

Haplotype Analysis of the Polymorphic Human Vascular Endothelial Growth Factor Gene Promoter¹

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ABSTRACT

The human vascular endothelial growth factor (*VEGF*) gene is unusually polymorphic, and there is evidence for inheritance of conserved haplotypes. One haplotype, carrying polymorphisms at $-460/+405$, is associated with enhanced production of *VEGF* *in vitro*. The *VEGF* promoter is activated by phorbol esters and, in endometrial cells, by estrogen. We have analyzed the impact of the common $-460/+405$ polymorphism on both basal and stimulated *VEGF* transcription using the human breast cancer cell line MCF7. Because the *VEGF* promoter is so highly polymorphic, haplotypes were established and analyzed. Carriage of the $-460/+405$ polymorphisms increased basal promoter activity by 71% compared with the wild-type sequence. However, this effect was dependent on colinearity with a series of further 5' sequence polymorphisms. The $-460/+405$ polymorphism also increased the mean induction by phorbol ester from 5-fold to 8.5-fold. In contrast to earlier studies in endometrial cells, none of the human *VEGF* promoter constructs was regulated by estrogen. Overexpression of the estrogen receptor did not confer estrogen regulation to *VEGF*, implying cell type-specific hormonal regulation. Therefore, carriage of the $-460/+405$ polymorphism significantly alters *VEGF* promoter activity and responsiveness. This has implications for the inherited susceptibility of common diseases.

INTRODUCTION

Angiogenesis only occurs in normal adult tissues during wound healing and in the female reproductive cycle but is activated in disease. *VEGF*³ is one of the most potent and specific angiogenic factors (1, 2), and increased *VEGF* expression and associated pathogenic angiogenesis are found in tumors (3), rheumatoid arthritis (4), and diabetic retinopathy (5).

Numerous studies have shown that growing tumors require the establishment of a blood supply (6), and *VEGF* is often up-regulated in cancer (1). Indeed interruption of *VEGF* action profoundly inhibits tumorigenesis (7, 8). Recent work has identified cell type-specific enhancers within the *VEGF* promoter that up-regulate *VEGF* expression in glioblastoma (9), a tumor type that abundantly expresses *VEGF* and is particularly dependent on *VEGF* for its aggressive growth (10).

The *VEGF* gene is located on chromosome 6p21.3 (11) and consists of eight exons exhibiting alternate splicing to form a family of proteins (12). The *VEGF* gene is reported to be regulated by estrogen, hypoxia, growth factors including epidermal growth factor, and cytokines including interleukin 6 (13–16). There is considerable variation between individuals in *VEGF* expression, and analysis of the 5'-

flanking region of the gene has shown the presence of many polymorphisms (17–19).

The rat *VEGF* gene contains two ER-binding sites. One of these was in the 3'-untranslated region and worked as a conventional enhancer, and the other, located in exon 1 (+410), appeared to repress transcription in the presence of estrogen (15). Transfection analysis of the human *VEGF* promoter, in contrast, did reveal a positive estrogen response, which appeared to be conferred by a sequence at between -1526 and -1514 (16).

One of the previously reported polymorphisms (+405 G to C) is located adjacent to the +410 ERE that was identified in the rat *VEGF* gene and is highly conserved in the human, and an 18-nucleotide insertion at -1512 is located close to a further, putative upstream ERE between -1514 and -1526 (17, 19). The sequence (-1526 -aatcagactgactgg-cctcagagccc) was found to bind ER in gel shift analysis; the site of the 18-nucleotide insertion identified by Brogan at -1512 is marked – (17; Fig. 1a). Therefore, it seemed likely that carriage of these polymorphisms would alter the estrogen responsiveness of the promoter, and, because they are frequent, this would have important implications for regulation of *VEGF* expression.

