

# Identification of Novel Alternatively Spliced Isoforms of RTEF-1 within Human Ocular Vascular Endothelial Cells and Murine Retina

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**PURPOSE.** Identification of transcription factors that regulate the transcription of the vascular endothelial growth factor (*VEGF*) gene may facilitate understanding of the etiology and progression of ocular neovascular diseases. The purpose of this study was to determine whether transcriptional enhancer factor 1-related (*RTEF-1*) was present within ocular vascular endothelial cells and whether it played a role in the control of the transcription of the *VEGF* gene.

**METHODS.** Primary cultures of human retinal vascular endothelial cells (RVECs) were maintained under normoxic or hypoxic conditions before isolation of mRNA. RT-PCR was performed to detect *RTEF-1* transcripts. Amplified products were cloned into an expression plasmid. Human VEGF promoter and deletion constructs were cloned into a pSEAP reporter vector. Various *RTEF-1* isoforms and VEGF promoter constructs were coelectroporated into human cells, and reporter expression levels were determined. Retinal tissue from a mouse model of retinopathy of prematurity (ROP) was analyzed by RT-PCR for the presence of *RTEF-1* transcripts.

**RESULTS.** Full-length 1305-bp and novel 936-bp *RTEF-1* transcripts were identified in cultured human RVECs under normoxic conditions. A novel 447-bp isoform was present in cells maintained in a hypoxic environment. Four of the 11 translated exons predicted to code for the 1305-bp product were spliced out of the 936-bp transcript. The 1305-bp product enhanced expression from the VEGF promoter 4-fold greater than background, whereas the 936-bp and the 447-bp isoforms enhanced expression 3× and 12×, respectively. Analysis with deletion promoter constructs determined that all isoforms required the presence of Sp1 elements for efficient activation and that the hypoxia response element (HRE) was not essential for enhancement. Transcripts for novel *RTEF-1* isoforms were also identified in neural retinal tissue of mice. Different murine-

specific isoforms were present at different stages of postnatal development.

**CONCLUSIONS.** Novel *RTEF-1* transcripts are present within human ocular vascular endothelial cells and mouse neural retina during normal and ROP development, and alternatively spliced products are produced under hyperoxic and hypoxic conditions. Alternatively spliced variants of human *RTEF-1* transcripts are able to potentiate expression from the *VEGF* 5' proximal promoter region. (*Invest Ophthalmol Vis Sci.* 2007;48:3775-3782) DOI: 10.1167/iovs.06-1172

**T**ranscriptional enhancer factor 1-related (*RTEF-1*) gene is a member of the TEA DNA binding domain gene family. The TEA DNA-binding domain gene family is highly conserved from *Aspergillus nidulans*, yeast, *Drosophila*, and mice to humans. The TEA DNA-binding family of proteins can be involved in the activation and repression of multiple genes, and their particular function can be modified by association with other proteins.<sup>1</sup> Expression of specific members of these genes has been identified in various mammalian tissues, including heart, skeletal muscle, pancreas, placenta, brain, and lung.<sup>2-4</sup> Isoforms arising from alternative splicing of mRNA from a single gene, for transcriptional enhancer factor-1 (*TEF-1*), have been identified within a single tissue such as the pancreas.<sup>5,6</sup> The expression profile of these genes within the mammalian eye has not been reported.

Transcripts of the *RTEF-1* gene were first identified in chicken tissue and were demonstrated to be enriched in cardiac and skeletal muscle.<sup>4</sup> Chicken *RTEF-1* binds to the myocyte-specific CAT (M-CAT) *cis* DNA elements, regulates the expression of muscle-specific genes, and requires muscle-specific cofactors for full transcriptional activation. Random screening of 2166 clones from a human colorectal cancer cDNA library identified a partial cDNA *RTEF-1* sequence that led to the isolation of a full-length human homolog of the avian *RTEF-1* from a heart cDNA library.<sup>2,7</sup> Northern blot analysis of human tissue indicated the highest levels of expression in skeletal muscle and pancreas, with lower levels in the heart, kidney, and placenta, whereas the message was not detected in the liver, lung, or brain.<sup>2</sup> Northern blot analysis of the mouse homolog of *RTEF-1* indicates a different tissue expression pattern when compared with that in humans. Adult mouse lung tissue expressed the highest level, with very low levels in kidney, heart, and skeletal muscle and undetectable amounts in liver, thymus, spleen, and brain, whereas *RTEF-1* message was abundant in mouse embryonic skeletal muscle.<sup>8</sup> An alternatively spliced mouse isoform of *RTEF-1* that lacks exon 5 compared with the full-length gene has been identified in mouse skeletal muscle cells.<sup>8</sup>

Recently, the full-length *RTEF-1* protein has been identified not only to bind to the VEGF promoter but also to upregulate the expression of VEGF under hypoxic conditions in bovine aortic endothelial cells (BAECs).<sup>9</sup> Microarray analysis revealed

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that RTEF-1 expression was upregulated by 3-fold in BAECs under hypoxic conditions. Surprisingly, RTEF-1 mediated VEGF gene activation through interaction with Sp1 elements within the VEGF promoter and not with M-CAT motifs. In addition, RTEF-mediated expression of VEGF is achieved independently of the hypoxia-inducible factor (HIF-1) and hypoxia responsive element (HRE) pathways of activation.<sup>9</sup>

Given that VEGF plays a key role in the development of various ocular neovascular diseases, it is plausible that RTEF-1 may also play a role in the pathogenesis of proliferative retinopathies. We examined human retinal vascular endothelial cells for the presence of RTEF-1 mRNA isoforms and tested whether these products could also affect transcription directed by sequences upstream of the VEGF gene. We also sought to determine whether RTEF-1 transcripts are present in vivo in retinal tissue from a mouse model of retinopathy of prematurity (ROP).

