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Serum, AP-1 and Ets-1 stimulate the human ets-1 promoter

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ABSTRACT

The ets-1 proto-oncogene codes for a transcription factor. In order to understand how ets-1 is regulated, we have cloned its promoter. We show that the promoter is inducible by serum and expression of c-Fos and c-Jun, and it is positively auto-regulated by its gene product. A 50 base-pair sequence is sufficient to confer c-Fos + c-Jun and c-Ets-1 responsiveness to a heterologous promoter. This element contains two AP1 and one Ets-1 like motifs. Striking, AP-1 and Ets-1 motifs are found in oncogene responsive units (ORU's) of other promoters, suggesting that combining these motifs is a common mechanism for generating mitogen responsive transcription elements.

INTRODUCTION

c-ets-1 is the cellular homologue of the v-ets oncogene found in the avian leukemia virus E26 (1). It belongs to a growing gene family whose products have important roles in the control of growth and development (2–4). Transcription of the human ets-1 gene gives a major 6.8 kb mRNA that is highly expressed in the hematological lineage, in particular T-cell (5). The Ets-1 protein may regulate the maturation and differentiation of T-cells (6). It is a nuclear phosphoprotein that binds and trans-activates through the PEA3 motif (7, 8). Associated PEA3 and AP1 motifs form the oncogene responsive units (ORUs) of the polyoma virus enhancer and the collagenase gene promoter (9).

To understand how ets-1 expression is regulated, we cloned the human ets-1 promoter and localized the transcription initiation sites. The promoter contains a GC rich domain with several Sp1-like boxes and a putative CCAAT box (in the non-coding strand). Footprinting experiments show that nuclear proteins bind to the AP-1 motif at −450 and an ORU-like element at −348. The ets-1 promoter responds efficiently to serum stimulation and the expression of c-Ets-1 and c-Jun+c-Fos in HeLa cells. The ets-1 ORU is sufficient for stimulation by c-Ets-1 and c-Jun+c-Fos. These data suggest that serum stimulation of the ets-1 promoter could be mediated by AP1 and sustained by positive auto-regulation.

MATERIALS AND METHODS

Cell lines, RNA and transfections

CEM and HSB2 cells [human T cell lines derived from ALL which exhibit high levels of ets-1 gene expression (10)] were routinely grown in RPMI 1640 media with 10% foetal calf serum at 37°C in 5% CO_2. Total RNA were prepared by the guanidine thiocyanate cesium chloride method. HeLa cells were grown in DMEM medium with 2.5% foetal calf serum (FCS) and 2.5% calf serum. Cells (10^6/10cm plate) were transfected by the calcium phosphate procedure (11). To performed serum stimulation, the cell were washed with DMEN without serum, 20 hours after the DNA addition and starved for 24 hours in DMEM + 0.05 FCS. Serum stimulation were performed by adding 1 ml of FCS to each 10ml dishes. The cells were harvested after 1, 3, 8 and 24 hours. Co-transfected RSVβGAL or pβCBx2 (S1 analysis) were used to control transfection efficiency. At least three independant transfections were performed. For CAT assays (12) acetylated chloramphenicol was separated by silica gel chromatography ($CHCl_3$/methanol; 95/1), detected by autoradiography and quantitated by scintillation counting. S1 mapping on total RNA were as described in (9).

RNase H mapping and primer extension

20 μg of CEM total RNA was annealed with 20 ng of a 20-mer oligonucleotide e_2 (3'ACCGTCAAAGAAGACCTTAA5'), RNase H digested, phenol extracted, ethanol precipitated, denatured in a formamide/formaldehyde buffer (5 min, 68°C), separated on a 1.2% denaturing agarose gel, transferred to an Hybond C membrane (Amersham) and hybridized with (alpha-^{32}P)dCTP labelled probe (see 13). The molecular weight marker was the 0.24–9.5 kb RNA ladder (BRL). 20 ng of a

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27-mer oligonucleotide (3'ACCCTTCTTTCAGCCTAAA-GGGGGCAG5') complementary to exon I was end-labelled with T_4 polynucleotide kinase, annealed to 20 μg of HSB2 total RNA, extended with AMV reverse transcriptase, and fractionated by denaturing 6% polyacrylamide gel electrophoresis, as described elsewhere (13) The size marker was HpaII digested pBR322.

