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The product of the c-*ets*-1 proto-oncogene and the related Ets2 protein act as transcriptional activators of the long terminal repeat of human T cell leukemia virus HTLV-1

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The c-*ets*-1 proto-oncogene and the related c-*ets*-2 gene encode related nuclear chromatin-associated proteins which bind DNA *in vitro*. To investigate the possibility that Ets1 and Ets2 are transcriptional activators, we analyzed the ability of these proteins to *trans*-activate promoter/enhancer sequences in transient co-transfection experiments. A CAT construct driven by the long terminal repeat of the human T cell leukemia virus, HTLV-1 was found to be *trans*-activated by both Ets1 and Ets2 in NIH3T3 and HeLa cells. The increased levels of CAT activity were paralleled by increased levels of correctly initiated CAT mRNA. Mutant Ets1 proteins unable to accumulate in the nucleus were found to be inactive. An *ets*-responsive sequence between positions −117 and −160 of the LTR was identified by analyses of a series of 5′ deletion mutants of the HTLV-1 LTR and of dimerized versions of specific motifs of the LTR enhancer region. Using a gel shift binding assay, Ets1 was found to bind specifically to an oligonucleotide corresponding to region −117 to −160. This sequence, which also contributes to Tax1 responsiveness of the HTLV-1 LTR, is characterized by the presence of four repeats of a pentanucleotide sequence of the type CC(T/A)CC. Competition experiments show that integrity of repeats 1 and 4 is important for Ets1 binding. These results show that Ets1 and Ets2 are sequence-specific transcriptional activators. In view of the high level expression of Ets1 in lymphoid cells, Ets1 could be part of the transcription complex which mediates the response to Tax1 and the control of HTLV-1 replication. More generally, Ets1 and Ets2 could regulate transcription of cellular genes.

Key words: ets oncogene/HTLV-1/transcriptional activator

Introduction

Avian leukemia virus E26 causes erythroblastosis and myeloblastosis in chickens and transforms erythroblasts and myeloblasts in tissue culture (Graf *et al.*, 1979; Moscovici *et al.*, 1981; Radke *et al.*, 1982). The genome of E26 contains two cell-derived oncogenes, v-*myb*^{E} and v-*ets* (Roussel *et al.*, 1979; Leprince *et al.*, 1983; Nunn *et al.*, 1983) which both contribute to its transforming properties (Beug *et al.*, 1984; Golay *et al.*, 1988; Nunn and Hunter, 1989). Several cellular genes homologous to the v-*ets* oncogene have been identified in vertebrate and invertebrate species. The *ets* gene family includes c-*ets*-1, the cellular progenitor of v-*ets* (Watson *et al.*, 1985; Gégonne *et al.*, 1987; Leprince *et al.*, 1988). c-*ets*-2 (Watson *et al.*, 1985, 1988; Boulukos *et al.*, 1988; Pribyl *et al.*, 1988), *erg* (Reddy *et al.*, 1987; Rao *et al.*, 1987), *elk*-1 and *elk*-2 (Rao *et al.*, 1989) and E74 (Burtis *et al.*, 1990).

Both the c-*ets*-1 and c-*ets*-2 gene products (Ets1 and Ets2) have been identified in cells (Chen, 1985; Ghysdael *et al.*, 1986a,b; Pognonec *et al.*, 1988; Leprince *et al.*, 1988; Fujiwara *et al.*, 1988) and shown to be short-lived phosphoproteins (Boulukos *et al.*, 1988; Fujiwara *et al.*, 1988; Pognonec *et al.*, 1988). Ets1 and Ets2 are nuclear, chromatin-associated proteins which display DNA binding activity *in vitro* in their non-phosphorylated state (Boulukos *et al.*, 1988; Pognonec *et al.*, 1988, 1990). These proteins share two domains of strong homology: the first region (70% identity) extends for 96 amino acid residues close to their amino-terminal end while the second domain (90% identity) includes 110 amino acid residues at their carboxy terminus (Boulukos *et al.*, 1988; Watson *et al.*, 1988). This latest domain is conserved in all members of the *ets* gene family identified so far and this high degree of conservation is in line with the essential role of this domain in targeting these proteins to the nucleus and in mediating their ability to bind to DNA *in vitro* (Boulukos *et al.*, 1989).

Several lines of evidence suggest that Ets1 and Ets2 have regulatory roles in cells and mediate in the nucleus molecular events which are initiated at the plasma membrane by the triggering of specific cell-surface receptors. First, expression of c-*ets*-2 has been shown to be induced rapidly and transiently during mouse liver regeneration (Bhat *et al.*, 1987) or treatment of macrophages with cMGF, a factor required for survival, proliferation and differentiation of chicken myeloid cells (Boulukos *et al.*, 1990) while expression of both c-*ets*-1 and c-*ets*-2 is induced upon growth stimulation of mouse fibroblasts by serum (Bhat *et al.*, 1987). Second, triggering of specific cell-surface receptor such as the T cell antigen receptor (TCR) by either mitogenic lectins or monoclonal antibodies to several TCR components has been shown to induce the rapid and transient calcium-dependent phosphorylation of Ets1 and Ets2 (Pognonec *et al.*, 1988, 1990; Fujiwara *et al.*, 1990) and the subsequent loss of affinity of these proteins for chromatin *in vivo* and for DNA *in vitro* (Pognonec *et al.*, 1989).

We report here that both Ets1 and Ets2 are able to *trans*-activate a chloramphenicol acetyltransferase (CAT) construct under the control of the LTR of the human leukemia virus HTLV-1 in transient co-transfection experiments. The Ets-responsive sequence is identified as a 44 bp sequence located

between residues −117 and −160 of the LTR. Furthermore, we show that the Ets1 protein specifically binds this Ets-responsive region.