## METHODS

### Primary Ocular Vascular Endothelial Cell Isolation and Culture

All use of human cells and tissue was in accordance with approved institutional review board protocols. Primary cultures of endothelial cells isolated from human retina were established using established protocols<sup>10,11</sup> and were used as a source for mRNA. Human cadaver eyes were obtained from anonymous donors (Lions Eye Bank, Portland, OR) within 24 hours of death. Donors had no history of cardiovascular or ocular disease and ranged in age from 16 to 42. Briefly, these retinal and iris tissues were aseptically dissected and separated from donor eyes and were digested in 0.2% collagenase (Sigma Chemical Co., St. Louis, MO). Endothelial cells (ECs) were isolated from other cell types with the use of mouse monoclonal anti-human CD31 antibody-coated magnetic beads (Dynal Biotech, Inc., Lake Success, NY). ECs were cultured in complete MCDB-131 medium (Clonetics/BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum and antibiotics. Cells were used at passages 2 to 5. After two rounds of magnetic bead separation, the EC cultures were greater than 99.5% pure, as evaluated by morphologic criteria, expression of CD31 and von Willebrand factor, and uptake of acetylated low-density lipoprotein.<sup>11</sup>

### Induction of Hypoxia

Retinal endothelial cells were cultured to 80% confluence in 60-mm-diameter culture dishes and then were placed in an air-tight chamber (Modulator Incubator Chamber; Billups-Rothenberg, Del Mar, CA). A gas mixture consisting of 1% O<sub>2</sub>, 5% CO<sub>2</sub>, and the remainder N<sub>2</sub> was flushed through the chamber for exactly 5 minutes, whereupon the chamber was sealed and placed in a humidified 37°C incubator. After 8 hours, the chamber was flushed again for 5 minutes with the hypoxic gas mixture, sealed, incubated for 8 more hours, flushed again, and incubated for another 8 hours, at which point total RNA was isolated.

### Total RNA Extraction and RT-PCR

Total RNA was isolated with the use of an RNA purification kit (RNAqueous; Ambion Inc., Austin, TX) according to manufacturer's protocol, and 50 ng of this RNA was used with oligo-dT primer first-strand synthesis (SuperScript II; Stratagene, La Jolla, CA). Human primers F1 (5'-ttggaggcaccggcga-3') and R1 (5'-tcattctcaccagcct-gta-3'), designed from the published human RTEF-1 sequence (accession no. U63824), were used for second-strand PCR amplification under standard conditions. Mouse primers F2 (5'-cctgggaccggtggaac-3') and R2 (5'-tgcagatggtgaagtc-3'), designed from published mouse sequence (accession no. D87965), were used for cDNA amplification. Amplified products were electrophoresed and visualized in a 1.5% agarose gel and subsequently purified from the gel (QIAquick Gel

Extraction; Qiagen, Valencia, CA) for standard dideoxynucleotide sequencing on an automated sequencer (ABI 310; Applied Biosystems, Foster City, CA).

### Reporter Gene Analysis

Full-length RTEF-1 isoforms were directionally cloned into the pcDNA 3.1 expression plasmid (Invitrogen, Carlsbad, CA). The predicted TTG start was converted to ATG within the forward primer sequence. Human VEGF 5' proximal promoter fragment of 1136 bp (F1-R3) containing 54 bp of 5'UTR and 1082 bp upstream of the transcription start site was directionally cloned 5' to the secretable alkaline phosphatase (SEAP) gene within the pSEAP reporter plasmid (Clontech, Mountain View, CA). A truncated human VEGF 5' proximal promoter fragment of 634 bp (F2-R3), containing 54 bp of 5'UTR and 580 bp upstream of the transcription start site, was also directionally cloned into the pSEAP plasmid. Promoter fragments with deletions were constructed by amplification of the 5' end of the promoter and the 3' end of the promoter and subsequent ligation of the amplified products. The ligated products lacking the region of interest were then amplified and directionally cloned into the promoterless pSEAP vector. All constructs were sequenced on both strands for verification before transfection studies.

### Transfection Assays

Transfection was performed using the Amaxa nucleofection device and reagents (Amaxa Inc., Gaithersburg, MD) according to the manufacturer's standard protocol. Briefly, 293T cells were cultured in 10% DMEM until they were 80% confluent, and then they were trypsinized and collected. Half a million cells were used for each nucleofection. Half a million cells were resuspended in 100  $\mu$ L solution (Nucleofect; Amaxa) and 5  $\mu$ L (containing 2  $\mu$ g) total plasmid DNA and electroporated (program A023 on Nucleofection Device; Amaxa) and then were immediately resuspended in 1 mL prewarmed media and seeded into a single well of a six-well plate. Cells were allowed to recover for 16 to 18 hours, and the media were carefully removed and replaced with exactly 500  $\mu$ L fresh media. After exactly 24 hours of incubation, 150  $\mu$ L media were carefully removed and 25  $\mu$ L of this was either assayed immediately or stored at -20°C for future SEAP analysis. Three separate 25- $\mu$ L media aliquots were used for SEAP analysis according to manufacturer's protocol (BD Biosciences, San Jose, CA), and the SEAP values for all three readings were averaged for comparison with triplicate repeat experiments.

Each cotransfection was repeated at least three times in a single experiment, and each experiment was repeated independently two more times with separate plasmid preparations ( $n = 9-12$ ). Results of one representative experiment performed in triplicate are presented. Statistical analysis was performed using a Student's *t*-test (two-tailed) to compare the three or four samples in a single experiment. Bonferroni correction for multiple testing was applied, and  $P < 0.01$  was considered significant.

For each cotransfection assay (when two plasmids were transfected together in the same tube), the copy number of each plasmid was adjusted to be equivalent to the copy number of the largest plasmid used. The pSEAP vector without a promoter and the pcDNA 3.1 expression plasmid with no insert served as negative controls. For each nucleofection experiment, two separate positive control plasmids, a SV40 promoter pSEAP plasmid and a pGFPmax vector, were transfected simultaneously to ensure efficient and equal transfection efficiencies. The pSEAP plasmid with an SV40 promoter served as a positive control for subsequent SEAP protein analysis. The pGFPmax vector also served as positive control for transfection for each batch of cells, allowing visual confirmation of consistent transfection efficiency. Nucleofection consistently gave 80% to 90% transfection efficiency in 293T cells in all experiments.