Several lines of evidence suggest the need for haplotype analysis of the human *VEGF* promoter. The human *VEGF* gene is highly polymorphic; some polymorphisms are frequent in the normal population (allele frequencies +405 G to C at 0.709; -1512 insertion at 0.675), and haplotype frequencies in the normal population suggest significant shared inheritance patterns (17, 19). Recent data suggest that linkage disequilibrium is highly structured into conserved blocks of sequence that are separated by recombination hot spots (20, 21). Within a conserved block, however, association studies will generally be insufficient to identify the causal polymorphism, and indeed, the overall function of a conserved haplotype may require interaction among polymorphisms for its final expression. It is proposed that within a conserved block, the precise location of a causal variant would be better identified based on biological function, rather than formal, statistical models (22). Case-control studies have shown associations between polymorphisms of the *VEGF* gene and diabetic retinopathy (23), preeclampsia (24), and acute renal allograft rejection (25).

This study sought to test the hypothesis that genetic polymorphisms in the 5' region of the *VEGF* promoter influence gene transcription. We analyzed haplotypes containing the common polymorphisms at -460 and $+405$ that have been associated with human disease in response to estrogen and phorbol esters.

MATERIALS AND METHODS

Genotyped individuals homozygous for the two common *VEGF* -460 and $+405$ haplotypes ($-460C/+405G$ and $-460T/+405C$) that account for 80.9% of the population were identified as described previously (19). The *VEGF* promoter was amplified from genomic DNA using Qiagen proofstart DNA polymerase and the primers 5'-caggactagtgcacgaatga and 3'-tctgtctgtctgtcctgccg. Conditions were 35 cycles of 94°C for 50 s, 61°C for 50 s, and 72°C for 5 min. The 2.3-kb band was excised from a 1% agarose gel (1× Tris acetate EDTA buffer) and cloned into a pGEM-T vector (Invitrogen). Clones were fully sequenced to exclude PCR errors, to confirm the presence of the predicted

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³ The abbreviations used are: *VEGF*, vascular endothelial growth factor; ER, estrogen receptor; TNF, tumor necrosis factor; ERE, estrogen response element; PMA, phorbol myristate acetate; PBML, peripheral blood mononuclear leukocytes; SERM, selective estrogen receptor modulator.

