Identification of Functional Estrogen Response Elements in the Gene Coding for the Potent Angiogenic Factor Vascular Endothelial Growth Factor

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis and a prognostic factor for many tumors including those of endocrine-responsive tissues such as the breast and uterus. We and others have previously shown that VEGF is regulated by estradiol and tamoxifen in the uterus and by estradiol in human breast cancer cells, and pharmacological evidence has suggested that this regulation was mediated by transcriptional activation of the estrogen receptor (ER). This prompted us to investigate whether the VEGF gene contains sequences that bind the ER and confer hormonal inducibility to reporter constructs in the presence of the two ER subtypes. These studies identified two sequences homologous to the consensus estrogen response element, GGTCA/AnnT-GACC, which bind both ER-α and ER-β. One of these elements is located in the 5'-untranslated region of the VEGF gene (GGGCAaagTGACT), and the other is located in the 3'-untranslated region (GAGC/ACC-T-GCC). Competition with excess unlabelled oligonucleotides indicates that these two elements bind both ERs specifically, mutations in either half-site of the two elements abolish receptor binding, and ER-α- and ER-β-specific antibodies interact with complexes formed with the corresponding receptor subtypes. In cells containing either ER-α or ER-β, the 3'-element behaves as a traditional enhancer that confers hormone inducibility to reporter constructs in an orientation-independent manner, and transcriptional activity is blocked by the pure antiestrogen ICI 182,780. The pattern of transcriptional activity of the element located in the 5'-flanking region is more complex. In the orientation found in the en-cancerres.aacrjournals.org Downloaded from cancerres.aacrjournals.org on April 12, 2017. © 2000 American Association for Cancer Research.

INTRODUCTION

Angiogenesis is the formation of new blood vessels from a pre-existing endothelium that involves proliferation of capillary endothelial cells and their migration toward the angiogenic stimulus (1, 2). The process is regulated by endothelial cell mitogens, of which VEGF is one of the most selective and potent angiogenic factors known (3). Numerous studies have demonstrated the importance of angiogenesis in cancer (see Ref. 4 for a recent review), and VEGF appears to be an important regulator of the process in this disease. For example, in vivo studies with nude mice demonstrated that suppression of VEGF inhibits tumor growth (5), and VEGF antibodies also decrease the metastasis of human tumors implanted in mice (6). Many studies have now demonstrated that VEGF transcript levels are elevated in human tumors, including those responsive to steroid hormones such as tumors of the breast, uterus, ovary, and prostate (7–11), and the vascularity and malignancy of these tumors correlate with their level of VEGF expression (10, 12). In some cases, VEGF production is so high that cancer patients even have measurably elevated levels of this angiogenic factor in their serum (13, 14).

In addition to such experimental studies, clinical studies have suggested a role of VEGF in cancers of tissues that are estrogen responsive such as the breast and endometrium. For example, in breast cancer, there is a correlation between VEGF expression and microvessel density in primary tumors (15), (b) a low relapse-free survival rate of patients with VEGF-rich tumors (15), and (c) an inverse relationship between VEGF levels and prognosis (16). Similarly, VEGF and its receptors are expressed in endometrial adenocarcinomas (17), and VEGF is produced by the Ishikawa (18) and HEC-1 (19) cell lines derived from human endometrial adenocarcinomas. Despite these observations, few studies have addressed the underlying mechanisms that regulate VEGF expression in these malignancies.

