A muscle-specific isoform of adenylosuccinase synthetase (AdSS1, EC 6.3.4.4) is one of three enzymes that constitute the purine nucleotide cycle, a muscle-specific metabolic cycle. Previously, we showed that the Adss1 gene was highly expressed in both skeletal muscle and heart of the adult mouse. Here we have shown that the Adss1 gene is initially activated early in embryonic development in skeletal muscle and heart precursors and is subsequently up-regulated perinatally. The earliest detectable gene expression corresponds with the establishment of the first myogenic and cardiac lineages. To allow identification of the genetic signals controlling this developmental pattern of expression, the Adss1 gene was cloned and its structure determined. Transgenic analysis has shown that 1.9 kilobase pairs of 5' flank can activate expression in skeletal muscle progenitors and direct enhanced expression to adult cardiac muscle. Sequence analysis of the promoter and 5' flanking region revealed the presence of numerous potential muscle-specific cis-regulatory elements.

Studies aimed at understanding muscle gene regulation have traditionally focused on three classes of tissue-specific genes, those encoding myogenic determination factors, those encoding contractile proteins, and those encoding enzymes of energy metabolism (1, 2). The myogenic transcription factors are instrumental in all phases of myogenesis (commitment, differentiation, and maturation) and as such are expressed in a very tightly controlled temporal fashion throughout skeletal muscle and cardiac development (3, 4). Members of the second class, the contractile proteins, are considered the building blocks of muscle and cardiac fibers and accordingly do not accumulate until the onset of myotube formation during the differentiation process (5). Unlike the myogenic factors, these structural proteins (specifically the actins and myosins) progressively accumulate throughout the prenatal and postnatal phases of development, some actually reaching levels as high as 30% of adult levels at birth. Several embryonic and/or fetal isoforms disappear around the time of birth, as the levels of the adult isoforms increase during the initial period of postnatal development. The third class of muscle-specific genes encodes enzymes of muscle energy metabolism. These genes are not highly expressed until late in embryogenesis (i.e. right before birth), after which the genes are highly up-regulated (20–30-fold) during the first weeks of postnatal development (6). Consistently, their cognate enzymes are abundant in adult cardiac and skeletal muscle. For several of these cardiac and skeletal muscle metabolic genes, it has been demonstrated that muscle-specific transcripts can be detected at low levels early in development (7–12). In fact, earliest detection of muscle-specific transcripts for the β-enolase gene corresponds with the presence of the first muscle progenitors and the primordial cardiac tube (8). This suggests that the increased expression of the muscle metabolic genes during postnatal development is preceded by a basal gene activation early in embryogenesis. The physiological significance, if any, of this early activation is not known, and the regulatory sequences that govern this process have not yet been identified.

We have chosen to focus our attention on understanding the biphasic expression pattern characteristic of the genes that encode enzymes of muscle energy metabolism. For this purpose we have been investigating the muscle-specific isoform of adenylosuccinate synthetase (AdSS1)1 (13), an enzyme that functions in the purine nucleotide cycle, along with myoadenylate deaminase and adenylosuccinate lyase. The deaminase is found in a skeletal muscle and a cardiac isoform, and one form of the lyase exists in both tissues. Genetic disorders affecting the purine nucleotide cycle are associated with specific myopathies and neuromuscular disorders (14, 15). This metabolic cycle, in conjunction with the myoadenylate kinase reaction, plays an important role in regulating adenine nucleotide metabolism in muscle in order to maximize ATP synthesis and utilization (Fig. 1). The direction of purine nucleotide flow through the cycle is governed by the physiological state of the muscle cell. During brief periods of intense muscle contraction, a large portion of the ADP that is produced is rapidly converted to IMP and ammonia by the combined action of myokinase and myoadenylate deaminase. The irreversible deamination of AMP serves to draw the myokinase reaction in the direction of ATP production, thereby helping to meet the immediate energy demands of the contracting muscle cell. Experiments have shown that during brief periods of maximum contraction, the concentration of IMP increases approximately 20-fold, reaching levels that exceed 1 mM. Therefore, it is necessary to have an efficient and properly regulated metabolic pathway to replen-
ADENINE NUCLEOTIDE METABOLISM IN MUSCLE

![Diagram of adenine nucleotide cycle](image)

**Fig. 1.** Schematic representation of the purine nucleotide cycle as part of adenine nucleotide metabolism in muscle. PNC, purine nucleotide cycle.

