Endothelial Argininosuccinate Synthase mRNA 5’-Untranslated Region Diversity

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INFRASTRUCTURE FOR TISSUE-SPECIFIC EXPRESSION*

Based on the integral role that argininosuccinate synthase (AS) plays in the production of nitric oxide in vascular endothelial cells and urea in liver, an analysis was carried out to determine whether signals reside in the AS mRNA to account for tissue differences in AS function and location. Reverse transcriptase-PCR and sequence analysis showed that the AS mRNA coding region was the same for both endothelial cells and liver; however, 5’-RACE analysis (rapid amplification of cDNA ends) identified AS mRNA species in endothelial cells in addition to a major 43-nucleotide (nt) 5’-untranslated region (UTR) AS mRNA with overlapping extended 5’-UTRs of 66 and 92 nt. Comparison to the genomic sequence immediately upstream of the reported transcription start site for the human and mouse AS gene suggested that expression of all three species of bovine endothelial AS mRNA are driven by a common promoter and that 5’-UTR diversity in endothelial cells results from three transcriptional initiation sites within exon 1. RNase protection analysis and real-time reverse transcriptase-PCR verified and quantitated the differential expression of the extended 5’-UTR species relative to the major 43-nucleotide (nt) 5’-untranslated region (UTR) AS mRNA. In vitro translation studies showed a less-pronounced but similar discordant expression. Sequential deletions starting from the 5’ terminus of the 92-nt 5’-UTR construct resulted in a corresponding increase in translational efficiency, but the most pronounced effect resulted from mutation of an upstream open reading frame, which restored translational efficiency of the 92-nt 5’-UTR AS mRNA. When the different AS mRNA 5’-UTRs, cloned in front of a luciferase reporter gene, were transfected into endothelial cells, the pattern of luciferase expression was nearly identical to that observed for the different 5’-UTR AS mRNAs in endothelial cells. Given the different roles ascribed for argininosuccinate synthase, urea versus NO production, these results suggest that sequence in the AS gene represented by position −92 to −43 nt from the translation start site in the extended AS mRNA 5’-UTRs plays an important role in differential and tissue-specific expression.

Argininosuccinate synthase (AS)1 catalyzes the reversible ATP-dependent ligation of citrulline and aspartate to produce argininosuccinate, AMP, and inorganic pyrophosphate. The primary role of AS is the detoxification of ammonia via the urea cycle in the liver (1). Although this essential physiological function occurs in the liver and to a lesser extent in the small intestine, virtually all other mammalian tissues possess detectable levels of AS and a second urea cycle enzyme argininosuccinate lyase (AL). Together these two enzymes have the net effect of generating arginine from citrulline and aspartate. In the kidney, AS and AL are responsible for the de novo synthesis of arginine to be released into the bloodstream (2). In other tissues, however, the function of this metabolic pathway remained obscure until the discovery of arginine-derived nitric oxide (NO) (3).

In endothelial cells, AS catalyzes the rate-limiting step (4) in the synthesis of l-arginine from 1-citrulline. Endothelial nitric-oxide synthase then utilizes the arginine, converting it back to citrulline to produce NO. Thus, available arginine is a prerequisite for NO production. Interestingly, intracellular levels of arginine in endothelial cells have been estimated to range from 0.1 to 0.8 mM (3–9), well above the reported $K_m$ of 5 μM for endothelial NO synthase (5). Yet an increase in extracellular l-arginine levels and/or an increase in the synthesis of arginine from extracellular citrulline will increase NO production from stimulated endothelial cells (3, 10–15). Moreover, during shear stress-induced NO synthesis (16) AS levels are up-regulated along with several other genes suggested to play a role in the regulation of NO production (17). These results have been taken to suggest that a separate pool of arginine is maintained for NO production in endothelial cells by either transport and/or the regeneration of arginine from citrulline (3, 10–15). The physiological importance of the arginine regeneration system also was suggested by a case report of two infants with a deficiency in argininosuccinate lyase (also essential for arginine regeneration from citrulline) who were shown to be hypertensive. Infusion of arginine resulted in the lowering of blood pressure in these infants, suggesting a critical role for arginine regeneration in the regulation of systemic blood pressure (18).