Sources of DNA, sequencing and Plasmid construction

2.8 and 1.3 kb HindIII fragments were isolated from a human genomic library from AM_0L blastic cells (patient CR) with a t(9;11)(q22;q23) translocation (14) and cloned into pBS(+), giving pE28 and pE13, respectively. Four SmaI and AvaI fragments from pE13 were inserted into the SmaI site of pBS(+) for sequencing by the dideoxy chain-termination method (15).

pHA reporter (−578 to +216 upstream from CAT): the HindIII-SmaI and blunt-ended SmaI-AvaI fragments of pE13 were sequentially cloned into the HindIII-SmaI and SmaI sites of pBluescript SK(+) and then transferred as a HindIII-BamHI fragment into the equivalent sites of pB9. pHHA: 2.8 kb HindIII fragment from pE28 in HindIII site of pHA.

pCAT B4 contains four copies of the oncogene responsive unit of the polyoma virus enhancer (8) upstream from the TK promoter of pBLCAT4. Insertion of a 43bp oligonucleotide (TCGAGCTACCACATGCCTCACGTCCTGTGTGTCAGT-CTTTGTG) usptream from the rabbit β-globin promoter of the pG1 reporter (9) yields the pGATA construct.

Footprint analysis

The HindIII-SmaI and SmaI-AvaI fragments were 5' end-labelled with T_4 polynucleotide kinase and purified by low melting point agarose gel electrophoresis. Nuclear extracts: a 0.35M NaCl nuclear wash was precipitated with ammonium sulfate (0.32 g/ml), dissolved and dialysed overnight against storage buffer (50 mM NaCl, 20 mM Hepes pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol and 20% glycerol) at 4°C. All solutions contained 0.5 mM phenylmethylsulfonylfluoride and benzamidine, 10 mM beta-glycerophosphate, and 0.1 mg/ml each of pepstatin A, aprotinin, leucopeptin and bestatin. Typically, nuclei containing 100 mg of DNA yielded 5 ml of nuclear extract. Footprint protection assays were performed as described (16). 2 μg of 5' end-labelled probe and 1 μg of poly(dI-dC)(dI-dC) were incubated with 0−80 μl of nuclear extract in a final volume

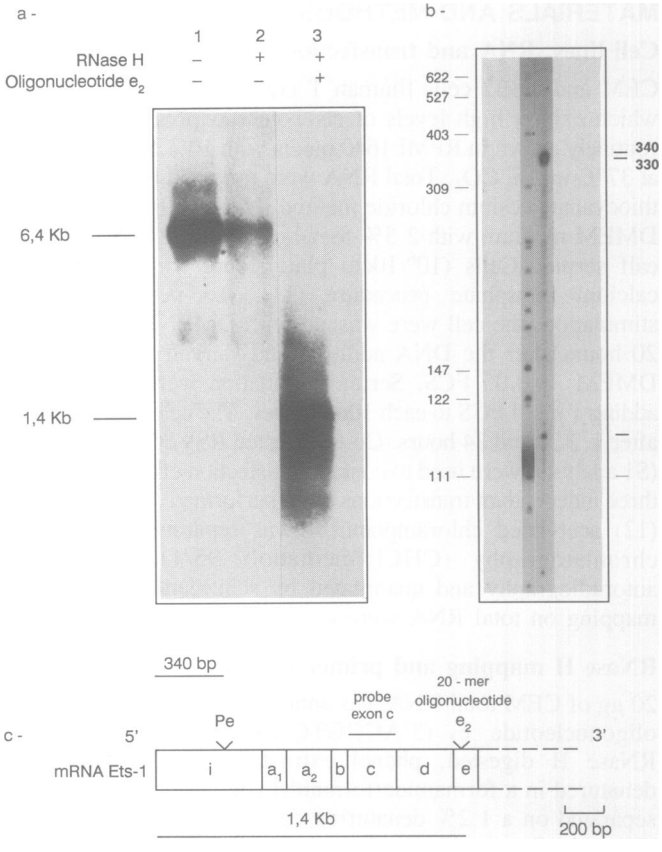

Fig. 1. 5' end mapping of the ets-1 transcript by Northern blots of RNase H experiments using total RNA of CEM cell line (a. 2−3 with RNase H treatment, 3, with oligonucleotide) and primer extension (b. PE oligonucleotide). 330 and 340 indicate the major extension products. c. The position of the oligonucleotides and the probe used in RNase H and primer extension experiments are indicated in the ets-1 transcript.