Given the established significance of estrogens in breast and endometrial cancer and the importance of angiogenesis in these diseases, we and others have recently initiated studies on the regulation of angiogenic factors such as VEGF by estrogens and antiestrogens. These studies have shown that estrogens increase VEGF expression in the rodent uterus (20–22), in human uterine tissue (19, 23), in Ishikawa (24) and HEC-1 cell lines derived from human endometrial adenocarcinomas (19), in 7,12-dimethylbenz(a)anthracene-induced rat mammary tumors (25), and in human breast cancer cells (26). There is thus an emerging body of evidence that estrogens and mixed estrogen agonists such as tamoxifen (26) may play a role in the regulation of angiogenic factors such as VEGF in both hormone-responsive cancers and normal target tissues (27, 28). Whereas the above-mentioned studies established that estrogens regulate VEGF expression in a number of systems, little is known about the underlying molecular mechanism(s) of this effect. Studies from our laboratory and the work of others have found that: (a) estrogens rapidly induce expression of VEGF mRNA (reviewed in Refs. 27 and 28); (b) this induction is blocked by the pure antiestrogen ICI 182,780 (29); and (c) elevations in transcript levels are sensitive to inhibitors of RNA, but not protein synthesis (21). Collectively, these findings suggest that the estrogenic regulation of VEGF expression is due at least in part to a direct transcriptional action of the ER. However, there have been no reports to date of the presence of a functional ERE in the VEGF gene, and this prompted us to search for such a regulatory sequence. We now report in this communication that an ERE analogous to the consensus element is located in the 5'-UTR of the VEGF gene and functions as a classical enhancer for both ER-α and ER-β. In addition, we have also identified a new type of ER binding site in the 5'-UTR of the gene. The biological activity of the 5'-element is dependent on its orientation upstream of the transcription start site in reporter constructs and on the ER subtype cotransfected into target cells. These findings further support the hypothesis.
that estrogens regulate VEGF expression by direct transcriptional actions of the ER.

MATERIALS AND METHODS

Materials. Enzymes were obtained from Boehringer Mannheim (Indianapolis, IN), and oligonucleotides were synthesized by Genosys (Woodlands, TX). Recombinant human ER-α (Ms 66,000) and ER-β (M, 53,000) were purchased from Panvera (Madison, WI). PAGE supplies were obtained from Bio-Rad (Richmond, CA). The antibody for the ER-α supershift experiments was purchased from Santa Cruz Biotechnologies (San Ramon, CA), and the antibody for ER-β was obtained from Affinity Bioreagents (Golden, CO).

All transfection studies were performed with HeLa cells obtained from American Type Culture Collection (Manassas, VA) using LipofectAMINE transfection reagent from Life Technologies, Inc. (Grand Island, NY). Fetal bovine serum was obtained from Life Technologies, Inc., and hormones were obtained from Steraloids (Newport, RI). DNA purification kits for the preparation of supercoiled plasmids used in transfections were purchased from Qiagen. ICI 182,780 was kindly provided by Dr. Alan Wakeling (Zeneca Pharmaceuticals, Maccelsfield, United Kingdom). All other materials used were of the highest grade commercially available.

Mobility Shift Assays. The sequences of the oligonucleotides derived from rat VEGF used for gel shift assays are shown in Fig. 1B and Fig. 1A, along with that of the ERE from the Xenopus vitellogenin gene (30). Oligonucleotides were labeled to a specific activity of 0.5–1.0 × 10⁶ cpm/ng with T4 polynucleotide kinase (Boehringer Mannheim) and [³²P]ATP (Amersham, Arlington Heights, IL), and gel shift assays were done with in vitro-synthesized receptors (Panvera) as described previously (31), with minor modifications. Briefly, 100 ng of the receptor protein (unless otherwise stated) were incubated with the polynucleotide dI-dC (1 μg/μg receptor) in 20 μl of TND buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM DTT] containing 1 mg/ml BSA at 4°C. ERs were diluted just before use to the desired concentration (usually 100 ng/μl) with storage buffer containing 1 mg/ml BSA as described in the manufacturer’s brochure. After 20 min on ice, 0.1–1.0 ng of a ³²P-labeled oligonucleotide containing an ERE sequence was added and incubated for 20 min at 4°C. The entire mixture was then loaded onto 4%–10% polyacrylamide gels (30:1) and run at 160 V for 90 min. When the ER antibody was used, it was added to the reaction mixture containing the receptor and incubated for 1 h before adding the probe. The position of the ER-DNA complexes is illustrated by the arrowheads in the figures.