More recently we have shown that AS and AL co-fractionate (along with endothelial NO synthase) with the endothelial caveolar fraction (10); therefore, regeneration of arginine from

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1 The abbreviations used are: AS, argininosuccinate synthase; UTR, untranslated region; nt, nucleotide; ORF, open reading frame; uORF, upstream ORF; AL, argininosuccinate lyase; BARC, bovine aortic endothelial cells; RACE, rapid amplification of cDNA ends; RPA, ribonuclease protection assay; RT, reverse transcriptase; TBST, Tris-buffered saline Tween 20; NO, nitric oxide.
citrulline is not only integral in endothelial NO production but also may involve different regulation and cellular localization relative to the hepatic AS and urea production. For this reason we have proposed that an endothelial isoform expressed from the same gene as the liver form (19) manifests some variations allowing AS to function in a different cellular location (10) and to be coordinately regulated with NO production (17). In support of this hypothesis, variations in the physical properties of the liver and endothelial cell AS protein were detected using different physical separation techniques and visualization by immunoblotting. 2

Based on these findings, we examined AS cDNA from liver and vascular endothelial tissue to determine whether differences in expression and in functional and physical properties could be accounted for at the level of the messenger RNA. In this report we show that the translatable sequence of AS mRNA is identical for both tissues but that the sequence represented by the diversity of endothelial AS mRNA 5'-UTRs may account in part for the specialized regulation of expression in nitric oxide production.

EXPERIMENTAL PROCEDURES

Cell Culture and RNA Isolation—Bovine aortic endothelial cells (BAEC) were cultured in Dulbecco's modified Eagle's medium (Mediatech) supplemented with 10% fetal bovine serum (HyClone Laboratories), penicillin, streptomycin, and amphotericin B (Mediatech). Total RNA was isolated from BAEC by the method of Chomczynski and Sacchi (20) using Tri Reagent (Molecular Research Center) according to the manufacturer's protocol. Total RNA from bovine liver was purified using the RiboPure kit ( Qiagen). A portion of the AS 5'-coding region was amplified by RT-PCR using primers ASL10 and ASR1338 to yield fragments of 1328, 763, and 338 bp, respectively. ASL572 was combined with ASR1209 to yield a 456-bp fragment. Primers were named based on the designation of position 1 as the first base of the AUG start codon.

All PCR reactions contained 1× assay Buffer B (10 mM Tris-HCl, pH 8.3, and 50 mM KCl), 200 μM each dNTP, 1.5 mM MgCl₂, 10 μl of cDNA, 5% Me₂SO, 50 pmol of each primer, and 2.5 units of Taq polymerase (Fisher Scientific) and consisted of 35 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min. Reactions were started by a 5-min denaturation step and ended with a 10-min extension step. PCR fragments were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

Rapid Amplification of cDNA Ends (RACE) RT-PCR—5'- and 3'-RACE analysis was carried out using the SMART RACE cDNA amplification kit (CLONTECH Laboratories). This technique involved the incorporation of a "Smart Oligo" onto the 5'-end of the reverse-transcribed cDNA for the 5'-RACE analysis. For the 3'-RACE analysis, the Smart Oligo was attached to an oligo (dT) primer to yield cDNA that had a complete 3'-UTR, a poly-A tail, and a Smart Oligo sequence hybridized onto the 5'-end of the reverse-tran-cribed cDNA for the 5'-RACE analysis. For the 3'-RACE analysis, the Smart Oligo was attached to an oligo (dT) primer to yield cDNA that had a complete 3'-UTR, a poly-A tail, and a Smart Oligo sequence extension. The 5'- and 3'-ends of AS were amplified using a standard PCR protocol. Primers were designed so that a short section of the coding region was amplified along with the 5'- or 3'-UTR. ASR348 was combined with the Smart Oligo primer to amplify the 5'-end, and ASL1188 was combined with the Smart Oligo primer to amplify the 3'-end. Primer sequences are listed in Table I. Amplified 5'- and 3'-RACE fragments were cloned and sequenced. To increase the efficiency of recovery of the specific RACE clones colony lifts were performed, and positive clones were selected by hybridization to the 1328-bp AS cDNA fragment generated by RT-PCR.

Ribonuclease Protection Assays (RPAs)—RPAs were performed using the RiboQuant multiprobe RNase protection assay system (BD Pharmingen). A portion of the AS 5'-UTR and 5'-coding region was amplified by RT-PCR using primers ASL82 and ASR94 (listed in Table I) and used as a template to produce the RPA probe. The 176-bp fragment was cloned using the TOPO TA cloning dual promoter kit (Invitrogen) and sequenced. To generate the antisense RNA probe, the plasmid construct
containing the 176-bp fragment was linearized with Xho I, purified by phenol/chloroform extraction and ethanol precipitation, and quantitated. The 32P-labeled RNA probe was synthesized using T7 RNA polymerase and hybridized to 100 μg of BAEC total RNA or 15 μg of liver total RNA following the manufacturer’s protocol. RNase digests were performed as per the standard protocol except that the RNase A was optimized to a final concentration of 38 ng/μl. Protected fragments were analyzed on a 6% acrylamide, 7 M urea sequencing gel. The gel was dried and exposed to film. Band densities were quantitated using ImageQuant software (Molecular Dynamics).

