Role of the 3′-Untranslated Region of Human Endothelin-1 in Vascular Endothelial Cells

CONTRIBUTION TO TRANSCRIPT LABILITY AND THE CELLULAR HEAT SHOCK RESPONSE*

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Endothelin-1 (ET-1) is a potent vasoconstrictor peptide expressed in the vascular endothelium. Stringent control over ET-1 expression is achieved through a highly regulated promoter and rapid mRNA turnover. Since little is known about mechanisms governing ET-1 post-transcriptional regulation, and changes in ET-1 mRNA stability are implicated in disease processes, we characterized these pathways using a variety of functional approaches. We expressed human ET-1 and luciferase transcripts with or without a wild type ET-1 3′-untranslated region (3′-UTR) and found that the 3′-UTR had potent mRNA destabilizing activity. Deletion analysis localized this activity to two domains of the 3′-UTR we have termed destabilizing elements 1 and 2 (DE1 and DE2). Mutational studies revealed that DE1 functions as an AU-rich element (ARE) dependent on a 100-nucleotide region. This activity was further localized to a 10-nucleotide region at position 978–987 of the 3′-UTR. Depletion of AUF1 by RNA interference up-regulated ET-1 in endothelial cells suggesting AUF1-dependent regulation. Since AUF1 functions through the ubiquitin-proteasome pathway, we disrupted this pathway with heat shock and proteasome inhibitor in endothelial cells and observed stabilization of endogenous ET-1 mRNA. Chimeric transcripts bearing wild type ET-1 3′-UTRs were also stabilized in response to proteasome inhibition whereas DE1 mutants failed to respond. Taken together, these findings suggest a complex model of ARE-mediated mRNA turnover dependent on two 3′-UTR domains, DE1 and DE2. Furthermore, DE1 functions as an ARE directing mRNA half-life through the proteasome. Finally, this data provides evidence for a novel pathway of ET-1 mRNA stabilization by heat shock.

The endothelium regulates local vascular tone and integrity through the coordinated release of vasoactive molecules. Secretion of endothelin-1 (ET-1) from the endothelium signals vasoconstriction and influences local cellular growth and survival (1–6). ET-1 has been implicated in the development and progression of vascular disorders such as atherosclerosis and hypertension (1–3). Endothelial cells upregulate ET-1 in response to hypoxia, oxidized LDL, pro-inflammatory cytokines, and bacterial toxins (7–10). Initial studies on the ET-1 promoter provided some of the earliest mechanistic insight into endothelial-specific gene regulation (11, 12). Numerous studies have since provided valuable insight into ET-1 promoter regulation under basal and activated cellular states (13).

The ET-1 mRNA is labile with a half-life of less than an hour (14, 15). Together, the combined actions of ET-1 transcription and rapid mRNA turnover allow for stringent control over its expression. We have previously shown that ET-1 mRNA is selectively stabilized in response to cellular activation by Escherichia coli O157:H7-derived verotoxins, suggesting ET-1 is regulated by post-transcriptional mechanisms (16). Regulatory elements modulating mRNA half-life are often found within 3′-untranslated regions (3′-UTR) (17–20). The 1.1-kb 3′-UTR of human ET-1 accounts for over 50% of the transcript length and features long tracts of highly conserved sequences including an AU-rich region (14, 15). Some 3′-UTR AU-rich elements (AREs) play important regulatory roles in cytokine and proto-oncogene expression by influencing half-life under basal conditions and in response to cellular activation (18, 20). Several RNA-binding proteins with affinities for AREs have been characterized including AUF1 (hnRNPD), the ELAV family (HuR, HuB, HuC, HuD), tristetraprolin, TIA/TIAR, HSP70, and others (19, 21–27). Although specific mechanisms directing ARE activity have not been fully elucidated, current models suggest ARE-binding proteins target specific mRNAs to cellular pathways that influence 3′-polyadenylate tail and 5′-cap metabolism (17–19).

Recent studies have revealed a functional link between AUF1, heat shock proteins and the ubiquitin-proteasome network (28). Proteasome inhibition by chemical inhibition or heat shock was shown to stabilize a model ARE-containing mRNA whereas promotion of cellular ubiquitination pathways was shown to accelerate ARE mRNA turnover (28–30). Studies with in vitro proteasome preparations suggest that the proteasome itself may possess ARE-specific mRNA destabilizing activity (31). The ARE-binding protein AUF1 has been linked to the ubiquitin-proteasome pathway. AUF1 mRNA destabilizing activity has been positively correlated with its level of polyubiquitination and has been shown to interact with a member of the E2 ubiquitin-conjugating protein family (28–30, 32–34). Further work is required to define the mechanisms by which AUF1 and its binding partners regulate ARE-mediated mRNA turnover.
thermore, under conditions of cellular heat shock AU1 associates with heat shock protein 70 (HSP70), which itself possesses ARE binding activity (27, 28, 34).

At present, very little is known about pathways regulating labile mRNA stability in endothelial cells. Given the important role of ET-1 in the vascular endothelium, we chose to study ET-1 as a labile mRNA. We present evidence that the 3′-UTR constitutively destabilizes the ET-1 transcript through two destabilizing elements, DE1 and DE2, and that DE1 functions through a conserved ARE by the AU1-proteasome pathway. We also provide evidence for a physiologically relevant pathway of ET-1 mRNA stabilization by the heat shock pathway.

