Amplification of an Adenylosuccinate Synthetase Gene in Alanosine-resistant Murine T-Lymphoma Cells

MOLECULAR CLONING OF A cDNA ENCODING THE "NON-MUSCLE" ISOZYME*

(Received for publication, July 29, 1993, and in revised form, October 12, 1993)

Ovin M. Guicherit, Bruce F. Cooper±, Frederick B. Rudolph†, and Rodney E. Kellemes§

From the Veraa and Marrs McLean Department of Biochemistry and The Institute for Molecular Genetics, Baylor College of Medicine, Houston, Texas 77030 and Department of Biochemistry and Cell Biology and The Institute of Biosciences and Bioengineering, Rice University, Houston, Texas 77005

Adenylosuccinate synthetase (EC 6.3.4.4) catalyzes the initial step in the conversion of IMP to AMP. Two isoforms of this enzyme have been observed in vertebrates. A muscle isozyme is highly abundant in cardiac and skeletal muscle tissue and is thought to play a role in muscle energy metabolism. The non-muscle isozyme, which is present at low levels in most tissues, likely functions in de novo AMP biosynthesis. The analysis of the non-muscle isozyme has been hampered by its low abundance and instability during purification. In this study a genetic selection scheme was used to generate a murine T-lymphoma cell line which was at least 100-fold enriched for the non-muscle isozyme, as a result of amplification of the non-muscle synthetase gene. This cell line made possible the purification of the non-muscle isozyme, and the subsequent isolation of isozyme-specific peptides. Based on peptide sequence information a degenerate oligonucleotide probe was designed and used to screen a mouse kidney cDNA library. A 1.6-kilobase cDNA encoding the non-muscle isozyme was cloned and found to contain an open reading frame of 1368 base pairs encoding 456 amino acids. Gene transfer experiments showed that the cDNA encoded a 50-kDa protein, the size expected for mammalian synthetases, that correlated with the presence of high levels of synthetase activity. The deduced amino acid sequence of the mouse non-muscle synthetase is ~75% identical to the previously reported mouse muscle synthetase. Southern blot analysis of mouse genomic DNA with the isozyme-specific cDNA probes revealed that the synthetase isoforms are encoded by separate genes. The non-muscle gene is expressed in most tissues but is virtually undetectable in striated muscle tissues. Three different transcripts (1.7, 2.8, and 3.4 kilobases) are detected for the non-muscle isozyme which show a similar tissue distribution. The availability of a cDNA for the non-muscle isozyme of adenylosuccinate synthetase will facilitate further comparative analyses with the previously cloned muscle isozyme.

Adenylosuccinate synthetase (AdSS) catalyzes the first of two steps leading to the synthesis of AMP from IMP. Two isoforms of AdSS have been observed in all mammalian species examined (Matsuda et al., 1977; Stayton et al., 1983). One isozyme (termed the non-muscle isozyme in this study) is widely distributed among mammalian tissues and functions at the branch point of purine nucleotide metabolism in de novo synthesis of AMP. The other isozyme (termed the muscle isozyme in this study) is highly abundant in striated muscle tissues and is part of the purine nucleotide cycle (Van Waarde, 1988; Lowenstein, 1990; Van den Berghe et al., 1992). This cycle, which involves AdSS as well as AMP deaminase and adenylosuccinate lyase, is active in cardiac and skeletal muscle where it is believed to play a role in muscle energy metabolism (Van Waarde, 1988; Lowenstein, 1990). Lower levels of the muscle isozyme of AdSS are found in kidney, brain, and testes, but the role of this isozyme in these tissues is unclear at this time.

Most previous biochemical studies (see Stayton et al. (1983) for review) have focused primarily on the muscle isozyme because its abundance in muscle allowed purification and characterization of this isozyme. To accurately identify muscle-specific features of this isozyme, however, it is necessary to have adequate quantities of the non-muscle isozyme for structural and functional comparison. Unfortunately, the analysis of the ubiquitous non-muscle isozyme has been hampered by low abundance and instability, making protein purification very difficult. The AdSS isoforms differ in their functional and physical properties (see Stayton et al. (1983); for review). In general, the muscle isozyme has a lower affinity for IMP and is less sensitive to AMP inhibition than its non-muscle counterpart. Also, there appears to be a difference in how divalent cations, especially Ca2+ and Mg2+, affect the isozymes (Cooper et al., 1982; Cooper, 1985). Furthermore, the muscle isozyme is functional as a dimer while the non-muscle enzyme appears to function as a monomer (Stayton et al., 1983). Additionally, the muscle enzyme has been shown to interact with purified myofibrils, a property which strengthens its proposed role in the muscle-specific purine nucleotide cycle (Manfredi et al., 1989). Further comparison of muscle and non-muscle isoforms of AdSS will be aided by a convenient source of the non-muscle isozyme.