ish the adenine nucleotide pool during periods of metabolic recovery. The return path of the purine nucleotide cycle, which involves adenylosuccinate synthetase and adenylosuccinate lyase, is believed to serve this purpose. Increased flux through this part of the cycle also contributes to the resynthesis of ATP by expanding the pool of tricarboxylic acid cycle intermediates through the production of fumarate from aspartate (14, 15). A striated muscle-specific isoform of adenylosuccinate synthetase, Adss1, catalyzes the first and rate-controlling step in this adenine nucleotide replenishment pathway. We previously showed that the gene (Adss1) encoding this muscle isozyme is highly expressed in both cardiac and skeletal muscle in the adult mouse (16). Here we report the cloning and structural analysis of the murine Adss1 gene, the identification of the endogenous expression pattern, as well as a functional study of the 5' flanking region in transgenic mice.

**MATERIALS AND METHODS**

**Genomic Library Screening and Isolation of λ Phage DNA**—To identify λ clones containing genomic fragments from the Adss1 locus, a commercial phage library (LAMBDA FIXII; Stratagene) was screened with the full-length Adss1 cDNA. The phage library was plated, and duplicate filter lifts were made according to standard protocol (17). Hybridizations and autoradiography were performed according to standard procedures (17). Genomic DNA subfragments were sequenced according to the dideoxy procedure of Sanger described (17). Genomic DNA dot blots were performed as described elsewhere (19).

**Southern Blot and DNA Dot Blot Analysis**—Southern blots of total genomic and/or λ phage DNA, utilizing full-length Adss1 cDNA as a probe, were performed as before (18). Similar blots utilizing oligonucleotide probes were hybridized and washed as recommended by the supplier (Bio-Rad) of the nylon membrane (Zeta-probe). Oligonucleotide probes were end-labeled with [γ-32P]ATP according to standard procedure (17). Genomic DNA dot blots were performed as described elsewhere (19).

**Exon Mapping and Sequence Analysis**—Standard restriction endonuclease digestes, cloning techniques, and DNA sequencing were as described (17). Genomic DNA subfragments were sequenced according to the dideoxy procedure of Sanger et al. (7). For mapping of exons polymerase chain reaction (PCR) assays were performed using one anchor primer with a known location in the vector and exon-specific primers to determine the distance of the exon from one end of the subclone. The following cycling conditions were established: 1 min 94°C, 25–30 cycles at 1 min 94°C, 1.5 min 50°C, 3 min 72°C, 10 min 70°C, store at 4°C.

**Primer Extension and Ribonuclease Protection Assays**—Total RNA was isolated with RNXazol as described (20), and its integrity was verified by formaldehyde/agarose gel electrophoresis (16). Primer extensions were performed as described previously (21) using the end-labeled 30-mer primer described under “Results.” The ribonuclease protection assay is described elsewhere (19). An [α-32P]UTP-labeled cRNA probe (riboprobe) was synthesized from the StyI-BamHI genomic fragment spanning exon 1 of the Adss1 gene (see “Results”).

**Generation of Riboprobes and in Situ Hybridization Analysis**—In situ hybridization to sections was performed as described (22). Antisense and sense riboprobes were generated from the Adss1 cDNA. Both probes were acid-hydrolyzed to an average size of 750 bp for 7 min at 60°C. Proteinase K treatment was 8 min at room temperature. Samples were hybridized overnight at 60°C and were treated as described (22). RNase T1 was added at a concentration of 20,000 units. Stringency washes were performed at 65°C. Slides were dipped in Kodak NTB-2 emulsion and exposed for 7–14 days. Sections were viewed and photographed with a Leitz Diaplan microscope (23).

**Isolation of RNA and Northern Blot Analysis**—Total cellular RNA from tissues was isolated by the acid guanidine thiocyanate procedure as described (20), fractionated electrophoretically on a 1.4% denaturing agarose-formaldehyde gel, and transferred to nylon membranes (GeneScreenPlus, DuPont NEN). Northern blot analysis was performed as described (17).

**Construction of CAT Fusion Genes**—Construction of the Adss1/cAT fusion genes was based on the backbone vector pSXS (24). The original rabbit β-globin intron II and SV40 polyadenylation signal remained intact. The CAT cDNA was cloned into the EcoRI site between the T7 promoter and the polyadenylation signal. The early SV40 promoter was removed and replaced with a Scal-SacI fragment containing 6.2-kb 5' flank of the Adss1 gene, resulting in the construct 6.2S.CAT. The construct, 1.9HS.CAT, was generated by digesting pS62C2CAT with HindIII and NdeI, blunt-ending with T4 polymerase, and religating. The third construct, 1.2XSCAT, was removed from the 1.9HS.CAT construct by an XhoI digestion.

