The human transmembrane fms-like receptor tyrosine kinase Flt-1 is one of the receptors for vascular endothelial growth factor, a growth factor which induces endothelial proliferation and vascular permeability. Flt-1 is expressed specifically in endothelium and is likely to play a role in tumor angiogenesis and embryonic vascularization. To elucidate the molecular basis for the endothelial specific expression of Flt-1, the promoter region has been isolated and functionally characterized. The promoter region contains a TATA box, a GC-rich region, and putative transcription factor binding elements such as cAMP response element binding protein/activating transcription factor (CREB/ATF) and etc. Adenovirus-mediated transient expression of the Flt-1 promoter/luciferase fusion gene in endothelial cells and other cell types demonstrated that a 1-kilobase fragment of the 5'-flanking region of flt-1 is involved in the endothelial-specific expression. A CREB/ATF element was found to be essential for basal transcription of the flt-1 expression. In addition, we also showed that the first intron negatively regulates flt-1 promoter activity. The flt-1 promoter will be useful in functional studies on the regulation of endothelial-specific gene expression and also as a tool in targeting the expression of exogenously introduced genes to the endothelium.

Vascular endothelial growth factor is not only a specific mitogen for vascular endothelial cells but also a potent mediator of vascular permeability (1, 2). We as well as other groups have shown that Flt-1 (fms-like tyrosine kinase) and Flk-1 (fetal liver kinase-1; mouse homologue of kinase insert domain containing receptor (KDR)) are receptors for vascular endothelial growth factor (3-6). These receptors and Flt-4 (7, 8) are members of a family of tyrosine kinases, which is characterized by proteins containing seven immunoglobulin-like domains, a single transmembrane region, and a kinase insert sequence. We have recently shown that flt-1 is expressed specifically in the endothelium in adult mouse tissues by in situ hybridization (9). We have also shown that flt-1 is expressed in the endothelium during neovascularization of healing skin wounds and in early vascular development in mouse embryos. Therefore, expression of flt-1 is highly restricted to vascular endothelial cells. However, little is known about the molecular regulation of endothelial-specific gene expression as yet.

As a first step to address this issue, we identified the promoter region of flt-1 and characterized this promoter in a transient expression assay. Here we show in a series of transfection assays that a 1-kb DNA fragment of a 5'-flanking sequence of flt-1 demonstrates functional activity in vascular endothelial cells but not in epithelial cells, vascular smooth muscle cells, or fibroblasts. The study also demonstrates that the flt-1 CREB/ATF element is essential for basal transcription and the first intron of flt-1 contains negative regulatory elements.

EXPERIMENTAL PROCEDURES

Materials—The recombinant adenovirus Adex1CAlacZ, the adenovirus cosmid vector pAdex1W and EcoT22I-digested adenoviral DNA-terminal protein complex were obtained from Dr. Izumi Saito at the Institute of Medical Science, the University of Tokyo.

Cell Cultures—Bovine adrenal endothelial cells (BAEC) were obtained from Dr. Richard Weiner at the University of California, San Francisco, and maintained in Dulbecco’s modified essential medium supplemented with 1 mg/ml glucose, 1 ng/ml basic fibroblast growth factor, and 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVEC), human aortic endothelial cells, human pulmonary arterial endothelial cells, human aortic smooth muscle cells, and human mammary epithelial cells were obtained from Clonetics and maintained according to the manufacturer’s recommendation. NIH-3T3 cells and human foreskin fibroblasts (HFF) were maintained in Dulbecco’s modified essential medium supplemented with 10% FBS. NC1-H292 human pulmonary mucoiderma carcinoma cells were maintained in RPMI 1640 supplemented with 10% FBS. Rat aortic smooth muscle cells (Sprague-Dawley rats) were isolated from explants as described previously (31) and maintained in Dulbecco’s modified essential medium supplemented with 10% FBS.

Cloning of the 5'-Flanking Region of the Human flt-1 Gene—A human placenta genomic library in EMBL-3 phage (Clontech) was screened with a 600-bp EcoRI/Acid fragment of the 5'-end of the flt-1 cDNA. After 6 rounds of screening, 13 positive clones were isolated. Two sets of overlapping synthetic oligonucleotides, 5'-GGACACTCTTTCGCGTCTCTCCCGGGAGGGCGGGCGGCTCGG-3' (oligo-E) and 5'-CCGCTGCGCGCTGCACCCGGAGCCCGAGCCCGCGCTCAGG-3' (oligo-F), corresponding to the 5'-end of the flt-1 cDNA between positions +3 and +79 (designated as probe A) and 5'-GGTCTTCTGCTGATGATGAGAAGGAGACG-3' (oligo-G) and 5'-CAGAGTTGGTCGAGTTCATGACT-3' (oligo-J) corresponding to the sequence of the flt-1 DNA between positions +427 and +502 (designated as probe B) were annealed, followed by filling-in with Klenow Fragment in the presence of dNTPs. Four of the 13

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) D66016.