Results

To examine whether Ets1 and Ets2 were able to *trans*-activate specific promoters *in vivo*, expression plasmids containing cDNAs encoding either chicken Ets1 or human Ets2 were co-transfected into NIH3T3 cells and HeLa cells together with a series of reporter plasmids containing the CAT gene under the control of different promoter/enhancer sequences. As negative controls, these reporter plasmids were either transfected alone or co-transfected with the same expression plasmid containing the c-*ets*-1 cDNA inserted in an anti-sense orientation. A recombinant plasmid containing the *Escherichia coli lacZ* gene under the control of the Rous sarcoma virus LTR (Gorman *et al.*, 1982) was also included in all transfection experiments to correct for variations in transfection efficiencies. Cells were incubated for 48 h and lysates were assayed for β-galactosidase and CAT activities. The results are expressed as fold stimulation of CAT activity as compared with the anti-sense control.

Using this procedure, we were unable to detect any stimulation by either Ets1 or Ets2 of CAT reporter plasmids containing the promoter/enhancer region of SV40 (pSV2-CAT; Gorman *et al.*, 1982), the LTR of bovine leukemia virus (Rosen *et al.*, 1985) and the LTR of human immunodeficiency virus (Siekevitz *et al.*, 1987) (data not shown). In contrast, we reproducibly observed a 3- to 4-fold stimulation of a CAT construct containing the LTR sequences of HTLV-1 (pU3R-CAT, Sodrosky *et al.*, 1984; see Figure 1 for a schematic of the plasmids) both in NIH3T3 cells and in HeLa cells (Figure 2). This *trans*-activation resulted from the expression of Ets1 or Ets2 rather than derepression of the reporter plasmid as the result of competition by the co-transfected expression vector for cellular factors, since the same expression vector containing the c-*ets*-1 cDNA in an inverse orientation failed to enhance the level of CAT activity (Figure 2). Similar results were obtained using an LTR−CAT construct derived from an independent HTLV-1 isolate (Fujisawa *et al.*, 1985; data not shown).

To delineate which region of the HTLV-1 LTR is required for the response to Ets1 and Ets2, we examined the effects of these proteins in co-transfection experiments using a series of 5′ deletion mutants of the HTLV-1 LTR. The structure of these mutants is shown schematically in Figure 1. Consistent with previous observations in CV1 cells (Brady *et al.*, 1987), progressive deletions from the 5′ end of the U3R region resulted in a decrease in basal promoter activity in both NIH3T3 and HeLa cells (Figure 3, panels A and B). When analyzed in co-transfection experiments as described before, only pU3R-CAT (−306) and pU3R-CAT (−242) were found to be specifically *trans*-activated by Ets1 and Ets2 (Figure 3, panels A and B). In contrast, reporter plasmid pU3R-CAT (−101), pU3R-CAT (−52) and pU3R-CAT (−21) failed to respond to either Ets1 or Ets2. We conclude from these experiments that sequences critical for *trans*-activation by Ets1 and Ets2 are localized in the −242 to −101 region of the HTLV-1 LTR. This region of the LTR contains several previously defined elements (see Figure 1) on the basis of binding of cellular factors and

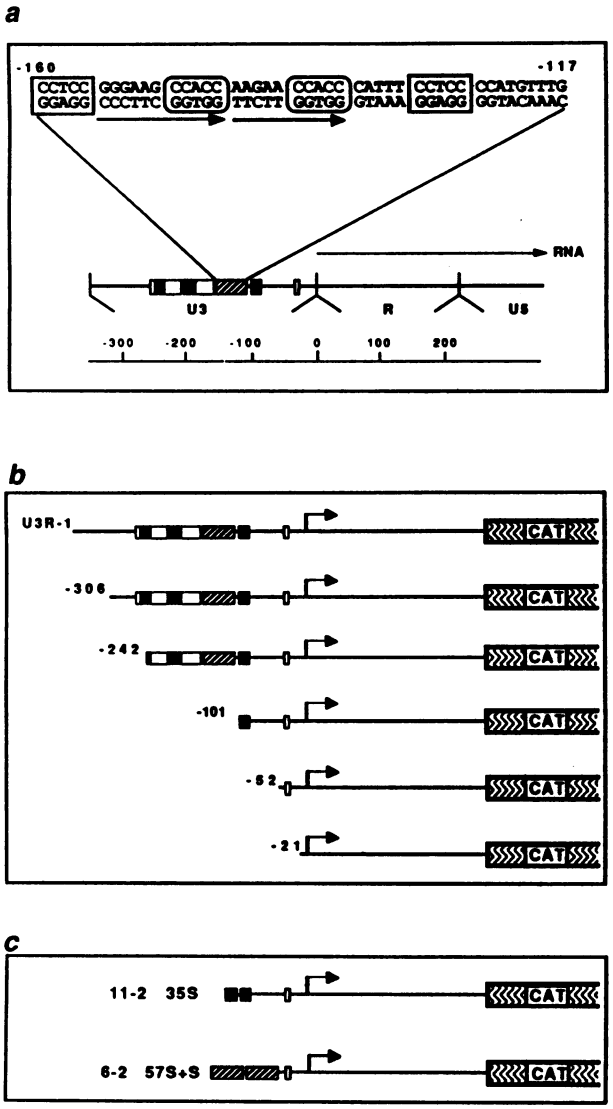

Fig. 1. Schematic of the reporter plasmids used in this study. **Panel a**, structure of the HTLV-1 LTR. Black boxes: 21 bp repeat sequences; large open boxes: 51 bp repeat sequences; hatched box: −117 to −160 region; small open box: TATA box. The sequence of the −117 to −160 region is shown and pentanucleotide repeats are squared (repeats 1 and 4) or circled (repeats 2 and 3). Arrows underline the 11 and 10 bp repeats. **Panel b**, structure of the pU3R-CAT construct and of 5′ deletion mutants derived thereof (Brady *et al.*, 1987). **Panel c**, structure of reporter plasmids containing either two copies of the 21 bp repeats (dl 11-2-35S; Brady *et al.*, 1987) or two copies of the −117 to −160 region linked to pU3R-CAT (−52)(6-2-57S+S).

