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Synergistic activation of the HTLV1 LTR Ets-responsive region by transcription factors Ets1 and Sp1

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Ets1 is the prototype of a family of transcriptional activators whose activity depends on the binding to specific DNA sequences characterized by an invariant GGA core sequence. We have previously demonstrated that transcriptional activation by Ets1 of the long terminal repeat (LTR) of human T cell lymphotropic virus type 1 is strictly dependent on the binding of Ets1 to two sites, ERE-A and ERE-B, localized in a 44 bp long Ets-responsive region (ERR1). We report here that the activity of ERR1 as an efficient Ets1 response element in HeLa cells also depends on the integrity of an Sp1 binding site localized immediately upstream of ERE-A. The response to Ets1 of an element restricted to the SP1/ERE-A binding sites is also strictly dependent on both the Ets1 and Sp1 binding sites. *In vitro*, Sp1 and Ets1 are shown to cooperate to form a ternary complex with the SP1/ERE-A element. Reconstitution experiments in *Drosophila melanogaster* Schneider cells show that Ets1 and Sp1 act synergistically to activate transcription from either the ERR1 or the SP1/ERE-A elements and that synergy requires the binding of both Sp1 and Ets1 to their cognate sites. SP1/ERE-A elements are found in the enhancer/promoter region of several cellular genes, suggesting that synergy between Ets1 and Sp1 is not restricted to the ERR1 region of the HTLV1 LTR. These results strengthen the notion that Ets1 as well as other members of the Ets family usually function as components of larger transcription complexes to regulate the activity of a variety of viral and cellular genes.

Key words: Ets1/HTLV1/Sp1/transcriptional factor cooperation

Introduction

The *c-ets1* proto-oncogene is the prototype of a gene family which includes *c-ets2* (Boulukos *et al.*, 1988; Watson *et al.*, 1988), *erg* (Rao *et al.*, 1987; Reddy *et al.*, 1987) *elk-1* and *elk-2* (Rao *et al.*, 1989), *spi-1*/Pu-1 (Klemsz *et al.*, 1990), *fli-1* (Ben-David *et al.*, 1991) and the gene encoding the α subunit of GABP (La Marco *et al.*, 1991), SAP-1 (Dalton and Treisman, 1992), Elf-1 (Thompson *et al.*, 1991), PEA3 (Xin *et al.*, 1992) and *Drosophila melanogaster* E74 (Burtis *et al.*, 1990).

Ets proteins form a novel class of sequence-specific DNA-binding proteins which bind specifically to purine-rich sequences characterized by an invariant GGA core sequence which, in the context of various promoters, have been shown to function as *cis*-acting DNA response elements for transcriptional activation by several members of this family (Bosselut *et al.*, 1990; Günther *et al.*, 1990; Ho *et al.*, 1990; Klemsz *et al.*, 1990; Wasylyk *et al.*, 1990; Reddy and Rao, 1991; Rao and Reddy, 1992; Xin *et al.*, 1992).

Several lines of evidence implicate Ets family members in developmental processes and in the response of cells to extracellular signals. First, expression of at least some members of this family is regulated in response to specific extracellular signals (Bhat *et al.*, 1987; Boulukos *et al.*, 1990; Thummel *et al.*, 1990) or is developmentally regulated in a given lineage (Bhat *et al.*, 1989). Secondly, binding sites for members of the Ets family contribute to transcriptional activation of responsive promoter/enhancer sequences in response to serum, phorbol esters, antigen and non-nuclear oncogenes (Wasylyk *et al.*, 1989; Yamaguchi *et al.*, 1989; Graham and Gilman, 1991; Thompson *et al.*, 1991). Thirdly, Ets proteins are subject to rapid and transient phosphorylation events in response to mitogenic stimuli, some of which appear to regulate distinct aspects of Ets proteins function (Fujiwara *et al.*, 1988; Pognonec *et al.*, 1988, 1989).

Ets proteins share a conserved domain characterized by the juxtaposition of a region with the potential to form an α helix and of a region rich in basic amino acid residues. This domain is sufficient for Ets family members to bind to specific DNA sequences (GABPα: Thompson *et al.*, 1991; Ets1: Gégonne *et al.*, 1992; Lim *et al.*, 1992; Wang *et al.*, 1992; SAP1: Dalton and Treisman, 1992; PEA3: Xin *et al.*, 1992). Since several members of the Ets family are often co-expressed in the same cells, the question arises as to how target gene selectivity is actually achieved. Some degree of selectivity is likely to exist at the level of DNA binding *per se* since the *in vitro* binding specificities of distinct members of the Ets family can be distinguished in some instances (Wang *et al.*, 1992; Hipskind *et al.*, 1991). Another level of specificity is likely to occur in the way Ets proteins interact with the components of the basal transcription initiation complex and their associated co-factors since the transcription activation domains of Ets1 (Gégonne *et al.*, 1992; Schneikert *et al.*, 1992) and Ets2 (Schneikert *et al.*, 1992) have recently been mapped to regions unique to either of these otherwise highly related proteins.

The recently determined *in vitro* DNA-binding specificity of Ets1 (Fisher *et al.*, 1991; Woods *et al.*, 1992) and *D.melanogaster* E74 (Urness and Thummel, 1990) show that extensive variation is authorized at both the 5′ and 3′ sides of the invariant GGA core sequence, indicating that potential binding sites for members of the Ets family are frequently represented in genomic DNA. Mechanisms are therefore likely to exist which restrict or condition the activity of these sites as effective Ets-responsive elements. Consistent with this notion, binding of various Ets family members to select promoter/enhancer sequences has been shown to occur in conjunction with other nuclear factors (Hipskind *et al.*, 1991; Thompson *et al.*, 1991; Dalton and Treisman, 1992;

Pongubala *et al.*, 1992) and activation of the Polyoma virus enhancer by Ets1 occurs in synergy with AP1 (Jun/Fos) (Wasylyk *et al.*, 1990).

In this paper, we report that the activity of a low-affinity Ets1 binding site in the long terminal repeat (LTR) of human lymphotropic virus type 1 (HTLV1) as an efficient Ets1-responsive element is dependent on the integrity of an adjacent GC-rich motif. Furthermore, we show that Sp1 and Ets1 cooperate to form a ternary complex with this composite element, and act synergistically in co-transfection experiments to activate transcription through this element. Finally, we propose that synergy between Ets1 and Sp1 is likely to be important to transcriptional regulation of other genes since similar elements can be identified in the promoter/enhancer region of several cellular genes.

Results

Ets1 activates transcription of the LTR region of HTLV1 through binding to two Ets1 binding sites, ERE-A and ERE-B, localized in a 44 nucleotide long Ets1-responsive region (ERR1, see Figure 1 for sequences; Bosselut *et al.*, 1990; Gitlin *et al.*, 1991). When inserted in a CAT reporter plasmid upstream from the basal promoter (−50 to +55; tkD) of the herpes simplex virus thymidine kinase gene, a dimer of ERR1 confers in HeLa cells efficient response to a co-transfected Ets1 expression vector (Figure 2, compare lanes 1−2 and 7−8). This activation requires the binding of Ets1 to ERR1 since mutations in ERE-A and ERE-B, which we showed previously to abolish binding of Ets1 to ERR1 (Gitlin *et al.*, 1991; see Figure 1 for a description of the mutant oligonucleotides), also suppress the response to Ets1 (Figure 3A, compare lanes 3−4 and 5−6). In contrast to the ERR1-containing reporter, tkD-derived reporter plasmids containing four direct repeated copies of the individual ERE-A or ERE-B Ets1 binding sites were found to be inefficiently transactivated when tested in the same conditions (Figure 2, compare lanes 3−8). This suggested that sequences in ERR1, distinct from the Ets1 binding sites, are important for full responsiveness to Ets1.

