Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 22, Issue of August 5, pp. 15267-15270, 1992

The Multiple ADP/ATP Translocase Genes Are Differentially Expressed during Human Muscle Development*

(Received for publication, March 23, 1992)

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The expression of the genes encoding the three isoforms of the human ADP/ATP translocase (T1, T2, and T3) has been analyzed at different stages of myogenic differentiation in an *in vitro* muscle cell system and compared with that in mature muscle. The results indicate that the three stages of muscle differentiation corresponding to myoblast proliferation, myotube formation, and mature muscle fibers are characterized by a different pattern of expression of the ADP/ATP translocase genes. In particular, the two T2-specific mRNAs are present at high, similar levels in myoblasts and myotubes and markedly decrease in amount in mature adult muscle. By contrast, the T3-specific mRNA is present in high amount in growing myoblasts, decreases markedly in myotubes, and is barely detectable in adult muscle. Finally, the T1-specific mRNA is present at a high level in adult muscle and is not detectable in either myoblasts or myotubes. Therefore, T1 gene expression appears to be a marker of a late stage in myogenesis. A parallel investigation of expression of the myosin heavy chain mRNA revealed absence of hybridization with the specific probe in RNA from proliferating myoblasts, a significant hybridization in myotube RNA, and a strong signal in adult muscle RNA.

The adenine nucleotide translocase, the most abundant protein of the inner mitochondrial membrane, is a nuclear coded protein that catalyzes the exchange of ADP with ATP between the cytosolic and the mitochondrial matrix (for reviews Vignais *et al.* (1985) and Klingenberg (1989)). The ADP/ATP translocase is therefore an essential component of the apparatus presiding over the aerobic energy metabolism of the cell.

The first evidence of tissue specificity of the bovine ADP/ATP translocase came from immunological studies (Schultheiss and Klingenberg, 1984). These observations were subsequently confirmed by molecular genetic studies which revealed the presence of at least three different genes in man (Battini *et al.*, 1987; Neckelmann *et al.*, 1987; Houldsworth and Attardi, 1988; Li *et al.*, 1989; Cozens *et al.*, 1989; Ku *et al.*, 1990). These three genes are designated here as T1, T2, and T3, following the terminology previously described (Lunardi and Attardi, 1991). Multiple nuclear genes for the ADP/ATP translocase have also been found in cow (Powell *et al.*, 1989), *Saccharomyces cerevisiae* (Lawson and Douglas, 1988; Kolarov *et al.*, 1990), and *Zea mays* (Bathgate *et al.*, 1989).

Mitochondrial energy production varies greatly among tissues, and it is likely that ATP/ADP transport parameters also differ between cells characterized by different energetic demands. It is therefore not surprising that the expression of the ADP/ATP translocase gene(s) has been found to be regulated in response to functional and developmental factors. In previous work on cultured human cells, changes in the levels or metabolic stabilities of the T2- and T3-specific mRNAs were observed in response to growth conditions, the differentiation state of the cells, and metabolic inhibitors (Lunardi and Attardi, 1991). Kinetic differences in ADP/ATP exchange have been reported between rat liver and beef heart mitochondria (Klingenberg, 1985).

In a wide variety of *in vitro* muscle cell systems, myogenic differentiation has been shown to be characterized by a series of physiological and metabolic changes. During this differentiation, skeletal muscle precursor cells, myoblasts, fuse to form multinucleate fibers, in which assembly of myofibrillar proteins takes place (Affara *et al.*, 1980; Medford *et al.*, 1983; Miller and Stockdale, 1986; Gunning *et al.*, 1987). This terminal differentiation is also characterized by both qualitative and quantitative changes in the expression of enzymes involved in energy metabolism (Gekakis *et al.*, 1989; Sabina *et al.*, 1989; Webster *et al.*, 1990). In particular, a biphasic and reciprocal expression of the mRNAs for the respiratory and glycolytic enzymes has been observed during myogenesis (Webster *et al.*, 1990). Therefore, it seems likely that the rate of exchange of the adenine nucleotides between the cytosolic and mitochondrial compartments will be different during the phase of proliferation of myoblasts, that of differentiation of myotubes and at the stage of mature muscle fibers. It is reasonable to ask whether these three different physiological situations require the activity of different isoforms of the ADP/ATP translocase, which may be characterized by different catalytic parameters. The present paper shows that, indeed, the multiple genes encoding the adenine nucleotide transport protein are differentially expressed at the mRNA level at different stages of myogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa S3 cells were grown in suspension as described (Amaldi and Attardi, 1968). Mass myoblast cultures were established, using a previously described procedure (Hurko *et al.*, 1986), from muscle biopsy specimens removed for diagnostic purposes from three individuals: a 26-year-old normal woman (6A), a 16-year-old male affected by Devic's syndrome (4Q), and a 40-year-old male affected by ocular myasthenia gravis (5A). Devic's syndrome is an idiopathic, but probably autoimmune, condition of optic neuropathy and spinal cord disease, which is thought to be related to multiple sclerosis. Myasthenia gravis is an autoimmune neuromuscular disease which