Real-time RT-PCR—Total RNA from bovine liver and BAEC was DNase-treated using DNA-free DNase treatment and removal reagent (Ambion) following the manufacturer’s protocol. 1 μg of RNA was reverse-transcribed with the Superscript first-strand synthesis system for RT-PCR (Invitrogen) following the protocol for high G+C content mRNAs. An AS-specific primer, ASR348, was used to reverse transcribe, and the resulting cDNA was amplified using the SYBR Green PCR master mix (Applied Biosystems). PCR conditions were 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The PCR products were detected in real time using the iCycler iQ detection system (Bio-Rad). Primer sets were designed to amplify regions of AS mRNA common to all species, ASL228 and ASR278, as well as to detect an AS-specific primer, ASR348, was used to reverse transcribed with the Superscript first-strand synthesis system for RT-PCR (Invitrogen) following the protocol for high G+C content mRNAs. An AS-specific primer, ASR348, was used to reverse transcribe, and the resulting cDNA was amplified using the SYBR Green PCR master mix (Applied Biosystems). PCR conditions were 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The PCR products were detected in real time using the iCycler iQ detection system (Bio-Rad). Primer sets were designed to amplify regions of AS mRNA common to all species, ASL228 and ASR278, as well as to detect an AS-specific primer, ASR348, was used to reverse transcribed with the Superscript first-strand synthesis system for RT-PCR (Invitrogen) following the protocol for high G+C content mRNAs. An AS-specific primer, ASR348, was used to reverse transcribe, and the resulting cDNA was amplified using the SYBR Green PCR master mix (Applied Biosystems). PCR conditions were 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The PCR products were detected in real time using the iCycler iQ detection system (Bio-Rad).

Western Blotting Analysis—Translated proteins were separated by SDS-PAGE on 12% Tris-HCl Ready Gels (Bio-Rad). Following electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore) using a wet transfer method (Bio-Rad). Following a blocking step of 1 h in TBST (20 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Tween 20), the membranes were incubated for 1 h at room temperature with Streptavidin-horseradish peroxidase antibody (Promega) or Jackson Immuno-Research Laboratories) at a final concentration of 0.025 μg/ml. The membranes were then washed three times in TBST and three times in nanopure water for 5 min per wash. The in vitro-translated proteins were visualized using an enhanced chemiluminescent reagent according to the manufacturer’s protocol (Amersham Biosciences) or Transcend reagent (Promega). Band densities were quantitated using ImageQuant software (Molecular Dynamics). Membranes were then placed in blocking buffer composed of 5% nonfat dry milk in TBST for 1 h at room temperature. Membranes were incubated with the primary antibody, anti-β-actin, mouse monoclonal, clone AC-15 (Sigma) in blocking buffer for 1 h at room temperature. Membranes were washed and incubated with the secondary antibody, peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) in blocking buffer for 1 h at room temperature. After washing with TBST, membranes were visualized using chemiluminescent reagent and exposed to film. Band densities were quantitated as before.

Mutational Analysis—Deletion mutants were constructed to include 88, 81, 76, and 71 nt of the AS 5′-UTR along with the T7 promoter sequence by amplification and subcloning as described previously. Primer sequences are listed in Table II. Mutations of the uORF AUG were carried out using a PCR strategy. A sense primer, ASL-70 Mut1, was designed to generate a single base mutation, converting the uAUG to AAC. A second primer, ASL-73 Mut2, was designed to alter the context of the uAUG by changing the surrounding nucleotides. The sequence GCC AUG GCG was mutated to AGG AUG GGC, changing the critical nucleotides at position −3 and +4 relative to the AUG to improve the function of the uAUG (21). These primers were combined with ASR429 to amplify fragments of 499 and 502 bp, following the protocol listed in the PCR section. PfuTurbo DNA polymerase (Stratagene) was used to reduce the frequency of error in the amplification. The amplified fragments were gel-purified, sequenced, and used as first primers in a second amplification with ASR-8777. Fragments were digested with BamHI and NarI and subcloned into the existing 92-nt 5′-UTR full-length AS cDNA construct. The deletion constructs and mutant AUG constructs were transfected, translated, and analyzed by Western blotting.

Transfection Analysis—Luciferase reporter constructs were designed to include each of the AS 5′-UTRs cloned directly after the simian virus

