The major control point for the hypoxic induction of the vascular endothelial growth factor (VEGF) gene is the regulation of the steady-state level of the mRNA. We previously demonstrated a discrepancy between the transcription rate and the steady-state mRNA level induced by hypoxia. This led us to examine the post-transcriptional regulation of VEGF expression. Actinomycin D experiments revealed that hypoxia increased VEGF mRNA half-life from 43 ± 6 min to 106 ± 9 min. Using an in vitro mRNA degradation assay, the half-life of VEGF mRNA 3′-untranslated region (UTR) transcripts were also found to be increased when incubated with hypoxic versus normoxic extracts. Both cis-regulatory elements involved in VEGF mRNA degradation under normoxic conditions and in increased stabilization under hypoxic conditions were mapped using this degradation assay. A hypoxia-induced protein(s) was found that bound to the sequence in the VEGF 3′-UTR which mediated increased stability in the degradation assay. Furthermore, genistein, a tyrosine kinase inhibitor, blocked the hypoxia-induced stabilization of VEGF 3′-UTR transcripts and inhibited hypoxia-induced protein binding to the VEGF 3′-UTR. These findings demonstrate a significant post-transcriptional component to the regulation of VEGF.

Hypoxia has been shown to be an important stimulus for the new blood vessel formation seen in coronary artery disease (1), tumor angiogenesis (2), and diabetic neovascularization (3). VEGF, 1 also known as vascular permeability factor (4–6), which is determined by the relative rates of mRNA synthesis and decay.

We have previously demonstrated that hypoxia induces VEGF steady-state mRNA 25.0 ± 11.4 and 12.0 ± 0.6 fold in rat primary cardiac myocytes (8) and rat pheochromocytoma PC12 cells (12), respectively. However, nuclear runoff transcription assays demonstrated that the transcription rate for VEGF was increased only 3.1 ± 0.6-fold by hypoxia in the PC12 cells (12). Rat genomic sequences encoding VEGF were cloned and a 28-bp element in the 5′ promoter was identified that mediates a significant portion of this hypoxia-inducible transcription in transient expression assays. This element was shown to have sequence and protein binding similarities to the hypoxia-inducible factor 1 binding site within the erythropoietin (Epo) 3′ enhancer (12). These studies demonstrated that, while increased transcription rate can account for a portion of the increase in the steady-state level of VEGF mRNA in the PC12 cells, it does not account for all of the increase and suggested that a post-transcriptional mechanism plays a significant role in the hypoxic induction of VEGF mRNA, as well.

Post-transcriptional mechanisms of regulation have previously been suggested for Epo (13–15) and demonstrated for tyrosine hydroxylase (16), two other hypoxia-inducible genes. In the present study we examine the post-transcriptional regulation of VEGF mRNA expression under both normoxic and hypoxic conditions. We have employed several complementary techniques including actinomycin D chase experiments, in vitro mRNA degradation studies, and RNA electromobility shift assays.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—PC12 rat pheochromocytoma cells were the generous gift of Dr. Eva J. Neer (Brigham and Women’s Hospital, Boston, MA). H9c2 rat heart myocytes were obtained from the American Type Culture Collection (Rockville, MD). The cells were routinely grown in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) with 10% fetal bovine serum and used for all experiments at ~70% confluence. Cells were cultured under either normoxic conditions (5% CO₂, 21% O₂, 74% N₂) in a humidified Napco incubator at 37°C or hypoxic conditions (5% CO₂, 1% O₂, 94% N₂) in an Espec triple gas incubator (Tabai-Espec Corp., Osaka, Japan). Genistein (Sigma) was prepared as a 100 mM stock in Me2SO and added to cells 30 min prior to placement in the hypoxia chamber at a final concentration of 50 μM.

Cloning and Sequencing of Rat VEGF cDNA—2 × 10⁶ bacteriophage clones were transferred to a λgt11 digoxigenin-primed PC12 cDNA library (Clontech, La Jolla, CA) and screened with two contiguous genomic fragments from the 3′-UTR of the VEGF gene (12), an 875-bp BamHI-EcoRI fragment (nucleotide 756–1642, GenBank™ accession no. U22372) and a 256-bp EcoRI-EcoRI fragment (nucleotide 1642–1855, GenBank™ accession no. U22372). Distinct VEGF cDNA clones hybridizing to both probes were isolated. The cDNA insert from each clone was isolated on a KpnI-SalI fragment (which contained both λgt11 and VEGF sequences) and cloned into the Bluescript vector (Stratagene). Sequencing of the cDNA inserts was performed by the dyeoxy chain-termination method using Sequenase (Stratagene) initially with oligonucleotide primers (5′-CCATCTGCTGCACGCGGAAGAAGGC-3′ and 5′-CCT-TACGCGAAATACGGGCAGACATG-3′) corresponding to a gII1 sequence adjacent to the insert and subsequently, in a progressive fashion, with oligonucleotides complementary to the sequences obtained.
from the respective clones. Both strands of all clones were sequenced.