### Mouse Model of Retinopathy of Prematurity

The procedure for the development of ROP in mice has been described previously.<sup>12,13</sup> Briefly, C57BL/6 (B6) mice were purchased (Simonsen

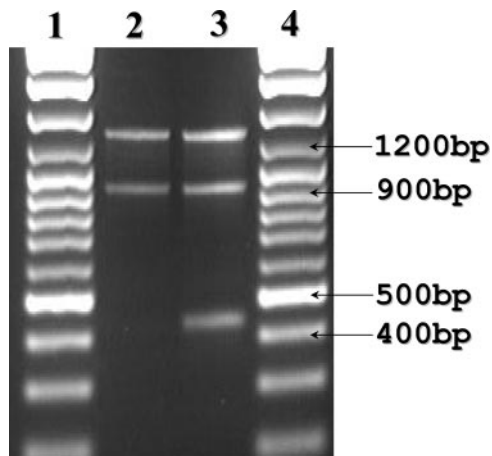
Laboratories, Gilroy, CA) and bred at the Oregon Health and Science University animal care facility in accordance with National Institutes of Health guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. To induce retinopathy, postnatal day (P)7 mice, with nursing mothers, were exposed to hyperoxic conditions (75% oxygen) for 5 days. At P12 the hyperoxic exposed mice were recovered in room air. Control litters were maintained at normal room air conditions. Pups exposed to high oxygen and control pups exposed to normal air were humanely euthanized on P8, a hyperoxic stage, and P17, a relatively hypoxic stage corresponding to peak time of neovascularization. Both eyes were immediately enucleated from each mouse and were dissected for careful removal of the neural retina. Retinal tissues from four eyes, two from each of two littermates at the same stage of development, were pooled for isolation of total RNA.

## RESULTS

### Novel Isoforms of RTEF-1 Exist within Hypoxic and Normal Ocular Vascular Endothelial Cells

Amplification from cDNA prepared from primary cultures of human retinal vascular endothelial cells (PRVECs) and iris VECs (PIVECs), using the F1 and R1 primer pair, gave products of approximately 1300 bp and 900 bp (Fig. 1). Identical primer pair amplification from cDNA isolated from PRVECs that had been cultured under hypoxic conditions for 24 hours, before isolation of mRNA, gave an additional product of approximately 450 bp (Fig. 1).

Sequencing analysis revealed that the largest product was identical with the full-length 1305-bp *RTEF-1* gene spanning from the start to the stop codon, whereas 900 bp and 450 bp were 936-bp and 447-bp alternate spliced transcripts of the 1305-bp product. The following description of codons will be numbered according to the sequence in the 1305-bp transcript, consisting of 435 codons, with the protein initiating codon numbered 1 and the stop codon numbered 435. Exons 5 to 8, four of the eleven exons that are predicted to code for the protein portion of the 1305-bp transcript, are spliced out of the 936-bp version (Fig. 2). Exon 5 was absent from the 447-bp isoform, and an unusual in-frame splice event occurs in the middle of exon 7, which splices out from Gln-83 in exon 7 to codon Gln-425 within exon 12 (Fig. 2).



**FIGURE 1.** Agarose gel electrophoresis showing RT-PCR of RTEF-1 from cDNA prepared from primary cultures of human RVECs. *Lanes 1 and 4*, DNA ladder; *lane 2*, cDNA prepared from RVEC under normoxic conditions gave 2 products (approximately 1300 and 900 bp); *lane 3*, cDNA prepared from RVECs under hypoxic conditions gave 3 products (approximately 1300, 900, and 450 bp).

The 1305-bp product shows identity to the transcriptional enhancer factor-1 related (*RTEF-1*) gene originally identified in human heart, skeletal muscle, pancreas, and lung tissue.<sup>2</sup> Two other RTEF isoforms—variant 2 (accession no. NM\_201441), which lacks exon 5 from Asp-119 to Gly-161, and variant 3 (accession no. NM\_201443), which makes use of a downstream protein initiation site at Met-130—have been reported. The 936-bp and 447-bp isoforms identified within human ocular vascular cells have not been identified in any other human tissue to date.

