Regulation of Endothelial Argininosuccinate Synthase Expression and NO Production by an Upstream Open Reading Frame*

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Argininosuccinate synthase (AS) catalyzes the rate-limiting step in the recycling of citrulline to arginine, which in endothelial cells, is tightly coupled to the production of nitric oxide (NO). In previous work, we established that endothelial AS mRNA can be initiated from multiple start sites, generating co-expressed mRNA variants with different 5′-untranslated regions (5′-UTRs). One of the 5′-UTRs, the shortest form, represents greater than 90% of the total AS mRNA. Two other extended 5′-UTR forms of AS mRNA, resulting from upstream initiations, contain an out-of-frame, upstream open reading frame (uORF). In this study, the function of the extended 5′-UTRs of AS mRNA was investigated. Single base insertions to place the uORF in-frame, and mutations to extend the uORF, demonstrated functionality, both in vitro with AS constructs and in vivo with luciferase constructs. Overexpression of the uORF suppressed endothelial AS protein expression, whereas specific silencing of the uORF AS mRNAs resulted in the coordinate up-regulation of AS protein and NO production. Expression of the full-length of the uORF was necessary to mediate a trans-suppressive effect on endothelial AS expression, demonstrating that the translation product itself affects regulation. In conclusion, the uORF found in the extended, overlapping 5′-UTR AS mRNA species suppresses endothelial AS expression, providing a novel mechanism for regulating endothelial NO production by limiting the availability of arginine.

Nitric oxide (NO) synthesized from arginine by endothelial nitric-oxide synthase is a potent vasodilator and a critical modulator of blood flow and blood pressure. In addition, it mediates vasoprotective actions through inhibiting smooth muscle proliferation, platelet aggregation, and leukocyte adhesion (1–3). Under pathophysiological conditions associated with endothelial dysfunction, such as heart failure (4), hypertension, hypercholesterolemia, atherosclerosis (5), and diabetes (6), the ability to produce NO seems to be impaired. Paradoxically, NO production can be impaired by limited availability of the substrate arginine, despite apparently saturating levels of intracellular and extracellular arginine (7–10). We have previously shown that under normal conditions, the essential arginine available for NO production is derived from the recycling of citrulline to arginine, catalyzed by two enzymes, argininosuccinate synthase (AS) and argininosuccinate lyase (AL) (11, 12). Although these two enzymes have been studied extensively in liver, where they participate in the urea cycle (13), it was not until the discovery of NO that their function in non-hepatic tissues was clarified. In endothelial cells, AS and AL play a critical role in the operation of a citrulline-NO cycle, which supports endothelial NO production (14–17).

Because AS catalyzes the rate-limiting step in the citrulline-NO cycle (15), our initial studies have focused on the molecular basis for the functional role of endothelial AS. Endothelial and hepatic AS appear to have the same primary structure (18, 19), but differ in cellular location and level of expression (11, 18). Hepatic urea cycle AS and AL are associated with the mitochondria (20), whereas in endothelial cells, AS and AL co-localize with endothelial nitric-oxide synthase in caveolae (11). AS expression in liver also differs from AS expression in endothelial cells as demonstrated by the diversity of co-expressed 5′-UTR AS mRNA species in endothelial cells (18). Three transcription initiation sites identified in endothelial cells result in overlapping 5′-UTR regions of 92, 66, and 43 nucleotides (nt). The longer forms make up ~7% of the total AS message, with the shortest 43-nt 5′-UTR AS mRNA being the predominant species in endothelial cells, and the only detectable form found in liver. Interestingly, the extended 92- and 66-nt 5′-UTR AS mRNAs contain an out-of-frame, upstream overlapping ORF that is terminated by a stop codon 70 nt past the in-frame start codon for the downstream ORF encoding AS. Previously we reported that in vitro translation of AS mRNA containing the extended 5′-UTRs was suppressed compared with the shortest and most predominant 43-nt 5′-UTR AS mRNA species (18). Moreover, we also showed that the translational efficiency of the extended 5′-UTR AS mRNA species was restored to the short form level when the uAUG was mutated to AAG, thus eliminating the uORF (18). This suppression of expression through cis effects was further demonstrated in vivo when the three forms of the AS 5′-UTR were placed in front of a luciferase ORF and transfected into endothelial cells. Here again, the presence of the uAUG found in the extended AS 5′-UTRs suppressed expression of luciferase in a cis-dependent manner.