**In Vitro Cell-free RNA Degradation Assay—**Cells were grown under either normoxic or hypoxic conditions and S100 cytoplasmic extracts were prepared according to the method of Wang et al. (17). Briefly, cells were washed twice with ice-cold phosphate-buffered saline and then scraped into 10 ml of phosphate-buffered saline. The cells were then pelleted and resuspended in 2 volumes of homogenization buffer (10 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol, 10 mM KCl, and 1.5 mM MgCl₂) and lysed with 20 strokes in a Dounce homogenizer (pestle B). 0.1 volume of extraction buffer (1.5 mM KCl, 15 mM MgCl₂, 100 mM Tris-HCl, pH 7.4, 5 mM dithiothreitol) was added, and the homogenate was centrifuged at 14,000 x g for 2 min to pellet nuclei. The supernatant from this centration and centrifugation step was then mixed with 100 ml of 10 M Tris-HCl, pH 8, 0.5 mM dithiothreitol, and stored at –70°C. Protein concentrations were determined by Bradford protein assay (Bio-Rad) and were routinely 3–5 mg/ml. The entire procedure was performed at 4°C.

**[32P]CTP-labeled,** capped, and polyadenylated transcripts were synthesized in vitro (18). The EcoRI site of psP64 polyA (Promega) was transformed into an Ase I restriction enzyme site by filling in EcoRI-digested psP64 polyA (with the Klenow fragment and then blunt-end ligating the vector. Restriction fragments containing the 3′-UTR of VEGF derived from clone 11.4 (12) were cloned into the multiple cloning site of this modified psP64 vector. A series of deletions were made from the 3′ end of these sequences using unique restriction sites in the VEGF 3′-UTR. Direction of these plasmids with Asd-generated DNA templates containing a poly(dT) sequence that was transcribed into a 30-base long poly(A) tail at the 3′ end. Capped transcripts were synthesized from these templates with SP6 RNA polymerase using the MEGAScript in vitro transcription kit (Ambion, Austin, TX) according to the manufacturer’s protocol with a 4:1 ratio of m7G5ppG (cap analog) to GDP. Labeled RNA transcripts were produced by inclusion of [α-32P]CTP (3000 Ci/mmol) in the reaction. 80,000 cpm were used for each degradation assay.

Degradation assays were performed by incubating the transcript (10⁶ cpm) with 130 μg of cytoplasmic extract in a total volume of 39 μl in a master mix at room temperature. At each time point the reaction was stopped by adding 3 μl of a 3 μl/ml solution of phenol:chloroform:isoamyl alcohol (25:24:1) and the supernatant was precipitated with 20 μl of isopropanol. The pellets were washed once with 80% ethanol and air-dried. Samples were then electrophoresed on a formaldehyde-agarose gel and transferred to GeneScreen (DuPont, NEN). Quantitation of the remaining primary (undegraded) level and the differences of the degradation was performed with a Molecular Dynamics PhosphorImager. All time points were performed in triplicate.

Measurement of VEGF mRNA Half-life in PC12 Cells—PC12 cells were grown under normoxic or hypoxic conditions in DMEM with 1% fetal bovine serum for 24 h prior to the addition of actinomycin D in T-25 flasks. Cells were grown under normoxic or hypoxic conditions in DMEM with 1% fetal bovine serum for 24 h prior to the addition of actinomycin D in T-25 flasks. Cells were grown under normoxic or hypoxic conditions in DMEM with 1% fetal bovine serum for 24 h prior to the addition of actinomycin D. Total RNA was prepared from the flasks using RNA STAT-60 (Tel-Test “B,” Inc., Friendswood, TX) and isolated according to the manufacturer’s protocol.

VEGF and 18 S rRNA were detected by RNase protection analysis of 10 μg of RNA isolated at the various time points. RNase protection assays were performed as described previously (8) to specifically detect the VEGF isoform and 18 S rRNA. After electrophoresis on 6% polyacrylamide, 7 m urea gels, the protected fragments were quantitated using a PhosphorImager (Molecular Dynamics). The quantity of VEGF mRNA was normalized to the amount of 18 S rRNA (19) by calculating a VEGF/18 S ratio for each sample. All time points were performed in triplicate. The entire experiment was repeated three separate times. The half-life of VEGF mRNA was calculated by drawing the best fit linear curve on a log-linear plot of the VEGF/18 S ratio versus time. The time at half-maximal VEGF/18 S ratio was taken to be the half-life.

