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Alternative polyadenylation sites of human endothelial nitric oxide synthase mRNA

Hideshi Ihara [a,*], Hiroyasu Tsutsuki [a], Tomoaki Ida [a], Shunji Kozaki [b], Shingo Tsuyama [b], Joel Moss [c]

[a] Department of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan
[b] Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan
[c] Pulmonary-Critical Care Medicine Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892-1590, USA

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Abstract

The mRNA 3′-untranslated region (3′-UTR) has been shown to have important roles in the regulation of mRNA function. In this study, we investigated the human endothelial nitric oxide synthase (eNOS) 3′-UTR to evaluate its potential regulatory role. 3′-RACE analysis revealed that the human eNOS mRNA has multiple alternative polyadenylation sites. Apart from the proximal site (418 bp downstream of the stop codon), we identified two additional distal sites approximately 770 and 1478 bp downstream of the stop codon. In addition, Northern analysis showed that the usage of these sites differed among human tissues. Further, amounts of these eNOS mRNAs were changed during growth of cultured human aortic endothelial cells; mRNAs with long 3′-UTRs decreased more rapidly than total mRNA, as cells approached confluency. Thus, the 3′-UTRs of human eNOS results from alternative polyadenylation sites and differ across tissues and during cell growth.

Keywords: 3′-Untranslated region; Alternative polyadenylation sites; eNOS

Nitric oxide (NO) is an important messenger molecule, which mediates many physiological processes in the cardiovascular, immune, and nervous systems [1]. The enzyme responsible for NO synthesis, i.e., nitric oxide synthase (NOS), converts L-arginine to L-citrulline and NO [1]. Three isoforms of NOS have been characterized; of these, one is of the inducible type (iNOS), while two of the isoforms, namely, neuronal NOS (nNOS) and endothelial NOS (eNOS), are of the constitutive type [1]. NO production by NOS is regulated by factors such as calcium/calmodulin and tetrahydrobiopterin, and by other mechanisms such as phosphorylation and protein–protein interactions [1,2].

The 3′-untranslated region (3′-UTR) of mRNA has important physiological and pathological roles in the regulation of mRNA level. In fact, the 3′-UTR of mRNA is often referred to as a molecular "hotspot" for pathology [3]. Regulation of gene expression through the 3′-UTR can include alternative polyadenylation, translational control, and differential mRNA stability [4,5]. The selection of a polyadenylation site involves specific *cis*-acting elements and *trans*-acting factors [5]. The terminal sequence of most mammalian mRNA contains the consensus AAUAAA hexamer or a close variant between 10 and 30 nucleotides upstream of the cleavage site, which serves as the binding site for a cleavage and polyadenylation specificity factor (CPSF). In addition, GU- or U-rich elements that are located downstream of the cleavage site are involved in directing polyadenylation by serving as the binding site for the cleavage stimulation factor (CstF) [5]. Together, the upstream polyadenylation signal and the downstream GU- or U-rich elements are thought to direct the polyadenylation reaction. Computational analyses suggested that a

* Corresponding author. Fax: +81 72 254 9932.
E-mail address: ihara@b.s.osakafu-u.ac.jp (H. Ihara).

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considerable number of human mRNA have alternative polyadenylation sites [6–8]. Selection of alternate polyadenylation sites is believed to be related to biological parameters such as cell type and disease state, and can produce mRNAs with variable length 3′-UTRs.