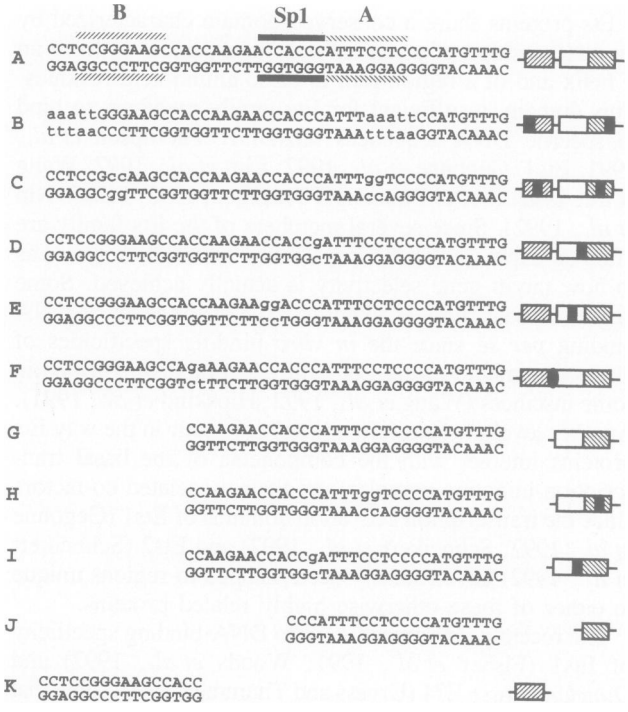

Fig. 1. Nucleotide sequence and schematic representation of oligonucleotides used in this study. The wild-type sequence of the ERR1 oligonucleotide (positions −117 to −160 of the HTLV1 LTR) and of oligonucleotide G (positions −117 to −147 of the HTLV1 LTR) are shown. The nature and position of the mutations in mutant oligonucleotides are shown as lower case letters. The Sp1 binding site is lined with a solid bar and the Ets1 binding site as a hatched bar. In the schematic representation of the nucleotides, the Sp1 binding site is shown as an open box and Ets1 binding sites as hatched boxes. Mutations in the Sp1 and Ets1 binding sites are depicted by black boxes. Mutation of the NF-$\varkappa$B site is marked as a black oval.

To analyse this question in further detail, we asked whether mutations introduced outside the Ets1 binding sites of ERR1 would affect the ability of Ets1 to activate ERR1 reporter constructs. In addition to members of the Ets family, ERR1 has been shown to bind at least three types of transcriptional regulators. First, footprinting analyses using nuclear extracts of both HeLa and HTLV1-infected cells have shown binding of a factor in a region localized between positions −129 and −146 of the HTLV1 LTR (Nyborg *et al.*, 1990). This region contains, in addition to ERE-A, a GC-rich motif localized immediately 5′ of ERE-A which, although deviating from the consensus Sp1 binding site, is likely to be responsible for the binding of purified Sp1 to this region *in vitro* (Nyborg *et al.*, 1990). Secondly, a binding site of NF-$\varkappa$B has been identified, which overlaps the ERE-B Ets1 binding site (Numata *et al.*, 1991). Finally, a factor has been purified from HeLa cell nuclear extracts which activates transcription from the HTLV1 LTR *in vitro* and which promotes the formation of a ternary complex between an as yet uncharacterized binding site in ERR1 and the HTLVI-encoded Tax_1 transactivator (Marriott *et al.*, 1990). The importance of the GC-rich sequence and of the NF-$\varkappa$B binding site in ERR1-mediated transcriptional activation by Ets1 was analysed using two types of reporter plasmids. ERR1 and mutant versions of ERR1 in either the GC element or $\varkappa$B site (see Figure 1 for a description of the mutations) were inserted as dimers upstream of the thymidine kinase promoter of plasmid tkD or as monomers upstream of position −101 of the promoter/enhancer region of reporter plasmid 11-2, a derivative of the HTLV1 LTR (Brady *et al.*, 1987). In addition to sequences required for basal promoter activity, this reporter contains one copy of the LTR 21 bp

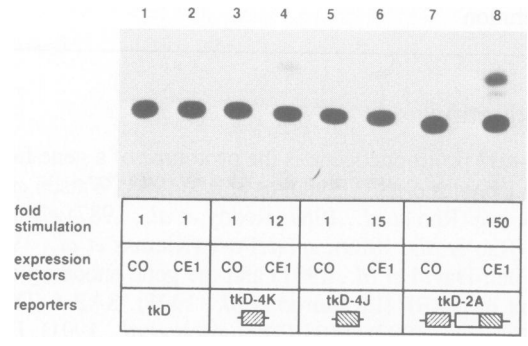

Fig. 2. Transcriptional activation by Ets1 of ERR1-derived reporter plasmids. 0.5 μg of the tkD reporter plasmid (lanes 1 and 2) or derivatives containing either four copies of oligonucleotide K (lanes 3 and 4), or four copies of oligonucleotide J (lanes 5 and 6), or two copies of oligonucleotide A (lanes 7 and 8) were co-transfected in HeLa cells along with 2 μg of either the control plasmid without insert (CO) or the Ets1 expression plasmid (CE1). CAT assays were performed as described in Materials and methods.

repeat elements which are known to bind select members of the CREB/ATF family of transcriptional activators (Yoshimura *et al.*, 1990). The results of Figure 3 show that Ets1 specifically activates CAT expression from either types of reporters containing the wild-type ERR1. Unexpectedly, reporter constructs mutated in the GC-rich element of ERR1 were impaired in their ability to respond to Ets1 (Figure 3A and B, compare lanes 3–4 and 7–8). In contrast, a mutation of the NF-κB site did not affect responsiveness to Ets1 (Figure 3B, compare lanes 3–4 and 9–10). The results of Figure 3C show that mutation in the GC-rich element of ERR1 was similarly found to impair the response to Ets2 and Fli-1, two other members of the Ets family (Boulukos *et al.*, 1988; Watson *et al.*, 1988; Ben-David *et al.*, 1991).

The effects of these mutations on the binding properties of ERR1 were examined by electrophoretic mobility shift assays. In agreement with the results of Nyborg *et al.* (1990), a bacterially expressed Sp1 protein was found to bind to ERR1 (Figure 4B, lane 2). This binding is specific since it is competed by an excess of unlabelled ERR1 (Figure 4A, lanes 1–3) or by an oligonucleotide corresponding to a high-affinity Sp1 binding site, but not by an oligonucleotide of random sequence (Figure 4A, lanes 1 and 8–10). Binding of Sp1 to ERR1 is mediated by the GC-rich motif since mutations introduced into this motif suppressed the ability of ERR1 to compete binding of Sp1 to an ERR1 probe (Figure 4A, lanes 4–7). In contrast, mutations in the Ets1