* These investigations were supported by National Institutes of Health Grants GM-11726 (to G. A.) and AR38231 (to O. H.), an Eleanor Roosevelt-International Union against Cancer Fellowship, and a grant from the Association Française Contre les Myopathies (to J. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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affects the acetylcholine receptors at neuro-muscular junctions. Neither of these diseases causes a myopathy, and none of the three individuals exhibited histological signs of myopathy. Secondary myoblast cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and harvested at 60–70% confluence as undifferentiated myoblasts. Myoblast differentiation was initiated by transferring cells to gelatin-coated plates, letting them grow in the medium described above up to 80% confluence, and then replacing the medium with Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated horse serum to induce the formation of multinucleated myotubes. The differentiated cultures were harvested when 50–70% of the myoblasts had undergone fusion.

Probes—A cDNA probe specific for T1 was subcloned by digesting the pHAT14 clone (Houldsworth and Attardi, 1988) with *Nci*I, filling in the ends of the fragment and ligating it into the *Hind*II site of the polylinker sequence of pUC9. The insert was isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after digestion of the recombinant pUC9 derivative with *Bam*HI and *Eco*RI. T2- and T3-specific cDNA probes were obtained by digesting, respectively, with *Ban*II the clone pHAT8 and with *Hind*III and *Mae*III the clone pHAT3 (Houldsworth and Attardi, 1988), and then isolating the fragments by agarose gel electrophoresis. Two oligonucleotides specific for the T1 sequences were synthesized, T1–1: 5′ GTCTGGTTTTATCCCAGATCTTT 3′ (corresponding to nt[1] 1094–1116 in the cDNA sequence reported by Neckelmann *et al.* (1987), and to nt 994–1016 according to the nucleotide numbering by Houldsworth and Attardi (1988)) and T1–2: 5′ TAGGAAGCTCCAAGCGTGATCACC 3′ (corresponding to nt 4–27 in the cDNA sequence reported by Houldsworth and Attardi (1988)). A mouse myosin heavy chain (MHC) probe was prepared by digestion with *Eco*RI of the clone pVZMHC kindly provided by Dr. H. Weintraub.

RNA Isolation and Gel Blot Analysis—A specimen was isolated for diagnostic purposes from the paraspinal muscle of a 13-year-old female who had a chronic progressive external ophthalmoplegia syndrome with trunk and limb muscle weakness. This syndrome can be caused by: (*a*) a myopathy affecting extraocular muscles, which is often associated with mitochondrial DNA deletions or (*b*) a lower motor neuron abnormality. Histologic examination of the muscle specimen from this patient revealed mild denervation (reflecting lower motor neuron abnormality), but no signs of myopathy; furthermore, biochemical studies showed no changes in the pattern of *in vitro* mitochondrial protein synthesis.[2] A portion of the specimen was used for the present analysis. Total cellular RNA from the various cell lines and from the muscle biopsy specimen was extracted by the guanidinium isothiocyanate method (Chirgwin *et al.*, 1979) and fractionated by electrophoresis through 1.4% agarose, 2.2 M formaldehyde gels in MOPS buffer (Davis *et al.*, 1986). The RNA integrity in the samples before fractionation was verified by running a portion of each sample on gel and staining it with ethidium bromide in order to check the ratio of the two cytosolic rRNAs. RNA was transferred from the gels onto Zetaprobe membranes (Bio-Rad) by electroblotting. RNA on the membranes was fixed by baking for 2 h at 80 °C in a vacuum oven, and the membranes were then washed, pretreated, and incubated for hybridization as described (Houldsworth and Attardi, 1988). Hybridizations were carried out for 18 h at 42 °C using 40% formamide in the presence of 5 × SSC (1 × SSC is 0.150 mM NaCl, 15 mM sodium citrate, pH 7.0) when cDNA-derived probes were used, and for 18 h at 52 °C in the presence of 6 × SSC when oligonucleotide probes were used. ^{32}P-Labeled probes were prepared by random priming labeling (T2 and T3 cDNA clone fragments and MHC cDNA) (Feinberg and Vogelstein, 1983) or filling in (T1 cDNA clone insert) (Cobianchi and Wilson, 1986) to a specific radioactivity of 3–5 × 10^8 cpm/μg. Purified synthetic oligonucleotides (T1–1 and T1–2) were 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. After hybridization, the filters were processed as described previously (Houldsworth and Attardi, 1988).