RNA Electromobility Shift Assay (EMSA)—The bacteriophage T7 RNA polymerase promoter sequence was appended to the 5′ end of sense polymerase chain reaction (PCR) primers used to generate template DNA (20, 21). For experiments involving the VEGF 3′-UTR Stul-NsI fragment (used for mapping the constitutive RNA-binding protein) oligonucleotide PCR primers were 5′-gattctAAATAGCCT- CATATAGGAGGCTGTAAGCTCCTCC-3′ (VEGF nucleotide 910–931, GenBank™ accession no. U22372) and 5′-GAGATGCTACTC- CATAAATG-3′ (VEGF nucleotide 1279–1259, GenBank™ accession no. U22372). For experiments involving the Nsi I-transcription termination site fragment (used for mapping the hypoxia-induced RNA binding protein), oligonucleotide primers were 5′-cctAAATAGCCTAC- TATAGGAGGCTGTAAGCTCCTCC-3′ (VEGF nucleotide 1251–1271, GenBank™ accession no. U22372) and 5′-TTTGGATGAGGAATCCTTCTGTATGAGAATCGG-3′ (VEGF nucleotide 1877–1841, GenBank™ accession no. U22372). PCR products were gel-purified and [32P]CTP-labeled RNA transcripts were generated by T7 polymerase using Maxiscript (Ambion) according to the manufacturer’s protocol. Nonradioactive transcripts used in competition experiments were similarly generated with Maxiscript. A 162-bp fragment of the tyrosine hydroxylase gene (nucleotide 1521–1682, GenBank™ accession no. M10244) (16) was generated by PCR, cloned into the psP73 (Promega) vector, and used to generate tyrosine hydroxylase RNA transcripts. A template used to generate an iron response element (IRE) transcript was kindly supplied by Dr. Beric R. Henderson (22).

A series of overlapping oligonucleotides was used to prepare templates for the generation of short transcripts containing the putative constitutive RNA-binding protein site. Competitor WT1 (VEGF nucleotide 1050–1080, GenBank™ accession no. U22372) was generated by overlapping oligonucleotides T7A (5′-ggaggctATACCTACCTAT- GATCCTGAGGCTGTAAGCTCCTCC-3′) and T7B (5′-GAGATGCTACTC- CATAAATG-3′) (VEGF nucleotide 1050–1080, GenBank™ accession no. U22372) and 5′-GAGATGCTACTTCATAAATGAGAATCGG-3′ (VEGF nucleotide 1080–1051, GenBank™ accession no. U22372). Competitor WT2 (VEGF nucleotide 1050–1091, GenBank™ accession no. U22372) was generated by overlapping oligonucleotides T7A and C (5′-cctAAATAGCCTAC- TATAGGAGGCTGTAAGCTCCTCC-3′) (VEGF nucleotide 1091–1051, GenBank™ accession no. U22372). Competitor M (VEGF nucleotide 1050–1080 containing a 3-bp change in the VEGF sequence, GenBank™ accession no. U22372) was generated by overlapping oligonucleotides T7A and D (5′-cctAAATAGGGATGCTACTC- TATAGGAGGCTGTAAGCTCCTCC-3′) (VEGF nucleotide 1050–1080, GenBank™ accession no. U22372). Equimolar amounts of each of the oligonucleotides was annealed with its competitor and then treated with Klenow fragment to fill in the overhang. These oligonucleotide generated templates then were used to make RNA transcripts with T7 RNA polymerase according to the manufacturer’s protocol for short transcripts (Ambion).

Ran-labeled RNA transcripts (100,000 cpm/reaction) with or without nonradioactive competitor were incubated with 20 μg of S100 cytoplasmic extract for 15 min at room temperature. 25 units of ribonuclease A (Boehringer Mannheim) were added and the reaction was incubated for an additional 10 min at 37°C. The reaction was then incubated for 1 h at 37°C. The gel was washed and exposed to x-ray film (Kodak). The RNA-protein bands were quantitated by PhosphorImager analysis (Molecular Dynamics).

**Statistical Analysis—**Where indicated, data are presented as the mean ± the standard error of the mean (23). Student’s unpaired t test was employed to assess differences between two groups.
would suggest the presence of two independent instability sequences in the VEGF 3'-UTR. Each of these instability regions co-localizes with one of the two nonamer consensus instability sequences (UUAUUUA(U/A)(U/A)) (25, 26) (Fig. 2A).

Mapping Elements Which Mediate the Hypoxic Stabilization of the VEGF 3' mRNA UTR in Vitro—S100 cytoplasmic extracts from hypoxic cells were prepared in an identical fashion to those from normoxic cells. In vitro RNA degradation assays (Fig. 3, A–C) were performed as described under "Materials and Methods" and demonstrated that VEGF 3'-UTR transcripts had a significantly longer half-life in vitro when incubated with hypoxic versus normoxic extracts (ratio 1.5 ± 0.1 n = 12) (Fig. 3A). Progressive 3' deletion analysis of the VEGF 3'-UTR demonstrated that this preferential stabilization by hypoxia was lost upon deletion of the NsiI-XbaI fragment. Similar results were obtained with both PC12 and H9c2 cells.

