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Expression of Three Stage-Specific Transcripts of AMP Deaminase during Myogenesis

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AMP deaminase, a ubiquitous enzyme in eucaryotes, plays a central role in energy metabolism. In the present study, RNase protection analyses and immunoprecipitation with tissue-specific antisera were used to examine the transcripts and peptides of AMP deaminase produced during myogenesis in vivo and during myocyte differentiation in vitro. In embryonic muscle and undifferentiated myoblasts, a 3.4-kilobase (kb) transcript encoded a 78-kilodalton (kDa) AMP deaminase peptide that cross-reacted with antisera raised to the AMP deaminase isoform purified from kidney of the adult animal. In perinatal muscle and myocytes at an intermediate stage of differentiation in vitro, a 2.5-kb transcript was produced, and it encoded a 77.5-kDa AMP deaminase peptide that cross-reacted with antisera to the isoform purified from adult heart muscle. At about the time of birth, another 2.5-kb AMP deaminase transcript that encoded an 80-kDa peptide became detectable. This peptide cross-reacted with antisera to the predominant isoform purified from adult skeletal muscle.

AMP deaminase is found in all vertebrate tissues, and tissue-specific isoforms have been identified in many species (6–8). In skeletal muscle, AMP deaminase may play a dual role, serving as a key component in a series of reactions involved in energy metabolism (4) and as an integral part of the myofibril, where it decorates the end of the A band in the sarcomere (1). The importance of this protein in muscle function is underscored by the myopathy which develops in patients with inherited deficiency of AMP deaminase (10a).

In the present study we have used a cDNA cloned from adult rat skeletal muscle (10) and antisera specific for different AMP deaminase isoforms (5, 6) to demonstrate stage-specific expression of AMP deaminase transcripts and peptides during skeletal muscle development. Myogenesis was examined both in vivo, with Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), and in vitro, with the myogenic rat cell line L6J1 (9).

As an initial step in this analysis, poly(A)$^+$ mRNA was prepared from rat hind-limb tissue and myocytes in culture at various stages of development and examined by Northern (RNA) analysis. While the predominant AMP deaminase transcript in adult skeletal muscle is 2.5 kilobases (kb) in length (10), undifferentiated L6 myoblasts produce a 3.4-kb AMP deaminase transcript (Fig. 1). The 3.4-kb transcript is also evident in perinatal hind-limb muscle. To examine the temporal expression of these transcripts in more detail, the more sensitive technique of RNase protection analysis was used. By using an adult rat skeletal muscle AMP deaminase cDNA cloned into the transcription vector Bluescribe (10), 5′ and 3′ cRNA probes were designed (Fig. 2). RNA from undifferentiated L6 myoblasts and hind-limb tissue obtained at an early stage of embryonic muscle development protected a 97-base fragment in the 341-base 3′ AMP-D1 cRNA probe (Fig. 3). Mapping experiments with progressively truncated 3′ cRNA probes demonstrated that this 97-base protected fragment is located approximately between bases 1930 and 2030 in the 3′ coding region of the adult skeletal muscle cDNA (location illustrated in Fig. 2).

Antiserum raised to the AMP deaminase isoform purified from kidney of the adult rat immunoprecipitates ≈95% of the activity in embryonic muscle and undifferentiated L6 myoblasts, while antisera to the predominant AMP deaminase isoform found in adult heart and skeletal muscle immunoprecipitate <10% of AMP deaminase activity in these tissues (Table 1). Previous studies from this laboratory have demonstrated that undifferentiated L6 myoblasts and embryonic muscle produce a 78-kilodalton (kDa) AMP deaminase peptide that is different on peptide mapping from the 80-kDa AMP deaminase peptide produced in adult skeletal muscle (5). We conclude from these observations that embryonic muscle and undifferentiated myoblasts produce a 3.4-kb AMP deaminase transcript, represented by the 97-base protected fragment in the 3′ cRNA probe, and that this transcript encodes a 78-kDa peptide of AMP deaminase which cross-reacts with antisera raised to the isoform purified from kidney of the adult animal.

In adult skeletal muscle and maximally differentiated L6 myotubes, the predominant AMP deaminase transcript is 2.5 kb in size (Fig. 1). RNA from these tissues protects the entire 341-base 3′ cRNA probe (Fig. 3). Approximately 90% of the AMP deaminase activity in adult mixed hind-limb tissue and 50% of the activity in differentiated myotubes can be immunoprecipitated with antisera raised to the predominant isoform purified from adult skeletal muscle (Table 1). Prior studies have shown that this peptide has a subunit molecular size of 80 kDa and a peptide map that is distinct from that of the AMP deaminase peptide produced in embryonic muscle and undifferentiated myoblasts (5).