RESULTS AND DISCUSSION

The three genes encoding the human ADP/ATP translocase are characterized by a high degree of sequence similarity in the coding region (Battini *et al.*, 1987; Neckelmann *et al.*, 1987; Houldsworth and Attardi, 1988; Cozens *et al.*, 1989). This could result in cross-hybridization in Southern blot or RNA transfer hybridization analysis. Therefore, cDNA and oligonucleotide probes highly specific for the three genes T1, T2, and T3 were prepared in order to follow variations of the expression of the individual genes (Fig. 1).

The possible changes in the levels of the mRNAs corresponding to the three ADP/ATP translocase genes occurring during the *in vitro* differentiation of human myoblasts were investigated by RNA transfer hybridization experiments. Two stages of the *in vitro* developmental process were analyzed. During the first phase, myoblasts were allowed to proliferate in mitogen-rich growth medium up to 60–70% of confluence; the second phase, during which the myoblasts differentiated to form multinucleated myotubes, was started by growing the cells to confluence and transferring them to mitogen-poor growth medium. The type(s) and level(s) of ADP/ATP carrier-specific mRNAs were then analyzed in the total cell RNA extracted from myogenic cells in the two developmental stages described above as compared with the RNA extracted from human adult skeletal muscle and from exponentially growing HeLa cells. Representative results are presented in Fig. 2.

As shown in Fig. 2*A*, the T1–1 oligonucleotide probe specific for the T1 gene hybridized strongly with a 1400-nt mRNA species expressed in the human adult muscle, whereas no detectable hybridization signals could be observed with RNAs isolated from human myoblasts or myotubes (derived from individual 5A) or HeLa cells. Similar results were obtained with the T1–2 oligonucleotide or the T1 cDNA probe (not shown). The hybridization patterns presented in Fig. 2*A* also show that the expression of the T2 and T3 genes is differentially regulated during the *in vitro* differentiation. T2 gene expression was characterized by the presence of two specific mRNAs with sizes of 1450 and 1600 nt. These two species were previously shown to correspond to the use of two different polyadenylation signals for the transcription of the T2 gene (Lunardi and Attardi, 1991). The levels of these two

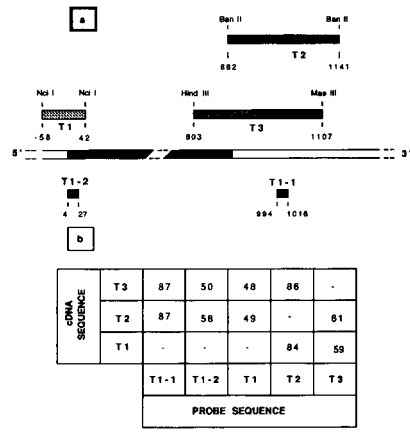

FIG. 1. **Isoform-specific probes used to analyze the ADP/ATP translocase transcripts.** The probes were isolated as described under "Experimental Procedures." *a*, probe T1, which corresponds to a 101-nt fragment (−58 to +42) of the pHAT14 cDNA generated by *Nci*I digestion, overlaps the 3′-end-proximal portion of the 5′-noncoding sequence and the first 42 coding nucleotides in exon 1 of the T1 gene. Probe T2, which is 259 nt long, overlaps the last 13 coding nucleotides and a portion of the 3′-noncoding sequence in exon 4 of the T2 gene. Probe T3, which is 304 nt long, overlaps the last 91 coding nucleotides and a portion of the 3′-noncoding region of the T3 gene. T1–1 and T1–2 oligonucleotides are complementary, respectively, to a portion of the 3′-noncoding region and to the beginning of the coding sequence of the T1 mRNA. *b*, binary comparisons of sequences of the different probes used for analysis of the ADP/ATP translocase genes. Indicated values represent the percentage differences between the sequences.