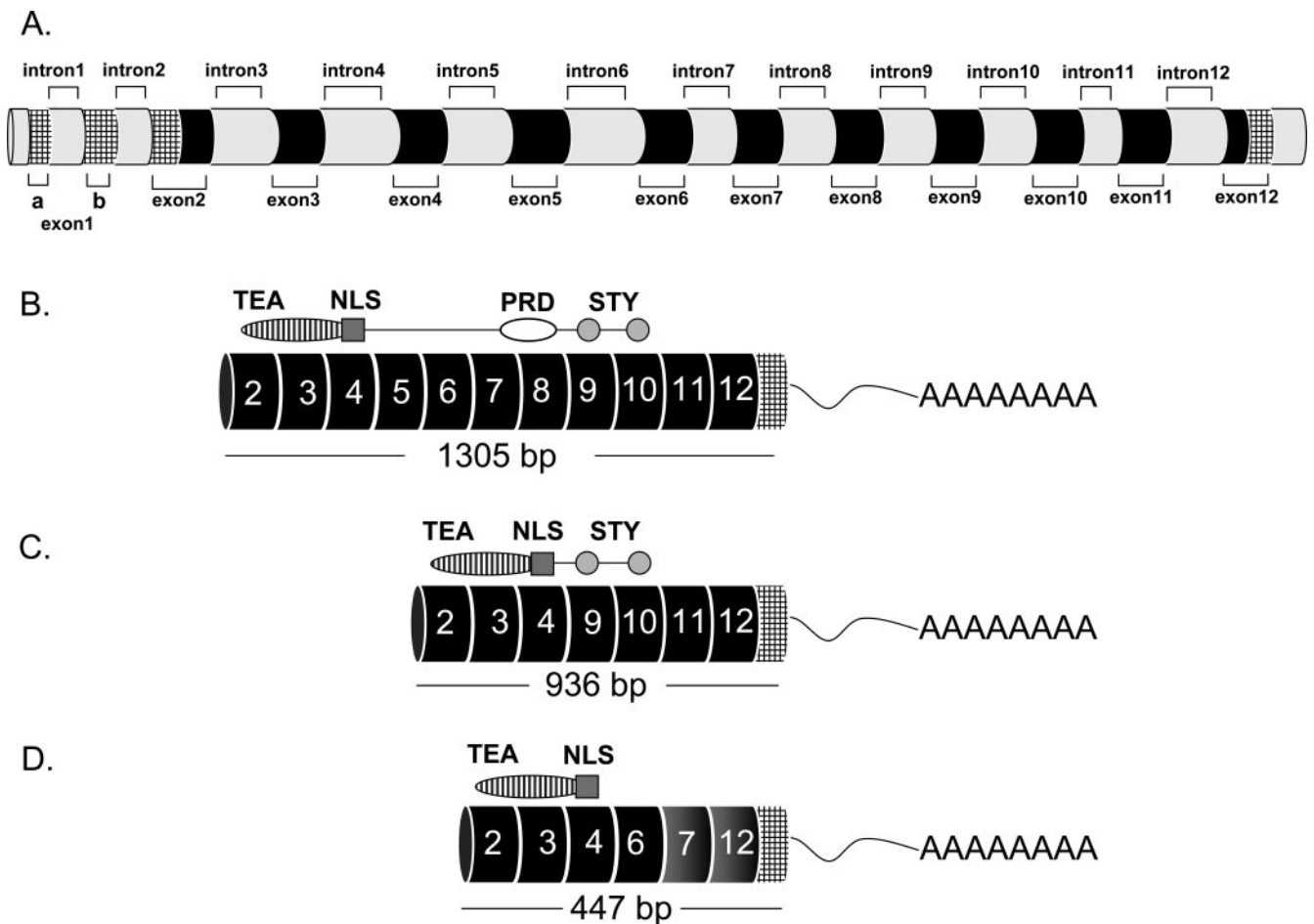
The predicted protein sequence for the 936-bp and 447-bp isoforms contain the 72-amino acid TEA domain (Asp-38 to Lys-109), which contains three predicted  $\alpha$ -helices and a putative nuclear localization signal (Leu-105 to Lys-109). However within the C-terminal domain, a proline rich-domain (Pro-189 to Pro-213) spanning the last six amino acids of exon 7 and the first 19 residues of exon 8 are missing from the 447-bp isoform (Fig. 2). In addition, two domains of STY (Ser-253 to Ser-271 and Ser-311 to Ser-336), a region laden with hydroxylated residues such as serine, threonine, and tyrosine—one located within exon 9 and the other within exon 10—are also lacking in the 447-bp isoform (Fig. 2).

### Novel Isoforms of RTEF-1 Are Able to Upregulate Expression from the VEGF Promoter

It has been shown that the 1305-bp isoform acts as a transcriptional stimulator of VEGF, in bovine aortic endothelial cells, by binding to an Sp1 site.<sup>9</sup> We investigated whether the new isoforms were also capable of stimulating expression from the human VEGF promoter. The 5' proximal promoter of the human VEGF gene, 54 bp of the 5'UTR and 1082 bp upstream of the transcription initiation site (Fig. 3A), was cloned into a pSEAP reporter plasmid, and the three RTEF isoforms were cloned into a pcDNA expression vector. Because of the difficulties in nucleotransfection of plasmid DNA into primary cultures of ocular vascular endothelial cells, 293T cells were used as a substitute cell line for transfection studies. Cotransfection of the VEGF promoter-reporter plasmid with any of the three isoforms indicated that all isoforms upregulated expression of the reporter from the VEGF promoter (Fig. 4). The full-length 1305-bp RTEF-1 product and the 936-bp isoform enhanced expression between 3-fold and 4-fold, significantly higher than background ( $P < 0.005$ ), and no difference was observed between these two isoforms ( $P > 0.01$ ) after correcting for multiple testing. The 447-bp isoform stimulated expression approximately 10- to 15-fold (average, 12 $\times$ ) above background expression ( $P < 0.001$ ). Each cotransfection experiment was repeated in triplicate on three separate occasions with the same results.

### Sp1 Elements Are Required for VEGF Promoter Activity but May Not Be Essential for RTEF-1 Enhancer Activity

Earlier studies demonstrated that the full-length RTEF-1 isoform binds to and requires an Sp1 element for augmentation of VEGF promoter activity. In a previous study, mutation of this Sp1 site situated at -97 to -89 bp, resulted in a loss of RTEF-1 enhancer activity.<sup>9</sup> In the same study, three other Sp1 sites within the same region, -86 to -58 bp, were found not to be essential for RTEF-1 enhancer activity. To test whether the new RTEF-1 isoforms required Sp1 sites for enhancer activity, the VEGF promoter, with all four Sp1 sites deleted (Fig. 3B), from -113 bp to -58 bp, was cloned into a pSEAP vector and was cotransfected with each isoform. Comparison of background reporter gene expression from the full-length and the Sp1-negative (Sp1<sup>-</sup>) VEGF promoter indicated that loss of Sp1 elements resulted in a dramatic 30-fold decrease in reporter



**FIGURE 2.** Schematic of the genomic structure of RTEF-1 and the three isoforms isolated from human retinal endothelial cells. (A) *Hatched* areas indicate 5' and 3' untranslated exons, and *solid black* areas indicate protein coding exonic regions. (B) Full-length RTEF-1 (1305 bp) from the initiating start codon within exon 2 to the stop codon in exon 12 contains the TEA DNA-binding domain (TEA, *oval with vertical stripes*), a putative nuclear localization signal (NLS), a proline rich domain (PRD) and 2 serine-threonine-tyrosine (STY) domains. The *square* shows the position of the NLS, the *open oval* shows the PRD, and the *circles* show the STY domains. (C) The 936-bp isoform lacks exons 5 to 8 and is missing the PRD. (D) The 447-bp isoform lacks the PRD and the STY domains and is spliced in such a way as to contain only the 5' end of exon 7 and the 3' end of exon 12.

expression (Fig. 5A), suggesting that at least one of the four Sp1 elements within the proximal promoter is essential for expression. The RTEF-1 isoforms could not enhance expression from the promoter without the Sp1 elements to levels observed with the F1-R3 promoter (Fig. 5A). However, a closer look at the effect of each isoform on the Sp1-negative promoter (Fig. 5B) relative to the background expression observed from the control indicated that a similar trend of enhancement was observed, representing 3-fold, 4-fold, and 12-fold enhancement above background control expression for the 1305-bp, 936-bp, and 450-bp isoforms, respectively. Thus, it would appear that the level of enhancement relative to the background control afforded by each isoform is comparable regardless of whether Sp1 elements are present within the VEGF promoter.

