Cloning and Functional Analysis of the Promoter for KDR/flk-1, a Receptor for Vascular Endothelial Growth Factor*

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KDR/flk-1 is one of two receptors for vascular endothelial growth factor, a potent angiogenic peptide. KDR/flk-1 is an early marker for endothelial cell progenitors, and its expression is restricted to endothelial cells in vivo. To investigate the molecular mechanisms regulating expression of KDR/flk-1, we cloned and characterized the promoter of the human KDR/flk-1 gene. The transcription start site was localized by primer extension and ribonuclease protection to a nucleotide 303 base pairs (bp) 5’ of the initiation methionine codon. The 5’-flanking sequence is rich in G and C residues and contains five Sp1 elements but no TATA consensus sequence. By reporter gene transfection experiments, we found that -4 kilobases of KDR/flk-1 5’-flanking sequence directed high level luciferase activity in bovine aortic endothelial cells; further deletion analysis revealed positive regulatory elements between bp -225 to -164, -95 to -77, -77 to -60, and +105 to +127. Mutation of an atypical GATA sequence between bp +105 and +127 did not affect promoter activity, suggesting that GATA elements are not essential for the high level promoter activity of this gene. Consistent with endothelial cell-restricted expression of KDR/flk-1 mRNA, we found that the 4-kilobase flanking sequence directed high level promoter activity in endothelial cells but not in other cell types. To our knowledge this is the first report characterizing the KDR/flk-1 promoter. Understanding the KDR/flk-1 promoter will allow us to investigate endothelial cell-specific gene regulation and to uncover methods for targeting gene delivery specifically to endothelial cells.

Vascular endothelial growth factor (VEGF)1 is a potent and specific endothelial cell mitogen (1, 2). Through interactions with its receptors KDR/flk-1 and flt1, VEGF has critical roles in the growth and maintenance of vascular endothelial cells and in the development of new blood vessels in physiologic and pathologic states (3–5). The patterns of embryonic expression of VEGF suggest that it is crucial for differentiation of endothelial cells from hemangioblasts and for development of blood vessels at all stages of growth (6, 7). Among many potentially angiogenic factors, VEGF is the only one whose pattern of expression, secretion, and activity suggests a specific angiogenic function in normal development (8).

High affinity receptors for VEGF are found only on endothelial cells, and VEGF binding has been demonstrated on macro- and microvascular endothelial cells and in quiescent and proliferating endothelial cells, suggesting that these receptors are important for both growth and maintenance of all endothelial cells (6, 9). The tyrosine kinases KDR/flk-1 and flt1 have been identified as candidate VEGF receptors by affinity cross-linking and competition binding assays (10–12). These two receptor tyrosine kinases contain seven similar extracellular immunoglobulin domains and a conserved intracellular tyrosine kinase domain interrupted by a kinase insert (10, 13, 14); they are expressed specifically by endothelial cells in vivo (11, 15–17). In situ hybridization in the developing mouse has demonstrated that KDR/flk-1 is expressed in endothelial cells at all stages of development, as well as in the blood islands in which endothelial cell precursors first appear (11), and that KDR/flk-1 specifies endothelial cell precursors at their earliest stages of development (17).

The vascular endothelium is critical for physiologic responses including thrombosis and thrombolysis, lymphocyte and macrophage homing, modulation of the immune response, and regulation of vascular tone. The endothelium is also intimately involved in the pathogenesis of vascular diseases such as atherosclerosis (18). Although a number of genes expressed in the endothelium have been characterized (19–22), expression of these genes is either not limited to vascular endothelium (e.g. the genes encoding von Willebrand factor, endothelin-1, vascular cell adhesion molecule-1, platelet/endothelial cell adhesion molecule-1, Tie2, another developmentally regulated endothelial cell receptor tyrosine kinase, has recently been shown to direct transgene expression in subpopulations of endothelial cells during mouse embryonic development but not in endothelial cells of adult mice (23). This suggests that the Tek/Tie2 promoter fragment used in this study is sufficient to direct gene expression to subpopulations of endothelial cells during specific periods of development, although functional elements within this promoter have not yet been identified.) In contrast with cells derived from the skeletal muscle and hematopoietic lineages, little is known about the mechanisms of specification and differentiation of endothelial cells. To understand the molecular mechanisms regulating cell type specificity and activation of...
the differentiation pathway in endothelial cells, we studied the control of KDR/flk-1 transcription. As a first step we cloned and characterized the promoter for the human KDR/flk-1 gene. We report here the sequence of the 5′-flanking region of the gene and identify a single transcription start site located 303 bp 5′ of the initiation methionine codon. Four kilobases of KDR/flk-1 5′-flanking sequence were found to have promoter activity similar to that of the potent SV40 promoter/enhancer in reporter gene transfection experiments. Deletion analysis in endothelial cells showed the presence of positive regulatory elements in regions from bp –225 to –164, –95 to –77, –77 to –60, and +105 to +127. We found that KDR/flk-1 mRNA was expressed specifically in endothelial cells in culture and that 4 kb of the KDR/flk-1 5′-flanking sequence had cell type-specific promoter activity in transient transfection assays.