**Construction of β-Galactosidase Fusion Genes**—Construction of the Adss1/lacZ fusion genes was based on the backbone vector pNASβ (24). Briefly, pNASβ contained a synthetic intron, based on SV40 splicing sequences, followed by a β-gal cassette and an SV40 polyadenylation signal sequence. A SacI-Sacl fragment and an EcoRI-EcoRI fragment from phage clone AGadSS1 1–7, spanning 6.2 and 9.0 kb of 5' flanking sequences of the Adss1 gene, respectively (see “Results”), were subcloned into the EcoRI site of pNASβ yielding the constructs 6.2SSβ and 5.0Eβ. Subsequently, an XbaI-XhoI fragment and a HindIII-HindIII fragment was excised from 6.2SSβ, resulting in the constructs 1.2XSβ and 1.9Hβ, containing 1.2 and 1.9 kb of 5' flanking sequences of Adss1, respectively. Finally, a 2.1-kb BamHI-HindIII fragment was subcloned into the HindIII site of 1.2XSβ to generate the
Fragments containing end of the gene were mapped and their overlap established. An additional clone containing exon 1 (AGAdSS1–1) was identified, but the overlap with the exon 2-containing clone (AGAdSS1–2) was not established (broken line). Based on restriction fragment analysis, Southern blot hybridizations, and a PCR-based strategy, exons 1–13 (black boxes) have been identified and mapped (see also Table I). The estimated size of each clone is indicated between parentheses. B, BamHI; E, EcoRI; H, HindIII; S, SacI; X, XbaI.

**Fig. 2. Structure of the murine Adss1 gene as deduced from λ clones spanning the gene.** Three overlapping clones (AGAdSS1–2, -13, and -1–1) spanning the gene from exon 2 to the 3′ end of the gene were mapped and their overlap established. An additional clone containing exon 1 (AGAdSS1–7) was identified, but the overlap with the exon 2-containing clone (AGAdSS1–2) was not established (broken line). Based on restriction fragment analysis, Southern blot hybridizations, and a PCR-based strategy, exons 1–13 (black boxes) have been identified and mapped (see also Table I). The estimated size of each clone is indicated between parentheses. B, BamHI; E, EcoRI; H, HindIII; S, SacI; X, XbaI.

**RESULTS**

The Murine Adss1 Gene Consists of 13 Exons Spanning a Region of 45 Kb—A lambda library containing genomic DNA inserts ranging from 10 to 20 kb was screened with a full-length Adss1 cDNA, encoding the muscle isozyme (16). Four λ clones containing Adss1 gene fragments, ranging from 13 to 19 kb, were isolated. To determine the extent of exon-containing fragments within the λ clones and the possible overlap between the clones, restriction enzyme analyses and Southern blot analyses were performed. The contig-map of the λ clones and a restriction map of the Adss1 locus that resulted from these analyses are shown in Fig. 2. In order to establish the exonic junctions and map the exons within lambda subclones (Table I), exon-containing genomic fragments were subcloned and subjected to sequence analysis. Sequencing was performed with nested oligonucleotide primers derived from sequence from the Adss1 cDNA. As listed in Table I, the murine Adss1 gene consists of 13 exons with sizes ranging from 50 to 380 bp. The positions of these exons within the Adss1 gene was determined via a PCR-based strategy (see “Materials and Methods”), with the aid of exon-specific primers. The 13 exons of the Adss1 structural gene are estimated to span at least 45 kb. The λ clones flanking the Adss1 gene contain sequences that extend by approximately 10 kb on either side of the gene.

Cardiac and Skeletal Muscle Utilize the Same Transcription Start Site—To map the transcription initiation sites at the 5′ boundary of the Adss1 gene, the 500-bp StyI-BamHI genomic fragment containing exon 1 was subcloned. The restriction map of this fragment with the relative position of exon 1 is shown in Fig. 3A. The subclone was used to generate a uniformly labeled antisense riboprobe spanning the entire Sty1-BamHI fragment across exon 1. This riboprobe was hybridized to total RNA from several murine tissues along with total yeast RNA as a negative control. After treatment with RNase the probe protected a predominant fragment of approximately 240 bp (Fig. 3B, arrow). Some minor protected fragments could sometimes be discerned. As expected, the major protected fragment was highly abundant in the muscle-enriched tissues (skeletal muscle, esophagus, heart, and tongue), confirming that the expression of the Adss1 gene is largely restricted to muscle (Fig. 3B, lanes 2, 7, 11, and 13). Furthermore, both cardiac and skeletal muscle tissues show an identical protected product (Fig. 3B, lane 2 versus lane 11) suggesting that both types of striated muscle tissues utilize the same transcription start site of the Adss1 gene.

