The \( \text{flt-1} \) gene encodes a transmembrane tyrosine kinase, Flt-1, a receptor for vascular endothelial growth factor. The expression of \( \text{flt-1} \) gene is restricted to endothelial cells \textit{in vivo}. To understand the molecular mechanism underlying endothelial-specific expression of this gene, we studied the functional significance of transcriptional motifs in the 200-base pair region of the human \( \text{flt-1} \) gene promoter, which has been identified to confer cell type specificity. By point mutation analysis using chloramphenicol acetyltransferase plasmids in \( 293E1 \) cells, which express significant levels of \( \text{flt-1} \) mRNA, we found that an Ets motif, E4, at -84 to -51 and a cAMP response element (CRE) at -83 to -76 are involved in the transcriptional regulation of this gene. Disruption of either this CRE or E4 within the promoter sequence of 90 base pairs resulted in a decrease in chloramphenicol acetyltransferase activity of 90\%, indicating that co-existence of both of CRE and Ets motif E4 is necessary for transcription of the \( \text{flt-1} \) gene. Co-transfection of an expression vector containing c-\textit{ets-1}, c-\textit{ets-2}, or c-\textit{erg} cDNA with this 90-base pair sequence yielded a 5-8-fold elevation of chloramphenicol acetyltransferase activity, further supporting the idea that Ets family protein(s) participates in the regulation of the \( \text{flt-1} \) gene. Gel shift assays using nuclear extracts of 293E1 and endothelial cells demonstrated the existence of protein factor(s) that specifically binds to CRE and Ets motif E4, respectively. Taken together, our results strongly suggest cooperation of a CRE and an Ets motif for the function of the \( \text{flt-1} \) gene promoter.

Vascular endothelial growth factor (VEGF) \(^1\) is a unique peptide growth factor that specifically stimulates the proliferation of endothelial cells (1) and is thought to play an essential role in angiogenesis during a variety of biological processes, including embryonal development (2), tissue repair and regeneration (3), and tumor growth, etc. (4–6). VEGF is also a potent inducer of microvascular extravasation (7–9).

Three tyrosine kinase genes, \( \text{flt-1} \) (10), \( \text{KDR/flk-1} \) (11, 12), and \( \text{flt-4} \) (13, 14), encode transmembrane receptors that share structural features, \textit{i.e.} seven immunoglobulin-like loops in the extracellular domain and a long kinase insert in the cytoplasmic kinase domain.

Recently, Flt-1 and KDR/Flk-1 were identified as high affinity receptors for VEGF (15–17), and analyses of mouse embryos homozygous for targeted disruption of the \( \text{flt-1} \) or \( \text{flk-1} \) gene revealed that both receptors are essential for the development of normal blood vessels (18, 19).

The expression of \( \text{flt-1} \) and \( \text{KDR/flk-1} \) genes is regulated to endothelial cells \textit{in vivo} (20), which at least partially accounts for the endothelial-specific action of VEGF. In a clinical application, a gene promoter method that is specifically activated in endothelial cells will have a great advantage as a genetic tool in the gene therapy for vascular diseases. Thus, it is quite interesting and important to elucidate the molecular mechanism underlying cell type-specific expression of the genes for these VEGF receptors.

The upstream sequences of these endothelial receptor tyrosine kinase genes have been characterized in both \( \text{flt-1} \) and \( \text{KDR/flk-1} \) (21–23). In the 5' flanking region of the \( \text{flt-1} \) gene, we found that a -200-base pair (bp) sequence upstream to exon 1 confers cell type specificity to this gene (22). This region contains several motifs for transcription factors including one cAMP response element (CRE), five Ets motifs, and two GC boxes (21, 22). It has also been reported that a transcriptional activator, c-\textit{ets-1} proto-oncogene, is highly expressed in endothelial cells during angiogenesis in embryogenesis and tumor growth (24–26).

