwitz for technical help. We are grateful to the Medical Research Council of Great Britain, the Muscular Dystrophy Association of the USA, the Muscular Dystrophy Group of Great Britain and Northern Ireland, the Medical Research Council of New Zealand, and the Wellcome Trust (Travelling Fellowship) for financial support.

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Received on July 17, 1987; revised on August 11, 1987