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Detection of Duchenne muscular dystrophy gene products in amniotic fluid and chorionic villus sampling cells

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We have examined the expression of several Duchenne muscular dystrophy (DMD) gene products in amniotic fluid (AF) and chorionic villus sampling (CVS) cells. Variable amounts of dystrophin could be detected in most CVS and AF samples by immunoprecipitation followed by Western blot analysis. PCR analysis demonstrated the presence of the muscle type dystrophin mRNA in all AF cell cultures. The brain type dystrophin mRNA was also detected in some of these cultures. These DMD gene transcripts are of fetal origin and are produced by most or all clonable AF cells. The results may facilitate the development of a method for prenatal diagnosis of DMD, based on the expression of the gene in AF and CVS cells.

Duchenne muscular dystrophy; Dystrophin; Prenatal diagnosis

1. INTRODUCTION

Duchenne muscular dystrophy (DMD) is one of the most frequent lethal X-linked diseases, characterized by progressive degeneration of the muscle. It occurs in about 1/3,500 newborn males. One-third of the cases are due to new mutations. A significant proportion of DMD patients also suffer from non-progressive mental retardation. Becker muscular dystrophy (BMD) is a milder form of X-linked dystrophy with a later onset, and is allelic to DMD (reviewed in [1]). The huge gene, which is defective in DMD and BMD, spans approximately 2,500 kb and is the largest gene known to date. The transcription product of the gene in the muscle is a 14 kb mRNA, encoding a 427 kDa rod-shaped protein dystrophin, which is associated with the sarcolemma [2–6]. Dystrophin consists of four domains: an N-terminal actin-binding domain; a domain of spectrin-like repeats; a cysteine-rich domain, which shares homology with the Ca^{2+}-binding domain of α-actinin; and a C-terminal domain with no similarity to any other known protein [2], except for the dystrophin-related protein (DRP) encoded by an autosomal gene [7,8].

A dystrophin isoform very similar to the muscle type dystrophin is present in the brain and is expressed in neuronal cells [9,10]. It is regulated by a different promoter and has a different first exon [11–14]. A third dystrophin isoform is expressed in Purkinje cells in the brain and is regulated by another promoter [15].

We have identified a novel mRNA which is the major DMD gene product in many non-muscle tissues, including neurons and glia cells [16–18]. This mRNA is transcribed from a small region of the DMD gene. Its promoter is located in the intron between exons 62 and 63 of the DMD gene, approximately 150 kb from the 3′ end of the gene [17,19]. It shares the 3′ untranslated region with dystrophin mRNA and codes for a 70.8 kDa polypeptide (Dp71), which contains the C-terminal and the cysteine-rich domains of dystrophin (with some modifications resulting from alternative splicing), but lacks the entire large domain of the spectrin-like repeats and the actin-binding N-terminal domain [16,19,21,22]. The function of Dp71 is unknown, as is the exact function of dystrophin. However, the absence of the spectrin-like repeats and the actin-binding domain makes it conceivable that the biological role of Dp71 is different from that of dystrophin.

The identification of the DMD gene facilitates the development of methods for prenatal diagnosis of the disease. About 55–65% of DMD cases are caused by partial deletions and 5% are due to duplications in the gene. Southern blotting, using cDNA probes covering the entire mRNA, or PCR (polymerase chain reaction) amplification of several exons along the gene, especially in the 'hot-spot' regions, would facilitate detection of these cases [23,24]. For the remaining 30–40% of cases, which lack detectable deletion mutations, linkage analysis by RFLPs (restriction fragment length polymorphisms) and microsatellite DNA segregation is the main tool used for carrier detection and prenatal diagnosis [25–28]. However, in a significant proportion of cases, the mutant haplotype can not be identified because of non-informativity, small family size and unavailability of DNA from deceased probands or other key family

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members. Furthermore, the high rate (12%) of intergenic recombination events between the polymorphic markers used and the site of mutation comprises another major problem [29]. To date, the only way to definitely determine the DMD status of the fetus is to obtain fetal muscle by intrauterine biopsy, which is difficult to perform and increases the risk of spontaneous abortions [30]. It is therefore important to be able to directly assay the expression of the gene in cells which are easily accessible for prenatal examination. Such a procedure could potentially also be used to detect those 30% of DMD cases which are due to de novo mutations. Amniocytes and chorionic villi are used routinely for the diagnosis of a number of genetically determined diseases, chromosomal abnormalities and prenatal sex determination. Therefore we investigated the expression of the DMD gene products in these cells. We show that Dp71 and its mRNA are present in both cell types in amounts which can be easily detected by Western blot analysis and RNase protection assay. The 14 kb mRNA of the muscle-type dystrophin is present in small amounts which are detectable by PCR. Most importantly, low levels of dystrophin could be detected by immunoprecipitation followed by Western blot analysis in most protein extracts obtained from cultured amniotic fliud (AF) and chorionic villus sampling (CVS) cells.

2. EXPERIMENTAL

2.1. *AF and CVS cell cultures*

Surplus normal or DMD-affected AF and CVS cells, obtained from pregnant women for prenatal diagnosis of various genetic traits (Ichilov Medical Center), were used for the present investigations.

