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# Molecular beacons can assess changes in expression and 3'-polyadenylation of human eNOS mRNA

Rachel Jones,<sup>1</sup> Meredith B. Baker,<sup>1</sup> Martina Weber,<sup>1</sup> David G. Harrison,<sup>1</sup> Gang Bao,<sup>2\*</sup> and Charles D. Searles<sup>1\*</sup>

<sup>1</sup>Division of Cardiology, Emory University School of Medicine, Atlanta, Georgia; and <sup>2</sup>Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia

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**Jones R, Baker MB, Weber M, Harrison DG, Bao G, Searles CD.** Molecular beacons can assess changes in expression and 3'-polyadenylation of human eNOS mRNA. *Am J Physiol Cell Physiol* 296: C498–C504, 2009. First published December 24, 2008; doi:10.1152/ajpcell.00462.2008.—The endothelium plays an essential role in maintaining vascular homeostasis, and it fulfills this role by modulating intracellular signaling and gene expression in response to chemical and mechanical stimuli. Assessing changes in endothelial gene expression is essential to understanding how physiological and pathophysiological processes modulate vascular homeostasis. Here we describe the use of molecular beacons to rapidly and quantitatively assess expression and 3'-polyadenylation of a gene that is important for vascular homeostasis, endothelial nitric oxide synthase (eNOS). Single- and dual-fluorescence resonance energy transfer (FRET) molecular beacon hybridization assays were developed to measure changes in mRNA levels and 3'-polyadenylation, respectively, in primary human endothelial cell cultures subjected to laminar shear stress or statin treatment. Optimized beacon hybridization assays took ~15 min to perform, and eNOS mRNA levels were validated by quantitative real-time RT-PCR. Competitive inhibition assays and posttranscriptional silencing of eNOS expression were used to verify the specificity of molecular beacon fluorescence. Finally, the dual-FRET method was used to assess eNOS polyadenylation in tissues isolated from mice subjected to exercise training. These data demonstrate that molecular beacons can be used to rapidly and efficiently measure endothelial gene expression and 3'-polyadenylation. This approach could easily be adapted for studies of other endothelial genes and has promise for applications in live endothelial cells.

endothelial nitric oxide synthase; gene expression; dual-fluorescence resonance energy transfer

IN BLOOD VESSELS, THE ENDOTHELIUM functions as both a physical barrier and a metabolically active secretory tissue that responds to various chemical and mechanical stimuli. To accomplish this it must tightly regulate expression of its genes. Endothelial nitric oxide synthase (eNOS) is an example of a gene whose expression is regulated and essential for vascular function. The human eNOS gene is composed of 26 introns spanning 21 kb of genomic DNA on chromosome 7q35–36 (14), and its expression has been shown to be modulated by a number of stimuli implicated in vascular pathophysiology (24). Commonly, the effect of a stimulus on eNOS mRNA expression is measured by quantitative real-time RT-PCR (qRT-PCR) of spliced transcripts, although recent studies have demonstrated

that there is a need to assess alternatively spliced (13) or polyadenylated transcripts (34). In the current study, we describe the use of molecular beacon-based methods to assess eNOS expression and 3'-polyadenylation.

qRT-PCR requires multiple steps, including a reverse transcriptase reaction to make cDNA, and PCR reaction that is tracked by dyes or fluorophores. This method may require some effort to obtain accurate results, and the necessary reagents are expensive. Recently, our group has described the use of ribonuclease protection assays to assess changes in expression of polyadenylated eNOS transcripts (34). This method is lengthy and is limited in its ability to provide a quantitative assessment of polyadenylated transcripts. Given these current methods' limitations, we sought to develop other sensitive, fast, and efficient methods to assess eNOS mRNA levels and 3'-polyadenylation.

Molecular beacons are oligonucleotide (DNA or RNA) hairpin probes designed to hybridize to specific mRNA sequences (31). These 15- to 25-nucleotide (nt) probes have fluorophore and quencher moieties on opposing ends of the oligonucleotide; in the absence of complementary mRNA target, the probe forms a stem-loop hairpin structure that leads to quenching of the fluorophore. Hybridization of the beacon with target RNA sequence opens the hairpin, thus physically separating the fluorophore from the quencher, and allows the fluorophore to fluoresce upon excitation (21). We designed molecular beacons that target both eNOS mRNA and its poly(A) tail. Subsequently, we developed solution assays that result in optimal beacon-target hybridization and found that this approach can rapidly and accurately quantify both the amount of eNOS mRNA and the extent of eNOS 3'-polyadenylation in a heterogeneous RNA sample. To our knowledge, this is the first report of a molecular beacon-based approach to quantify changes in mRNA processing.

