Illegitimate Transcription

Application to the Analysis of Truncated Transcripts of the Dystrophin Gene in Nonmuscle Cultured Cells from Duchenne and Becker Patients

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Abstract

We have previously demonstrated that there is a low level of transcription of tissue-specific genes in every cell type. In this study, we have taken advantage of this phenomenon, called illegitimate transcription, to analyze the muscle-type dystrophin mRNA in easily accessible cells such as lymphoid cells, fibroblasts, and peripheral blood cells from Duchenne and Becker muscular dystrophies with known internal gene deletion. The results showed that, in the studied regions surrounding the deletions, processing of truncated transcripts is identical in specific (muscle tissue) and in nonspecific cells (lymphoid cells). In Becker cases with out-of-frame deletions, the already described alternatively spliced species found in muscle samples were also found in nonspecific cells. These results demonstrate that illegitimate transcripts are a bona fide version of tissue-specific mRNA, and that they represent a useful material to investigate the qualitative consequences of gene defects at the mRNA level. (*J. Clin. Invest.* 1991. 88:1161–1166.) Key words: mRNA processing • reading frame • deletion • ectopic transcription • cDNA-polymerase chain reaction

Introduction

Molecular characterization of unknown mutations in disease genes based on DNA analysis is laborious, especially when the gene is very large, with numerous exons and undefined exon/intron junctions (e.g., the genes responsible for Duchenne (DMD)[1] and Becker (BMD) muscular dystrophies, cystic fibrosis, neurofibromatosis type 1). In theory, searching for unknown mutations is much easier if mRNA is used as a source of genetic material, since the coding sequence is more compact in mRNA than in DNA. We have already demonstrated that the defects found in transcripts from a ubiquitously expressed gene, such as α subunit of β-hexosaminidase A, faithfully reflect the DNA abnormality (1, 1a). However, this strategy is restricted by difficulties in obtaining mRNA from tissue-specific genes (e.g., muscle, brain, liver, pancreas, heart).

We have developed a cDNA-polymerase chain reaction (PCR) method to detect and quantitate minute amounts of dystrophin mRNA (2). With this method, we could detect and characterize dystrophin transcripts in specific cells (striated, smooth muscle, and brain), and also in nonspecific cells, such as cultured lymphoblastoid cells. The unexpected finding of a ubiquitous basal level of transcription of a tissue-specific gene was also observed with other genes, such as the genes for β-globin, anti-hemophilic factor VIIIC, and antimüllerian hormone (3). This phenomenon was coined "illegitimate" transcription by our group (3), and "ectopic" transcription by Sarkar et al. (4). We have previously demonstrated that normal illegitimate transcripts found in lymphoblasts, lymphocytes, and fibroblasts are a bona fide version of normal tissue-specific mRNAs. However, the processing of illegitimate transcripts produced by abnormal genes had not yet been investigated.

In this study we report the results of analysis performed on cDNA-PCR-amplified dystrophin mRNA in fibroblasts, blood cells, or lymphoblasts from eight Duchenne and three Becker patients with internal deletion of the dystrophin gene. Qualitative analysis consisted in sequencing the truncated transcripts resulting from internal deletions of the dystrophin gene, using appropriate combinations of primers amplifying across the predicted junction points.

We found that the sequence of truncated transcripts around the junction point of the deletion corresponds precisely to the one deduced from the genomic deletion and is identical in legitimate (muscle) and in illegitimate (non-muscle cells) transcripts. In BMD cases with deletion transgressing the reading frame rule (5) (see Discussion), analysis performed on illegitimate transcripts reveals, as in muscle RNA (6), the presence of alternatively spliced transcripts restoring the reading frame. This indicates that the illegitimate transcription allows us to study easily the consequences of a gene mutation on the processing in tissues other than their normal sites of expression.

Methods

Clinical classification of patients and deletion analysis. The patients were classified as Duchenne (wheelchair-bound by age 12), or Becker (wheelchair-bound at age 16 or later). In all cases, the diagnosis was ascertained by serum creatine kinase activity and muscle histological pattern. Deletion breakpoints in the dystrophin gene were mapped relative to exons by Southern blot analysis of HindIII digests of genomic DNA, using cDNA probes covering 9 kb of dystrophin cDNA (7). In some cases, additional studies included EcoRI and BglII maps and PCR amplification of specific exons.