AF and CVS cells were taken at the 16th and 8–10th week of gestation, respectively. Unless otherwise indicated, the cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum, 0.02 mM glutamine, 100×10^3 U/l penicillin, 100 mg/l streptomycin. The cultures were maintained at 36.5°C in a humidified CO_2 incubator. A human hepatoma cell line (HepG2) and HeLa cells were grown in DMEM supplemented with 15% fetal calf serum.

2.2. *Preparation of RNA*

Total RNA was prepared from cell cultures and tissues by the lithium chloride/urea extraction procedure [31]. The integrity of the RNA was tested on agarose/formaldehyde gels.

2.3. *RNase protection assay*

A 266 bp DNA fragment containing 194 bp shared by the dystrophin and Dp71 mRNAs and 72 bp from the specific Dp71 mRNA first exon was amplified by reverse transcription (RT)/PCR and cloned in the *Sma*I site of the vector Gemini 3 (Promega). A cRNA probe was synthesized on the linearized plasmid using the Sp6 RNA polymerase. The RNase protection assay was performed as described by Melton et al. [32] with minor modifications [17].

2.4. *RT/PCR analysis*

cDNA was synthesized on 10–15 μg total RNA samples using the relevant antisense primer and MMLV reverse transcriptase (BRL). The cDNA was amplified by PCR using *Taq* DNA polymerase (USB) and suitable primers, as indicated in the legends to the figures. The PCR program included 35 cycles of 1 min at 94°C, 2 min at 56°C (48°C for the brain-type dystrophin mRNA specific primers) and 10 min at 72°C. In the quantitative PCR only 25 cycles were performed. The primers were 20–30 nucleotides long and had 40–60% GC content. The PCR products were analyzed by Southern blotting, using an internal oligonucleotide as the radiolabeled probe.

2.5. *Immunoprecipitation and Western blot analysis*

Protein samples were prepared from AF cell cultures as follows. Cultures were washed with PBS and cells were collected using rubber policeman in 1 ml/9 cm plate lysis buffer (TNNE + PMSF: 50 mM Tris pH 8, 150 nM NaCl, 5 mM EDTA, 1 mM PMSF and 0.4% Nonidet-40). After sonication, cell debris was removed by 15 min centrifugation (Eppendorf microfuge, maximum speed). The extracts were then mixed and shaken with protein A–Sepharose beads for 10 mins (20 ml/1 ml extract). The beads were removed by centrifugation. 40 μl of protein A–Sepharose resin in 1 ml of TNNE were shaken at room temperature for 20 min with 4 μl of rabbit anti-mouse γ-globulin antibodies. The resin was pelleted and washed with TNNE. The resin was then added to 1 ml cell extract samples that were shaken for 2 h with 20 μl of the anti-dystrophin monoclonal antibodies (mAb's) MANDRA1 or MANDRA6. Shaking continued for 18 h. The beads were then pelleted, washed, resuspended in 50 μl electrophoresis sample buffer and boiled for 5 min. The resin was removed by centrifugation. All the steps, except where otherwise indicated, were done at 0–4°C.

Western blot analysis was done using 3–10% polyacrylamide/SDS gels as previously described by Laemmli [33] and modified by Pons et al. [34]. The blots were stained with the mAb, NCL dys 1. Positive signals were detected by the ECL system (Amersham).

2.6. *Antibodies*

MANDRA1 and MANDRA6 are mAb's raised against two different eiptopes in the C-terminal domain of human dystrophin. The two Abs do not react with DRP [35]. NCL Dys1 is a mAb against an epitope in the mid-rod domain of human dystrophin (Novocastra Laboratories).

3. RESULTS

3.1. *Expression of the DMD gene products in amniotic fluid and CVS cells*

RNase protection analysis of total RNA from CVS and AF cells, using probes which differentiate between the Dp71 and dystrophin mRNAs, revealed substantial amounts of Dp71 mRNA in both AF and CVS cells. The 194 bp band which is diagnostic for the 14 kb mRNA, was not detected in this assay (Fig. 1). Likewise, Western blot analysis using a monoclonal antibody (mAb) directed against an epitope which is common to Dp71 and dystrophin (MANDRA1) detected a protein co-migrating with the Dp71 marker but not in the region of the dystrophin marker (427 kDa). When staining the blot with another mAb, NCL-dys1, which is directed against an epitope in the spectrin-like repeats, we observed in some samples a very faint staining of a protein co-migrating with dystrophin (Fig. 2). In order to increase the sensitivity of the assay we introduced an immunoprecipitation step which allowed us to load immunoreactive proteins derived from approximately 50-fold more cell extract in each slot. The introduction of the immunoprecipitation step before the Western blot analysis also made it possible to increase