eNOS is a key enzyme involved in the regulation of vascular function. NO that is produced by eNOS is physiologically important for maintaining vascular homeostasis. The production of NO by the endothelium is regulated by changes in eNOS enzyme activity and/or eNOS gene expression [9,10]. An abnormality of eNOS activity and/or expression can cause several diseases. The association between the sequence variations in the human eNOS gene and vascular diseases has been investigated [11,12]. Although eNOS was initially considered to be a constitutive enzyme, it was shown later that eNOS expression was regulated by exogenous stimuli. Of note, stability of the mRNA can be controlled by different compounds and stimuli via interaction with the 3′-UTR [13–21]. Bovine eNOS mRNA stability is regulated during the proliferative phase of endothelial cells through binding of a 51-kDa cytosolic protein to the 3′-UTR [15]. In bovine endothelial cells, destabilization of eNOS mRNA induced by TNF-α results from specific binding of a cytosolic 60-kDa protein to the 3′-UTR [13]. Similarly, in endothelial cells of the human umbilical vein, a 56-kDa cytosolic protein is involved in TNF-α-induced destabilization of eNOS mRNA [20], which contains two AU-rich elements (AUUUA) [22] and eight CCUCC or CCUCU motifs [20]; these signals have been implicated in the modulation of mRNA stability [23,24]. Thus, there has been increasing evidence that the 3′-UTR of eNOS mRNAs may play an important role in the regulation of mRNA level by affecting mRNA stability.

To evaluate the potential regulatory roles of the human eNOS 3′-UTR, we examined alternate polyadenylation sites, as well as amounts of the mRNAs with variable length of the 3′-UTR in human tissues, and the mRNAs during growth of human aortic endothelial cells.

Materials and methods

3′-Rapid amplification of cDNA ends (3′-RACE). A 3′-RACE-ready cDNA of the human aorta (Clontech, Mountain View, CA, USA) was used. eNOS-specific primers are illustrated in Fig. 1B. For the first amplification, eNOS-specific F0 primer (5′-CCTCCCGGCTCAGAC ACCAACAG-3′) and adapter primer 1 (AP1: 5′-CCATCCTAATAC GACTCACTATAGGG-3′) were used, and for the second nested amplification, eNOS-specific F1 (5′-GGCTTTCCCTTCCAGTTCCG-3′), F2 (5′-CCTCCACCCCACAAGTTCAA-3′), and F3 (5′-CCTACACGCCA CTTGACCCTGC-3′) primers and nested adaptor primer 2 (AP2: 5′-ACT CACTATAGGGCTCGAGCGGC-3′) were used. The products were separated using agarose gel electrophoresis and stained with ethidium bromide. Each band was cut from the gel and purified using QIAex kit (Quiagen, Valencia, CA, USA). The purified DNA was subcloned into the TA vector (Invitrogen, Carlsbad, CA, USA) and subjected to sequence analysis. The primers (F0 and adapter primer 1) with CAUCAUCAU CAU at the 5′-end were used to clone the first PCR products, and these products were then subcloned into the pAMP1 vector using the CloneAmp system (Invitrogen).

Northern blotting. Total RNA from normal human tissues (Clontech) (20 μg/lane) was subjected to formaldehyde–agarose gel electrophoresis, stained with ethidium bromide, transferred to a Hybond-N^+ membrane (Amersham Biosciencs, Piscataway, NJ, USA), and hybridized with oligonucleotide probes (239R: 5′-ATCTAACATTCGACTAAGAAA CAGGAAGCGGGTGGCAGTAGGCCCTGGGG-3′ and 511R: 5′-GG CTGAGGCAGGAGAATCACTTGAACTTGTGGGGTGGAGGTTG CAGTGA G-3′) labeled with [α-^{32}P]dATP (Amersham Biosciencs) at the 3′-end and detected by autoradiography. Band intensities were analyzed using NIH Image software. Expression of human eNOS was normalized using 28s rRNA.

