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Genomic structure and promoter activity of the E1AF gene, a member of the ETS oncogene family

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Abstract

E1AF is a member of the ETS oncogene family and is thought to be a human homologue of mouse PEA3. We have isolated a genomic clone of E1AF and analyzed the promoter activity of its 5′-flanking region. We identified a variation in exon 1, which depends on the cell type. There was no typical TATA box in the 5′-flanking region, but putative binding sites of a number of transcription factors including PEA3 as well as CAAT boxes were seen. A luciferase reporter assay indicated that the 5′-flanking region possesses promoter activity. Northern blot studies demonstrated significant expression of the E1AF gene in restricted tissues such as the pituitary gland, placenta, and fetal kidney. Moreover, the E1AF promoter was activated by E1AF itself and estrogen receptor. These findings suggest that E1AF is a housekeeping gene, whose expression is controlled in specific tissues.

Keywords: E1AF; ETS family; PEA3; Genome; Promoter; TATA-less; Estrogen receptor

ETS-related genes encode a family of transcription factors, which contain a DNA-binding motif consistent with approximately 85 amino acids named the ETS domain [1]. These genes are thought to be essential for many biological events such as normal development, cell growth, differentiation, immune response, and apoptosis.

E1AF was first identified as a transcription factor that binds to enhancer motifs of the adenovirus E1A gene. Since amino acid sequences of the ETS domain of E1AF were 100% identical to those of PEA3 and several features of PEA3 were also conserved in E1AF, E1AF is a human homologue of mouse PEA3 [2,3]. E1AF/PEA3, ERM, and ER81 were defined as forming the PEA3 subfamily due to their similar amino acid sequences [4].

E1AF is associated with the invasion and metastasis of tumor cells [5–7] and it stimulates the transcription from multiple matrix metalloproteinase genes [8]. Overexpression of the E1AF gene induced the MMP gene expression and conferred invasive phenotypes on MCF7 breast cancer cells [5].

In Ewing sarcoma and Ewing sarcoma-related peripheral neuroectodermal tumor (PNET), the EWS gene is fused to an ETS gene by a specific chromosome translocation [9,10]. The translocation resulted in fusion of the N-terminal domain of the EWS gene (NTD-EWS) and the ETS domain of ETS family genes, replacing the C-terminal RNA-binding domain of EWS with the DNA-binding ETS domain [9]. The E1AF gene was fused with EWS in Ewing sarcoma by t(17;22)(q12;q12) chromosome translocation

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[11,12] and the fusion gene product is able to activate telomerase activity via upregulating TERT gene expression [13]. These results suggest that the deregulation of ETS protein may contribute to tumor development.

To further investigate the physiological role of E1AF, we characterized the genomic organization of the E1AF gene and surveyed the function of the promoter region. We found that the E1AF gene varies in the length and position of the first exon which depend on the cell type properties. Promoter activity was confirmed in the 5′-flanking region of the E1AF gene by luciferase assay. Sequence analysis of the promoter region showed many putative E1AF-binding sites. Expression analysis revealed that E1AF is expressed in specific tissues. Furthermore, the E1AF promoter was activated by E1AF itself and estrogen receptor.

Materials and methods

Cells. Human prostatic adenocarcinoma PC3, human neuroblastoma GOTO, human oral squamous-cell carcinoma SAS, human embryo lung fibroblast MRC-5, and mouse mammary carcinoma FM3A (Health Science Research Resources Bank) were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum and penicillin/streptomycin.