RNA EMSA—RNA transcripts of different regions of the VEGF 3'-UTR incubated with S100 extract allowed for the identification by EMSA of both constitutive and hypoxia-induced VEGF mRNA binding proteins. The constitutive protein complex was found to map between the NsiI and Stul restriction enzyme sites (Fig. 4A). This complex could be completely inhibited by competition with excess unlabeled transcript from this region, but was not competed out with 500-fold excess β-actin or IRE transcripts (Fig. 4B). Proteinase K treatment of the extracts completely inhibited formation of the complex. Interestingly, 100-fold excess of a 162-base RNA transcript of the tyrosine hydroxylase 3'-UTR, corresponding to the region previously demonstrated to bind a hypoxia-inducible protein (16), completely inhibited formation of this complex. A region of the VEGF Stul-NsiI fragment was identified which is highly homologous to a region within a 28-base fragment specifically protected by the tyrosine hydroxylase RNA-binding protein (16) and to a region within the Epo 3'-UTR demonstrated to be the site for an RNA-binding protein (15) (Fig. 4C). Oligonucleotides were constructed from this region as described under "Materials and Methods" to define the binding site by competition studies. RNA derived from templateWT1 or WT2 was capable of specifically competing with the protein complex binding to Stul-NsiI RNA transcripts, whereas template M, which contains a 3-nucleotide substitution in this region of homology, did not efficiently compete with the complex.

A hypoxia-inducible protein complex was mapped by EMSA between the NsiI site and the transcription termination site (Fig. 4A) using a template generated by a strategy described under "Materials and Methods." The RNA-protein complex was induced 2.2 ± 0.2-fold (n = 12) by EMSA using hypoxic versus normoxic S100 extracts (Fig. 4D). This complex could be inhibited by excess unlabeled transcript from this region, but was not displaced by a 500-fold excess of IRE transcript (Fig. 4D) or Epo transcripts. Proteinase K treatment of the extracts completely inhibited formation of the complex. 3' truncated forms of this template were generated using restriction endonucleases XbaI, EcoRI, HinfI, and Msel (Fig. 4A). RNA transcripts from these truncated templates allowed the binding site for this hypoxia-inducible species to be further defined within a Msel-XbaI fragment (nucleotide 1412–1754, GenBank™ accession no. U22372) (Fig. 4D).

Genistein Blocks Hypoxic Stabilization of VEGF 3'-UTR Transcripts in Vitro—PC12 or H9c2 cells were incubated with 500 μM genistein, a tyrosine kinase inhibitor, for 30 min prior to their placement in the hypoxia chamber. Cells were exposed to hypoxia or normoxia for 4 h. Hypoxic and normoxic S100 extracts were then prepared in parallel from cells exposed to genistein and cells that were not exposed to genistein. As demonstrated in Fig. 5, A and B, genistein inhibited the pref-
Post-transcriptional Regulation by Hypoxia

Hydrogen peroxide acts on VEGF gene expression by increasing VEGF steady-state mRNA levels. Previous work has strongly suggested that an increase in transcription rate of the VEGF gene cannot account for all of the observed increase in the steady-state VEGF mRNA levels induced by hydrogen peroxide (12). These studies provide further evidence for a post-transcriptional mechanism contributing to VEGF mRNA induction by hydroxyp. Several cis-acting elements that may mediate the turnover of VEGF mRNA under normoxic and hypoxic conditions are identified.

The half-life of the VEGF mRNA, determined using actinomycin D, is increased 2.5 ± 0.4-fold by hypoxia. Steady-state kinetics (27) would predict that the increase in steady-state mRNA with hypoxia would be the product of the increase in the transcription rate and the increase in the mRNA half-life. These data therefore provide an adequate explanation for the discrepancy between the increase in the steady-state mRNA (12.0 ± 0.6) and the increase in the transcription rate (3.1 ± 0.6) in PC12 cells.

We have demonstrated using an in vitro RNA degradation assay that there are two distinct cis-acting instability elements in the VEGF 3’-UTR. The VEGF 3’-UTR contains two consensus nonamer sequences 5’-UUAAUUA(U/A)(U/A)-3’ (25, 26) that have been demonstrated to mediate the rapid turnover of multiple cytokine mRNAs. These nonamer consensus sequences fall within the fragments shown to significantly affect transcript stability in the in vitro degradation assays. We cannot rule out however that other sequences contained within these fragments are responsible for or contribute to the RNA instability.

The increased stability of VEGF 3’-UTR transcripts in vitro in the presence of hypoxic versus normoxic extracts has allowed us to map an element that when deleted from the UTR abrogates this increased stability with hypoxic extracts. From these studies one may hypothesize that a trans-acting factor that mediates stability binds to this region or, alternatively, the region is necessary for formation of a RNA secondary structure that mediates the change in RNA stability.