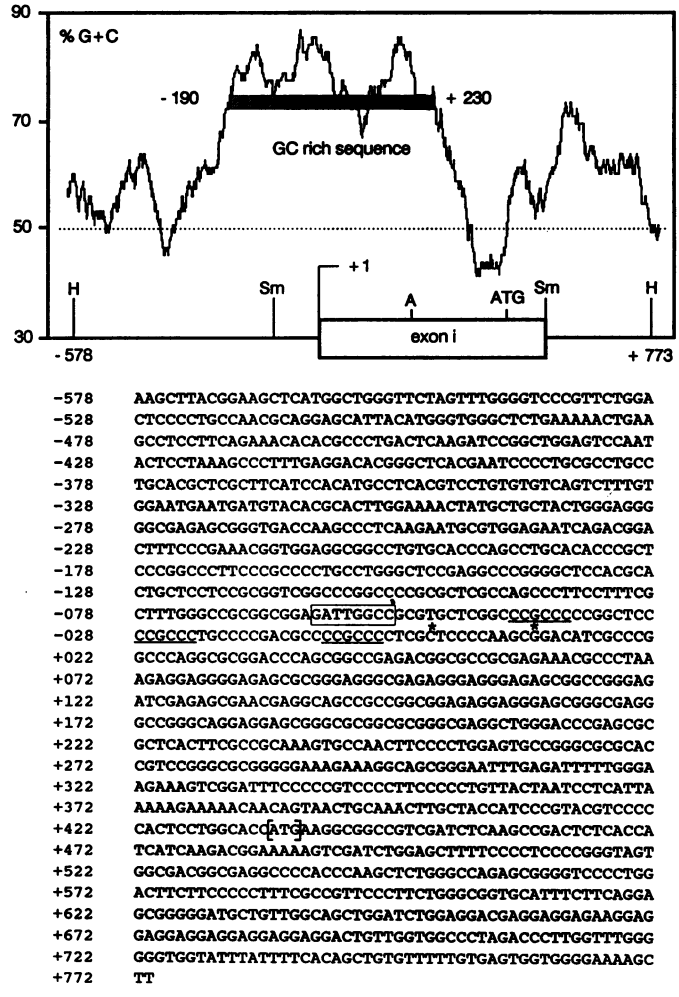

Fig. 2. Nucleotide sequence of the human ets-1 promoter and percentage of G+C base pairs along the 1.3 kb Hind III genomic fragment; H= Hind III, Sm = Sma I, A= AvaI. In the nucleotide sequence, the transcription initiation site and the AUG are indicated by a star and brackets, respectively. The box indicates the position of a putative CCAAT element in the non-coding strand. Putative Sp1 binding sites are underlined.

of 100 µl of storage buffer for 60 min on ice. After limited digestion with DNase 1 at 22°C for 30 secondes, samples were purified and separated by denaturing 6% polyacrylamide gel electrophoresis. Markers were prepared from the 5′ end-labelled probes by the G+A chemical sequencing reaction.

RESULTS

Mapping of the human ets-1 transcription initiation site

We localized the 5′ end of ets-1 mRNA by RNase H mapping followed by primer extension. A 1.4 kb band was detected when total CEM RNA was hybridized to a 20-mer oligonucleotide complementary to exon e (e2), digested with RNase H and analyzed on Northern blots with an exon c probe (fig. 1a, lane 3). The signal is broad because the band is in fact a doublet. The lower band corresponds to RNase H digestion of a transcript about 250 bp shorter due to alternative splicing of exon d (17) which occurs with high frequency in this cell line (Kerckaert J.-P. personnal communication). A control without oligonucleotide gave the expected 6.8 kb ets-1 transcript (fig. 1a, lanes 2). Consequently, oligonucleotide e2 hybridizes to a sequence about 1.4 kb from the 5′ end (Fig 1c), suggesting that exon I is about 500 bp in size. Extension with a primer complementary to a sequence 93 bp upstream from the ATG (fig. 1c) gave major 330 and 340 nucleotide products, (fig. 1b), showing that the 5′ untranslated region is about 430 nucleotides long. The significance of the minor shorter products is not known. The mapping of the transcription initiation sites were done in human T cell because these cell lines expressed high level of ets-1 transcript whereas in epithelial cell the level of expression is much lower.