EXPERIMENTAL PROCEDURES

Restriction and DNA-modifying enzymes were obtained from New England Biolabs (Beverly, MA) and Roche Diagnostics (Laval, QC). PCR amplifications were carried out using TaqDNA polymerase (In Vitrogen, Burlington, ON). Oligonucleotides were synthesized using a Beckman Oligo 1000 DNA synthesizer (Beckman Instruments, Fullerton, CA). DNA constructs were sequenced using an ABI Prism 377 DNA sequencer (ABI, Foster City, CA). All plasmid constructs were generated using standard recombinant DNA methodologies.

Luciferase Reporter Constructs

The firefly luciferase open reading frame was excised from pSP-luc (Promega, Madison, WI) with HindIII and XhoI and ligated into the XhoI/BstBI site of pET1Ctrl. Wild type and mutant 3′-UTRs were excised from pCRII-based plasmids (see below) by complete or partial EcoRI digestion and ligated into the EcoRI site of pLuc-Ctrl immediately downstream of the luciferase ORF.

Wild Type and Mutant 3′-UTR Constructs

ET-1 3′-UTR reagents are numbered relative to the first nucleotide of the 3′-UTR (GenBank™ accession number J05008 [14]). The 3′-UTR was amplified by PCR using human genomic DNA as a template and primer set ET1-UTR-2S (5′-CAGACCTTCTGAGAGGCTG-3′)/ET1-UTR-2AS (5′-TTTCTAAAGTCATTACCTTGACAGGCA-3′), and subcloned into pCRII (Invitrogen) to generate pET1-3′UTR. Ligation into the EcoRI site of pLuc-Ctrl, immediately downstream of the luciferase open reading frame, generated plLuc-ET1WT. Deletion mutants were generated by strategic use of restriction enzyme sites in 3′-UTR-containing plasmids. Briefly, plLuc-A1–272, plLuc-A1–603 and plLuc-A1–924 were generated by partial or complete excision from pET1–3′UTR and ligated into the EcoRI site of pLuc-Ctrl. Wild type and mutant 3′-UTRs were excised from pCRII-based plasmids (see below) by complete or partial EcoRI digestion and self-ligation to generate 3′-UTR internal deletions, followed by excision and ligation into the EcoRI site of plLuc-Ctrl. Linker-scanning mutants were created as previously described (35). Briefly, using pET1–3′UTR as a template, two adjacent but slightly overlapping amplicons were created corresponding to upstream (amplon A) and downstream (amplon B) regions of the 3′-UTR relative to the mutation target site. The sense primer of amplon A and antisense primer of amplon B were specific to regions on the host vector outside of the 3′-UTR. The mutagenic antisense primer for A and mutagenic sense primer for B for each contained 20 nt of 3′-UTR-specific sequences and an additional common 10 nt mutant sequence (5′-CAGATCATTCATCTG-3′) creating a 10 nt overlap region between amplons A and B. Amplons A and B were restriction-digested with BamHI/AccI or BamHI/XhoI, respectively, and 3′-way ligated into AccI/ XhoI-digested pET1–3′UTR, thereby regenerating the complete 3′-UTR with a 10 nt mutation at the site of BamHI recombination. This procedure generated pCRII-based plasmids from which mutant 3′-UTRs were excised by partial EcoRI digestion and ligated into pLuc-Ctrl. To generate a full-length mutant 3′-UTR, the fragment was amplified from human genomic DNA using primer set GCMSCF-UTR-1S (5′-AATAATTATTACGG-3′)/GCMSCF-UTR-2AS (5′-CGTGGCGGCTTACCTCGACCG-3′), subcloned into pCRII, EcoRI excised and ligated into pLuc-Ctrl to give pLuc-WTGMCSF. The GM-CSF linker-scanning mutant pLuc-MutGMCSF (10 nt mutation at position 234 of the 3′-UTR) was generated using a similar strategy as with ET-1 mutants.

Full-length ET-1 cDNAs

ET-1 cDNAs are numbered according to the spliced sequence arising from human ET-1 genomic sequences (GenBank™ accession number J05008 [14]). Two PCR products corresponding to nucleotides 1–544 and 425–1309 of the ET-1 cDNA were amplified from reverse-transcribed human umbilical vein endothelial cells (HUVEC) total cellular RNA using primer sets ET1–1S (5′-AGACGGGCTCTGCACTTGC-3′)/ET1–544AS (5′-TCTTTGGAACCTGGTCCTC-3′) and ET1–1248S (5′-TCTTTGGAACCTGGTCCTC-3′)/ET1–1309AS (5′-GCGTTTCTCGTGAGGAGG-3′), respectively. Subcloning of PCR amplicons into pCRII generated phuET1FL5′ and phuET1FL3′. XhoI/AccI (from phuET1FL5′) and AccI/BstBI (from phuET1FL3′) fragments were ligated into the XhoI/BstBI site of pET1–3′UTR to generate a full-length human ET-1 cDNA corresponding to nucleotides 1–2010 (pCRII-huET1FL). ET-1 cDNAs were excised from pCRII-huET1FL by partial EcoRI digestion (wild type) or partial EcoRI digestion (3′-UTR deletion), blunt-ended using the Klenow fragment and ligated into pcDNA3 to generate pET1-WT and pET1-3′UTR, respectively.