To better understand the functional significance of the AdSS isoforms in mammalian tissues, it also will be useful to analyze

---

* The work was supported by National Institutes of Health Grant GM42436, National Cancer Institute Grant CA14030, Robert A. Welch Foundation grants C-1041 and Q-893, and a grant from the Muscular Dystrophy Association. Peptide and nucleic acid sequencing and synthesis, provided by core facilities at Baylor College of Medicine, were supported by Baylor Mental Retardation Research Center Grant 2 P30 HD24064. 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.

† To whom correspondence should be addressed. Fax: 713-796-9438; e-mail: rkellemes@bcm.tmc.edu.

‡ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L24554.

§ To whom correspondence should be addressed. Fax: 732-396-9438; e-mail: rkellemes@bcm.tmc.edu.

1 The abbreviations used are: AdSS, adenylosuccinate synthetase; Blotto, nonfat dried milk; AICR, 5-aminoimidazole-4-carboxylic acid ribonucleoside 5'-phosphate; SAICAR, 4-(N-succino)-5-aminoimidazole-4-carboxylic acid ribonucleoside 5'-phosphate; kb, kilobase.

2 The non-muscle isozyme was previously called the L or acidic isozyme. The muscle isozyme was previously called the M or basic isozyme.
Amplification and Cloning of Adenosuccinate Synthetase

their pattern of expression. For this purpose it will be necessary to have available cDNAs and/or antibodies specific for each isozyme. Isozyme-specific antibodies would also enable the cellular and subcellular localization of each isozyme to be determined. Furthermore, the availability of isozyme specific cDNAs would enable wild type or mutant forms of each isozyme to be produced in a prokaryotic or eukaryotic expression system for subsequent structural and functional analysis.

We have previously reported the cloning of a mouse cDNA encoding the muscle isozyme of AdSS (Guicherit et al., 1991). Here we present the isolation and initial analysis of a mouse cDNA encoding the non-muscle isozyme. Because of the low abundance of this isozyme in mammalian tissues and available cell lines we developed a genetic selection scheme to isolate a cell line that is enriched for the non-muscle synthetase. Enrichment appeared to result from gene amplification. The availability of this cell line facilitated the purification of the non-muscle enzyme and the subsequent isolation of isozyme-specific peptides. Degenerate oligonucleotide probes based on these peptide sequences were used to screen a mouse cDNA library. Data presented here show that the cloned cDNA encodes a protein with extensive sequence similarity to the previously reported muscle isozyme. The present study shows for the first time that the muscle and non-muscle isozymes of AdSS are encoded by different genes, which have contrasting patterns of expression. Northern analysis showed multiple transcripts for the non-muscle isozyme which appear to be ubiquitously expressed.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Selection Scheme—The YAC-1 cell line was obtained from the American Type Culture Collection (ATCC TIB 160). Cells were grown in RPMI 1640 media (Life Technologies, Inc.) supplemented with 10% iron-supplemented calf serum (Seru-Max 4, Sigma), 50 µg/ml gentamicin (Life Technologies Inc.), and 10 mM HEPES (Sigma). Cells were split into fresh media every 3-4 days. The synthetase-overproducing YAC-A16 was derived from YAC-1 by a stepwise selection in increasing concentrations of the drug alanosine (National Cancer Institute), beginning with 0.5 µM up to 16 µM. At each step the drug was increased by 2-fold, and selection was maintained for at least three culture splits. The alanosine-resistant YAC-A16 cells were maintained in the presence of 16 µM alanosine.

Purification of the Synthetase from YAC-A16 Cells and Isolation of Synthetase-specific Peptides—Approximately 3 liters of YAC-A16 cells grown in the presence of 16 µM alanosine were harvested by centrifugation. The cell pellet was resuspended in lysis buffer (10 ml) containing 20 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.2% detergent (Nonidet P-40, Sigma), and 1 mM phenylmethylsulfonyl fluoride (Sigma), and cells were lysed by sonication. The crude lysate was subjected to ultracentrifugation at 100,000 x g for 1 h. The synthetase activity was precipitated with (NH₄)₂SO₄ to 15% saturation from the clarified supernatant (S-100 fraction). The ammonium sulfate pellet obtained after centrifugation was dissolved in the lysis buffer (2.5 ml) and desalted over a PD-10 (Pharmacia LKB Biotechnology Inc.) column. Synthetase activity was applied to an ion-exchange (DEAE-Bio-Gel (Bio-Rad)) column (2.5 x 10 cm) and eluted with a salt gradient of 0-200 mM KC1 (120 ml). The synthetase-containing column fractions were identified by a activity assay (see below) and by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). The AdSS-containing fractions were combined and applied to a Procion Red column (Sepharose 4B (Sigma) derivatized with Procion Red H-3B (Sigma) according to protocol (Low and Pearson, 1984). The Procion Red column (1 x 7 cm) was eluted with a 0-3 mM KC1 gradient (40 ml) in lysis buffer, at 0.4 ml/min. The AdSS-containing fractions were again pooled and finally concentrated over a Centricon (30,000 molecular weight cutoff, Amicon) spin-column.