In this study, we have prepared various chloramphenicol acetyltransferase (CAT) plasmids containing mutant forms of the \( \text{flt-1} \) promoter sequence in which several putative binding sites for transcription factors were disrupted by introducing point mutations. Then, we examined the functional significance of these transcription motifs by transient DNA transfection experiments, especially focusing on the role of the multiple Ets motifs.

MATERIALS AND METHODS

\textit{Cell Culture—}293E1, HeLa, and A431 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5\% fetal bovine serum. A cell line of endothelial origin, designated as AG1, was established from human benign hemangioma in our laboratory. These hemangioma cells, immortalized by SV40 large T antigen, were grown in F-12 medium with 10\% fetal bovine serum. Human umbilical vein endothelial cells (HUVEC) were obtained from Morinaga (Japan) and cultured in MCDB104 medium with 5\% fetal bovine serum according to the manufacturer's recommendation. By Western blot analysis, these HUVEC were confirmed to express Flt-1 kinase at passage 5 (data not shown).

\textit{Northern Blot Analysis—}Preparation of RNA and Northern blot analysis were performed as described previously (27, 28). A \(^{32}\text{P}\)-labeled 1.6-kilobase (kb) \( \text{Pst-I-PstI} \) fragment of the human \( \text{flt-1} \) cDNA corre-
sponding to the extracellular domain was used as a probe.

Nuclear Run-on Assay—A nuclear run-on assay was performed according to the standard method (29). Plasmid DNAs containing cDNA for human flt-1 or β-actin were denatured and transferred to the filter. Nuclei isolated from 5 × 10⁷ cells were incubated with [α-32P]dUTP (Amersham; >3000 Ci/mmol) to label RNA transcripts. Labeled RNA was purified and hybridized with the linearized cDNA fixed on the filter. The amount of RNA used for each hybridization was adjusted so that it contains the same level of radioactivity (3 × 10⁶ cpm).

Construction of CAT Plasmids—CAT plasmids carrying various lengths of human flt-1 upstream sequence were prepared as described previously (22). The p195WTCAT, which contains the upstream sequence −195 to +8, was generated by utilizing Smal site at −192.

For point mutation analysis, several mutants for transcription factors were disrupted by site-directed mutagenesis using polymerase chain reaction (30). First, we examined five Ets motifs in p195WTCAT (E1 at −147 to −144, E2 at −130 to −127, E3 at −105 to 102, E4 at −54 to −51, and E5 at −41 to −38) by converting core sequence GGAAT to CCAAT. The roles of CRE at −83 to −76 and E4 were studied using p90WTCAT, which carries the upstream sequence −90 to +8, as a wild-type control. CRE was mutated in two ways: TCGAGTCA to TCT-CTCA or to TGACGGAT. All these mutations were confirmed by DNA sequencing.

To determine the sufficiency of the DNA sequence containing CRE and E4 motif for driving cell type-specific expression of flt-1 gene, we fused a single copy of a 50-bp fragment of the flt-1 upstream region (−90 to −41) to SV40 promoter of pCAT-Promoter Vector (Promega, Madison, WI).

DNA Transfection and CAT Assay—Plasmid DNA was introduced to cells by the calcium phosphate precipitation method described by Graham and van der Eb (31). Cells were plated at 1 × 10⁸/10-cm plate and, after 24 h, transfected with 20 μg of reporter plasmid and 3 μg of internal control pSPEI-lacZ plasmid (32) for 293E1, or pCAGGS-IacZ (33) for HeLa and A431. After 48 h, cell lysates were prepared by freeze-thawing, and CAT activity was measured as described previously (22). For stimulation experiments, forskolin (1 to 100 μM) was added to culture medium 4 or 24 h prior to harvest. The CAT conversion rate was calculated by dividing the radioactivity of acetylated chloramphenicol by that of total chloramphenicol (BAS2000, Fuji, Japan). In the co-transfection experiments, 10 μg of pS8G-based expression vectors (34) for human c-ets-1, c-ets-2 (35), or c-erg (36) were transfected with 20 μg of reporter plasmids to investigate whether the trans-activation mechanism was specific to the Ets family proteins, pRSV-ets-c (rat) and pRSV-ets-cjun (rat) (37), 10 μg each, were co-transfected with reporter plasmids. In each experiment, CAT activity elicited by 0.2 μg of PEFS21CAT, kindly provided by Dr. Sumio Sugano (Institute of Medical Science, University of Tokyo), was used as a positive control.