Cell Culture

All cell culture reagents were obtained from Invitrogen unless otherwise noted. Primary human aortic Primary human aortic endothelial cells (BAEC) were isolated from calf aortas, cultured on 0.2% gelatin and maintained in RPMI 1640 medium supplemented with 15% bovine calf serum and antibiotics, as previously described (9). BAEC were utilized at passages 3–7. HUVEC were isolated from umbilical cords, cultured in M199 supplemented with 20% fetal bovine serum, endothelial mitogen (Bio- medical Technologies Inc., Stoughton, MA) and antibiotics, as previously described (36). HUVEC were utilized at passages 3–5. Human dermal microvascular endothelial cells (HDMEC) were obtained from Cambrex (East Rutherford, NJ) and maintained in EGM-MV supplemented with 5% fetal bovine serum and EGM2 supplements (Cambrex). HeLa (human cervical epithelial adenocarcinoma) and HepG2 (human hepatocellular carcinoma) cells were obtained from ATCC (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. All cells were incubated at 37 °C and 5% CO2, MG132 and actinomycin D (Sigma-Aldrich, Oakville, ON) treatments were carried out as indicated in figure legends.

Transfections

Plasmids utilized in DNA transfections were purified by two rounds of cesium chloride gradient ultracentrifugation. DNA concentrations were determined by UV spectrophotometry and confirmed by analytical gel electrophoresis. For transient transfections, cell cultures were seeded and grown to 50–80% confluence 24 h prior to transfection. For stable transfections, HepG2 cells in 100-mm dishes were transfected with 9 μg of DNA per dish. DNA transfection efficiency was determined by UV spectrophotometry and confirmed by analytical gel electrophoresis. For state experiments, cell number and/or RNA was harvested 48–48 h post-transfection. For stable transfections, HepG2 cells in 100-mm dishes were transfected with 9 μg of DNA per dish. DNA transfection efficiency was determined by UV spectrophotometry and confirmed by analytical gel electrophoresis. Gene Expression Assays—Triplicate samples were extracted and luciferase activities were determined using the Dual Luciferase Reporter System (Promega). Protein content was determined using the BCA protein assay kit (Pierce Biotechnology). For transient
assays, Firefly luciferase activity was normalized to Renilla luciferase activity to correct for transfection efficiency. For stably transfected cells, Firefly luciferase activity was normalized to protein content.

**Northern Blot**—Total cellular RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (37). Northern blots were performed as described (16) using UltraHyb hybridization solution (Ambion, Austin, TX). Gel-purified cDNA probes were labeled with [α-32P]dCTP to a minimum specific activity of 10⁸ dpm/μg by random primer labeling methods (Amersham Biosciences, Baie d’Urfe, QC). For Northern blot analysis of RNA from transient transfection assays, Firefly luciferase or human ET-1 signals were normalized to signals from the neomycin resistance gene (neoR or aminoglycoside 3'-phosphotransferase) to correct for transfection efficiency. neoR is expressed from all pLuc and pET-1 series plasmids based on pcDNA3 (Invitrogen). cDNA probe templates were as follows: luciferase, 1.7-kb HindIII/XhoI fragment from pLuc-Ctrl containing the full luciferase ORF; human ET-1, 0.54-kb EcoRI fragment from pCrihuET1FL5' spanning the 5' -UTR and partial ORF; neoR, 0.14 kb EcoRI fragment of pCRII-neoR140 specific to the ORF. The human-specific ET-1 probe detects human ET-1 mRNA and does not detect endogenous bovine ET-1 in BAEC. Radioactive signals were detected with a Storm PhosphorImager and quantified with ImageQuant 2 for Mac (Molecular Dynamics, Sunnyvale, CA).

**Real-time RT-PCR**—First-strand cDNA was synthesized from 5 μg of DNase-treated total cellular RNA using random primers and SuperScript II reverse transcriptase (Invitrogen) using manufacturer's protocols. Real-time PCR assays were performed with 100 ng of RNA equivalent cDNA in triplicate on an ABI 7900HT Sequence Detection System and analyzed with SDS2.1 software (Applied Biosystems Inc.). PCR was carried out under the following conditions: 95 °C, 10 min; 40 cycles of 10 s at 95 °C and 1 min at 60 °C. SYBR Green or TaqMan formulations were utilized according to manufacturer's protocols (Applied Biosystems Inc.). All real-time PCR assays were performed in the presence of serial dilutions of reference plasmids for determination of template copy number. Primer pairs and probes for ET-1 and endothelial nitric-oxide synthase (eNOS) real-time PCR analysis cross-exon boundaries. ET-1 TaqMan assays were performed with primer set hueET1RT-S (5'-TGGACATCATTTGGCTACAA-3'/huET1RT-AS (5'-TCCTCTGGATCTACTGGTC-3') and detected with hueET1RT-Probe (5'-FAM-CCCGAGACCTGTTCTCAGATGACTCAG(38)). eNOS TaqMan assays were performed with primer set hueNOSRT-S (5'-GGCATACACAGGAAGAAC-3'/hueNOSRT-AS (5'-TACCTCGTCGACGATCC-3') and detected with hueNOSRT-Probe (5'-FAM-CACACGGCGTTAGATCAGC(38)). 18S SYBR Green assays were performed with primer set 18SF (5'-GGACATCTAAAGGGCATTACA-3'/18SR (5'-AGAATTACGGACAGGAC-3'). Luciferase SYBR Green assays were performed with primer set Luc590S (5'-AC-TCCTCGGATCATTGCGTC-3'/Luc840AS (5'-GTTAATCGGAGGCTCCTCA-3').