Source of RNA and RNA analysis. Total RNA was extracted from the following sources: (*a*) EBV-immortalized lymphoblastoid cell lines; (*b*) peripheral blood lymphocytes; (*c*) cultured fibroblasts. Total cellular RNAs were purified by the method of Chirgwin et al. (8) and were

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1. *Abbreviations used in this paper:* BMD, Becker muscular dystrophy; c, complementary; DMD, Duchenne muscular dystrophy; i, identical; LCL, lymphoblastoid cell lines; PCR, polymerase chain reaction.

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checked by agarose gel electrophoresis followed by ethidium bromide staining.

Oligonucleotide primers were synthetized according to the dystrophin sequence published by Koenig et al. (9). Nucleotide sequence of primers complementary (c) and identical (i) to mRNA sequence are indicated in figures and legends.

cDNA-PCR coamplification of several fragments of the dystrophin transcript was performed as described in detail in Chelly et al. (10). cDNA was synthetized (using 5–10 μg total RNA) by simultaneous extension of different dystrophin-specific primers (10c, 44c, and 62c). In some cases, the cDNA was synthetized by random priming using hexanucleotide primers as a template to the reverse transcriptase enzyme (MMLV, BRL). After this first step, coamplification was carried out with three couples of primers corresponding to the dystrophin transcript (see Fig. 1). Reaction products (after 30 or 40 cycles) were subjected to polyacrylamide gel electrophoresis, transferred to a nylon membrane, and hybridized to labelled internal oligoprobes or cDNA probes (see figures).

Cloning and sequence analysis of abnormal transcripts. Truncated transcripts of the dystrophin gene from DMD and BMD patients were amplified by PCR using primers flanking the junction points. For illegitimate transcripts present in nonspecific cells (lymphoid cells and fibroblasts), it was necessary to perform an additional run of 30 cycles of amplification using either the same primers or nested primers (see figures). The amplified fragments were separated by electrophoresis in a low melting agarose gel (4% NuSieve; FMC Corp., Rockland, ME) or in polyacrylamide gel (8%, wt/vol). Gels were stained with ethidium bromide, and the specific fragments, previously attested by specific hybridization and restriction map analysis, were cut out, purified, phosphorylated, and ligated into SmaI digested M13 mp10 vector. Single stranded M13 DNA from recombinant clones was sequenced by the 2-dideoxy method using [^{35}S]-dATP and T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH). In each case, at least three independent clones were sequenced.