To more precisely map the 5′ termini of the Adss1 transcripts, primer extension experiments were performed with a 30-bp oligonucleotide primer positioned at nucleotides −16 to +14 relative to the ATG start codon (Fig. 3A). The oligo-probe was end-labeled and hybridized to similar murine tissue RNA samples as above and extended by reverse transcription. A major product was observed (51 bp), which was only present in the muscle tissues, skeletal muscle, esophagus, heart, and tongue (Fig. 3C, arrow). Again, minor products were sometimes observed. As shown with the protection assay, cardiac and skeletal muscle revealed an identical major extended product,
confirming that the same gene is active in both tissues, producing identical transcripts. Because the size of the primer extension product could be more accurately sized (51 nucleotides) than the protected fragment from the RNase protection experiments, we assigned the initiation site according to the extended products (Fig. 3C, and Fig. 4).

**Table I**

| Exon | Position in open reading frame | Sequence of exon-intron junctions |
|------|--------------------------------|----------------------------------|
| 1    | 1–229                         | CTCGCT ... (228) ... TGCCAAGtgcc |
| 2    | 230–332                       | ttcagGGGGGCC ... (102) ... TCATTGtgag |
| 3    | 333–396                       | tggcagGCAATG ... (62) ... AGAAAGtgagg |
| 4    | 396–446                       | ctcgagGCTCTGA ... (50) ... ACCTTGtgtag |
| 5    | 447–513                       | cccagTGTTCCG ... (66) ... GAAGAGtgag |
| 6    | 514–621                       | ttcagTATCGCC ... (107) ... TGCCAGtgat |
| 7    | 622–697                       | gccaagATTCGAA ... (75) ... AAAAGGttag |
| 8    | 698–830                       | gttcctCTCAAG ... (132) ... ATTTCGtgag |
| 9    | 831–985                       | ccctcaGGACCT ... (154) ... ATCAATtgag |
| 10   | 986–1110                      | acacagGAAAATT ... (124) ... CACTCGtgatc |
| 11   | 1111–1207                     | gcacagGCTGGC ... (96) ... TTCCCTggtat |
| 12   | 1208–1357                     | ttccacAGCTAAC ... (149) ... TTCCAGtgatg |
| 13   | 1358–1738                     | cttcacGAAAAAT ... (380) ... TCACTG |

**Fig. 3.** Identification of the transcription start site of the Adss1 gene in striated muscle. A, restriction map of the Sty1-BamHI fragment containing the proximal 5′ flanking region and exon 1 of the Adss1 gene. The arrow indicates the position of the 30-bp oligonucleotide used for primer extension analysis. The fragment was subcloned in the vector pBlueScript KS(+) (Stratagene) and used for generating an antisense cRNA probe (solid line) used for RNase protection analysis. The ATG start codon and relevant restriction sites are indicated. B, BamHI; S, SacI; X, XbaI. B, for RNase protection analysis a uniformly labeled cRNA probe of approximately 550 base pairs (see A) was hybridized to 20 μg of total RNA from relevant mouse tissues. The major protected product as observed after gel electrophoresis and autoradiography is indicated by an arrow. Total RNA from yeast served as a negative control. C, primer extension analysis was performed with a 30-nucleotide primer described in the text. For analysis 20 μg of total RNA from each of the relevant mouse tissues was hybridized with 5′-end-labeled primer. The extended products were fractionated over a denaturing polyacrylamide gel (6%) and visualized by autoradiography. The position of the primary product is indicated by an arrow. Between parentheses is the estimated size of the product, in base pairs. M, DNA size markers, in base pairs.