Electrophoretic Gel Mobility Shift Assay—Crude nuclear extracts were prepared from 293E1, AG1, HeLa, and A431 cells using hypotonic buffer as described by Dignam et al. (38). For HUVEC and sinusoidal endothelial rat liver cells, and 293E1 cells derived from human embryonic kidney express flt-1 mRNA at significant levels. As shown in Fig. 1A, the pattern of flt-1 mRNA expression in 293E1 cells is similar to that of placental tissues: i.e. 8.0- and 7.5-kilobase transcripts for full-length receptor molecule, 3.0- and 2.2-kilobase shorter mRNAs that encode for only the extracellular domain, and an additional 31 amino acids encoded by intron 1. Human hemangiooma cells also express detectable levels of flt-1 mRNA. In contrast, flt-1 transcripts were not detected in HeLa cells by Northern blot analysis.

Nuclear run-on assay revealed that the difference in the amount of flt-1 mRNA among these cell lines is mainly due to the high rate of transcription in 293E1 cells (Fig. 1B). Thus, we used 293E1 cells as a target for transient DNA transfection experiments for flt-1 promoter/enhancer activity.

One Ets Motif E4 at −54 to −51 Is Involved in the Transcriptional Regulation of the flt-1 Gene—We have recently found that about the 200-base pair region of the flt-1 promoter contributes to cell type-specific expression of this gene (22). Since one of the striking features of this region is that it contains multiple Ets motifs, we first examined the role of these Ets motifs by point mutation analysis. As shown in Fig. 2, introduction of point mutation into one Ets motif E4 at −54 to −51 resulted in a marked decrease in CAT activity by 65%. On the other hand, CAT activities remained relatively unchanged after disruption of any one of the three Ets motifs (E1, E2, and E3) located upstream to CRE. Mutation of the fifth Ets motif E5 yielded elevated CAT activity; however, nucleotide sequence flanking this Ets motif is considerably different from the consensus sequence of the typical Ets-binding sites (41, 42). The biological significance of this elevation in CAT activity remains to be seen. Combinalional mutations of E3 and E4, which surround CRE, did not bring about remarkable change in CAT activity compared with that of single E4 mutation. Thus, we concluded that of these five Ets motifs, E4 is most significantly involved in the transcriptional control of the flt-1 gene as a positive regulatory element.

Both CRE and Ets Motif E4 Are Necessary for the Promoter Activity of the flt-1 Gene—The data described above prompted us to carry further deletion analysis of the upstream sequence to determine the minimum components required for promoter activity.
activity of the \( \text{flt}-1 \) gene. As shown in Fig. 3, the short 90-bp-long segment (p90WTCAT) retained a promoter activity at higher levels. After further deletion to –77, which disrupts CRE sequence, CAT activity markedly decreased.

Introduction of point mutation into either CRE or Ets motif E4 in p90WTCAT resulted in a strong suppression of CAT activity by \(-90\%\) (Fig. 4), indicating that both CRE and Ets motif are indispensable for the promoter activity of the \( \text{flt}-1 \) gene. When these motifs are mutated simultaneously, CAT activity declined to the background level.

However, in stimulation experiments, we could not detect elevation of CAT activity in response to forskolin, dibutyryl-cAMP, and calcium ionophore A23187 in 293E1 cells transfected with p90WTCAT (Fig. 5). This CRE motif may not be strongly ralated to CAMP- or calcium-dependent signal transduction pathway.