Plasmids and Transient Transfection Assays. The TK-Luc and ERE-TK-Luc plasmids were generously provided by Dr. Vincent Giguere (University of Montreal, Montreal, Quebec, Canada). These contain the minimal promoter from the TK gene linked to the Luc reporter plasmid. In the case of ERE-TK-Luc, a single copy of the cERE is placed upstream of the TK promoter in the TK-Luc plasmid. The ER-α expression plasmid has been described previously (32), and the ER-β plasmid, which encodes a 447-amino acid form of the protein under the control of the RSV promoter (33), was a kind gift of Dr. Donald McDonnell (Duke University, Durham, NC).

The transfection of HeLa cells was performed as described previously (34). Briefly, cells were maintained in DMEM supplemented with 10% fetal bovine serum. Twenty-four h before transfections, cells were plated at 3 × 10⁵ cells/well in Falcon 6-well dishes in 5% dextran-coated charcoal stripped serum. Cells were transfected with the indicated plasmids using LipofectAMINE transfection reagent (Life Technologies, Inc.) according to the manufacturer’s guidelines. Cells were washed, DMEM containing 5% charcoal stripped serum was added, and hormones were added to the media. Cells were harvested 24 h later and assayed for Luc activity using the Luc assay system from Promega (Madison, WI). Luc activity is expressed as light units/unit protein, which we (34) and others (e.g., see Ref. 35) have established yields values similar to those based on internal β-galactosidase transfection standards. Data are presented as the means of three or more determinations, and all experiments were repeated at least twice with similar results. Where indicated in the text and figure legends, the means were analyzed for statistical significance by using Student’s t test.

RESULTS

The VEGF Gene Contains Numerous Sequences Homologous to the cERE. Fig. 1A is a representation of the genomic structure of the VEGF gene. It consists of eight exons and spans approximately 14 kb of the genome. Because the most commonly reported location of EREs is in the 5′-flanking region of target genes, we initially transfected 1 kb of the rat VEGF gene promoter and its 5′-flanking sequence linked to the Luc promoter (kindly provided by Dr. Mark...
Goldberg, Brigham and Women’s Hospital, Boston, MA) into HeLa cells and cotransfected the cells with an ER expression plasmid. This region of the VEGF gene did not confer hormonal inducibility by estradiol concentrations of up to 10 nM in this commonly used test system.

Because EREs have also been identified in the untranslated and exonic regions of some hormone target genes, we next examined all of the available VEGF genomic sequence for regions homologous to the cERE, which is also referred to as the vitellogenin ERE because it was initially identified in that gene. The consensus element is a perfect palindrome with the sequence GGTCAnmTGACC, and we initially searched for sequences with identity in at least 6 of 10 bases in the two half-sites. We have previously used this strategy to identify EREs and ER binding sites in other genes (36, 37), and this approach would identify the majority of known EREs that are homologous to the consensus element (38). This approach identified 11 sequences with a minimum 60% homology to the cERE. These sequences are shown in Fig. 1B, and their location in the VEGF gene is indicated in Fig. 1A. The base mismatches from the consensus element in the core of the cERE sequence are shown in lowercase letters in Fig. 1B.

**Sequences Present in the 5'- and 3'-Regions of VEGF Selectively Bind the ER.** All of the oligonucleotides shown in Fig. 1B were then labeled with 32P and tested for ER binding in a gel shift assay using recombinant human ER-α (Fig. 2A) or ER-β (Fig. 2B). The cERE from the vitellogenin gene (Lane V in Fig. 2, A and B) was used as a positive control. As seen in Fig. 2, these studies showed that only the oligonucleotides used in Lanes 4 and 9 bind ER efficiently. Although some binding was also seen with the DNA sequence used in Lane 7, other experiments indicated that this binding is nonspecific because it is not competed by excess unlabeled oligonucleotide (data not shown). This is in contrast to the results of competition experiments with the sequences used in Lanes 4 and 9 (see below).