EXPERIMENTAL PROCEDURES

Screening of Human and Mouse Genomic Libraries—A 567-bp human KDR/flk-1 cDNA fragment was generated from human umbilical vein endothelial cell (HUVEC) total RNA by the reverse transcriptase polymerase chain reaction (PCR) (24). This fragment was radiolabeled with [α-32P]dCTP and used to screen a phage library of human placenta genomic DNA in the vector λFixII (Stratagene, La Jolla, CA) as described (24). Likewise, a 451-bp mouse KDR/flk-1 cDNA was generated by reverse transcriptase PCR from mouse lung total RNA and used to screen a phage library of mouse placenta genomic DNA in the vector λDalII (Stratagene). Hybridizing clones were isolated and purified and used for smooth muscle cells was supplemented with 25 mM Hepes (Sigma) and that HUVEC were cultured as above. Primary culture cells, HeLa human epidermoid carcinoma cells, HepG2 human hepatoma cells, human fibroblasts, U937 human histiocytic lymphoma cells, RD human embryonal rhabdomyosarcoma cells, MCF7 human breast adenocarcinoma cells, JEG-3 human choriocarcinoma cells, A7r5 fetal rat aortic smooth muscle cells, and NIH 3T3 mouse fibroblasts were obtained from the American Type Culture Collection. Primary culture HUVEC were obtained from Clonetech Corp. (San Diego) and were grown in EGM medium containing 2% fetal calf serum (Clonetech Corp.). Primary culture human aortic and intestinal smooth muscle cells were also obtained from Clonetech Corp. All cells were cultured in conditions identical to those for BAEC, with the exception that medium used for smooth muscle cells was supplemented with 25 mM Hepes (Sigma) and that HUVEC were cultured as above. Primary culture cells were passaged every 4–6 days, and cells from passages 3–5 were used in the experiments described here. Total RNA from cells in culture was prepared by guanidinium isothiocyanate extraction and centrifugation through cesium chloride (24).

DNA Sequencing—Restriction fragments derived from the human and mouse KDR/flk-1 genomic phage clones were subcloned by standard techniques into pSP72 (Promega, Madison, WI) or pBlueScript SK (Stratagene) and sequenced from alkaline-denatured double-stranded plasmid templates by the dideoxy chain termination method (26) with Sequenase 2.0 DNA polymerase (U. S. Biochemical Corp.). DNA was sequenced from both directions at least twice, and both dGTP and dITP sequencing protocols were used to resolve compression artifacts in the highly GC-rich 5′-flanking region of the human and mouse KDR/flk-1 genes. Sequence analysis was performed with the GCG software package (Genetics Computer Group, Madison, WI).

Primer Extension Analysis—Primer extension analysis was performed as described (25). A synthetic oligonucleotide primer (5′-CT-GTCTAGAAGAGAGGCGGGAGGTGAACT-3′) complementary to the 5′ end of the human KDR/flk-1 cDNA (Fig. 1A) was end labeled with [γ-32P]ATP and hybridized to 20 μg of each RNA sample, which was then subjected to reverse transcription. Extension products were analyzed by electrophoresis on an 8% denaturing polyacrylamide gel.

Ribonuclease Protection Assay—We used a 559-bp PstI-PstI fragment of the human KDR/flk-1 gene (Fig. 2B) cloned in pSP72 as the template for in vitro transcription and analysis by ribonuclease protection assay to confirm that the antisense RNA probe complementary to the 3′ noncoding region of the KDR/flk-1 gene was specific.
for in vitro transcription of an α-32P-labeled antisense RNA with T7 RNA polymerase (Boehringer Mannheim). Gel-purified riboprobe (5 x 10^5 cpm) was hybridized with 20 μg of total RNA or 3 μg of poly(A) RNA plus 17 μg of yeast tRNA at 55°C for 16 h in an annealing buffer containing 20 mM Tris-HCl, pH 7.40, 400 mM NaCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate in 75% formamide. After the RNA had been annealed the unhybridized RNA was digested for 45 min at room temperature with 200 units of RNase T1 (Boehringer Mannheim) and 0.3 unit of RNase A (Boehringer Mannheim) in a buffer containing 10 mM Tris-HCl, pH 7.50, 300 mM NaCl, 5 mM EDTA. The digestion products were then treated with proteinase K, extracted with phenol-chloroform, and analyzed by electrophoresis on a 4% denaturing polyacrylamide gel.