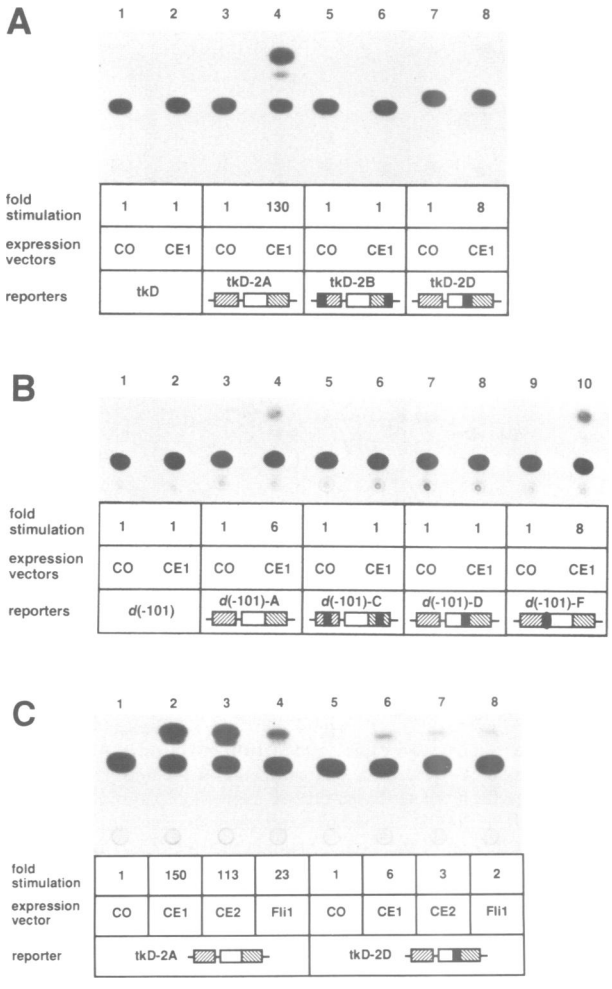

Fig. 3. A GC box element is important for Ets1 transcriptional activation of the ERR1 region. (**A**) 0.5 μg of the tkD reporter plasmid (lanes 1 and 2) or its ERR1-based derivatives as schematized (lanes 3–8) were co-transfected in HeLa cells along with 2 μg of the control expression vector (CO) or the Ets1 expression vector (CE1). (**B**) 0.5 μg of the −101 deletion derivative d(−101) of pU3R CAT or recombinants obtained thereof and containing one copy of oligonucleotides A (lanes 3–4), C (lanes 5–6), D (lanes 7–8) and F (lanes 9–10) were co-transfected in HeLa cells along with 2.5 μg of either the control expression vector (CO) or the Ets1 expression vector (CE1). (**C**) 0.5 μg of the tkD reporter plasmids containing two copies of oligonucleotides A (lanes 1–4) or D (lanes 5–8) were co-transfected in HeLa cells along with 2 μg of the control expression vector (CO) or the expression vectors for Ets1 (CE1) or Ets2 (CE2) or Fli-1 (Fli-1). CAT assays were performed as described in Materials and methods.

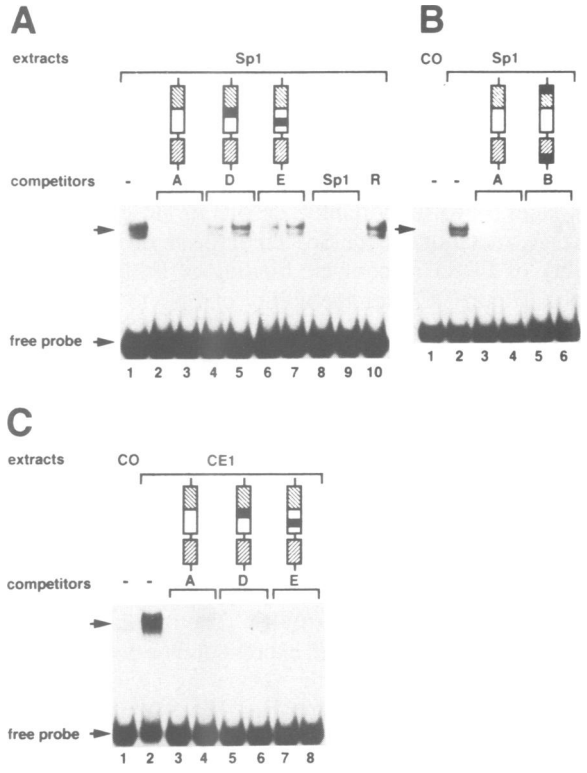

Fig. 4. Binding of Sp1 and Ets1 to ERR1 and mutant versions derived thereof. (**A**) Binding of Sp1 to ERR1 is specific. Electrophoretic mobility shift assays were performed with a ^{32}P-labelled wild-type ERR1 probe (oligonucleotide A) and a bacterially expressed Sp1 protein. Binding was performed either in the absence of competitor oligonucleotide (−) or in presence of either a 25-fold molar excess (lanes 3, 5, 7 and 9) or a 200-fold molar excess (lanes 2, 4, 6, 8 and 10) of competitor oligonucleotides. Competitor oligonucleotides used are oligonucleotide A (lanes 2–3), oligonucleotide D (lanes 4–5), oligonucleotide E (lanes 6–7), an Sp1 consensus binding site oligonucleotide (lanes 8–9) or a competitor oligonucleotide of random sequence (R). (**B**) Mutations in the Ets1 binding sites do not affect Sp1 binding to ERR1. Competition electrophoretic mobility shift assay was performed with oligonucleotide A as probe and either control bacterial extract (CO) or Sp1-containing extract (Sp1). Binding reactions were performed either in the absence of competitor (−) or in the presence of either a 25-fold (lanes 4 and 6) or a 200-fold molar excess (lanes 3 and 5) of competitor oligonucleotides A and B. (**C**) Mutations in the Sp1 binding site do not affect Ets1 binding to ERR1. Competition electrophoretic mobility shift assays were performed with oligonucleotide A as probe and extracts of non-infected SF9 cells (CO) or SF9 cells infected with an AcNPV Ets1 recombinant (CE1). Binding reactions were performed either in the absence of competitor (lanes 1–2) or in the presence of either a 25-fold (lanes 4, 6, and 8) or a 200-fold (lanes 3, 5 and 7) molar excess of competitor oligonucleotides. Competitors used were either the wild-type oligonucleotide A or Sp1 site mutant oligonucleotides D (lanes 5–6) and E (lanes 7–8).

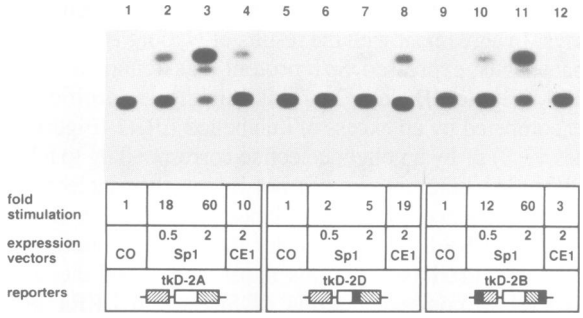

Fig. 5. Sp1 and Ets1 activate transcription from the ERR1 region in *Drosophila* Schneider cells. 1 μg of the tkD reporter plasmids containing two copies of oligonucleotides A (lanes 1–4), D (lanes 5–8) or B (lanes 9–12) were co-tranfected in *Drosophila* SL2 cells along with 2 μg of the actin promoter-based control expression vector (CO) or the indicated amounts of either the Sp1 expression vector (0.5 μg in lanes 2, 6 and 10; 2 μg in lanes 3, 7 and 11) or 2 μg of the Ets1 expression vector (lanes 4, 8 and 12).

binding site do not affect binding of Sp1 to ERR1 (Figure 4B, lanes 5–6). Importantly, despite its close proximity to ERE-A, mutations in the Sp1 binding site do not affect the ability of ERR1 to compete binding of Ets1 to an ERR1 probe (Figure 4C) nor the ability of Ets1 to bind directly to ERR1 (data not shown). We conclude from these experiments that integrity of the Sp1 binding site is important to the specific activation of ERR1-containing reporter plasmid by Ets1.

To establish that the Sp1 binding site of ERR1 effectively mediates Sp1 responsiveness *in vivo*, the tkD-based reporter constructs described above were co-transfected, together with an expression vector for Sp1, into *D.melanogaster* SL2 cells, which are devoid of endogenous Sp1 and have therefore provided a useful system to study Sp1 function (Courey and Tjian, 1988; Courey *et al.*, 1989). The results of Figure 5 show that Sp1 activates CAT expression in a dose-dependent manner from the tkD reporter harbouring a wild-type ERR1 and that stimulation is impaired by a mutation which suppresses binding of Sp1 to ERR1 (Figure 5, compare lanes 2–3 to 6–7). Consistent with their lack of effect on Sp1 binding to ERR1, mutation of the Ets1 binding sites does not affect transcriptional activation by Sp1 (Figure 5, compare lanes 2–3 to 10–11). Ets1 also activates CAT expression of the wild-type ERR1 reporter in SL2 cells (Figure 5, lane 4). Stimulation is impaired by a mutation suppressing binding of Ets1 to ERR1 (Figure 5, lane 12), but not by mutation of the Sp1 binding site (Figure 5, lane 8). Similar results were obtained with an Ets1 deletion mutant limited to the DNA binding and transcriptional activation domains of Ets1 (Ets1Δ5; Gégonne *et al.*, 1992; data not shown), indicating that Ets1 functions equivalently in SL2 cells and HeLa cells.