The two elements that exhibit specific DNA binding are located in the 5’- and 3’-UTRs of the gene, and both have sequences very similar to the cERE. The oligonucleotide represented in Lane 4 in Fig. 2 is located in the 5’-UTR of the VEGF gene (present in exon 1 between the transcription start site and the ATG codon used to initiate translation) and has one mismatch from the cERE in both half-sites. The oligonucleotide used in Lane 9 is located in the 3’-UTR between the first and second polyadenylation sites and has two mismatches from the consensus element in the 5’ half-site and one mismatch in the 3’ half-site. For ease of description, these two sequences are referred to as VEGF-5’ and VEGF-3’ sequences or EREs in the remainder of this article.

**The Binding of ER-α and ER-β to the VEGF-5’ and 3’ Sequences Is Specific.** We next tested the two oligonucleotides showing binding of ER-α and ER-β for specificity by using excess unlabeled oligonucleotide for competition and examining the effects of receptor antibodies in gel shift studies. Fig. 3A shows that both VEGF-5’ and VEGF-3’ sequences bound ER-α and ER-β and that binding in both cases was competed by excess unlabeled oligonucleotide. Similar experiments with the oligonucleotide used in Lane 7 of Fig. 2, A and B, failed to show competition when either receptor was used, indicating that the binding of this sequence is nonspecific (data not shown).

In addition to competition studies with excess cold oligonucleotides, we examined the effect of receptor antibody on the retarded bands. Fig. 3B illustrates that an ER-α-specific antibody supershifts the oligonucleotide complex formed on incubation with that receptor subtype. Similarly, antibodies to ER-β supershift the complex formed with that receptor subtype and either VEGF sequence (Fig. 3C). Other experiments showed that the ER-α antibody used in Fig. 3B did not supershift complexes formed with ER-β and that the ER-β antibody used in Fig. 3C did not supershift complexes formed with the ER-α receptor (data not shown).

**Mutations in the VEGF 5’ and 3’ Sequences Abolish ER Binding.** To establish the exact identity of the sequence in the 5’- and 3’-regions of the VEGF gene that bound the ER in the preceding experiments, we mutated bases in the first (mt1) or second (mt2) candidate half-sites of the 27-bp oligonucleotides (see Fig. 1B) used above for ER binding. These mutated sequences are shown in Fig. 4A. These mutated oligonucleotides were labeled with 32P and examined for binding to both receptor subtypes in gel shift experiments. Mutations in either the first or second half-site of both elements abolished ER-α binding (Fig. 4B) and also abolished ER-β binding (Fig. 4C).

**Transcriptional Activity of VEGF 5’- and 3’-Elements.** To assess whether the two regions in the VEGF gene that bind ER also confer hormone responsiveness, we ligated tandem copies of the elements upstream of the TK promoter linked to a luc reporter. These constructs were then transfected into HeLa cells (which lack endogenous ER) together with an expression plasmid for either ER-α (Fig. 5A) or ER-β (Fig. 5B) to test whether the VEGF sequences conferred
We inserted the VEGF sequences into the reporters in both their naturally occurring and opposite orientations, illustrated in Fig. 5 by arrows in the forward (→) or reverse (←) directions, respectively. This was done to determine whether any observed activity of the VEGF sequences was similar to that of classical enhancer elements, which is orientation independent. As a positive control in each series of experiments, we transfected a Luc reporter ligated downstream of the cERE, and vectors lacking any ERE sequence (TK-Luc) were also included in each series.

The results obtained with the VEGF 3'-element are relatively straightforward and indicate that this element confers hormonal inducibility to reporter constructs. As shown in the far right panel of Fig. 5A, the VEGF 3'-sequence functions as a classical estrogen-dependent enhancer, i.e., it confers estrogen-induced transcription when present in either the forward or reverse orientation in cells containing ER-α. This receptor subtype increases reporter activity 3–4-fold, and induction is blocked by ICI 182,780. This element also confers an induction of activity with ER-β that is abolished by the pure antiestrogen (Fig. 5B, far right panel). As with the α receptor, the β receptor induces Luc activity when the VEGF 3'-sequence is present in either orientation. The induction with the β receptor is reproducible and statistically significant in both orientations, but the

![](image1.png)