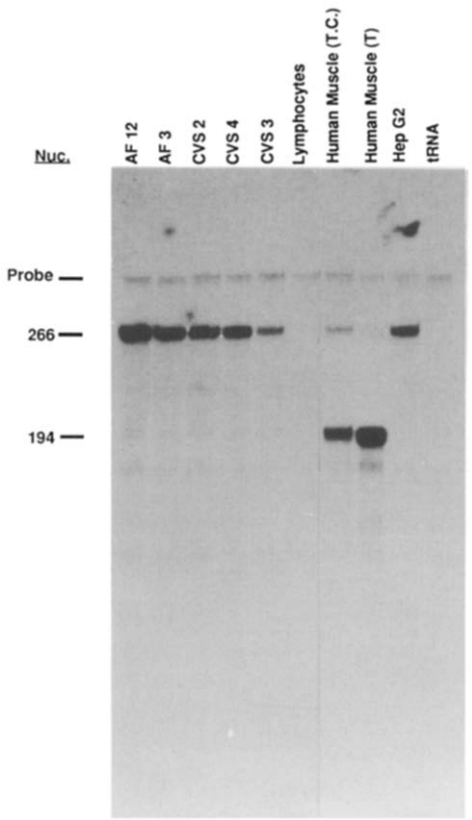

Fig. 1. RNase protection analysis of Dp71 and dystrophin mRNAs in AF and CVS cell cultures. Assays were performed on 15 μg total RNA samples. The 266 and 194 nucleotide protected fragments are diagnostic for Dp71 and dystrophin mRNAs, respectively. The human muscle RNA was prepared from either muscle tissue (T) or from differentiated muscle cell cultures (T.C.)

the specificity of the assay by using a different anti-dystrophin antibody in each step. Indeed, when the immunoprecipitation was performed with MANDRA1 and the blot was stained with NCL-dys1, a band co-migrating with dystrophin could be clearly detected in seven of nine different normal AF cell samples (Fig. 2A). Such a band was not detected in extracts from AF cells derived from a DMD-affected fetus (Fig. 2A). In all the extracts of CVS cultures that were tested, which were derived from six different normal fetuses, a band co-migrating with dystrophin was detected after immunoprecipitation. No such band was detected in extracts of the two samples of CVS from DMD-affected fetuses. In general, the signal produced with the extracts from CVS cells was weaker than that obtained from AF cells (Fig. 2B). Similar results were obtained when using a mAb directed against a different epitope in the C-terminal domain of dystrophin, MANDRA6, for the immunoprecipitation (data not shown).

These results indicate that small and variable amounts of dystrophin are present in most AF and CVS cell samples. To further analyze the expression of the DMD gene transcripts in AF and CVS cells, we employed the very sensitive PCR method for the detection of dystrophin mRNA. Several combinations of primers were used for the synthesis of cDNA and PCR amplification (Fig. 3A). One group of primers was from the region of divergence of the sequences of the Dp71 and dystrophin mRNAs. These primers were designed to differentiate between sequences specific to the dystrophin mRNAs and sequences specific for the mRNA encoding Dp71 (primers 1, 2 and 2L). Another combination of primers was designed to amplify sequences in the region encoding the N-terminal (actin-binding) domain common to the muscle- and brain-type dystrophin mRNAs and the recently discovered Purkinje cell dystrophin, but absent in Dp71 mRNA (primers 3, 4 and 5; results not shown). A third group of primers from the 5′ end of the 14 kb mRNAs was designed to differentiate between the muscle and brain-type dystrophin mRNAs (Primers 7, 8M and 8B). RNA samples from cultures of AF cells of 13 fetuses and CVS cells of 6 fetuses were analyzed. As expected from the RNase protection assay, primers diagnostic for Dp71 mRNA produced a strong signal with all the cDNA preparations (Fig. 3B). In addition, all cDNA preparations derived from AF cells produced signals with primers diagnostic for sequences common to the muscle and brain dystrophin 14 kb mRNAs and lacking the Dp71 mRNA (Fig. 3C). It should be pointed out that these sets of primers cannot differentiate between Dp71 and Apo dystrophin 3 mRNAs, and between dystrophin and Dp116 mRNAs. Apo dys 3 and Dp116 were recently

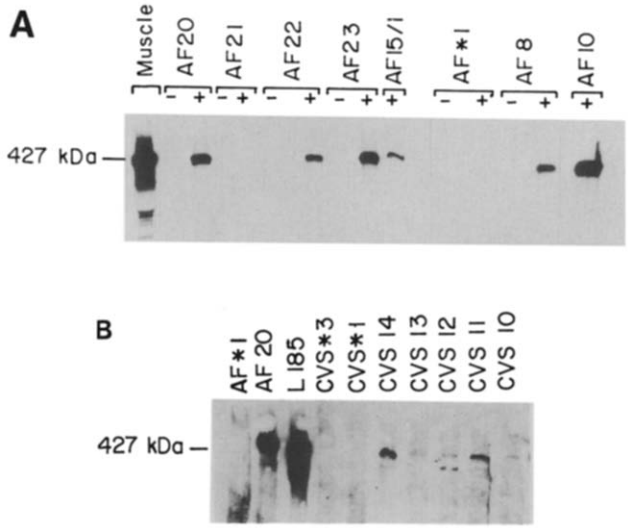

Fig. 2. Immunoprecipitation and Western blot analysis of dystrophin from (A) AF cell cultures (AF) or (B) CVS cell cultures (CVS). Preparation of cell extracts, immunoporecipitation and Western blot analysis are described in section. 20 μl of immunoprecipitated protein samples, equivalent to 40% of one confluent 9 cm Petri dish were loaded on the gel. In the untreated sample approximately 40 μg protein were analyzed (equivalent of approximately 2% of the protein extracted from one plate). The muscle control samples contained 25 μg protein. AF 15/1 = extract of an AF cell culture derived from a single clone of a male fetus. AF*1 and CVS* are cultures of cells obtained from DMD-affected donors.

described [36,37]. However, the sets of primers described below are specific for dystrophin mRNAs.