transcriptional activation by the HTLV-1 encoded *tax*1 gene product and several pharmacological agents. These include, first, one copy of an imperfect 21 bp repeat element which binds a variety of cellular factors (Nyborg *et al.*, 1988; Jeang *et al.*, 1988; Tan *et al.*, 1989a; Montagne *et al.*, 1990) and contributes to the response to Tax1, cAMP and phorbol myristate acetate (PMA) through distinct mechanisms (Fujisawa *et al.*, 1989; Poteat *et al.*, 1989; Radonovich and Jeang, 1989; Tan *et al.*, 1989b). Second, a 44 bp sequence localized between residues −117 and −160 and which confers responsiveness to Tax1 (Brady *et al.*, 1987; Marriott *et al.*, 1989) and is characterized by the presence of four repeats of a pentanucleotide sequence (CCT/AAC), the

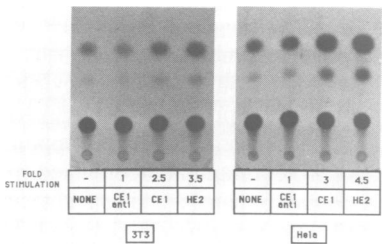

Fig. 2. CAT assays following co-transfection of pU3R-CAT. Plasmid pU3R-CAT (2 μg) was transfected into NIH3T3 cells and HeLa cells either in the absence of expression plasmid (NONE) or in the presence of expression plasmids (5 μg) for chicken Ets1 (CE1) or human Ets2 (HE2) or with the same expression plasmid containing the c-*ets*-1 cDNA insert in an antisense orientation (CE1 anti). All transfection mixtures also included 3 μg of pRSV-lacZ. CAT assays were performed on an amount of extract containing the same level of β-galactosidase activity (Herbomel *et al.* 1984).

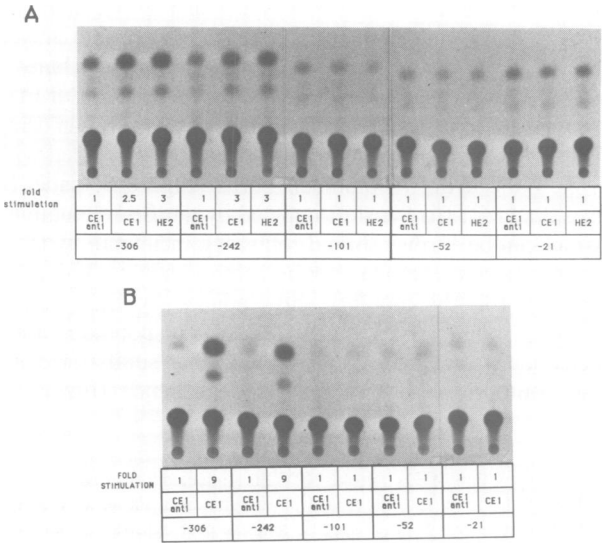

Fig. 3. CAT assays following co-transfection of 5′ deletion mutants of the U3 region of pU3R-CAT. Plasmids (2 μg) harboring 5′ deletions in the U3 region of pU3R-CAT were co-transfected into NIH3T3 (**panel A**) or HeLa cells (**panel B**) either in the presence of a chicken Ets1 (CE1) or a human Ets2 (HE2) expression plasmids (5 μg), or the same expression plasmid (5 μg) containing the c-*ets*-1 cDNA insert in an anti-sense orientation (CE1 anti). All transfection mixtures also included 3 μg of pRSV-lacZ. CAT assays were performed on an amount of extract containing the same level of β-galactosidase activity (Herbomel *et al.*, 1984).

second and third pentanucleotide repeats being part of larger direct 11 and 10 bp repeats (Figure 1, panel a).

To identify more precisely the Ets-responsive sequences in the U3 region of the HTLV-1 LTR, we analyzed duplicate copies of either the 21 bp repeats or of the −117 to −160 region, linked to minimal unresponsive HTLV-1 promoter in co-transfection experiments (see Figure 1, panel c). A CAT construct containing two copies of a 21 bp element linked to position −101 of the HTLV-1 LTR proved completely unresponsive to either Ets1 or Ets2, although it showed the expected induction when co-transfected with a Tax1 expression vector (Figure 4, panel A). In contrast, the level of CAT activity of a construct containing a duplicate copy of the −117 to −160 element linked to position −52 of the HTLV-1 LTR (6-2-57S+S) was induced by a factor of ~20 as the result of the expression of Ets1 or Ets2