Upstream ORFs can affect the translation of a downstream ORF in a variety of ways (21). In higher eukaryotes, initiation of translation generally occurs at the first AUG that resides in a favorable context. When the first AUG context is suboptimal, a portion of the scanning ribosomes may continue past the first AUG and initiate translation downstream at subsequent AUGs.

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‡ The abbreviations used are: NO, nitric oxide; AS, argininosuccinate synthase; AL, argininosuccinate lyase; UTR, untranslated region; uORF, upstream open reading frame; nt, nucleotide(s); BAEC, bovine aortic endothelial cells; dnasUG, downstream AUG; upsStop, upstream stop; GFP, green fluorescent protein; uORFs, upstream open reading frame shift; siRNA, small interfering RNA.
via leaky scanning (22). Several eukaryotic mRNAs have been shown to contain one or more ORFs that affect the translational efficiency of the main, downstream ORF (21). Depending on factors such as intercistronic length and secondary structure, scanning ribosomes, upon initiation at the uAUG, can either translate the uORF and reinitiate downstream or stall on the mRNA during elongation, thus preventing initiation at other sites (21). In other cases, partial translation of the nascent peptide prevents downstream re-initiation by interaction of the peptide with a protein or RNA in the ribosome preventing termination from proceeding efficiently (23). However, another less common event is for the uORF to be translated and for the peptide product to affect translation of the downstream cistron via a trans mechanism (24). Based on these examples and our previous findings, we show in this report that the uORF in the extended 5'-UTR AS mRNA species is functional and acts to limit overall AS expression as well as NO production, thus providing a novel mechanism for regulating endothelial NO production.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Bovine aortic endothelial cells (BAEC) were cultured in Dulbecco's modified Eagle's medium (1 g/liter glucose, Mediatech) supplemented with 10% fetal bovine serum (Hyclone Laboratories), 100 units/ml penicillin, and 100 μg/ml streptomycin (Mediatech) at 37 °C and 5% CO₂.

**Preparation of AS Constructs**—Full-length AS cDNA was constructed to contain either the 92- or 43-nt 5'-UTR, shown in Fig. 1, and subcloned into the vector pPDM-2 (Epicerent Technologies) as previously described (18). Mutations were created in the constructs by multiple rounds of PCR amplification using Pfu Turbo DNA Polymerase (Stratagene) in a reaction containing 1× Pfu reaction buffer (10 mM KCl, 10 mM (NH₄)SO₄, 20 mM Tris-Cl, pH 8.75, 2 mM MgSO₄, 0.1% Triton X-100, and 0.1 μg/ml bovine serum albumin), 200 μM each dNTP, 50 μM of each primer, 10 μg of digested plasmid template, and 2.5 units of Pfu Turbo DNA polymerase. PCR cycles included denaturation at 95 °C for 45 sec, annealing at 60 °C for 90 sec, and extension at 72 °C for 1 min. PCR products were then purified using minispin G-50 Sephadex (CPG) and 5% CO₂.

**Preparation of Luciferase Constructs**—Luciferase reporter constructs were designed to include different sections of the AS 5'-UTR cloned directly after the simian virus 40 promoter and before the start codon of the luciferase gene. One set of clones contained truncated forms of the 5'-UTR, the sequence spanning the region from either the 66- or 92-nt positions to the uAUG at position −57 relative to the AS AUG. Left primers LucASL-66 (5'-AGAAGCTTACCGAAGACGCCG-3') and LucASL-92 (5'-AGAAGCTTACCGAAGACGCCG-3') both contain a HindIII site on the 5'-end, AS 5'-UTR sequence (underlined), and the first 17 bases of the luciferase gene after the AUG on the 3'-end. These primers were combined with RTLuc18 (5'-CACTGATATGTGATCCTG-3') to amplify a small fragment of the luciferase gene that was then cloned into pGL3Control vector (Promega), using HindIII and NcoI, so that the various AS 5'-UTR segments recombined into the luciferase 5'-UTR.