Nucleotide sequence of the 5′-flanking region of the ets-1 gene

The sequence of the 1.3 kb Hind III genomic fragment contains the open reading frame for the first 27 amino acids of Ets-1 (+435, +516)(17) and the donor splice site (CGG/gtgagt). The sequence has a number of features: 1) There is no TATA box. 2) On the non-coding strand at −60 there is a CCAAT motif that is nearly identical to the consensus sequence for CP1 (18, Fig 2). 3) Between −190 and +230 the sequence is greater than 75% G + C, with a frequency of CpG's close to expected. The frequency of GpC's and CpG's is roughly equivalent, similar to HpaI tiny fragment (HTF) islands (19,20). This region contains three Sp1 motifs (GGGCGG, 21), grouped near the start sites. 4) Downstream from the initiation codon there is a repeat of GGA (+650, +690) that is also found in the untranslated region of the first exon of chicken c-ets-1 (22). Similar sequences are found in several human genes; 5′ of the first exon in c-erbB-2 (23, 24) and trk (25), and 3′ of the first intron in IGF (26). The phylogenetic conservation and proximity to promoters suggest that it might have a role, possibly in transcription. 5) Lastly, there is a consensus AP1 site at −452, and two similar sequences at −354 and −340 that surround a putative PEA3 motif (28).

Protein binding to the ets-1 promoter

To detected ubiquitous nuclear factors that interact with the promoter, DNase I protection experiments were performed with nuclear extracts from a mouse epithelial cell line (SN 161). There

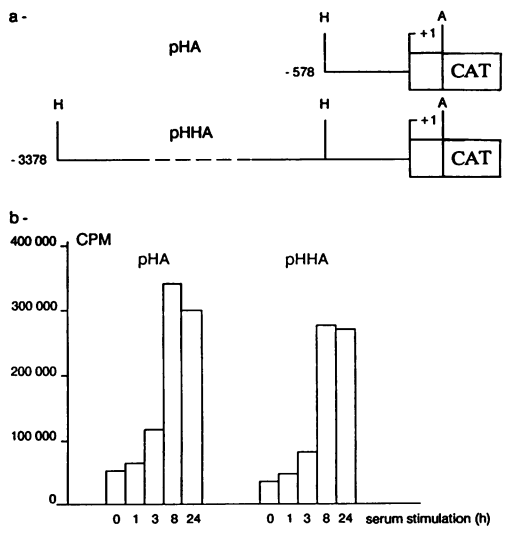

Fig. 3. Footprint analysis of the ets-1 promoter. We used the HindIII-SmaI restriction fragments (−578, −140, Fig. 2) 5′ end labelled on the non-coding strand. Lane 1: G + A sequencing reaction of the probe. Lane 2 and 3: probes after limited DNase 1 digestion without or with nuclear extracts, respectively. The protein binding sites are represented by boxes. The AP-1 and PEA3 like motifs are indicated.

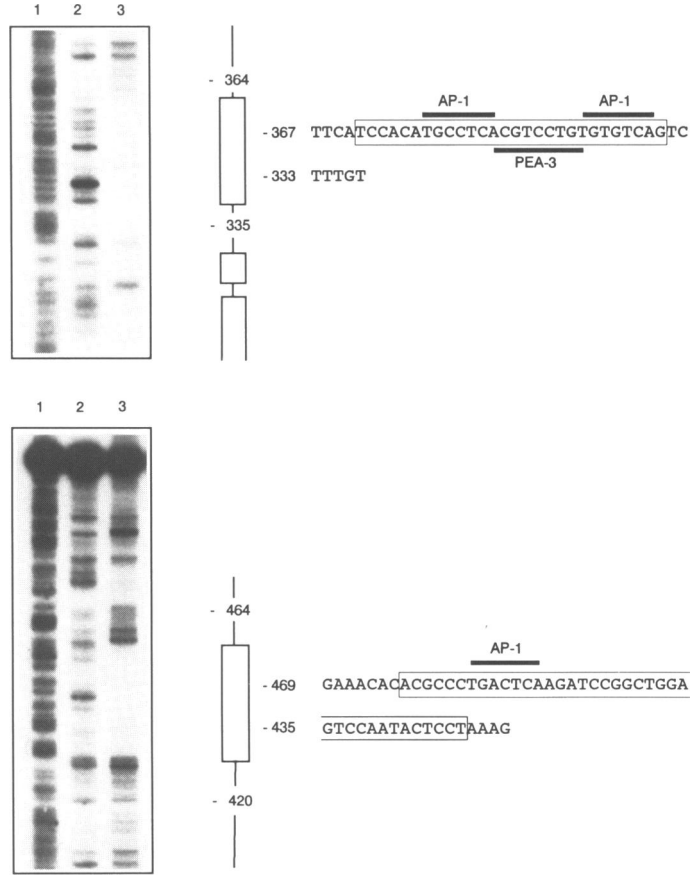

Fig. 4. Analysis of the ets-1 promoter activity following serum stimulation of HeLa cells. a. pHA contains the Hind III-AvaI restriction fragments (see fig.2) cloned upstream from the CAT gene. pHHA contains in addition the 2.8kb Hind III genomic fragment, extending the promoter to −3378. b. The CAT activity of the pHA and pHHA constructs, corrected for variations in the internal control, are expressed in arbitrary units (c.p.m./OD).