Although we detected a prominent 2.5-kb transcript (Fig. 1) and a 341-base protected fragment with the 3′ cRNA probe in RNA obtained from perinatal muscle and myocytes at an intermediate stage of differentiation (Fig. 3b, lane 3, and 3a, lane 2), <10% of the AMP deaminase activity in

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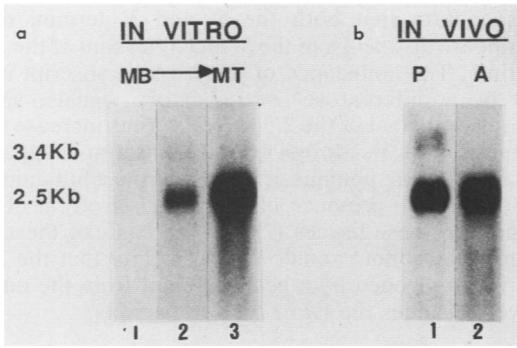

FIG. 1. Northern analysis of RNA from different stages of myogenesis. (a) In vitro myogenesis. Lane 1, RNA from undifferentiated, proliferating L6 myoblasts maintained in Dulbecco modified Eagle medium with 10% horse serum; lane 2, RNA from L6 cells 3 to 4 days after reaching confluence in 1% serum, i.e., intermediate stage of differentiation; lane 3, RNA from L6 cells after 10 to 12 days in 1% serum, i.e., well-differentiated myotubes. MB, Myoblasts; MT, myotubes. (b) In vivo myogenesis. Lane 1, RNA from perinatal hind-limb tissue (15 to 16 days of gestation); lane 2, RNA from hind-limb tissue of adult animal. P, Perinate; A, adult. Poly(A)$^+$ RNA (10 μg) was applied to each lane except for the adult hind-limb sample lane, to which 20 μg of total RNA was applied. Experimental conditions for electrophoresis, transfer, and hybridization to AMP deaminase cDNA were as described elsewhere (10), except that filters were washed at 55°C.

these tissues could be immunoprecipitated with antisera specific for the predominant AMP deaminase isoform found in adult skeletal muscle (Table 1). These data suggest that either the 2.5-kb transcript in perinatal muscle and myocytes at an intermediate stage of development is subject to post-transcriptional regulation or there is another stage-specific transcript of the same size that shares a common 3′ terminus with the predominant 2.5-kb transcript found in adult muscle.

To distinguish between posttranscriptional regulation and multiple 2.5-kb transcripts, a 215-base cRNA probe (Fig. 2, AMP-D2) was used to analyze RNA obtained at the different stages of myogenesis (Fig. 4). These experiments demonstrate that the 3′ 341-base protected fragment identifies two transcripts that can be distinguished by the 5′ cRNA probe.

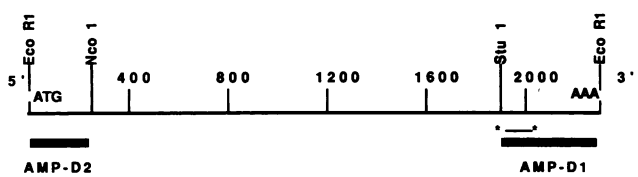

FIG. 2. AMP deaminase cRNA probes. The sequence of this AMP deaminase cDNA, cloned from the library made from adult skeletal muscle of the rat, is reported elsewhere (10). The 341-base cRNA probe complementary to the 3′ terminus (AMP-D1) was synthesized with the T3 promoter in the pBS transcription plasmid truncated at the *Stu*I site. This transcript includes 18 bases of 3′ untranslated sequences in addition to the 3′ coding sequences. The short overline (*) indicates the location of the 97-base fragment of this probe protected by RNA from embryonic muscle and undifferentiated myoblasts. The 215-base cRNA probe complementary to the 5′ terminus was synthesized by subcloning the *Eco*RI-*Nco*I restriction fragment after blunt-ending into the *Sma*I site of pBS. The 215-base cRNA produced from this construct contains 15 bases of 5′ untranslated sequences, and the remainder is complementary to the 5′ coding region of the predominant transcript in adult muscle.