Isolation of genomic clones. A human genomic library of human peripheral blood cells (from Japanese Cancer Research Resources Bank, Tokyo, Japan) was screened using E1AF cDNA [2] as a probe to obtain a genomic DNA clone (λF1-20). To isolate the further upstream region of the λF1-20 clone, a human genomic library in P1 phage (Genome Systems, St. Louis, USA) was screened by PCR using E1AF primers (FcSI5; 5′-TGTCCCACTTGGATGAGAGC-3′, FcA; 5′-TTTTCCTTCCCAATG ACTCC-3′). As the last step in the isolation of genomic clones, long PCR was performed using a primer (PEAATG; 5′-CGGGATCCATGACTAA GTCTTCCAACCAC-3′, FD1450AS; 5′-CGCGGATCCTTTCGGG CG CAGCAGACAGTT-3′) that matches the first ATG in the mouse homologue of E1AF, PEA3.

5′-RACE analysis. We designed a primer for 5′-RACE (rapid amplification of cDNA end) analysis (5AI; 5′-GAAGAT CTGCCGATCAGC GCTTCGCGCAAGTCTCCCATTT-3′). cDNA was synthesized from poly(A)$^+$ RNA by Super Script TM II RNase H—Reverse Transcriptase (Gibco-BRL) using oligo(dT) primer or primers specific for the E1AF cDNA sequence (FNa36; 5′-CGCGGATCCTGAAGGTGTAGGGCA CTTGCTGGTCCA-3′, FN35; 5′-CGCGGATCCTATCCGGCTTTCA TCCTCCGCTCCAT-3′, FD1470AS; 5′-CGCGGATCCCCAGCGCA CCGACTTGTTT-3′, FD1450AS; 5′-CGCGGATCCTTTCGGG CGCA GCAGACAGTT-3′), to analyze 5′-end of E1AF. RACE [14] was performed using sequential antisense primer sets following the manufacturer's protocol (Clontech).

Luciferase assay. The 5′-upstream region of E1AF was fused to the luciferase gene present in pGL2-Basic (Promega). The 1789 base *Bgl*II–*Bss*HII fragment was inserted into pGL2-Basic to generate pFpro. The 5′-deleted mutants pFpro-1439, pFpro-790, pFpro-300, and pFpro-238 were constructed from pFpro using *Stu*I, *Eco*47III, *Pvu*II, and *Sma*I restriction endonucleases, respectively (Fig.2A). These plasmids were co-transfected with or without effector expression plasmids, pcDNA3E1AF [13] and estrogen receptor α and β expression plasmids [15], into several cell lines by the $CaPO_4$ method [16] or FuGENE6 (Roche Molecular Biochemicals, USA), and 48 h later the cells were harvested to measure luciferase activity. Luciferase activities were determined as described [13].

RNA blot analysis. A multiple tissue blot containing poly(A)$^+$ RNAs from different human tissues was obtained from Clontech and probed with a cDNA fragment corresponding to bp 190–760 according to the previously published sequence [2]. The hybridization reaction and washing step were performed as described previously [5].