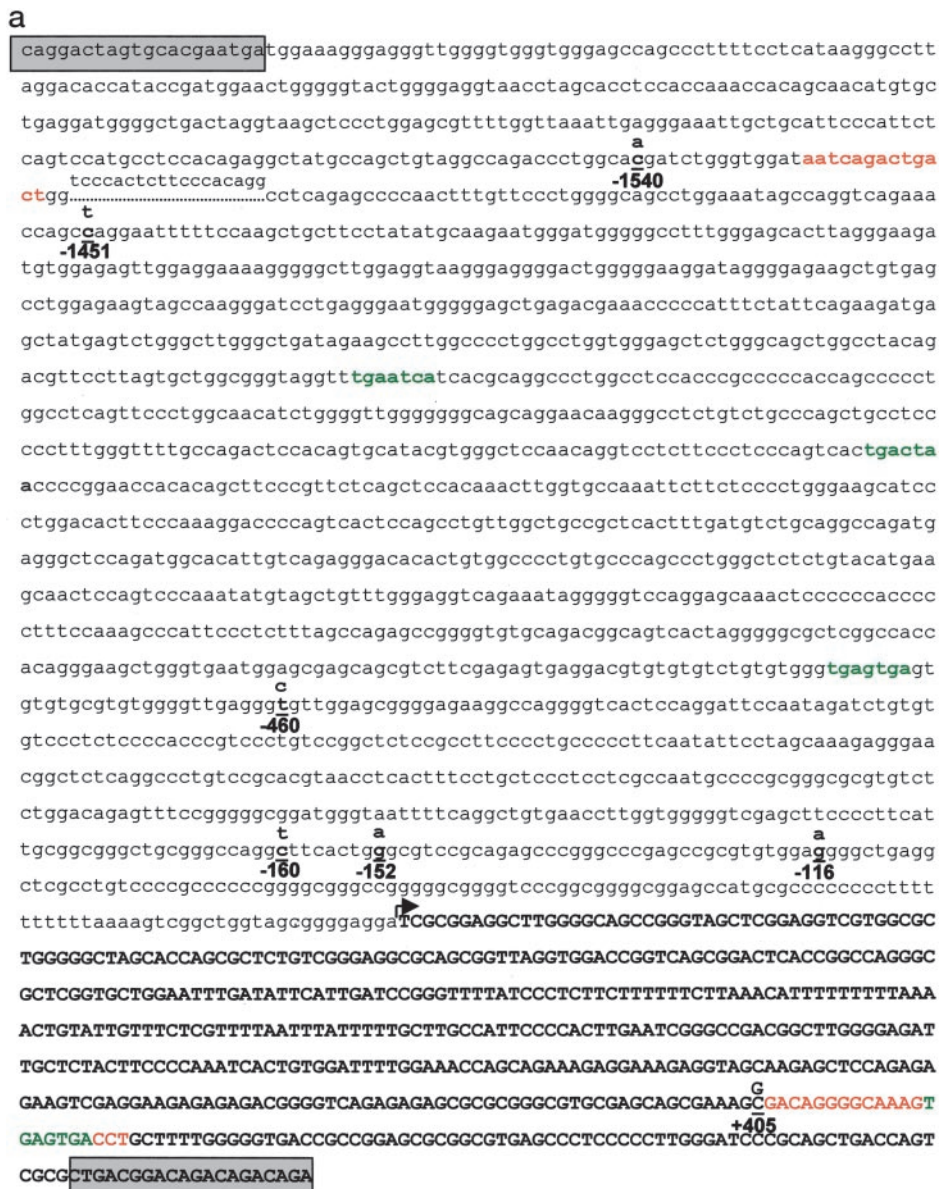
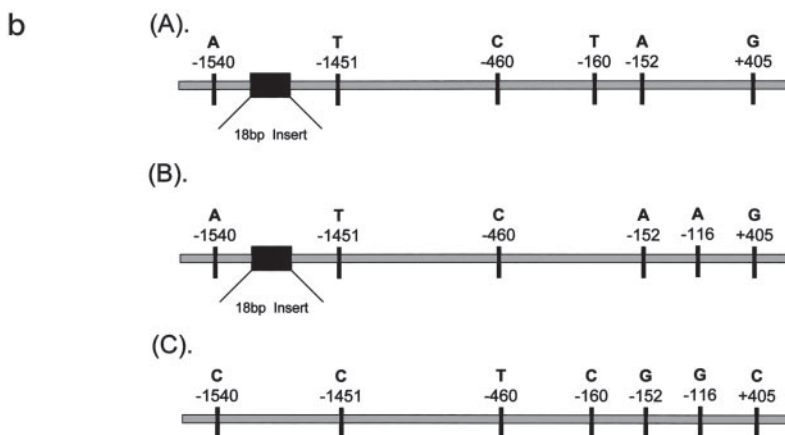


Fig. 1. *a*, diagram of the human *VEGF* promoter region. The amplification primer sequences are boxed; polymorphisms are marked with numbers relative to the transcription start site. The putative EREs are marked in red, and the AP-1 binding sites are marked in green. The transcription start site is marked with an arrow, and the transcribed nucleotides are marked in capital letters. *b*, diagram of *VEGF* gene haplotypes A (A), B (B), and C (C; wild type). The relationship between the polymorphisms identified is shown. Haplotype C (the wild-type promoter) was found to contain no sequence variation compared with the published sequence.



polymorphisms, and to allow the haplotype to be established. The promoter was excised using *Hind*III and *Xho*I before ligation into pGL3-luc (Promega). Clones were resequenced to confirm the haplotypes.

MCF7 cells were obtained from European Collection of Animal Cell Cultures and cultured in DMEM with 10% FCS (Life Technologies, Inc.). Cells

were transfected using GeneJuice (Novagen), according to the manufacturer's recommendations. All transfections were performed in sextuplicate, and cells were cotransfected with VEGF-luc or empty-luc and a cytomegalovirus-*Renilla* vector to control for transfection efficiency.

After transfection, cells were washed with serum-free, phenol red-free

DMEM (Life Technologies, Inc.) and incubated with the same medium for 24 h. Cells were then treated with vehicle, PMA (Sigma, Poole, United Kingdom), 17 β -estradiol (Sigma), dexamethasone (Sigma), TNF- α (R&D Systems), or cptcAMP (Sigma) for 16 h before harvest. There was no effect seen with any of the vehicle concentrations used in this study.