## METHODS

**Tissue culture.** Human umbilical vein endothelial cells (HUVECs; Genlantis) were cultured in endothelial cell growth medium (Genlantis) supplemented with 20% fetal calf serum, 13.3 U/ml heparin, 40 µg/ml endothelial mitogen (Biomedical Technologies), 1% L-glutamine, and penicillin-streptomycin. Postconfluent HUVECs between passages 2 and 6 were used for experiments. A cone-in-plate viscometer with a 1° angle (2) was used to shear cells (15 dyn/cm<sup>2</sup>), or cells were treated with mevastatin (10 µM, 24 h).

**RNA isolation.** Total cellular RNA was isolated using the RNeasy Mini and QIAshredder kits (Qiagen), including the DNase I digestion

\* G. Bao and C. Searles contributed equally to this work.

Address for reprint requests and other correspondence: C. Searles, Div. of Cardiology, Emory Univ. School of Medicine, Atlanta, GA 30322 (e-mail: csearle@emory.edu); G. Bao, Dept. of Biomedical Engineering, Georgia Inst. of Technology and Emory Univ., Atlanta, GA 30322 (e-mail: gang.bao@bme.gatech.edu).

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step. RNA was isolated from mouse tissues using TRIzol reagent (Invitrogen). Total RNA concentration was measured by ultraviolet absorbance at 260 and 280 nm and subsequently stored in aliquots at  $-80^{\circ}\text{C}$ .

**Real-time RT-PCR.** Real-time RT-PCR was performed as described previously (29). All results were normalized to GAPDH. PCR primers for eNOS were 5'-CACATGTTTGTCTGCGG-3' and 5'-GAGG-GCCTTCCAGATTAAG-3'. GAPDH primer sequences were 5'-ATGGCACCCTCAAGGCTGAGAAC-3' and 5'-GTTGCTGTAGC-CAAATTCGTTGTC-3'. SuperScript III First-Strand Supremix was used for RT reactions, and qRT-PCR was performed with Platinum Sybreen qPCR Supremix (Invitrogen) on a LightCycler (Roche).

**Molecular beacon design and synthesis.** For targeting eNOS mRNA, a single molecular beacon was designed using the mFOLD program (36) to analyze secondary structures of human eNOS mRNA for open, single-stranded sequence. On the basis of this analysis, a 20- to 30-nt region in the eNOS 3'-untranslated region (UTR) was selected and subjected to Basic Local Alignment Search Tool analysis (National Center for Biotechnology Information) to rule out any significant homology with other genomic sequence. This region of eNOS was then used to create an oligonucleotide primer (Sigma-Genosys) for RT-PCR. RT-PCR was performed using Ready-to-Go RT-PCR beads (Amersham) and total RNA from HUVECs. This step was performed to verify the specificity of the primer for its target; the primer was judged to be specific for eNOS if RT-PCR produced a single band matching the size of the positive control on subsequent agarose gel electrophoresis (not shown). Once these criteria were fulfilled, the sequence was incorporated into a molecular beacon as the loop segment. A 5-nt stem sequence (containing at least 75% G/C base-pairs) was added to each end of the loop to form the stem. The fluorophore 6-carboxyfluorescein (6-FAM; excitation 488 nm, emission 520 nm) and quencher black-hole quencher 1 (BHQ-1) were added to have the following final molecular beacon design: 5'-6FAM-CCTGGAGTCCCTTATGGTAAATCTTCCCAGG-BHQ1-3'; underlined bases indicate the stem sequences.

To assess eNOS 3'-polyadenylation, dual-fluorescence resonance energy transfer (FRET) molecular beacons were designed to be complementary to the distal 3'-sequence of the eNOS 3'-UTR and the poly(A) tail. The dual-FRET fluorophores were Cy3 (donor beacon, with excitation wavelength of 545 nm and emission wavelength of 570 nm) and Cy5 (acceptor beacon, with excitation wavelength of 645 nm and emission wavelength of 670 nm); both beacons had the Black-Hole Quencher 2 (BHQ-2) as the quencher. The sequences of these beacons are as follows: 5'-Cy5-CCTAAAGTTTTTGTAGCTGGGGTAGGCACITTAGG-BHQ2-3' (eNOS beacon) and 5'-BHQ2-GAGCGTTT-TTCGCTC-Cy3-3' (poly-T beacon); underlined bases indicate the stem sequences.

The eNOS-targeting molecular beacons were synthesized by Sigma-Genosys, and the poly-T beacon was synthesized by Tri-Link Biotechnologies (San Diego, CA).