Reverse transcription-polymerase chain reaction (RT-PCR). The expression of RNAs with various lengths of 3′-UTR in human aortic endothelial cells during cell growth was analyzed using quantitative PCR. Normal human aortic endothelial cells (Clonetics, Walkersville, MD, USA) were cultured in an endothelial cell growth medium (BioWhittaker, Walkersville, MD, USA). The cells were then split in a ratio of 1:6. Proliferating, semiconfluent, and confluent cells were analyzed 24, 70, and 144 h after plating. Total RNA was extracted from the cells using the RNeasy kit (Qiagen) and was treated with amplification-grade DNase I (Invitrogen). Oligo(dT) primed cDNA was prepared from this RNA using Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed with SYBER green supermix (Bio-Rad, Hercules, CA, USA) using three sets of human eNOS primers (F1–R1, F2–R2, and F3–R3, R1: 5′-GGAAGCGGGTGGCAGTAGG-3′, R2: 5′-ACTTTGGGAGGCTG AGGCG-3′, R3: 5′-CTAAATGGAAGGGCGACAGTGA-3′) and one set of rRNA primers (forward: 5′-CGGCGGCTTTGGTGACTCTA-3′, reverse: 5′-CTTGGATGTGGTAGCCGTTTCTC-3′). The amount of each human eNOS RNA was standardized by using rRNA. Statistical analyses were performed by Student's *t*-test.

Results

Sequence analysis

Sequence analysis of human eNOS genomic DNA (GenBank Accession No. AF519768) downstream of the human eNOS stop codon showed several putative functional motifs (Fig. 1). Although there were no polyadenylation signal sequences (AATAAA), four AATAAA-like sequences (two ACTAAA, one AGTAAA, and one TATAAA) were observed; these sequences are found in other human genes [7] and function as polyadenylation signals [25]. Further, three GT-rich regions that contribute to polyadenylation were found in the downstream region, consistent with the existence of additional polyadenylation sites in this region. Four AT-rich elements (ATTTA) and 18 CCTCC or CCTCT (CCTCC/T) motifs were noted; four AU-rich elements (AREs) and 18 CCUCC or CCUCU (CCUCC/U) motifs may affect mRNA stability [23,24]. Previous reports have demonstrated that human eNOS mRNA possessed two AREs and eight CCUCC/U motifs in the 3′-UTR [20,22]. In this analysis, two additional AREs and 10 CCUCC/U motifs were found in the downstream region.

3′-RACE analysis

Fig. 2 shows the results of the 3′-RACE analysis carried out using human aortic cDNA. Products with variable sizes were amplified using the F1 primer for qualitative