Northern Analysis—RNA blots were hybridized as described (27). Total RNA (10 μg) from cells in culture was fractionated on a 1.3% formaldehyde-agarose gel and transferred to a nitrocellulose filter. The human KDR/flk-1 cDNA probe was labeled with 32P by random priming and used to hybridize the filter. The filter was then autoradiographed for 16 h on Kodak XAR film at −80°C.

Plasmids—Plasmids pGL2 Basic and pGL2 Control contained the firefly luciferase gene (Promega). pGL2 Basic had no promoter, whereas pGL2 Control was driven by the SV40 promoter and enhancer. The plasmid pSVβgal (Promega) contained the β-galactosidase gene driven by the SV40 promoter and enhancer.

Reporter constructs containing fragments of the human KDR/flk-1 5'-flanking region were inserted into pGL2 Basic and named according to the length of the fragment (from the transcription start site) in the 5' and 3' directions. (For example, plasmid pGL2 -4kb+296 contained a human KDR/flk-1 promoter fragment extending from approximately −4 kb 5' of the transcription start site position +296 inserted into pGL2 Basic.) Plasmids pGL2 -4kb+296 and pGL2 -900+296 were created by restriction digestion of purified phage DNA by using 5' BamHI and PvuII sites, respectively, and the 3' XhoI site at position +296. Plasmids pGL2 -716+268, pGL2 -570+268, pGL2 -323+268, pGL2 -225+268, pGL2 -164+268, pGL2 -37+268, pGL2 -225+127, pGL2 -225+105, pGL2 -225+56, and pGL2 -225+5 were created from promoter fragments generated by PCR of human KDR/flk-1 phage DNA. Plasmids pGL2 -116+268, pGL2 -95+268, pGL2 -77+268, pGL2 -60+268, and pGL2 -12+268 were created by digesting the promoter fragment contained in plasmid pGL2 -164+268 from the 5' end with exonuclease III (Pharmacia Biotech Inc.). Plasmid pGL2 GATA-MUT was identical to pGL2 -225+268 except that bp +108 to +110 were mutated in the former (see below). All constructs were sequenced from the 5' and 3' ends to confirm orientation and sequence.

Mutagenesis—Site-directed mutagenesis of the atypical GATA sequence located in the first exon of the human KDR/flk-1 5'-flanking region was performed by PCR according to the method of Higuchi et al. (28). A DNA fragment containing human KDR/flk-1 bp −225 to +268 was used as a template. The sequence TGGATATC was mutated to GGTGCGTC by using one set of mismatched primers (5'-TCTGGCAGC-TGGTCGTC-3') and one set of primers flanking both ends of the template (5'-GCTGCTTCTCGAGTTGTTGCTCTGGGATGTT-3' and 5'-TGCCTCGAGTTGTTGCTCTGGGATGTT-3'). The sequence of the mutated PCR fragment was confirmed by the dideoxy chain termination method.

Transfections—All cell types were transfected by the calcium phosphate method as described (22) with the exception of A7r5 cells, which were transfected with DOTAP (Boehringer Mannheim) as instructed by the manufacturer. In all cases, 20 μg of the appropriate reporter construct was transfected along with 2.5 μg of pSVβgal to correct for variability in transfection efficiency. Cell extracts were prepared 48 h after transfection by a detergent lysis method (Promega). Luciferase activity was measured in duplicate for all samples with an EG&G Autolumat 953 luminometer (Gaithersburg, MD) and the Promega luciferase assay system. β-Galactosidase activity was assayed as described (22).

The ratio of luciferase activity to β-galactosidase activity in each sample served as a measure of the normalized luciferase activity. The normalized luciferase activity was divided by that of pGL2 Control and expressed as a relative luciferase activity. Each construct was transfected at least six times, and data for each construct are represented as the mean ± S.E. Relative luciferase activity among constructs was compared by a factorial analysis of variance followed by Fisher's least significant difference test. Statistical significance was accepted at p < 0.05.

**RESULTS**

Isolation and Characterization of Human and Murine KDR/flk-1 Genomic Clones—Our initial screening of a human placental phage library with a human KDR/flk-1 cDNA probe yielded a positive clone that was examined by restriction en-
zyme DNA mapping, subcloning, and sequencing. The 780-bp sequence of the promoter and first exon is shown in Fig. 1A. Likewise, a murine KDR/flk-1 cDNA probe was used to screen a murine placental phage library, and one clone was similarly identified and characterized. The sequence of the mouse KDR/flk-1 promoter is shown in Fig. 1B.