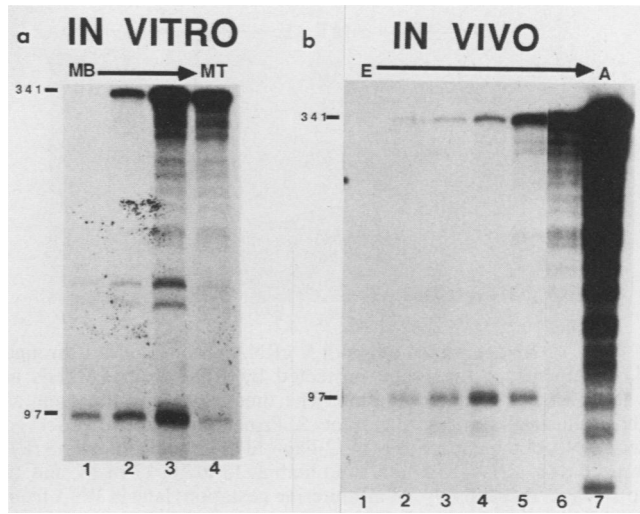

FIG. 3. RNase protection with 3′ cRNA probe. (a) Fragments of the probe protected by RNA from L6 cells at various stages of differentiation, beginning with undifferentiated myoblasts (MB) (lane 1) and progressing to days 3, 6, and 12 in culture (lanes 2, 3, and 4, respectively). Lane 4, RNA from well-differentiated myotubes (MT). A 50-μg portion of total RNA was used at each time point. (b) Protected fragments observed with 50 μg of RNA obtained from 11- to 12-day-old embryonic hind limb (E; lane 1); fetal hind limb at 13 to 14, 15 to 16, 17 to 18, and 19 to 20 days of intrauterine gestation (lanes 2 to 5, respectively); and hind limb of 1-day-old neonate (lane 6) and adult (A; lane 7) rats. Only 10 μg of RNA from adult muscle was used for the studies. RNase protection was performed essentially as described by Krieg and Melton (3). The resulting fragments were resolved on an 8% urea–acrylamide gel.

RNA from perinatal muscle (Fig. 4, lane 6) or myocytes at an intermediate stage of differentiation (lane 2) protects a 170-base fragment in the 215-base 5′ cRNA probe. This transcript precedes by several days the appearance of an AMP deaminase transcript that protects the full-length 215-base 5′ cRNA probe (Fig. 4).

We conclude from these analyses that the 215-base 5′ protected fragment represents the 2.5-kb AMP deaminase transcript that encodes the 80-kDa peptide of AMP deaminase produced in adult skeletal muscle and fully differenti-

TABLE 1. Immunoreactivity of AMP deaminase at different stages of myogenesis

Antiserum	% Enzyme activity precipitated[a]					
	In vitro[b]			In vivo		
	MB	→	MT	Embryo	Perinate	Adult
Antikidney	95	75	57	95	67	<5
Antiheart	<5	27	22	<5	35	<10
Antimuscle	<10	<10	50	<10	<10	90

[a] Immunoreactivity of AMP deaminase in tissue extracts (5) was determined by incubating extract with polyclonal antisera raised against rat muscle AMP deaminase (5), rat kidney AMP deaminase (6), or rat heart AMP deaminase (6). Antibody-antigen complexes were precipitated with Pansorbin (Calbiochem-Behring, La Jolla, Calif.). Residual AMP deaminase activity in the supernatant was assayed as previously described (5), except that the product (IMP) and substrate (AMP) were resolved and quantitated by high-performance liquid chromatography (11). Each experiment included a control with nonimmune serum. Residual activity following incubation with the indicated antiserum is expressed as a percentage of the activity in the supernatant following incubation with nonimmune serum.

[b] Abbreviations: MB, myoblast; MT, myotube. The arrow represents the transitional stage.

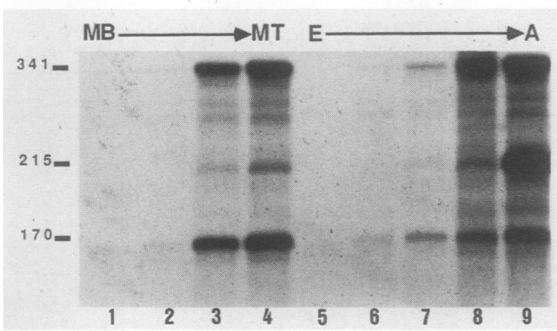

FIG. 4. RNase protection with 5′ cRNA probe. Lanes 1 through 4, Fragments of the probe protected by RNA from L6 cells at various stages of differentiation. The time points are the same as those illustrated in Fig. 3a. Lane 5, Protected fragment observed with RNA obtained from 11- to 12-day-old embryonic hind limb (E); lanes 6 to 8, RNA from fetal hind limb at 15 to 16, 17 to 18, and 19 to 20 days, respectively, of intrauterine gestation; lane 9, RNA from hind limb of adult rat. A 50-μg portion of total RNA was used at each time point, except for the adult sample (10 μg). Abbreviations are the same as in Fig. 3. The 3′ cRNA probe described in the legend to Fig. 3 was also included as an index of total amount of 2.5-kb AMP deaminase transcripts in each sample (i.e., 341-base protected fragment).

ated L6 myocytes. The 170-base 5′ protected fragment represents a second 2.5-kb transcript. It encodes an AMP deaminase peptide which cannot be immunoprecipitated by antisera raised to the predominant isoform in adult skeletal muscle but which can be precipitated by antisera raised to the predominant isoform produced in adult heart muscle (Table 1). On the basis of its time of appearance (i.e., during intermediate stages of development), we assume that this transcript and its cognate peptide correspond to the 77.5-kDa perinatal isoform of AMP deaminase described previously (5).