**Beacon hybridization assay.** For all single-beacon hybridization experiments, buffer containing 2.5 M NaCl was used, while 5 M NaCl was used for all dual-beacon experiments (dual-FRET). This high NaCl concentration of the assay buffer was necessary to achieve good detection specificity (Supplemental Fig. 1; supplemental material for this article is available online at the *American Journal of Physiology-Cell Physiology* website). For the single-beacon assays, 500 ng of total RNA was used, and the final concentration of beacon in the binding mixture was 500 nM. In dual-FRET beacon experiments, the concentration of both beacons was 250 nM. Negative control experiments were performed with yeast RNA or a nonspecific beacon (Supplemental Fig. 2). Background signal was assessed by performing the assays with beacon alone; specific signal was calculated by subtracting this background fluorescence from total fluorescence (beacon plus endothelial RNA). For the hybridization, reagents were mixed in flat-bottomed, black, nonbinding sterile 384-well plates

(Fisher Scientific) and were incubated for 15 min at  $37^{\circ}\text{C}$ . This incubation time was based on a separate experiment where beacon fluorescence was assessed after different incubation periods (Supplemental Fig. 3). Beacon fluorescence was determined immediately with a Safire fluorescent microplate fluorometer (Tecan, Zurich) with dual excitation-emission functions. Each hybridization assay was performed in triplicate, and each set of experiments was repeated three to six times. A schematic depiction of the molecular beacon hybridization assays is shown in Fig. 1.

**Blocking oligonucleotide.** The blocking oligonucleotide sequence was 5'-AGTCCCTTATGGTAAATCTTC-3', and, in experiments to assess specificity of beacon signal, it was used in threefold molar excess to that of beacon.

**Posttranscriptional silencing of eNOS.** Prevalidated small interfering RNA (siRNA) for eNOS and control siRNA were purchased from Qiagen. Twenty micromolar siRNA targeting eNOS or 20  $\mu\text{M}$  control siRNA (scrambled) was incubated with Oligofectamine (Invitrogen) and Opti-Mem reagent (Invitrogen) and was then added to HUVECs for 4 h. The media were then replaced, and cells were cultured normally overnight. The transfection process was repeated the following morning; RNA was extracted 24 to 48 h after the second transfection.

**Animals.** Animal studies were approved by the Emory University Institutional Animal Care and Use Committee in accordance with the guidelines set forth by the National Institutes of Health in the *Guide*

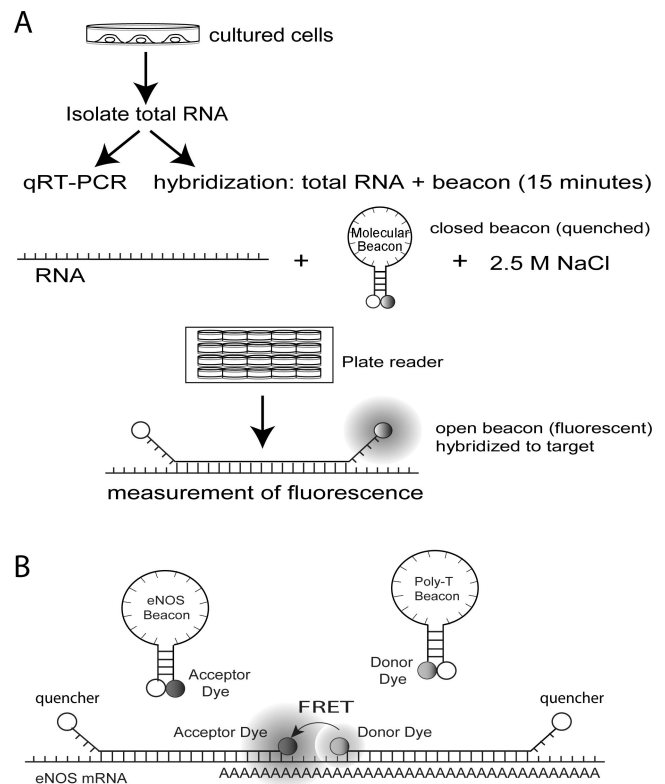


Fig. 1. **A:** single-beacon hybridization assay. Total RNA was extracted from endothelial cells and hybridized to a molecular beacon targeting endothelial nitric oxide synthase (eNOS) mRNA. Hybridizations were performed in a 384-well microplate with buffer containing 2.5 M NaCl, and fluorescence was assessed in a microplate fluorometer. qRT-PCR, quantitative real-time RT-PCR. **B:** schematic of dual-fluorescence resonance energy transfer (FRET) beacon hybridization assay for assessing eNOS 3'-polyadenylation. The FRET signal requires simultaneous hybridization of eNOS beacon (acceptor) and poly-T beacon (donor) to adjacent sequences on the same eNOS mRNA, since only the donor dye on the poly-T beacon is directly excited, and the emission from the acceptor dye on the eNOS beacon due to FRET is detected.

for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996). Twelve- to eighteen-week-old mice (B6129PF2/J) were exercised as described previously (1).

**Data analysis and statistics.** For both single and dual-FRET molecular beacon assays, background fluorescence values were expressed relative to control values. Unpaired *t*-tests were used to analyze differences between sheared or statin-treated cells and control cells.