Identification of the Transcription Start Site of Human KDR/flk-1-To identify the transcription start site of the human KDR/flk-1 gene, we performed primer extension with a complementary oligonucleotide probe corresponding to bp +212 to +243 (underlined with arrow, Fig. 1A). Primer extension was performed on total RNA from HUVEC and HeLa cells and on poly(A) RNA from HUVEC. Gene transcription was initiated only in endothelial cells (Fig. 2A). A single transcription start site, corresponding to an A nucleotide located 303 bp 5' of the site of translation initiation, was identified. We designated this nucleotide as +1 for our remaining experiments involving the human KDR/flk-1 gene. The 5'-CA-3' nucleotide pair at this position is the most common site for transcription initiation (29).

To confirm the results of the primer extension studies, we performed ribonuclease protection analysis with an antisense riboprobe generated from a 559-bp genomic PstI-PstI fragment extending 5' from position +145 (Fig. 2B; the PstI sites are double underlined in Fig. 1A). Incubation of this probe with HUVEC poly(A) RNA and HUVEC total RNA, but not with total RNA from HeLa cells, resulted in protection of a single fragment corresponding in length to the distance between the 3' PstI site and the transcription start site identified by primer extension (Fig. 2C). Despite the absence of a TATA consensus sequence, transcription of the human KDR/flk-1 gene appears to begin from a single site located 303 bp 5' of the translation initiation codon (Fig. 1A, curved arrow).

Identification of Potential cis-Acting Sequences—The 5'-flanking sequence of the human KDR/flk-1 gene contains islands rich in G and C residues and lacks TATA and CCAAT boxes near the transcription start site (Fig. 1A). Comparison of this 5'-flanking sequence with sequences in the Transcription Factors Data Base revealed a series of five Sp1 sites (30) located between human KDR/flk-1 nucleotides -124 and -39. There are two AP-2 consensus sites (31, 32) at positions -95 and -68 and two inverted NF-κB-binding elements (33, 34) at -130 and -83 interspersed among the Sp1 sites. Two atypical GATA consensus sequences (both GGATAT) are present in the KDR/flk-1 promoter, one at position -759 and the other at position +107 within the untranslated portion of the first exon. In addition, multiple CANNTG elements are found in the promoter at positions -591, -175, +71, and +184; CANNTG elements can be bound by E-box-binding proteins (35-37). The sequence AAACAAA, which is conserved among genes expressed preferentially in keratinocytes (38), is present at human KDR/flk-1 position -508.

We also compared the human and mouse KDR/flk-1 promoters to identify conserved consensus sequences for nuclear proteins (Fig. 1B). Elements conserved between the two species include two Sp1 sites located at positions -244 and -124 relative to the 5' end of the reported mouse cDNA sequence (13), two AP-2 sites at positions -168 and -148, a noninverted NF-κB site at position -153, and the keratinocyte element AAACAAA at position -195. An atypical GATA element (GGATAA) is found in the untranslated portion of the first exon of the mouse promoter at position +18; an atypical GATA element (GGATAT) is located similarly in the human promoter. Also, a CANNTG sequence is present 12 bp 5' of the G- and C-rich sequences of the promoter at mouse KDR/flk-1 position -257, a location analogous to that of the CANNTG element at position -175 of the human promoter. Conservation of these elements across species suggests that some may have functional significance.

Deletion Analysis of the Human KDR/flk-1 Promoter—To identify DNA elements important for basal expression of KDR/flk-1 in endothelial cells, we constructed a series of luciferase reporter plasmids containing serial 5' deletions through the promoter region (Fig. 3). These plasmid constructs in pGL2 Basic were cotransfected into BAEC with pSV40gal (to correct for differences in transfection efficiency), and the luciferase activity was normalized to that of the pGL2 Control vector driven by the SV40 promoter/ enhancer. The activity of the longest human KDR/flk-1 genomic fragment, spanning bp -4 kb to +296, was similar to that of the powerful SV40 promoter/ enhancer and consistent with the high level of KDR/flk-1 mRNA expression in endothelial cells. Similar levels of activity were produced in constructs containing as much as 15.5 kb of 5'-flanking sequence (data not shown). Serial 5' deletions from bp -4 kb to -225 caused no significant change in promoter activity, implying that elements in this region are not important for basal activity of the KDR/flk-1 promoter. Deletion of sequences between bp -225 and -164 significantly reduced KDR/flk-1 promoter activity to 63% that of the full promoter fragment (p < 0.05), suggesting the presence of positive regulatory elements in this region. Deletion of bp from -95 to -77, a sequence that contains one AP-2 site and one NF-κB site, resulted in a further significant decrease in activity to 20% that of pGL2 -4 kb to +296 (p < 0.05). Further deletion of bp from -77 to -60, an area containing an overlapping AP-2/Sp1 site, significantly reduced KDR/flk-1 promoter activity to less than 5% that of pGL2 -4 kb to +296 (p < 0.05). Thus, 5' deletion analysis revealed that many positive regulatory elements in the KDR/flk-1 promoter are necessary for high level expression of the gene.