Another luciferase construct, described previously (18), was designed to contain the entire 92 nt of the AS 5'-UTR in front of the luciferase gene. This construct was mutated, using the three round PCR method described in the preparation of AS constructs, to contain a single base insertion at position −39 (Ins 2 in Fig. 1). Similar to the AS Ins 2 mutation, this mutation put the AS uAUG and the luciferase AUG in-frame. Constructs were verified by sequencing.

**Luciferase Assays**—BAEC were used for transfections were plated at 6 × 10⁴ cells per well in a 24-well plate, 24 h prior to transfection. 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 transcribed and synthesized into capped RNA using T7 RNA polymerase. Transcription reactions were incubated at 37 °C for 5 min, dried on a gel dryer for 2 h, and exposed to film. Band intensities were quantitated using a Chemilumager 4400 (Alpha Technet).

**Preparation and Transfection of ASuORF Constructs**—For transfection studies with the AS uORF, AS sequence covering the region from −92 to +70 relative to the AS AUG was cloned into pcDNA 3.1/V5-His B expression vector (Invitrogen). Primers ASL92BamHI (5'-AAGCTTACCGAAGACGCCGAG-3') and ASR168MStop (5'-AGGCCCC CGCTTAAACCGGATGGAAAGCGCCAAAATATA-3') both carry a HindIII site on the 5'-end, AS 5'-UTR sequence (underlined), and the first 17 bases of the luciferase gene after the AUG on the 3'-end. These primers were combined with RTLuc18 (5'-CACTGATATGTGATCCTG-3') to amplify a small fragment of the luciferase gene that was then cloned into pGL3Control vector (Promega), using HindIII and NcoI, so that the various AS 5'-UTR segments recombined into the luciferase 5'-UTR.

Additional constructs of the AS uORF were used to investigate the effects of sequence and/or length of the uORF relative to its ability to suppress AS expression. Mutations were made that deleted a residue at
position −53 and inserted a residue at position +69 relative to the AS AUG to cause a frameshift in the peptide sequence of the AS uORF. Primers uORFleft (5′-ACCCGCGAGTGGCGCGCAATA-3′) and uORFright (5′-GAGCATCTGACGAGGATGTA-3′) were used to amplify by PCR the mutated fragment. The deletion and insertion sites are marked by a slash and an underline, respectively. A Small site in the left primer and EcoRI site in the right primer were used to clone the fragment into the ASuORF expression vector in place of the wild-type fragment. Similarly, mutations were introduced to move the AS uORF start codon downstream to position +1 relative to the AS start codon and to move the AS uORF stop codon upstream to position −11. Using primers uORFDnsAUG (5′-GCTGGTCGTTACGCAATTGAGGACTACT-3′) and uORFStop (5′-GCTGCACCTTACTCAGGATGTCGGCGCAATAGGCTG-3′) combined with AS73EcoRI, the mutated fragments were amplified and cloned using the BstEII site in the forward primers and the EcoRI site in the reverse primer. The dnasAUG (downstream of AUG) mutation fragment was cloned into the AAGNegC construct that was lacking the normal uAUG. The upStop (upstream stop) fragment was cloned into the wild-type ASuORF construct. BAEC were transfected as described in the previous section.

AS uORF constructs were developed that allowed the protein product to be easily resolved and visualized by SDS-PAGE analysis. Green fluorescent protein (GFP) was amplified from the pGween plasmid (Invitrogen) using the primers GFPleft (5′-ACTGGCGGCGCCTCCGC-CCCAATCGAGCAGAACG-3′) and GFPright (5′-CTAGAGCCGGCCCTACATTGTA-3′). The left primer contained a NotI site for cloning and a base in the middle of the site and the AUG to place GFP and the AS uORF in-frame. The right primer also contained a NotI site for cloning and deleted a base at the GFP stop codon to mutate out the stop codon and also to put GFP in-frame with the V5 and His tags. GFP was cloned into the ASuORF construct at the NotI site between the uORF and the V5/His tags. GFP was also spliced into the uORFs (frameshift) construct in the same manner. Constructs were verified by sequencing, and BAEC were transfected as described in the previous section.