The Fractions Probe-Design™ Peptide Separation System was used to isolate cyanogen bromide-cleaved peptides from the purified synthetase, according to the recommended protocol. Two synthetase-specific peptides were isolated (see Results) and subjected to sequence analysis.

AdSS Activity Assay—The assay was similar to that described previously (Guicherit et al., 1991). The reaction mixture contained 20 µM HEPES, pH 7.0, 2.5 mM aspartate, 5.0 mM magnesium acetate, 0.2 mM IMP, and 0.1 mM GTP. The reaction was started by addition of IMP/GTP mixture and monitored at 30°C. The difference extinction coefficient was 11.7 x 10³ M⁻¹ cm⁻¹.

Isolation and Sequencing of cDNA Clones—A mouse kidney cDNA library was constructed in the vector pSPORT1 (Life Technologies, Inc.). Approximately 2,000,000 colonies were screened with a [γ-³₂P]ATP-end-labeled oligonucleotide probe (see Results). Peptides were isolated from one of the isolated synthetase-specific peptides. Peptides were hybridized in a mixture of 5 x SSC (0.15 M sodium chloride, 0.015 M sodium citrate), 20 mM NaH₂PO₄ (pH 7.5), 2% SDS, 5 x Denhardt's solution, 0.5% Biotin, and 100 µg/ml denatured salmon sperm DNA at 50°C. Filters were washed twice at 65°C for 30 min in 0.5 x SSC, 0.5 x Denhardt's solution, 2% SDS, 20 mM NaH₂PO₄ (pH 7.5) and once at 50°C for 30 min in 1.5 x SSC, 0.25 x Denhardt's solution, 1% SDS, 12.5 mM NaH₂PO₄ (pH 7.5). Washed filters were exposed to film for 48 h at -70°C with an intensifier screen. Positive colonies were further amplified and the cDNA clones were isolated and sequenced according to the dideoxy procedure (Sanger et al., 1977).

Expression of a cDNA Encoding the Non-muscle AdSS Isozyme—For expression of the AdSS cDNA from the kidney library the eukaryotic expression vector pBS (Stratagene) which contains a SV40 origin/promoter, a β-globin intron, and a SV40 poly(A) sequence was used. Standard cloning techniques were applied (Sambrook et al., 1989) to check for transfection efficiency. For expression of the cloned cDNA we transfected COS-7 cells (American Type Culture Collection), African green monkey kidney cells) with a calcium phosphate-based procedure (Chen and Okayama, 1987). Cells were harvested after 48 h, and cell lysates were analyzed for AdSS activity (see above) and by SDS-PAGE. Furthermore, cells were co-transfected with a β-galactosidase expression vector (pcMV-β) (MacGregor and Caskey, 1989) as a control for transfection efficiency.

Northern Analysis of Cell Lines and Tissues—Isolation, fractionation, and transfer of total cellular RNA from the cell lines and tissues was as described previously (Guicherit et al., 1991). Probes were randomly labeled with [α-³²P]CTP according to the supplier (Boehringer Mannheim). Hybridization was at 60°C in 4 x SSC, 0.5% SDS, 5 x Denhardt's solution, 0.5% Biotin, and 100 µg/ml denatured salmon sperm DNA, for 16-24 h. Subsequently, membranes were washed at 60°C, twice for 30 min in 2 x SSC, 0.1% SDS, once for 30 min in 0.5 x SSC, 0.1% SDS, and twice for 30 min in 0.1 x SSC, 0.1% SDS. After washing, the membranes were exposed to film for several days at -70°C with intensifier screens.