A band diagnostic for the muscle-type-specific first exon was detected with all the AF cDNA samples (Fig. 3D). The variability in the strength of the signals produced with various RNA preparations was greater than that obtained with primers complementary to sequences in the common region. The results with the cDNA prepared from RNA extracted from CVS cells were similar to those obtained with the AF cell preparations, but were less consistent, and with some RNA samples the band diagnostic for the 14 kb mRNAs was not detected. Sequences specific for the brain-type dystrophin mRNA were only detected in a few RNA samples using the same AF and CVS RNA extracts and PCR conditions (Fig. 3E).

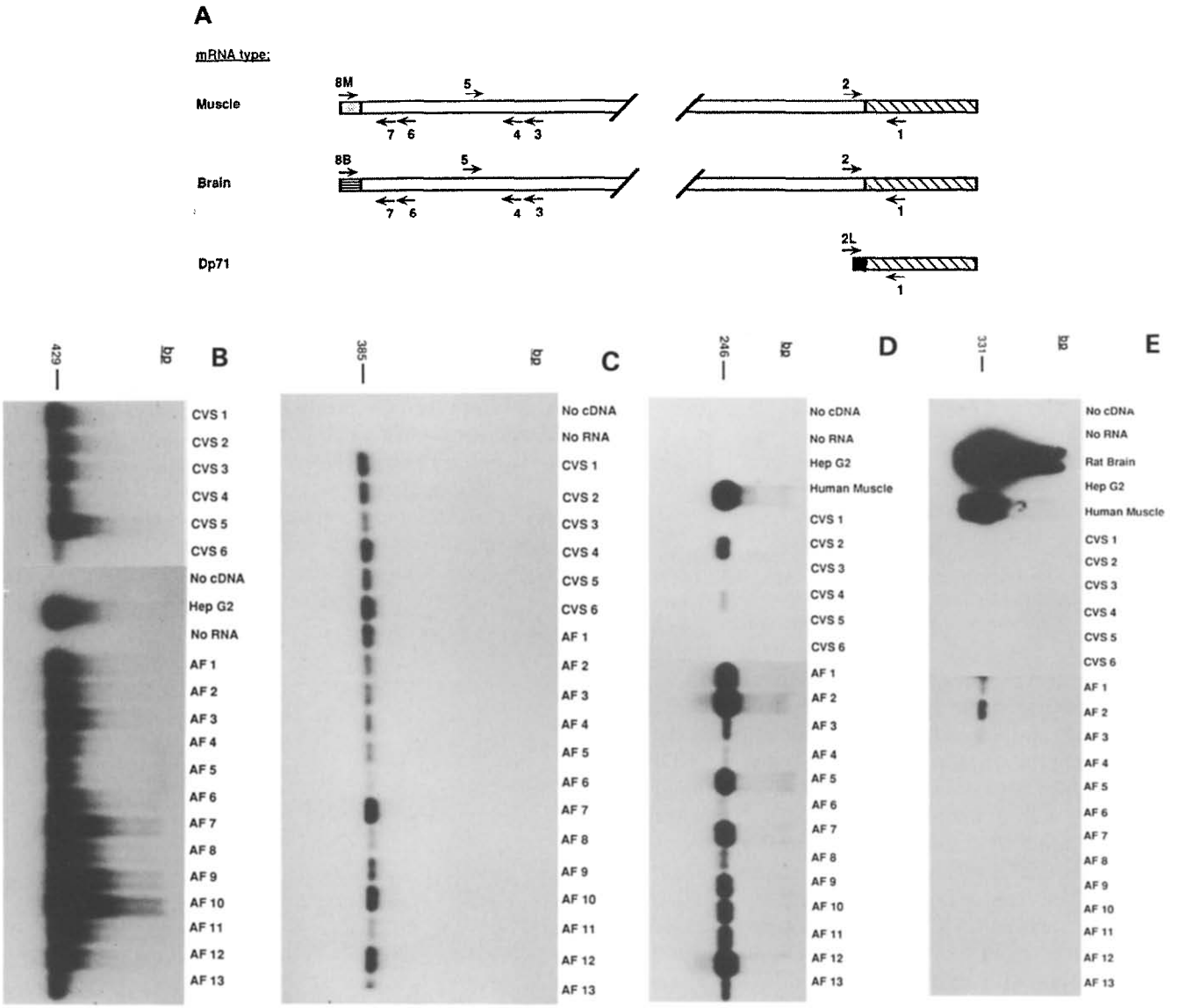

Fig. 3. PCR analysis of dystrophin and Dp71 mRNAs in AF and CVS cells. (A) Schematic presentation of the primers used for cDNA synthesis and PCR. Open bars, coding sequences specific to dystrophin mRNA; slanted, striped bars, coding sequences common to dystrophin and Dp71 mRNAs; dotted and horizontally striped bars, first and specific exons of muscle and brain dystrophin mRNA, respectively; black bar, first and specific exon of Dp71 mRNA. The arrows under the bar show the position of the antisense primers used for cDNA synthesis (1,3,6) and PCR (1,4,7). Arrows above the bars indicate the position of the sense primers used in the PCR amplification (2, 2L, 5, 8M, 8B). (B) PCR amplification of Dp71 mRNA. Primer 1 was used for cDNA synthesis. Primers 1 and 2L were used for PCR. (C) PCR amplification of dystrophin mRNA. Primer 1 was used for cDNA synthesis and primers 1 and 2 for PCR. (These primers will also amplify Dp116 mRNA if present; [37].) (D) PCR amplification of muscle-type dystrophin mRNA. Primer 7 was used for cDNA synthesis and primers 7 and 8M for PCR amplification. (E) PCR amplification of brain-type dystrophin mRNA. The cDNA described in D was amplified with primers 7 and 8B. The PCR products were analyzed by Southern blotting using suitable internal oligonucleotide probes. The no RNA and no cDNA controls were intended to detect contamination introduced during RT/PCR. HepG2, rat muscle and rat brain RNAs were used as positive controls for Dp71, muscle- and brain-type dystrophin mRNAs, respectively. Similar results were obtained when nested PCR was performed using different primers for the cDNA synthesis in PCR amplification (e.g. primers 6, 7, 8M).