**Western Blot**—HUVEC monolayers were lysed with boiling sample buffer (62.5 mM Tris-HCl [pH 7.4], 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol). Cell lysates were boiled for 10 min, centrifuged at 12,000 × g for 5 min, cellular supernatant collected, and protein concentration determined by the Bradford method (Protein Assay Dye Reagent, Bio-Rad, Mississauga, ON). Protein samples were electrophoresed in denaturing 10% polyacrylamide-SDS gels and transferred by electroblotting onto polyvinylidene difluoride membranes (Amer sham Biosciences). Comassie Blue staining of duplicate gels confirmed equivalent loading of samples. AUF1 immunoblots were incubated with anti-human AUF1 polyclonal antibody (2 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) followed by peroxidase-conjugated anti-rabbit secondary antibody (1:12,000 dilution, Amersham Biosciences). Lamin immunoblots were incubated with anti-human lamin monoclonal antibody (0.2 μg/ml, Santa Cruz Biotechnology) followed by peroxidase-conjugated anti-mouse secondary antibody (1:12,000 dilution, Pierce Biotechnology). Chemiluminescent reactions were carried out using the Supersignal West Fico substrate (Pierce Biotechnology) and detected using a Fluor-8 Max Multimager (Bio-Rad Laboratories).

**Statistics**—All experiments were performed a minimum of three times. Data points represent the mean ± S.E. of multiple independent experiments. Statistically significant differences for data points, half-life values, and half-life curves represent p < 0.05 and calculated using either the unpaired t test or analysis of variance (Dunnet or Bonferroni post-tests). Statistical analyses were performed using GraphPad Prism V4.0 software (GraphPad Software Inc., San Diego, CA).

![Image](319x228 to 561x737)

**Fig. 1.** The ET-1 3'-UTR is a potent regulator of human ET-1 mRNA stability. BAEC were transiently transfected with human ET-1 expression plasmids as described under “Experimental Procedures.” A, schematic representation of full-length ET-1 wild type (ET1-WT) and 3'-UTR deletion mutant (ET1-ΔUTR) transcripts. Black and gray lines represent ET-1 and vector-derived sequences, respectively. Dotted line represents deleted region. B, total cellular RNA was harvested from transfected BAEC and human ET-1 expression determined by Northern blot with a human-specific ET-1 probe. Blots were stripped and reprobed for neoR expression (to control for transfection efficiency) and relative ET-1 expression calculated (ET1-neoR). C, transfected BAEC were subjected to transcriptional arrest with 10 μg/ml Act D, total cellular RNA extracted at times indicated, ET1-neoR expression determined as above and mRNA decay curves calculated using exponential regression. Half-lives in hours were WT, 1.9 ± 0.3 and ΔUTR, 13.4 ± 3.5. All data points represent the average ± S.E. of 4 independent experiments (*, p < 0.05 versus ET1-WT).
The ET-1 3'-UTR destabilizes a reporter mRNA. BAEC were transiently transfected with chimeric luciferase expression plasmids as described under "Experimental Procedures." A, schematic representation of luciferase transcripts bearing a control (Ctrl) or wild type ET-1 (ET1-WT) 3'-UTR. Black and gray lines represent ET-1 and vector-derived sequences, respectively. Dotted line represents deleted region. B, cellular protein lysates were harvested from transfected BAEC and dual-luciferase assays performed. Chimeric reporter activity (firefly luciferase) was normalized to a secondary reporter (Renilla luciferase) to control for transfection efficiency. C, total cellular RNA was harvested from transfected BAEC and firefly luciferase expression determined by Northern blot with a luciferase-specific probe. Blots were stripped and reprobed for neoR expression to control for transfection efficiency and relative luciferase mRNA calculated (luciferase/neoR). D, transfected BAEC were subjected to transcriptional arrest with 10 μg/ml Act D, total cellular RNA extracted at times indicated, luciferase/neoR expression determined as above and mRNA decay curves calculated using exponential regression. Half-lives in hours were Ctrl, 19.5 ± 2.8 and WT, 1.8 ± 0.2. E, multiple endothelial and non-endothelial cell-types were transfected and luciferase expression determined as described in panel B. HDMEC, primary human dermal microvascular endothelial cells; HeLa, human cervical epithelial adenocarcinoma cells. Data points represent the average ± S.E. of three (panel E), four (panels B and C), or five (panel D) independent experiments (*, p < 0.05 versus Ctrl).
cular endothelium are a function of both promoter activity and mRNA turnover. However, very little is currently known about the post-transcriptional mechanisms regulating ET-1. To characterize these pathways, we expressed human ET-1 transcripts in bovine aortic endothelial cells (BAEC) with or without the 3'UTR (ET1-WT or ET1-3'UTR, respectively, Fig. 1A). Northern blot analysis of RNA from transfected cells revealed that removal of the 3'UTR resulted in a significant increase in steady-state ET-1 mRNA expression (Fig. 1B). Half-life studies with actinomycin D-mediated transcriptional arrest demonstrated that removal of the 3'UTR significantly increased mRNA half-life (Fig. 1C). We also tested the ability of the 3'UTR to transfer mRNA destabilizing activity to a reporter transcript. Chimeric luciferase transcripts with control or ET-1 3'UTRs were expressed in endothelial cells (Luc-Ctrl and Luc-ET1-WT, respectively, Fig. 2A). Luciferase activity, steady-state mRNA levels, and mRNA half-life were all decreased in the presence of the ET-1 3'UTR (Fig. 2, B–D). Taken together, these findings demonstrate that the ET-1 3'UTR possesses potent mRNA destabilizing activity and is necessary for the lability of ET-1 mRNA.

The ET-1 3'UTR Regulates mRNA Stability in Multiple Cell Types—Some pathways regulating mRNA turnover are cellspecific (39–42). To assess the cell specificity of ET-1 3'UTR activity, we extended our study to include other primary endothelial cells that express abundant ET-1 (HUVEC; human dermal microvascular endothelial cells, HDMEC), as well as two non-endothelial human cell lines that do not express appreciable amounts of ET-1 (HeLa and HepG2). The ratio of chimeric luciferase reporter activities (Luc-Ctrl versus Luc-ET1-WT) in the varied cell types was comparable to BAEC (Fig. 2E). This suggests that the ET-1 3'UTR functions in a variety of cell types, independent of whether the cells express ET-1 endogenously.