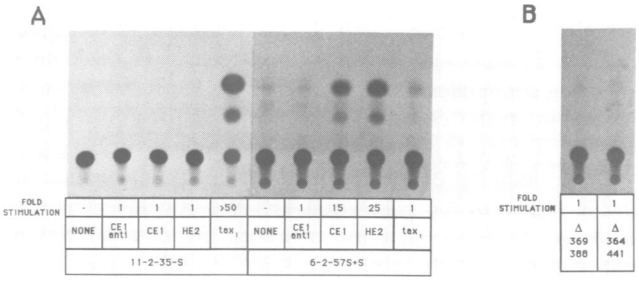

Fig. 4. CAT assays following co-transfection of reporter plasmids containing two copies of the 21 bp sequence or two copies of the −117 to −160 region and analysis of Ets1 deletion mutants. **Panel A**: reporter plasmids (2 μg) 11-2-35S or 6-2-57S+S were co-transfected with 5 μg of either the Ets1 expression plasmid (CE1), the Ets2 expression plasmid (HE2), or the same expression plasmid containing the c-*ets*-1 cDNA insert in the inverse orientation (CE1 anti), or 2 μg of an expression plasmid for tax1 (pMTPX; Seiki *et al.*, 1986) and 3 μg of pRSV-lacZ. CAT assays were performed on an amount of extract containing the same level of β-galactosidase activity. **Panel B**: reporter plasmid 6-2-57S+S was co-transfected with expression vectors for chicken Ets1 deletion mutants Δ364−441 and Δ369−388 (Boulukos *et al.*, 1989). Conditions of transfection and CAT assays are as described in panel A.

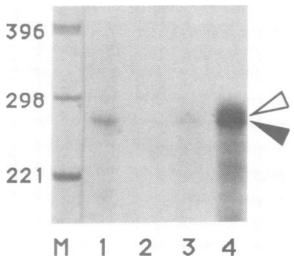

Fig. 5. RNase protection assay. Reporter plasmid 6-2-57S+S (7 μg) was co-transfected with the Ets1 expression vector (5 μg) (lane 4) or the same vector containing the c-*ets*-1 cDNA insert in an anti-sense orientation (lane 3). Either non-transfected cells (lane 2) or cells transfected with pU3R-CAT alone (lane 1) were used as negative or positive controls, respectively. Total RNA was extracted 48 h after transfection and the level of CAT mRNA was measured using an RNase protection assay (see Materials and methods). Protected fragments were analyzed by electrophoresis on a 3.5% polyacrylamide sequencing gel. The protected fragment corresponding to the correctly initiated transcript (filled arrowhead) and that initiated upstream (open arrowhead) are marked. M: size markers.

(Figure 4, panel A). We conclude from these experiments that the region between residues −117 and −160 of the HTLV-1 LTR defines an Ets1 and Ets2 responsive sequence.

To establish that the increase in CAT activity observed in these experiments actually reflects an increase in the steady state level of CAT mRNA and results from the translation of a correctly initiated transcript, we performed RNase protection analyses to measure the level of CAT RNA in the presence or absence of Ets1. Total RNA was extracted from cells co-transfected with the 6-2-57S+S construct and either the Ets1 expression vector or the same expression vector containing the c-*ets*-1 cDNA inserted in an anti-sense orientation. RNA extracted from non-transfected cells and RNA extracted from cells transfected with pU3R-CAT were used as negative and positive controls, respectively. A major band of 265 nucleotides and a minor band of ~280 nucleotides were detected at low levels in cells transfected with the anti-sense plasmid which were not observed in the non-transfected controls cells (Figure 5, compare lanes 2 and

3). The intensity of these bands is considerably enhanced in cells co-transfected with the Ets1 expression plasmid (Figure 5, lane 4). The major protected 265 nucleotide fragment comigrates with the fragment protected by the same probe in cells co-transfected with pU3R-CAT alone (Figure 5, lane 1). The size of this fragment is consistent with an initiation of transcription at the first nucleotide of the R region of the HTLV-1 LTR. We conclude that the increase in CAT activity observed in co-transfection experiments with the Ets1 expression plasmid is paralleled by an increase in the level of correctly initiated CAT mRNA.

Previous analyses of Ets1 deletion mutants have demonstrated that the carboxy-terminal domain of the protein, which is highly conserved in all members of the c-*ets* gene family, is essential for both nuclear targeting and the ability of the protein to bind to non-specific DNA *in vitro* (Boulukos *et al.*, 1989). An Ets1 protein deleted in either its 78 carboxy-terminal residues (Δ364−441) or in residues 369−388 (Δ369−388), which suppresses both nuclear accumulation in cells and binding to DNA *in vitro* (Boulukos *et al.*, 1989) failed to *trans*-activate the 6-2-57S+S construct (Figure 4, panel B). Thus, the Ets1 *trans*-activating property apparently requires the protein to accumulate in the nucleus of transfected cells. Similar data were obtained in co-transfection experiments using the complete LTR as a reporter construct (data not shown).

To investigate whether Ets1 is able to bind directly to the −117 to −160 region, we made use of an Ets1 protein produced in *Spodoptera frugiperda* cells (SF9 cells) infected by an Ac-NPV-Ets1 recombinant baculovirus. Insect SF9 cells infected by this recombinant baculovirus synthesize large amounts of a 54 kd protein indistinguishable by size and two-dimensional tryptic peptide mapping from the endogenous Ets1 protein immunoprecipitated from chicken lymphoid cells (Figure 6, panel a and data not shown). First, an exonuclease III assay was performed on an HTLV-1 probe which has been 5′ end-labeled with polynucleotide kinase at position +285. Incubation of the probe with the Ets1-containing, but not the control extract, resulted in the appearance of a strong exonuclease III stop at −160 (data not shown). We next compared the ability of extracts of either non-infected SF9 cells or SF9 cells infected with Ac-NPV-Ets1 or a control Ac-NPV-ErbA recombinant virus to bind a ^{32}P-labeled oligonucleotide probe corresponding to residues −117 to −160 in electrophoretic mobility shift assays. The results of Figure 6 show that Ets1-containing extracts specifically induce a mobility shift of the labeled oligonucleotide probe (Figure 6, panel b, compare lanes 1 and 4). This mobility shift results from the binding of Ets1 to the labeled probe since (i) no shift is observed when using matched extracts of non-infected cells or cells infected with an Ac-NPV-ErbA recombinant virus (Figure 6, panel b, lanes 2 and 3); (ii) the shift induced by the Ets1-containing extract can be further shifted following incubation with an Ets-specific antiserum but not by control antisera (Figure 6, panel c); (iii) the mobility shift induced by Ets1 can be competed out by an excess of the corresponding oligonucleotide but not by an oligonucleotide of similar size and base composition but of unrelated sequence (Figure 6,