Using the same primers and PCR conditions, we did not detect dystrophin sequences in human hepatoma RNA (HepG2) and HeLa cell RNA.

3.2. *Origin of the cells expressing the dystrophin mRNA sequence*

In view of the extreme sensitivity of the PCR and the variability in the intensity of the signal, we addressed the possibility that the 14 kb mRNA sequences detected by PCR in AF and CVS cells could be due to the contamination of the biopsies with cells of maternal origin (e.g. smooth muscle). To test this possibility we examined the expression of the DMD gene in AF cells obtained from the biopsy of an affected fetus which has a deletion spanning exon 7–34 of the DMD gene. RNA from AF cells of this fetus and from AF cells of normal fetuses were analyzed by RT/PCR, using two sets of primers, which are described in Fig. 4A. cDNA was synthesized using either primer A or primer C. Primers B and D were expected to amplify cDNA of the mutated dystrophin mRNA only (of fetal origin), and primers C and D of normal dystrophin mRNA only (of maternal origin). The first set of primers (B,D) amplified only cDNA which was synthesized with an RNA template derived from the AF cells from the affected fetus. No fragment diagnostic for maternal dystrophin mRNA was obtained using the same cDNA and the second set of primers (Fig. 4B). On the other hand, when we used RNA from normal AF cells as a template for the cDNA, no band was obtained with the primers diagnostic for the deleted gene product (due to the great distance between the locations of the complementary sequences in normal dystrophin mRNA). However, a strong band was obtained with the primers diagnostic for the normal dystrophin mRNA. Thus, in the AF cells of the DMD-affected embryo, only transcripts derived from the deleted DMD gene were detected and they contained the sequence of the muscle-type dystrophin mRNA.

The DMD-affected embryo expressed normal amounts of Dp71 (not shown). This result is expected since the promoter of Dp71 is located between exons 62 and 63, i.e. 3′ to the deleted region of the DMD gene and is therefore unaffected by the deletion.

3.3. *Expression of dystrophin sequences is not confined to a small subset of AF cells*

In order to obtain further insight into the cellular origin of the low levels of dystrophin mRNA in AF cultures we attempted to test whether the transcript is produced by a small fraction of the AF cell population. Cultures of AF cells, derived from one male and one female fetus, were plated in cloning densities on a feeder layer. Ten days following plating, single well-isolated colonies of cells were marked, transferred separately to new plates, and amplified to mass culture. The cultures were harvested, RNA was extracted, and cDNA prepared and subjected to PCR analysis using three sets of primers described in Fig. 3A (primers 1 and 2, primers 6 (cDNA), 7 and 8M (PCR) and primers 6 (cDNA), 7 and 8B (PCR)). The muscle-type dystrophin mRNA sequence was detected in all RNA preparations (not shown).

Interestingly, while in all four clones derived from one AF sample no brain-type dystrophin mRNA was detected, all six clones derived from the other AF sample were positive for the brain-type dystrophin mRNA (not shown).

These results suggest that muscle-type dystrophin mRNA is produced by most or all of the clonable AF cells and is not limited to a small subset of the cell population. Growing the cells in nutritional medium which enhances the differentiation of myoblasts into muscle fibers (2HI medium) did not result in noticeable morphological differentiation and did not affect the PCR results.

Since one of the two donor fetuses used in this experiment was a male, we could also check whether the cloned cells were of fetal or maternal origin, by testing their karyotype. The analysis showed that all four cloned cell populations which we examined had an XY karyotype. Immunoprecipitation followed by Western blot analysis of protein extracts of one of these clones (AF15/1) demonstrated the presence of dystrophin in these cells (Fig. 2A). These results corroborate the conclusion derived from the PCR analysis of the AF cells of an embryo harboring a deletion in the DMD gene, that the dystrophin is expressed in cells which originated from the fetus and not from the mother.

3.4. *The relative abundance of dystrophin mRNA in AF cells*

To obtain a rough estimation of the amounts of dystrophin mRNA in the AF cells, we performed a quantitative PCR analysis of RNA from several samples of AF cells, HepG2 and HeLa cells and several dilutions of cDNA prepared from muscle RNA. cDNA was prepared with primer 6 (Fig. 3A), and a pair of primers diagnostic for muscle dystrophin (8M and 7) were used for PCR (Fig. 5). The results indicate that the amounts of muscle dystrophin mRNA in the AF cells is between 1/10 and 1/100 of the amount of dystrophin mRNA in the muscle. Under the same conditions, no dystrophin mRNA was detected in HepG2 and HeLa cells. At the protein level, a rough estimation based on Western blot analysis suggests that the amount of dystrophin in AF and CVS cells is less than 1% of that in muscle.