Cells were analyzed using the Dual Luciferase kit (Promega), according to the manufacturer's recommendations. All luciferase results were corrected for *Renilla*, which varied by less than 10% within each experiment.

RESULTS

Establishment of Haplotypes. We amplified DNA from an individual who was homozygous for the -460C/+405G polymorphisms. From this individual, two alleles were amplified both with the predicted polymorphisms and the 18-nucleotide insertion and the -1540, -1451, and -152 polymorphisms (Fig. 1). The expected population frequencies of the +405G allele (0.709) and the -1540C to A/18-nucleotide insertion allele (0.675) are high, and therefore colinearity in a haplotype is not unexpected. However, the sequences differed at two other polymorphisms, -160C to T (allele frequency, 0.017) and -116G to A (allele frequency, 0.3). Both alleles also contained the unusual polymorphism -152G to A (allele frequency 0.017) and a novel variant, -1451C to T. The allele frequency of -1451C was 0.43 (determined from analysis of 36 individuals; GenBank accession number AY102626). Sequencing of multiple clones identified two alleles and excluded PCR errors. These two polymorphic haplotypes were termed A and B (Fig. 1).

Genomic DNA from one individual, genotyped as homozygous -460T/+405C (wild-type sequence), was amplified and found to correspond to the published sequence throughout. This was termed haplotype C.

Basal Promoter Activity of Polymorphic (VEGF Promoter. Initial experiments compared the promoter activity of the empty plasmid pGL3-luc against the three different *VEGF* haplotypes. The *VEGF* DNA increased luciferase activity by greater than 100-fold, indicating the presence of significant promoter activity. Haplotype A showed consistently higher promoter activity compared with haplotypes B and C (Fig. 2).

Phorbol Ester Regulation of the Polymorphic VEGF Promoters. The human *VEGF* gene promoter (haplotype C) used in these experiments contains multiple predicted AP-1 sites (Fig. 1) and has previously been shown to be responsive to phorbol esters (16). We found that the empty reporter gene *pGL3-luc* was not responsive to PMA but that haplotypes B and C both showed a 5-fold induction

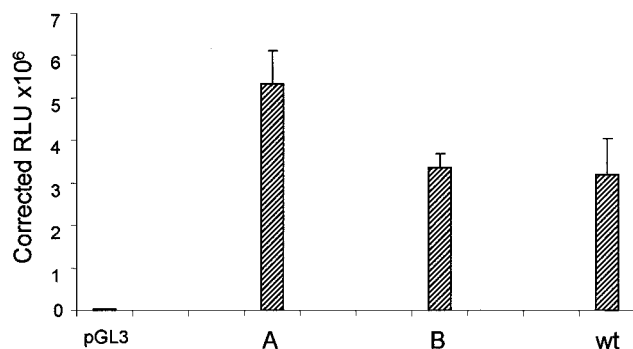


Fig. 2. Expression of human *VEGF* haplotypes in human MCF7 cells. MCF7 cells were transfected with pGL3-luc constructs containing the *VEGF* gene haplotypes A (A), B (B), or wild-type (C) for comparison against empty pGL3-luc (pGL3). Cells were cotransfected with cytomegalovirus-*Renilla* to control for transfection efficiency. This experiment was performed in sextuplicate and is representative of experiments performed on three occasions. Results are expressed as corrected relative light units (RLU; mean \pm SD).