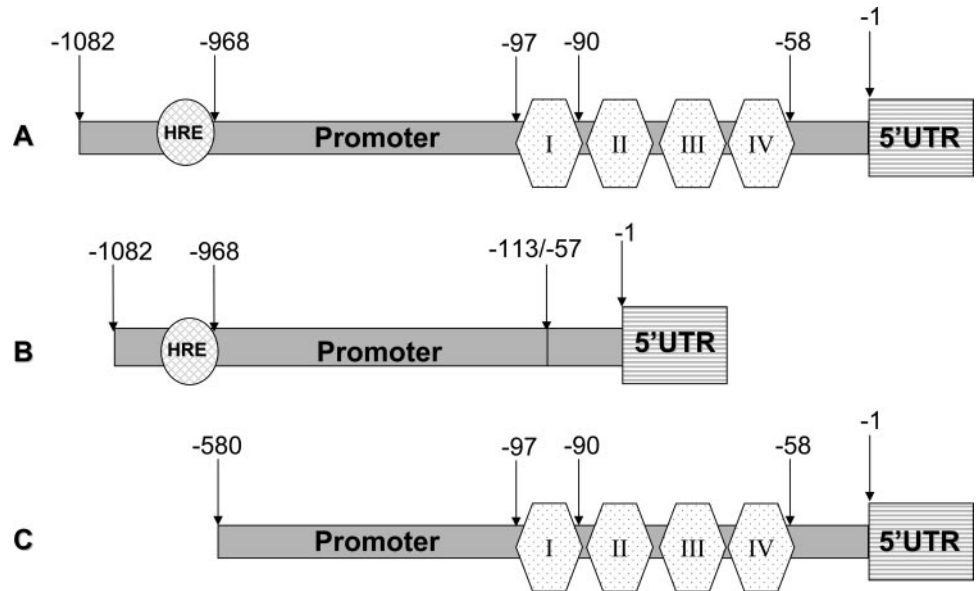
#### Novel Isoforms of RTEF-1 Do Not Require the HRE

The hypoxia response element (HRE) sequence, situated between -985 and -939 bp with a core sequence (-975 and -968 bp) within this region, is essential for the binding of hypoxia-inducible factor (HIF)-1 $\alpha$ , which is responsible for the enhancement of VEGF expression under conditions of low oxygen.<sup>14</sup> We investigated whether the RTEF isoforms, specifically the 450-bp isoform, observed under hypoxic conditions and

identified in this study, require the presence of the HRE within the VEGF promoter for enhancement activity. A truncated version of the 5' proximal promoter region of the human VEGF gene, from -580 bp spanning 54 bp of 5' UTR, was cloned into a pSEAP plasmid (F2-R3; Fig. 3C). Cotransfection of this promoter with each RTEF-1 isoform implies that all isoforms are able to stimulate expression of the VEGF promoter lacking the HRE region above background expression observed from the control (Fig. 6). Only the 936-bp and 447-bp isoforms were thought to show a significant difference compared with the no insert control ( $P < 0.0001$ ) and enhanced between 2- to 3-fold and 8- to 12-fold above the control. Again, the 447-bp isoform gave the most robust stimulation compared with the other isoforms. The full-length 1305-bp product only gave a slight enhancement above background (less than 2-fold) and might have required the HRE or other nearby sequences upstream of -580 bp for efficient enhancement, as observed with the F1-R3 promoter.

#### Novel RTEF-1 Isoforms Are Present within Murine Retinal Tissue

Amplification from cDNA prepared from neural retinal tissue isolated from normoxic (control) and hyperoxic exposed mice



**FIGURE 3.** Schematic of the human VEGF promoter cloned for expression analysis (not to scale). (A) Native promoter (F1-R3) containing 54 bp of the 5' untranslated region (5'UTR) 1082 bp upstream of the transcription initiation site (-1 bp) with the position of the HRE and 4 Sp1 sites (I, II, III, IV). (B) Sp1-deleted promoter (F1-R3 Sp1<sup>-</sup>), which lacks a 56-bp region between -57 and -113-bp and contains four Sp1 sites. (C) F2-R3 VEGF promoter construct lacks the HRE motif and starts at -580-bp.

at P8 and P17, using the F2 and R2 primer pair, demonstrated the presence of the 1305-bp RTEF-1 isoform. This was confirmed by sequence analysis (Fig. 7). Interestingly, additional amplified products were present, a 920-bp fragment in the P8 control, an 850-bp fragment in the P17 control, and 1100-bp and 610-bp bands in P17 hyperoxia exposed samples (Fig. 7). Sequencing analysis showed that all extra fragments were alternatively spliced RTEF-1 isoforms (data not shown). The mouse RTEF-1 protein coding exonic structure was identical to the human RTEF-1 exon architecture; thus, Figure 2B can be used as a reference. The 1100-bp isoform, which lacks exon 5, has been identified in craniofacial tissue (not including brain or eye tissue) from 12.5-day-old mouse embryos.<sup>3</sup> The other three isoforms are novel and unique to mouse. The 920-bp isoform identified in P8 normoxic retina lacks exons 5, 7, and 8, whereas the 850-bp isoform within the P17 normoxic retina

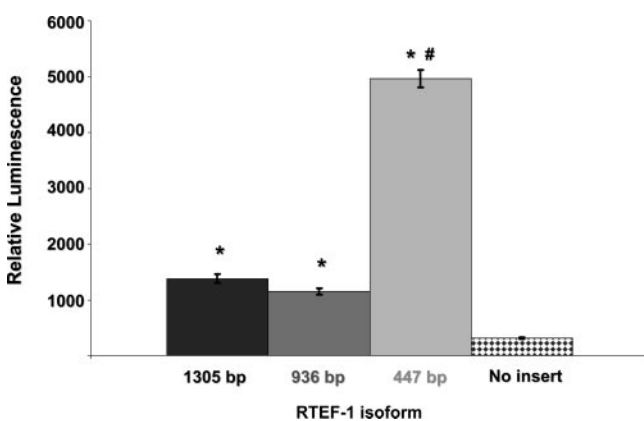
lacks exons 5, 8, and 9. The 920-bp isoform lacks the entire PRD region (contained within exons 7 and 8) and retains the 2 STY domains within exons 9 and 10, whereas the 850-bp isoform lacks exon 8. Thus, the 850-bp isoform has only 7 of the 13 proline residues contained within the PRD and retains only one of the STY domains, which is in exon 9. The 610-bp P17 ROP-specific isoform is spliced from within exon 2, just before the start of the TEA domain, and links again to exon 8. Thus, the 610-bp isoform lacks most of exon 2 and all of exons 3 to 7, which means the entire TEA domain, the nuclear localization signal (NLS), and five proline residues of the PRD are missing.