When a 50-bp fragment of the \( \text{flt}-1 \) promoter containing CRE and E4 motifs was ligated upstream to the SV40 promoter of pCAT-promoter vector, CAT activity was elevated by 3–4-fold in 293E1 cells, while this sequence had little effect using A431 pCAT-promoter vector, CAT activity was elevated by 3–4-fold and E4 motifs was ligated upstream to the SV40 promoter of**

**CRE and Ets Motifs Cooperate in \( \text{flt}-1 \) Promoter**

As indicated in Fig. 7, co-transfection of expression vectors for \( c\text{-ets}-1, c\text{-ets}-2, \) and \( c\text{-erg} \) showed trans-activation of the promoter activity of p90WTCAT in HeLa cells by 5–8-fold. This indicates that Ets family protein(s) could participate in the regulation of the \( \text{flt}-1 \) gene. This trans-activation appeared to be specific to Ets family proteins, since co-transfection of c-fos and c-jun expression vectors had little or no effect.

Similar experiments were also carried out using 293E1 cells that express a significant level of \( \text{flt}-1 \) gene. We observed only a minor elevation of CAT activity by \( c\text{-ets}-1, c\text{-ets}-2, \) and \( c\text{-erg} \), suggesting that the E4 Ets motif was already occupied in 293E1 cells by an endogenous Ets family protein(s) (data not shown).

To further analyze the mechanism of trans-activation by Ets family proteins, we transfected \( c\text{-ets}-1 \) expression vectors with reporter plasmids containing the wild type (p90WTCAT) or mutant form (p90M2CAT or p90M3CAT) of \( \text{flt}-1 \) promoter sequences. As shown in Fig. 8, overexpression of \( c\text{-ets}-1 \) gene could not stimulate the promoter activity of either the p90M3CAT-carrying mutation in Ets motif E4 or p90M2CAT in which the CRE motif was disrupted. This suggests that these two motifs are required for the \( \text{flt}-1 \) gene promoter.

**Nuclear Extracts of 293E1 and Endothelial Cells Contain Protein Factors That Specifically Bind to CRE and Ets Motif E4**

Finally, we examined the binding of cellular proteins to the transcription motifs in the \( \text{flt}-1 \) promoter sequence. Gel shift assays using nuclear extracts obtained from experiments using HeLa cells as the target for transient DNA transfection. This cell line expresses almost undetectable levels of the \( \text{flt}-1 \) gene; thus, it appeared to be suitable for induction of this gene. As indicated in Fig. 7, co-transfection of expression vectors for \( c\text{-ets}-1, c\text{-ets}-2, \) and \( c\text{-erg} \) showed trans-activation of the promoter activity of p90WTCAT in HeLa cells by 5–8-fold. This indicates that Ets family protein(s) could participate in the regulation of the \( \text{flt}-1 \) gene. This trans-activation appeared to be specific to Ets family proteins, since co-transfection of c-fos and c-jun expression vectors had little or no effect.

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CRE and Ets Motifs Cooperate in flt-1 Promoter

293E1 and HUVEC demonstrated the existence of protein factors that specifically bind to CRE and Ets motif E4, respectively (Fig. 9A and B). For both motifs, the binding of these proteins to DNA probes is dependent on the intact core motifs, because excess mutant probes failed to compete their binding to the wild-type probes.

By Western blot analysis using anti-Ets-1 antibody, we were able to show a clear band of 51 kDa in 293E1, AG1, HUVEC, and sinusoidal endothelial cells of rat liver, but at very low levels in A431 cells (Fig. 9, C and D). Further, 293E1 and HeLa cells expressing exogenously introduced c-ets-1 gene showed an increased intensity of these bands (Fig. 9D), suggesting that Ets-1 or Ets-1-like protein is involved in the expression of the flt-1 gene in 293E1 and endothelial cells.

**DISCUSSION**

The expression patterns of VEGF receptors, Flt-1 and KDR/Flk-1, are quite similar to each other in cell type specificity and in temporal course; it is (i) restricted to endothelial cells in vivo (43), (ii) up-regulated in embryonic vascular system (17), and (iii) up-regulated during tumor angiogenesis (44).