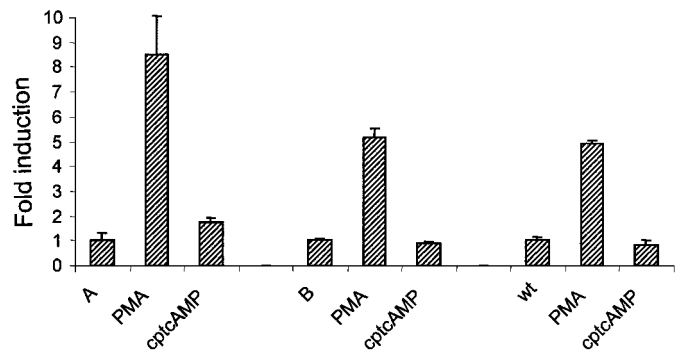


Fig. 3. Regulation of human *VEGF* haplotypes by phorbol esters and cAMP. MCF7 cells were transfected with the *VEGF* haplotype reporter genes A, B, and C, and after transfection, cells were divided into treatment groups of 6. Cells were incubated with 10^{-7} M PMA, 250 μ M cptcAMP, or nothing for 16 h before harvest. Results are expressed as fold induction over the control for each haplotype (mean \pm SD).

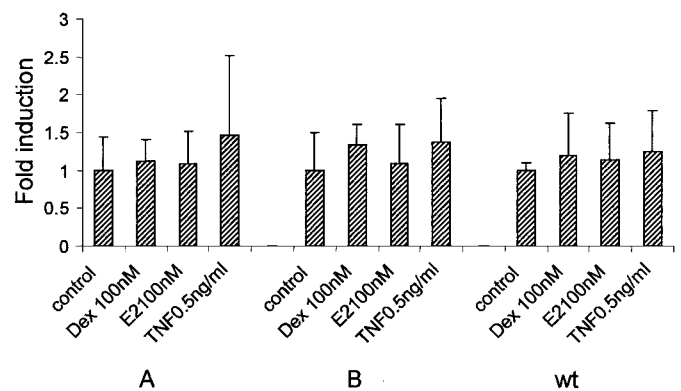


Fig. 4. Regulation of *VEGF* haplotypes by glucocorticoid, estrogen, and TNF- α . MCF7 cells were transfected with the *VEGF* haplotype reporter genes, and after transfection, they were divided into treatment groups of 6. Cells were incubated with the indicated concentrations of dexamethasone (Dex), 17 β -estradiol (E2), or TNF- α (TNF) for 16 h before harvest. Results are expressed as fold induction over the control for each haplotype (mean \pm SD).

(Fig. 3). Haplotype A, which had a higher basal activity, showed an 8.5-fold induction in activity in response to PMA.

Estrogen Regulation of Polymorphic VEGF Promoters. The *VEGF* gene promoter does not contain a consensus ERE, but two candidate atypical EREs have been described. MCF7 is an estrogen-responsive, human breast cancer cell line. None of the *VEGF* promoters showed any induction of activity in response to 100 nM estradiol, nor did the pGL3-luc control. A previous report suggested that overexpression of ER might be required to manifest estrogen regulation of the *VEGF* promoter, and so we cotransfected an ER α expression vector with the *VEGF* constructs. Transfected cells were divided into treatment and control groups to eliminate differences in transfection efficiency. Again there was no estrogen response. The same ER α expression vector had been used to confer estrogen responsiveness to another human cancer cell line, confirming its authenticity (26).

Regulation of VEGF Promoter by Other Agents. In an effort to identify other signaling pathways that might differentially impact the polymorphic promoters, we examined the action of the synthetic glucocorticoid dexamethasone (100 nM; Sigma), the proinflammatory cytokine TNF- α (0.5 ng/ml; R and D Systems), and the cell-permeable, long-acting, cAMP analogue cptcAMP (250 μ M; Sigma). None of these three agents altered the promoter activity of any of the three *VEGF* promoters (Fig. 4).

DISCUSSION

The human *VEGF* gene 5'-flanking region is very highly polymorphic, with some of the polymorphisms occurring at high frequency, and it is unclear which *VEGF* sequence is the most appropriate for analysis. This is important because significant associations are noted between genotype and both VEGF secretion and disease (19, 23, 25). In this work, we define three haplotypes and identify differences in both basal promoter activity and responsiveness to stimulation. Furthermore, we have determined that the 2.3-kb segment of the human *VEGF* gene examined is not estrogen responsive in the ER-positive human breast cancer cell line MCF7, in contrast to previously published work in endometrial cells (16).