RNA Duplex Preparation and Transfection—Ambion’s Silencer siRNA Construction Kit was used to synthesize 21-nucleotide RNA duplexes. Target sequences were chosen following the guidelines described by Elbashir et al. (25). The siRNA sequence specific to AS corresponds to nt −65 to −47 (Fig. 1) relative to the first nucleotide of the AS start codon (5′-CCCCGGGGAUGCGCGCAATTG-3′). A control siRNA was designed by scrambling the bases of the AS siRNA (5′-ACAGAGGGACUCGCCGCGCT-3′). Both sequences were subjected to BLAST search to rule out homology to mRNAs encoding known proteins. Twenty-four hours prior to transfection, BAEC were seeded in a 24-well plate at 1 × 105 cells per well. Twenty-four hours after transfection of the siRNA, mRNA was isolated from BAEC by the method of Chomczynski and Sacchi (26) using TriReagent (Molecular Research Center) according to the manufacturer’s protocol. Pellet Paint Co-Precipitant (Novagen) was added to help visualize the small RNA pellets. RNA was treated with DNase using the DNA-free kit (Ambion) and quantitated prior to reverse transcription with oligo(dT) primers using the Superscript First Strand cDNA Synthesis Kit (Invitrogen) following the manufacturer’s protocol. Real time quantitative PCR was performed as previously described using AS-specific primer sets ASL228 and ASR278 for detecting all mRNA, and ASL-62 and ASR-12 for detecting the extended 5′-UTR forms of AS mRNA (18). Results were normalized to β-actin using primers β-actin forward (5′-AGTCGGCGGCCGCTAGAGGA-3′) and reverse (5′-TCCATGGTCGCCCGCCTAGG-3′) and present as nitrite produced per 1 × 105 cells.

RESULTS

Functional activity of the AS uAUG in an In Vitro Transcription/Translation System—Because the extended AS 5′-UTRs, containing an uAUG, were shown to act in cis to down-regulate the translation of AS mRNA (18), studies were carried out to determine whether the cis effect was due to initiation at the uAUG and/or translation of the uORF. To examine the functionality of the uAUG in the extended 5′-UTR AS mRNAs, two different mutations were made in a full-length AS construct containing the 92-nt 5′-UTR. A single nucleotide was inserted between the uAUG and the downstream AS AUG, placing the uORF in-frame with the AS ORF so that initiation at either AUG would produce AS protein. One insertion was placed in close proximity to the AS AUG (Ins 1), whereas the other was placed more distal, 39 nt upstream from the AS AUG (Ins 2) as shown in Fig. 1. The two different nucleotide insertions were chosen such that secondary structure (folding pattern) of the RNA was predicted to be essentially the same for mutated and wild-type versions. Transcribed RNA from each construct was verified to be a single band by agarose gel electrophoresis and ethidium bromide staining (Fig. 2). All constructs were transcribed and translated in vitro in the presence of [35S]methionine. Translated products were separated on SDS-PAGE as shown in Fig. 2. The wild-type 92-nt 5′-UTR construct yielded a single product of ~47 kDa, the expected size of AS (29). Both of the insertion mutant constructs yielded doublets of ~47 and ~49 kDa, where the 49-kDa band represented the calculated size of the AS protein if translation was initiated using the uAUG. The amount of label observed in the ~47-kDa bands reflects not only the cis negative influence of the uORF as observed in the intact 92-nt extended AS mRNA, but also the relative efficiency of downstream initiation observed in the case of the two insertion mutations. In addition, the slight decrease in the Ins 1 ~47-kDa band may indicate the influence of...
was verified by agarose gel electrophoresis (in-frame were transcribed and translated mutations (Ins 1 and Ins 2) that put the AS AUG and the uAUG containing the 92-nt 5′-UTR were transcribed and translated in vitro in the presence of label, and the translated products were analyzed by SDS-PAGE analysis (Fig. 3). Translation of the 43-nt 5′-UTR AS mRNA construct yielded a single band of the correct size (~47 kDa) for AS, showing that the mutations (two amino acid changes) did not affect translation. Translation of the 92-nt 5′-UTR AS mRNA construct, however, resulted in two L-[35S]methionine-labeled bands; an ~47 kDa and a second smaller protein product at ~21 kDa, demonstrating that the out-of-frame uORF is functional. The broad darkened region at about 30 kDa was considered to be unrelated to the in vitro translation of the extended 92-nt 5′-UTR AS mRNA species because it was most predominant in the translation of the 43-nt 5′-UTR AS mRNA. These results provide further evidence that ribosomes can translate the entire uORF rather than prevent the translation of the downstream ORF encoding AS by stalling at the uAUG (21, 30).