The upstream sequences that confer cell type specificity to these tyrosine kinases have been studied in both flt-1 and KDR/Flk-1 (21–23). Surprisingly, the DNA sequences of the 5′-flanking region of these two genes have few common features. The human flt-1 gene contains one TATA box, one CRE, two GC boxes, and multiple Ets motifs in the upstream 200-bp region (21, 22), while the promoter region of KDR/Flk-1 gene contains putative binding sites for AP2, SP1, and NFκB; GATA consensus motifs; and E boxes (23). It seems that, although closely related to each other in their structure and functions, flt-1 and KDR/Flk-1 genes are regulated by different sets of transcription factors. In human embryos, Kaipainen et al. (45) showed overlapping, but distinct expression patterns of the receptor tyrosine kinases encoded by the flt family genes (flt-1, KDR, and flt-4); i.e. although most of the endothelial cells expressed all of these genes, certain endothelia lacked one or two of the three receptor mRNAs. As shown in this report, human cell line 293E1 expresses considerable concentrations of flt-1 mRNA, but we could not detect KDR transcripts in these cells by Northern blot analysis (data not shown). Differential regulation mechanisms of transcription of flt-1 and KDR/Flk-1 genes may account for some discrepancy in the cell type specificity of the expression of these receptor molecules.

As mentioned above, one of the remarkable features of the flt-1 gene promoter is the existence of multiple Ets motifs. Ets family proteins are a group of transcription factors characterized by the unique DNA binding domain, which specifically interacts with DNA sequences containing the purine-rich core motif, GGAAT. Ets proteins are implicated in the regulation of gene expression during many important biological phenomena, such as cell growth, differentiation and transformation, particularly in hematopoietic cells and in endothelial cells (41, 42).

In this report, we show that in the human flt-1 promoter, one Ets motif at nucleotide residue −54 to −51 from the transcription initiation site functions as an important positive regulatory element for this gene. This Ets motif is located just downstream of CRE at −83 to −76. The crucial role of this CRE motif in the flt-1 promoter has recently been reported by Morishita et al. (21), and our results are consistent with their report; introduction of point mutations to the CRE motif brought out a marked decrease in CAT activity. However, in this study, we demonstrated that CRE motif alone is not enough for the promoter activity of this gene. The results of the point mutation analysis clearly indicated that the adjacent motifs of CRE and Ets E4 are necessary for the promoter activity of the flt-1 gene. Although the functional significance of

**Fig. 6.** Cell type-specific promoter activity of DNA fragment carrying CRE and Ets motif E4. The 50-bp DNA fragment corresponding to nucleotides −90 to −41 of the flt-1 upstream sequence was subcloned into the BgIII site of pCAT-Promoter Vector (Promega). 293E1 or A431 cells were transiently transfected with reporter plasmids, and CAT activities were measured. Fold activation rate is the mean ± S.D. of three experiments.

**Fig. 7.** Co-transfection of expression vectors for Ets proteins with the 90-bp flt-1 promoter sequence. Ten μg of expression vectors as indicated and 20 μg of reporter plasmids, p90WTCAT, were transiently transfected to HeLa cells. Fold activation represents the average of double or triple experiments.

**Fig. 8.** Co-transfection of pSG5-c-ets-1 with the mutant form of the 90-bp sequence. To HeLa cells, 10 μg of pSG5 vector or c-ets-1 expression vectors were transfected with 20 μg of reporter plasmids as indicated. In p90M2CAT and p90M3CAT, CRE sequence or Ets motif E4 was disrupted as indicated in Fig. 4. Fold activation is the mean ± S.D. of double or triple experiments.
other transcriptional motifs including GC boxes in the -200-bp region has not yet been extensively studied, combined mutations of CRE and Ets motif almost completely abolished CAT activity, indicating that the intact CRE and Ets motif E4 are the essential components of *flt*-1 promoter. Accumulating evidence also suggests that some Ets proteins act synergistically with other transcription factors in the regulation of many viral and cellular genes (46–49). Details about the molecular mechanism concerning the interaction between CRE and Ets motifs in the *flt*-1 promoter await further investigation. However, disruption of only one CRE or Ets motif resulted in a significant decrease in CAT activity by 90%. Accordingly, as shown in co-transfection experiments (Fig. 8), when the CRE sequence was mutated, exogenously induced Ets-1 protein failed to trans-activate the 90-bp *flt*-1 promoter even if the Ets motif E4 remained intact. These results strongly suggest the existence of intimate cooperative relations between CRE and Ets motif E4 in the *flt*-1 promoter.