The half-life of each construct was calculated from the regression line extrapolated to zero. In the representative experiment shown, the half-life of construct A (full-length, □) was 2.6 min, B (XbaI, ○) was 2.0 min, C (NsiI, △) was 4.0 min, and D (StuI, ▽) was 8.0 min.

**Fig. 2.** Mapping instability elements in the VEGF 3’-UTR under normoxic conditions. [32P]CTP-labeled, capped, and polyadenylated transcripts were generated in vitro as described under “Materials and Methods.” A, restriction map of constructs in pSP64A (AseI). Linear fragments (A–D) were cloned in the pSP64A (AseI) vector and used to generate sense 3’-UTR transcripts in vitro. Deletions from the 5’ end of the UTR were produced with the designated restriction enzymes. Construct A (full-length) (nucleotide 1–2201, GenBank™ accession no. U22372) contains the entire 3’-UTR and yields a RNA of 2.2 kb. Construct B (XbaI) (nucleotide 1–1754, GenBank™ accession no. U22372) is derived by deletion of the XbaI from construct A and yields a RNA of 1.7 kb. Construct C (NsiI) (nucleotide 1–1255, GenBank™ accession no. U22372) is derived by deletion of the NsiI-XbaI fragment from construct B and yields a RNA of 1.2 kb. Construct D (StuI) (nucleotide 1–913, GenBank™ accession no. U22372) is derived by deletion of the StuI-XbaI fragment from construct B and yields a RNA of 900 bases. The locations of the nonamer instability consensus signals are depicted by lines with open circles. The half-life in minutes obtained for each construct was expressed relative to the half-life of construct A for each individual experiment. Results are expressed as the mean ± S.E. of four different experiments. In the experimental conditions described under “Materials and Methods” with S100 extract from normoxic cells, the half-life of construct A was approximately 3 min. B, representative autoradiograph of products from a cell-free degradation assay of constructs A (Full-length) and D (StuI) as described under “Materials and Methods.” Time refers to the time after the addition of normoxic cytoplasmic extract to the RNA. The arrow points to the undegraded transcript. C, log-linear regression lines of VEGF RNA degradation quantitated by PhosphorImager analysis. The half-life of each construct was calculated from the regression line extrapolated to zero. In the representative experiment shown, the half-life of construct A (full-length, □) was 2.6 min, B (XbaI, ○) was 2.0 min, C (NsiI, △) was 4.0 min, and D (StuI, ▽) was 8.0 min.

**Fig. 3.** Determination of instability elements with corresponding UTR untraduced maps. Linear fragments (A–D) were cloned in the pSP64A (AseI) vector and used to generate sense 3’-UTR transcripts in vitro. Deletions from the 5’ end of the UTR were produced with the designated restriction enzymes. Construct A (full-length) (nucleotide 1–2201, GenBank™ accession no. U22372) contains the entire 3’-UTR and yields a RNA of 2.2 kb. Construct B (XbaI) (nucleotide 1–1754, GenBank™ accession no. U22372) is derived by deletion of the XbaI from construct A and yields a RNA of 1.7 kb. Construct C (NsiI) (nucleotide 1–1255, GenBank™ accession no. U22372) is derived by deletion of the NsiI-XbaI fragment from construct B and yields a RNA of 1.2 kb. Construct D (StuI) (nucleotide 1–913, GenBank™ accession no. U22372) is derived by deletion of the StuI-XbaI fragment from construct B and yields a RNA of 900 bases. The locations of the nonamer instability consensus signals are depicted by lines with open circles. The half-life in minutes obtained for each construct was expressed relative to the half-life of construct A for each individual experiment. Results are expressed as the mean ± S.E. of four different experiments. In the experimental conditions described under “Materials and Methods” with S100 extract from normoxic cells, the half-life of construct A was approximately 3 min. B, representative autoradiograph of products from a cell-free degradation assay of constructs A (Full-length) and D (StuI) as described under “Materials and Methods.” Time refers to the time after the addition of normoxic cytoplasmic extract to the RNA. The arrow points to the undegraded transcript. C, log-linear regression lines of VEGF RNA degradation quantitated by PhosphorImager analysis. The half-life of each construct was calculated from the regression line extrapolated to zero. In the representative experiment shown, the half-life of construct A (full-length, □) was 2.6 min, B (XbaI, ○) was 2.0 min, C (NsiI, △) was 4.0 min, and D (StuI, ▽) was 8.0 min.