In Vivo Functionality of the AS uAUG When Placed Immediately Upstream of a Luciferase ORF—To demonstrate the functionality of the AS uAUG in endothelial cells, luciferase constructs were generated that replaced the luciferase 5′-UTR with forms of the extended AS 5′-UTRs, spanning the region from either the −66- or −92-nt positions to the uAUG. As shown in Fig. 4, the construct containing the sequence from position −66 to −57 (the site of the uAUG) expressed luciferase activity at ~60% of control, whereas the −92 to −57-nt construct expressed luciferase activity at a lower level, ~36% of control. The fact that luciferase expression with the extended AS 5′-UTRs was lower than that of the control may reflect differences in the influence of the normal 5′-UTR versus the replacement AS 5′-UTRs. These results demonstrated that the uAUG is sufficient to support luciferase expression in endothelial cells.

In Vivo Functionality of the AS uAUG in Relation to a Downstream Luciferase ORF—To determine the in vivo functionality of the AS uAUG in the presence of a downstream ORF, a full-length luciferase ORF construct was modified to contain the 92-nt AS 5′-UTR with the out-of-frame uAUG. An additional luciferase construct was generated that contained an Ins 2 mutation in the 92-nt AS 5′-UTR, which positioned the AS upstream AUG and the downstream luciferase AUG in-frame. As shown in Fig. 5, there was no significant difference in luciferase activity levels for the control construct and the insertion mutation (Ins 2) luciferase construct. However, the luciferase activity level for the 92-nt AS 5′-UTR luciferase construct containing an out-of-frame uAUG was ~20% of the control activity. Western blot analysis to follow luciferase protein levels showed a single band of ~61 kDa for the control construct and a barely detectable 61-kDa band for the 92-nt AS 5′-UTR/luciferase construct containing the out-of-frame uAUG. In contrast, the 92-nt AS 5′-UTR/luciferase construct with the Ins 2 mutation placing the uAUG in-frame, showed a doublet of ~61 and ~63 kDa. The 61-kDa protein corresponded to the luciferase ORF initiated from the downstream AUG, whereas the 63-kDa protein corresponded to a luciferase protein initiated from the in-frame uAUG in the 92-nt AS 5′-UTR Ins 2 construct. These results demonstrated that the uAUG in the extended 5′-UTRs of AS mRNA can function in the presence of a functional downstream ORF in endothelial cells.

The Effect of Overexpression of the AS uORF on Endothelial AS Expression and NO Production—To investigate possible