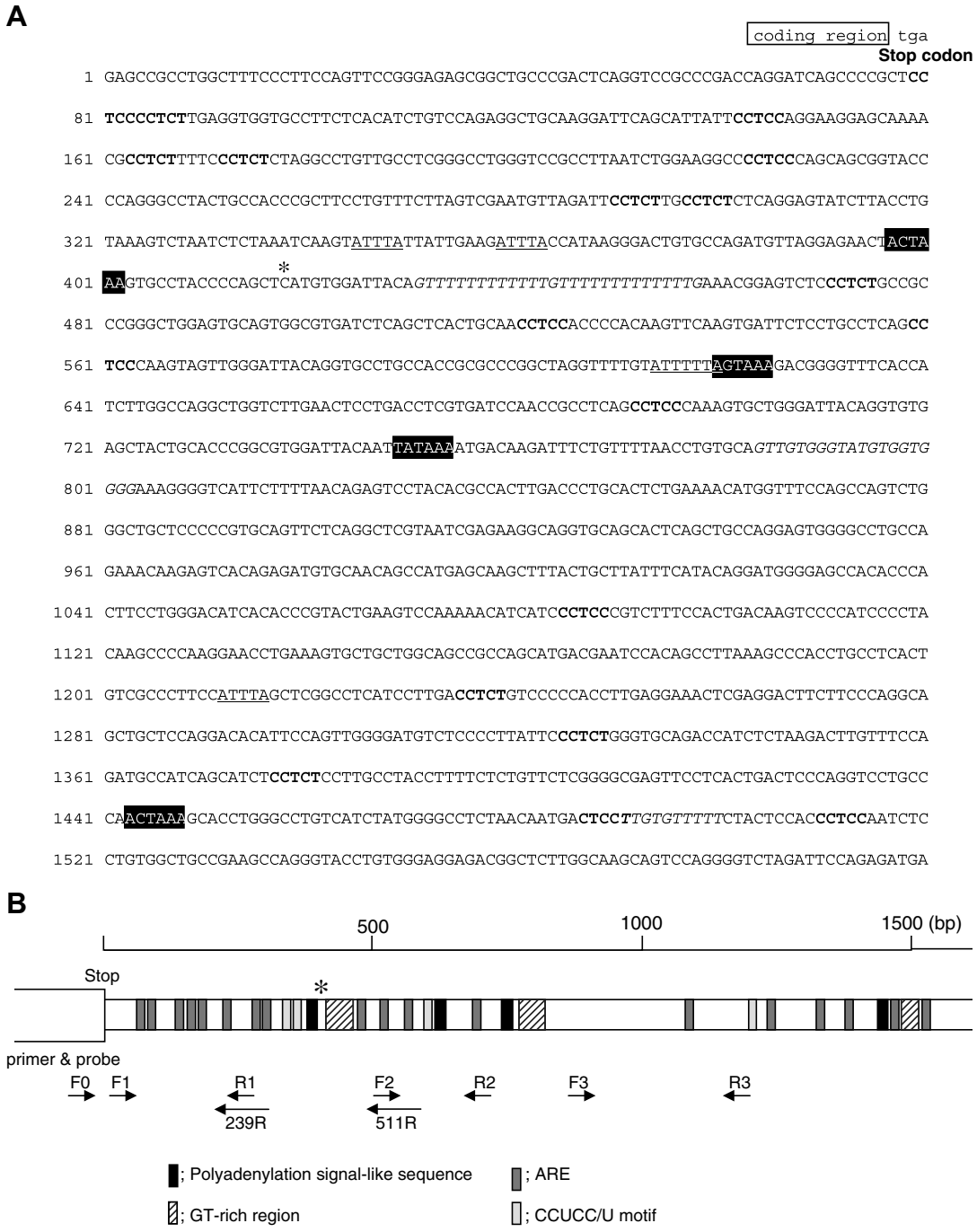

Fig. 1. Structure of genomic DNA downstream of human eNOS stop codon. (A) Nucleotide sequence (GenBank Accession No. AF519768); an asterisk indicates previously reported polyadenylation site. Polyadenylation signal-like sequences, GT-rich regions. AREs, and CCUCC/U motifs are inverted, italicized, underlined, and bolded, respectively. (B) Schematic representation; an asterisk indicates a previously reported polyadenylation site. Polyadenylation signal-like sequences, GT-rich regions. AREs, and CCUCC/U motifs are represented by closed-, hatched-, shaded-, and dark-boxes, respectively.

nested amplification. A 450-bp product (Fig. 2A-a) was predicted from a reported polyadenylation site [22], termed the ''proximal site''. Some products were amplified using other nested primers located downstream of a reported polyadenylation site (F2 and F3). A 300-bp product in the F2 amplification corresponded to a 800-bp product (Fig. 2A-b) in the F1 amplification, consistent with the existence of an polyadenylation site located 800-bp downstream from the stop codon, which is termed here distal site 1. A 880-bp product of the F3 amplification corresponded to a 1200-bp product in the F2 amplification and a 1600-bp product (Fig. 2A-c) in the F1 amplification, suggesting the existence of an polyadenylation site located 1600-bp downstream from the stop codon, termed here distal site