| Primer Name | Bam HI site | T7 Promoter Sequence | Spacer Region | AS specific sequence |
|-------------|-------------|----------------------|---------------|---------------------|
| ASL-9277    | GGA TCC     | TAA TAG GAC TCA CTA TAG GGG | A ACA G       | CC CTG CCC CCC GGC CCC GAG |
| ASL-6677    | GGA TCC     | TAA TAG GAC TCA CTA TAG GGG | A ACA G       | AC CGC GGA TGC GCG CCA AAA |
| ASL-4377    | GGA TCC     | TAA TAG GAC TCA CTA TAG GGG | A ACA G       | GC CCT GCT CCG CCG ACT GCT |
| ASL+177 Control | GGA TCC     | TAA TAG GAC TCA CTA TAG GGG | A GCC ACC     | ATG TCC GGC AAA GGC TCC GTG GT |
| *ASL-8677   | GGA TCC     | TAA TAG GAC TCA CTA TAG GGG | A ACA G       | CC CCC GGC CCC GAG CTT ATA |
| *ASL-8177   | GGA TCC     | TAA TAG GAC TCA CTA TAG GGG | A ACA G       | GG CCC CGA GCT TAT AAC CCG |
| *ASL-7877   | GGA TCC     | TAA TAG GAC TCA CTA TAG GGG | A ACA G       | CG AGC TTA TAA CCC GGG ATG |
| *ASL-7177   | GGA TCC     | TAA TAG GAC TCA CTA TAG GGG | A ACA G       | TT ATA ACC CGG GAT GGC GGC |

Sequence written in 5′→3′ notation. Primers with *−* denote sequence in the 5′-UTR and increasing numbers indicate sequence upstream from the start AUG. The Kozak consensus sequence (23) for the control primer is shown in bold type. *Indicates primers designed for deletion mutants.

5′-UTR Diversity of Endothelial Argininosuccinate Synthase mRNA

TABLE II

Primer Design for In Vitro Transcription and Translation

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Fig. 1. Amplification of reverse-transcribed AS mRNA from bovine liver and cultured endothelial cells by polymerase chain reaction. A, RT-PCR products from endothelial cells (E) and liver (L) fractionated on a 1.5% agarose gel. Numbers on the left designate size (bp) of molecular standards. B, expected fragment sizes (bp) and location with respect to AS mRNA (bottom).

40 promoter and before the start codon of the luciferase gene. Left primers ASL-92HindIII, ASL-66HindIII, and ASL-43HindIII were combined with ASRluc (Table I) to amplify the different sized AS 5'-UTRs with a HindIII site on the 5'-end and 45 bases of the luciferase gene attached to the 3'-end. This strategy took advantage of a NarI site within the luciferase gene close to the start codon. The amplified fragments were digested with HindIII and NarI and cloned into pGL3Control in place of the existing 5'-UTR. Constructs were verified by sequencing. BAEC to be used for transfections were plated at 2 x 10⁴ cells per well in a 24-well plate. Control plasmids (Promega) included pGL3Control as a positive control, pGL3Basic as a promoterless negative control, and pRL-TK, a renilla expression vector, as an internal transfection control. Control, Basic, and experimental plasmids (200 ng each) and pRL-TK (50 ng) were transiently transfected into BAEC using Transit-LT1 (Panvera) in serum-free medium. After 4.5 h, the medium was replaced with complete medium, and cells were cultured for 48 h. Lysates generated with Passive lysis buffer (Promega) were assayed for luciferase and renilla activity using Promega’s Dual-Luciferase Reporter Assay System according to the manufacturer’s recommendations. Luciferase and renilla activity were measured as relative light units using a luminometer (Turner Designs). Experiments were carried out three times in triplicate. Luciferase expression was normalized to renilla activity.

RESULTS

Comparison of RT-PCR Fragments from Bovine Liver and Endothelial Cell AS mRNA—To investigate possible differences in the coding region of AS mRNA, total RNA from bovine liver and cultured bovine aortic endothelial cells was reverse-transcribed and amplified by PCR using a series of oligonucleotide primer sets designed to cover the entire coding region of the mRNAs. The resulting amplified fragments were separated by agarose gel electrophoresis, shown in Fig. 1. As can be seen, the relative mobility of each set of amplified fragments was identical, indicating that there were no substantial differences in the two AS messages that would indicate splicing variations of the ~13 exons that define the AS gene (22). To confirm this finding sequence analysis was carried out, which showed that there were no differences (data not shown) within the translatable sequence of endothelial and liver AS mRNA.

RACE Analysis of the 5’- and 3’-Ends of Liver and Endothelial Cell AS mRNA—Because the UTRs of mRNAs can influence stability, localization, and translation, rapid amplifications of the reverse-transcribed 5’- and 3’-ends of AS mRNA were carried out on bovine liver and cultured endothelial cell total RNA. The products of the RACE analyses were subcloned and sequenced.

For the 3’-RACE analysis, minimal differences attributed to minor polymorphisms at the level of the gene were observed. Direct sequence analysis further confirmed this finding (data not shown). In contrast, the 5’-RACE analysis of endothelial AS mRNA yielded clones containing different length extensions of the 5’-UTR. The majority of clones represented AS mRNA with 43 nucleotides of sequence upstream of the AUG start codon common to liver and endothelial cells (Fig. 2). A single isolated clone contained the identical 43 nt in the 5’-UTR but with an additional 23 nt of upstream sequence. A second clone verified the sequence of the 66-nt 5’-UTR but contained an additional 26 nt of upstream sequence for a total of 92 nt of 5’-UTR. Importantly, the 66- and 92-nt 5’-UTR clones were only identified by 5’-RACE analysis with endothelial cell total RNA. 5’-RACE analysis of liver total RNA yielded only the 43-nt AS 5’-UTR.