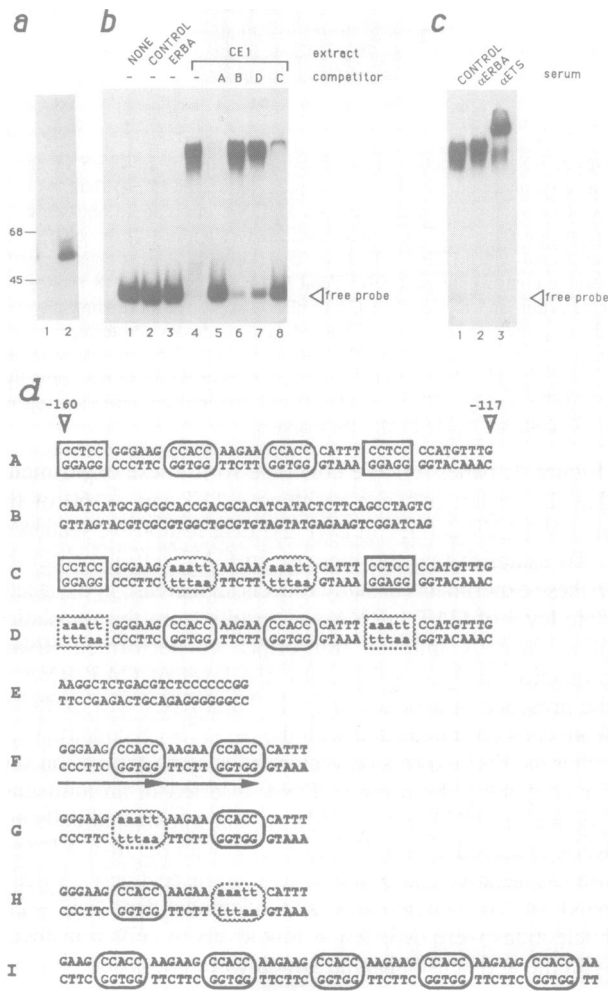

Fig. 6. Expression of Ets1 in Ac-NPV-Ets1 infected cells and electrophoretic mobility shift assays of Ets1 binding. **Panel a**: Western blotting analysis using an *ets*-specific antiserum of extracts (20 μg of protein) of uninfected (lane 1) or Ac-NPV-Ets1 infected (lane 2) insect cells. The non-phosphorylated form of 54 kd of Ets1 is seen in lane 2; the slowly migrating species represent phosphorylated forms of Ets1 (Pognonec *et al.*, 1988 and data not shown). **Panel b**: electrophoretic mobility shift assays using oligonucleotide A as ^{32}P-labeled probe (25 fmol/assay) and either no extract (lane 1); an extract (4 μg) of uninfected insect cells (lane 2); an extract (4 μg) from insect cells infected with an Ac-NPV-ErbA recombinant baculovirus (lane 3); an extract (4 μg) of insect cells infected with the Ac-NPV-Ets1 recombinant baculovirus (lanes 4−8). Assays contained either no competitor (lanes 1−4) or a 200-fold molar excess of either oligonucleotides A (lane 5), B (lane 6), D (lane 7) or C (lane 8). **Panel c**: electrophoretic mobility shift assays using an extract from Ac-NPV-Ets1 infected cells and oligonucleotide A as a probe (25 fmol/assay) followed by incubation with a control non-immune serum (lane 1), an *erbA*-specific antiserum (lane 2) and an *ets*-specific antiserum (lane 3). **Panel d**: sequences of oligonucleotides used in electrophoretic mobility shift assays. (**A**) oligonucleotide corresponding to the wild-type sequence of the −117 to −160 region; (**B**) oligonucleotide of the same composition but of unrelated sequence; (**C**) oligonucleotide corresponding to the sequence −117 to −160 mutated in repeats 2 and 3; (**D**) oligonucleotide corresponding to the sequence −117 to −160 mutated in repeats 1 and 4; (**E**) oligonucleotide corresponding to the 21 bp of the HTLV-1 LTR; (**F**) oligonucleotide including the 11 bp and 10 bp repeats which contain CCACC motifs 2 and 3 (underlined by arrows) of the −117 to −160 region; (**G**) same as F, except that CCACC repeat 2 is mutated; (**H**) same as F, except that CCACC repeat 3 is mutated; (**I**) pentamer version of the region surrounding the second pentanucleotide repeat. Pentanucleotide repeats are either squared (repeats 1 and 4) or circled (repeats 2 and 3). Mutated versions of the repeats are shown as lower case letters. Each of these oligonucleotides is flanked at its extremities by additional sequences containing restriction enzyme sites.