![Fig. 2. Mutational analysis to determine the functionality of the AS uAUG by in vitro transcription/translation. AS constructs containing the 92-nt 5′-UTR (92 nt) and two single base insertion mutations (Ins 1 and Ins 2) that put the AS AUG and the uAUG in-frame were transcribed and translated in vitro. Transcribed RNA was verified by agarose gel electrophoresis (panel A, top). Translated L-[35S]methionine-labeled proteins were separated by SDS-PAGE, and gels were dried and exposed to film (panel A, bottom). The quantitation of the bands is shown in panel B. Expression levels are normalized to the 92-nt band.](image-url)
trans effects of the AS uORF, AS sequence from −92 to +70, relative to the AS AUG, was cloned into pcDNA3.1 vector so that the uORF was fused to a V5/His tag (ASuORF). For a negative control construct, the uAUG at position −59 was mutated to AAG, thereby rendering the AS uORF non-functional (AAGNegC). Equal amounts of protein from endothelial cells transfected with 0.8, 1.6, and 2.4 µg of ASuORF, AAGNegC, and vector plasmid DNA, along with a Lipofectamine-alone control, were analyzed by Western blot analysis with anti-V5 and anti-AS antibodies. The putative product of the uORF, ~7 kDa protein with the V5/His tag, could not be visualized by Western blotting with the V5 antibody. However, transfection of the AS uORF reduced endogenous AS protein levels, in a dose-dependent fashion when compared with transfection reagent alone (Fig. 6). Aliquots were removed 2 h after stimulation to measure nitrite as an indicator of cellular NO production. At the highest plasmid concentration of the ASuORF transfected, NO production was decreased to 5% of a control with Lipofectamine alone (Fig. 6). Although the empty vector and the AAG mutant showed some negative effect relative to the Lipofectamine control, the magnitude and dose-dependent decrease in NO production correlated significantly only with the loss of AS protein in ASuORF-treated cells. These results were also in keeping with those from previous work demonstrating the essential role of AS in endothelial NO production (12), despite excessive levels of intracellular and extracellular arginine.

Requirement for AS uORF Sequence and Length for Suppression of Endothelial AS Protein Levels and NO Production—To investigate whether sequence and/or length of the uORF are prerequisites for the trans-suppressive effects of the uORF on endogenous AS expression, we examined the overexpression of point mutation constructs with altered uORF structures. The first mutation was constructed with the initiation codon of the uORF left unchanged, but a deletion in the third codon and an insertion at the last codon caused a frameshift in the amino acid sequence. This frameshift mutation (uORFfs) yielded an ORF potentially encoding the same length peptide (44 amino...
acids), but where only the first two amino acids of the AS uORF were conserved. A second mutation was constructed with a new start codon for the AS uORF introduced 60 nucleotides downstream of the original, so as to potentially encode only 23 amino acids of the C terminus of the putative peptide (dnsAUG). A third mutation generated a construct where the stop codon was moved upstream to potentially encode 23 amino acids from the N terminus (upsStop) of the putative peptide.

As shown in Fig. 7, none of the mutated constructs showed the degree of suppression of AS expression or NO production exhibited by the wild-type AS uORF. These series of experiments demonstrated that both the sequence and the length of the AS uORF found in the extended 5'-UTR AS mRNAs are necessary to elicit negative trans effects on endothelial AS expression and NO production.

Regulation of AS Expression by the Translation Product of the AS uORF—To demonstrate that the translation product of the AS uORF suppresses overall AS expression in endothelial cells and to facilitate detection of the translation product, the protein was tagged by cloning GFP between the AS uORF and the V5/His tags of the ASuORF pcDNA3.1/V5-His B construct. An additional construct involved GFP cloned into the AS uORF frameshift construct (uORFs). Equal amounts of protein from lysates of endothelial cells transfected with 0.8, 1.6, and 2.4 µg of ASuORF-GFP and uORF6-GFP, in addition to a Lipofectamine alone control, were analyzed by Western blot analysis. As shown in Fig. 8, a dose-dependent increase in the ASuORF-GFP-V5/His tag fusion protein (~37 kDa) directly correlated with a decrease in endogenous AS protein levels. Expression of the frameshift uORF construct, which produced a protein of equal size but different amino acid content had no effect on AS protein levels. These results demonstrated that the protein encoded by the AS uORF mediates the negative trans effects on endothelial AS expression.