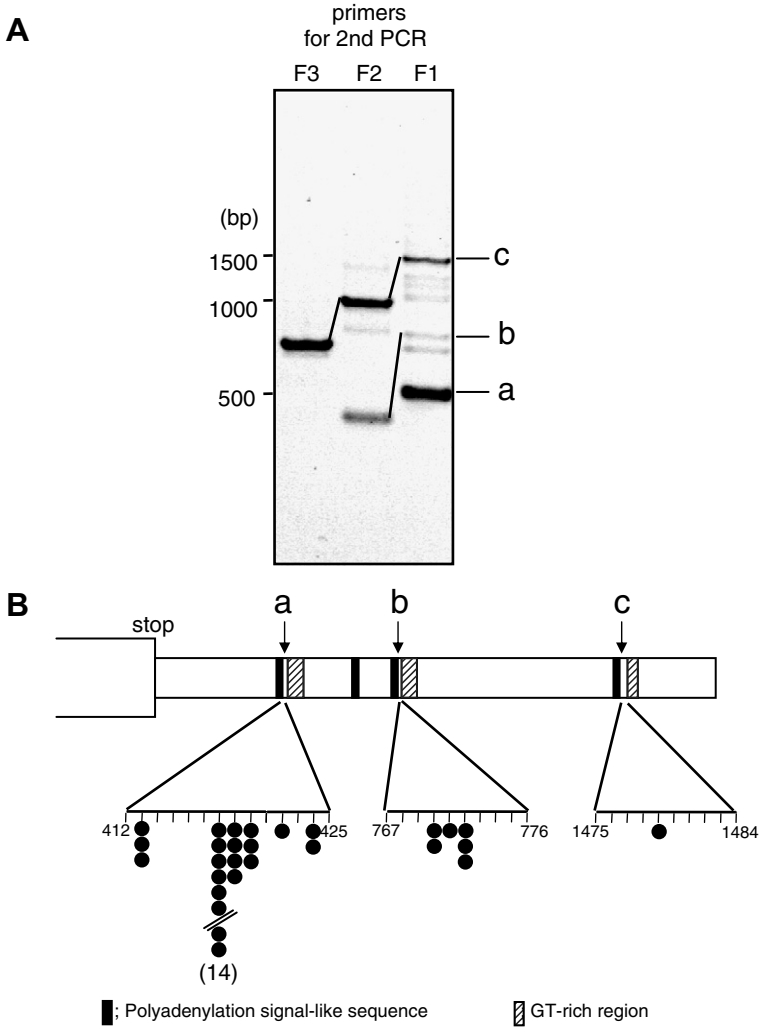

Fig. 2. 3′-RACE analysis of human eNOS mRNA. (A) Nested PCR analysis: after amplification of human eNOS with primers F0-AP1 from human aortic cDNA, primers F1–AP2, F2–AP2, and F3–AP2 were used for nested PCR. The products with same polyadenylation sites are linked with line. Bands a, b, and c were the products with the proximal site, distal site 1, and distal site 2, respectively. (B) Sequence analysis: the products of the first amplification were cloned and sequenced to determine their polyadenylation sites. The polyadenylation site of each clone is indicated by the closed circle. Of 34 clones, 14 have previously reported polyadenylation site.

2. Sequence analysis confirmed that these products were specific to human eNOS and were polyadenylated (data not shown).

In the cloning experiment using the F0 primer, polyadenylation sites were determined for a total of 34 independent polyadenylated eNOS-specific clones (Fig. 2B). A predominant polyadenylation site was seen in 14 of 36 clones 418 bp downstream from the stop codon, as had been previously reported [22]. Further, other additional sites in the vicinity of this predominant site were observed, plus six clones near distal site 1 and at distal site 2, all consistent with the results of nested 3′-RACE analysis and predictable from sequence analysis (Fig. 1).

Northern blotting

Fig. 3 shows a Northern analysis of human eNOS mRNA with variable lengths of 3′-UTR in different tissues. Amounts of total human eNOS mRNA (hybridized with probe 239R) were in descending order, placenta, lung, kidney, heart, liver, brain, trachea, cerebellum, skeletal muscle, and pancreas. Amounts of human eNOS mRNA with long 3′-UTR (hybridized with probe 511R) were in descending order, placenta, cerebellum, lung, brain, kidney, heart, liver, trachea, pancreas, and skeletal muscle. Ratios of transcripts with long 3′-UTRs to the total eNOS mRNA differed among tissues. In particular, the ratio of the transcripts with long 3′-UTR to total eNOS was notably higher in the human brain and cerebellum than other tissues, consistent with specific tissue-related functions of eNOS mRNA isoforms.