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clones hybridized with probe A, but not with probe B. In contrast, the other clones hybridized with probe B, but not with probe A. Three different clones which hybridized with probe A were selected for restriction endonuclease and Southern blot analyses. The 3-kb EcoRI/Xhol fragments from all three clones and a 7-kb EcoRI fragment from clone 5-11 were subcloned into Bluescript KS+ (Stratagene) to generate pBKS3.0. These plasmids were used for further restriction enzyme mapping, nucleotide sequencing analysis, subcloning, and expression studies as described above.

Construction of p(-2.5k/ +284)-luc Containing the 5'-Flanking Region of flt-1 and Luciferase-fusion Gene—The plasmid pMC-luc was generated by cloning annealed complementary oligonucleotides including the promoter region of flt-1 and Luciferase-fusion gene—pCMV-β-galactosidase. The luciferase gene was inserted into pCMV-β-galactosidase vector under the control of the TATA box containing the 5'-flanking region of flt-1 and a downstream polyadenylation signal element. The resulting plasmid p(-2.5k/ +284)-luc was digested with SacI, and the flanking region of flt-1 and the 3'-portion of the polyadenylation signal were ligated to form the pMC-luc vector.

Construction of 5'-Deletion Mutant Plasmids—The plasmid p(-1.9k/ +284)-luc was constructed by digesting p(-2.5k/ +284)-luc with PstI followed by self-ligation. The plasmid p(-1.9k/ +284)-luc was constructed by digesting p(-2.5k/ +284)-luc with PstI, BstXI, treating with T4 DNA polymerase, and self-ligating to generate a series of deletion mutants. The plasmid p(-2.5k/ +284)-luc was treated with BstXI, T4 DNA polymerase, and PstI, and digested with exonuclease III from the 5'-end followed by self-ligation using the Erase-a-Base kit (Promega). Several 5'-deletion mutants were produced: p(-962/ +284)-luc, p(-748/ +284)-luc, p(-550/ +284)-luc, p(-338/ +284)-luc, p(-239/ +284)-luc, p(-151/ +284)-luc, and p(-1/ +284)-luc. To construct another deletion mutant, p(-2.5k/ +284)-luc was digested with AatII and PstI, treated with T4 DNA polymerase, and then self-ligated to generate p(-75/ +284)-luc.

Construction of Luciferase-fusion Plasmids Containing a 5'-Flanking Region, Exon 1, and a Hybrid Intron—The plasmid p(-2.5k/ +550)-sp-luc, which contains the 5'-flanking region of flt1, the first intron of flt-1 and the 3'-portion of mouse immunoglobulin heavy chain gene (10) was constructed by cloning annealed complementary oligonucleotides corresponding to a mouse immunoglobulin heavy chain variable region (5'-TTCAGCTCTTGCAGCTTTGGGAGTCATATCCGCTAG-CAATGCATTCACATCCGCTATGCAGGGCGCTGCACAGTACAGGTCA-ACCGTTCGAGGAGAAGGAAAATGTGATCATGTACCATCATGATG-3') into the Xhol and SrfI sites of pMC-luc fragment. The resulting plasmid p(-2.5k/ +550)-sp-luc was treated with T4 DNA polymerase, and then self-ligated and digested with NcoI, Xhol, and mung bean nuclease followed by self-ligation.

Sequence Analysis of the Promoter Region of flt-1—The nucleotide sequence and restriction enzyme site of the promoter region of flt-1 were determined by the dideoxy chain termination method using dye-terminator (Perkin Elmer) and the ABI 377 (Perkin Elmer) automatic sequencer. The deletion mutants were sequenced using the dideoxy chain termination method using the ABI 377 (Perkin Elmer) automatic sequencer. Unique restriction enzyme sites were determined by DNA analysis software (Genex, Michigan) and gel analysis.