[1] The abbreviations used are: nt, nucleotide(s); MHC, myosin heavy chain.

[2] A. Chomyn, unpublished observations.

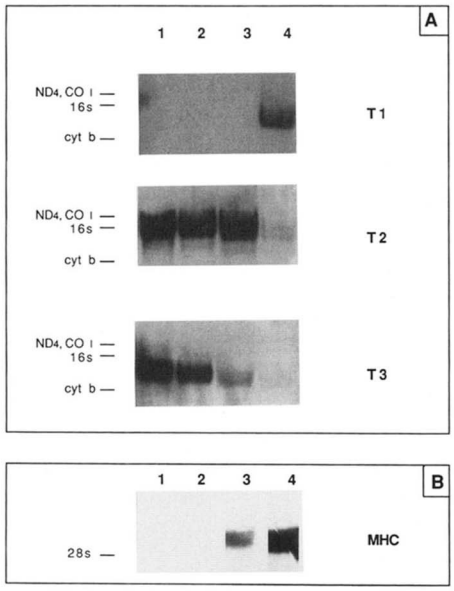

FIG. 2. **RNA transfer hybridization analysis of the ADP/ATP translocase mRNA species and of the MHC mRNA expressed at different stages of muscle development.** Samples (12 μg) of total RNA extracted from exponentially growing HeLa cells (*lane 1*), proliferating human myoblasts (*lane 2*), early differentiated myotubes (*lane 3*), and adult human muscle (*lane 4*) were fractionated by electrophoresis in a 2.2 M formaldehyde, 1.4% agarose gel, electroblotted onto a Zetaprobe membrane, and hybridized with the specific ^{32}P-labeled probes, as described under "Experimental Procedures." *Panel A*, hybridization with ADP/ATP translocase probes: T1–1, 6×10^6 cpm/lane, 48-h exposure; T2, 4×10^6 cpm/lane, 70-h exposure; T3, 4×10^6 cpm/lane, 70-h exposure. *Panel B*, hybridization with MHC probe: 6×10^6 cpm/lane, 72-h exposure (*lanes 1–3*) and 36-h exposure (*lane 4*). Mitochondrial RNA species labeled in isolated organelles (Gaines and Attardi, 1984) were used as molecular weight markers, and the migration positions of relevant species are indicated. The size of 16 S rRNA (excluding the oligo(A) tail (Dubin *et al.*, 1982) is 1559 nt (Ojala *et al.*, 1980), and those of ND4, COI and cytochrome *b* mRNAs (including the poly(A) tail) are ~1,725, 1,675, and 1,200 nt, respectively (Ojala *et al.*, 1980).

transcripts appeared to be very similar in myoblasts and myotubes, whereas they were strongly decreased in the adult muscle fibers. The analysis of the T3 ADP/ATP translocase-specific mRNA revealed a single mRNA species with a size of 1300 nt. The T3 gene appeared to be expressed at a high level in growing myoblasts, whereas the T3 mRNA level decreased strongly in myotubes and was barely detectable in the adult muscle. Similar results to those shown in Fig. 2*A* were obtained with myoblast and myotube cultures derived from individual 4Q and from the normal individual 6A (not shown), indicating that the observed differences in the expression of the three ADP/ATP translocase isoforms were not related to the disease condition of the donor.

The differentiation of myoblasts to myotubes was monitored by measuring the level of MHC mRNA in the different cell systems. MHC mRNA has been shown not to be expressed in growing myoblasts, whereas it accumulates rapidly during the first days of differentiation (Medford *et al.*, 1983). As shown in Fig. 2*B*, a significant hybridization was observed with myotube RNA (from individual 5A) and a strong hybridization with adult muscle RNA, whereas no signal was detected with either myoblast RNA or HeLa cell RNA.