Extended 5’-UTR Sequence Comparison—To determine the possible genomic origin of the endothelial-specific 5’-UTR species, these sequences were compared with the 5’-UTR and genomic 5’-flanking regions from human (23) and mouse (24). Comparison of the 43-nt bovine AS mRNA 5’-UTR with exon 1 from human and mouse AS gene sequences demonstrated 72 and 64% homology, respectively. A sequence of 10 nt surrounding the TATTA box was identical in all three species. Importantly, the upstream AUG found in the extended 5’-UTR AS mRNAs from bovine endothelial cells was distinctly identified in the 5’-flanking genomic regions of both human and mouse AS genes.

Ribonuclease Protection Assays of the 5’-UTR of Liver and Endothelial Cell AS mRNA—To verify and quantitate these results, RPs were carried out on total RNA isolated from BAEC and liver using an antisense RNA probe complementary to the AS sequence extending from 82 nt upstream of the AUG start codon to 94 nt downstream of the start codon. Consistent with the 5’-RACE analysis, RNase protection analysis of the endothelial RNA yielded three different sized fragments (Fig. 4) corresponding to the predicted sizes of the different 5’-RACE AS mRNA sequences (43, 66, and 92 nt) identified previously. Quantitation by densitometry showed that 78% of the total AS mRNA contained the shortest 43-nt 5’-UTR sequence followed by 12% for the 92-nt 5’-UTR sequence and 10% for the 66-nt 5’-UTR. Notably, the RPA for liver AS mRNA only demonstrated the existence of the 43-nt 5’-UTR sequence.

Real-time RT-PCR Analysis of the 5’-UTR of Liver and Endothelial Cell AS mRNA—A second approach to quantitate relative levels of 5’-UTR AS mRNA species took advantage of the increased sensitivity of real-time RT-PCR analysis. As shown in Fig. 5, real-time RT-PCR analysis corroborated the RPA results, demonstrating again the disproportionate expression of three AS mRNA 5’-UTR species found in endothelial
cells. Because of the linear response of the technique over a large dynamic range, more accurate quantitation was generated for the percentages of each mRNA species. Plasmid DNA standards were detected in duplicate over a range of 9 orders of magnitude, easily allowing for the precise quantitation of mRNA levels differing by a factor of 100 or more. The percentage of the 43-, 66-, and 92-nt 5′-UTR AS mRNA species were determined to be 93.79 ± 1.24, 3.75 ± 1.70, and 2.46 ± 0.93%, respectively, of the total AS mRNA. The percentages are lower than those detected by ribonuclease protection analysis, probably because of the limitations of quantitation from x-ray film over a wide range of signal. Although the 66- and 92-nt 5′-UTR species were detected in liver above negative controls, levels were significantly lower than in endothelial cells, representing less than 0.1 ± 0.06% of total liver AS mRNA. Real-time RT-PCR analysis also confirmed differences in AS mRNA expression relative to total RNA, showing that in liver there is an ~40-fold higher level of expression compared with endothelial cells.

In Vitro Translational Efficiency of the 5′-UTR AS mRNAs—Sequence analysis of the diverse 5′-UTR AS mRNAs showed that each contained >70% G+C content and that the two longer forms contained a uORF frame. These features have been noted by other investigators to affect the translational efficiency of mRNAs (25). For this reason, experiments were designed to examine the influence that the diversity of endothelial AS 5′-UTRs might have on the translation of AS mRNA.

Full-length AS mRNA containing the 92-nt 5′-UTR sequence was cloned and then amplified to yield each of the other AS mRNA 5′-UTR species. An additional clone of AS mRNA referred to as the control was constructed to contain a 5′-UTR with optimal features relative to AUG start codon recognition and initiation (21). These constructs were transcribed and translated initially in a coupled transcription/translation system that visualized the protein products by immunodetection of biotinylated lysine incorporated during translation. Protein expression, analyzed by SDS-PAGE and Western blotting, showed that the AS mRNA with the shortest 43-nt upstream sequence translated as well or better than the control. The most dramatic effect on translational efficiency was observed with the longest endothelial 5′-UTR of 92 nt. A protein product was essentially undetectable even after longer exposures, whereas the intermediate length 5′-UTR of 66 nt translated nearly as well as the 43-nt 5′-UTR (data not shown).