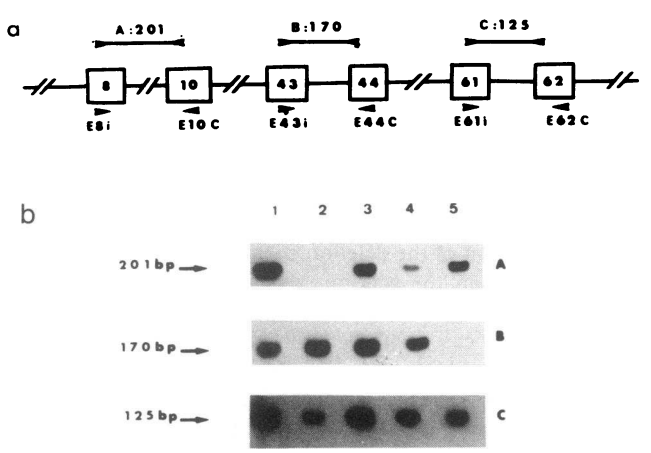


Figure 1. Coamplification of three different segments (A, B, and C) of the dystrophin mRNA originating from lymphoblastoid cell lines. (*a*) Position of primers and their relation to the target gene region and size of cDNA fragments coamplified. The primers, complementary (c) and identical (i), are chosen in different exons to distinguish amplification of mRNA fragments from amplification of contaminating DNA. The following primers were used: in exon 8 (E8i: 5'CATCAAATGCACTATTCTCAACAG3') and in exon 10 (E10c: 5'CTCCATCAATGAACTGCCAAATGA3') for segment A; in exon 43 (E43i: 5'GACATTATTCATACCAAGAAG3') and in exon 44 (E44c: 5'CCATTTCTCAACAGATCTGTC3') for segment B; in exon 61 (E61i: 5'CTTTCCACGTCTGTCCAGGGTCCC3') and exon 62 (E62c: 5'CTGGTAGAGCTCTGTCATTTTGGGA3') for segment C. Primers were designated according to the dystrophin sequence published by Koenig et al. (9). (*b*) Autoradiograms of Southern blots performed on cDNA-PCR coamplified products (30 cycles) of dystrophin (segments *A, B,* and *C*) transcripts, hybridized with dystrophin cDNA probes (11). In this figure we showed the dystrophin transcript analysis in lymphoblasts from patients 1–5 (Table I).

Results

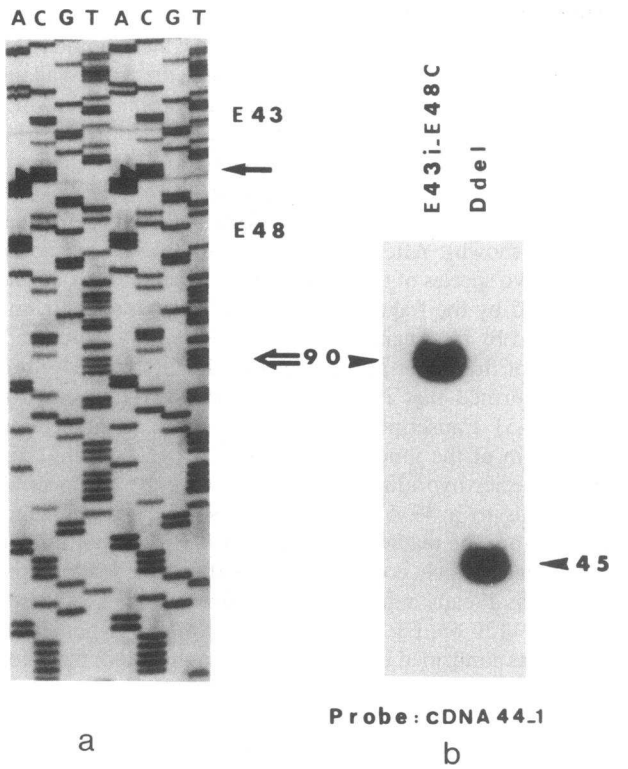
Detection of mature transcripts of the dystrophin gene in nonmuscle cultured cells from DMD and BMD patients. We have screened by PCR coamplification three different portions of the dystrophin mRNA (see Fig. 1 *a*) from cultured lymphoblastoid cell lines (LCL), fibroblasts, and peripheral blood cells: (*a*) a 201-nt segment in the 5' region; (*b*) a 170-nt segment in the middle part of the transcript; (*c*) a 125-nt segment in the 3' region. In all samples from these 11 patients (8 DMD and 3 BMD) with previously detected internal gene deletions, the three dystrophin mRNA segments were detected, only if the primers were outside of the deleted region of the transcript (Table I and Fig. 1 *b*). Owing to the very low level of dystrophin illegitimate transcripts in these cells, 30 PCR cycles had to be performed, and the amplified specific fragments were only detectable by hybridization with a radioactive probe, or visualized after a second run using nested primers. No dystrophin mRNA quantitation could be performed in these nonspecific cells (see Discussion).