**Fig. 3.** Binding characteristics of ER-α and ER-β to candidate EREs in the VEGF gene. A, specificity of ER-α and ER-β binding. ER-α (left lanes) or ER-β (right lanes) was incubated with labeled oligonucleotides containing the cERE present in the vitellogenin gene (Vit-ERE), the putative ERE in the 5'-UTR of VEGF (top lanes), or the putative ERE in the 3'-UTR of VEGF (bottom lanes) as described in “Materials and Methods.” The arrows on the left and right sides of the figure illustrate the position of the complexes formed. Additions: all lanes contained a labeled oligonucleotide as indicated; ER-α or ER-β, and a 100-fold excess of the unlabeled oligonucleotide (COMP) as indicated. B, antibodies to ER-α supershift complexes formed with the corresponding receptor. Gel shift analysis was performed by incubating the labeled oligonucleotides as indicated with ER-α (ER) in the presence or absence of an antibody (Ab) specific for that receptor. The arrow marked F illustrates the position of free probe. C, antibodies to ER-β supershift complexes formed with the corresponding receptor. Gel shift analysis was performed as described in B using ER-β and an antibody specific for that receptor.

...estrogen inducibility in the presence of the two ER subtypes. We inserted the VEGF sequences into the reporters in both their naturally occurring and opposite orientations, illustrated in Fig. 5 by arrows in the forward (→) or reverse (←) directions, respectively. This was
response is quantitatively less than that seen with ER-α. This difference between the magnitude of induction by the two receptors has been reported previously for reporters containing the cERE (39, 40), and we observed a similar effect with our positive controls, i.e., note that the fold induction of the cERE over background is greater in the presence of ER-α (Fig. 5A, far left panel) than ER-β (Fig. 5B, far left panel).

In contrast to the results obtained with the VEGF-3′ ERE, the element that we identified in the 5′-UTR of the gene demonstrated properties not previously observed in other EREs. In the presence of ER-α, this sequence confers estrogen inducibility when present in the reverse orientation (Fig. 5A, center panel), and this effect is blocked by ICI 182,780, as expected for a receptor-mediated event. However, when placed upstream of the promoter in the forward or naturally occurring orientation, the element represses basal transcription on the addition of hormone. This effect is partially reversed by ICI 182,780, suggesting that it is mediated at least in part by the ER. Depending on its orientation, this element thus activates or inhibits transcription in the presence of ER-α when estradiol is added. It is important to emphasize that in these experiments, the VEGF 5′-sequence was inserted into the two reporter constructs in exactly the same position, i.e., the spacing of the element relative to the TK-Luc sequences is identical in both orientations.

The 5′-element also exhibits rather unusual properties in cells cotransfected with ER-β. When present in the naturally occurring orientation, the element is inert in the presence of this receptor subtype (Fig. 5B, center panel). In the reverse orientation, estradiol activates transcription (Fig. 5B, center panel), and control experiments (data not shown) verified that this hormone-dependent activation is absolutely dependent on ER-β cotransfection. Surprisingly, estradiol activation is not blocked by the pure antiestrogen ICI 182,780. One possible explanation of this observation was that the ICI compound behaves as an agonist in this experimental context and thus fails to inhibit the effect of estradiol. However, a separate set of measurements showed that ICI 182,780 alone does not induce Luc activity from this reporter in the presence of ER-β and the antiestrogen does not affect the basal activity of this construct (Fig. 5A, far left panel), and Luc activity was determined 24 h later.

The overall results of our transactivation studies are summarized in Table 1. The VEGF 3′-element behaves as a classical enhancer element in the presence of either ER subtype. In contrast, the activity of Table 1. The VEGF 3′-sequence is reverse orientation-dependent.

Table 1  Receptor- and orientation-selective actions of VEGF ERE transcriptional activity

<table>
<thead>
<tr>
<th>ER</th>
<th>5′ VEGF</th>
<th>3′ VEGF</th>
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<tbody>
<tr>
<td>α</td>
<td>Inhibition (→)</td>
<td>Activation (→, →)</td>
</tr>
<tr>
<td>β</td>
<td>Inert (→)</td>
<td>ICI 182,780-insensitive activation (→)</td>
</tr>
</tbody>
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*Arrows indicate endogenous (→) or reverse orientation (→ →).
of the 5′-sequence is both orientation and receptor dependent, and the activity of this element in the presence of ER-β is completely unresponsive to ICI 182,780.