## RESULTS

**Changes in eNOS expression in response to laminar shear stress.** Laminar shear stress has been shown to be a potent regulator of eNOS mRNA expression both in vitro (19, 30, 32) and in vivo (15, 18, 25). We wanted to determine whether molecular beacons could accurately quantify the effect of laminar shear stress on eNOS mRNA levels. HUVECs were exposed to laminar shear (unidirectional, 15 dyn/cm<sup>2</sup>) or static conditions for 24 h, and RNA extracted from these cells was subjected to a single-beacon hybridization assay. Figure 2A shows a three- to fourfold increase in beacon signal that was detected in RNA from sheared cells compared with static cells. This signal was due to beacon hybridization to eNOS mRNA, and the efficacy of the beacon-based approach for measuring eNOS mRNA was verified by qRT-PCR. The shear-induced

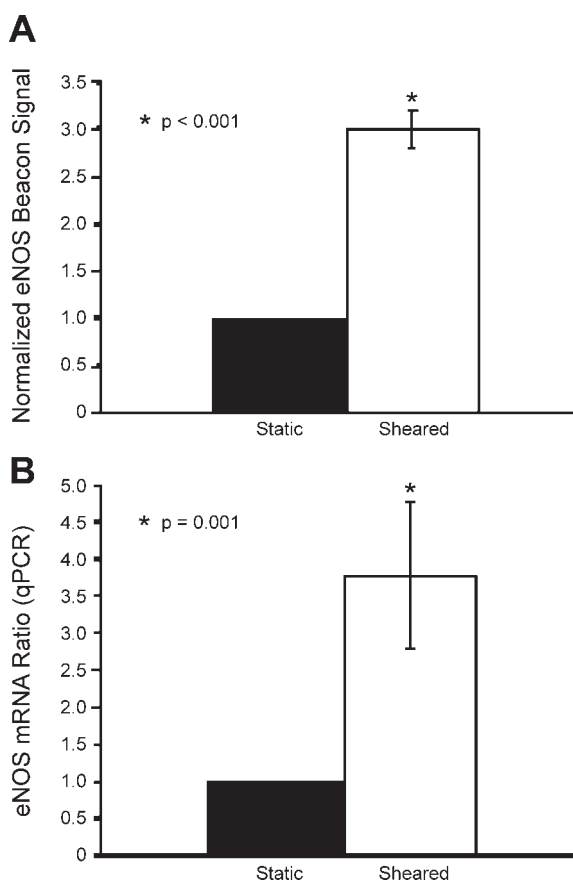


Fig. 2. Changes in eNOS mRNA expression in human umbilical vein endothelial cells (HUVECs) exposed to laminar shear stress. *A*: fluorescence signal from single beacons hybridized to eNOS mRNAs extracted from static control and sheared cells (15 dyn/cm<sup>2</sup>, 24 h), expressed as ratio to control (RNA from static cells) beacon signal ( $n = 3$ ). *B*: qRT-PCR (qPCR) of the same mRNA sample used for beacon hybridization assays, expressed as ratio to static control values.

increase in eNOS mRNA observed by the two methods was similar (Fig. 1B).

**Specificity of molecular beacon signal.** We used a competitive inhibition strategy to examine the signal specificity of single-beacon hybridization to eNOS mRNA. A blocking oligonucleotide composed of the same sequence as the beacon loop section (probe sequence) but lacking the fluorophore, stem, and quencher was added in threefold molar excess to the beacon-target mixture. The addition of blocking oligonucleotides resulted in a dramatic attenuation of fluorescence intensity (Fig. 3A), suggesting that the beacon was specific for eNOS mRNA.

To further assess beacon specificity in detecting eNOS mRNA, siRNA was used to knock down eNOS mRNA in endothelial cells. As shown in Fig. 3B, specific knockdown of eNOS mRNA resulted in a large decrease in beacon signal compared with controls. The magnitude of eNOS silencing was verified by qRT-PCR (Fig. 3C).

**eNOS expression in response to mevastatin.** In both cell culture and animal studies, 3-hydroxy-3 methylglutaryl coenzyme A reductase inhibitors (statins) have been shown to increase eNOS expression (1, 5, 10–12, 27). To determine whether the single-beacon binding assay could be used to accurately measure changes in eNOS expression in response to statin treatment, beacons were incubated with RNA from HUVECs that had been incubated with regular media or media supplemented with mevastatin (10  $\mu$ M) for 24 h. We found a twofold increase in beacon signal due to hybridization to eNOS mRNA from mevastatin-treated cells compared with that from control cells (Fig. 4A). The magnitude of change obtained by this method was similar to that obtained by qRT-PCR (Fig. 4B).

**Dual-FRET beacons to quantify 3'-polyadenylation of eNOS mRNA.** Figure 1 illustrates the dual-FRET molecular beacon strategy to assess 3'-polyadenylation of eNOS mRNA. The donor beacon is a "poly-T" beacon with a 40-nt hybridization domain that binds to long poly(A) tails of an mRNA. The acceptor beacon, an eNOS-specific beacon, targets the first 6 nt of the poly(A) tail and adjacent sequence in the eNOS 3'-UTR. In the presence of eNOS mRNA with a long poly(A) tail (>25 nt), both beacons will bind, and excitation of the donor dye (on poly-T beacon) leads to an emission from the acceptor dye (on eNOS beacon) due to FRET. In the presence of eNOS mRNA with a short (<25 nt) poly(A) tail, the donor beacon will not be able to hybridize to the target; thus no FRET signal is possible under donor excitation.