Delineation of the *VEGF* promoter haplotypes is difficult because the sequence is highly polymorphic. Previously, tight linkage disequilibrium has been reported for some groups of polymorphisms. Notably, the -460/+405 polymorphisms are found together at a higher frequency than predicted from their allele frequencies, and the haplotype -460C/+405C was found in only 1 of 230 chromosomes examined (19). Further upstream from the promoter, a -1540 C to A conversion was always found with the adjacent 18-nucleotide insertion at -1512 (17), and -460C was always found with -152A in a further study (23). Therefore, the identity of a few, preserved haplotypes is proposed, with superimposition of more recent polymorphisms (20–22). For this reason, we sought to analyze the functional consequences of haplotypes containing the common -460C/+405G polymorphisms, which are in linkage disequilibrium (19), and which have been found to be associated both with disease and altered VEGF production (19, 23). The *VEGF* sequence obtained from a -460C/+405G individual showed two haplotypes, both with the 18-nucleotide insertion, -1540C to A, -1451C to T, and -152 G to A (17, 19). However, one allele contained the -160C to T, and the other contained the -116G to A. The haplotype -460C/-152A/-116G/+405G occurs at a frequency of 16% in a Japanese population, and -460C/-152A/-116A/+405G occurs at a frequency of 13% (23). All of the other polymorphisms have been reported previously, but it was surprising to find them in the same haplotype as the -460C/+405G on both chromosomes. Both chromosomes from the -460T/+405C individual had wild-type *VEGF* sequence [estimated haplotype frequency in a Japanese population, 40% (23)].

Because two haplotypes had been identified in the individual with -460C/+405G, both were compared against the wild-type *VEGF* promoter. Under basal conditions, haplotype B did not differ from wild-type *VEGF* (haplotype C), suggesting that either carriage of the numerous polymorphisms did not significantly alter promoter function or that the polymorphisms represented two classes, one activating, and the other repressing. Therefore, the net impact of the polymorphisms would be dependent on colinearity, frequency, and nature. Haplotype B contained a frequent polymorphism -116G to A (allele frequency, 0.3), and haplotype A contained a different, unusual change, -160C to T (allele frequency, 0.017). Previous work has shown a correlation of the +405 genotype with production of VEGF *in vitro* (19) and *in vivo* (23); however, the extended haplotype of the +405 homozygotes in these studies was not determined. It is interesting that haplotype A consistently showed markedly higher *VEGF* promoter activity compared with either haplotype B or the wild-type promoter. It is not possible from this analysis to be certain whether this change is due to loss of the -116 polymorphism or gain of the -160 polymorphism. However, it is clear that some haplotypes containing the +405G allele are high VEGF expressors.

The earlier report showing higher VEGF production from PBML cells isolated from +405G individuals is compatible with the current data. Carriage of the +405G genotype has been shown to be in

linkage disequilibrium with carriage of other polymorphisms (19) and, in some combinations (*i.e.*, haplotype A), certainly results in substantially higher promoter activity.

It was of interest that none of the three human VEGF promoter sequences tested were estrogen responsive in MCF7 cells. Even overexpression of ER, sufficient to confer an estrogen response in other cell types, did not make VEGF responsive. The endometrium and breast are well known to differ in their responses to partial agonist estrogens (SERMs), with full agonist activity in endometrium compared with antagonist activity in breast. The mechanistic explanation for this appears to be differential expression of cofactor molecules (27). Because VEGF does not have a consensus ERE, it is likely that the imperfect sequence only supports estrogen regulation in certain cell types. It is important that a functional ERE in the rat VEGF gene is located 3' to the gene coding sequence, a region not present in the constructs used in the current study (15).

In summary, we find that carriage of common VEGF polymorphisms alters both basal and post-stimulation promoter activity. There is clear evidence for a haplotype effect. A comprehensive analysis of recombination hot spots and possible haplotypes across this locus would be of interest and would aid association studies with human disease and ultimately gene linkage analysis (22). The relative contributions of the colinear polymorphisms are also of interest. It seems likely that the highly polymorphic VEGF gene will contribute to the inherited predisposition to diseases in which angiogenesis plays a part.

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