Sequence analysis of the junction point of truncated dystrophin mRNA in lymphoblasts from DMD patients. To investigate the structure of the truncated dystrophin mRNAs, we used primers flanking the deleted exons to amplify the transcript of lymphoblastoid cells from DMD patients. In Fig. 2, we show typical results obtained in a DMD case with an internal out-of-frame deletion encompassing exons 44–47 (patient 5, Table I and Fig. 2). Using primers in exons 43 and 48, we amplified a sequence of ~ 90 bp, which hybridized to the 44.1 cDNA probe (11) and was cut by DdeI, as expected (Fig. 2 *a*). This

Table I. Dystrophin DNA and mRNA Analysis in Patients with Duchenne and Becker Muscular Dystrophy

Patient	Phenotype	Deleted exons	Predicted reading frame	Analyzed cells	mRNA fragment detected		
					A	B	C
1	Duchenne	46 → 49	Frameshift	LCL	+	+	+
2	Duchenne	4 → 17	Frameshift	LCL	−	+	+
3	Duchenne	46 → 48	Frameshift	LCL	+	+	+
4	Duchenne	17 → 21	Frameshift	LCL	+	+	+
5	Duchenne	44 → 47	Frameshift	LCL	+	−	+
6	Duchenne	20 → 44	Frameshift	LCL	+	−	+
7	Duchenne	8 → 17	Frameshift	LCL	−	+	+
8	Duchenne	7 → 13	Frameshift	LCL	−	+	+
9	Becker	3 → 7	Frameshift	LCL	+	+	+
10	Becker	3 → 7	Frameshift	Lym	+	+	+
11*	Becker	50	Frameshift	Fib	+	+	+

Phenotypes are classified as described in Methods. The predicted consequence of deletion on the reading frame was determined by examining the remaining two exons flanking the deletion (the exon borders were defined by Koenig et al., 7). * Patient 11 is designated as P22 on Fig. 5; for fragments A, B, and C, see Fig. 1. Lym, blood lymphocytes; Fib, cultured fibroblasts.



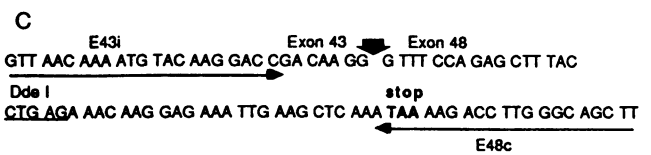


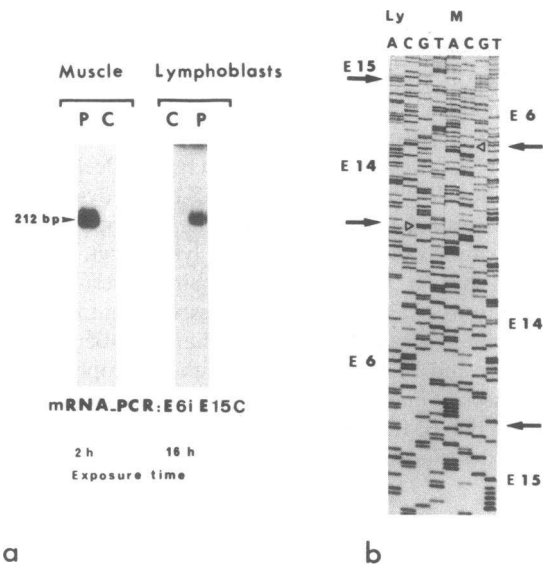
Figure 2. Sequence analysis of the junction point of truncated dystrophin mRNA in lymphoblasts from DMD patient 5 with an out-of-frame deletion. (*a*) Sequencing gel of two different clones corresponding to the abnormal segment. Triangle and arrow indicate the junction point of the truncated cDNA and the limits of exons, respectively. The sequence shown here is complementary to the coding strand described in *c*. (*b*) Specific hybridization before and after DdeI restriction of the abnormal dystrophin cDNA segment amplified from lymphoblast total RNA. (*c*) Complete nucleotide sequence of the abnormal cDNA segment and position of primers used for the PCR, and of the DdeI restriction site used to check the amplified segment. Note that the new reading frame is interrupted by a stop codon.

fragment was subcloned, and three independent clones were sequenced. Each of them gave the expected sequence and showed that exons 43 and 48 were precisely abutted (Fig. 2 *b*). As shown in Fig. 2 *c*, there is a stop codon 49 nucleotides downstream of the junction point.