DISCUSSION

VEGF is an important angiogenic molecule that is regulated by estrogens both in vivo (20, 21) and in primary cultures derived from the reproductive tract (19, 23), in endometrial cancer cell lines (24), and in breast cancer cells (26). In addition to stimulating the growth of new capillaries from existing vessels, VEGF is also the most potent known regulator of capillary permeability (41). In vivo, this biological activity of the growth factor could thus play a role in the proliferation of uterine, ovarian, and breast cells by causing changes in capillary permeability to water, small molecules, and proteins that have been reported previously in response to estrogens (27). Increased angiogenesis and changes in permeability enhance the perfusion of tissues and lead to an increase in nutrient supply and the local tissue concentration of plasma proteins. In addition, endothelial cells are also known to synthesize a variety of growth factors (42), and VEGF stimulation of endothelial cell proliferation or growth factor secretion thus has the potential to initiate paracrine signaling pathways that could also stimulate proliferation.

The seminal work of Folkman (1) has established that tumors cannot grow beyond a certain volume without the formation of new blood vessels. This raises the possibility that estrogens may regulate the growth and spread of cancer cells in part by stimulating VEGF production and thus increasing the density of the microvasculature needed for tumor expansion and metastasis. It is known, for example, that the capillary density of breast tumors correlates with their level of VEGF production (28), and estrogens increase VEGF production in 7,12-dimethylbenz(a)anthracene-induced rat mammary cancer (25). Other studies have also shown that androgen-dependent (43) prostate growth correlates with VEGF production and that progestins stimulate VEGF production in some human breast cancer cells (44). Thus, VEGF may mediate certain actions of androgens and progestins, as well as those of estrogens. Steroid hormone control of angiogenesis and vascular permeability thus seems likely to play a number of physiological and pathological roles, and this has stimulated our group and others to investigate the mechanisms by which sex steroids regulate the production of VEGF and other angiogenic factors (19, 20, 23, 26).

We (21, 29) and others (19, 20, 23) have shown that VEGF mRNA levels are regulated in vivo by estrogens as well as by partial estrogen agonists such as tamoxifen (21, 26, 45). This regulation is very rapid, is sensitive to actinomycin D but independent of new protein synthesis, and is blocked by the pure antiestrogen ICI 182,780 (21, 29). All this evidence suggests that estrogens directly regulate VEGF expression at the transcriptional level and led us to search for a functional ERE within the gene. Given the increasing evidence that angiogenesis is important for the growth and metastasis of many hormone-dependent tumors, we felt this question was of fundamental importance in understanding potential mechanistic bases for the effects of estrogens on this process.

Because many EREs are present in the 5′-flanking region of hormone-responsive genes within 1–2 kb of the transcription start site, we initially tested this region of the rat VEGF gene for biological activity. These results were negative, but they do not rule out the possibility that sequences in the 5′-flanking region might affect estrogen responsiveness in other cell backgrounds or that other elements in this region contribute to the overall control of VEGF transcription in vivo (46).

However, based on sequence homologies to the cERE we were able to identify two putative ERE sequences within the rat VEGF gene. These two DNA sequences bind both ER subtypes with the characteristics expected of authentic hormone response elements. With both receptors, binding is specific, mutations in either half-site destroy binding, and complexes formed with either ERE are supershifted by the appropriate receptor antibody. However, these elements are located in the 5′- and 3′-UTRs of the gene, unlike most EREs identified to date, which are more commonly located in the 5′-flanking region of hormone target genes. However, it is important to note that EREs have previously been identified in the UTRs and exonic regions of other genes (36, 47, 48). More novel was the discovery that functional EREs were present in both the 5′-UTR and 3′-UTR of the gene. To our knowledge, VEGF is thus the first example of a gene containing EREs that flank the coding sequence of a target gene. This spatial arrangement raises the interesting possibility that ER molecules bound to both upstream and downstream regions of the gene may interact to regulate its overall pattern of expression. Because the 5′- and 3′-flanking regions of the gene are also likely to contain binding sites for additional regulatory factors, this would theoretically increase the combinatorial options for integration of estrogenic and other signals for the overall control of VEGF expression. The possibility of such multifactorial control could have special significance by providing multiple options for VEGF production and subsequent tumor expansion and metastasis because both these processes are absolutely dependent on angiogenesis.