To determine whether sequences in the first exon of human KDR/flk-1 are important for basal expression, we created a series of 3' deletion constructs from the vector pGL2 -225 +268, which is the smallest construct that possessed full promoter activity (Fig. 4). A fragment was identified between bp +105 and +127 which, when deleted, caused a 5-fold reduction in promoter activity (p < 0.05), indicating the presence of a positive regulatory element in this region.

Because GATA-2 is a key regulatory factor in endothelial cell-specific gene expression (21, 39), we examined the functional importance of the atypical GATA site located between bp +105 and +127 of human KDR/flk-1. Three bp of the GATA motif in the fragment -225 to +268 were mutated to GTCG by PCR (28) to create pGL2 GATA-MUT. Mutation of these bp in the GATA motif has been observed to eliminate GATA-2 binding activity in the endothelin-1 gene promoter.2 In comparison with the native pGL2 -225 +268 promoter construct, the pGL2 GATA-MUT construct containing the mutated atypical GATA sequence did not have significantly decreased promoter activity in BAEC (p > 0.05; Fig. 5).

High Level Expression Induced by the KDR/flk-1 Promoter Is Specific to Endothelial Cells—Although KDR/flk-1 expression is restricted to endothelial cells in vivo (11, 16), it does not necessarily follow that its expression would be limited to endothelial cells in culture. To determine whether a tissue culture system is suitable for studying cell type-specific regulation of the KDR/flk-1 gene, we performed Northern analysis of RNA extracted from various cells in culture. KDR/flk-1 message was detected in HUVEC but not in primary culture cells (human aortic and intestinal smooth muscle cells and fibroblasts) or

2 M.-E. Lee and T. Quertermous, unpublished observations.
human cell lines (RD, HeLa, HepG2, MCF7, and U937; Fig. 6). Similarly, we have not detected the presence of a KDR/flk-1 message by reverse transcriptase PCR in HeLa, A7r5, or 3T3 cells (data not shown). Thus, expression of KDR/flk-1 message in tissue culture appears to be restricted to endothelial cells, as it is in vivo.

Fig. 3. 5' deletion analysis of the KDR/flk-1 promoter in BAEC. Panel A, representation of deletion sites in relation to consensus sequences for known nuclear proteins are discussed under “Results.” Panel B, functional analysis of the human KDR/flk-1 promoter by transfection into BAEC of luciferase reporter constructs containing serial 5' deletions. Constructs are described in the text. All constructs were cotransfected with pSVβ-gal to correct for transfection efficiency, and luciferase activity was expressed as a percentage of pGL2 Control (mean ± S.E.). Significant differences are noted under “Results.”

Fig. 4. 3' deletion analysis of the KDR/flk-1 promoter in BAEC. Panel A, representation of deletion sites in relation to consensus sequences for known nuclear proteins discussed in the text. Panel B, analysis effect of 3' deletions on KDR/flk-1 promoter activity in BAEC. Constructs are described in the text, and luciferase activity is presented as a percentage of pGL2 Control. Significant differences are noted under “Results.”

Fig. 5. Mutation of the GATA site at +107 does not decrease the ability of the KDR/flk-1 promoter to direct transcription. When transfected into BAEC, the plasmid pGL2-225-268 directed luciferase expression comparable to that directed by pGL2 Control, which contains the SV40 promoter and enhancer. When 3 bp of the GATA motif at +107 were mutated to create pGL2 GATA-MUT there was no significant difference in promoter activity.

To determine whether 5'-flanking sequences of the KDR/flk-1 gene confer endothelial cell-specific expression in cultured cells, we transfected pGL2-4kb+296, which contains more than 4 kb of the human KDR/flk-1 5'-flanking sequence and includes most of the untranslated portion of the first exon, into a variety of cell types in culture (Fig. 7). In accord with our previous experiments in BAEC, reporter gene expression driven by the pGL2-4kb+296 promoter fragment was similar...
Analysis of the KDR/flk-1 Promoter

Fig. 6. KDR/flk-1 RNA expression is restricted to endothelial cells in culture. RNA was extracted from cells in culture and analyzed by Northern blotting as described under “Experimental Procedures” with a human KDR/flk-1 cDNA probe. HASMC, human aortic smooth muscle cells; H1SMC, human intestinal smooth muscle cells; Fibroblast, human cultured fibroblasts. A photograph of ethidium bromide-stained ribosomal RNA of the agarose gel is provided to assess loading.