**Fig. 4.** Determination of instability elements with corresponding UTR untraduced maps. Linear fragments (A–D) were cloned in the pSP64A (AseI) vector and used to generate sense 3’-UTR transcripts in vitro. Deletions from the 5’ end of the UTR were produced with the designated restriction enzymes. Construct A (full-length) (nucleotide 1–2201, GenBank™ accession no. U22372) contains the entire 3’-UTR and yields a RNA of 2.2 kb. Construct B (XbaI) (nucleotide 1–1754, GenBank™ accession no. U22372) is derived by deletion of the XbaI from construct A and yields a RNA of 1.7 kb. Construct C (NsiI) (nucleotide 1–1255, GenBank™ accession no. U22372) is derived by deletion of the NsiI-XbaI fragment from construct B and yields a RNA of 1.2 kb. Construct D (StuI) (nucleotide 1–913, GenBank™ accession no. U22372) is derived by deletion of the StuI-XbaI fragment from construct B and yields a RNA of 900 bases. The locations of the nonamer instability consensus signals are depicted by lines with open circles. The half-life in minutes obtained for each construct was expressed relative to the half-life of construct A for each individual experiment. Results are expressed as the mean ± S.E. of four different experiments. In the experimental conditions described under “Materials and Methods” with S100 extract from normoxic cells, the half-life of construct A was approximately 3 min. B, representative autoradiograph of products from a cell-free degradation assay of constructs A (Full-length) and D (StuI) as described under “Materials and Methods.” Time refers to the time after the addition of normoxic cytoplasmic extract to the RNA. The arrow points to the undegraded transcript. C, log-linear regression lines of VEGF RNA degradation quantitated by PhosphorImager analysis. The half-life of each construct was calculated from the regression line extrapolated to zero. In the representative experiment shown, the half-life of construct A (full-length, □) was 2.6 min, B (XbaI, ○) was 2.0 min, C (NsiI, △) was 4.0 min, and D (StuI, ▽) was 8.0 min.
**FIG. 3.** Mapping an element in the VEGF 3'-UTR that mediates stabilization by hypoxia. [32P]CTP-labeled, capped, and polyadenylated transcripts were generated in vitro as described under "Materials and Methods." A, restriction map of constructs A–D in pSP64A (Asa) as described in the legend to Fig. 2A. A half-life for each construct was determined with normoxic and hypoxic extracts using the identically labeled transcript. The results are expressed as a ratio of the transcript half-life using hypoxic to normoxic extracts. All of the time points were performed in triplicate. Each transcript was assayed three different times with different extracts. B, representative autoradiograph of products from the degradation assay of constructs A (Full-length) and D (Stu I). Time refers to time after the addition of the normoxic or hypoxic extract to the labeled transcript. The arrow points to the undegraded transcript. One of the RNA pellets in the triplicate 5 min 1% O2 time point for the Stu I RNA fragment was lost in processing, and the data from this sample are not included. C, log-linear regression lines of VEGF RNA degradation quantitated by PhosphorImager analysis. The half-life of each construct was calculated from these regression lines using normoxic [■] and hypoxic [□] extracts. This is a representative experiment of the data summarized from three independent experiments in Fig. 3A. Each time point was performed in triplicate.
Identification of constitutive and hypoxia inducible RNA-protein complexes by EMSA. A, map of the VEGF mRNA 3'-UTR demonstrating location of templates used for generation of riboprobes for EMSA and to map the cis-elements with which the RNA binding proteins interact. A T7 promoter was appended to the sense primer for generation of templates as described under “Materials and Methods.” The Stu-NsiI template corresponds to nucleotide 909-1279 of the VEGF 3'-UTR, GenBank™ accession no. U22372. The NsiI transcription termination (TT) site template includes nucleotide 1251-1877 of the VEGF 3'-UTR, GenBank™ accession no. U22372. Restriction endonuclease MseI, HinfI, EcoRI, and XbaI sites in the NsiI-TT site template are located at nucleotides 1412, 1566, 1632, and 1754, respectively, of the VEGF mRNA 3'-UTR, GenBank™ accession no. U22372. TGA is the translation termination codon of VEGF and is located 6 bp 5' to nucleotide 1 in GenBank™ accession no. U22372. TT is the transcription termination