## DISCUSSION

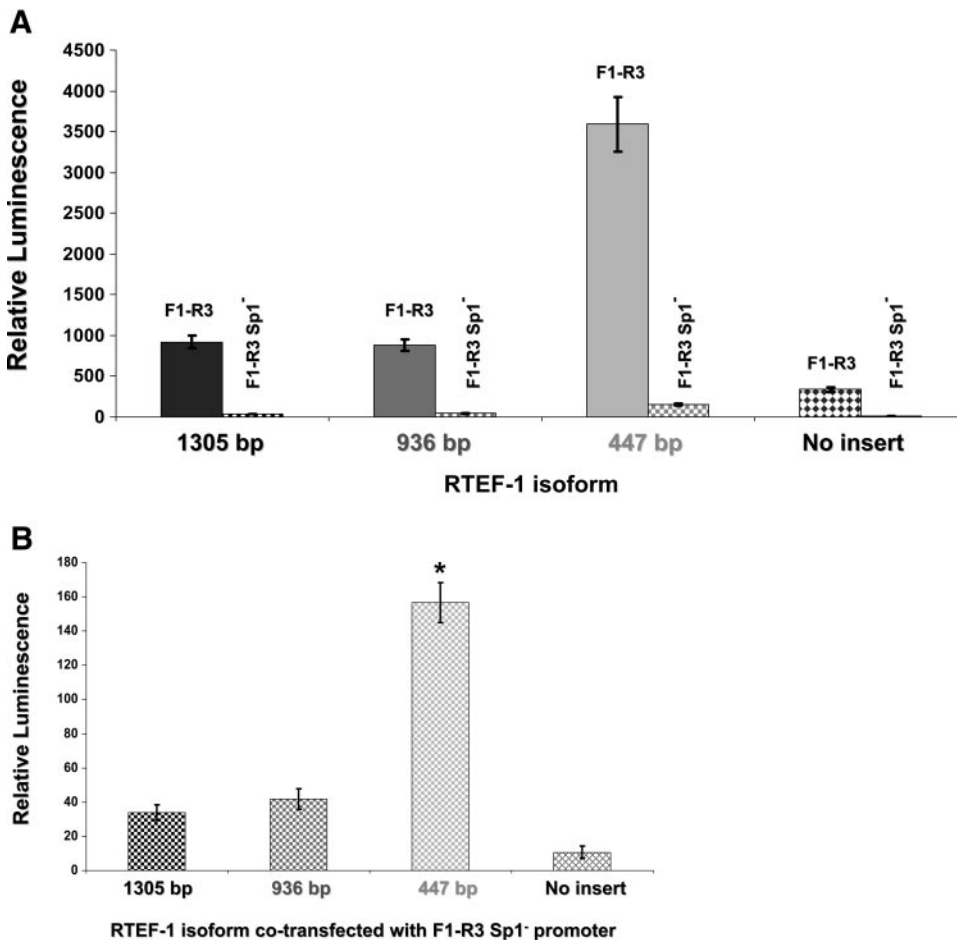
In this study we examined whether RTEF-1 is expressed within the human ocular vascular endothelial cells. We demonstrated that RTEF-1 and other previously undescribed alternatively spliced isoforms are expressed within human ocular vascular endothelial cells. The 447-bp isoform is present under hypoxic conditions. We showed that all isoforms are capable of enhancing expression from the human VEGF 5' proximal promoter and that 447-bp "hypoxic" isoform exhibits the most potent effect of the three isoforms tested.

To be able to design useful therapies, we must first hope for an understanding of the molecular events that lead to the onset and progression of disease. It is well established that VEGF plays an important role in the development and severity of ROP and other ocular neovascular diseases.<sup>15-18</sup> Thus, understanding how VEGF gene expression is regulated and which factors are involved in this process will allow us to better understand the etiology of neovascular disease and to develop new targets for therapeutic intervention. The RTEF-1 protein is able to bind to the VEGF promoter and to upregulate expression of the VEGF gene under hypoxic conditions in BAECs.<sup>9</sup> We investigated whether RTEF-1 mRNA is present within human retinal endothelial cells in culture under normoxic and hypoxic conditions.

The discovery of two new isoforms, one of which is present only under hypoxic conditions in human retinal vasculature, suggests that tissue-specific and possibly disease-specific isoforms exist for RTEF-1. Another member of the TEA domain family of genes, TEF-1, is also alternatively spliced into multiple



**FIGURE 4.** All RTEF-1 isoforms enhance expression from the full-length VEGF (F1-R3) promoter. The 1305-, 936-, or 447-bp isoform or a pcDNA no insert control was cotransfected with a human VEGF (F1-R3) promoter pSEAP reporter construct ( $n = 12$ ). One representative triplicate experiment is shown. Bars indicate reporter protein levels driven from the F1-R3 promoter when cells were cotransfected with pcDNA containing no insert or one of the RTEF-1 isoforms. All products showed significant differences (\*) when compared with the no insert control ( $P < 0.005$ ). No difference was observed between the 1305- and 936-bp isoforms. The 447-bp isoform was significantly different (#) from either of the two larger isoforms ( $P < 0.001$ ).