Since mutations which suppress binding of Sp1 to ERR1 also inhibit the response of ERR1-containing reporters in HeLa cells, and since both Sp1 and Ets1 function as transcriptional activators in *Drosophila* SL2 cells, we next sought for a synergistic action of these activators in SL2 cells. Suboptimal amounts of expression vectors for Ets1 and Sp1 were therefore introduced in these cells, either alone or in combination along with the ERR1-containing tkD reporter plasmid. Representative CAT assays are presented in Figure 6A, as well as the compiled results of several similar experiments in the form of histograms. It appears clearly

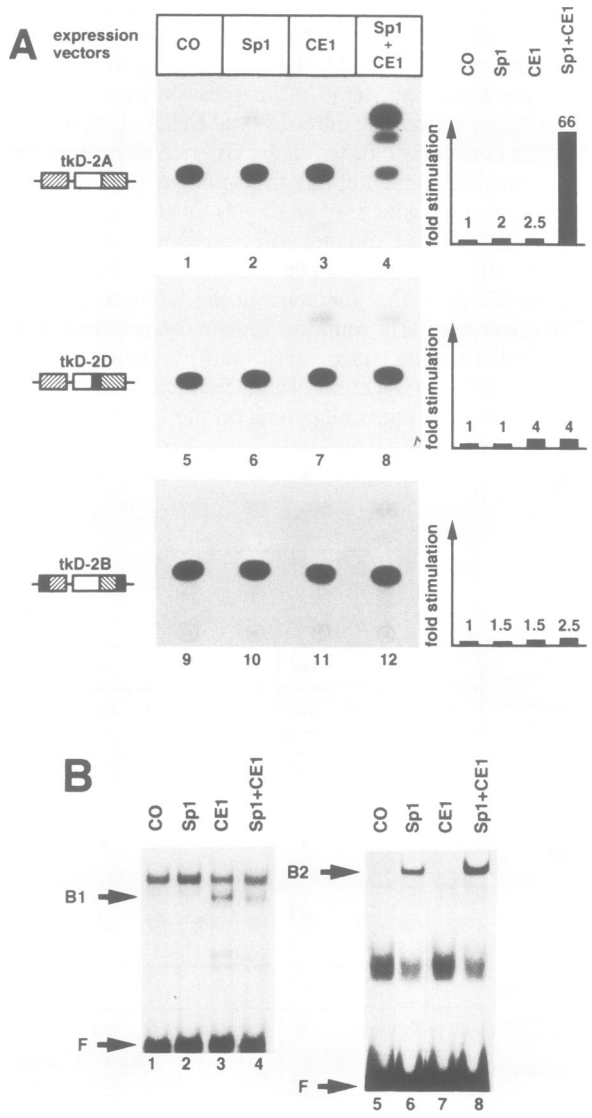

Fig. 6. Synergistic activation of ERR1-derived reporter plasmids by Ets1 and Sp1. (**A**) Representative CAT assay following co-transfection of *D.melanogaster* SL2 cells with 1 μg of the tkD-based reporter plasmids containing two copies of either oligonucleotide A (top), or oligonucleotide D (centre) or oligonucleotide B (bottom) along with either the actin promoter-based control expression plasmid (CO: lanes 1, 5 and 9) or 100 ng of the Sp1 expression plasmid (Sp1: lanes 2, 6 and 10) or 500 ng of the Ets1 expression plasmid (CE1: lanes 3, 7 and 11) or both 100 ng of the Sp1 and 500 ng of Ets1 expression plasmids (lanes 4, 8 and 12). The total amount of expression plasmid was made up to 600 ng using the actin promoter-based control expression plasmid. Diagrams represent the average stimulation values with respect to the control expression vector of a set of three similar experiments. (**B**) Electrophoretic mobility shift assays using extracts of SL2 cells co-transfected with the tkD-2A reporter plasmid and either control (lanes 1 and 5) or Sp1 (lanes 2 and 6) or Ets1 (lanes 3 and 7) or both Sp1 or Ets1 (lanes 4 and 8) expression plasmids as described in panel A. Binding reactions were carried out on the same amount of extract (normalized for total protein concentration) using either a high-affinity Ets1 binding site oligonucleotide probe (lanes 1–4) or an Sp1 binding site oligonucleotide probe (lanes 5–8). The positions of the free probes (F) and of the Ets1 (B1) and Sp1 (B2) DNA complexes are indicated by arrows. The amount of radioactivity in complexes B1 and B2 was quantified and found to be 2200 c.p.m. B1 lane 3, 2210 c.p.m. B1 lane 4, 1750 c.p.m. B2 lane 6, 2800 c.p.m. B2 lane 8.

that amounts of Ets1 and Sp1 expression vectors chosen to give an ~2-fold stimulation when introduced alone in SL2 cells result in a >60-fold increase in CAT activity when

combined (Figure 6A, lanes 1−4 and histogram). To exclude the possibility that the increased CAT activity resulted from the activation *in trans* of the Sp1 expression vector by Ets1 or vice versa, electrophoretic mobility shift assays were performed using the extracts used in Figure 6A and oligonucleotide probes specific for either Ets1 or Sp1. As shown in Figure 6B, plasmid-encoded Ets1 and Sp1 generate specific complexes B1 (Figure 6B, compare lanes 1 and 3) and B2 (Figure 6B, compare lanes 5 and 6), respectively. These complexes were shown to be specific by competition electrophoretic mobility shift assays similar to those of Figure 4 (data not shown). It appears clearly that expression of Sp1 does not affect in a significant way the binding activity of Ets1 to an Ets1-specific probe (Figure 6B, compare lanes 3 and 4) and that expression of Ets1 does not affect binding of Sp1 to an Sp1-specific probe (Figure 6B, compare lanes 6 and 8). We conclude from these experiments that Ets1 and Sp1 synergistically activate CAT activity from ERR1-containing tkD reporter plasmid. Synergy requires binding of both Ets1 and Sp1 to ERR1 since it is suppressed by mutation of either the Sp1 binding site (Figure 6A, lanes 5−8) or the Ets1 binding site (Figure 6A, lanes 9−12).

Since the 44 nucleotide long ERR1 oligonucleotide contains binding sites for other cellular transcription factors, of which only some have been identified so far, the results presented do not necessarily imply a direct interaction between Ets1 and Sp1. To investigate this point further, we analysed the properties of tkD-based reporter plasmids containing either two copies of a deletion variant of ERR1 restricted to its Sp1 and ERE-A Ets1 binding sites or of mutant versions in either the Ets1 or Sp1 binding sites (oligonucleotides G, H and I, see Figure 1 for sequence). In *Drosophila* SL2 cells, transfection of 2 μg of either Ets1 or Sp1 expression vectors alone was found to activate CAT expression of the wild-type tkD-2G reporter. As expected, mutation of either the Ets1 or the Sp1 sites was found to inhibit activation by Ets1 and Sp1, respectively (Figure 7A, panel a). Suboptimal quantities of either expression vectors activate CAT expression only marginally, whereas their combination results in a 40-fold induction (Figure 7A, panel b). As described for ERR1, mutation of either the Sp1 or Ets1 binding sites in this reporter abolished synergy (Figure 7A, panel b). In line with these results, Ets1 was found to activate CAT expression from the tkD-2G reporter in HeLa cells and activation was impaired following mutation of either the Ets1 or Sp1 binding sites (Figure 7B).