The 3'UTR Requires Two Domains, DE1 and DE2, for Destabilizing Activity—To identify cis-elements directing 3'UTR destabilizing activity, we undertook a comprehensive mutational study in primary endothelial cells. Chimeric reporter transcripts bearing wild type and deletion mutant 3'UTRs were expressed and assayed for both luciferase activity and steady-state mRNA expression (Fig. 3A). Deletion of either the 5' or 3' half of the 3'UTR (Δ1–603 or Δ573–1109) abolished destabilizing activity, as evidenced by an increase in both luciferase activity and mRNA expression (Fig. 3, B and C). Further deletion of varied regions of the 3'UTR led to a complex

### Table I

| 3'UTR       | Destabilizing activity | DE1 | DE2 |
|-------------|------------------------|-----|-----|
| WT          | +                      | +   | +   |
| Δ1–603      | +                      | +   | +   |
| Δ573–1109   | +                      | +   | +   |
| Δ1–272      | +                      | +   | +   |
| Δ272–570    | +                      | +   | +   |
| Δ571–935    | +                      | +   | +   |
| Δ935–1104   | +                      | +   | +   |
| Δ1–924      | +                      | +   | +   |

**Fig. 3. Deletion analysis of the 3'UTR.** BAEC were transiently transfected with chimeric luciferase expression plasmids bearing wild type or deletion mutant ET-1 3'UTRs. A, schematic representation of Ctrl, WT and mutant 3'UTRs. B, cellular protein lysates were harvested from transfected BAEC and dual-luciferase assays performed. C, total cellular RNA was harvested from transfected BAEC and firefly luciferase and neoR expression determined by Northern blot. Data points represent the mean ± S.E. of three independent experiments (*, p < 0.05 versus WT).
expression pattern (Fig. 3, B and C and Table I). Specifically, we noted that luciferase expression similar to wild type 3'-UTR levels (destabilizing activity) was always associated with the presence of nucleotides 272–570 and 935–1104, respectively, whereas deletion of either of these two regions (3'-H9004272–570 and 3'-H9004935–1109) resulted in expression levels similar to control (no destabilizing activity). Therefore these regions represent important functional regulatory domains of the 3'-H11032-UTR that we refer to as DE1 and DE2 (destabilizing elements 1 and 2 corresponding to nucleotides 935–1104 and 272–570 of the 3'-H11032-UTR, respectively). It is interesting to note that although deletion of DE1 resulted in the strongest increase in reporter expression, the presence of DE1 alone (3'-H90041–924) could not reconstitute destabilizing activity. These findings indicate that although DE1 is necessary, it alone is not sufficient for destabilizing activity. Together, our findings suggest that DE1 and DE2 function together to promote ET-1 mRNA destabilization.

**DE1 Functions through a 10-Nucleotide AU-rich Element**—Sequence inspection of DE1 and DE2, and comparison across bovine and murine genes, revealed several interesting features (Fig. 4). Firstly, both domains are highly conserved across species. Secondly, DE1 features five highly conserved and clustered AU motifs with the sequence A(U)\text{n}A, where 3 ≤ n ≤ 7 (Figs. 4 and 5A). These sequences resemble those bound by AU-rich element RNA-binding proteins implicated in the regulation of mRNA half-life (18, 19). To further characterize DE1, we developed two mutants to assess the contribution of conserved AU motifs (Fig. 5B). Deletion of a 100-nt region encompassing all five DE1 AU motifs (3'-H9004938–1037), or site-specific mutation of all five motifs (Multi) resulted in a significant increase in luciferase activity (Fig. 5C) and mRNA expression (data not shown) over the wild type 3'-H11032-UTR. This data not only establishes DE1 as a functional AU-rich element (ARE), it also implicates one or more AU motifs within DE1 as a critical regulator of mRNA stability.

To further localize functional regions within DE1, we expressed five linker-scanning mutants (M938, M952, M968, M978, and M1028), each representing 10 nt mutations of individual AU motifs (Figs. 4 and 5A). As non-DE1 mutant controls, we also mutated two 10-nt regions including a non-DE1 AU motif (M613) and a non-AU motif (M761) (Fig. 4). We first confirmed the feasibility of our approach by assessing the functional properties of a mutation on the GM-CSF 3'-H11032-UTR, a well-studied model ARE (20, 43). The 10-nt mutation effectively abolished destabilizing activity of a chimeric luciferase-GM-CSF 3'-H11032-UTR reporter transcript, as assessed by luciferase activity and mRNA half-life (Fig. 6, A–C). Expression studies with ET-1 linker-scanning mutants revealed that one mutant, M978, significantly enhanced luciferase activity and mRNA expression above wild type 3'-UTR levels (Fig. 6, D–F). All other ET-1 linker-scanning mutants failed to change expression levels.

Half-life studies using functionally important DE1 mutants (3'-H9004988–1037, Multi and M978) demonstrated that increases in
motifs. The Multi (H11032) motifs are marked by expanded representation of DE1. The relative positions of DE1 AU stabilization of the wild type ET-1 3'-UTR contains a 100-nt deletion (dotted line) that removes all five DE1 AU sequences 5'-CAGGAUCCAU-3' and are individually named according to their 5'-nucleotide position within the 3'-UTR. B, schematic representation of the wild type ET-1 3'-UTR highlighting DE1 and DE2 and an expanded representation of DE1. The relative positions of DE1 AU motifs are marked by black vertical bars (WT). The Δ938–1037 3'-UTR contains a 100-nt deletion (dotted line) that removes all five DE1 AU motifs. The Multi 3'-UTR mutant replaces 10 nt of every DE1 AU motif with the mutant sequence 5'-CAGGAUCCAU-3' and is indicated by hatched vertical bars. C, cellular protein lysates were harvested from transfected BAEC and dual-luciferase assays performed. Data points represent the mean ± S.E. of three independent experiments (*, p < 0.05 versus WT).

steady-state expression were accompanied by significant increases in mRNA half-life (Fig. 6G).