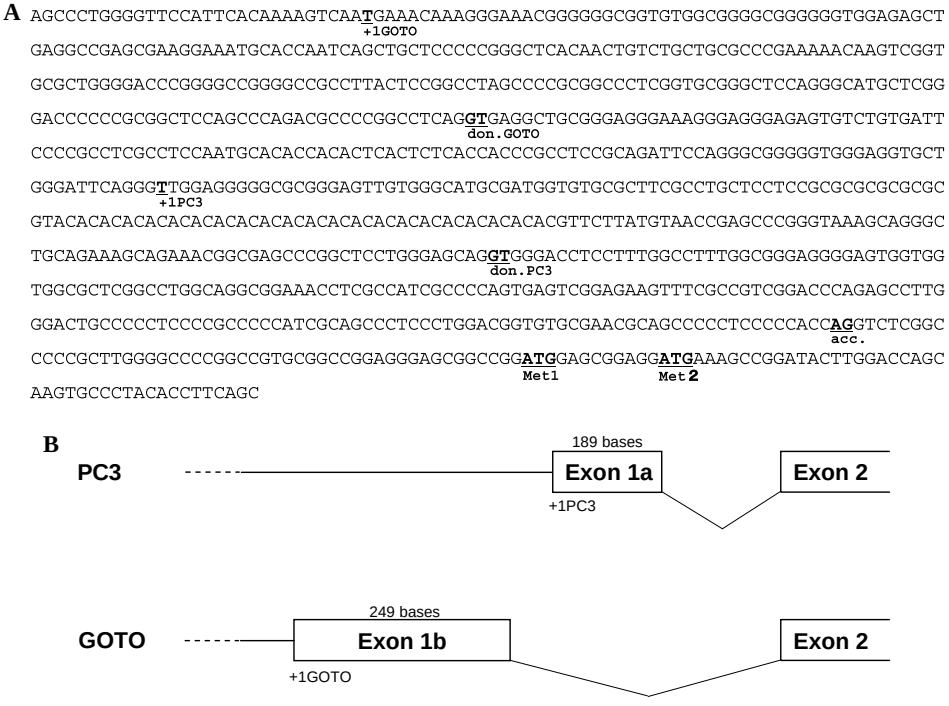


Fig. 1. Structure of the exon 1 region of the human E1AF gene. (A) The 5′-donor GT (don.GOTO and don.PC3), 3′-acceptor AG (acc.), and transcription start sites (+1GOTO and +1PC3) derived from the genome DNA of GOTO and PC3 cells are shown. Putative translation start codons are indicated as Met1 and Met2. (B) Schematic representation of the exon 1 region. The exons are represented by a box. The length of each exon is indicated.

Fig. 2. Promoter activity of the 5′-flanking region of the E1AF gene. (A) Nucleotide sequence of the 5′-flanking region of the E1AF gene. Nucleotide numbering is relative to the transcription initiation start point (+1PC3) indicated by a curved arrow. Putative binding sites of transcription factors, PEA3, AP1, SP1, and estrogen receptor (ER), are underlined. CAAT boxes are shown by a box. The putative translation start codon is indicated in boldface. (B) The size of the 5′-flanking region cloned in front of the luciferase coding sequence and the mutated positions of each reporter construct are shown in a schematic representation. (C) Each reporter construct was introduced into GOTO and PC3 cells. Luciferase activities relative to those obtained from the control pGL2 vector are shown in the histograms. (D) Luciferase activity from the pFpro reporter was cotransfected with different amounts of E1AF expression plasmid as indicated in 293 cells.

Results and discussion

Genome organization of the human E1AF gene

To obtain a genomic clone of *E1AF*, we screened a genomic library of human peripheral blood cells and a P1 phage system using primers corresponding to the 5′-end of E1AF cDNA [2] (see Materials and methods). The *E1AF* gene spanned approximately 30 kb and contained 14 exons as shown previously [17]. To clarify the start site, we carried out 5′-RACE (rapid amplification of the cDNA end) analysis using several cell lines such as PC3, SAS, MRC-5, and GOTO. Sequence analysis by comparing a nucleotide sequence between the genome and 5′-RACE products revealed a variation in exon 1 which depends on the cell type properties (Figs. 1A and B). In the case of PC3 cells, which express E1AF at a high level, the most extended 5′-RACE product showed a putative start site (Fig. 1A, +1PC3) of E1AF mRNA expressed in the cells and showed an open reading frame 189 bases long of exon 1a (Fig. 1B, upper panel). All clones obtained from PC3 cells showed the same splicing feature. SAS and MRC-5 cells showed the same splicing pattern as those of PC3 cells; however, GOTO cells had differences in their genome structure (Fig. 1A, +1GOTO). Exon 1b was 249 bases long (Fig. 1B, lower panel), which is longer than exon 1a and exists upstream of exon 1a. These introns start from different donor sites (Fig. 1A, don.GOTO and don. PC3), whereas they share the same splicing acceptor site (Fig. 1A, acc.). Variations of exon 1 were also reported in ERM genome, another PEA3 family transcription factor [18]; however, we have no information about the biological significance of these exon variations.