Southern Analysis of Genomic DNA—High molecular weight genomic DNA was isolated from the murine YAC-1 cell line. Cells were lysed in 20 mM EDTA, pH 8, 50 mM Tris/HCl, pH 8, 0.5% SDS, and 400 µg/ml Proteinase K (Boehringer Mannheim) at 55°C for at least 3 h (to overnight). Subsequently samples were extracted very mildly with phenol, phenol/chloroform, and finally chloroform. Genomic DNA was precipitated with isopropanol, washed with 70% ethanol, and resuspended in 10 mM Tris/HCl, 1 mM EDTA, pH 8. After digestion with several restriction enzymes, the DNA was fractionated on a 0.8% agarose (Life Technologies, Inc.) gel and transferred to nylon membranes (Zeta-probe; Bio-Rad) in the presence of 10 x SSC, for 2 h. Hybridizations, washes, and autoradiography were as described for the Northern analysis (see above).

RESULTS

Selective Overproduction of AdSS in Alanosine-resistant Murine T-Lymphoma Cells—The purification of the non-muscle isozyme of AdSS has been hampered by its low abundance in tissues and cell lines. To circumvent this problem and to facilitate the purification of the non-muscle isozyme we isolated a cultured cell line that produces large quantities of this enzyme. This was accomplished by selecting a murine T lymphoma cell line, YAC-1, for resistance to increasing concentrations of alanosine, an aspartic acid analog (Fig. 1). Alanosine is metabolized to alanosyl-AICAR by SICAR synthetase (Fig. 1), an enzyme of de novo purine nucleotide biosynthesis (Tyagi and Cooney, 1984). Alanosyl-AICAR functions as an IMP analog

* S. Chong and M. Hughes, manuscript in preparation.
Amplification and Cloning of Adenylosuccinate Synthetase

FIG. 1. Enzyme catalyzed reactions of purine nucleotide metabolism relevant to the action of alanosine. The SAICAR synthetase reaction is before the branch point (IMP) for both AMP and GMP biosynthesis. The AdSS reaction is the first of two steps involved in AMP biosynthesis. The aspartate-analog alanosine is a substrate for the SAICAR synthetase which converts it to alanosyl-AICAR, a compound that competes for IMP in the AdSS reaction.

and competitively inhibits AdSS (Tyagi and Cooney, 1984; Stayton et al., 1983). We used a stepwise selection procedure to obtain a population of cells, YAC-A16, that grew in 16 µM alanosine. As shown by the killing curves in Fig. 2 the drug-resistant cells were approximately 1,000-fold more resistant to alanosine than the parental cells. Furthermore, the YAC-A16 cells were characterized by the presence of a very high level of AdSS activity (~500 nmol/min/mg) as compared to the original YAC-1 population (not measurable, with lower limit of detection <5 nmol/min/mg). SDS-PAGE analysis of lysates from YAC-1 and YAC-A16 cells showed that the alanosine resistant cells were specifically enriched in a protein of approximately 50 kDa (Fig. 3A, arrow), the size expected for AdSS (Stayton et al., 1983). As shown in Fig. 3A the 50-kDa protein appears to be the only protein enriched in the alanosine resistant cell line (YAC-A16). The chromatographic profile (see below) and kinetic properties of the synthetase activity in the drug-resistant cells were characteristic of those expected for the non-muscle isozyme. Furthermore, the 50-kDa protein overproduced in YAC-A16 cells showed no significant cross reactivity with antibody to the muscle isozyme. Additionally, a cDNA probe of the mouse muscle isozyme did not detect elevated message levels in the AdSS overproducer. These results indicate that YAC-A16 cells are considerably enriched for the non-muscle isozyme of AdSS and that these cells could be a convenient source from which to purify this enzyme.

4 O. M. Guicherit, B. F. Cooper, F. B. Rudolph, and R. E. Kellems, unpublished observations.

![Diagram of enzyme catalyzed reactions of purine nucleotide metabolism relevant to the action of alanosine.]

Fig. 2. Survival curves for the parental cell line (YAC-1) and its selected derivative (YAC-A16) in increasing amounts of alanosine. The parental cell line (YAC-1) and its alanosine-selected derivative (YAC-A16) were seeded in increasing concentrations of alanosine at an initial density of approximately 2 x 10^6 cells/ml. Cell survival was calculated as the cell density present after 7 days in culture and plotted as a percentage of the initial cell density. The range of error at each alanosine concentration is indicated as vertical bars.

![Survival curves for the parental cell line (YAC-1) and its selected derivative (YAC-A16) in increasing amounts of alanosine.]