Effect of Silencing of the Extended 5'-UTR AS mRNAs on Endothelial AS Expression—To further demonstrate the trans suppressive effect of the uORF on endothelial AS expression, an siRNA was designed to selectively knockdown the 92- and 66-nt 5'-UTR AS mRNA species. Analysis of AS mRNA in transfected endothelial cells by real-time reverse transcriptase-PCR demonstrated that a scrambled form of the siRNA (control) had no effect on the levels of the extended forms of AS mRNA. In contrast, an siRNA directed against the extended 5'-UTR AS mRNA species decreased both the 92- and 66-nt 5'-UTR AS mRNAs to ~20% of transcription reagent alone (Fig. 9). Importantly, the level of total AS mRNA was essentially unaffected, consistent with the fact that the extended 5'-UTR forms of AS mRNA containing the uORF represent less than 7% of the total message. Equal amounts of protein from the extended AS 5'-UTR siRNA and from scrambled siRNA transfected endothelial cells were examined by Western blot analysis using anti-AS antibody. AS protein levels, normalized to glyceraldehyde-3-phosphate dehydrogenase expression, were
markedly increased in response to selective silencing of the 92- and 66-nt 5'-UTR AS mRNAs. An ~2.3-fold increase in expressed AS protein was seen compared with the scrambled siRNA at the 25 nM concentration of siRNA (Fig. 9). These results suggest that the trans effects of the uORF found in the extended 5'-UTR AS mRNA forms are mediated post-transcriptionally, and most likely at the translational level.

**Effect of Silencing of the Extended 5'-UTR AS mRNAs on NO Production**—Based on the previous results demonstrating that AS expression levels are coordinately linked to the production of NO in endothelial cells, we examined whether the knockdown of the endogenous extended 5'-UTR AS mRNA species and the accompanying increase in AS protein had an effect on the NO produced in these cells. Aliquots of media were removed 24 h after transfection of 25 nM siRNA specific for the extended AS 5'-UTR or a scrambled negative control siRNA, and nitrite as an indicator of cellular NO production was measured. As shown in Table I, an ~2.2-fold increase in NO production was observed compared with the scrambled siRNA-treated cells. This increase in NO production correlated closely with the increased expression of AS in response to the knockdown of extended 5'-UTR AS mRNA forms (Fig. 9).

**DISCUSSION**

We previously established that the recycling of citrulline to arginine is essential to provide the substrate arginine for NO production, even in the presence of saturating levels of intracellular and extracellular arginine (11, 12). We demonstrate in this study that expression of the extended 5'-UTR forms of AS mRNA, containing an uORF, mediates a trans effect, suppressing overall endothelial AS expression and causing a corresponding suppression of endothelial NO production. This suppression of AS expression requires a functional, out-of-frame uORF represented in the 5'-UTR regions of the co-expressed extended forms of endothelial AS mRNA (18). The uORF AUG was shown to be functional both in vitro and in vivo. When the uAUG was put in-frame with the downstream AUG by inserting a nucleotide, two in vitro translated 35S-labeled products were evidenced by electrophoretic SDS-polyacrylamide gel analysis. The larger AS species (~49 kDa) was initiated from the uAUG, whereas the smaller (~47 kDa) species represented the translation product initiated from the normal, downstream reading frame encoding AS. Interestingly, the ratio of products in this case favored use of the uAUG. Moreover, when the context of this uAUG was altered to better match the Kozak consensus initiation sequence (22), translation significantly improved from the uAUG. To demonstrate that this uORF, when positioned out-of-frame, was still translated, two putative stop codons for the uORF were mutated to allow production of a larger, more easily identifiable translation product (~21 kDa). Although the difference in methionine content did not permit a quantitative comparison by 35S labeling, the results clearly demonstrated a 21-kDa product, confirming the functionality of the uORF in its natural context.
With the support of in vitro results, we then assessed the in vivo functionality of the uORF in endothelial cells using a luciferase reporter assay. Expression of luciferase from the uAUG demonstrated that the context of the uAUG is sufficient to support initiation of translation. Moreover, when the AS uAUG start codon was positioned in-frame, in the context of the entire 5'-H11032-UTR and preceding the normal start codon for a luciferase gene, our results again demonstrated functionality. In this case, two luciferase products were identified by Western blot analysis consistent with the interpretation that both the uAUG and the downstream luciferase AUG are recognized in endothelial cells.