RESULTS

Restriction Map and Exon-Intron Organization of 5'-Specific Human flt-1 Genomic Clones—Genomic clones from a human placental genomic library were obtained by using the human flt-1 cDNA 5'-end 600-bp DNA fragment (described under “Experimen- tal Procedures”). Three overlapping but not identical genomic clones were selected for further analysis based on the result of Southern analyses using the human flt-1 cDNA 5'-end oligo DNA probe. The restriction maps of these clones were determined by the partial restriction method (Fig. 1). The 3-kb EcoRI/Xhol fragments from all three clones were subcloned into pBluescript-KS(+) (10). Detailed restriction maps and partial sequences showed that these 3-kb fragments were identical. Although the restriction pattern of the 5' region of clone 4-18 was different from that of 5-21A and 5-11, the reason for this diversity remains unclear.

Sequence Analysis of the Promoter Region of flt-1—The nu-
The flt-1 Promoter

**Fig. 1.** Structure and restriction map of the flt-1 genomic clones containing a 5′-flanking region, exon 1, and a 5′ portion of intron 1. Three clones contained overlapping genomic segments. Solid boxes indicate the position of exon 1. The 3-kb EcoRI/Xhol fragments from all three clones were subcloned into pBluescript and restriction sites were determined. Restriction patterns of the three fragments were identical. The nucleotide sequence of the 5′-flanking region of flt-1 between positions −1195 (BstXI) and +550 (Xhol) indicated by the dotted line was determined by the Sanger method. Restriction sites for enzymes are indicated as follows: Ap, Apal; Al, AatII; B, BamHI; Bs, BstXI; E, EcoRI; N, NaeI; Nc, Ncol; P, PstI; S, SmaI; Sp, Sphi; X, Xhol.

The flt-1 Promoter Activity Is Endothelial Cell Specific—To determine the sequences essential for efficient transcription of the flt-1 promoter, a DNA segment extending from −284 bp to −2.5 kb was fused to a luciferase gene in the pMC-luc vector. The construct designated as p(−2.5κ/−284)-luc contains 2.5 kb of the promoter region, 230 bp of exon 1, and 54 bp of the 5′-end of the first intron. This construct was used to generate a series of 5′-end deletions (Figs. 4 and 6A). The resultant constructs are referred to as p(X/Y)-luc. For each, X and Y represent the 5′- and 3′-end positions in nucleotides. Each construct was transfected into BAEC.

Deletion mutant p(−748/+284)-luc showed the highest activity. Transfection of the series of constructs deleted from −2.5 kb to −151 suggested the presence of at least two regions, 2500 to −1195 and −356 to −333, containing negative regulatory sequences, and two regions, −748 to −583 and −239 to −75, containing positively regulatory sequences. Deletion to +151 decreased luciferase activity to the level of the promoterless plasmid pMC-luc.

To determine if the 2.5-kb promoter region used in these experiments was sufficient to confer cell-type specificity, the deletion constructs were also transfected into NIH 3T3 cells and HFF. Relative luciferase activities in these cells were much weaker than those in BAEC (Fig. 4).

To clarify endothelial specificity of the flt-1 promoter further, we employed various human primary cells. However, because we could not efficiently transfet primary cells by conventional methods, we introduced the construct using a replication-deficient recombinant adenovirus. The recombinant adenovirus AdexFLTP-luc carrying the flt-1 promoter (−748/+284)-luciferase fusion gene was used to infect various human primary cells and established cell lines. Following infection, relative luciferase activities seen in human primary endothelial cells such as aortic endothelial cells, pulmonary arterial endothelial cells, umbilical vein endothelial cells, and bovine adrenal endothelial cells were much higher than activities seen in human primary mammary epithelial cells, human primary aortic smooth muscle cells, NCI-H292 cells, human foreskin fibroblasts, rat primary aortic smooth muscle cells, and NIH-3T3 cells (Fig. 5). These results showed that the flt-1 promoter region between positions −748 and +284 conferred endothelial-specific gene expression.

The CREB/ATF Element Is Essential for flt-1 Promoter Activity—In some genes, a CREB/ATF element has been shown to be involved in not only transcriptional activation by a cAMP-dependent protein kinase A but also basal transcription. To characterize the CREB/ATF element of the flt-1 promoter, we constructed a deletion mutant in the CREB/ATF element. Deletion of 4 internal bases in the CREB/ATF element of the flt-1 promoter (ACGT out of TGACGTCA) diminished relative luciferase activity in BAEC by 85% (Fig. 6B). However, we also failed to detect any stimulation of luciferase activity in response to forskolin/IBMX and dibutyryl-cAMP/IBMX in BAEC transfected with either p(−962/+284)-luc or pCRE(−962/+284)-luc (data not shown). Therefore, the CREB/ATF element of the flt-1 promoter is important for basal transcription of flt-1, but may not be important in the transcriptional activation in...
response to cAMP elevation.