Purification and Partial Sequence Analysis of AdSS from YAC-A16 Cells—The synthetase was purified from the alanosine resistant cells by a combination of high speed centrifugation, ammonium sulfate precipitation, and column chromatography as described under "Experimental Procedures." The resulting product, as shown in Fig. 3B (arrow), was >95% pure. The protein was further purified by gel electrophoresis and subjected to chemical cleavage with cyanogen bromide to gen-
erate peptide fragments. Fragments were isolated by gel electrophoresis and two such fragments of approximately 9–10 kDa were partially sequenced. The sequence of the first 30 amino acids of each peptide was determined (Fig. 4, underlined sequences) and compared with the previously published sequence of the mouse muscle synthetase (Guicherit et al., 1991). The deduced sequence of each peptide showed extensive identity with the previously published sequence. Differences between the amino acid sequences presumably reflect isozyme-specific differences between the muscle and non-muscle isozymes.

**Molecular Cloning and Expression of a cDNA Encoding the Non-muscle Isozyme of AdSS—**A degenerate oligonucleotide probe was designed based on amino acid sequence from one of the peptide fragments of the synthetase: 5'-'TTG/A/TCC/T/GCC/TG/T/GA/GAA/GG/A/GGC/G/C/GCC/AAT/GA/CC/C/AAC-3'. The oligonucleotide mixture was end-labeled and used as a hybridization probe to screen a mouse kidney cDNA library. The oligonucleotide probe hybridized to 10 colonies from a total of 2,000,000 colonies screened. One of these positive cDNA clones was further amplified and completely sequenced in both directions. Sequence analysis revealed an open reading frame of 1368 base pairs (Fig. 4) capable of encoding a poly-
FIG. 5. Comparison of amino acid sequences of the muscle and non-muscle isozymes of AdSS from mouse. The sequences of the mouse muscle (mAdSS; 457 residues) and non-muscle (nmAdSS; 456 residues) isozymes of AdSS are aligned according to the algorithm of Smith and Smith (1990). Identical amino acids are indicated by vertical dashes. Regions (G-1, G-2, G-3, and G-4) which show homology to known GTP-binding motifs are in boldface type and underlined (see "Discussion" for explanation). The numbering on the right is adjusted for each sequence.

Muscle and non-muscle Isozymes of AdSS Are Encoded by Separate Genes That Are Differentially Regulated in Mouse Tissues—Nucleotide sequence comparison of cDNAs encoding the mouse muscle (Guicherit et al., 1991) and non-muscle (Fig. 4) isozymes of AdSS revealed a significant number of nucleotide mismatches throughout the protein coding region and no significant sequence similarity in the 5'- and 3'-non-coding regions. These findings suggest that although the two isozymes are clearly related by sequence similarity, they are not likely to be the products of the same gene. To test directly the two gene hypothesis, we probed duplicate genomic Southern blots with either the muscle or non-muscle cDNA probes. As shown in Fig. 7, cDNA probes specific for each isozyme hybridized to very distinct patterns of restriction fragments which clearly differed for each isozyme, providing strong evidence that the murine isozymes of AdSS are encoded by separate genes.

We have previously shown that the mouse muscle isozyme of AdSS is highly abundant in skeletal and cardiac muscle (Guicherit et al., 1991). Northern analysis was used to assess the pattern and level of expression of the non-muscle isozyme in a variety of murine tissues. The results in Fig. 8 indicate that three transcripts (1.7, 2.8, and 3.4 kb) were detected with the non-muscle cDNA. These transcripts were present in all tissues tested, with relatively higher levels in the brain, thymus, kidney and uterus, and lower levels in the liver. The non-muscle synthetase mRNAs were barely detectable in cardiac and skeletal muscle. Thus, the non-muscle synthetase gene shows a distinctly different pattern of expression than that of the muscle-specific AdSS gene (Guicherit et al., 1991).

Amplification of an AdSS Gene in the Alanosine-resistant Cells—To determine the genetic basis for overproduction of AdSS in alanosine-resistant cells, total RNA and DNA was isolated from YAC-1 and YAC-A16 cells and analyzed by blot transfer hybridization using the full-length non-muscle cDNA as a probe. The results of Northern analysis (Fig. 9) indicate that the cDNA probe hybridized to three transcripts of 1.7, 2.8, and 3.4 kb that are very abundant in the YAC-A16 cells. The same three transcripts were present in the parental YAC-1 cells (and in the mouse tissues, Fig. 8), although at much lower levels. A dilution series of YAC-A16 RNA suggests that all three transcripts are overproduced by approximately 100-fold in the

presented in Figs. 4–6 we conclude that the kidney cDNA (as in pSGnmAdSS, Fig. 6) contains the protein coding region for a non-muscle isozyme of murine AdSS.
Amplification and Cloning of Adenylosuccinate Synthetase