One important question is whether these data obtained from 293E1 cells could apply to primary endothelial cells. Our preliminary data from DNA transfection experiments using human AG1 cells, which are thought to be derived from endothelial cell and express some level of *flt*-1 mRNA, showed an almost identical pattern; the 90-bp fragment retains enough CAT activity, and both CRE and Ets motifs contained in this fragment are important for the promoter activity (data not shown). Therefore, we consider that the same elements play essential roles in the regulation of the *flt*-1 gene in endothelial cells.

Electrophoretic gel mobility shift assays demonstrated that the nuclear extracts of 293E1 and endothelial cells contain protein factor(s) that specifically bind to DNA probes containing CRE and Ets motif E4, respectively. The nature of these protein factors remains unknown.

The DNA sequence of Ets motif E4 and its flanking region does not coincide with formerly reported Ets-binding sites (41, 42). This DNA-protein complex formation involving the DNA

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**Fig. 9.** Electrophoretic gel mobility shift assay and Western blot analysis. A, gel shift assay using DNA probes containing CRE motif. DNA probes were mixed with 293E1 or HUVEC nuclear extracts containing 3 μg of protein. For competition study, 50 molar excess cold competitors or 200 molar excess mutant competitors were added. The positions of a specific retarded band, non-specific complex, and free probes are indicated. B, gel shift assay for Ets motif E4. Nuclear extracts containing 2 μg of protein were mixed with labeled DNA probes with or without excess cold competitor probes indicated in the panel. C, Western analysis for Ets-1 protein in nuclear extracts. Lane 1, A431; lane 2, AG1; lane 3, 293E1 cells. Arrows, positions of Ets-1 protein; ordinate, size markers (kDa). D, 293E1 and HeLa cells were transiently transfected with pSG5 vector or pSG5-c-ets-1 expression vector as described under "Materials and Methods"; then, nuclear extracts were prepared from these cells and analyzed by Western blotting with anti-Ets-1 antibody. Lanes 1 and 3, 293E1 and HeLa cells transfected with pSG5 vector, respectively; lanes 2 and 4, 293E1 and HeLa cells transfected with pSG5-c-ets-1, respectively; lane 5, HUVEC; lane 6, sinusoidal endothelial (NP) cells of rat liver. Arrowheads, positions of 51-kDa Ets-1 protein; ordinate, size markers (kDa).
probes containing Ets motif E4 was sequence specific, because mutant probes failed to compete the binding to wild-type probes. Further, co-transfection of Ets expression vectors activated the promoter activity of the 90-bp fragment in HeLa cells. Thus, it is plausible that Ets-related protein(s) are involved in the transcriptional regulation of the flt-1 gene.

c-ets-1 proto-oncogene is reported to be differentially expressed in the primitive endothelial cells in chicken embryos (24), and recently, Wernert et al. demonstrated, by in situ hybridization, that the c-ets-1 gene expression is up-regulated in blood vessels in embryos and tumor angiogenesis (25, 26).

By Western blot analysis, we could detect Ets-1 protein in the nuclear extracts of 293E1, AG1, HUVEC, and sinusoidal endothelial cells of rat liver (Fig. 9, C and D). Further, in the co-transfection experiments using HeLa cells, exogenously induced c-ets-1 gene potentiated the promoter activity of the 90-bp fragment in the flt-1 upstream sequence in accordance with the increase of Ets-1 protein contents in nuclear extracts (Figs. 7 and 9D). Therefore, our results suggest that Ets-1 or Ets-1-like protein is involved in the transcriptional regulation of flt-1 gene.

Identification of protein factors involved in the transcriptional regulation of the flt-1 gene is considered to be quite important in understanding the molecular mechanism underlying the growth control of endothelial cells and angiogenesis.

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