**FIGURE 5.** (A) All RTEF-1 isoforms require an Sp1-rich region within the VEGF promoter for efficient enhancement of expression. The 1305-, 936-, or 447-bp isoform or a pcDNA no insert control was co-transfected separately with a pSEAP reporter construct containing a human VEGF (F1-R3) promoter or a VEGF promoter lacking a 56-bp region containing four Sp1 sites (F1-R3 Sp1<sup>-</sup>). One representative triplicate experiment is shown ( $n = 12$ ). *Solid columns* show relative reporter protein levels driven from the full-length promoter (F1-R3), and *hatched columns* juxtaposed immediately to the right of the *solid columns* indicate relative reporter levels from the promoter lacking the Sp1 sites (F1-R3 Sp1<sup>-</sup>) when cells were cotransfected with the equivalent isoform. All isoforms showed a significant reduction in enhancement from the F1-R3 Sp1<sup>-</sup> promoter compared with the full-length F1-R3 promoter ( $P < 0.004$ ). Thus, all RTEF-1 isoforms require an Sp1-rich region for full-strength enhancement. (B) Comparison of the effect of each RTEF-1 isoform induced enhancement from the human VEGF promoter lacking Sp1 sites (F1-R3 Sp1<sup>-</sup>). The 1305-, 936- or 447-bp isoform or a pcDNA no insert control was cotransfected with an F1-R3 Sp1<sup>-</sup> promoter pSEAP reporter construct ( $n = 12$ ). Although overall enhancement is drastically reduced relative to the full-length F1-R3 VEGF promoter (Fig.

5A) the trend of enhancement by each isoform relative to the background expression, as indicated by the no insert control, is similar. The 1305-bp ( $P = 0.027$ ) and 936-bp ( $P = 0.035$ ) isoforms gave approximately 3- to 4-fold higher enhancement above background, whereas the 447-bp ( $P = 0.009$ ) isoform increased expression by approximately 12-fold. In this study, the 447-bp isoform was the only isoform considered to give a significant enhancement difference (\*) when compared with the no insert control ( $P < 0.01$ ).

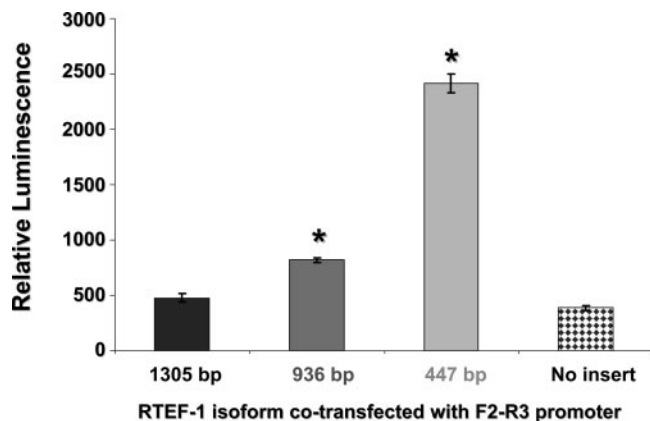
isoforms. These isoforms differ between normal and cancerous pancreatic cells.<sup>5</sup> Thus, it is possible that the 447-bp RTEF-1 isoform is expressed under conditions of disease. Whether this isoform is specific to retinal vascular tissue or is also present within other ocular cells or other human tissue remains to be determined. It would be of interest to determine whether RTEF-1 isoforms play a role in the etiology of human neovascular eye disease. To show that RTEF-1 exists *in vivo* within the mammalian retina, we looked for and identified transcripts (Fig. 7) of alternatively spliced RTEF-1 isoforms during mouse neural retina development and ROP disease development (Appukuttan B, et al. *IOVS* 2005;46:ARVO E-Abstract 3117). Not only are some of the mouse isoforms novel, they are unique to mouse tissue. In addition, specific isoforms are only present during particular stages of disease or normal development (Fig. 7). It is possible that retinal-specific isoforms exist that are unique to mice and humans and, though species specific, may have similar roles during disease and normal retinal development.

Human RTEF-1 was originally cloned from a heart cDNA library and was shown, by Northern blot analysis, to be abundantly expressed in human skeletal muscle and pancreas, less expressed in heart and kidney, and absent from brain, liver, and lung.<sup>2</sup> Subsequently, RTEF-1 has been shown to regulate gene expression through myocyte-specific CAT elements and may play a role in  $\alpha 1$ -adrenergic-induced hypertrophy of cardiac myocytes.<sup>19,20</sup> Mouse RTEF-1 also binds to M-CAT ele-

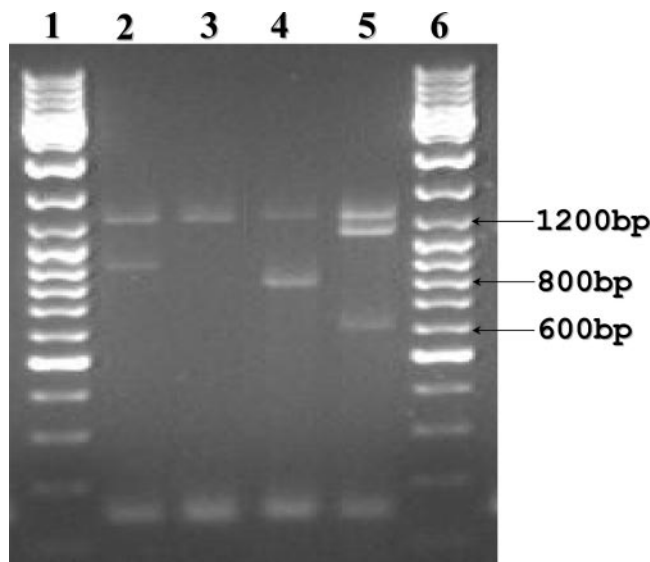
ments and is involved in skeletal muscle-specific expression and embryogenesis.<sup>8,21</sup> In a previous report, RTEF-1-mediated VEGF gene activation required interaction with an Sp1 element within the VEGF promoter and not M-CAT motifs within BAECs.<sup>9</sup> Mutation of the Sp1 site, situated at -97 to -89 bp, resulted in the abolishment of RTEF-1 enhancer activity, resulting in reporter gene expression equivalent to background expression levels. We observed a sharp decrease in overall expression from the VEGF promoter when Sp1 sites were deleted (Fig. 5A). Others have also shown that mutation of these Sp1 sites results in a natural reduction in VEGF promoter activity compared with the native promoter. Interestingly, enhancer activity is completely abolished for certain factors, such as platelet-derived growth factor, that normally requires these Sp1 sites for activity.<sup>22</sup> In contrast, all RTEF-1 isoforms still mediated some level of enhancement from the VEGF Sp1-negative promoter compared with the control, even though the SP1 sites were deleted (Fig. 5B). Although not conclusive, it is possible that these isoforms use sites other than Sp1 sites for enhancement. It is also possible that RTEF-1 regulates expression through an Sp1 site in BAECs but that RTEF-1 and isoforms modify expression in 293T cells through an alternative mechanism. Bovine endothelial cells may contain cell-specific cofactors necessary for RTEF-1/Sp1-mediated expression that are lacking in 293T cells. It would be of interest to determine whether RTEF-1 isoforms have the same effect on the VEGF promoter in various cell types.