**Fig. 7. Identification of sequences encoding the muscle and non-muscle isozymes of AdSS.** Genomic DNA from the cell line YAC-1 was digested with three different restriction enzymes followed by separation on a non-denaturing agarose (0.8%) gel. Duplicate digests were transferred to a nylon membrane and independently probed with either the cDNA encoding the muscle isozyme (A, AdSS1) or the kidney cDNA encoding the non-muscle isozyme (B, AdSS2). The relevant restriction enzyme is shown above each lane. Each digest contained 30 µg of genomic DNA. M, DNA size-markers (kb).

drug-resistant cells. The relative abundance of the three transcripts appears to be the same in the parental and drug-resistant cells. The smallest transcript appears to correspond in size to the isolated cDNA clone in pSGnmAdSS (Fig. 6). The relationship between the multiple transcripts is currently under investigation.

Southern blotting analysis of genomic DNA, using the non-muscle cDNA as a probe, was performed to assess the role of gene amplification in the increased production of AdSS in alanosine-resistant cells. The results of a dilution series comparing YAC-A16 DNA with DNA from YAC-1 cells (Fig. 10) indicate an approximately 100-fold amplification of an AdSS gene in the alanosine-resistant cells. From these results we conclude that the overproduction of AdSS in alanosine-resistant cells is the result of a corresponding amplification of the non-muscle AdSS gene.

**DISCUSSION**

Gene amplification is one of the most successful strategies for achieving high levels of protein production in mammalian cells (Kelles, 1993). In general this is accomplished by selecting cells for the ability to grow in increasing concentrations of a cytotoxic compound, usually an inhibitor or antimetabolite, which is specific for the enzyme of interest. Our initial attempts to select cells with amplified copies of AdSS genes involved the use of hadacidin, an aspartic acid analog inhibitor of the enzyme (Stayton et al., 1983). Stepwise selection for resistance to
concentrations as high as 10 mM hadacadin resulted in the isolation of cells in which synthetase levels were increased approximately 30-fold (specific activity in S-100 cell lysate: $\sim 175$ nmol/min/mg). A more effective selection scheme involved the use of alanosine, also an aspartic acid analog, but not itself an inhibitor of AdSS. Alanosine is converted by an enzyme of de novo purine nucleotide biosynthesis to alanoyl-AICAR (Fig. 1), a cytotoxic metabolite that binds AdSS with high affinity (Tyagi and Cooney, 1984; Stayton et al., 1983). Most cells are killed by as little as 0.1 $\mu$M alanosine, making this compound a highly selective agent. A stepwise selection for resistance to increasing concentrations of drug up to only 16 $\mu$M alanosine resulted in the isolation of cells which produced AdSS at least 100-fold (specific activity in S-100 cell lysate: $\sim 500$ nmol/min/mg). The increased amounts of synthetase produced by alanosine-resistant cells is fully accounted for by a corresponding amplification of the synthetase gene. The alanosine-resistant mouse lymphoma cells we isolated made possible the purification and amino acid sequence analysis of the non-muscle isozyme of murine AdSS.

The existence of multiple AdSS isoforms has been known for many years (Stayton et al., 1983). However, the genetic basis for the multiple isoforms has not been determined previously. We addressed this issue by isolating cDNAs encoding muscle AdSS (Guicherit et al., 1991) and non-muscle (this work) isoforms of the murine enzyme and directly comparing the nucleotide sequence of the two cDNAs. The results revealed nucleotide mismatches throughout the open reading frame, with an overall identity of $\sim 65\%$. No significant sequence identity was observed in the 5'- and 3'-untranslated regions. These findings indicate that the mouse AdSS isoforms are not likely to be the products of the same gene, a conclusion that is also supported by Southern blot analysis. In the latter case, duplicate Southern blots of mouse genomic DNA were probed with the full-length isozyme-specific cDNAs and clearly showed different hybridization patterns for each cDNA, indicating that the two mouse proteins are encoded by separate genes. We propose to designate the gene encoding the muscle enzyme, AdSS1, and the gene encoding the non-muscle enzyme, AdSS2.