Since synergy between Ets1 and Sp1 is strictly dependent on the integrity of their respective binding sites, we asked whether this property reflected a cooperation between Ets1 and Sp1 in their ability to bind the SP1/ERE-A element. Electrophoretic mobility shift assays show that, as expected, both Ets1 and Sp1 bind to oligonucleotide G to form complexes C1 and C2, respectively (Figure 8B, lanes 1 and 2). These complexes are specific by the same criteria as those used for binding of these proteins to the ERR1 probe (Figure 4; data not shown). When the amounts of Ets1 and Sp1 used in this experiment are mixed together, a new complex of lower electrophoretic mobility is formed (complex C3; Figure 8B, lane 3) at the expense of complexes C1 and C2. Complex C3 is specific since its formation is suppressed in the presence of an excess of non-labelled oligonucleotide G used as competitor, but not by an excess of an unrelated oligonucleotide of similar length (Figure 8B, compare lanes

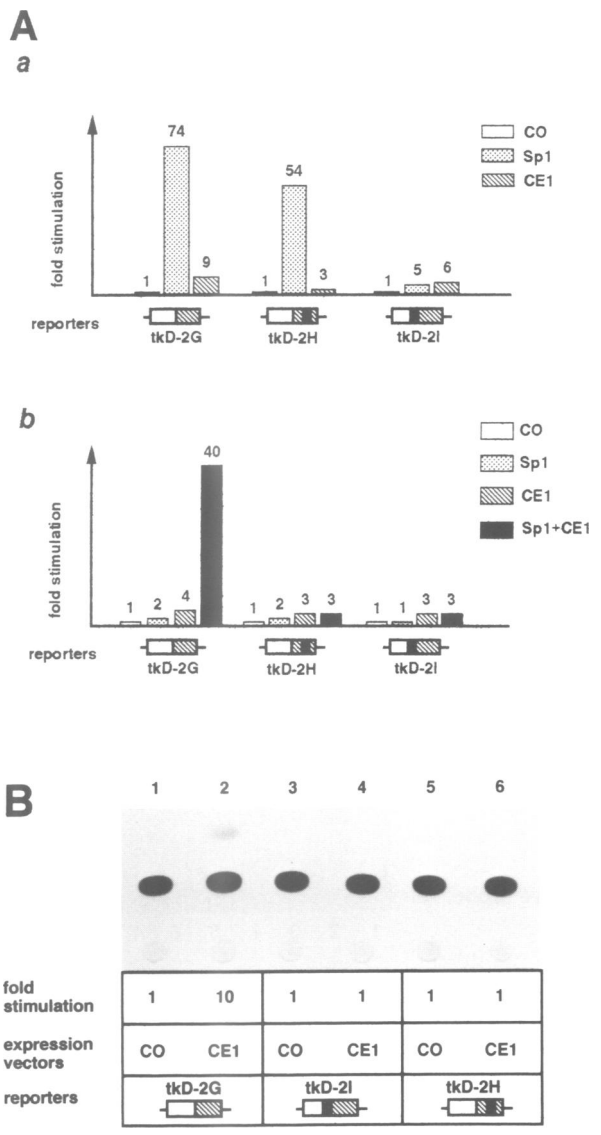

Fig. 7. Synergy between Ets1 and Sp1 is maintained in oligonucleotide G-derived reporter plasmids. (**A**) Panel a: The diagram represents the average fold stimulation obtained from CAT assays following co-transfection in SL2 cells of the tkD-2G reporter plasmid or mutants derived thereof (1 μg) with 2 μg of either the actin promoter-based control expression vector or expression vectors for either Sp1 or Ets1. Panel b: Diagrams of the average fold stimulation obtained from CAT assays following co-transfection in SL2 cells of the tkD-2G reporter plasmid or mutants derived thereof (1 μg) with either the actin promoter-based control expression vectors (CO), or expression vectors for either Sp1 (100 ng) or Ets1 (500 ng), or a mixture of the Sp1 and Ets1 expression vectors. The total amount of expression plasmid was kept constant (600 ng) using actin promoter-based control expression plasmid. (**B**) Representative CAT assay following co-transfection in HeLa cells of 0.5 μg of the tkD-2G reporter plasmid (lanes 1−2) or the indicated mutants derived thereof (lanes 3−6) with 2 μg of either the control plasmid without insert (CO) or the Ets1 expression plasmid (CE1). CAT assays were performed as described in Materials and methods.

3, 7 and 8). Complex C3 results from the formation of a ternary complex between Ets1, Sp1 and the probe since competition by an oligonucleotide corresponding to a high-affinity Ets1 binding site resulted in the conversion of complex C3 into complex C2, whereas competition by an excess of an oligonucleotide corresponding to a high-affinity Sp1 binding site resulted in the conversion of complex C3

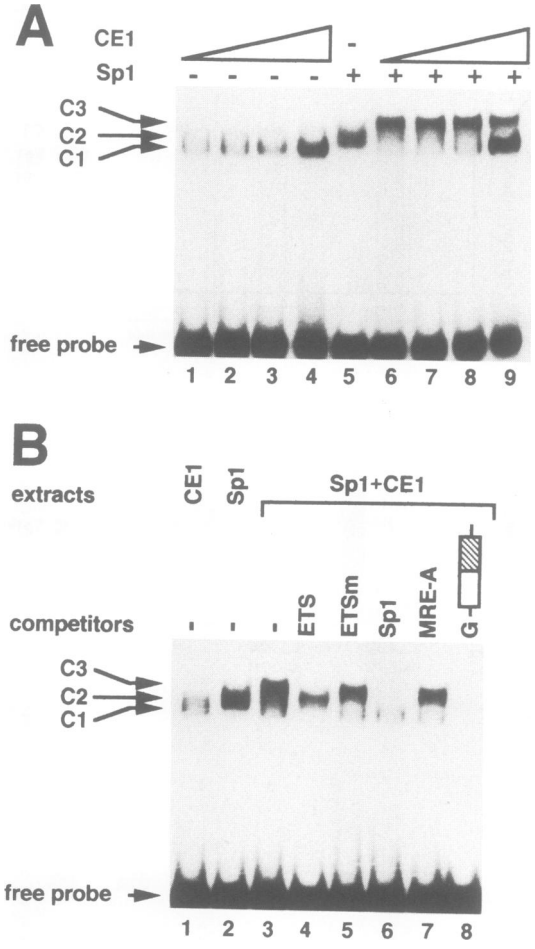

Fig. 8. Electrophoretic mobility shift analysis of the binding of Ets1 and Sp1 to oligonucleotide G. (**A**) The ^{32}P-labelled oligonucleotide probe G was incubated first with either the bacterial control (−) or the Sp1 (+) extracts. Increasing amounts of Ets1 (lanes 1−4 and 6−9) or SF9 control extracts (lane 5) were next added. Complexes were separated by non-denaturing polyacrylamide gel electrophoresis. The Ets1 complex is labelled C1, the Sp1 complex is labelled C2. The newly formed complex in the presence of both Ets1 and Sp1 is labelled C3. The amount of Sp1 used in all indicated reactions is ~20 ng. The amounts of Ets1 used are ~1 ng (lanes 1 and 6); 1.2 ng (lanes 2−7); 2 ng (lanes 3−8); 5 ng (lanes 4−9). (**B**) Electrophoretic mobility shift assay was performed with ^{32}P-labelled oligonucleotide G as probe and either Ets1 (CE1) to generate complex C1, or Sp1 (Sp1) to generate complex C2, or a mixture of both Ets1 and Sp1 (Sp1 + CE1) to generate complex C3 (lanes 1−3). Competitions were carried out using 100-fold molar excess of an unlabelled high-affinity Ets1 binding site oligonucleotide (lane 4), or a mutant version derived from it (lane 5), or an Sp1 consensus binding site oligonucleotide (lane 6), or a Myb binding site oligonucleotide (lane 7) or oligonucleotide G (lane 8).