The 447-bp RTEF-1 isoform isolated in this study was the most powerful stimulator of expression. This isoform was isolated from hypoxic cells, and it is well documented that VEGF mRNA levels are increased in human and rodent eyes under hypoxic disease conditions.<sup>18,23-25</sup> Thus, this isoform is still able to stimulate enhanced expression of a VEGF promoter that lacks an HRE region, the presence of which is normally crucial for VEGF expression induction under hypoxic conditions (Fig. 6). How an alternatively spliced isoform that codes for a protein lacking 65% of the "normal" full-length content is capable of augmenting function remains unclear. The 447-bp isoform contains the complete 72-amino acid TEA domain with nuclear localization signal, as do the other two larger isoforms. The 447-bp and the 936-bp isoforms lack exons 5 and 8, implying that the moieties responsible for the differences in enhancement lie elsewhere. A proline-rich domain (PRD), an activation domain common to other TEA domain proteins, exists within RTEF-1 between amino acids Pro-189 and Pro-213, encoded mainly by exons 7 and 8. This 25-amino acid stretch contains 12 proline residues. The PRD in TEF-1 is crucial for the activation of gene expression but is not required for the function of RTEF-1 in mouse skeletal muscle or human cervical carcinoma (HeLa) cells.<sup>8</sup> Thus, although the 936-bp isoform lacks the PRD, this is unlikely to be the cause of the variation given that the 1305-bp isoform containing the PRD exhibits levels of enhancement similar to the latter. Deletion and chimeric constructs of RTEF-1 and TEF-1 imply that the  $\alpha$ 1-adrenergic transactivation function of RTEF-1 is located within the conserved 200 amino acids at the carboxyl-terminal end.<sup>20</sup> This 200-amino acid region spans exons 9 to 12, and the 447-bp isoform contains only nine amino acids of exon 12 (Fig. 2). Of the three human RTEF-1 isoforms isolated, and considering that the 447-bp isoform is the most potent enhancer of the VEGF promoter, it seems that a combination of the loss of the carboxyl-terminal end and the PRD is somehow responsible for the amplification of activation.

Another plausible explanation for the function of the 447-bp isoform is the lack of two STY-rich domains. The STY-rich domains are regions rich in serine, threonine, and tyrosine residues, which are putative activation do-



**FIGURE 6.** RTEF-1 isoforms do not require the presence of the HRE sequence for the enhancement of expression from the VEGF promoter. Columns indicate relative luminescence of reporter protein levels driven from the F2-R3 promoter when cells were cotransfected with pcDNA containing no insert or one of the RTEF-1 isoforms. Results of one experiment performed in triplicate are shown ( $n = 9$ ). Asterisk: the 936-bp and 447-bp isoforms showed a significant difference compared with the no insert control ( $P < 0.0003$ ), whereas the 1305-bp product showed no difference ( $P = 0.027$ ;  $P > 0.01$ ). A significant difference was also observed between the 936-bp isoform and the shortest isoform ( $P < 0.0001$ ).



**FIGURE 7.** Agarose gel electrophoresis showing RT-PCR of RTEF-1 from cDNA prepared from mouse neural retina under normal development and conditions of ROP. Lanes 1 and 6, DNA ladder; lane 2, cDNA prepared from P8 mice under normoxic conditions gave 2 products (approximately 1300 and 920 bp); lane 3, cDNA prepared from P8 ROP mice gave 1 product (approximately 1300 bp); lane 4, cDNA prepared from P17 mice under normoxic conditions gave 2 products (approximately 1300 and 800 bp); lane 5, cDNA prepared from P17 ROP mice gave 3 products (approximately 1300 bp, 1100 bp, and 610 bp).

ains.<sup>26-28</sup> The STY-rich domains within RTEF-1 are situated between amino acids Ser-253 and Ser-271, within exon 9 and between Ser-311 and Ser-336 within exon 10.<sup>8</sup> The 447-bp isoform, unlike the other two isoforms, is missing exons 9 and 10. Serine, threonine, and tyrosine residues all have the potential to be phosphorylated, which can profoundly affect the biological activity of a protein. It is possible that the loss of potential phosphorylation sites within RTEF-1 may be responsible for the enhanced ability of the shortest isoform to stimulate expression. The STY-rich domain within exon 10 conforms to a consensus MAPK site.<sup>29</sup> Mutation of this MAPK site results in the loss of potentiation of an  $\alpha$ 1-adrenergic response in cardiac myocytes,<sup>20</sup> and the deletion of only the STY-rich domain in exon 9 results in loss of transactivation within mouse skeletal muscle and HeLa cells.<sup>8</sup> Further investigation into the new isoforms is required to discern the molecular mechanisms of expression enhancement.

In summary, the *RTEF-1* gene is expressed within human ocular-derived vascular cells and mouse retina. Novel murine-specific isoforms have been identified that may play a role in normal retinal development or in the development of retinal disease. Alternatively spliced novel human RTEF-1 isoforms have been identified that confer different transcriptional enhancer activity of the human VEGF promoter. A novel 447-bp isoform present within hypoxic retinal endothelial cells shows greater activation of expression from the VEGF promoter than other human isoforms. Developing therapies that target the 447-bp product for the treatment of ocular neovascular disease may prove beneficial.

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