**Sequence Analysis of the Promoter and 5′ Flanking Region Reveals Numerous Potential Muscle Regulatory Elements**—The 1.9-kb HindIII-SacI fragment was sequenced and screened for potentially important cis-elements (Fig. 4). The region upstream of the transcription initiation site is very G/C-rich with an overall G + C content of 69%, within 200 bp of the transcription start. This region contains a TATA box-like element (28) within 25–30 bp upstream of the start site, an organization that is usually observed in TATA box-containing eukaryotic genes. In addition, the sequence spanning the transcription start matches the consensus for the initiator element (29) that is often observed for eukaryotic genes. Other potentially important skeletal muscle or cardiac regulatory elements are noted.

**Expression of the Murine Adss1 Gene Is Developmentally**
Regulated—Northern (16) and Western (13) analyses have established that Adss1 RNA and protein, respectively, are restricted to adult striated muscle tissues, where they are present at relatively high levels. Little is known about the developmental timing of expression of the Adss1 gene. In order to determine the expression pattern of Adss1 at the level of transcription, total RNA from mouse muscle tissue at later stages of development was collected and subjected to Northern blot analysis. The results show that Adss1 transcripts are virtually undetectable in day 13 and day 16 embryos (Fig. 5, lanes 5 and 4). After birth, however, the Adss1 message is clearly present (Fig. 5, lane 3) and significantly up-regulated during further postnatal development. After long term exposure of similar Northern blots, the Adss1 transcript was also detectable, albeit at very low levels, in skeletal muscle of day 16 embryos. These results suggest that the expression of the Adss1 gene is highly up-regulated perinatally and that this regulation most likely takes place at the level of transcription.

The Adss1 Gene Is Activated in Skeletal Muscle and Heart Progenitors Early in Embryonic Development—To determine whether the Adss1 gene is expressed during early development, embryos were collected at various developmental points, and in situ hybridization analysis was performed. The heart develops from a discrete mesodermal lineage, the splanchnic mesoderm, 8–9 d.p.c. is the point in cardiac development when the inactive primordial endocardial tubes fuse in the central pericardial cavity to form a beating linear heart tube (30, 31). Earliest expression of Adss1 transcripts was detected in the atrial and ventricular walls of the developing heart in 9.5 d.p.c. embryos (Fig. 6A). No signal above background was detected with sense riboprobes (data not shown). Cardiac expression increases through 10.5 and 13.5 d.p.c., becoming clearly visible in both the atrium and ventricle walls (Fig. 6, C and D). Thus, the

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2 A. L. Lewis, O. M. Guicherit, S. K. Datta, G. R. Hanten, and R. E. Kellems, unpublished observations.

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**Fig. 4.** Nucleotide sequence of the promoter and immediate 5’ flanking region of the Adss1 gene. The arrow indicates the major transcription initiation site as determined by primer extension. The bold regions indicate matches with consensus sequences for known transcription factors described in the text. Important restriction enzyme sites are in italics.

**Fig. 5.** Northern analysis of total RNA from mouse skeletal muscle at different stages of development. Total RNA (30 μg) from muscle tissue was fractionated on a denaturing 1.4% agarose gel and transferred to a nylon membrane. The Northern blot was probed with the Adss1 cDNA. The stages of development are listed above each lane. The ribosomal RNA markers (18S, 28S) are included for size reference and were visualized by ethidium bromide staining prior to and following blot transfer to ensure equal loading and efficient transfer of RNA.

Adss1 gene is expressed during cardiogenesis as the embryonic heart develops and begins to function. Somites are the earliest structures currently identifiable as muscle precursors that develop from the dorsal mesoderm in a rostral to caudal gradient beginning at approximately 8.0 d.p.c. (3). Adss1 RNA was detected in caudal somites on either side of the neural tube in 9.5 d.p.c. embryos (Fig. 6B). Expression patterns in a 10.5-d.p.c. embryo illustrate myoblast migration away from the somite and initial myotube fusion as formation of thoracic body wall muscle begins in the developing myotome (Fig. 6C). At 13.5 d.p.c., Adss1 transcripts were readily detected in the developing intercostal body wall muscles (Fig. 6D). Expression was not seen by 10.5 d.p.c. in the head mesenchyme and branchial arches that give rise to the mandibular and
facial muscles and tongue, respectively (Fig. 6C). However, by 13.5 d.p.c. the signal was clearly present in the muscle of the newly formed tongue and in the facial and mandibular muscles (Fig. 6D). These data show that Adss1 is activated early in embryonic development at the time when skeletal and head muscle-specific lineages are established and maturing.

Low levels of Adss1 transcripts were detected in the livers of 10.5- and 13.5-d.p.c. embryos (Fig. 6C and 6D), a finding consistent with low levels of Adss1 expression in the adult tissue (16). Low Adss1 expression was also observed in the midgut at 9.5 d.p.c. (Fig. 6B).