Whereas the exact biological significance of this spatial arrangement of these sequences in the VEGF gene remains to be established, the receptor binding characteristics of both elements are similar to those of other EREs. In contrast, the transcriptional activities of the 5′- and 3′-elements are complex and dependent on both receptor subtype and orientation. These are summarized briefly in Table 1, which illustrates that these elements have unique transcriptional properties that have not been observed in other EREs identified to date.

The most straightforward results were obtained with the VEGF 3′ sequence. This element behaves as a traditional enhancer in the presence of both ER-α and ER-β, i.e., it activates transcription in an orientation-independent manner. As expected, hormonal induction from this element is blocked by the pure antiestrogen ICI 182,780. The magnitude of induction of transcriptional activity from reporters containing this element is somewhat greater with ER-α than ER-β, and this is consistent with previous reports showing similar quantitative effects of the two receptor types in studies using the cERE (40). The transcriptional effects of the ERE in the VEGF 5′-UTR are more complex. When present in the reverse orientation, this element functions with ER-α as a traditional hormone response element to activate the Luc activity of reporter constructs (Fig. 5A, middle panel). This indicates that the element can confer hormonal responsiveness to ER-α similar to that of a traditional ERE when present in one orientation.

When present in the reverse orientation, the 5′-element also confers hormonal inducibility with ER-β. However, one of the most surprising results from our study was that this activity was not blocked by the pure antiestrogen ICI 182,780. This led us to test whether ICI 182,780 alone had agonist activity with ER-β in the context we used for these experiments. The results of that study indicated that ICI 182,780 is devoid of either agonist or antagonist activity, indicating that it cannot activate ER-β nor block activation by estradiol from reporters containing the 5′-ERE of the VEGF gene. It is important to note that ICI 182,780 completely blocks transcriptional activity from reporters containing either the cERE (Fig. 5B, far left panel) or the VEGF 3′-ERE (Fig. 5B, far right panel) in the presence of ER-β. This is an important point because it demonstrates unequivocally that in the experimental system used for these studies, the antiestrogen inhibits ER-β-mediated transcription in the absence of the VEGF 5′-element.
We do not understand the mechanistic basis for this unusual effect, but there are several possible explanations. One possibility is that the binding of ER-β to the 5′ VEGF DNA sequence alters the conformation of the receptor’s ligand binding site so that it cannot bind estradiol (see the center panel of Fig. 5B illustrating reporter activation of the 5′-ERE in the reverse orientation). Another possibility is that the binding of ER-β to this particular DNA sequence distorts the receptor surface so that it fails to bind or interact correctly with a corepressor on antiestrogen binding. Finally, estradiol may activate ER-β by a novel mechanism (e.g., via a phosphorylation cascade) that selectively stimulates transcription from the VEGF 5′-ERE but not other elements, and this pathway is insensitive to ICI 182,780 inhibition. Regardless of the molecular mechanism, this effect may have special implications for the growth of tumor cells containing ER-β because it raises the possibility that pure antiestrogens such as ICI 182,780 may not block estrogenic induction of VEGF in such cases. In this regard, it is interesting to note that a recent report identified a subset of breast tumors containing high levels of ER-β that is not responsive to tamoxifen (49).