Fig. 7. High level activity of the KDR/flk-1 promoter is specific to endothelial cells. The luciferase reporter construct pGL2-4kb+296 was transfected into cells in culture, and transfection efficiency was corrected by cotransfection with pSV 

Luciferase activity (% of pGL2 Control)

Panel A

Panel B

Fig. 8. Deletion analysis of the KDR/flk-1 promoter in JEG-3 cells. Panel A, effect of 3' deletions on KDR/flk-1 promoter activity in JEG-3 cells. Constructs are described in the text, and luciferase activity is presented as a percentage of pGL2 Control. Deletion of bp −164 to −95 and −77 to −60 significantly reduced luciferase activity in JEG-3 cells (p < 0.05). No other significant differences were noted. Panel B, effect of 3' deletions on KDR/flk-1 promoter activity in JEG-3 cells. Deletion of bp +127 to +105 significantly reduced luciferase activity in JEG-3 cells (p < 0.05).

DISCUSSION

As a receptor for VEGF, KDR/flk-1 plays an essential role in angiogenesis and endothelial cell growth, and it is among the earliest markers of endothelial cell differentiation during development. Moreover, in situ analysis and immunocytochemistry have shown that KDR/flk-1 expression is restricted to endothelial cells in vivo; presumably this restricted pattern of expression determines the pattern of VEGF activity. Despite the importance of the KDR/flk-1 gene in endothelial cell growth, the mechanisms that regulate and restrict its expression are not known. We report for the first time the cloning and characterization of the human and mouse KDR/flk-1 promoters, and we identify regions containing positive regulatory elements within the 5'-flanking region of the human gene.

Analysis of the human KDR/flk-1 5'-flanking region reveals that the transcription start site is located 303 bp 5' of the methionine initiation codon. Like constitutive endothelial nitric oxide synthase (40), another gene expressed in endothelial cells, KDR/flk-1 lacks a TATA box, is rich in G and C residues, and has numerous putative binding sites for Sp1, a ubiquitous nuclear protein that can initiate transcription of TATA-less genes (41). We identified by deletion analysis three sequences within the 5'-flanking region of the KDR/flk-1 gene which appear to contain elements important for its expression in endothelial cells. Deletion of sequences between bp −225 and −164 reduced activity to 63% that of the full-length promoter, deletion between −95 and −77 further reduced promoter activity to 20%, and deletion from −77 to −60 reduced promoter activity to that driven by the potent SV40 promoter/enhancer. In JEG-3, Saos-2, A7r5, 3T3, and HeLa cells, however, expression driven by the pGL2-4kb+296 promoter was markedly lower, demonstrating that induction of high level expression by this promoter is specific to endothelial cells. We observed a similar expression pattern with a reporter plasmid containing 15.5 kb of KDR/flk-1 5'-flanking sequence (data not shown).

Finally, in an attempt to establish the function of regulatory elements within the KDR/flk-1 5'-flanking sequence in other cell types, we transfected into JEG-3 and Saos-2 cells the promoter constructs that defined positive regulatory elements in endothelial cells. In JEG-3 cells, promoter activity was reduced significantly (p < 0.05) when elements from bp −77 to −60 and +127 to +105 were removed (Fig. 8). Because similar reductions were obtained in endothelial cells, these two positive regulatory elements do not appear to be endothelial cell specific. In contrast, no significant changes were noted after deletion of the elements from −225 to −164 and −95 to −77, suggesting that these fragments may define endothelial cell-specific regulatory elements. (Deletion of the region from −164 to −95 resulted in a reduction in promoter activity in JEG-3 cells but not BAEC, which may reflect differential usage of core promoter elements in nonendothelial cells.) Identical studies were done in Saos-2 cells, and the results were similar (data not shown). Because promoter activity in nonendothelial cells is so low, we are reluctant to overinterpret the cell type specificity of regulatory elements in the KDR/flk-1 promoter. However, these results exclude the possibility that the cell type specificity of this promoter is due to the presence of silencer elements in the 5'-flanking region of this gene.
activity to less than 5%. Because potential binding sites for Sp1, AP-2, NFκB, and E-box proteins located within these three positive regulatory elements in the human KDR/flk-1 gene are also present in the mouse 5′-flanking sequence, they may represent functional binding domains. AP-2 is a developmentally regulated trans-acting factor (42) without a demonstrated role in endothelial cell gene regulation. NFκB, however, trans-activates the inducible expression of vascular cell adhesion molecule-1 and tissue factor in endothelial cells (20, 43) and is known to be a mediator of tissue-specific gene regulation (33). Nuclear proteins that bind the E-box motif include the basic helix-loop-helix family of trans-acting factors. E-box-binding proteins have not been clearly associated with endothelial cell gene expression, although members of this family are critical for proper maturation of many cell types, including skeletal muscle and B lymphocytes (36, 44). Further experiments will be necessary to determine if these or other unidentified nuclear proteins specifically trans-activate the KDR/flk-1 gene.