5’ Flanking Sequences of Adss1 Target Cardiac Muscle Expression in Transgenic Mice—To determine if important regulatory elements lie upstream of the promoter region, various amounts of the 5’ flanking region of the Adss1 gene were fused to the bacterial chloramphenicol acetyltransferase (CAT) gene and were tested in adult transgenic mice. In 6.2SSCAT and 1.9HSCAT transgenic mice, CAT expression was consistently highest in the heart (Fig. 7A, lane 2). Lower levels of expression were noted in skeletal muscle tissues. However, when the 1.2XSCAT construct was tested, muscle-specific enhancement was abolished. Fig. 7B depicts an average and quantitation of multiple experiments. The results show that this 1.9-kb fragment consistently directs very high levels of transgene expression specifically to cardiac tissue. All 1.9HSCAT muscle tissues expressed higher levels of reporter activity than the 6.2-kb parent construct. This may be attributed to the loss of a negative regulatory element in the deletion construct. Low level expression was noticed in the spleen and thymus consistently for all three constructs (Fig. 7A, lanes 9 and 10). We speculate that this expression could be controlled by multiple transcription factor binding sites present around the start site. The overall results suggest, however, that sequences necessary for high levels of adult heart expression lie within 1.9 kb of 5’ flanking, with a critical region lying within the 700-bp HindIII-XbaI fragment.

5’ Flanking Sequences of Adss1 Also Activate Expression in Skeletal Muscle Precursors—In order to determine whether the 5’ flank also controls gene expression in muscle progenitors during prenatal development, Adss1/lacZ fusion constructs were generated and tested. Because of histochemical detection capabilities, the lacZ reporter is well-suited for prenatal expression analysis. All transgenic mice contained multiple copies of the construct, ranging from 5 to 70 copies. Multiple embryos of varying age were analyzed for β-galactosidase expression by whole mount 5-bromo-4-chloro-3-indolyl β-D-galactoside staining. Staining was clearly visible by 9.0 d.p.c. in the somites of 6.2SSβ transgenic mice (Fig. 8). By 11.5 d.p.c. the staining of the thoracic somites had extended well into the trunk area, presumably reflecting precursors of body wall muscle. β-Gal activity was also detected in the forelimb buds, neck, and facial areas. From about 14 d.p.c., the staining was very
strong in the tongue and the peripheral musculature of the embryo. This pattern reflected the endogenous skeletal muscle pattern of Adss1 expression delineated by in situ analysis. Two deletion constructs 5.0ESβ (data not shown) and 1.9HSβ, containing 5.0 and 1.9 kb of 5’ flank of Adss1, respectively, reproduced the staining pattern of the parent 6.2SSβ construct. Of the three lines containing the 1.2XSβ construct, none of the embryos showed any detectable β-gal activity. Since this was the case for multiple lines, it most likely reflects the inability of the transgene to properly function, rather than the effect of chromosomal integration site(s) on the transgene. A final construct, 4.0BH.XSβ, which contained 4.0 kb of 5’ flank with a 700-bp deletion from the HindIII site to the XbaI site, also showed no expression in multiple transgenic embryos (data not shown).