Figure 5 shows the results of both single-beacon and dual-FRET hybridization assays, using the beacons described above. The single-beacon assay with the eNOS-specific (acceptor) beacon (Cy5 excitation and emission) was performed to verify its specificity, and its fluorescence signal indicated changes in total (short and long polyA) eNOS expression. Similar to our earlier finding, there was a threefold increase in beacon signal due to hybridization to eNOS mRNA from sheared cells compared with static control cells. Figure 5B shows the results from the dual-FRET beacon hybridization assay (Cy3 excitation, Cy5 emission); the FRET signal represents only those eNOS transcripts with long poly(A) tails (>30 adenosines). Compared with control (RNA from nonsheared cells), there appeared to be a 3.5-fold increase in dual-FRET beacon fluorescence in assays of sheared cells, indicating significant en-

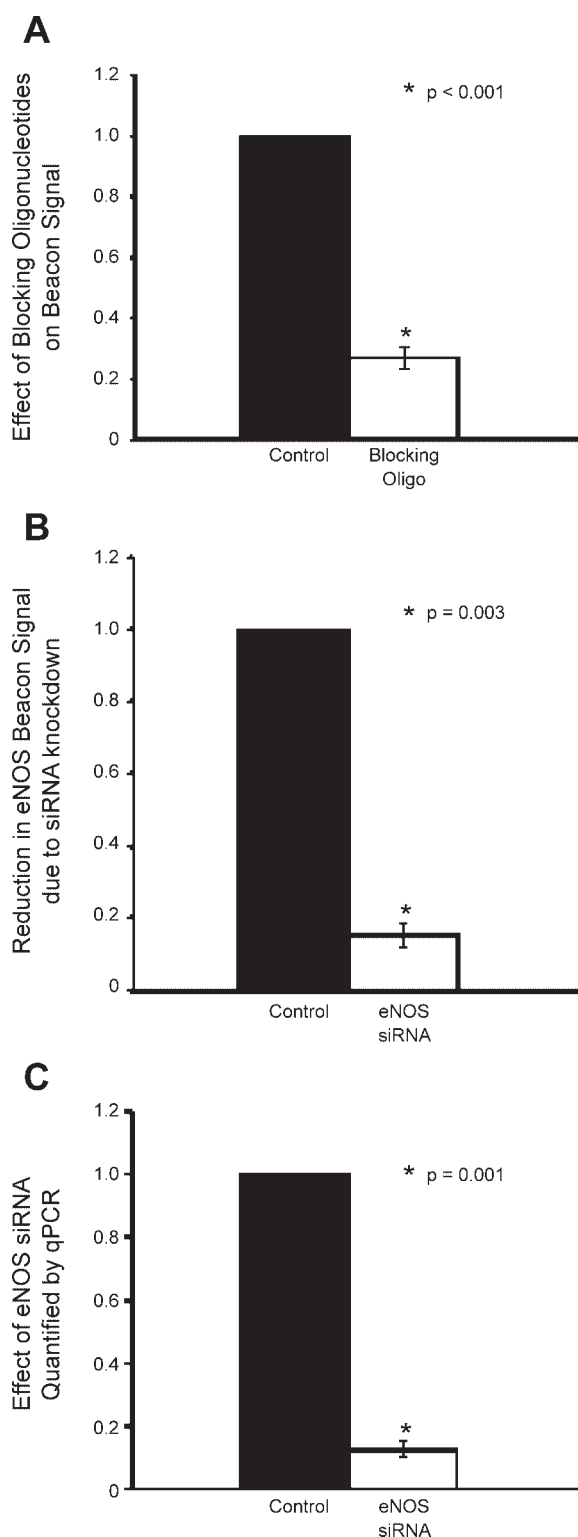


Fig. 3. Specificity of single-beacon signal for eNOS mRNA. *A*: fluorescence signal from single beacons hybridizing to mRNA from HUVECs with and without blocking oligonucleotides added to the assay mixture. The result is expressed as ratio to signal from beacon hybridization in absence of blocking oligonucleotides ( $n = 4$ ). *B*: fluorescence signal from single beacons binding to mRNA from HUVECs in which eNOS had been posttranscriptionally silenced, expressed as ratio to beacon signal from control mRNA [from cells transfected with nonspecific small interfering (si)RNA]. *C*: qRT-PCR of RNA from siRNA-transfected cells, expressed as ratio to control mRNA values.