Since mutation of all five DE1 AU motifs (Δ938–1037 and Multi) or just one AU motif (M978) abolished ET-1 3'-UTR destabilizing activity, we conclude that DE1, when present together with DE2, functions as an AU-rich element.

ET-1 mRNA Is Up-regulated in AUF1-depleted Cells and Stabilized in Response to Heat Shock or Proteasome Inhibi-

tion—Recent studies have suggested a functional link between ARE-dependent mRNA decay and pathways involving the ARE-binding protein AUF1, the proteasome, and disruption of this pathway by heat shock (28). Furthermore, a strong correlation exists between ARE mRNA decay and amounts of AUF1 protein and ARE binding activity (29, 32, 44). Since the ET-1 3'-UTR functions through an AU-rich element, we considered the possibility that its expression is regulated by AUF1. To test this possibility, we employed RNA interference (RNAi) to knockdown AUF1 expression in primary human endothelial cells (Fig. 7A). AUF1 consists of four protein isoforms of 37, 40, 42, and 45 kDa generated by alternative mRNA splicing of the AUF1 gene (45). These isoforms differ in their N- and C-terminal domains, which confer different functional properties and sites for post-translational modification (29, 46, 47). Western blot analysis demonstrated that under control conditions all four AUF1 isoforms were evident in human endothelial cells (Fig. 7A). It is of interest to note that the p37 and p40 isoforms were present in lower relative amounts than p42 and p45 isoforms. This may reflect the fact that p37 and p40 have been shown to be ubiquitinated and subject to protein degradation pathways (28, 29). AUF1 or Lamin knockdown by RNAi resulted in a greater than 90% decrease in expression for the respective proteins (Fig. 7A). We quantitated both ET-1 and eNOS mRNA levels in these cells using real-time RT-PCR. ET-1 mRNA levels were significantly elevated in AUF1-depleted cells relative to Lamin-depleted cells (included as a control) or a scrambled control (Fig. 7B). In contrast, eNOS mRNA failed to respond to AUF1 knockdown.

To further characterize the involvement of this pathway, we subjected primary bovine and human endothelial cells to heat shock (42 °C) or proteasome inhibition with the inhibitor MG132 and measured ET-1 mRNA expression using Northern blot analysis and real-time RT-PCR. Significantly elevated ET-1 mRNA levels were observed after four hours of heat shock (Fig. 7, C and D). ET-1 also responded to proteasome inhibition by MG132 in a concentration- and time-dependent manner (Fig. 7, C, E, and F). In contrast, eNOS mRNA failed to respond to heat shock or MG132 in HUVECs (Fig. 7, D–F). To elucidate the mechanism of ET-1 induction by these pathways, heat-shocked or MG132-treated endothelial cells were subjected to transcriptional arrest with actinomycin D and mRNA half-lives assessed. ET-1 mRNA was significantly stabilized by either heat shock or proteasome inhibition (Fig. 7G). This data not only provides evidence that ET-1 mRNA turnover is regulated in part by the proteasome, it also reveals a novel and physiologically relevant mechanism of ET-1 induction by the heat shock pathway.

Induction of ET-1 mRNA expression by AUF1 knockdown, heat shock, and proteasome inhibition suggests ET-1 is post-transcriptionally regulated by the AUF1-proteasome pathway. The ET-1 3'-UTR Functions through the Proteasome—We next investigated the interaction between the ET-1 3'-UTR and proteasome-dependent mRNA decay pathways. HepG2 cell lines were generated that stably expressed chimeric luciferase transcripts with control, WT, M978, or Δ938–1037 ET-1 3'-UTRs. Steady-state expression patterns in HepG2 stable cell lines were assessed by luciferase activity, Northern blot, and real-time RT-PCR and were comparable to results observed in transient transfection assays of endothelial cells (data not shown). MG132 treatment of HepG2 stable cell lines led to an induction of both luciferase activity and mRNA in cells expressing WT ET-1 3'-UTR chimeric transcripts (Fig. 8, A and B). In contrast, M978 and Δ938–1037 mutants failed to respond to MG132. Half-life studies under control or MG132 treatment conditions confirmed that steady-state increases in expression...
FIG. 6. Mutation of DE1 AU motifs. BAEC were transiently transfected with chimeric luciferase plasmids bearing wild type and mutant GM-CSF or ET-1 3'-UTRs. A and B, as a positive control, luciferase transcripts bearing wild type GM-CSF (WT GMCSF) or linker-scanning mutant (Mut GMCSF) 3'-UTRs were expressed in BAEC. The sequence of the human GMCSF 3'-UTR is shown with stop codon underlined and functionally important AU motifs bolded. The boxed region represents wild type nucleotides replaced with the mutant sequence 5'-CAGGAUCCAU-3' at position 234 of the 3'-UTR. Cellular protein lysates were harvested and dual-luciferase assays performed. C, transfected cells were treated with 10 μg/ml Act D, total cellular RNA extracted at times indicated, luciferase/neoR expression determined and mRNA decay curves calculated using exponential regression. Half-lives in hours were Ctrl, 17.5 ± 3.1*; WT GMCSF, 2.3 ± 0.2; Mut GMCSF, 8.0 ± 1.2*. D, schematic representation of the wild type ET-1 3'-UTR highlighting DE1 and DE2 and expanded representations of control and DE1 regions. Black vertical bars mark the relative positions of DE1 AU motifs (M938, M952, M968, M978, and M1028), a non-DE1 AU motif (M613), and a non-DE1 non-AU motif (M761). Linker-scanning mutants have 10 nucleotides of individual wild type AU motifs replaced with mutant sequence (5'-CAGGAUCCAU-3') indicated by hatched vertical bars. E, chimeric luciferase plasmids bearing wild type or linker-scanning mutant ET-1 3'-UTRs were transfected into BAEC, cellular protein lysates harvested, and dual-luciferase assays performed. F, total cellular RNA was harvested from transfected BAEC and Firefly luciferase and neoR expression determined by Northern blot. G, transfected cells were treated with 10 μg/ml Act D, total cellular RNA extracted at times indicated, luciferase/neoR expression determined as above and mRNA decay curves calculated using exponential regression. Half-lives in hours were Ctrl, 17.5 ± 3.1*; WT, 1.8 ± 0.1; M978, 7.9 ± 1.3*; Multi, 19.5 ± 5.1*; ΔM938-1037, 21.4 ± 5.6*. Data points represent the mean ± S.E. of three (panels B and C) or four (panels E-G) independent experiments (*, p < 0.05 versus WT).
were mirrored by similar changes in mRNA half-life (Fig. 8C). Taken together, these studies suggest that DE1 regulates ET-1 mRNA half-life through a proteasome-dependent pathway.