Northern analysis revealed that the murine AdSS genes have a very distinct and almost mutually exclusive pattern of expression. The muscle-specific AdSS1 gene encodes a 1.8-kb transcript found predominantly in skeletal and cardiac muscle (Guicherit et al., 1991). The AdSS2 gene, encoding the non-muscle isozyme, is active at lower levels in a wide range of tissues. There appear to be three different transcripts (1.7, 2.8, and 3.4 kb) specific for the non-muscle synthetase (AdSS2) gene which show the same relative abundance in all tissues and cell lines tested, with the largest of the transcripts being the most abundant. The structural relationship among the three AdSS2 transcripts is unknown at this time. AdSS2 gene expression is virtually undetectable in striated muscle. This may indicate the importance of keeping the two isozymes separated because they could potentially interfere with each other's role in metabolism, as a consequence of their regulatory differences. On the other hand it has been reported that muscle has a significant capacity for de novo AMP biosynthesis (Sheehan et al., 1977), a function normally assigned to the non-muscle isozyme. Additionally, the purine nucleotide cycle which apparently is important for proper muscle physiology has also been shown to operate in non-muscle tissues such as brain (Bogusky et al., 1976; Moss and McGivan 1975; Schultz and Lowenstein 1976). In order to better understand the tissue specific role of each isozyme it will be important to investigate the cellular and subcellular localizations of the isozymes in the relevant tissues. With isozyme-specific cDNAs and antibodies it will be possible to more precisely determine the tissue-specific expression and cellular localization of each isozyme during development and in the adult mouse. This will hopefully lead to a better understanding of the relevance of each isozyme of AdSS in the physiology of the different mammalian tissues.

Powell and co-workers (1992) recently identified a human liver AdSS cDNA clone which, like the mouse non-muscle AdSS cDNA, detected multiple transcripts. Even though the human liver cDNA clone was isolated based on cross-hybridization with sequences encoding the murine muscle AdSS, they were unable to assign the human liver synthetase as either a muscle or non-muscle isozyme, because liver is believed to contain low levels of each. When we compared the amino acid sequences of the two murine cDNAs with that encoded by the human liver cDNA we found that the deduced amino acid sequence of the human synthetase shares over 90% identity with the murine non-muscle synthetase and less than 75% identity with the murine muscle synthetase. Based on these findings it is likely that the human liver cDNA clone described by Powell et al. (1992) encodes the non-muscle isozyme of human AdSS. Lai et al. (1991) have made use of somatic cell hybrids between human lymphocytes and AdSS-deficient hamster cells to map a human AdSS gene. They show that a region on human chromosome 1 (1cen-1q12) cosegregates with synthetase activity, suggesting that this region contains an AdSS gene (Lai et al., 1991). Based on our findings and the assumption that the muscle isozyme is not expressed in human lymphocytes it is most likely that the human non-muscle AdSS gene maps to the region of chromosome 1 identified by the studies of Lai et al. (1991).

Multiple genes have also been identified for AMP deaminase, which encode muscle and non-muscle isoforms (Mahnke-Zisel-mann and Sabina, 1992; Morisaki et al., 1990; Sabina et al., 1990). The muscle isoform of AMP deaminase, like AdSS, is also a component of the purine nucleotide cycle (Sabina et al., 1989). Several organ-specific and stage-specific transcripts have been identified for AMP deaminase. In adult rat tissue a non-muscle
transcript (3.4 kb) appears to be widely expressed but not in muscle tissue, while two alternatively spliced muscle transcripts (each 2.5 kb) are present at relatively high levels in muscle tissue (cardiac and skeletal muscle) and at much lower levels in non-muscle tissues. This is a pattern of expression which is very similar to that of the AdSS transcripts, suggesting that the expression of both enzymes may be regulated by common tissue-specific and possibly developmentally-regulated signals. Additionally, it has been shown that both AdSS and the deaminase can interact with myofibrillar (Sabina et al., 1989; Manfredi et al., 1989) which suggests that the enzymes of the purine nucleotide cycle might be co-localized along muscle fibers. Thus, to maintain a precise stoichiometry of the muscle and non-muscle AMP deaminase genes, AMPD1 and AMPD2, have been shown to be located on the same chromosome in rat, mouse, and man (Sabina et al., 1990). We have no evidence for linkage of the AdSS1 and AdSS2 genes at this time.

The mouse non-muscle isozyme of AdSS, like all previously identified synthetases, contains typical guanine nucleotide-binding motifs (G-regions; 30GDEGKK, 260DFGYTPFVT, 292EVYG, 361TKLD), common to all GTP-binding proteins (Bourne and Sanders, 1991; Dever et al., 1987): the N-terminal phosphate-binding loop GXXXXK (G-1 region), the Mg2+-binding sequence D-(X)-T (G-2 region), the central phosphate-binding sequence (D/E)XXX(A/G) (G-3 region), and the C-terminal guanine-recognition sequence (N/T/Q)KXD (G-4 region). The G-1 and G-4 regions are completely conserved in the mouse non-muscle synthetase (Fig. 5, Table I), as in all the other published synthetases, consistent with their proposed functional roles. Furthermore, site-directed mutagenesis studies on other genes of interest and in this way obtain mammalian cell lines producing large quantities of desired proteins. The alkaline selection scheme described here represents a significant addition to the list of amplifiable systems (Kellem, 1993) available to the mammalian genetic engineer and will be useful alone or in combination with other amplifiable systems to genetically engineer mammalian cells to produce high levels of proteins of academic interest and/or commercial value.