into complex C1 (Figure 8B, lanes 4−6). As expected from these results, mutation of either the Ets1 or the Sp1 binding sites of oligonucleotide G abolishes ternary complex formation in direct binding assays (data not shown). We next analysed the effect of titrating the Ets1 protein either in the absence or in the presence of a constant amount of Sp1 using oligonuleotide G as a probe. If formation of complex C3 is cooperative, Ets1 would be expected to bind preferentially to a preformed Sp1-specific complex C2 rather than to the protein-free probe, whereas non-cooperative complex formation would result in a non-discriminative linear recruitment both from complex C2 to form a ternary complex and from the free probe to form complex C1. The results

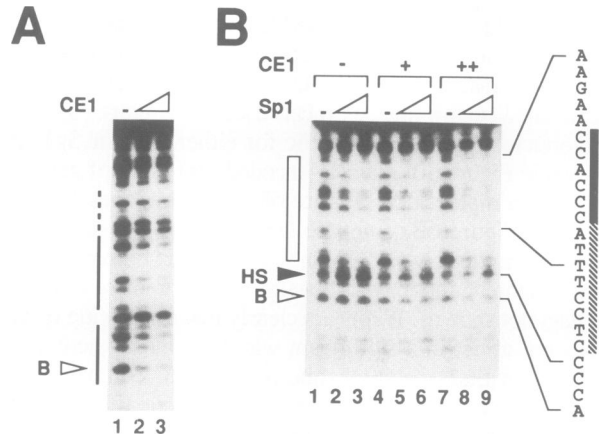

Fig. 9. DNase I footprinting analysis of Ets1 and Sp1 binding to oligonucleotide G. (**A**) DNase I footprinting analysis of Ets1 binding to oligonucleotide G (upper strand). Binding reactions were performed with either SF9 control extract (lane 1) or increasing amounts of Ets1-containing extract (lane 2: 10 ng Ets1; lane 3: 20 ng Ets1). All reactions were made up to contain the same amount of insect cell proteins. The complete or partial Ets1-protected DNA sequences are shown as solid and dashed bars, respectively. B points to an Ets1 specific protected band. (**B**) DNase I footprinting analysis of Ets1 and Sp1 binding to oligonucleotide G. Binding reactions were performed with increasing amounts of purified human Sp1 alone (0, 1 and 2 μl; lanes 1−3) or the same concentrations of Sp1 in the presence of either 2 ng of Ets1 (lanes 4−6) or 5 ng of Ets1 (lanes 7−9). The open box indicates the Sp1-protected DNA sequences and HS points to the Sp1-specific hypersensitive site. The upper strand sequence on the right (5′ on top) is derived from a parallel G + A sequence ladder of the probe.

of Figure 8A show that the addition of increasing amounts of Ets1 to the preformed complex C2 results in the immediate appearance of complex C3 at the expense of complex C2 (Figure 8A, lanes 5−9). Binding of Ets1 to the free probe is only observed at higher Ets1 concentration after complete recruitment of complex C2 into C3. In the absence of Sp1, the amounts of Ets1 protein used in the titration experiments were found to bind the probe to exclusively form complex C1 (Figure 8a, lanes 1−4). In parallel experiments in which the amount of Sp1 is titrated in the presence of a constant amount of Ets1, Sp1 was found to bind preferentially to the preformed Ets1−probe complex as compared to the free probe (data not shown). Further evidence for the preferential binding of Ets1 to a preformed Sp1−probe complex was obtained by DNase I footprinting analyses. Binding of increasing amounts of Sp1 modifies the digestion pattern of an oligonucleotide G probe by inducing the dose-dependent appearance of both a DNase I hypersensitive site and a protected region centred over the GC-rich motif (Figure 9B, lanes 1−3). Consistent with our previous results (Gitlin *et al.*, 1991), binding of high concentrations of Ets1 generates a large DNase I protected area which overlaps the region protected by Sp1, but extends further to the 3′ side of the probe (Figure 9A). Low concentrations of Ets1 do not (Figure 9B, lane 4) or minimally (Figure 9B, lane 7) alter the DNase I digestion pattern of the probe. Of note, at the lowest Ets1 concentration used in this experiment, no effect is observed on the Sp1-specific DNase I hypersensitive site (Figure 9B, compare lanes 1 and 4). In contrast, addition of low concentrations of Ets1 to Sp1 modifies Sp1 binding as evidenced by the disappearance of the Sp1-specific DNase I hypersensitive site (Figure 9B, compare lanes 1−3 to

4−9). Since in our experimental conditions the DNA probe is in excess as compared to Ets1 and Sp1, these results show that Ets1 binds preferentially to preformed Sp1−probe complexes rather than to the free probe. We conclude from these experiments that Ets1 and Sp1 cooperate in ternary complex formation with the SP1/ERE-A element.

Discussion

The DNA-binding specificity of Ets1 is of rather relaxed specificity *in vitro* (Fisher *et al.*, 1992; Woods *et al.*, 1992), indicating that determinants other than their nucleotide sequence are likely to decide whether Ets1 binding sites will act as effective Ets1-responsive elements. Consistent with this notion, transcriptional activation by Ets1 and other members of the Ets family does not appear to depend in a simple way on the binding of the respective proteins to their specific DNA recognition sequence. For example, multimers of oligonucleotides specifically bound by different members of the Ets family, when inserted upstream of minimal unresponsive promoters, generally convey a low level of activation by the respective proteins (this study; Klemsz *et al.*, 1990; Wasylyk *et al.*, 1990; Reddy and Rao, 1991. Lim *et al.*, 1992; Rao and Reddy, 1992; Thompson *et al.*, 1992). Furthermore, in some instances, high-affinity binding sites for Ets1 have turned out to be either unaffected by Ets1 (MSV LTR, B.Graves, personal communication) or to mediate Ets1 repression both in their natural context (TCRβ enhancer: Prosser *et al.*, 1992) and in the context of heterologous promoters (Prosser *et al.*, 1992; our unpublished results). In contrast to these obervations, the Ets1-responsive region of the HTLV1 LTR (ERR1: Bosselut *et al.*, 1990; Gitlin *et al.*, 1991), when multimerized 5′ of either a −109 (unpublished data) or a −50 HSV thymidine kinase promoter (this study), is efficiently activated by Ets1. We show here that Ets1 activation through ERR1 depends not only on the integrity of the ERE-A and ERE-B Ets1 binding sites, but also of an Sp1 binding site localized immediately 5′ of ERE-A. Reconstitution experiments in *Drosophila* SL2 cells show that this requirement actually reflects a synergistic interaction between Ets1 and Sp1 to activate transcription from either the ERR1 region or a DNA response element restricted to the adjacent Sp1 and ERE-A binding sites (SP1/ERE-A). In these contexts, the presence of an ajdacent Sp1 binding site and binding of Sp1 to this site are essential determinants in the ability of ERE-A to function as an efficient Ets1-responsive element.

Synergy between Ets1 and Sp1 is likely to require multiple protein−protein and protein−DNA interactions. The carboxyterminal DNA-binding (Ets domain) and the transcriptional activation domain of Ets1 appear sufficient for synergy with Sp1 since an Ets1 protein limited to its 110 carboxyterminal amino acid residues fused to the Ets1 activation domain (EtsΔ5 in Gégonne *et al.*, 1992) also synergizes with SP1 in *Drosophila* SL2 cells (our unpublished data) and is an efficient transcriptional activator of ERR1-based reporters in HeLa cells (Gégonne *et al.*, 1992). Furthermore, synergy does not appear to be restricted to Ets1 as efficient transcriptional activation of ERR1-based reporters in HeLa cells by Ets2, or the more distantly related Fli1 protein, similarly depends on the integrity of the Sp1 binding site. This, together with the fact that synergy between Ets1 and Sp1 strictly depends on the integrity of their respective binding sites and that Ets1 and Sp1 cooperate *in vitro* to form a ternary complex with the SP1/ERE-A element, supports a model in which synergy depends on similar interactions between these factors *in vivo*. Additional studies will be required to determine how events downstream from complex formation contribute to synergy between Ets1 and Sp1. Transcriptional activation by Sp1 through a single GC element is dependent on two glutamine-rich transcriptional activation domains (Courey and Tjian, 1988; Pascal and Tjian, 1991) which have been shown to act through co-activators tightly associated with the TATA box binding protein (Pugh and Tjian, 1990; Tanese *et al.*, 1991). Whether the synergistic action of Sp1 and Ets1 involves co-activators specific for each of these factors, or occurs through a shared co-activator, remains to be analysed.