We checked that the truncated dystrophin transcript found in lymphoblastoid cells from DMD patients was identical to that found in skeletal muscle. Fig. 3 shows results obtained in a DMD case (patient 8), in which exons 7–13 were deleted. Primers in exons 6 and 15 amplified a fragment of ~ 210 bp which hybridized to the 30.2 cDNA probe (11), both in muscle and in lymphoblastoid cells from this patient, and not from controls (see legend to Fig. 3). The sequence of this fragment was found identical in muscle and in lymphoblastoid cells, showing the precise splicing between exon 6 and exon 14 (Fig. 3 *b*), with a stop codon 143 nucleotides downstream of the junction point in the new reading frame.

Analysis of dystrophin transcripts in cases in which the phenotype (BMD) is in contradiction with the reading frame hypothesis. We have applied the strategy of PCR amplification of truncated transcripts to study the dystrophin transcripts in three unrelated BMD with an out-of-frame deletion (patients 9, 10, and 11, table I).

Patient 9, 21 yrs old, had a dystrophin gene deletion removing exons 3–7 which disrupts the reading frame (12). The dystrophin transcripts were investigated in lymphoblastoid cells



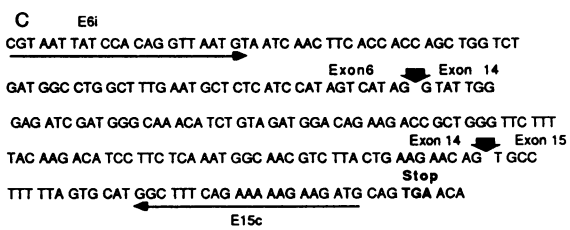


Figure 3. Identical sequence of the junction point of truncated dystrophin mRNA in muscle and in lymphoblasts from DMD patient 8 with an out-of-frame deletion. (*a*) Specific hybridization to the 30.2 cDNA probe of the abnormal cDNA segment amplified from muscle and lymphoblast total RNA. The normal fragment amplified between primers in exons 6 and 15 is 1.2 kb long and was not visualized in the 8% acrylamide gel used to allow migration of the truncated 212-bp segment. (*b*) Sequencing gel of the abnormal segment obtained in lymphoblasts (*Ly*) and skeletal muscle (*M*). Arrows indicate limits of exons, and triangles the position of the junction point. Note that the sequence is identical in muscle and in lymphoblasts, and complementary to the coding strand. (*c*) Complete nucleotide sequence of the abnormal cDNA segment with position of primers, and position of the stop codon generated by the new reading frame.

and in muscle. To explore the expected truncated transcript, as well as other species resulting from conceivable alternative splicing, cDNA-PCR amplification was performed using two couples of primers, located in exons 1 and 8, and exons 1 and 10, respectively. In lymphoblastoid cells, results obtained after 30 cycles and confirmed by specific hybridization are the following: in addition to the expected truncated transcript in which exons 2 and 8 are juxtaposed, we found three additional species illustrated by a 144-bp and an 81-bp segment (Fig. 4 *a*) with the E1i-E10c primer couple and by a 93-bp segment (data not shown) with the primers located in exons 1 (E1i) and 8 (E8c).

The sequencing and exon-specific hybridization data showed that the three additional transcript species correspond to different splicing patterns: between exons 2 and 10 (Fig. 4, *a* and *c*), between exons 1 and 10 (Fig. 4 *a*), and between exons 1 and 8 (data not shown). In each case, the exons were precisely spliced, without any sequence abnormality, other than the random errors due to the Taq polymerase. Among the different splicing patterns observed, only the juxtaposition of exon 1 to exon 8 and of exon 2 to exon 10 restore the reading frame. The species corresponding to alternative splicing were not detected in control lymphoblastoid cells (data not shown).

Patient 10 (12) is another patient with a typical Becker phenotype and a deletion removing exons 3–7 of the dystrophin gene which disrupts the reading frame. The dystrophin transcripts were explored in peripheral blood lymphocytes, using the same primers as in the preceding case. Similar results were obtained, but in this case the species corresponding to the in-frame 2–10 splicing was more abundant than in patient 9.