Two in-frame ATG codons were identified in exon 2 (Fig. 1A, Met1 and Met2). Met2 is well conserved among the PEA3 subfamily [19] and Met1 is located 4 amino acids upstream from Met2. Both ATG appear to be encompassed with nucleotides for optimal translation by eukaryotic ribosomes. Both possess either a G at position +4 or

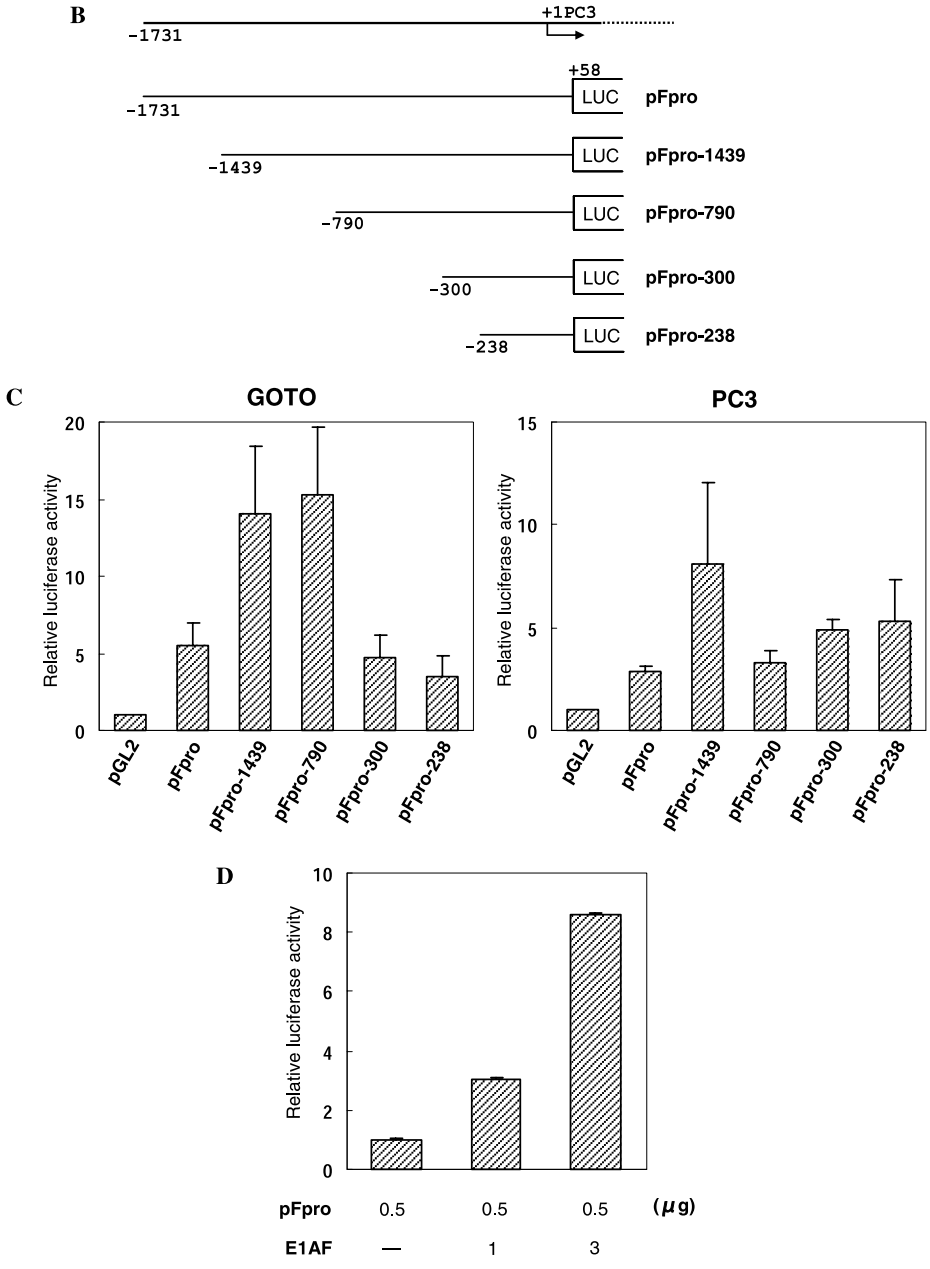


Fig. 2. (*continued*)