The First Intron of flt-1 Negatively Regulated Transcription—Transfection of the p(-2.5k/+550)-luc construct, which contains 220 bp of the first intron of flt-1 (containing the 5’ splice site but not the 3’ splice site), resulted in no luciferase activity (Fig. 7). This may be due to the production of an undesirable protein instead of luciferase since the first intron contains an ATG at +1286 which is upstream of the initiation codon of the luciferase gene and may not be spliced out because of the lack of a 3’ splice site. When a 3’ splice site from a mouse immunoglobulin gene (10) was introduced downstream of the first intron to generate a hybrid intron (p(-2.2k/550)sp-luc), luciferase activity was partially restored. The idea to make this construct was based on results obtained with a hybrid intron consisting of a 5’ splice site from the first exon of the adenovirus tripartite leader and a 3’ splice site from a mouse immunoglobulin gene on pMT2 expression vector (14). These studies showed that the hybrid intron was completely spliced out when eukaryotic initiation factor 2 was expressed on pMT2 vector. Thus, it appears likely that the decrease of luciferase activity seen in p(-2.5k/550)sp-luc (Fig. 7) does not result from a deficiency in splicing. Therefore, we conclude that the first intron of flt-1 negatively regulated the transcription.

DISCUSSION

In this report, we identified the flt-1 promoter region and showed that a 1-kb fragment of the 5’-flanking sequence of the flt-1 gene demonstrated functional activity in vascular endothelial cells but limited activity in epithelial cells, vascular smooth muscle cells, and fibroblasts. Deletion studies of the flt-1 sequence indicated that the regions from -2500 to -1195 and -356 to -333 contained negative regulatory sequences and regions from -748 to -583 and -239 to -75 contained positive regulatory sequences.

We determined that the transcription initiation site is located 25 bp downstream of a TATA box by primer extension and S1 mapping. The regions surrounding the transcription initiation site is similar to other initiator sequences. These
The flt-1 Promoter

The flt-1 Promoter

**Fig. 8.** Comparison between the sequence surrounding the transcription arrest site in the first intron of the adenosine deaminase (30) and the homologous region in the first intron of flt-1. Asterisks indicate identical bases. The core sequence of the transcription arrest site is boxed. The underlined bases have been shown to be important for full transcription arrest activity by point mutational analysis (30).

![Diagram](image)

**A**

The flt-1 Promoter

**B**

The flt-1 Promoter

![Diagram](image)

**Fig. 6.** Effect on flt-1 promoter activity by: (A) further deletion of a 5′-flanking region and (B) internal deletion of CREB/ATF element. A constructs containing shorter promoter regions than the constructs shown in Figs. 4 and 5 were analyzed. B, four internal bases of the CREB/ATF element (ACGT out of TGACGTCA) were deleted in ΔCREB(−962/+284)-luc. These constructs were transiently transfected into BAEC. The data are presented in the same manner as in Fig. 4. Each value is the mean of two independent experiments. Filled triangles indicate the CREB/ATF element.

**Fig. 7.** Negative effect of intron 1 on the flt-1 promoter activity. Both (−2.5k/+550)-luc and (−2.5k/+4.5k)-luc contain a flt-1 promoter oriented in the reverse direction. These constructs were transiently transfected into BAEC. The data are presented in the same manner as in Fig. 4. Each value is the mean of two independent experiments. The shaded box indicates a 3′ splice site of a mouse immunoglobulin gene (10).

The 5′-flanking sequence of flt-1 exhibits common features of a promoter because it contains a TATA box, a GC-rich region, and potential binding sites for transcription factors. Internal deletion studies showed that the consensus CREB/ATF element (TGACGTCA) located at −74 was essential for basal transcription of flt-1. However, many attempts have failed to transactivate the flt-1 promoter in response to CAM elevation by treatments with forskolin/IBMX and dibutyryl-cAMP/IBMX, suggesting that CREB is not involved in regulation of the flt-1 expression. As many transcription factors are known to bind the consensus CREB/ATF element, other members of the basic region-leucine zipper protein family may bind and activate transcription of flt-1 (15). The CREB/ATF-like element of the TGF-β2 promoter has been shown to be essential for basal level promoter activity, but does not confer responsiveness from either protein kinase A or C (16).