Because the coupled system did not produce capped (7-methylguanosine, m7G) AS mRNA, the experiments were repeated using AS mRNA that contained a 5′-terminal m7G cap. The translation products containing biotinylated lysine generated from the different AS 5′-UTR mRNA species were separated by SDS-PAGE and visualized by immunodetection (Fig. 6). This time using capped AS mRNA, a product for the 92-nt 5′-UTR AS mRNA was detectable, probably reflecting the increased efficiency of translation observed for each of the capped species examined. The intermediate length 5′-UTR of 66 nt translated nearly as well as the control containing the Kozak sequence (25); however, the 43-nt 5′-UTR construct translated more efficiently than either the 66-nt 5′-UTR construct or the control. Examining the comparative level of AS protein produced, where the ratio to control remained essentially identical to that observed using uncapped AS mRNA, supported this conclusion. These differences demonstrated that the 5′-UTR species of endothelial AS mRNA influenced in vitro translational efficiency.

Deletion and Mutational Effects on Endothelial 5′-UTR AS mRNAs Translational Efficiency—To determine the basis for the decreased translational efficiency of the longer 5′-UTR, deletion and mutational analysis was carried out. As shown in Fig. 7, sequential deletion of the 92-nt 5′-UTR yielded results demonstrating a direct correlation of 5′-UTR length with translational efficiency. However, mutation of the upstream AUG to eliminate the uORF restored the translational efficiency of the 92-nt 5′-UTR AS mRNA to essentially that observed for the 66-nt 5′-UTR species. When the context of the upstream AUG
Protein expression was quantitated by subtracting the negative reaction for immunodetection with streptavidin horseradish peroxidase (Streptavidin-HRP) and translated. Biotinylated lysine was incorporated into the reaction for immunodetection with streptavidin horseradish peroxidase. Protein expression was quantitated by subtracting the negative control (no RNA added to the translation reaction) and normalizing to β-actin. B, expression level relative to the 43-nt 5'-UTR AS mRNA.

![Graph](Image)

**Fig. 7.** Deletion and mutational analysis of the 5'-UTR AS mRNAs. Full-length AS cDNA deletion mutants were constructed to include 86, 81, 76, and 71 nt in the 5'-UTR. Constructs were also created in which the upstream uAUG was mutated to AAG (Mut1) or altered to a more favorable context (25) by changing nucleotides at −3 and +4 relative to the uAUG (GGG AUG CGC to AGG AUG GGC, Mut2). Constructs were transcribed and capped (Ribo m7G Cap Analog), and then translated. Biotinylated lysine was incorporated into the reaction for immunodetection with streptavidin horseradish peroxidase. Protein expression was quantitated by subtracting the negative control (no RNA added to the translation reaction) and normalizing to β-actin. Expression levels of the mutants are shown relative to the 43-nt 5'-UTR AS mRNA.

was mutated to conform to an optimal consensus Kozak sequence, there was no significant decrease in translational efficiency observed with the 92-nt 5'-UTR containing AS mRNA. These results suggest that the uORF is an important feature affecting translational efficiency and that at least 22 nt of sequence 5' to the uORF are required for maximum suppression.

**Effects of AS mRNA 5'-UTRs on Luciferase Expression in Transfection Studies**—To isolate the effects of the different AS mRNA 5'-UTRs on expression, each 5'-UTR sequence was cloned in front of a luciferase reporter gene. Constructs were transfected into BAEC, and luciferase expression measured. Relative to the 43-nt 5'-UTR construct, expression of the 66- and 92-nt 5'-UTRs were 8- and 6-fold lower, respectively (Fig. 8). These results were essentially consistent with the discordant expression of the various 5'-UTR AS mRNAs in endothelial cells, demonstrating that the sequence from position −92 to −43 in the AS gene relative to the translational start codon is sufficient to affect differential expression of the three 5'-UTR AS mRNA species in endothelial cells.

**DISCUSSION**

Although the physiological role for AS was originally identified with the urea cycle in the liver, virtually all other mammalian tissues possess detectable levels of AS and a second urea cycle enzyme, AL. In nitric oxide-producing tissue, these two enzymes together have the net effect of regenerating arginine from citrulline and aspartate, providing arginine for NO production (2). We had previously shown that endothelial AS differs from liver AS, not only in its relationship to NO production but also by its caveolar co-localization with nitric oxide synthase (10).

Based on these findings, we examined whether an endothelial isoform of AS may exist that is encoded by the same respective hepatic urea cycle AS gene (19) but that plays a specialized role in regenerating arginine from citrulline for NO production. Although physical differences (mobility and isoelectric point) were observed, a comparison of the coding regions of liver and endothelial AS mRNA by RT-PCR analysis suggested that alternative splicing could not account for these physical differences or for the functional and localization differences. This result was further confirmed by direct sequence analysis.