site of VEGF mRNA. The nonamer instability consensus signals are depicted by lines with open circles. The small open box at nucleotide 1070 is the proposed site to which the constitutive protein complex binds. B, EMSA of the constitutive RNA-protein complex. RNA EMSA using the NsiI-StuI fragment as template was performed as described under “Materials and Methods.” Unlabeled RNA transcripts for competition studies were generated from the following templates: NsiI-StuI (VEGF), IRE (22); tyrosine hydroxylase (TH) 162-bp fragment (16); oligonucleotides WT1, WT2, and M as described under “Materials and Methods.” Proteinase K (PK) indicates extracts were first treated with proteinase K before adding the probe. The arrow points to the constitutive RNA-protein complex. The bracket encompasses free and degraded probe. C, sequence homology. Region of homology between the rat VEGF 3'-UTR, rat tyrosine hydroxylase 3'-UTR, and human Epo 3'-UTR. This region of the NsiI-Stul fragment of the VEGF 3'-UTR (nucleotide 1066-1075) was demonstrated to bind a protein(s) in S100 cytoplasmic extracts. The tyrosine hydroxylase sequence is within a 28-bp sequence of the tyrosine hydroxylase 3'-UTR (nucleotide 1552-1579) (32) that is protected by a hypoxia-inducible protein (16). The Epo sequence (nucleotide 2831-2841) (33) is within a 120 bp sequence of the Epo 3' UTR shown to bind a Epo mRNA binding protein that is up-regulated by hypoxia in brain and spleen (15). Nucleotide sequence for rat VEGF, rat tyrosine hydroxylase, and human Epo refer to GenBank™ accession nos. U22372, M10244, and M11319, respectively. D, EMSA of the hypoxia-inducible complex. RNA EMSA using the NsiI-transcription termination site of VEGF mRNA. The nonamer instability consensus signals are depicted by lines with open circles. The small open box at nucleotide 1070 is the proposed site to which the constitutive protein complex binds. The bracket encompasses free and degraded probe.
FIG. 5. **Genistein inhibits hypoxic stabilization of VEGF 3'-UTR transcripts in vitro.** Cells were pretreated with 500 μM genistein, an inhibitor of the hypoxic induction of VEGF mRNA (32), for 30 min prior to beginning the hypoxic exposure. After 4 h extracts were prepared from normoxic and hypoxic cells. Degradation of full-length VEGF 3'-UTR transcript (construct A, Fig. 2A) was assessed as described under “Materials and Methods.” A, representative autoradiograph of decay kinetics of the VEGF 3'-UTR with genistein treated or control cells using hypoxic and normoxic extracts. The arrow points to undegraded transcript. One of the RNA pellets in the triplicate 10 min 1% O₂ time point for genistein treated cells and one of the RNA pellets in the triplicate 10 min 21% O₂ time point for control cells were lost in processing and the data from these samples are not included. B, regression analysis of A demonstrating an inhibition in the stabilization of VEGF 3'-UTR transcripts in extracts prepared from genistein-treated cells. The experiment was performed three times with different preparations of extract. In the representative experiment shown, the ratio of the half-lives in hypoxia (■) to normoxia (▲) of the VEGF 3'-UTR transcript is decreased from 1.4 in control cells to 0.5 in genistein-treated cells. C, genistein inhibited formation of the hypoxia-inducible RNA-protein complex on EMSA. The NsiI-transcription termination template was used for EMSA analysis as described under “Materials and Methods.” The competitor was an RNA transcript derived from the NsiI-transcription termination template (VEGF) or the IRE element (22). 1 G or 21 G indicates that the S100 extract was made under hypoxic or normoxic conditions, respectively, from cells treated with genistein. The arrow points to the hypoxia-inducible species.
In EMSA studies, the region of the VEGF mRNA 3'-UTR to which the hypoxia-induced protein complex bound correlated with the RNA degradation assays and points toward an important role for this complex in mediating the increased stabilization of VEGF mRNA by hypoxia. The hypoxia-inducible complex is occasionally seen to migrate as a doublet using the entire NsiI transcription termination site riboprobe (Fig. 4D). This has not been observed with truncated forms of the template (NsiI-EcoRI), although hypoxia-inducible binding is still seen. In addition, further truncation of this fragment (NsiI-HinfI) still results in binding of a protein by EMSA, but the complex is no longer hypoxia-inducible.