Previous work from our laboratory suggested that AS mRNA species containing the uORF in the extended 5'-H11032-UTR sequence do not express AS well, either in vitro or in vivo, because of cis effects of the uORF (18). In this article, we have clarified not only the functionality of the uORF, but also its trans-mediated effects, showing that overexpression of this uORF resulted in a dramatic decrease in AS expression in endothelial cells. This result suggested that the co-expression of the extended 5'-UTR forms of AS mRNA, containing an out-of-frame uORF, may play a role in suppressing the overall expression of endothelial AS. Additionally we showed that NO production is significantly reduced when the AS uORF is overexpressed, further linking the requirement for AS expression to NO production in endothelial cells. The fact that AS expression was not suppressed when the uORF was rendered nonfunctional, via loss of an operational start codon, or by overexpression of ASuORF containing either a frameshift mutation, or altered start or stop codons, demonstrated that the entire sequence of the uORF is required to mediate the trans effects that decrease endothelial AS expression and NO production. Furthermore, a direct effect was observed that related expression of the translational product encoded by the uORF to the suppression of endothelial AS expression.

When expression of the endothelial extended 5'-UTR AS mRNA species were specifically silenced by siRNA treatment,

### FIG. 8

**Regulation of AS expression by the translation product of the AS uORF.** An AS uORF construct was prepared in which GFP was cloned into the ASuORF pcDNA3.1V5/His construct between and in-frame with the AS uORF and the V5/His tags (ASuORF-GFP). GFP was also inserted into the uORFs (frameshift) construct (uORFfs-GFP). The GFP constructs were transfected into BAEC and compared with a Lipofectamine alone control (Lipo). Twenty-four hours after transfection, equal amounts of protein were separated by SDS-PAGE and standard Western blotting was performed using anti-V5, anti-AS, and anti-β-actin antibodies (panel A). Quantitation of AS protein expression, normalized to β-actin, is indicated as a fraction of the Lipofectamine alone control (panel B).

### FIG. 9

**Effect of silencing of the extended 5'-UTR AS mRNAs on endothelial AS expression.** BAEC were transiently transfected with siRNA specific for the 92- and 66-nt 5'-UTR species of AS mRNA (●) and a scrambled negative control siRNA (■). Total RNA was isolated and AS mRNA was detected by real time reverse transcriptase-PCR. Primer sets were designed to specifically amplify the 66- and 92-nt 5'-UTR species (panel A), or total AS message (panel B). Equal amounts of protein were separated by SDS-PAGE and standard Western blotting was performed using anti-AS and anti-glyceraldehyde-3-phosphate dehydrogenase antibodies. Quantitation of AS protein expression from four separate experiments, normalized to glyceraldehyde-3-phosphate dehydrogenase, is indicated as a fraction of the transfection reagent alone (panel C).

### TABLE I

**Effect of silencing of the extended 5'-UTR AS mRNAs on NO production**

| Condition | Relative NO produced |
|-----------|---------------------|
| 25 nm scrambled siRNA | 1.0 |
| 25 nm siRNA | 2.2 ± 0.33 |

BAEC were transiently transfected with siRNA specific for the 92- and 66-nt 5'-UTR species of AS mRNA and a scrambled negative control siRNA. Basal NO production was determined over a 24-h period. NO was measured as nitrite produced per 1 x 10⁶ cells and normalized to scrambled siRNA levels.
expression of AS increased dramatically (~2-fold), despite the fact that these species represent less than 7% of the total AS mRNA. Consistent with the rate-limiting role of AS in recycling citrulline to arginine and in maintaining the essential arginine for NO production, knockdown of the extended 5’-UTR AS mRNA species containing this uORF resulted in an increased capacity of endothelial cells to produce NO. Thus, the overall results suggest that the uORF found in the extended 5’-UTR forms of endothelial AS mRNA is functional, and as such expresses a protein product that acts to suppress expression of the predominant short form of the AS mRNA.

In summary, a small protein produced through expression of the uORF of the extended 5’-UTRs of two minor forms of AS mRNA, unique to endothelial cells, suppresses AS expression. The overall effect of this suppression of AS expression is to decrease NO production in endothelial cells by limiting the availability of the substrate arginine. These results provide evidence for a novel mechanism for the regulation of endothelial AS protein expression and further support the essential role of the citrulline-NO cycle in endothelial NO production.

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