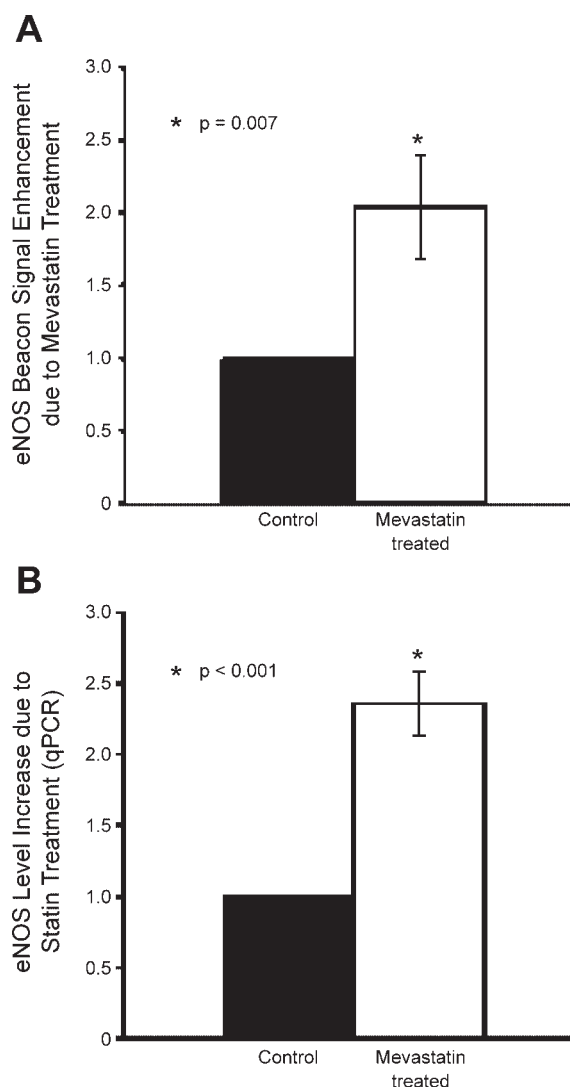


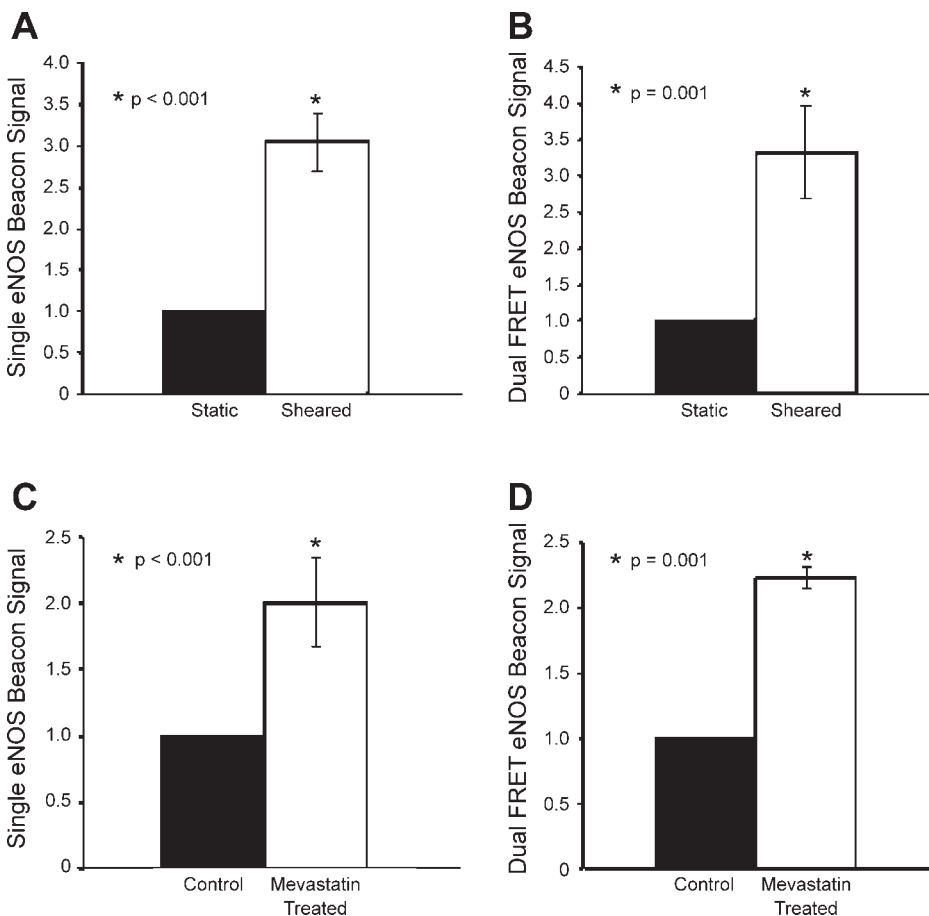
Fig. 4. Changes in human eNOS mRNA expression in response to statin treatment. *A*: fluorescence signal from single beacons hybridizing to eNOS mRNA extracted from either untreated or statin-treated HUVECs (mevastatin, 10  $\mu$ M, 24 h,  $n = 3$ ), expressed as ratio to beacon signal from control cells (mRNA from untreated cells). *B*: qRT-PCR result of the same RNA sample used for the beacon hybridization assays, expressed as ratio to control.

hancement of eNOS 3'-polyadenylation in response to shear stress.