Four zinc finger-containing transcription factors in the GATA protein family bind to the consensus sequence (A/T)GATA(A/G) and regulate cell type-specific gene expression in many cell lineages (45); among these GATA-2 has been most closely linked to endothelial cell gene expression. GATA-2 functions as an enhancer of endothelin-1 gene expression (39) and acts to restrict expression of von Willebrand factor to endothelial cells (21). We observed that the human KDR/flk-1 5′-flanking region has two potential GATA-binding sequences, at positions −759 and +107, and that loss of the element located at −759 had no effect on expression of KDR/flk-1 in endothelial cells. The potential GATA element at position +107 is in a region of the first exon which we have identified as a powerful positive regulatory element. Although this GATA sequence (GGATA) differs from the GATA-binding sequences of endothelin-1 and von Willebrand factor and from the consensus GATA sequence (A/T)GATA(A/G), we speculated that it might be the functional motif in the region between +105 and +127 because the functional GATA site in the von Willebrand factor gene is located similarly in the first exon and because a similar GATA element is found in the first exon of the mouse KDR/flk-1 gene. To our surprise, mutation of 3 bp in this element (GATA to GTGC), which had been observed to prevent trans-activation of the GATA cis-acting element in the endothelin-1 promoter,2 had no significant effect on KDR/flk-1 promoter activity (Fig. 5). Thus, our deletion analysis and mutagenesis studies do not support a functional role for the two GATA sequences in the human promoter in its high level activity in endothelial cells. These observations are consistent with the finding that early stages of endothelial cell development are normal in mice deleted for KDR/flk-1 (46). The KDR/flk-1 5′-flanking region contains an enhancer element that is required for expression of the KDR/flk-1 gene in vivo (47). In contrast, transgenic constructs containing the KDR promoter are not sufficient to drive expression of KDR/flk-1 in vivo (48). The KDR promoter contains an enhancer element that increases MyoD expression in many cell types in culture, even though MyoD expression is specific to skeletal muscle in vivo (47). Therefore, a complete explanation of the mechanisms of endothelial cell-specific expression of KDR/flk-1 may require integration of in vivo and in vitro observations.

Identification of the regulatory mechanisms responsible for KDR/flk-1 expression is likely to provide important information about the specification and differentiation of endothelial cells early in embryogenesis. Moreover, knowledge about DNA elements that restrict gene expression to endothelial cells may be useful for deciphering the function of proteins in this cell type and, potentially, for directing or preventing expression of genes specifically in endothelial cells.

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REFERENCES
1. Connolly, D. T., Heuvelman, D. M., Nelson, R., Olander, J. V., Eppley, B. L., Delfino, J. J., Leininger, R. M., and Feder, J. (1989) J. Clin. Invest. 84, 1470–1478
2. Leung, D. W., Cachianes, G., Kuang, W.-J., Goeddel, D. V., and Ferrara, N. (1989) Science 246, 1206–1209
3. Aiello, L. P., Avery, R. L., Arrigo, P. G., Keyt, B. A., Jampel, H. D., Shah, S. T., Pasquale, L. R., Thieme, H., Iwamoto, M. A., Park, J. E., Nguyen, H. V., Atlavio, M. L., Ferrara, N., and King, G. L. (1994) N. Engl. J. Med. 331, 1480–1487
4. Shewki, D., Itin, A., Soffer, D., and Keshet, E. (1992) Nature 359, 843–845
5. Berkman, R., Merrill, M., and Reinhold, W. (1993) J. Clin. Invest. 91, 153–159
6. Jaken, L. B., Armanini, M., Phillips, H. S., and Ferrara, N. (1993) Endocrinology 133, 848–859
7. Breier, G., Allbrecht, U., Sterrer, S., and Risau, W. (1992) Development 114, 521–532
8. Klagsbrun, M., and Soker, S. (1993) Curr. Biol. 3, 699–702
9. Jaken, L. B., Winer, J., Bennett, G. L., Altar, A., and Ferrara, N. (1992) J. Clin. Invest. 89, 244–253
10. de Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N., and Williams, L. T. (1992) Science 255, 898–911
11. Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., Rasmussen, B., Eddy, R., and Shows, T. B. (1993) J. Biol. Chem. 268, 843–846
12. Terman, B. I., Dougher-Vermaazien, M., Carrion, M. E., Dimotrost, D., Armeldino, D. C., Gospodarowicz, D., and Bohlen, P. (1992) Biochem. Biophys. Res. Commun. 187, 1579–1586
13. Matthews, W. J., Ordan, C. T., Gavins, M., Jenkins, N. A., Copeland, N. G., and Lenischka, I. R. (1993) Proc. Natl. Acad. Sci. U.S.A. 88, 9026–9030
14. Terman, B., Carrion, M., Kovacs, E., Rasmussen, B., Eddy, R., and Shows, T. B. (1993) Oncogene 6, 1677–1683
15. Peters, K., de Vries, C., and Williams, L. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 8915–8919
16. Quitas, T. P., Peters, K. G., de Vries, C., Ferrara, N., and Williams, L. T. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7533–7537
17. Yamaguchi, T. P., Dumont, D. J., Conlon, R. A., Breitman, M. L., and Rossant, J. (1993) Development 113, 489–495
18. Ross, R. (1993) Nature 362, 801–809
19. Collins, T., Williams, A., Jokhsht, G. I., Kim, J., Eddy, R., Shows, T., Gimbrone, M. A., and Bevilacqua, M. P. (1991) J. Biol. Chem. 266, 2466–2473
20. Iademarco, M. F., McQuillan, J. J., Rosen, G. D., and Dean, D. C. (1992) J. Biol. Chem. 267, 16323–16329
21. Jovourd, N., and Lynch, D. C. (1981) Mol. Cell. Biol. 14, 999–1008
22. Lee, M., Bloch, D. K., Clifford, J. A., and Quertermous, T. (1990) J. Biol. Chem. 265, 10446–10450
23. Schlaeger, T. M., Qiu, H., Fujihara, Y., Magran, J., and Satow, T. N. (1995) Development 121, 1098–1098
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
25. Fen, Z., Dhady, M. S., Yoshizumi, M., Hikiter, R. J., Quertermous, T., Eddy, R. L., Shows, T. B., and Lee, M.-E. (1993) Biochemistry 32, 7932–7938
26. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463–5467
Analysis of the KDR/ flk-1 Promoter