4. DISCUSSION

It was previously shown that Dp71 is expressed in many non-muscle cell types, including very early embryos. Therefore, its detection in AF and CVS cells in

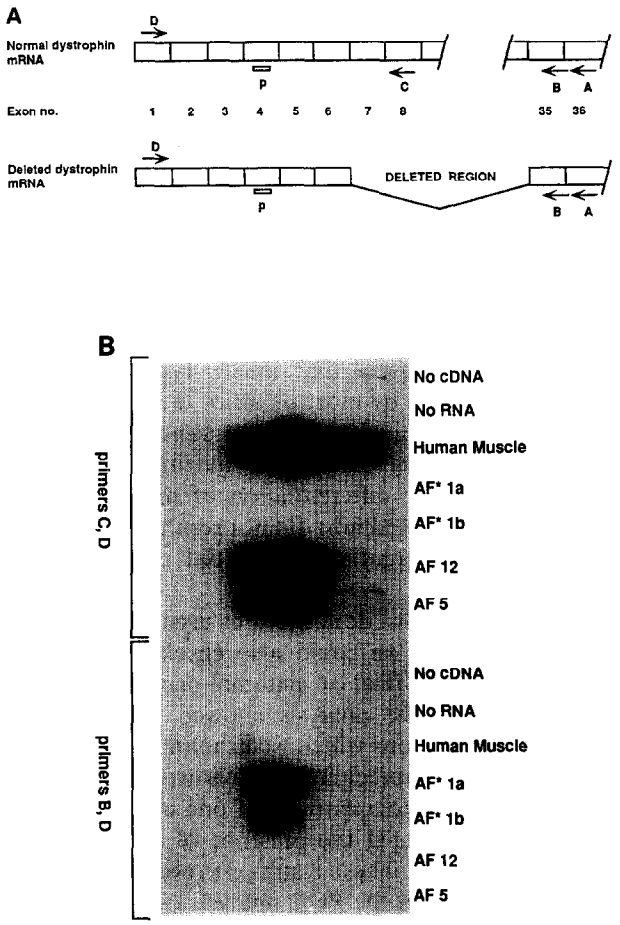

Fig. 4. PCR analysis of dystrophin mRNA from AF cells of a fetus with a deletion in the DMD gene. (A) Schematic representation of the primers used for cDNA synthesis and PCR. The DMD gene exons are indicated by numbers. The arrows below the bars indicate the positions of antisense primers used for cDNA synthesis and PCR. The arrows above the bars indicate the position of the sense primer used for PCR. The small open bar designated P indicates the position of the probe used in the Southern blot analysis. (B) Southern blot analysis of the PCR products. DNA synthesis and PCR were performed on RNA samples (15 μg) from normal AF cells (AF 5, 12) and AF cells from a fetus harboring a large deletion in the DMD gene (exons 7–34; AF*1a and AF*1b). The following combination of primers were used: cDNA, primer A; PCR, primers B and D; cDNA, primer C; PCR, primers C and D. The PCR products were analysed by the Southern blot technique using olignucleotide probe P.

significant amounts by Western blot analysis and the detection of the mRNA by RNase protection analysis were not surprising. However, the application of this finding for prenatal diagnosis is rather limited. As the promoter of Dp71 is located just 150 kb from the 3′ end of the huge DMD gene and it is transcribed from only 6% of the gene, only a small fraction of the mutations in the DMD gene are expected to affect the expression of Dp71. Thus while the lack of normal Dp71 can be used as a clear indication for a defect in the DMD gene, the presence of Dp71 does not necessarily indicate a normal genotype.

The detection of the dystrophin protein and the muscle-type dystrophin mRNA in AF and CVS cells is of special biological interest and may facilitate the development of a prenatal diagnosis procedure. Previous PCR analysis of cloned cell populations indicated that the expression of the dystrophin mRNAs is very stringently controlled. The muscle-type isoform is expressed in multinucleated muscle fibers and in glia cells, but is undetectable in mononucleated myoblasts before cell fusion or in neurons. The brain-type dystrophin mRNA is present in neuronal cultures but is undetectable in myogenic cells [38]. Here we show that the muscle-type dystrophin mRNA is present in low levels in AF and CVS cells. In some samples the brain-type dystrophin mRNA is also detectable.

The identification of the protein comigrating with dystrophin as a genuine product of the DMD gene was established using two dystrophin-specific mAb's, raised against different epitopes in the C-terminal domain of dystrophin for the immunoprecipitation step, and a 3rd mAb raised against an epitope in the spectrin like repeats for staining the blotted protein. The identification of the mRNA was done using several sets of specific primers and different probes for the amplification and detection of the amplified products in the PCR analysis.

RT/PCR analysis of an RNA sample from AF cells taken from an embryo with a deletion in the DMD gene confirmed that dystrophin is expressed in fetal cells and is not of maternal origin. PCR analysis also demonstrated that, in AF cultures containing dystrophin, apparently all clonable cells expressed dystrophin mRNA, and those cells were of fetal origin.