Another surprising finding was the properties of the VEGF 5′-element when present in the forward orientation (i.e., its orientation in the endogenous gene). In the presence of ER-α, the element in this orientation decreases basal transcription by 70% (see Fig. 5A, far left bars), and this effect is highly significant (P < 0.01). This inhibition is significantly reversed by the ICI 182,780 compound (P < 0.001 for the E2 versus E2 + I groups), which suggests receptor involvement, although the antiestrogen does not restore the activity completely to the basal level (see below). In contrast to ER-α, the ER-β receptor is transcriptionally inert when this element is ligated upstream of the TK promoter in the forward orientation. The 5′-element thus provides an example of a sequence that binds both ER subtypes well in gel shift assays but shows receptor-selective transcriptional effects and could thus have biological implications for understanding the effects of estrogens on tumor cells with varying ratios of the two ER subtypes.

As noted above, ICI 182,780 diminishes but does not completely reverse the inhibition of ER-α-mediated transcription from the VEGF 5′-element in the forward orientation (see Fig. 5A, center panel). Note, however, that the activity of all the reporter constructs (including the one with the cERE) co-transfected with ER-α is less than the basal level when both estradiol and the antiestrogen are added to the medium (Fig. 5A). This is often observed in transfection studies with ER-α and is generally attributed to either low amounts of residual estrogens (which could “artificially” elevate the basal level of transcription) or the well-established decrease in ER-α protein levels caused by the antiestrogen (50). Thus, whereas ICI 182,780 does not completely restore activity to the basal level, it does restore the activity to the same level observed with all of the constructs in the presence of estradiol and the antiestrogen (see the E2 + I activity for all reporters containing the cERE or either VEGF element). Because this level of activity is probably a true measure of basal transcription, these results are quite consistent with the interpretation that the inhibition of reporter activity from the VEGF 5′-element is mediated by ER-α.

To our knowledge, the VEGF 5′-ERE is the first example of a functional ERE that mediates transcriptional activation in one orientation and repression in the opposite orientation. When present in the orientation of the endogenous gene, it is also the first example of an element that binds both ER subtypes but exhibits a selective transcriptional response to one of them, i.e., ER-α. These observations raise many questions about the molecular basis for these observed effects, and we have initiated studies to determine the mechanisms involved. In any case, these observations also raise many important issues about the estrogenic regulation of VEGF-induced angiogenesis in hormone-responsive cancers. For example, VEGF production in a normal cell may be regulated by an interplay of positive and negative signals emanating from different ERE elements that could be differentially altered in hormone-responsive tumors, and the level of VEGF production in a given tumor might depend on the relative levels of receptor subtypes because these appear to exert different transcriptional activities at the two VEGF elements we have identified.

Our results do not exclude the possibility that estrogens may also regulate VEGF via other mechanisms, e.g., by protein-protein interactions, via kinase-mediated pathways, or by binding to one ERE half-site followed by cooperation with other transcription sites (51–53). Also, our data do not exclude the possibility of other classical EREs that may be present at relatively long distances away from the promoter, as has been noted for other estrogen-responsive genes (47, 54). Rather, the main significance of our results is that we have identified for the first time elements within the VEGF gene that can confer transcriptional regulation in intact cells, thus supporting the hypothesis of direct hormonal control of VEGF production.

In summary, our results have identified functional EREs in the two UTRs of the VEGF gene. Previous studies from our group and others have demonstrated that estrogens regulate VEGF expression in normal tissues, endometrial cancer cells, and breast cancer cells, but this is the first report of a functional ERE(s) in a gene coding for a major angiogenic growth factor. This supports the hypothesis that estrogens regulate VEGF expression at least in part via transcriptional effects of either ER-α or ER-β. This in turn raises the possibility that estrogens can directly stimulate the production of this key regulator of angiogenesis in hormone-responsive tumors. Although there have been no definitive studies linking the expression of VEGF to steroid receptors in endocrine-dependent cancers, one recent report indicates that VEGF expression may correlate with ER levels (55) in certain types of human breast tumors. This would make angiogenesis a potential target for antihormone therapy in a subset of human breast cancers and potentially in other endocrine diseases.

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# Identification of Functional Estrogen Response Elements in the Gene Coding for the Potent Angiogenic Factor Vascular Endothelial Growth Factor

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*Cancer Res* 2000;60:3183-3190.

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