27. Li, J., Perrella, M., Tsai, J.-C., Yet, S.-F., Hsieh, C.-M., Yoshizumi, M., Patterson, C., Endege, W., Zhou, F., and Lee, M.-E. (1995) J. Biol. Chem. 270, 308–312
28. Higushi, R., Krummel, B., and Saiki, R. (1988) Nucleic Acids Res. 16, 7351–7367
29. Bucher, P., and Trifonov, E. N. (1986) Nucleic Acids Res. 14, 10009–10026
30. Briggs, M., Kadonaga, J., Bell, S., and Tjian, R. (1986) Science 234, 47–52
31. Mitchell, P., Wang, C., and Tjian, R. (1987) Cell 50, 847–861
32. Imagawa, M., Chiu, R., and Karin, M. (1987) Cell 51, 251–260
33. Lenardo, M., and Baltimore, D. (1989) Cell 58, 227–239
34. Leung, K., and Nabel, G. (1988) Nature 333, 776–778
35. Ephrussi, A., Church, G. M., Tonegawa, S., and Gilbert, W. (1985) Science 227, 134–140
36. Buskin, J. N., and Haushka, S. D. (1989) Mol. Cell. Biol. 9, 2627–2640
37. Church, G. M., Ephrussi, A., Gilbert, W., and Tonegawa, S. (1985) Nature 313, 798–801
38. Blessing, M., Zentgraf, H., and Jorcano, J. (1987) EMBO J. 6, 567–575
39. Lee, M. E., Temizer, D. H., Clifford, J. A., and Quertermous, T. (1991) J. Biol. Chem. 266, 16188–16192
40. Marsden, P. A., Heng, H. H. Q., Scherer, S. W., Stewart, R. J., Hall, A. V., Shi, X.-M., Tsai, L.-C., and Schappert, K. T. (1993) J. Biol. Chem. 268, 17478–17488
41. Pugh, B., and Tjian, R. (1990) Cell 61, 1187–1197
42. Mitchell, P., Timmons, P., Hebert, J., Rigby, P., and Tjian, R. (1991) Genes & Dev. 5, 105–119
43. Mol, T., Czyz, M., Holzmuller, H., Hofer-Warbinek, R., Wagner, E., Winkler, H., Bach, F., and Hofer, E. (1993) J. Biol. Chem. 270, 3849–3857
44. Murre, C., McCaw, P., Vaessin, H., Caudy, M., Jan, L., Jan, Y., Cabrera, C., Buskin, J., Haushka, S., Lassar, A., Weintraub, H., and Baltimore, D. (1989) Cell 58, 537–544
45. Orkin, S. (1992) Blood 80, 575–581
46. Tsai, F., Keller, G., Kuo, F., Weiss, M., Chen, J., Rosenblatt, M., Alt, F., and Orkin, S. (1994) Nature 371, 221–226
47. Goldhamer, D., Faerman, A., Shani, M., and Emerson, C. (1992) Science 256, 538–542
48. Sassoon, D., Lyons, G., Wright, W., Lin, V., Lassar, A., Weintraub, H., and Buckingham, M. (1989) Nature 341, 303–307
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