Expression of eNOS mRNA isoforms during cell growth

To evaluate the functional significance of human eNOS mRNA with 3′-UTR of different lengths, expression in

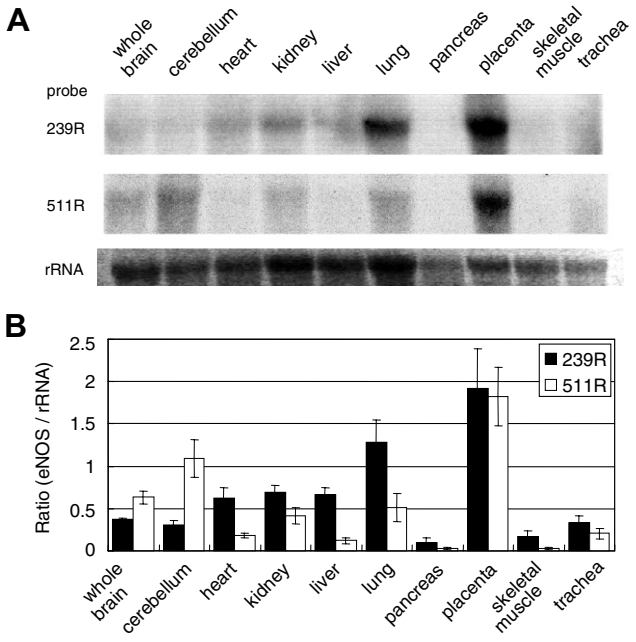

Fig. 3. Expression of human eNOS mRNA with long 3′-UTR in human tissues. (A) Northern blot were hybridized with [α-^{32}P]dATP-labeled probes 239R and 511R for the detection of total eNOS mRNA and that with long 3′-UTR, respectively. 28s rRNA were visualized with ethidium bromide staining. (B) Band intensities were normalized using 28s rRNA.

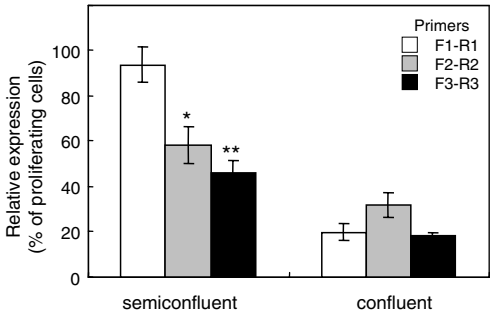

Fig. 4. Regulation of human eNOS mRNA with different lengths of 3′-UTR during cell growth. RNA from proliferating, semiconfluent, and confluent normal human aortic endothelial cells was reverse transcribed. Human eNOS mRNA 3′-UTR of different lengths was quantified by real-time PCR. The amount of each human eNOS mRNA was standardized using rRNA. The relative expression is given as percentage of that of proliferating cells. The data represent mean of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

human aortic endothelial cells during cell growth was examined using quantitative PCR, with specific primers for each region (Fig. 4). Total eNOS mRNA expression, amplified using primers F1-R1, was less in confluent than in proliferating cells. Compared to the proliferating state, the expression levels of eNOS mRNA had decreased to 90% and 20% at the semiconfluent (70–90% confluent) and confluent states, respectively. Similar results were obtained with eNOS mRNA with long 3′-UTRs, that had been amplified using primers F2–R2 and F3–R3. These mRNAs, however, decreased more dramatically than did total eNOS mRNA, as cells reached confluence. Compared to the proliferating state, eNOS mRNA levels were 55% and 30% (F2–R2 amplification), or 46% and 18% (F3–R3 amplification), in semiconfluent and confluent states, respectively.

Discussion

We investigated human eNOS 3′-UTR to evaluate its potential regulatory roles and report here that human eNOS mRNA has multiple alternative polyadenylation sites. In addition, we demonstrated that the relative amounts of individual mRNAs differed among human tissues and their levels were differentially altered during cell growth.