A similar series of assays were performed with mRNA from cells treated with mevastatin (Fig. 5, *C* and *D*). Again, we observed an increase in probe hybridization signal for both total eNOS transcripts and eNOS transcripts with long poly(A) tails (Fig. 5*D*), findings that are compatible with our previous results (7). These data indicate that the sensitivity and specificity of the dual-FRET approach is sufficient for accurate assessment of changes in mRNA 3'-polyadenylation that occur in response to different stimuli.

To validate the specificity of the poly-T beacon for transcripts with poly(A) tails >30 adenosines, a series of hybridization assays was performed on synthetic poly-A oligomer targets that were 10 to 40 adenosines in length. The results of these hybridization assays are shown in Fig. 6. Points on a curve depict fluorescence signal level of the poly-T beacon

Fig. 5. Fluorescence signal from single and dual-FRET beacons hybridizing to eNOS mRNA from HUVECs exposed to laminar shear stress or treated with mevastatin. *A* and *C*: fluorescence signal due to hybridization of eNOS beacon to mRNA from sheared or statin-treated cells, expressed as ratio to control signal ( $n = 3$ ). This beacon is the same as the acceptor beacon in the dual-FRET beacon assays and should hybridize to all eNOS transcripts. *B* and *D*: fluorescence signal due to dual-FRET beacon hybridizing to eNOS mRNA from sheared or statin-treated cells, expressed as ratio to control signal ( $n = 3$ ). The signal detected in this assay was produced by simultaneous hybridization of the eNOS beacon and the poly-T beacon to polyadenylated eNOS transcripts.



when assayed with progressively longer poly-A oligomer targets, and each curve is based on fluorescence signal grouped by the concentration of the poly-A oligomer in the assay mixture (normalized to the molar concentration of the 10 A- oligomer). For poly-A targets of 10 to 20 adenosines in length, poly-T

beacon fluorescence signal level was very low, regardless of oligomer concentration. Fluorescence signal significantly increased when the target oligomer had 25 adenosines or more, particularly at higher oligomer concentrations (8–32 ng). These data support the specificity of the poly-T beacon for eNOS transcripts with tails >25 adenosines in length. In fact, the transcripts that elicit dual-FRET signal in our assay must

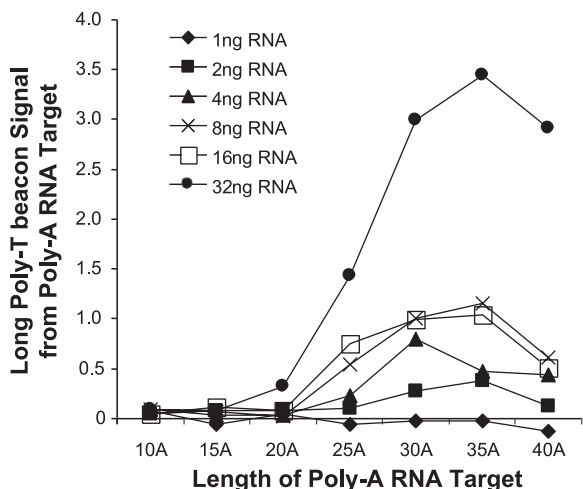


Fig. 6. Fluorescence signal due to poly-T beacon hybridizing to poly-A oligomers of varying length. Seven different poly-A oligomers (10–40 adenosines) were assessed. Assays were performed on increasing concentrations of poly-A oligomer (1 ng–32 ng;  $n = 3$  for each concentration). The concentration of each different-length poly-A oligomers was equimolar to the concentration of the 10-adenosine oligomer.

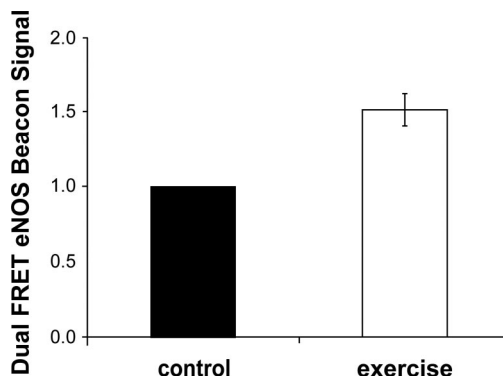


Fig. 7. Fluorescence signal from dual-FRET beacons hybridizing to eNOS mRNA from lungs of mice subjected to 2 wk of exercise training. Mice were exercised as described previously (1). Assay conditions were similar to those described above for human endothelial cells. Freshly isolated mouse lung RNA was hybridized with the poly-T beacon and an acceptor beacon (Cy5) designed to target the distal 3'-sequence of the mouse eNOS 3'- untranslated region. Fluorescence intensity is expressed as ratio to signal from control (sedentary) mice ( $n = 2$ ).

have tails >30 adenosines, because the acceptor beacon occupied the first 6 adenosines of the poly(A) tail.