**Fig. 7. 5’ flanking sequences of Adss1 target cardiac muscle expression in adult transgenic mice.** A, representative CAT assays for the three constructs shown in panel B. 6.2SSCAT showed cardiac-specific enhancement in 5/5 transgenic animals; 1.9HSCAT showed cardiac-specific enhancement in 3/4 animals; and 1.2XSCAT showed no expression in 2/2 animals. B, schematic representation of the CAT transgenes, containing various amounts of the Adss1 5’ flanking region. Relevant restriction sites are shown, and the transcription initiation site is indicated. CAT activities are expressed as pmol of chloramphenicol acetylated per min/mg protein/transgene copy number. I1, β-globin intron II. CAT, chloramphenicol acetyltransferase reporter. pA, SV40 polyadenylation signal. S, SacI; H, HindIII; X, XbaI. Tissues examined were skeletal muscle (SKM), heart (HT), tongue (TO), esophagus (ESO), kidney (KY), liver (LIV), small intestine (SM I), stomach (STO), spleen (SPL), thymus (THY), and bladder (BL).
Fig. 8. 5' flanking sequences of Adss1 have the ability to activate reporter gene expression in muscle progenitors in transgenic embryos. A, schematic representation of the lacZ transgenes, containing various amounts of the Adss1 5' flanking region. Relevant restriction sites are shown, and the transcription initiation site is indicated. Stippled box, Adss1 5' flank; dark stippled box, SV40 intron 1; white box, β-galactosidase reporter; dotted box, SV40 polyadenylation signal. Asterisk indicates that the animals that were identified as transgenic had copy numbers greater than 5 to allow for proper expression of the transgene. Sc, ScaI; E, EcoRI; P, PvuII; B, BamHI; H, HindIII; X, XbaI; Xh, XhoI; St, StyI; S, SacI. B, 6.2SSβ transgenic embryo stained at 9.5 d.p.c. Expression is clearly localized to the somites. C, 6.2SSβ transgenic embryo stained at 11.5 d.p.c. Transgene expression is expanding into the developing myotome. D, 6.2SSβ transgenic embryo stained at 14 d.p.c. β-Gal staining is apparent in the skeletal muscles extending through the body, in the facial musculature, and in the tongue (black arrowhead). E, 1.9HSβ transgenic embryo stained at 11.5 d.p.c. Expression again is localized to the developing myotome and faint expression can be seen in the atrium of the heart (white arrow).
shown). The data above show that the 1.9-kb 5′ flank of the Adss1 gene contains regulatory sequences that are able to properly target skeletal muscle progenitors.

Surprisingly, β-gal activity was not detected in the heart of the 6.2SSβ transgenics at any time during development, but faint staining was noticed in cardiac tissue of 11.5 d.p.c. 1.9HSβ transgenic mice (Fig. 8E, white arrow). This difference is consistent with the ability of the 1.9HSCAT construct to drive higher expression than the 6.2SSCAT construct in adult heart. This faint cardiac expression could be attributed to a penetration problem of the X-gal.

**DISCUSSION**

Cloning and Structural Analysis of the Murine Adss1 Gene—Previous studies have established that the muscle isozyme of AdSS as well as its cognate transcript are predominantly expressed in striated muscle tissues (16, 13). We report here the molecular cloning and structural analysis of the murine Adss1 gene. We defined 13 exons with their respective intron/exon boundaries and mapped these exons along four lambda clones, which span about 45 kb. Sequence analysis of the proximal 5′ flanking sequence of Adss1 revealed an organization of cis-elements around the transcription start that is commonly observed for eukaryotic genes (32). In addition, we identified several putative binding sequences for known muscle-specific transcription factors. The messages expressed in both cardiac and skeletal muscle showed identical 5′ ends, suggesting that in the adult the same Adss1 transcripts are expressed in both types of striated muscle tissue. This is unlike the muscle structural proteins, which are commonly encoded by separate cardiac and skeletal muscle-specific genes (5, 33). Another scenario is seen with the aldolase A gene in which separate promoters, pM and pH, direct gene expression to skeletal muscle and cardiac tissues, respectively (34). However, the Adss1 gene appears to utilize separate cis-regulatory elements for tissue-specific expression of a single promoter in the heart and skeletal muscle (see below).

Endogenous Expression of Adss1 During Mouse Development—Adss1 gene expression is developmentally controlled, being activated prenatally around the time of establishment of the first myogenic and cardiac lineages and then significantly up-regulated perinatally, with a pronounced increase in gene expression during postnatal development. In situ analysis revealed the presence of Adss1 transcripts in 9.5-d.p.c. caudal somites as well as the atrium and ventricle walls of the beating linear cardiac tube. Expression was detected throughout embryonic development (10.5 and 13.5 d.p.c.) in the maturing muscles, heart, and tongue. Perinatally, an apparent phase of enhancement of Adss1 was identified, which correlates with increased skeletal muscle usage. Expression gradually increased until it reached adult levels around the time of weaning. Therefore, Adss1 is present in the earliest muscle precursors and is subject to developmental up-regulation in mature, functional muscle.

Other muscle genes encoding metabolic enzymes, such as β-enolase and creatine kinase, which are generally highly expressed in the adult, are active prenatally, at very low or basal levels (7, 8). Adss1 expression pattern resembles that of the β-enolase gene, as the muscle-specific gene is activated early in embryonic development and continues to be expressed in adult muscle tissues (8). This differs from another metabolic gene, creatine kinase, where the expression of the nonmuscle gene precedes that of the muscle-specific gene in prenatal development (7). Since the heart is beating around 9.0 d.p.c. and fetal movement can be detected, it is not unreasonable that the purine nucleotide cycle may be functioning in these tissues to aid in energy metabolism. Whether these muscle metabolic genes are functionally important during embryonic development or whether, at the time of commitment, all muscle genes are basally activated are issues that remain largely unsolved. In this regard, one purine nucleotide cycle enzyme, myoadenylate deaminase, is not critical for prenatal development since individuals lacking this enzyme are born with no apparent developmental abnormalities (14).