Genistein, a tyrosine kinase inhibitor, was recently shown to inhibit the hypoxic induction of VEGF mRNA (28) through its action on Src. A signal transduction cascade leading to the hypoxic induction of VEGF through Raf was demonstrated using the dominant inhibitory Raf-1 mutant Raf(301) (28, 29). In other systems this cascade has been shown to proceed through mitogen-activated protein kinase and ultimately to modulate transcription of specific genes and phosphorylation of specific gene products (30, 31). We have shown here that genistein interferes with the post-transcriptional induction of VEGF by hypoxia. 500 μM genistein had no effect on the hypoxic induction of VEGF by hypoxia. 500 μM genistein had no effect on the hypoxic induction of VEGF through Raf was demonstrated using the dominant inhibitory Raf-1 mutant Raf(301) (28, 29).

An understanding of the molecular basis of the regulation of VEGF by hypoxia forms the essential groundwork for the rational design of pharmacological agents to modulate VEGF expression and thereby augment or inhibit neovascularization. The in vitro degradation assays and EMSA described here should allow for the rapid and economic assessment of multiple agents that may affect VEGF mRNA stability.

Acknowledgment—We thank Dr. H. Franklin Bunn for critical review of this manuscript.

REFERENCES
1. Sabri, M. N., DiSciascio, G., Cowley, M. J., Alpert, D., and Vetrovec, G. W. (1991) Am. Heart J. 121, 876–880
2. Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992) Nature 359, 843–845
3. Aiello, L. P., Avery, R. L., Arrigg, P. G., Keyt, B. A., jampel, H. D., Shah, S. T., Pasqua, L. R., Thieme, H., Iwamoto, M. A., Park, J. F., Nguyen, H. V., Aiello, L. M., Ferrara, N., and King, G. (1994) N. Engl. J. Med. 331, 1480–1487
4. Leung, D. W., Casichianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. (1989) Science 246, 1306–1309
5. Levy, A. P., Tamargo, R., Bren, H., and Nathans, D. (1989) Growth Factors 2, 9–19
6. Senger, D., Ven De Water, L., Brown, L., Nagy, J., Yeo, K.-T., Yeo, T.-K., Berse, B., Jackman, R., Dvorak, A., and Dvorak, H. (1993) Cancer Metastasis Rev. 12, 303–324
7. Goldberg, M. A., and Schneider, T. (1994) J. Biol. Chem. 269, 4355–4359
8. Levy, A. P., Levy, N. S., Liscio, J., Calderone, A., Takahashi, N., Yeo, K.-T., Koren, G., Colucci, W., and Goldberg, M. (1995) Circ. Res. 76, 758–766
9. Sharma, H., Sassen, L., Verdouw, P., and Schaper, W. (1992) J. Mol. Cell. Cardiol. 24, Suppl. V, S10 (abstr.)
10. Sharma, H. S., Wunsch, M., Schmidt, M., Schott, R. J., Kandolf, R., and Schaper, W. (1992) Exper. Suppl. 61, 255–260
11. Sharma, H. S., Wunsch, M., Brand, T., Verdouw, P. D. and Schaper, W. (1992) J. Cardiovasc. Pharmacol. 20, S23–S31
12. Levy, A. P., Levy, N. S., Wegner, S., and Goldberg, M. A. (1995) J. Biol. Chem. 270, 13333–13340
13. Goldberg, M. A., Gaut, C. C., and Bunn, H. F. (1991) Blood 77, 271–277
14. Ho, V., Acquaviva, A., Duh, E., and Bunn, H. F. (1995) J. Biol. Chem. 270, 10084–10090
15. Rondon, I. J., MacMillan, L. A., Beckman, B. S., Goldberg, M. A., Schneider, T., Bunn, H. F., and Malter, J. S. (1991) J. Biol. Chem. 266, 16594–16598
16. Czyzyk-Krzeska, M. F., Dominski, Z., Krol, R., and Millhorn, D. E. (1994) J. Biol. Chem. 269, 9940–9945
17. Wang, X., Kiledjian, M., Weiss, I. M., and Liebhaber, S. A. (1995) Mol. Cell. Biol. 15, 1769–1777
18. Gorospe, M., and Baglioni, C. (1994) Science 269, 11845–11851
19. Khalili, K., and Weinmann, R. (1984) J. Biol. Chem. 259, 10084–10090
20. Mullis, K. B., and Faloona, F. (1987) Methods Enzymol. 155, 335–350
21. Stoffet, E. S., Koeberl, G., Sarkar, G., and Sommer, S. S. (1988) Science 239, 491–494
22. Mulliner, E. W., Neupert, B., and Kuhn, L. C. (1989) Cell 58, 373–382
23. Skoog, D. A., and West, D. M. (1980) Analytical Chemistry, 3rd Ed., W. B. Saunders, Philadelphia
24. Sheets, M. D., Oggi, S. C., and Wickens, M. P. (1990) Nucleic Acids Res. 18, 5799–5805
25. Lagnado, C. A., Brown, C. Y., and Goodall, G. J. (1994) Mol. Cell. Biol. 14, 7984–7995
26. Zubiaga, A. M., Belasco, J. G., and Greenberg, M. E. (1995) Mol. Cell. Biol. 15, 2219–2230
27. Rodgers, J. R., Johnson, M. L., and Rosen, J. M. (1985) Methods Enzymol. 109, 572–592
28. Mukhopadhyay, D., Tsikas, L., Zhou, X.-M., Foster, D., Brugge, J. S., and Sukhatme, V. P. (1995) Nature 375, 577–581
29. Kolch, W., Heidecker, G., Lloyd, P., and Rapp, U. R. (1991) Nature 349, 426–428
30. Aurruch, J., Zhang, X. F., and Kyriakis, J. M. (1994) Trends Biochem. Sci. 19, 279–283
31. D’Lima, G., Eisenman-Tappe, I., Fries, H. W., Trupmair, J., and Rapp, U. R. (1994) Trends Biochem. Sci. 19, 474–480
32. Grima, B., Loumoum, A., Blanot, F., Biquet, N. F., and Mallett, J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 617–621
33. Lin, F. K., Suggs, S., Lin, C. H., Browne, J. K., Smalling, R., Egejje, J. C., Chen, K. K., Fox, G. M., Martin, F., Stabinsky, Z., Badrawi, S. M., Lai, P. H., and Goldwasser, E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7580–7584