panel b, lanes 5 and 6). We conclude from these experiments that Ets1 binds specifically to the −117 to −160 oligonucleotide. In order to identify further important sequences within the −117 to −160 region which are responsible for Ets1 binding, competition experiments were performed with a series of oligonucleotides corresponding to specific regions of the HTLV-1 LTR and mutant oligonucleotides of the −117 to −160 region (see Figure 6, panel d for the description of these oligonucleotides). A −117 to −160 oligonucleotide containing mutations in repeats 1 and 4 was unable to compete for binding of Ets1 to the wild-type oligonucleotide probe (Figure 6, panel b, lane 7). This oligonucleotide was also unable to be shifted by Ets1-containing extracts (data not shown). In contrast, a −117 to −160 mutant oligonucleotide in repeats 2 and 3 was an efficient competitor for the binding of Ets1 to the wild-type probe (Figure 6, panel b, lane 8). The results of Figure 7 show that the gel shift complex observed with Ets1 is not competed by the addition of a 25-fold molar excess of an oligonucleotide containing the HTLV-1 21 bp repeat (Figure 7, lane 3). This shift was also not competed by a 25-fold molar excess of an oligonucleotide (oligonucleotide F in Figure 6, panel d) corresponding to the 11 bp and 10 bp repeats of the HTLV-1 LTR, which includes CCACC motifs 2 and 3 (Figure 7, lane 5), nor by mutant versions of this oligonucleotide containing only CCACC motif 3 (Figure 7, lane 6) or CCACC motif 2 (Figure 7, lane 7). The shift was also not efficiently competed by a 25-fold molar excess of an oligonucleotide containing five tandem copies of the second pentanucleotide motif (Figure 7, lane 8). As expected from our previous results, the shift was efficiently competed by a 25-fold molar excess of oligonucleotides A and C (Figure 7, lanes 4 and 9). We conclude that the integrity of repeats 1 and 4 is required for efficient binding of the −117 to −160 oligonucleotide by Ets1.

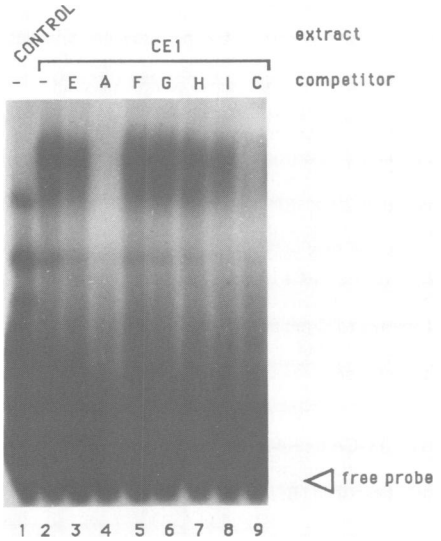

Fig. 7. Mobility shift assay of Ets1 binding. Mobility shift assays were performed using oligonucleotide A as ^{32}P-labelled probe (530 fmol/assay) and either an extract (10 μg) of non-infected insect cells (lane 1) or an extract from insect cells infected with an Ac-NPV-Ets1 recombinant baculovirus (lanes 2−9). Assays contained either no competitor (lanes 1 and 2) or a 25-fold molar excess of: lane 3, oligonucleotide E; lane 4, oligonucleotide A; lane 5, oligonucleotide F; lane 6, oligonucleotide G; lane 7, oligonucleotide H; lane 8, oligonucleotide I; lane 9, oligonucleotide C. Description of oligonucleotides is in Figure 6d.

Discussion

The results described have shown that the product of the c-*ets* proto-oncogene (Ets1) and the related Ets2 protein act as transcriptional activators of the LTR of the human leukemia virus HTLV-1. First, Ets1 and Ets2 *trans*-activation of the HTLV-1 pU3R-CAT construct requires Ets proteins to accumulate in the nucleus. Second, this *trans*-activation is mediated by a *cis*-acting sequence localized at positions −160 to −117 of the LTR. Third, Ets1 binds specifically to this sequence. The CCTCC pentanucleotide repeats 1 and 4 are apparently important for the binding of Ets1 to the −160 to −117 HTLV-1 regulatory sequence.

During preparation of this manuscript, two sets of experimental data were published which also suggest that Ets1 and Ets2 may act as regulators of transcription. First, a 271 amino acids long carboxy-terminal polypeptide derived from mouse Ets1 is able to bind to specific DNA sequences in the enhancer region of the MSV LTR (Gunther *et al.*, 1990). Second, the carboxy-terminal domain of PU-1, a transcription factor specific to the purine-rich sequence of the promoter region of SV40, bears in its carboxy-terminal domain a 40% identity with that of Ets1 and Ets2 (Klemsz *et al.*, 1990). It is interesting to note that repeat 4 of the −160 to −117 sequence contains a perfect Pu-box core sequence (Figure 8). We also note the presence between repeats 1 and 2 of the −160 to −117 region, of a sequence which displays homology to the binding site recently described for mouse Ets1 (Gunther *et al.*, 1990) (Figure 8). Therefore, it appears possible that the −160 to −117 region of the HTLV-1 LTR may contain binding sites for several transcription factors of the Ets family and the related PU-1 factor. Additional experiments are required to investigate the precise sequence requirement for Ets1 binding and the possible importance of spacing between binding motifs for optimal binding of Ets1.