Regulation of Adss1 in Adult Cardiac Muscle—The results of our transgenic studies indicate that cardiac elements reside in the 1.9 kb of 5′ flanking DNA and are distinct from skeletal muscle regulatory elements. 1.2 kb of flank showed no tissue-specific enhancement. Therefore, the 700-bp HindIII-Xba1 fragment appears to contain regulatory sequences that are needed for strong enhancement in adult cardiac tissue. Sequence analysis of this 700-bp fragment revealed potential cardiac transcription factor binding sites. GATA-4 sites may function in series to regulate the expression of several cardiac-specific genes (35). The MEF2 factor also regulates several cardiac-specific genes through its MADS domain (36). The E boxes could possibly bind the newly discovered cardiac bHLH factors, e/dHAND (37). Finally, the loss of the homeobox-containing Tinman protein disrupts cardiac development, but the target genes controlled by this factor are still unknown (31, 38). Mutational analysis of this fragment is required to delineate the sites that control cardiac gene expression.

Transgenic Expression in Skeletal Muscle Progenitors—Further analysis of the 5′ flanking region using a lacZ reporter revealed the ability to direct expression to embryonic skeletal muscle progenitors, specifically. Constructs containing only 1.9 kb of 5′ flank produced whole mount embryo staining patterns that reproduced the endogenous prenatal Adss1 in situ pattern in somites from 9.5 d.p.c. and tongue from 13 d.p.c. 1.2 kb of flank did not express in transgenic embryos and neither did 4.0 kb of flank with an internal deletion. We conclude that we have initially identified a 700-bp fragment that contains regulatory elements necessary for gene activation in skeletal muscle progenitors.

This Adss1 fragment also contains several potential skeletal muscle regulatory elements, specifically a MEF2 site and a group of four E boxes (Fig. 4). The timing and pattern of transgene expression are almost identical to that observed for the myogenic transcription factor myogenin (39, 40). Transcripts for myogenin appear in the myotome at approximately 8.5 d.p.c. This is the first bHLH factor that is detected in skeletal muscle development in the mouse; therefore, it could play a role in activating Adss1 expression. Another potential regulatory element present in the 700-bp fragment is an MEF2 binding site. This factor can interact with the MyoD bHLH family to regulate the expression of various skeletal muscle genes (41). These genetic elements (E boxes and MEF2 site) could possibly play a role in controlling the expression of Adss1 in both cardiac and skeletal muscle tissue, either by binding different factors or by using different combinations of the sites. An example of such complex regulation occurs as the muscle creatine kinase gene is expressed in both striated muscle tissues and utilizes the same 290-bp enhancer. However, elements within this enhancer are differentially used in each tissue (33). Further analysis will decipher which sites play a role in skeletal muscle versus cardiac regulation of the murine Adss1 gene.

Little is known about the molecular mechanisms involved in regulating the perinatal enhancement of skeletal muscle metabolic genes. Our analysis of the 5′ flanking region of the Adss1 gene has identified a 700-bp fragment that contains elements required to activate reporter gene expression in embryonic skeletal muscle progenitors but unable to continue expression.
into adult muscle tissues. Information about elements that control the enhancement of muscle metabolic genes in transgenic animals is lacking. Studies in either skeletal muscle established lines or primary muscle cells have revealed limited information, but it is becoming more apparent that the elements necessary for gene activation are separate from the elements that control enhancement during development. One element in the desmin gene confers high levels of transgene expression in myoblasts, whereas a separate element controls expression in myotubes (42). An enhancer in the β-enolase gene allows high levels of expression in myoblasts, but its activity declines in myotubes (43). Our transgenic results suggest that the regulatory elements required for postnatal enhancement of the Adss1 gene in skeletal muscle lie outside of the immediate 1.9 kb of 5′-flank and are separate from the elements required for prenatal activation. Unraveling the mechanism(s) by which the early-activation regulatory elements function in the Adss1 gene could teach us more about processes that take place at the time that cells are committed to a myogenic lineage.

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