**DISCUSSION**

The highly dynamic environment of the blood vessel demands that the endothelium respond rapidly to changes in local hemodynamics, cellular traffic and blood-derived signals. As a potent vasoconstrictor and cellular mitogen, the ET-1 gene has evolved multiple levels of regulation from the production and destruction of its mRNA to the proteolytic maturation and secretion of its peptide (1–3,13). Because of its constitutively short mRNA half-life, changes in the rate of ET-1 transcription are rapidly mirrored by changes in steady-state mRNA levels. Together, this system functions as an early and influential step in ET-1 biosynthesis. However, recent studies have implicated mRNA stabilization as an important determinant of ET-1 expression in response to E. coli O157:H7-derived verotoxins and other cellular activators (16, 48–51). Whether these changes in mRNA half-life reflect a cellular strategy to increase ET-1 production or pathophysiologic perturbations in mRNA turnover pathways remains unclear.

To gain insight into human ET-1 mRNA turnover, we considered the 3’-UTR as a potential regulatory element. The 3’-UTR comprises over 50% of the mRNA length and features multiple regions of sequence conservation across species. In our
FIG. 7. ET-1 mRNA expression is regulated by the AUF1-proteasome pathway in endothelial cells. HUVEC were transfected with siRNAs targeting AUF1 or Lamin A/C, or Scramble (nonspecific) siRNAs as described under "Experimental Procedures." A, representative Western blot of protein lysates from siRNA-transfected HUVEC using AUF1 (top) or Lamin A/C (bottom) antibodies. B, ET-1 and eNOS mRNA expression were determined using real-time RT-PCR and normalized to 18S RNA as described under "Experimental Procedures." Data points (panel B) represent the mean ± S.E. of three independent experiments (*, p < 0.05 versus scramble control). C, confluent BAEC and HUVEC monolayers were heat-shocked (42 °C for 0, 2, and 4 h) or treated with MG132 at (0, 5, 25, and 50 μM for 4 h) and ET-1 mRNA expression measured by Northern blot. D–F, HUVEC were heat-shocked or treated with MG132 as in panel C and ET-1 and eNOS mRNA expression determined by RT-PCR (ET-1/18S or eNOS/18S). For MG132 studies, cells were treated for 4 h at indicated concentrations (panel E) or with 25 μM MG132 for indicated times (panel F). G, control, heat-shocked (HS, 42 °C, 4 h;  ), or MG132-treated (25 μM, 4 h;  ) HUVEC were subjected to transcriptional arrest with 10 μg/ml actinomycin D. Total cellular RNA was extracted at times indicated, ET-1/18S expression determined as above and mRNA decay curves calculated using exponential regression. Half-lives in hours were: Ctrl, 0.46 ± 0.06; MG132, 1.35 ± 0.30; HS, 1.65 ± 0.40. Data points (panels D–G) represent the mean ± S.E. of three independent experiments (*, p < 0.05 versus non-treated control).
study, we found the 3′-UTR to possess potent destabilizing activity in the context of the ET-1 5′-UTR and ORF, as well as a luciferase reporter transcript. Although our study does not exclude the possibility of 5′-UTR or ORF determinants of mRNA stability, it defines a clear role for the 3′-UTR. We concluded that the 3′-UTR utilizes a ubiquitous pathway of mRNA destabilization since it was active in primary bovine and human endothelial cells, as well as non-endothelial human cell lines (Fig. 2). Using 3′-UTR deletions, we mapped functional activity to two domains that we refer to as DE1 and DE2 (Fig. 3 and Table I). Both domains feature large tracts of nucleotides that are conserved across human, bovine and murine species (Fig. 4). DE1 was of particular interest because of five clustered and highly conserved AU motifs. Mutation of AU motifs identified nucleotides 978–987 of the 3′-UTR as a critical region for destabilizing activity (Figs. 5 and 6). The presence of ET-1 3′-UTR AU motifs were first reported with the genomic organization of the human ET-1 gene (14, 15). However to date, there have been no studies functionally characterizing determinants of ET-1 mRNA stability. Furthermore, several studies have shown that not all AREs are active and most have proven to be unique in their sequence or function (52, 53).