**Dual-FRET beacons to quantify 3'-polyadenylation of eNOS mRNA in mouse tissues.** To determine whether we could assess eNOS polyadenylation in freshly isolated tissue samples, we isolated total RNA from the lungs of mice subjected to 2 wk of exercise training. Exercise training, which increases cardiac output, has been used as an in vivo model for increased laminar shear stress in dogs, mice, rats, and pigs (6, 25, 26, 28, 35), and we have observed that exercise training results in increased eNOS expression in mice (1). Similar to the effect of laminar shear stress on eNOS polyadenylation in vitro, we found that exercise enhanced eNOS polyadenylation in lung tissues of exercised mice (Fig. 7). This increase in eNOS polyadenylation was associated with an increase in eNOS protein levels (not shown).

## DISCUSSION

In the present study, we describe molecular beacon-based methods for the study of eNOS expression and mRNA 3'-polyadenylation. The expression of eNOS is known to be modulated by several physiological and pathophysiological stimuli (24), and we demonstrate that a well-designed molecular beacon can reliably quantify changes in eNOS expression, no matter whether the stimulus produces a large change (laminar shear stress) or a more modest change (statin treatment) in eNOS expression. In addition, we describe the use of a dual-FRET method to quantify mRNA 3'-polyadenylation and show enhanced 3'-polyadenylation of human eNOS mRNA in response to laminar shear stress and statin treatment. Our findings indicate that molecular beacons could be useful for the assessment of other genes in heterogeneous RNA samples derived from either cell cultures or whole tissues.

We anticipate that our dual-FRET approach for assessing mRNA 3'-polyadenylation will prove to be a very useful method for the study of gene expression. In mammalian cells, the 3'-poly(A) tail plays an important role in regulating mRNA stability and translation (16, 20, 23). In general, transcripts with long poly(A) tails are more stable and more actively translated (3, 16, 33). The process of adding a poly(A) tail to primary transcripts is highly regulated and dependent on the interaction between *cis*-acting elements in the 3'-UTR and *trans*-acting factors whose activities are coordinated by RNA polymerase II. We have recently demonstrated that bovine eNOS mRNA has a short 3'-poly(A) tail under static or low laminar shear conditions, suggesting that bovine eNOS has a relatively inefficient polyadenylation signal at baseline (34). Subsequently, we found that enhanced eNOS mRNA stability and translation in response to higher levels of laminar shear stress was associated with 3'-poly(A) tail lengthening. There are other examples of stimulus-induced modulation of 3'-polyadenylation (4, 8, 9), and it is likely that this mechanism is important for regulation of mRNAs that have inefficient polyadenylation signals or have otherwise become translationally inactive (17). Thus, as we have shown here for human and mouse eNOS, the dual FRET beacon approach will be helpful in assessing the role of 3'-polyadenylation in the expression of genes that undergo changes in mRNA stability or translation.

Once we had established that molecular beacons could accurately measure changes in eNOS mRNA expression, the

dual-FRET approach was used to assess 3'-polyadenylation because of the need for signal specificity (22) and limitations in the availability of target sequence for beacon design. In particular, the beacons had to not only be specific for eNOS but also specific for eNOS transcripts with long poly(A) tails. As shown in Fig. 4, FRET signal in our assays required simultaneous and adjacent hybridization of a beacon specific for eNOS and a beacon specific for poly(A) sequence >30 nt in length. Previously, we have shown that short eNOS 3'-poly(A) transcripts have tails of <25 nt in length (34). Although the probe domain of the poly-T beacons could have been longer than 40 nt to better match the tail length of the long poly(A) eNOS transcripts of 75 to 160 adenosines that we observed in our previous study (34), potential issues with probe secondary structure required us to limit the size of our poly-T beacon to 40 thymidines. Despite this limitation, we believe that this beacon is able to distinguish between short and long poly(A) transcripts, because the poly-T beacon did not appear to produce significant signal when presented with poly(A) targets of <25 nt (Fig. 6). In addition, to enhance the specificity of the acceptor beacon, it was designed to be a shared stem beacon, complementary to the target at its 5'-end (Fig. 4).

While real-time RT-PCR (qRT-PCR) is currently the most common method for quantifying a specific RNA species from a cellular source, the molecular beacon-based method detailed in this report is more rapid and likely more cost-effective. Because the method only has a few steps, it also minimizes interexperiment pipetting variability. The dual-FRET method does not provide information about the absolute length of the poly(A) tail, but it does provide a quantitative assessment of poly(A) tails above a certain length. Previously, we have used ribonuclease protection assays to assess mRNA 3'-polyadenylation, but this method is cumbersome and has a limited ability to reliably quantify changes. The dual-FRET molecular beacon method is innovative because it is fast, relatively simple, and 3'-polyadenylation can be quantified by beacon fluorescence. Other potential applications of these methods are detection of alternatively spliced transcripts, analysis of large mRNA segments, and tracking of polyadenylated mRNA in live endothelial cells.

## GRANTS

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