The molecular basis for AMP deaminase transcript diversity will not be known for certain until cDNAs for each of the transcripts and their respective genes are cloned and sequenced. These studies are in progress. In the meantime, certain inferences can be made from the RNase mapping results and patterns of transcript expression presented here. The two 2.5-kb transcripts are closely related. They have apparently identical 3′ termini and share an additional segment of sequence similarity in their 5′ regions. We suspect that both of these mRNAs are derived from a common primary transcript, possibly through alternative exon splicing, a common mechanism used by skeletal muscle to generate peptide diversity (2).

The 3.4-kb AMP deaminase transcript is different from the two 2.5-kb transcripts in a number of respects. While it encodes a 78-kDa peptide which is similar in size to the 77.5- to 80-kDa peptides encoded by the 2.5-kb transcripts, the 5′ or 3′ or both noncoding regions of this transcript must be longer to account for its greater size. RNase protection studies confirm that both the 5′ and 3′ termini of this transcript are distinct from the 5′ and 3′ termini of the 2.5-kb transcripts. The abundance of the 3.4-kb transcript is controlled by a different set of regulatory signals, since it decreases while that of the 2.5-kb transcripts increase during myogenesis (Fig. 3). Moreover, this transcript is apparently expressed in many nonmuscle tissues of the adult animal, as evidenced by the presence of the 78-kDa isoform of AMP deaminase in these tissues (7). On the basis of these comparisons, we cannot exclude the possibility that the 3.4-kb transcript is encoded by a gene different from the one that putatively encodes the two 2.5-kb transcripts.

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LITERATURE CITED

1. **Ashby, B., C. Frieden, and R. Bischoff.** 1979. Immunofluorescent and histochemical localization of AMP deaminase in skeletal muscle. J. Cell Biol. **81**:361–373.
2. **Breitbart, R. E., A. Andreadis, and B. Nadal-Ginard.** 1987. Alternative splicing: a ubiquitous mechanism for the generation of multiple protein isoforms from single genes. Annu. Rev. Biochem. **56**:467–495.
3. **Krieg, P. A., and D. A. Melton.** 1987. *In vitro* RNA synthesis with SP6 RNA polymerase. Methods Enzymol. **155**:397–415.
4. **Lowenstein, J. M.** 1972. Ammonia production in muscle and other tissues: the purine nucleotide cycle. Physiol. Rev. **52**: 382–414.
5. **Marquetant, R., N. M. Desai, R. L. Sabina, and E. W. Holmes.** 1987. Evidence for sequential expression of multiple AMP deaminase isoforms during skeletal muscle development. Proc. Natl. Acad. Sci. USA **84**:2345–2349.
6. **Ogasawara, N., H. Goto, and T. Watanabe.** 1975. Isozymes of rat AMP deaminase. Biochim. Biophys. Acta **403**:530–537.
7. **Ogasawara, N., H. Goto, Y. Yamada, and T. Watanabe.** 1978. Distribution of AMP-deaminase isozymes in rat tissues. Eur. J. Biochem. **87**:297–304.
8. **Ogasawara, N., H. Goto, Y. Yamada, T. Watanabe, and T. Asano.** 1982. AMP deaminase isozymes in human tissues. Biochim. Biophys. Acta **714**:298–306.
9. **Ringertz, N. R., V. Krondahl, and J. R. Coleman.** 1978. Reconstitution of cells by fusion of cell fragments. I. Myogenic expression after fusion of minicells from rat myoblasts (L6) with mouse fibroblast (A9) cytoplasm. Exp. Cell Res. **113**:233–246.
10. **Sabina, R. L., R. Marquetant, N. M. Desai, K. Kaletha, and E. W. Holmes.** 1987. Cloning and sequence of rat myoadenylate deaminase cDNA: evidence for tissue-specific and developmental regulation. J. Biol. Chem. **262**:12397–12400.

10a. **Sabina, R. L., J. L. Swain, and E. W. Holmes.** 1989. Myoadenylate deaminase deficiency, p. 1077–1084. *In* J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown (ed.), Metabolic basis of inherited disease, 6th ed. McGraw-Hill Book Co., New York.

11. **Swain, J. L., R. L. Sabina, P. A. McHale, J. C. Greenfield, Jr., and E. W. Holmes.** 1982. Prolonged myocardial nucleotide depletion after brief ischemia in the open-chest dog. Am. J. Physiol. **242**:H818–H826.