Several mechanisms are used to generate a variety of transcripts from a single gene, including the alternative splicing of pre-mRNAs, selection of alternative polyadenylation sites, and the use of different transcription start sites [26–28], but there are few reports of diversity with respect to human eNOS mRNA [2]. Recent computational analysis of the 3′ ends of the expressed sequence tags identified alternative polyadenylation sites in human and mouse tissues [29], and a database for mammalian mRNA polyadenylation, namely, PolyA_DB (http://polya.umdnj.edu) [8], was established. All human eNOS polyadenylation sites in this database are located near the reported proximal site, 418 bp downstream from the stop codon [8]. They are 413, 415, 417, and 424 bp downstream from the stop codon. Generally, polyadenylation sites are defined by upstream and downstream elements [5]. The best-known upstream element is the hexameric polyadenylation signal, which is 10–30 nucleotides upstream of the cleavage sites. Although AAUAAA (AATAAA in gene) is the most common polyadenylation signal, single-nucleotide variants have been demonstrated to play similar roles in polyadenylation [6]. No AATAAA hexamers were observed downstream of the human eNOS gene termination codon; however, there are four single-nucleotide variants (two ACTAAA, one AGTAAA, and one TATAAA) that can function as polyadenylation signals [25]. A GU (GT in gene)-rich region 20–40 nucleotides downstream of the cleavage site has been identified as the downstream element for polyadenylation [5]. Our sequence analysis revealed three GT-rich elements in the region downstream of the human eNOS gene. Apart from reporting the proximal sites, we identified two additional distal sites approximately 770 and 1478 bp downstream of the stop codon using 3′-RACE analysis. Both these sites are between the upstream AATAAA-like signal and the downstream GT-rich region. Therefore, two newly identified polyadenylation sites in human eNOS mRNA are quite consistent with the rules of polyadenylation.

We found that amounts of eNOS mRNA was differentially changed during growth of cultured human aortic endothelial cells, and the mRNAs with long 3′-UTRs decreased more rapidly than the total mRNA, as cells approached confluency. Generally, there are *cis*-elements

in the 3′-UTRs of mammalian mRNA that control mRNA levels [4,24]. In bovine eNOS mRNA of endothelial cells, the UC-rich region at the 5′-end of the 3′-UTR was identified as the *cis*-element involved in TNF-α-induced destabilization through its binding of a 60-kDa cytosolic protein, which was increased by TNF-α treatment [13,14]. It was also shown that bovine eNOS mRNA was less in confluent than in growing bovine aortic endothelial cells [30]. A 43-nucleotide region at the origin of the bovine eNOS 3′-UTR is critical in destabilization of the bovine eNOS mRNA, and a 51-kDa cytosolic proteins, recently identified as globular actin [21], binds to this region [15]. In the endothelial cells of the human umbilical vein, three ribonucleoprotein complexes (RNPs) of approximately 66, 56, and 53 kDa were identified in cytoplasmic extracts [20]. Amounts of the 56 kDa protein, which has an affinity for a CU-rich RNA, were increased by TNF-α treatment of cells and binding of this protein to the CU-rich region in human eNOS 3′-UTR was required for the TNF-α-induced mRNA downregulation. The long 3′-UTRs in human eNOS mRNA that are identified in this study contains two additional AREs and eight CCUCC/U elements. These elements could be involved in the control of human eNOS mRNA levels, due to differences in stability of mRNA with 3′-UTRs of various lengths.

This study provides evidence of alternative polyadenylation sites in human eNOS mRNA, which result in 3′-UTRs of variable lengths that can contribute to the control of human eNOS activity. Regulation of eNOS gene expression has been associated with several human diseases, and the regulation of alternative polyadenylation of human eNOS mRNA might well contribute to disease phenotypes. Further clarification of the molecular mechanisms that control human eNOS mRNA structure, function, and amount is clearly important.

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