Quantitative and qualitative analysis of the dystrophin gene products (transcript and protein levels) performed on muscle biopsy of these two BMD patients and on normal control muscle have been described in detail elsewhere (6).

Patient 11 had a Becker clinical pattern and a dystrophin gene deletion removing exon 50 only (13), which should result in an out-of-frame transcript. In this case, the only available source of dystrophin mRNA was cultured fibroblasts, in which the truncated dystrophin transcript was amplified by PCR (40 cycles) using primers located in exons 49 and 52. A segment of ~ 350 bp, hybridizing to cDNA probe 44.1 (Fig. 5 *a*), was detected. This fragment was cut by RsaI and DdeI as expected (data not shown). After a second run of PCR using nested primers, two species of transcripts were obtained: a major one, represented by the fragment of ~ 300 bp, and a minor one, represented by the fragment of ~ 70 bp long. The nucleotide sequence of the major species detected in the patient's fibroblasts confirmed that it corresponds precisely to an out-of-frame 49–51 transcript, with a stop codon 30 nucleotides downstream of the junction point (Fig. 5 *c*). The minor and smaller species hybridizes to 44.1 cDNA probe and possibly corresponds to a 49–52 alternatively spliced species which would restore the reading frame (Fig. 5 *a*). In normal muscle, the fragment which corresponded to this 49–52 alternative splicing was already detected after the first round of PCR (segment of ~ 120 bp, Fig. 5 *a*). The nature of this hypothesized splicing was confirmed by hybridization to the internal primers E49i. 2 and E52c. 2 and by nucleotide sequencing (6).

Discussion

Usefulness of illegitimate transcription of the dystrophin gene to characterize abnormal transcripts in DMD and BMD patients. It has been recently shown that any gene may be transcribed in any cell, whatever its tissue specificity, yielding a bona fide mature transcript (3, 4). In addition, preliminary results indicate that the initiation of these illegitimate transcripts in non-specific cells is similar to that occurring in legitimate tran-

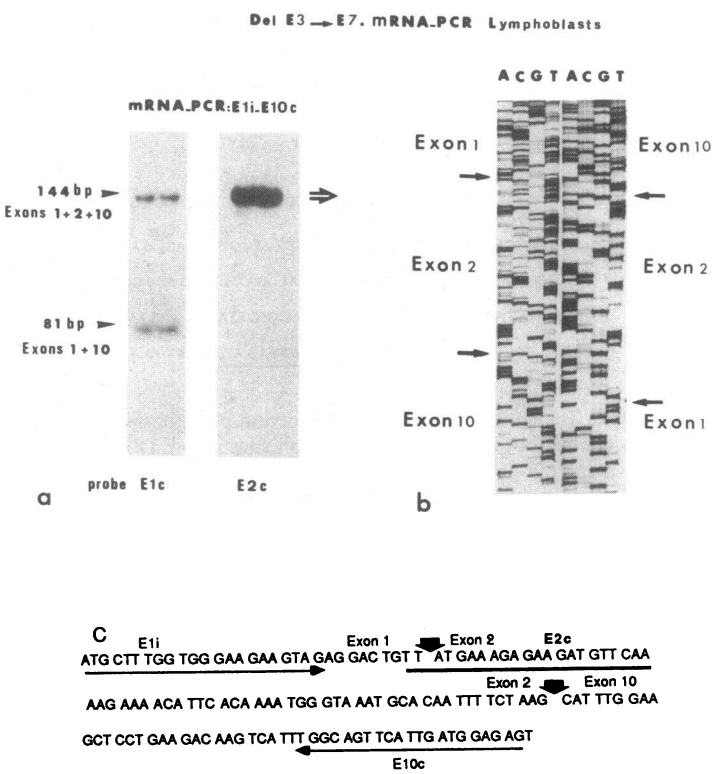


Figure 4. Alternative splicing of dystrophin transcript in lymphoblasts from Becker patient 9 with a predicted out-of-frame deletion (exons 3–7). (*a*) Southern blot analysis of two identical aliquots of cDNA-PCR amplified products of dystrophin transcripts after two runs of amplification (30 cycles each). (*b*) and (*c*) Sequencing gel and nucleotide sequence of the 1-2-10 in-frame segment showing alternative splicing of exons 8 and 9 in the patient's lymphoblasts. The sequence is identical to the coding strand.