were a number of footprints between +215 and −140 on the non-coding strand, including a large 50 bp protection over the three Sp1 motifs (data not shown). Several footprints were detected between −578 and −140 on the non-coding strand, upstream from the HTF. In particular, there was a marked protection between −335 and −365 (fig. 3a), on the sequence with a PEA3 motif surrounded by AP1 motifs that ressembles an ORU (9, 28). The consensus AP-1 motif further upstream (−464 to −420) was also protected (fig 3b).

Serum stimulation of the ets-1 promoter

ets-1 promoter activity was studied with the reporters pHHA and pHA, that contain 3378 or 578 bp, respectively, of ets-1 5′ flanking sequences upstream from the bacterial CAT gene (fig. 4a). HeLa cells were transfected with these constructs and serum starved for 24 hours before serum treatment for different times. pHHA and pHA were about 20 fold more active than the promoterless construct, showing that the ets-1 gene sequences have promoter activity. Their activities were 7 to 8 fold higher after 8 hours of serum stimulation (Fig 4b), showing that sequences between −567 and the cap site are sufficient to confer serum responsiveness to the ets-1 promoter.

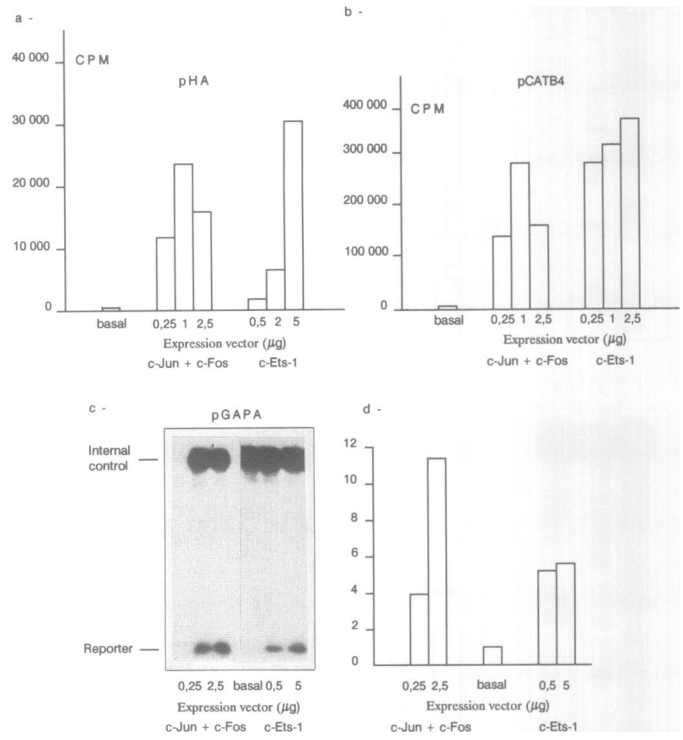

Fig. 5. c-Fos+c-Jun and c-Ets-1 activate the ets-1 promoter through an element containing AP1 and PEA3-like motifs. 4μg reporters (pHA, pCATB4, pGAPA), 4μg internal control (RSVGAL for CAT assays, pCBx2 for S1 analysis) and the indicated quantities of expression vectors for c-Jun, c-Fos and c-Ets-1 were transfected into HeLa cells by the calcium phosphate technique. a, b. CAT activities are corrected for variations in the internal control. c. Internal control and Reporter indicate the bands expected from specific RNA initiated on the control and reporter recombinants, respectively. d. Activation of pGAPA by c-Fos+c-Jun and c-Ets1 relative to basal activity, taking into account variations in the internal control. The expression of c-Fos+c-Jun and c-Ets-1 did not affect the activity of pG1, the reporter without the ets-1 ORU (data not shown).