One unexplained observation is the great variability in the amounts of dystrophin protein mRNA in different AF cell samples. This variability is greater in the cultured CVS cells. In addition, while in most samples only muscle-type dystrophin mRNA was identified, in some samples both muscle- and brain-type dystrophin mRNAs were detected. The biological nature of this variability is still unknown. It might reflect differences in the genetic milieu between the donor embryos; differences in the composition of the cell population in AF or CVS samples which are intrinsic to the donors' AF or CVS cell population; or a population drift caused by the amplification of small samples of cells. Compatible with the latter possibility is the observation that cloned cells derived from the same AF samples were qualitatively and quantititively similar in their expression of dystrophin isoforms, while clones of two different donors differed in dystrophin isoform expression. Differences in expression might also result from differences in the history of the cell cultures; e.g. we have noticed a decrease in the amounts of muscle dystrophin mRNA after several cell passages in one of the cloned populations of AF cells. The understanding of this variability in expression will be important for the application of the present observations for prenatal diagnosis of DMD.

Solving this problem will enable the development of two independent and complementary methods, based

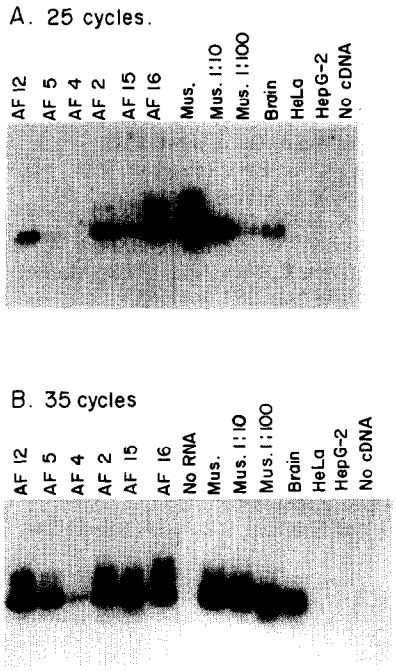

Fig. 5. Quantitative PCR analysis of muscle-type dystrophin mRNA in AF cells. cDNA synthesis and PCR used 15 μg of total RNA from the indicated tissues and cell cultures; primer 6 was used for cDNA synthesis and primers 7 and 8M for the PCR. An internal oligonucleotide probe was used in the Southern analysis. (A) 25 cycles of PCR; (B) 35 cycles of PCR. Blots A and B were exposed overnight and for 3.5 h, respectively. Mus., rat skeletal muscle cDNA. 1:10 and 1:100 indicate the fold of dilution of this cDNA.

on the expression of the gene, for prenatal diagnosis of DMD. (1) The detection of dystrophin in AF or CVS cell extracts by immuno-precipitation or other enrichment procedures followed by Western blot analysis. Using a panel of mAb's raised against epitopes in the relevant domains of dystrophin, this method could provide information on the integrity of the protein. (2) Examination of the transcripts of the DMD gene in AF or CVS cells by a combination of PCR and in vitro transcription and translation of the PCR products. Since it is impossible to amplify the entire 14 kb mRNA in one piece, a series of primers for RT/PCR reactions which amplify partially overlapping nested regions covering the entire coding sequence of dystrophin mRNA can be synthesized. The 5′ primer of each pair will include a sequence for the T7 or SP6 RNA polymerase and an in-frame translation initiation sequence. The PCR products containing the polymerase initiation sites can then be subjected to in vitro transcription and translation. The translation products can be analyzed on a gel to check whether all expected peptides are present. It may be possible to combine all PCR products into one sample which will then be separated on isoelectric focussing (IEF)-SDS 2D gels which will be screened automatically against a reference of a 2D gel of the products of normal CVS or AF mRNA. The feasibility of direct transcription and transplantation of PCR products has been demonstrated [39].

Diagnosis based on the expression of the gene has the advantage that it is not dependent on availability of informative genetic or cytological data from previous cases in the family. It can also potentially be applied to the detection of new mutations in the DMD gene, which comprise about 30% of all cases.