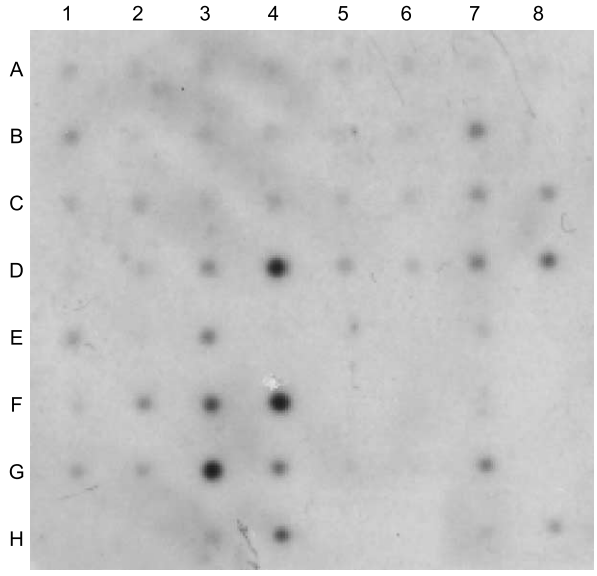
an A at position −3 which is essential for efficient translation [20].

The sequence alignment of mouse PEA3 and human E1AF indicates that the novel isolated 5′-upstream region of E1AF cDNA is 74% identical to the corresponding region of PEA3. Although the 5′-end region of E1AF cDNA was elongated sufficiently by the 5′-RACE method, we could not isolate cDNA clones which contain additional 7 amino acids and a 5′-untranslated region corresponding to PEA3 cDNA [21].

Sequence and functional analysis of the 5′-flanking region

The nucleotide sequence of the 5′-flanking region of the E1AF gene was determined to analyze its promoter function (Fig. 2A). No typical TATA box sequence was found, but two CAAT boxes were present at approximately 80 and 310 bp from the transcriptional start site shown in PC3 cells (Fig. 2A, +1PC3). A number of potential binding sites for transcription factors such as PEA3/E1AF, AP1, Sp1, and estrogen receptor (ER) were identified by TFBIND [22] (Fig. 2A). There are many PEA3 binding motifs (9 sites) in the region, suggesting the possibility of self-regulating E1AF gene expression.

To identify the regions required for E1AF gene expression, a series of deletion fragments of a putative E1AF promoter was ligated to the luciferase reporter gene in the pGL2 vector (Fig. 2B) and transfected into human cancer cells. pFpro containing the longest 5′-flanking region (+58 to −1731) showed significant luciferase activity



	1	2	3	4	5	6	7	8
A	whole brain	amygdala	caudate nucleus	cere-bellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
B	occipital lobe	putamen	substanti a nigra	temporal lobe	thalamus	sub-thalamic nucleus	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary galnd
E	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
F	appendix	lung	tracheae	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	
H	yeast total RNA 100ng	yeast tRNA 100ng	E.coli rRNA 100ng	E.coli DNA 100ng	Poly n(A)	human DNA 100ng	human DNA 100ng	human DNA 500ng

Fig. 3. Tissue expression of the E1AF gene detected by RNA blot analysis. (Left) A multiple tissue blot containing poly(A)$^+$ RNAs from different human tissues was probed with a partial cDNA fragment of E1AF [2]. (Right) Tissue blot table corresponding to the RNA blot.

relative to the parent vector pGL2, indicating that this fragment contains a functional eukaryotic promoter activity in GOTO and PC3 cells (Fig. 2C). Since deletion of the 5′-end 292 bases increased promoter activity (Fig. 3C; pFpro-1439), this region may contain a negative regulatory element. A different pattern of luciferase activity was obtained when GOTO cells, which showed a different splicing pattern, were used. In the case of GOTO cells, luciferase activity was the highest even if pFpro-790 was used (Fig. 2C, left panel), whereas that was not the case in PC3 cells. Approximately 240 bases upstream from +1PC3 were sufficient for basic E1AF promoter activity in both cell lines (Fig. 3C; pFpro-238).