An important and surprising caveat to our findings is that unlike the majority of AREs characterized, DE1 cannot destabilize a reporter transcript alone, functioning only when present with another domain, DE2 (Fig. 3 and Table I). Several mRNAs have been identified that utilize multiple 3′-UTR domains that act independently of each other including cox-2, VEGF, and c-Fos mRNAs (54–57). However, very few mRNAs have been shown to require two mutually dependent domains for full destabilizing activity. One useful example is c-Jun, which contains a U-rich 3′-UTR element requiring a downstream domain for mRNA destabilization (58). Because RNA molecules have the ability to form complex secondary and tertiary structures, long-range RNA domain interaction between DE1 and DE2 must be considered. For example, long-range interaction of two domains within the insulin-like growth factor II (IGF-II) 3′-UTR regulates expression by a mechanism involving endonucleolytic cleavage of the mRNA (59). Whether ET-1 3′-UTR destabilizing activity requires direct interaction of DE1 and DE2 through RNA-RNA hybridization or indirect

![Figure 8](image-url)

**Fig. 8. Destabilizing activity of the ET-1 3′-UTR is dependent on the proteasome.** HepG2 cell lines stably expressing chimeric transcripts with a variety of 3′-UTRs (Ctrl, WT ET-1, M978, or Δ938–1037) were generated as described under “Experimental Procedures.” MG132 treated (40 μM for 8 h) or untreated HepG2 cells were assayed for (A) luciferase activity (normalized to protein content) and (B) luciferase mRNA expression by real-time RT-PCR (normalized to 18 S RNA). In both panels A and B, fold-induction of reporter expression in response to MG132 relative to controls is presented. Data points (panels A and B) represent the mean ± S.E. of three independent experiments (*, p < 0.05 versus Ctrl). C, untreated () or MG132-treated (40 μM for 8 h) HepG2 cells were subjected to transcriptional arrest with 10 μM/ml actinomycin D and total cellular RNA extracted at indicated times. Luciferase/18 S expression was determined as above and mRNA decay curves calculated using exponential regression. Half-lives in hours were Ctrl (MG132), 9.5 ± 2.4; Ctrl (+MG132), 9.5 ± 0.4; WT (MG132), 1.9 ± 0.3; WT (+MG132), 6.4 ± 0.4; M978 (–MG132), 5.0 ± 1.6; M978 (+MG132), 8.0 ± 0.8; Δ938–1037 (–MG132), 7.5 ± 0.8; Δ938–1037 (+MG132), 12.2 ± 2.6. Data points (panel C) represent the mean ± S.E. of 3 independent experiments (*, p < 0.05 versus untreated controls).
interaction through a bridging ribonucleoprotein complex remains unclear at this time. It should also be noted that the requirement for DE2 provides evidence for a unique non-ARE cis-element. Studies to localize and define functional DE2 regions will provide further insight into this complex 3′-UTR.

In some instances, 3′-UTRs have been shown to regulate translational efficiency (60). We closely examined the effects of ET-1 3′-UTR sequences on both reporter mRNA and protein levels and found no evidence for regulation of translational efficiency suggesting the 3′-UTR regulates ET-1 mRNA expression primarily through mRNA stability.

Since the ET-1 3′-UTR functions through an AU-rich element, we considered the possibility that it functions via the AUF1-proteasome pathway. In this study, we provide several lines of evidence to implicate this pathway in ET-1 mRNA turnover (Figs. 7 and 8). First, ET-1 expression was up-regulated by RNAi knockdown of AUF1 in endothelial cells. Secondly, endothelial cells treated with heat shock or proteasome inhibitors induced ET-1 expression through a mechanism of mRNA stabilization. Finally, only transcripts bearing wild type 3′-UTRs were stabilized by proteasome inhibition. Together, these studies provide the first evidence that the AUF1-proteasome pathway functions in vascular endothelial cells and regulates ET-1 expression through a specific 3′-UTR ARE. We also tested eNOS mRNA levels and found no significant effect in response to AUF1 knockdown, heat shock or proteasome inhibition in vascular endothelial cells. An important question for future studies will be whether AUF1 mRNA and protein levels are regulated by exogenous stimuli and its influence on endothelial cell phenotype in health and disease. In other cell types, the AUF1-proteasome pathway has been shown to be rate-limiting suggesting that modulation of AUF1 expression may alter cellular mRNA turnover pathways (30, 32). Moreover, how varied components of the AUF1 pathway and enzyme components of the ubiquitin cycle (deubiquitinating enzymes) are regulated and interdependent remains to be defined.

We have provided evidence for a pathway of ET-1 induction and mRNA stabilization by heat shock. The induction of heat shock proteins (HSPs) confers protective properties against cellular stress and prevents apoptosis in endothelial cells (61, 62). The heat shock pathway was initially characterized in cellular models of thermal stress. It is now appreciated that this pathway represents a broader cytoprotective response to cellular stress typified by the induction of heat shock proteins (HSPs) (63, 64). Endothelial cells respond to heat shock with the expression of stress-inducible HSPs (62, 65). Endothelial HSP expression is also induced in response to oxidized LDL, inflammatory cytokines, hypoxia and oxidative stress (66, 67). Furthermore, HSP60 and HSP70 expression have been defined. The role of HSPs in the regulation of ET-1 and other labile mRNAs will provide a strong foundation for understanding how perturbations in these pathways influence the health and disease of the vasculature.

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