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REFERENCES

[1] Moser, H. (1984) Hum. Genet 66, 17–40.
[2] Koenig, M., Monaco, A.P. and Kunkel, L.M. (1988) Cell 53, 219–228.
[3] Arahata, K., Ishiura, S., Ishiguro, T, Tsukahara, T, Suhara, Y., Eguchi, C., Ishihara, T., Nonaka, I., Ozawa, E. and Sugita, H. (1988) Nature 333, 861–863.
[4] Sugita, H., Arahata, T., Ishiguro, Y., Tsukahara, T., Ishiura, S., Eguchi, C., Nonaka, I. and Ozawa, E. (1988) Proc. Japan Acad. 64, 37–39.
[5] Watkins, S.C., Hoffman, E.P., Slayter, H.S. and Kunkel, L.M. (1988) Nature 333, 863–866.
[6] Zubrycka-Gaarn, E.E., Bulman, D.-E., Karpati, G., Burghes, A.H.M., Belfall, B., Klamuy, H.J., Talbot, J., Hodges, R.S., Ray, P.N. and Worton, R.G. (1988) Nature 333, 466–469.
[7] Love, D.R., Hull, D.P., Dickson, G., Speerr, N.K., Byth, B.C., Marsden, R.S., Walsh, P.S., Edwards, Y.H. and Davies, K.E. (1989) Nature 339, 55–58.
[8] Khurana, T.S., Hoffman, E.P. and Kunkel, L.M. (1990) J. Biol. Chem. 265, 16717–16720.
[9] Nudel, U., Robzyk, K. and Yaffe, D. (1988) Nature 331, 635–638.
[10] Chamberlain, J.S., Pearlman, J.A., Muzny, D.M., Gibbs, R.A., Ranier, J.E., Reeves, A.A. and Caskey, C.T. (1988) Science 239, 1416–1418.
[11] Nudel, U., Zuk, D., Einat, P., Zeelon, E., Levy, Z., Neuman, S. and Yaffe, D. (1989) Nature 337, 76–78
[12] Boyce, F.M., Beggs, A.H., Feener, C. and Kunkel, L.M. (1991) Proc. Natl. Acad. Sci. USA 88, 1276–1280.
[13] Makover, A., Zuk, D., Breakstone, J., Yaffe, D. and Nudel, U. (1991) Neurom. Dis. 1, 39–45.
[14] den Dunnen, J.T., Casula, L., Makover, A., Bakker, B., Yaffe, D., Nudel, U. and Van Ommen, G.-R.B. (1991) Neuromus. Dis. 1, 327–331.
[15] Górecki, D.C., Monaco, A.P., Derry, M.J., Walker, A.P., Barnard, E.A. and Barnard, P.J. (1993) Hum. Mol. Genet. 1, 505–510.
[16] Bar, S.E., Barnea, E., Yaffe, D. and Nudel, U. (1990) Biochem J. 272, 557–560.
[17] Rapaport, D., Lederfein, D., den Dunnen, J.T., Grootscholten, P.M., Van Ommen, G.-J.B., Fuchs, O., Nudel, U. and Yaffe, D. (1992) Differentiation 49, 187–193.
[18] Rapaport, D., Fuchs, O., Nudel, U. and Yaffe, D. (1992) J. Biol. Chem. 267, 21289–21292.
[19] Hugnot, J.P., Gilgenkrantz, H., Vincent, N, Chafey, P., Morris, G.E., Monaco, A.P., Berwald-Netter, Y., Koulakoff, A., Kaplan, J.C., Kahn, A. and Chelly, J. (1992) Proc. Natl. Acad. Sci. USA 89, 7506–7510.

[20] Lederfein, D., Yaffe, D. and Nudel, U. Hum. Mol. Genet. (in press).
[21] Lederfein, D., Levy, Z., Augier, N., Mornet, D., Morris, G., Fuchs, O., Yaffe, D. and Nudel,U. (1992) Proc. Natl. Acad. Sci. USA 89, 5346–5350.
[22] Blake, D.J., Love, D.R., Tinsley, J., Morris, G.E., Tuley, H., Gatter,K., Dickson, G., Edwards, Y.H. and Davies, K.E. (1992) Hum. Mol. Genet. 1, 103–109.
[23] Beggs, A.A., Koenig, M., Boyes, F.M. and Kunkel, L.M. (1990) Hum. Genet.
[24] Chamberlain, J.S., Gibbs, R.A., Ranier, J.E. and Caskey, C. (1990) in: PCR Protocols (Innis M., Gelfand, D., Sninsky, J. and White, T. (eds.) pp. 277–281, Academic Press, NY.
[25] Davies, K.E., Pearson, P.L., Harper, P.S., Murray, J.M., O'Brien, T., Sarfarazzi, M. and Williamson, R. (1983) Nucleic Acids Res. 11, 2303–2312.
[26] Kingston, H.M., Sarfarazzi, M., Thomas, N.S.T. and Harper, P.S. (1984) Hum. Genet. 67, 6–17.
[27] Feener, CA., Boyce, F.M. and Kunkel, L.M. (1991) Am. J. Hum. Genet. 48, 621–627.
[28] Oudet, C., Heilig, R. and Mandel, J.L. (1990) Hum. Genet. 84, 284–285 and 85, 677.
[29] Oudet, C., Heilig, R., Hanauer, A. and Mandel, J.L. (1991) Am. J. Hum. Genet. 49, 311–319.
[30] Kuller, J.A., Hoffman, E.P., Fries, M.H. and Golbus, M.S. (1992) Hum. Genet. 90, 34–40.
[31] Auffray, C.R., Nageotte, R., Chambraud, B. and Rougeon, F. (1980) Nucleic Acids Res. 8, 1231–1241.
[32] Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Nucleic Acids Res. 12, 7035–7056.
[33] Laemmli, U.K. (1970) Nature 227, 680–685.
[34] Pons, F., Augier, N., Leger, J., Robert, A., Tome, F.S.M., Fardeau, M., Voit, T., Nicholson, L.V.B., Mornet, D. and Leger, J.J. (1991) FEBS Lett. 282, 161–165.
[35] Nguyen, T.M., Ginjaar, I.B., van Ommen, G.J.B. and Morris, GE. (1992) Biochem J. 288, 663–668.
[36] Tinsley, J.T., Blake, D.J. and Davies, K.E. (1993) Hum. Mol. Genet. 2, 521–524.
[37] Byers, T.J., Lidov, H.G.W. and Kunkel, L.M. (1993) Nature Genet. 4, 77–81.
[38] Barnea, E., Zuk, D., Simantov, R., Nudel, U. and Yaffe, D. (1990) Neuron 5, 881–888.
[39] Sarkar, G. and Sommer, S.S. (1989) Science 244, 331–334.