We have previously shown that integrity of the 78 carboxy-terminal amino acids of Ets1 is required for this protein to bind DNA *in vitro* (Boulukos *et al.*, 1989). This, together with the fact that a domain which includes the 271 carboxy-terminal residues of Ets1 binds DNA (Gunther *et al.*, 1990), suggests that the DNA binding domain of members of the *ets* gene family corresponds to the highly conserved 83 amino acid residues conserved in all members of this family

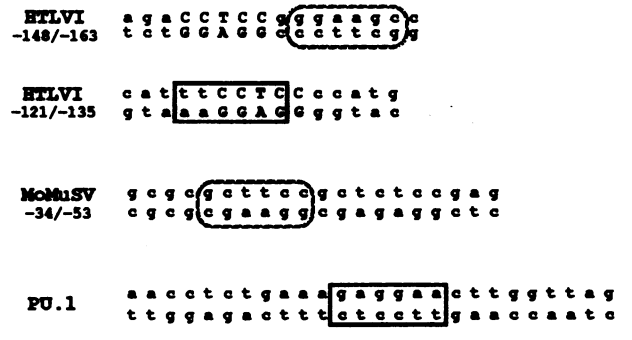

Fig. 8. Comparison of HTLV-1 regulatory sequence with reported Ets1 and PU-1 binding sites. Sequences of the HTLV-1 LTR surrounding repeats 1 (−148/−163) and 4 (−121/−135) are shown. Pentanucleotide repeats 1 and 4 are in upper case letters. Homology to either the Mo-MuSV Ets1 binding site (Gunther *et al.*, 1990) and the PU-1 core recognition sequence (Klemsz *et al.*, 1990) are shown as dotted circles and boxes, respectively.

(Boulukos *et al.*, 1988; Watson *et al.*, 1988; Rao *et al.*, 1989). Interestingly, unlike the products encoded by other known members of the *ets* gene family in which this domain is at their carboxy-terminal end, this domain is found at the amino terminus of the *elk*-1 gene product (Rao *et al.*, 1989). This change in protein structure, together with the tissue-specific expression of *elk*-1 (Rao *et al.*, 1989) suggests that the physiological role of Elk1 might be different from that of Ets1/Ets2. As we previously noted (Boulukos *et al.*, 1990), this domain does not present homology to known DNA binding motifs (zinc fingers, helix-turn-helix, SPKK motifs). It does however, contain a high density of basic amino acid residues, a feature reminiscent of the DNA binding domain of proteins containing a leucine-zipper motif (Landschultz *et al.*, 1989). This Ets1 domain also includes three regularly spaced tryptophan residues, a feature also shared by the DNA binding domain of the products encoded by members of the c-*myb* gene family (Anton and Frampton, 1988). The significance of these repeats in the Myb and Ets DNA binding domains remains to be established.

We have shown that Ets1 and Ets2 are able to *trans*-activate the HTLV-1 LTR and that this activity is dependent upon the −160 to −117 region. It is not clear at present whether Ets1 and Ets2 contain one or several intrinsic transcriptional activation domain(s) or whether their ability to *trans*-activate depends upon the association with other proteins.

Ets1 is expressed at high levels in both B and T lymphoid cells (Chen, 1985; Ghysdael *et al.*, 1986a), and phosphorylation of Ets1 is rapidly and transiently induced following triggering of the T cell antigen receptor of either CD4+ or CD8+ mature T cells (Pognonec *et al.*, 1988, 1990), suggesting a role for Ets1 in lymphoid activation. The promoter region of several lymphokine genes includes a purine-rich box sequence. For example, the IL-2 promoter region contains two purine-rich boxes which are important for promoter activity (Shaw *et al.*, 1988; Serfling *et al.*, 1989), and which have a core sequence similar to that found in repeat 4 of the HTLV-1 LTR. The NFAT-1 complex which binds to this purine box (Shaw *et al.*, 1988) could include members of the Ets gene family. The promoter region of the c-*ets*-2 gene has recently been identified (Mavrothalassitis *et al.*, 1990) and is characterized by multiple repeats of a CCTCC motif. Thus, Ets2 or other *ets*-related gene products may act as regulators of their own transcription.

It is interesting to speculate on the importance of Ets1 in the regulation of HTLV-1 gene expression. Several protein factors have been identified which bind to regulatory sequences within the LTR. These include a 180 kd cellular protein (Jeang *et al.*, 1988), multiple members of the ATF or CRE family of proteins (Tan *et al.*, 1989a; Maekawa *et al.*, 1990), HEB1 and HEB2 (Montagne *et al.*, 1990), SP1 (Nyborg *et al.*, 1990) and TIF-1 (Marriott *et al.*, 1990). At present, Ets1 is somewhat unique to this series of factors in that the experiments presented here functionally demonstrate that the protein is able to activate transcription of HTLV-1 LTR *in vivo* in the absence of Tax1. This observation is important because cellular factors such as Ets1, may be important in activating early viral transcription and thus the synthesis of mRNA coding for viral regulatory proteins Tax1 and Rex1. In addition, the level of mRNA synthesized in ATL cells is extremely low. Thus, one could suggest that modulation of Ets1 expression might be important in activating the virus from this latent state.

The fact that Ets1 activates transcription in the absence of Tax1 does not preclude the possibility that the activity of the protein is modulated by Tax1. We have previously shown that the region between −160 and −117 is important for Tax1 responsiveness of the HTLV-1 LTR (Marriott *et al.*, 1989). We have further demonstrated that Tax1 binds indirectly to this regulatory region via interaction with sequence-specific cellular transcription factors. One of these proteins is a transcription factor present in HeLa cells and HTLV-1 transformed cells, p36 or TIF-1 (Marriott *et al.*, manuscript submitted). The fact that the DNA binding sequences overlap for TIF-1 and Ets1 may suggest that they are related transcription factors or may cooperate in Tax1 *trans*-activation. The possibility that Ets1 could also facilitate the indirect interaction of Tax1 with the LTR is presently under investigation. Alternatively, it is possible that the presence of Tax1 in the cell increases the level or specific activity of Ets1, resulting in the increase in HTLV-1 transcription. In either case, it is important to remember that full transcriptional activity of the LTR is probably also dependent on proteins that interact with the 21 bp repeats. Thus, it is of importance to understand and investigate the possible interaction of these two groups of transcription factors.