Induction of the ets-1 promoter by AP1 and Ets-1 by cotransfection

c-Jun+c-Fos and c-Ets-1 expression stimulated the ets-1 promoter in pHA about 70 fold and 90 fold, respectively (figure 5a). As expected, pCATB4, with four copies of the ORU of the polyoma virus enhancer, was stimulated about 100 fold (Fig 4b) (28). The promoter was activated 10 times more efficiently by the expression plasmids than by serum. The −325 to −365 region, that contains AP-1 and PEA3 like motifs, may play a role in the induction of the ets-1 promoter. This sequence was inserted upstream from the rabbit β-globin promoter of the pG1 reporter, to give pGAPA. Quantitative S1 nuclease mapping was used to measure the amount of specific RNA initiated from the reporters. Transcription of pGAPA increased 11 and 5 fold due to expression of c-Jun+c-Fos and c-Ets-1 respectively (figures 4c and 4d), in experimental conditions where the control vector pG1 was unaffected (data not shown). These results show that the ets-1 ORU responds to transcription activation by c-Fos+c-Jun and c-Ets-1.

DISCUSSION

The transcription start sites and the minimal promoter of the human ets-1 gene were localized on a 1340 bp region. The promoter ressembles so-called 'housekeeping' genes, in that it is very GC-rich sequences and lacks a TATA box. The sequence between −190 and +230 is particulary GC-rich (>75% GC). There are three Sp1 motifs proximal to the cap sites that are protected from DNase1 digestion by nuclear extracts. A CCAAT box on the non-coding strand at −60 is nearly identical to the consensus sequence for the CCAAT-binding proteins CP1 (18).

CP1 consensus	Y	N	N	N	N	N	N	N	R	R	C	C	A	A	T	C	A	N	Y	T/G
ets-1 −46 −65	G	A	G	C	A	C	G	C	G	G	C	C	A	A	T	C	T	C	C	G

CCAAT boxes in this orientation are found in many eucaryotic mRNA coding genes (29−31).

The −567 to +216 promoter fragment was sufficient in transfection assays to give basal promoter activity and to respond to serum (fig 3). This region lacks a palindrome that was described previously in the ets-1 promoter (33) to ressemble the serum response element of the c-fos gene (32), showing that it cannot account for serum inducibility. Interestingly, an SRF motif (or CArG box, CCTAAAGAGG; 34, 35) is present in the minimum promoter, at position +72. It appears unlikely that this motif mediates the serum response, since the c-fos SRF motif is not sufficient to mediate serum activation in HeLa cells (36).

Footprint protection assays were used to locate potential transcription regulatory elements in the miminum promoter. The AP-1 motif at −452 and an ORU-like element containing two AP-1 like motifs surrounding a PEA3 like motif are clearly footprinted. The AP-1 motif at −452 is identical to a canonical AP-1 recognition (TGACTCA) whereas both AP-1 motifs surrounding the PEA3 motif differ in one nucleotide (TGCC-TCA and TGACACA). Furthermore the ets-1 PEA3 motif (C-AGGACGT) differ only in one nucleotide from a canonical ets-1 recognition sequence (CAGGAAGT) (28, 37) However the combination of these cis-elements probably has a biological relevance in the ets-1 promoter in analogy with the ORU of the polyoma virus and the collagenase promoter (9). The ets-1 promoter is induced by the expression of c-Fos+c-Jun (AP-1) and c-Ets-1 (PEA3) (fig 4). The ets-1 ORU is sufficient to confer

responsiveness to the heterologous β-globin promoter. However, transactivation of the promoter by c-Fos+c-Jun may also involve the consensus AP-1 sequence at −440. Interrestingly, in contrast to the Py ORU (8), cFos+c-Jun and c-Ets-1 do not co-operate in transactivation through the ets ORU (data not shown), perhaps because of the particular arrangement or sequence of the AP-1 and PEA3 motifs. Both the spacing and the sequence of the PEA3 and AP-1 motifs modulate transcription activation by oncogene expression (9). The ORU could mediate the serum responsiveness of ets-1 promoter in HeLa cells. Induction of c-Fos and c-Jun synthesis by serum could lead to stimulation of the ets promoter. Previous promoter assays performed independently in Papas T. S. laboratory (33), have revealed that deleting the AP-1 and ORU sequences decreases ets-1 promoter activity six-fold. Consequently, these elements probably act at a constitutive level for basal transcription and to enhance transcription in response to c-Fos+c-Jun and c-Ets-1. Overexpression of Ets-1 has been shown to activated expression of the endogenous ets-1 gene (38). The ORU may play a role in this positive autoregulation at the transcriptional level.

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