Association between Ets1 and Sp1 does not appear to exist in the absence of the SP1/ERE-A element since complexes with the same electrophoretic mobility are observed on binding of either Sp1 or Ets1 to oligonucleotides corresponding to their cognate binding sites, irrespective of the presence or absence of the other protein partner (Figure 6B and data not shown). In this respect, cooperation between Ets1 and Sp1 appears to be distinct from the recently described homotypic synergy between Sp1 binding sites (Anderson and Freytag, 1991; Pascal and Tjian, 1991) or the synergistic action of Sp1 and the BPV enhancer E2 protein (Li *et al.*, 1991) which are proposed to depend on stable protein−protein interactions. The dependence of Ets1 on Sp1 for efficient binding to the SP1/ERE-A element is reminiscent of recently described examples in which other members of the Ets family have been shown to efficiently bind their target DNA sequence *in vitro* only in the presence of a second, adjacently bound, factor. For example, *in vitro* binding of GABPα to its dimerized target site in the enhancer of the HSV ICP4 and ICP27 immediate early genes is facilitated by its association with an unrelated β subunit to form a tetrameric bound factor in which both types of subunits ultimately contribute to DNA binding (Thompson *et al.*, 1991). Similarly, efficient binding of Elk1 (Hipskind *et al.*, 1991) and SAP1 (Dalton and Treisman, 1992) to the *c-fos* SRE is dependent on binding of SRF to an adjacent site, an association which appears instrumental in the response of *c-fos* to at least some extracellular stimuli (Graham and Gilman, 1991). Also, binding of Pu-1 to a purine box in the immunoglobulin κ 3′ enhancer has recently been shown to allow the recruitment of a second factor to an adjacent site to form a complex essential to enhancer activity (Pongubala *et al.*, 1992). Although the mechanisms involved in these different systems are different and remain to be characterized in their details, these results suggest that Ets family members are often—if not always—found as subunits of larger transcription complexes which are involved in the fine regulation of viral and cellular promoter/enhancer sequences.

The association between Ets and Sp1 binding sites in a configuration similar to that of the SP1/ERE-A element of the HTLV1 LTR is found in the promoter/enhancer regions of several cellular genes (Figure 10), suggesting that the synergistic activation between Ets1 and Sp1 described here is not restricted to the ERR1 region of the HTLV1 LTR. This list is likely to expand into a larger repertoire as more is learned about the influence of the respective orientation and spacing of the Ets and Sp1 binding sites on their

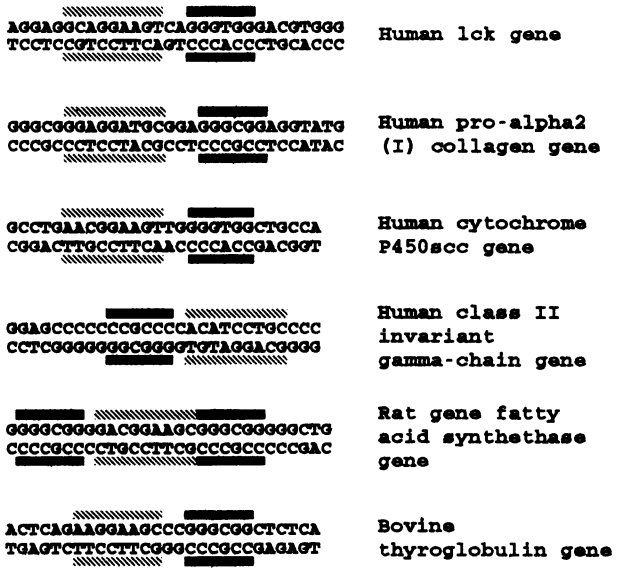

Fig. 10. Occurrence of SP1/ETS binding sites in cellular promoter/enhancer sequences. The list only includes cellular promoter/enhancers displaying Sp1 and Ets1 binding sites with sequences and relative orientations similar to that of the SP1/ERE-A element of the HTLV1 LTR. These include the −38 to −8 region of the type II promoter of the human protein tyrosine kinase *lck* gene (Takadera *et al.*, 1989); the −296 to −266 region of the human proα2(I) collagen gene (Dickson *et al.*, 1985); the −1878 to −1851 region of the human P450scc promoter/enhancer (Moore *et al.*, 1990); the −170 to −143 region of the human MHC class II invariant γ chain gene promoter (Kudo *et al.*, 1985); the −242 to −214 promoter region of the rat fatty acid synthase gene (Amy *et al.*, 1990); the −1740 to −1766 region of the bovine thyroglobulin gene promoter (Ledent *et al.*, 1990). Numbering is with respect to the (major) start site of transcription as +1. The Ets sites are lined as hatched bars and the Sp1 sites as solid bars.

synergistic transcriptional activity. As for the SP1/ERE-A element of the HTLV1 LTR, the activity of these elements could be exquisitely sensitive to variations in the intracellular concentrations of either Sp1 and/or a particular member of the Ets family. Although the picture is far from being complete, expression of Ets family members is developmentally regulated (Bhat *et al.*, 1989; Vanderbunder *et al.*, 1989; Boulukos *et al.*, 1990) and, although Sp1 is usually referred to as a ubiquitous factor, its expression also varies widely between different tissues (Saffer *et al.*, 1991). Furthermore, the activity of both Ets family members and Sp1 is regulated by post-transcriptional modification events (Pognonec *et al.*, 1988, 1989, 1990; Fujiwara *et al.*, 1988, 1990; Jackson *et al.*, 1990). This suggest that Ets family members, which individually are not restricted to a particular cell type, could nevertheless participate in the tissue-restricted expression of specific genes when acting in synergy with non-tissue-restricted factors such as Sp1. In that respect, recent experimental evidence suggests that Ets factors do contribute to high-level expression of promoters such as the type I *lck* promoter in T cells (Leung *et al.*, 1993) or the glycoprotein IIB promoter in the megakaryocytic lineage (Lemarchandel *et al.*, 1993). On the other hand, tissue-specific expression of the cardiac α actin gene in muscle cells requires Sp1 in addition to MyoD1 and an SRF-like factor (Sartorelli *et al.*, 1990). Mutational analyses and reconstitution experiments similar to those described here are under way to clarify the role of the SP1/ETS elements in the activity of cellular promoters.

Materials and methods

Plasmid constructions

The tkD series of plasmids, containing the different versions of the −117/−160 region of the HTLV1 LTR, were derived from pTK-50 (Ham *et al.*, 1991; a generous gift of Dr M.Yaniv, Institut Pasteur, Paris). pTK-50 contains the −50 to +55 region of the HSV thymidine kinase promoter region, linked to the CAT gene. A polylinker containing *Hin*dIII, *Sac*I, *Kpn*I and *Xba*I sites was inserted between the *Hin*dIII and *Xba*I sites of pTK-50 to obtain the ptkD plasmid used in this study. The oligonucleotides A, B, D, J and K bordered with *Ava*I restriction site were phosphorylated with T4 polynucleotide kinase and ligated *in vitro*. Dimerized direct-repeated versions of oligonucleotides A, B and D, and tetramerized direct-repeated versions of oligonucleotides J and K, were purified by non-denaturing acrylamide gel electrophoresis and inserted at the *Ava*I site of the PVo plasmid (Fromental *et al.*, 1988; a generous gift of Dr C.Egly, LGME, Strasbourg). The recombinant plasmids were digested with *Sac*I and *Xba*I, and fragments were ligated to the *Sac*I/*Xba*I-restricted ptkD. Dimers of oligonucleotides G, H and I were synthesized directly with bordering *Sac*I and *Xba*I sites, and inserted in *Sac*I/XbaI-restricted ptkD to generate ptkD-2G, ptkD-2H, ptkD-2I. The d(−101) series of recombinant plasmids were derived from a −101 5′ deletion mutant of pU3R CAT (dl 11-2; Brady *et al.*, 1987). Oligonucleotides A, C, D and F bordered with *Xba*I site were ligated to the unique *Xba*I site of dl 11-2, and recombinants with oligonucleotides inserted in the correct orientation were selected. Orientation and proper sequence at the recombinant constructs were verified by DNA sequence analysis using the dideoxy chain termination method.