A quantitative analysis of the hybridization patterns obtained for each RNA species at different stages of muscle development is shown in Fig. 3. The data clearly show that T1 gene transcripts are the major ADP/ATP translocase mRNA species expressed in human adult muscle. The absence

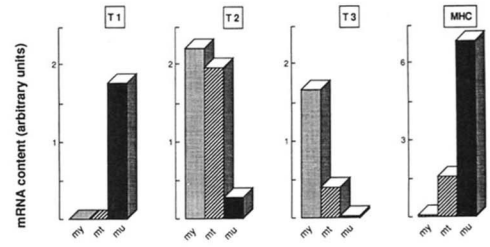

FIG. 3. **Quantitative behavior of the mRNA species for each of the three ADP/ATP translocase isoforms and for MHC at different stages of muscle development.** Appropriate exposures of the autoradiograms were scanned with an Ultroscan XL laser densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden). Complete area scanning of the sample tracks was performed to compensate for artifactual band variations and automatically integrated. Values are given in arbitrary units and are comparable only within each set of hybridization reactions carried out with the same probe.

of expression of the T1 gene in proliferating myoblasts observed in the present work agrees with the results by Webster *et al.* (1990). However, the authors reported the appearance of relatively low levels of T1 mRNA in the earlier phase of myoblast differentiation to myotubes, in contrast to the present observations, which have pointed to a late activation of the T1 gene. The reason for this discrepancy is not known, although it may reflect the "age" of the myoblasts at the time of induction of differentiation. In any case, it is clear that the T1 gene is subject to a strong developmental regulation, reaching its full expression in the late stages of the myogenic differentiation. In addition, the T1 gene has been shown to have a high tissue specificity of expression, being active primarily in muscle and heart tissues (Neckelmann *et al.*, 1987; Powell *et al.*, 1989). Indeed, the human T1 gene has some features, such as the presence of CCAAT and TATA boxes and Sp1 binding motifs, that have been associated with tissue-specific genes. Interestingly, Li *et al.* (1990) have described the presence of a positive transcriptional element for the T1 gene, which appears to be specific for muscle cells.

The T2 ADP/ATP translocase gene has features in its 5′-region that suggest that it might be a housekeeping gene (Cozens *et al.*, 1989). However, the present results indicate that the expression of this gene varies during myogenesis, the level of the corresponding mRNAs being high in myoblasts and myotubes and decreasing markedly in adult muscle. The T3 ADP/ATP gene was first described as a growth-regulated gene since its mRNA level in Balb/c/3T3 was sensitive to growth factors (Battini *et al.*, 1987). Consistent with these observations is the finding that the T3 mRNA level decreases progressively during the late exponential and stationary phases of HeLa cell growth, in contrast to the T2 mRNA level (Lunardi and Attardi, 1991). In addition, the present observations, indicating that the T3 gene is expressed at a high level in multiplying myoblasts, whereas its mRNA level decreases strongly when myoblasts are induced to fuse to form myotubes and is barely detectable in adult muscle, support the conclusion that T3 is a growth-regulated gene active during myoblast multiplication.

From the data presented here it can be concluded that adult muscle fibers are characterized by a different pattern of expression of the ADP/ATP translocase gene as compared to myoblasts or newly formed, non-innervated myotubes. In particular, it would appear that expression of the T1 gene is a marker of a late stage in myogenesis. If one considers the energetic demands of muscle cells at different stages of development, it is reasonable to assume that mature muscle fibers will require high levels of cytosolic ATP, which can be main-

tained only by a high rate of the ADP/ATP exchange between cytosol and mitochondria. By contrast, myoblasts, which do not exhibit contractile movements, may require a lower ADP/ATP exchange rate. It is conceivable that the ADP/ATP exchange is the rate-limiting step in ATP production by oxidative phosphorylation, and therefore the switching "on" or "off" of the expression of the different ADP/ATP translocases, possibly characterized by different catalytic parameters, could be an efficient way for the muscle cells to adapt to changing energetic requirements during their development. Different *cis*-acting and *trans*-acting factors, including factors specifically involved in myogenesis, may play a role in such a regulation, and studies are under way to characterize these factors and the sequences involved.

Acknowledgments—We thank Dr. H. Weintraub for the gift of the MHC clone. We gratefully acknowledge the technical assistance of Arger Drew, Linda McKee, Benneta Keeley, and Lisa Tefo.

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