Materials and methods

Cell culture, transfection and CAT assays

NIH3T3 and HeLa cells were grown in Dulbeccos modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. For transfection experiments, cells were seeded at 1.5×10^6 cells per 100 mm Petri dish and, 24 h later, they were transfected by the calcium phosphate co-precipitation technique (Graham and Van der Eb, 1973). Cells were incubated with the DNA co-precipitate for 16 h, washed and further incubated in complete medium for 24 h. Cell monolayers were washed in PBS, collected and lysates were assayed for CAT activity as described (Gorman *et al.*, 1982). Quantification of CAT assays was performed by scintillation counting of the appropriate areas of the chromatography plate. Fold stimulation is measured with respect to the anti-sense control plasmid.

The *S.frugiperda* cell line SF9 was grown in TC100 medium (Gibco) supplemented with 10% fetal calf serum. For infection with the *Autographa californica* nuclear polyhedrosis virus c-*ets*-1 recombinant (Ac-NPV-Est1) or ErbAα recombinant (Ac-NPV-ErbA) (Ghysdael *et al.*, in preparation), cells were seeded at 20×10^6 cells per 150 mm Petri dish and infected at a multiplicity of infection of 10. Two days after infection, cells were collected, washed in PBS and whole cell lysates prepared by Dounce homogenization in Tris−HCl 10 mM pH 7.4; EDTA 1 mM; DTT 1 mM; Triton X-100 0.1%; paramethylsulfonylfluoride 100 μg/ml; leupeptin 10 μg/ml and aprotinin 1% (all from Sigma). The homogenates were clarified by centrifugation at 100 000 *g* for 30 min and supernatants collected and stored in liquid nitrogen.

Plasmids

The plasmids pU3R-CAT (−440), 5′ deletion mutants derived thereof and plasmid 11-2-35S, containing two copies of the 21 bp repeat enhancer element were previously described (Brady *et al.*, 1987). The HTLV-1 6-2-57S+S plasmid was constructed by insertion of a tandem copy of oligonucleotide −117 to −160 into pU3R-CAT deletion mutant dl 6-2 (Brady *et al.*, 1987). Expression plasmids for chicken Ets1 (sense and anti-sense orientation) and deletion mutants of Ets1 derived thereof (Δ369−388; Δ364−441) were previously described (Boulukos *et al.*, 1988, 1989). The expression plasmid for human Ets2 was obtained by subcloning of a 3.6 kb *Eco*RI fragment containing the entire coding sequence of Hu-c-*ets*-2 (A.Gegonne, unpublished) in the *Eco*RI site of pKCR3 (Breathnach and Harris, 1983).

RNase protection assays

RNA was isolated from control and transfected cells by the guanidium-isothiocyanate method followed by centrifugation through a CsCl cushion

(Chirgwin *et al.*, 1979). The probe used for RNase protection experiments was obtained by *in vitro* transcription by SP6 polymerase of a recombinant pSP64 plasmid containing the 700 bp *Xho*I–*Hin*dIII fragment corresponding to the LTR portion of pU3R-CAT. RNase protection was carried out on 20 μg of total RNA as described by Zinn *et al.* (1983). Protected fragments were separated on a 3.5% denaturing polyacrylamide gel containing 8.3 M urea, followed by autoradiography.

Electrophoresis mobility shift assays

Mobility shift assays were performed essentially as described (Fried and Crothers, 1981). Oligonucleotide corresponding to region −117 to −160 of the HTLV-1 LTR and mutant oligonucleotides derived thereof were labeled by 5′ phosphorylation with T4 polynucleotide kinase. DNA binding reactions were carried out for 10 min at 0°C in a final volume of 16 μl containing 25 fmol of end-labeled probe in a final buffer concentration of 10 mM HEPES, pH 7.4; 25 mM KCl; 1.25 mM Na phosphate; 0.175 mM EDTA; 0.075 mM EGTA; 1 mM DTT; 5 mM $MgCl_2$; 1.5 μg poly[d(I-C)]; 0.4 μg salmon sperm DNA and 4 μg whole cell extracts from baculovirus infected or non-infected cells (Figure 6) or with 530 fmol of labeled probe for 20 min at 24°C in 10 mM Tris–HCl pH 7.5; 40 mM NaCl; 1 mM DTT; 1 mM EDTA and 10 μg whole cell extracts (Figure 7).

In competition experiments, competitor oligonucleotides were included in the reaction mixture as indicated in the text and figure legends. Samples were loaded on a 5% polyacrylamide gel (acrylamide:bisacrylamide ratio of 30:1) in 0.25 × TBE (1× TBE = 0.089 M Tris, 0.089 M boric acid, 2.5 mM EDTA, pH 8.3) and electrophoresed at 140 V for 90 min at room temperature. Gels were fixed, dried and exposed to Kodak X-O-Mat S film at −20°C using an intensifying screen. For incubation with serum, 4 μl of either a control non-immune serum, an *erbA*-specific antiserum (Goldberg *et al.*, 1988) or an *ets*-specific antiserum (Ghysdael *et al.*, 1986a) were added to binding reactions and incubation was continued for 20 min on ice before loading the gels.

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