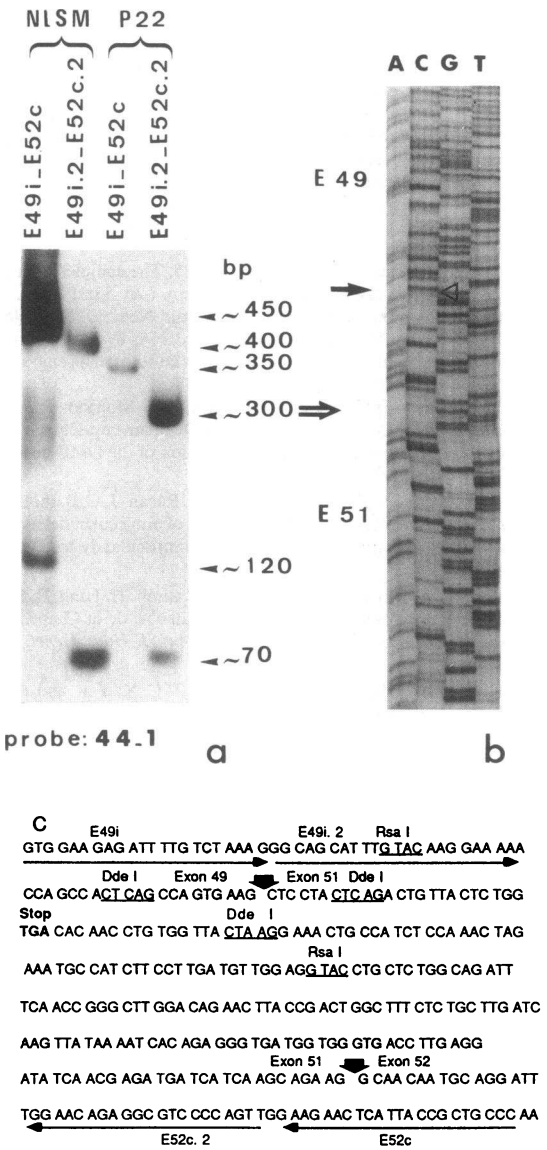


Figure 5. Analysis of truncated dystrophin transcripts in fibroblasts from a Becker patient with out-of-frame deletion of exon 50. (P 22 is designated as patient 11 in Table I). (*a*) Autoradiograms of Southern blots performed on cDNA-PCR coamplified products (40 cycles) of dystrophin transcripts, hybridized with 44.1 dystrophin cDNA probes. The dystrophin transcript was analyzed in normal muscle biopsy (*NI SM*) and in patient's fibroblasts (P 22 corresponds to patient 11 in Table I) using primer couple E49i-E52c for cDNA-PCR and nested primer couple E49i.2-E52c.2 for the second run of PCR. In normal muscle the expected fragment comprising exons 49–52 is the 450-bp species (400 bp with nested primers). The additional 120-bp fragment (70 bp with nested primers) corresponds to alternative splicing of exons 50 and 51. In the patient's fibroblasts, the 350- and 300-bp fragments correspond to the expected truncated transcript (exons 49-51-52). The in-frame spliced species (exons 49–52) is only seen after reamplification between nested primers (70-bp fragment). (*b*) Sequencing gel of the abnormal 300-bp cDNA segment obtained after amplification using nested primers. The triangle indicates the junction point. The sequence is complementary to the coding strand. (*c*) Complete nucleotide sequence of the abnormal 300-bp cDNA fragment with position of primers used for PCR and sites of restriction enzymes used to characterize the abnormal fragment. Nucleotide sequence of the fragment corresponding to the alternatively spliced species was already published (6).