The SV40 promoter-based ΔEB expression vector for Ets1 (chicken) has been described previously (Boulukos *et al.*, 1989). The expression vectors for Fli-1 and Ets2 were constructed by insertion of the mouse Fli-1 cDNA (Ben-David *et al.*, 1991; a generous gift from Dr A.Bernstein) and the human Ets2 cDNA (A.Gégonne, unpublished) at the *Eco*RI site of ΔEB. The *D.melanoganster* actin 5C promoter-based Ets1 expression plasmid was constructed as follows. pPacNdeI was constructed by inserting a *Nde*I−*Xho*I−*Bam*HI polylinker just downstream of the initiation codon of the pPacU plasmid (Biggin *et al.*, 1988). The chicken *c-ets1* cDNA (P54) was then cloned into pPacNdeI in phase with the ATG initiator codon provided by the *Nde*I site of the polylinker in two steps: a *Bam*HI ended *c-ets1* cDNA was first inserted in the *Bam*HI site of pPacNdeI to obtain a plasmid referred to as pPacE; a 5′ *Nde*I−*Hpa*I *c-ets1* subfragment was then obtained by polymerase chain reaction (PCR) amplification of the *c-ets1* cDNA (Boulukos *et al.*, 1988) between positions −16 and +123. The 5′ oligonucleotide included an *Nde*I restriction enzyme site overlapping the ATG codon and the sequence of the 3′ oligonucleotide spanned the *Hpa*I site localized at position +108 in the *c-ets1* cDNA. PCR amplification was performed according to the specifications of the manufacturer (Perkin-Elmer-Cetus). The amplified fragment was digested with *Nde*I and *Hpa*I, and then inserted in *Nde*I−*Hpa*I digested pPacE to give pPac Ets1. The integrity of the *c-ets1* open reading frame was checked by DNA sequence analysis using the dideoxy chain termination method. The pPac Sp1 (Courey and Tjian, 1988) was a generous gift of Dr R.Tjian (University of California, Berkeley).

Transfection and CAT assays

HeLa cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum. For transfection experiments, HeLa cells were seeded at 5×10^5 cells/60 mm Petri dish and co-transfected 24 h later with 2 or 2.5 μg of expression plasmid as indicated and 0.5 μg of reporter plasmids using the calcium phosphate co-precipitation method (Gégonne *et al.*, 1992). *Drosophila melanogaster* SL2 cells were maintained in Schneider medium supplemented with 10% fetal calf serum and 2 mM glutamine. One day before transfection, cells were seeded at 10^7 cells/100 mm Petri dish and co-tranfected with the indicated amount of expression plasmids and 1 μg of reporter plasmid using the calcium phosphate co-preciptiation method. The total amount of DNA was kept constant (20 μg) by the addition of non-recombinant expression plasmid and carrier plasmid DNA. After the addition of DNA, the plates were left undisturbed until the time of harvest 48 h later. Cells were harvested by pipetting, washed once with PBS and resuspended in 150 μl of 0.25 M Tris−HCl (pH 7.8). Cells were lysed by four cycles of freezing and thawing, and the lysates were cleared by centrifugation at 10 000 *g* for 10 min. Conditions for CAT assays were as described previously (Bosselut *et al.*, 1990). 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 control plasmid.

Electrophoretic mobility shift assay

Analysis of Ets1 binding made use of whole cell extracts of *Spodoptera frugiperda* SF9 cells infected with a recombinant AcNPV Ets1 baculovirus (Bosselut *et al.*, 1990). Extracts from non-infected cells were used as controls. Analysis of Sp1 binding made use of extracts of *Escherichia coli* cells transformed by a pET8C recombinant plasmid expressing Sp1-516C (Kadonaga *et al.*, 1987; P.Chavrier and P.Charnay, unpublished) or a pET8C plasmid without insert as control. These extracts were kindly provided by Dr P.Charnay (Ecole Normale Supérieure, Paris). Oligonucleotide probes were labelled by filling in with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dCTP. DNA binding reactions (Figures 4 and 8) were carried out for 10 min at 0°C in a final volume of 16 μl containing 200 fmol of labelled 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 dithiothreitol (DTT); 5 mM $MgCl_2$; 1.5 μg poly d(I-C); 0.4 μg salmon sperm DNA. Competitor oligonucleotides were added as indicated in the Figure legends. Binding reactions were started by the addition of the cellular extracts and incubated for 10 min on ice. Electrophoresis was as described previously (Bosselut *et al.*, 1990).

For electrophoretic mobility shift assays using extracts of SL2 transfected cells (Figure 6B), cells were collected 2 days after transfection, washed in phosphate-buffered saline (PBS) and two-thirds were lysed in 3 vol of 10 mM Hepes (pH 7.9), 0.3 M NaCl, 1 mM DTT, 0.1 mM EDTA, 0.5% Triton X-100, 1% aprotinin, 100 μg/ml phenylmethylsulphonylfluoride, 10 μg/ml leupeptin and centrifuged at 10 000 *g* for 20 min. Binding reactions were performed on the supernatants of these centrifugations in a final volume of 16 μl for 10 min at 0°C in the presence of non-specific competitors (1.5 μg poly [d(I-C)], 0.4 μg salmon sperm DNA), followed by the addition of 200 fmol of the ^{32}P-labelled oligonucleotide probe for 10 min at 25°C. The remaining one-third of the extract was processed for CAT assays as described above.

The Ets1 oligonucleotide (ETS) corresponds to an optimized high-affinity Ets1 binding site ATAAACAGGAAGTGGT flanked by *Ava*I restriction sites. The mutant Ets1 binding site (ETSm) has the sequence ATAAACACCAAGTGGT flanked by the same restriction site. The Sp1 consensus binding site (SP1) has the sequence GGGCGGGGCGGGTTAGAC. Non-specific competitors included a random oligonucleotide (R) with the sequence CATCATGCAGCGCACCGACGCACATCATACTCTTCAGCCTAGTC and the Myb binding site oligonucleotide TCGACACATTATAACGGTTTTTAGC (MRE-A; Ness *et al.*, 1989).

DNase I footprint analysis

Purified human Sp1 (Promega) and baculovirus-produced Ets1 were used in these experiments. The ptkD-2G plasmid was digested with *Xba*I and 3′ labelled by filling in with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dCTP and digested with *Hin*dIII. The labelled fragment was isolated by polyacrylamide gel electrophoresis. DNase footprint reactions were based on previously described procedures (Gitlin *et al.*, 1991; Booselut *et al.*, 1992). Binding reactions were carried out for 30 min at 0°C in a final volume of 20 μl containing 100 fmol of labelled probe in a final buffer concentration of 10 mM Tris-HCl (pH 7.5), 40 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 μg poly (d(I-C). After incubation, the concentrations of $MgCl_2$ and $CaCl_2$ were adjusted to 5 mM and 2.5 mM, respectively, and 1 μl of DNase I (10 U, Stratagene) was added and incubation continued for 1 min at room temperature. The digestion was stopped by 100 μl of 20 mM EDTA, 1% sodium dodecyl sulphate (SDS), 0.2 M NaCl, 250 μg/ml tRNA, extracted once with phenol-chloroform (1:1) and ethanol precipitated before loading onto a 12% sequencing polyacrylamide gel for electrophoresis.

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