Acknowledgments—We are grateful to Bill Fanslow who initiated the selection of the cells. We thank Dr. Surjit Datta for his contribution to Fig. 2. We also thank Dr. Richard Cook and his core facility for peptide sequence analysis. Alanosine was kindly supplied to us by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. The kidney cDNA library was a generous gift from Sam Chong in the laboratory of Dr. Mark Hughes. We are also thankful for the technical support of Donna Muzny and Dr. Richard Gibbs at the sequence core facility at Baylor College of Medicine for assisting us in generating the cDNA sequence. We thank Dr. John Winston and Shera Kas for their critical reading of the manuscript and their helpful suggestions.

REFERENCES

Bogusky, R. T., Lowenstein, L. M., and Lowenstein, J. M. (1976) J. Clin. Invest. 58, 326-335

S. Datta, O. M. Guicherit, and R. E. Kellemes, manuscript in preparation.
Amplification and Cloning of Adenylosuccinate Synthetase

Bourne, H. R., and Sanders, D. A. (1991) Nature 349, 117-127

Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745-2752

Cooper, B. F. (1985) Metal Studies of Adenylosuccinate Synthetase: Metal Activation, Isotope Exchange and Kinetic Characterization, Ph.D. thesis, Rice University.

Cooper, B. F., Clark, S. W., and Rudolph, F. B. (1982) Fed. Proc. 41, 2756

Dever, T. E., Glynias, M. J., and Memck, W. C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1814-1818

Guicherit, O. M., Rudolph, F. B., Kellems, R. E., and Cooper, B. F. (1991) J. Biol. Chem. 266, 22582-22587

Kellems, R. E. (1993) Gene Amplification in Mammalian Cells: A Comprehensive Guide, Marcel Dekker, Inc., New York

Laemmli, U. K. (1970) Nature 227, 680-65

Lai, L.-W., Hart, I. M., and Patterson, D. (1991) Genomics 9, 322-328

Liu, F., Dong, Q., and Fromm, H. J. (1992) J. Biol. Chem. 267, 2388-2392

Lowenstein, J. M. (1990) Int. J. Sports Med. 11, S36-S46

Mahnke-Zizelman, D. K., and Sabina, R. L. (1992) J. Biol. Chem. 267, 20866-20877

Manfredi, J. P., Maguetant, R., Magid, A. D., and Holmes, E. W. (1989) Am J. Physiol. 257, C29-C35

Matsuda, Y., Ogawa, H., Fukutome, S., Shiraki, H., and Nakagawa, H. (1977) Biochem. Biophys. Res. Commun. 78, 766-771

Nonnemek, T., Sabina, R. L., and Holmes, E. W. (1990) J. Biol. Chem. 265, 11482-11486

Moss, K. M., and McGivan, J. D. (1975) Biochem. J. 150, 275-283

Powell, S. M., Zalkin, H., and Dixon, J. F. (1995) FEBS Lett. 353, 4-10

Sabina, R. L., Swain, J. L., and Holmes, E. W. (1980) in Metabolic Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Vallee, D. eds) Vol. I, pp. 1077-1084, McGraw-Hill, New York

Sabina, R. L., Morisaki, T., Clarke, F., Eddy, R., Shows, T. B., Morton, C. C., and Holmes, E. W. (1990) J. Biol. Chem. 265, 9423-9433

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467

Schultz, V., and Lowenstein, J. M. (1976) J. Biol. Chem. 251, 485-492

Schulman, T. G., Buckley, B. M., and Tully, E. R. (1977) Biochem. Soc. Trans. 5, 1753-1755

Smith, R. F., and Smith, T. F. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 118-122

Stouten, M. M., Rudolph, F. B., and Fromm, H. J. (1983) Curr. Top. Cell. Regul. 22, 103-141

Tzag, A. K., and Cooney, A. (1984) Adv. Pharmacol. Chemother. 20, 69

Van den Berghe, G., Bontemps, F., Vincent, M. F., and Van den Bergh, P. (1992) Prog. Neurobiol. 35, 547-561

Van Waarde, A. (1988) Biol. Rev. 63, 259-266

Wiedemuller, L., Witzbrodt, J., Noegel, A. A., and Schleicher, M. (1991) J. Biol. Chem. 266, 2480-2485

Wolfe, S. A. and Smith, J. M. (1988) J. Biol. Chem. 263, 19147-19153