Expression of genes that play a key role in metabolic processes are often regulated at multiple levels, and differences in regulation and localization have been attributed in some cases to differences in UTRs of an mRNA (26–31). We therefore compared sequences of both the 5'- and 3'-UTR of AS mRNA from liver and endothelial cells to assess the possible importance of these regions in the regulation of AS expression in endothelial cells. In this report we have shown that AS mRNA from bovine liver and bovine aortic endothelial cells differs in the relative level of expression and in the diversity observed for the 5'-UTR region. 5'-RACE analysis demonstrated unique expression of three different 5'-UTR AS mRNAs in endothelial cells. In contrast, only the shortest 5'-UTR of 43 nt was detected in liver.

Sequence inspection of the various 5'-UTRs of AS mRNA defined by 5'-RACE, RT-PCR, RNase protection analysis, and real-time RT-PCR highlighted the following features. First, the expression of the various AS mRNA 5'-UTR species is disproportionate. The AS mRNA with the shortest 43-nt 5'-UTR represents the major form (~94%) of the total AS mRNA, whereas the longer 5'-UTRs of 66 and 92 nt represent ~4 and
2%, respectively. Second, all 5′-UTRs for endothelial AS mRNA fall within the average length of eukaryotic 5′-UTRs (50–100 nt) (21). Third, an upstream AUG codon was detected in the 66- and 92-nt extended 5′-UTRs that is out-of-frame with the downstream AUG start codon but in-frame with multiple ORFs in the coding sequence of AS mRNA. Finally, composition analysis revealed that all three 5′-UTR sequences are highly enriched in G+C content (~76%), suggesting that all are capable of forming stable complex secondary structures known to affect translational efficiency (30).

Sequence analysis also suggested the possible origin of the 5′-UTR AS mRNA species. Comparison to the 5′-UTRs from both human (23) and mouse (24) AS mRNA demonstrated a high degree of homology. This similarity increased when the extended 5′-UTR sequences from bovine endothelial AS mRNA were compared with the genomic sequence immediately upstream of the reported transcription start site for the human and mouse AS gene. Our findings suggest that expression of all three species of bovine endothelial AS mRNA are driven by a common promoter, and that 5′-UTR diversity in endothelial cells results from three transcriptional initiation sites within exon 1.

Considerable research related to the 5′-UTR region of mRNAs has suggested a regulatory role in translational efficiency in part through cis effects at the primary (e.g. upstream short ORFs affecting ribosome scanning), secondary (e.g. hairpins and stem-loop structures), or tertiary (e.g. pseudoknots) levels (21, 25–30, 32). Such effects may also involve the contribution of RNA-binding proteins (32). Some general principles by which uORFs have been proposed to participate in translational control include the process of recognition of uORFs, regulation of reinitiation at downstream cistrons after translation of uORFs, and regulatory effects of peptides encoded by uORFs (32).

In endothelial cells there are only two species of AS mRNA that contain an identical 5′-UTR, and they represent at most no more than 4% of the total AS mRNA. But because these features are regarded as important determinants of translational efficiency, this suggested that the diversity at the 5′-end of AS mRNA transcripts may have important biological implications on the regulation of translation and may possibly explain observed differences between steady-state mRNA levels and protein expression.2

For this reason, after defining the diversity of 5′-UTR AS mRNAs in endothelial cells, an examination was carried out to determine to what extent the various 5′-UTRs of AS mRNA influence the efficiency of translation. Results from these experiments clearly showed that the nature of the AS mRNA 5′-UTR influenced translation. Based on the in vitro translation results for the 92-nt 5′-UTR, we expected that the intermediate length 66-nt 5′-UTR that still contained the uORF would yield similar results. However, the 66-nt 5′-UTR AS mRNA expression was nearly 70% of that observed for the 43-nt 5′-UTR AS mRNA. Improvement in the translational efficiency of all AS mRNA 5′-UTRs was observed in vitro when the message was capped, although disproportionate expression was still evident for the 92- and 66-nt 5′-UTR AS mRNAs. We had not anticipated that the control in these experiments, which possessed no AS mRNA 5′-UTR and was constructed to contain what may be considered an optimal consensus sequence around the start codon, would not express protein as well as the 43-nt 5′-UTR AS mRNA. This result indicated that features of the 43-nt 5′-UTR as well as features in the translated sequence of AS mRNA compensate for the lack of optimal consensus sequence within the context of the AUG start codon (25). The fact that loss of the uORF restored the translational efficiency of the 92-nt 5′-UTR to that observed for the 66-nt 5′-UTR and that the 66-nt 5′-UTR containing this uORF expressed protein at ~70% of the 43-nt 5′-UTR also suggested that attenuation of translation required ~22 nt of sequence 5′-to the uORF for maximum effect.

Transfection studies, which focused on the effect of AS 5′-UTR sequence diversity on luciferase expression in endothelial cells, produced results essentially consistent with the levels of expression observed in endothelial cells for the different 5′-UTR AS mRNAs. These findings suggested that sequence in the AS gene encoding positions 92 to 43 in the extended 5′-UTRs of AS mRNAs was sufficient to affect differential expression.