The promoters of genes whose expression is limited to endothelial cells and certain other cells, such as endothelin-1 (17), endothelial-leukocyte adhesion molecule 1 (18), P-selectin (19), vascular cell adhesion molecule 1 (20–22), and thrombomodu-

**REFERENCES**

1. Ferrara, N., Houck, K., jaken, L., and Leung, D. W. (1992) Endocr. Rev. 13, 18–32
2. Klagsbrun, M., and Soker, S. (1993) Curr. Biol. 3, 699–702
3. De Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N., and Williams, L. T. (1992) Science 255, 898–901
4. Quinn, 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
5. Millauer, B., Wizigmann-Voss, S., Sznürich, H., Martinez, R., Maller, N. P. H., Risau, W., and Ullrich, A. (1993) Cell 72, 835–846
6. Terman, B. J., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D., and Böhle, P. (1992) Biochem. Biophys. Res. Commun. 187, 1579–1586
7. Pajusola, K., Aprikianova, O., Korhonen, J., Kaipainen, A., Pertovaara, L., Alitalo, R., and Alitalo, K. (1992) Cancer Res. 52, 5738–5743
8. Galland, F., Karamysheva, A., Mattei, M. G., Rosnet, O., Mardetcho, S., and Birnbau, D. (1992) Genomics 13, 475–478
9. Peters, K. G., De Vries, C., and Williams, L. T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6915–6919
10. Bothwell, A. L. M., Faskind, M., Reth, M., Imanishi-Kari, T., Rajewsky, K., and Baltimore, D. (1981) Cell 24, 625–637
11. Seed, B. and Shen, J.-Y. (1988) Gene (Amst.) 67, 271–277
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) Current Protocols in Molecular Biology, pp. 4.6.1–4.6.13. Wiley, New York
14. Kaufman, R. J., Davies, M. V., Pathak, V. K., and Hershey, J. W. B. (1989) Mol. Cell Biol. 9, 946–958
15. Mayer, T. E., and Habener, J. F. (1993) Endocr. Rev. 14, 269–290
16. O’Reilly, M. A., Geiser, A. G., Kim, S. J., Bruggeman, L. A., Lue, A. X., Roberts,
A. B., and Sporn, M. B. (1992) J. Biol. Chem. 267, 19938–19943
17. Lee, M.-E., Bloch, K. D., Clifford, J. A., and Quertermous, T. (1990) J. Biol. Chem. 265, 10446–10450
18. Montgomery, K. F., Odborn, L., Hession, C., Tizard, R., Goff, D., Vassallo, C., Tarir, P. I., Bomsztyk, K., Lobb, R., Harlan, J. M., and Pohlan, T. H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6523–6527
19. Pan, J., and McEver, R. P. (1993) J. Biol. Chem. 268, 22600–22608
20. Iademarco, M. F., McQuillan, J. J., Rosen, G. D., and Dean, D. C. (1992) J. Biol. Chem. 267, 16323–16329
21. Iademarco, M. F., McQuillan, J. J., and Dean, D. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3943–3947
22. Neish, A. S., Williams, A. J., Palmer, H. J., Whitley, M. Z., and Collins, T. (1992) J. Exp. Med. 176, 1583–1593
23. von der Ahe, D., Nischan, C., Kunz, C., Otte, J., Knies, U., Oderwald, H., and Wasylyk, B. (1993) Nucleic Acids Res. 21, 5636–5643
24. Macleod, K., Leprinse, D., and Stehelin, D. (1992) Trends Biochem. Sci. 17, 251–256
25. Vandenbunder, B., Pardanaud, L., Jaffredo, T., Mirabel, M. A., and Stehelin, D. (1989) Development 106, 265–274
26. Wernert, N., Raes, M.-B., Lassalle, P., Dehouck, M.-P., Gosselin, B., Vandenbunder, B., and Stehelin, D. (1992) Am. J. Pathol. 140, 119–127
27. Plate, K. H., Breier, G., Weich, H. A., and Risau, W. (1992) Nature 359, 845–848
28. Krystal, G., Armstrong, B. C., and Battey, J. F. (1990) Mol. Cell. Biol. 10, 4180–4191
29. Kimmelman, D., and Kirschner, M. W. (1989) Cell 59, 687–696
30. Kash, S. F., Innis, J. W., Jackson, A. U., and Kellems, R. E. (1993) Mol. Cell. Biol. 13, 2718–2729
31. Ross, R. (1971) J. Cell Biol. 50, 172–186
32. Nakamura, Y., Wakimoto, H., Abe, J., Kanegae, Y., Saito, I., Aoyagi, M., Hirakawa, K., and Hamada, H. (1994) Cancer Res. 54, 5757–5760
33. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Gene (Amst.) 108, 193–200
A Novel Promoter for Vascular Endothelial Growth Factor Receptor (flt-1) That Confers Endothelial-specific Gene Expression
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