To examine whether E1AF regulates its own transcription, 293 cells, which do not express E1AF, were cotransfected with pFpro and the E1AF expression plasmid. The promoter was activated in a dose-dependent manner (Fig. 3D). These data suggest that the E1AF gene is positively regulated depending on the quantity of its own gene product.

A promoter lacking a TATA box is found for many housekeeping genes in which multiple initiation sites were detected. Since the TATA box defines the point at which transcription will start [23], absence of the TATA sequence might account for the multiple start points observed in 5′-RACE analysis of the E1AF gene (Fig. 1).

Expression of human E1AF mRNA

We examined the expression of E1AF in many tissues using RNA blot analysis. Poly(A)$^+$ RNA blots from human tissues were hybridized with a probe specific for E1AF. As shown in Fig. 3, human E1AF mRNA is expressed in the pituitary gland, placenta, and fetal kidney at very high levels. A modest expression was observed in the trachea and mammary glands. The data suggest that E1AF is expressed in many endocrine tissues and its expression may be suppressed in immune-related tissues.

In this study, human E1AF mRNA was expressed in restricted tissues regardless of its housekeeping gene-like features of the promoter region (Fig. 2A). The expression profile was different from another PEA3 family of the gene. ERM was highly expressed in the brain as well as in the placenta, lung, pancreas, and heart [19]. The expression level of ER81 was very high in the brain and high in the testis, lung, and heart [24].

The promoter of E1AF is activated by estrogen receptor

As E1AF is expressed in many endocrine tissues such as the pituitary and mammary glands, this prompted us to examine the response of the E1AF promoter to the

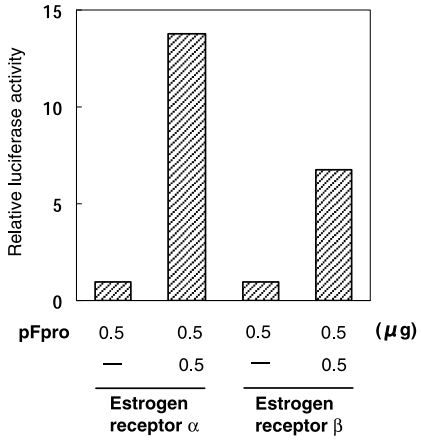


Fig. 4. Activity of the E1AF promoter mediated by the estrogen receptor. The pFpro reporter was cotransfected with the expression constructs of estrogen receptors (α and β) as indicated in COS1 cells. Luciferase activity relative to that obtained from the control empty vector of the estrogen receptor is shown in the histograms.

hormone receptor. To this end, we performed a luciferase assay to observe the effect of estrogen receptor on E1AF promoter activation. The expression constructs of estrogen receptor α (ERα) and β (ERβ) [15] were cotransfected with pFpro into COS1 cells. The promoter was activated by a factor of 13.8 and 6.8 when ERα and ERβ expression plasmids were cotransfected, respectively (Fig. 4). These data suggest that the E1AF promoter is responsible for estrogen receptors.

Interestingly, the estrogen-receptor binding site (ER) is close to the PEA3 binding sites about 115 bases upstream of +1PC3 (Fig. 2A). The cooperative activation of various promoters by ETS family transcription factors and other transcription factors has been reported [25]. The presence of these binding sites suggests tissue-specific regulation in the expression of E1AF mRNA. Further studies are required to determine whether these factors bind to the E1AF promoter.

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