In summary, there is a remarkable diversity and disproportionate expression of each of the 5′-UTR AS mRNA transcripts that clearly differentiate AS expression in vascular endothelial cells from that in liver. We have demonstrated that expression of a 43-nt 5′-UTR AS mRNA is robustly enhanced in liver versus endothelial cells and that the unique diversity of 5′-UTR AS mRNAs in endothelial cells reflects sequence in the AS gene that permits selective modulation and tissue-specific expression.

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REFERENCES

1. Morris, S. M., Jr. (1992) Annu. Rev. Nutr. 12, 81–101
2. Wu, G., and Morris, S. M., Jr. (1998) Biochem. J. 336, 1–17
3. Hecker, M., Stebbins, W. C., Harris, H. J., Anggard, E. E., and Vane, J. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 87, 8612–8616
4. Xie, L., and Gross, S. S. (1997) J. Biol. Chem. 272, 16624–16630
5. Harrison, D. G. (1997) J. Clin. Invest. 100, 2153–2157
6. Block, E. R., Herrera, H., and Couch, M. (1995) Am. J. Physiol. 269, L574–L580
7. Baydoun, A. R., Emery, P. W., Pearson, J. D., and Mann, G. E. (1990) Biochem. Biophys. Res. Commun. 173, 940–948
8. Mitchell, J. A., Hecker, M., Anggard, E. E., and Vane, J. R. (1990) Eur. J. Pharmacol. 182, 573–576
9. Gold, M. E., Bush, P. A., and Ignarro, L. J. (1989) Biochem. Biophys. Res. Commun. 164, 714–721
10. Flam, B. R., Hartmann, P. J., Harrell-Both, M., Molsonom, L. P., and Eichler, D. C. (2001) Nitric Oxide 3, 187–197
11. Xie, L., Hattori, Y., Tame, H., and Gross, S. S. (2000) Semin. Perinatol. 24, 42–45
12. McDonald, K. K., Rouhani, M. R., Handlogen, M. E., Block, E. R., Griffith, O. W., Allison, R. D., and Kilberg, M. S. (1997) Biochem. Biophys. Acta 1324, 135–141
13. Greene, B., Pacitti, A. J., and Souba, W. W. (1993) Am. J. Physiol. 264, L351–L356
14. McDonald, K. K., Zharikov, S., Block, E. R., and Kilberg, M. S. (1997) J. Biol. Chem. 272, 31213–31216
15. Shuttleworth, C. W., Burns, A. J., Ward, S. M., O'Brien, W. E., and Sanders, K. M. (1995) Neuroscience 68, 1295–1304
16. Dimmel, S., Fleming, I., Fahlhafer, R., Herrmann, C., Busse, R., and Zeiher, A. M. (1999) Nature 399, 605–608
17. McCormick, S. M., Esen, S. G., McEntire, L. Y., Teng, C. L., Lu, M. C., Russell, C. G., and Chittur, K. K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8955–8960
18. Falkler, C. R., Kafan, H. A., and Nelin, L. D. (1995) Acta Paediatr. Scand. 84, 460–462
19. Freytag, S. O., Bock, H. G., Beaudet, A. L., and O'Brien, W. E. (1984) J. Biol. Chem. 259, 3160–3166
20. Chomczynski, P., and Sacchi, N. (1987) Annal Biochem. 162, 156–159
21. Kozak, M. (1987) Nucleic Acids Res. 15, 8125–8148
22. Freytag, S. O., Beaudet, A. L., Bock, H. G., and O'Brien, W. E. (1984) Mol. Cell. Biol. 4, 1978–1984
23. Jinno, Y., Matsuo, S., Nomiyama, H., Shimada, K., and Matsuda, I. (1985) J. Biochem. (Tokyo) 98, 1395–1403
24. Suh, L. C., Beaudet, A. L., and O'Brien, W. E. (1991) Gene 99, 181–189
25. Kozak, M. (1991) J. Cell Biol. 115, 887–903
26. Sonenberg, N. (1993) Genes Exp. 3, 217–223
27. Gray, N. K., and Hendz, W. M. (1994) EMBO J. 13, 3882–3891
28. Gray, N. K., and Wickens, M. (1998) Annu. Rev. Cell Dev. Biol. 14, 399–458
29. van der Velden, A. W., and Thomas, A. A. (1999) Int. J. Biochem. Cell Biol. 31, 87–106
30. Kozak, M. (2000) Genomics 36, 396–406
31. de Moor, C. H., and Richter, J. D. (2001) Int. Rev. Cytol. 203, 567–608
32. Morris, D. R., and Geballe, A. P. (2000) Mol. Med. Cell. Biol. 20, 8695–8642
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