scripts from specific tissues (13a). This faithful illegitimate transcription offers the invaluable advantage of giving access to abnormal transcripts using easily available cells. In this study we generally used EBV-established LCL, and, occasionally, peripheral blood lymphocytes and cultured fibroblasts. We sequenced the illegitimate dystrophin transcripts across the junction points in nonspecific cells from five cases with internal deletions of the dystrophin gene. In all of them, we detected the expected transcript corresponding precisely to the one deduced from DNA analysis using cDNA probes. We checked that the sequence of the illegitimate truncated dystrophin transcripts found in lymphoid cells from three patients (two BMD and one DMD, this study and results already described in 6) was identical to that of the legitimate truncated transcripts present in muscle.

Possible contamination inherent in the PCR procedure was eliminated on the following basis: (*a*) an RNA sample prepared from lymphoblasts of a patient with a 4-megabase deletion encompassing the whole dystrophin gene (patient JM, described in 14) was regularly used as a negative control; (*b*) in two cases (patients 5 and 11), for which we had no sample other than lymphoblastoid cells or fibroblasts, the truncated illegitimate transcript, never found in normal material, could not originate from a contaminant.

The difficulty encountered in this type of analysis is the scarcity of the illegitimate transcripts, which correspond to far less than one copy per cell (3). However, we have observed that their abundance could be increased by translation inhibitors such as cycloheximide (13a). To get enough material for qualitative studies including sequence analysis, it is usually necessary to perform a second run of PCR amplification using nested primers. It should be emphasized that, in standard culture conditions, the abundance of the illegitimate transcripts is variable (13a), which precludes any use of illegitimate transcription to quantitate the abundance of abnormal mRNAs.

Monaco et al. (5) postulated that internal deletions disrupting the reading frame of the dystrophin gene result in the Duchenne phenotype, whereas those which maintain the reading frame result in the Becker phenotype. This has been verified in 92% of the cases (7, 15). In this study, among three BMD cases representing apparent exceptions to the reading frame rule, two exceptions concerning exons 3–7 out-of-frame deletions were analyzed (patients 9 and 10). This deletion is the most frequent one in the 5′ region of the gene giving rise to Becker disease (16). In both cases, the out-of-frame transcript with the expected truncation, i.e., containing exons 1, 2, and 8, was found; however, three minor additional transcripts, resulting from alternative splicing, were also found. Two of them, joining exons 2 and 10 and exons 1 and 8, respectively, restore the reading frame, while the third one, less abundant, joins exons 1 and 10 and is still out-of-frame. We have also looked for alternative splicing in one patient with an out-of-frame deletion of exon 50 and clinically classified as BMD (case 11). In fibroblasts, the only material available from this patient, we found the expected out-of-frame 49–51 transcript as a major species, and minute amounts of an alternatively spliced in-frame 49–52 transcript.

These results, which are qualitatively identical to those obtained on muscle from the BMD patients with an out-of-frame deletion that removes exons 3–7 (6), demonstrate that the impact of gene abnormalities on mRNA processing is similar in specific and in presumably nonexpressing cell types and could

suggest that the milder BMD phenotype may be due to translation of the minor alternatively spliced in-frame transcripts.

The major expected application is the direct analysis of point mutation in nonspecific accessible cells of tissue-specific transcripts produced by mutant genes. Berg et al. (17) and Knebelman et al. (18) have taken advantage of this illegitimate or ectopic transcription to detect a novel point mutation in the FVIIIC gene causing hemophilia A and a nonsense mutation in the antimüllerian hormone gene associated with the persistent müllerian duct syndrome, respectively. Schlösser et al. (19) and Roberts et al. (20) have used this phenomenon to characterize pathological dystrophin transcripts from the lymphocytes of a muscular dystrophy carrier.

Our data on abnormal transcripts investigated in nonmuscle tissue specimens from DMD and BMD patients extend the practical usefulness of the analysis of illegitimate transcripts corresponding to tissue-specific genes and suggest that illegitimate transcription could also be exploited (*a*) to characterize mRNA splicing defects that may occur at high frequency in complex genes, and (*b*) to understand correlations between gene abnormalities and clinical phenotype.

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