Nucleic Acids Research, 1994, Vol. 22, No. 19 3871–3879

A residue of the ETS domain mutated in the *v-ets* oncogene is essential for the DNA-binding and transactivating properties of the ETS-1 and ETS-2 proteins

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Received July 7, 1994; Accepted August 14, 1994

ABSTRACT

The *c-ets-1* locus encodes two transcription factors, p54$^{c\text{-}ets\text{-}1}$ and p68$^{c\text{-}ets\text{-}1}$ that recognize purine-rich motifs. The *v-ets* oncogene of the avian retrovirus E26 differs from its cellular progenitor p68$^{c\text{-}ets\text{-}1}$ by two amino acid substitutions (alanine 285 and isoleucine 445 in *c-ets-1* both substituted by valine in *v-ets*, mutations A and B respectively) and its carboxy-terminal end (mutation C). The B mutation affects a well conserved residue in the carboxy-terminal 85 amino acids, ETS DNA-binding domain. To address the biological relevance of the B mutation found between *v-ets* and *c-ets-1*, we have randomly mutagenized isoleucine 445 of p68$^{c\text{-}ets\text{-}1}$ by polymerase chain reaction. Using *in vitro* gel mobility shift assays, we show that this residue is crucial for the binding properties of *c-ets-1* since the 12 mutations we have generated at this position, all diminish or even abolish the binding, to the 'optimized' Ets-1 binding site (EBS), of 35 kDa proteins corresponding to the 311 carboxy-terminal residues of *c-ets-1*. Among them, substitutions of isoleucine to glutamic acid, glycine or proline have the highest inhibitory effects. Similar results were obtained when the same mutations were introduced either in full-length p68$^{c\text{-}ets\text{-}1}$ protein or into a carboxy-terminal polypeptide of 109 amino acids encompassing the ETS-domain which has previously been shown to display a very high binding activity as compared with the full-length protein. Consistent with the *in vitro* results, point mutations in p68$^{c\text{-}ets\text{-}1}$ that decrease binding activity to EBS abrogate its ability to transactivate reporter plasmids carrying either the TPA Oncogene Response Unit of the Polyoma virus enhancer (TORU) or a sequence derived from the HTLV-1 LTR. Furthermore, as this isoleucine residue is rather well-conserved within the ETS gene family, we show that mutation of the corresponding isoleucine of *c-ets-2* into glycine also abrogates its DNA-binding and hence, transactivating properties. Thus, the *v-ets* B mutation highlights the isoleucine 445 as an essential amino acid of the *c-ets-1* and *c-ets-2* DNA-binding domains.

INTRODUCTION

The first characterized member of the ETS gene family was the *v-ets* oncogene (1,2), one of the two cell-derived sequences, along with a truncated c-*myb* gene, transduced in the avian acute leukemia virus E26, which is able to transform erythroid and myeloid cells *in vivo* and *in vitro* (3–5). In addition to the cellular chicken locus *c-ets-1* from which *v-ets* arose, a number of ETS genes have been identified in various species ranging from lower metazoan species to human (6,7 and references therein). Ets proteins are DNA-binding proteins and most of them are proved to be transcription factors acting either alone or in synergy with various cofactors to regulate numerous viral and cellular promoters (8,9 and references therein). To achieve this function, ETS family members interact in a sequence-specific manner with *Ets*-binding sites (referred to as EBS; 10,11). In most cases, the EBS are centered over a GGAA/T core sequence (10–12) but accept different flanking sequences, notably for Elf1 (13) or E74 (14). Strikingly, PU-1 which is most distantly related to the founder *c-ets-1* gene, was shown to bind to and transactivate via a GGAA core but also in the immmunoglobulin J chain promoter through a motif centered over a AGAA core (15).

All Ets proteins share sequence homology in a domain of about 85 amino acids, called the ETS domain. This ETS domain is necessary and sufficient for the binding to EBS in gel retardation assays (12). Even though the DNA-binding domains studied so far exhibit a great deal of structural diversity, most of them use an α-helix to make sequence specific major-groove contacts (16). However, the ETS gene family represents a novel class of DNA-binding proteins since they produce a unique pattern of DNA contacts and display no structural homology with already described DNA-binding motifs such as the zinc finger, the basic leucine zipper, the helix–turn–helix (HTH), the helix–loop–helix or the recently described β-strand–turn–β-strand

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motif (17). Clearly, such a fundamental issue will be solved by NMR analyses and/or crystal structure of an ETS domain bound to DNA. However, although far from the resolution of these powerful techniques, several approaches have been fruitful for our knowledge of the ETS domain. They fall into two distinct but complementary classes, namely, computer-assisted sequence alignments and structure predictions or site-specific mutagenesis.

Sequence alignments of the various ETS domains whose identities to *ets-1* range from 98 to 37% highlight several invariant amino acids scattered all along the domain as well as clusters of high conservation. These include a highly conserved 10 amino-acids region rich in leucine residues and predicted to adopt an α-helix structure in the N-terminal and a basic region located in the C-terminal half of the domain (Fig. 1). The first identified similarity between the ETS domains was a repetition of tryptophan residues spaced by 17–18 amino acids and it was highly reminiscent of the one observed in the DNA-binding domain of Myb proteins (18). Recently, more sophisticated computer analyses with several ETS family members predicted two or three α-helices in the basic domain (19,20) in addition to the α-helix already described in the N-terminal domain (13). Together with methylation interference studies indicating that interaction between the ETS domain and DNA occurs in the major groove of the DNA helix (12), these analyses suggest that the ETS domain could adopt an HTH-like conformation (19–21). In another recent study, the HCA (Hydrophobic Cluster Analysis) method combined with known three-dimensional NMR data suggest that the ETS domain may be related to the Myb DNA-binding domain and possibly to the HMG (High Mobility Group) one (22). In that case, the ETS domain is likely to contain two HTH motifs.

Site-specific mutagenesis experiments have stressed the crucial role played by the HTH motif and specially by the 3′ helix which is referred to as the CRIII motif (19,21). Mutations throughout the ETS domain have a detrimental effect on binding activity but half of the mutations that reduce it below 10% of the original activity are clustered within the HTH motif. Furthermore, four residues namely lysine 379, lysine 381, arginine 391 and arginine 394 in the human ETS-1 protein absolutely required for the DNA-binding activity have been identified within this motif (19,21). Similarly, a single amino acid substitution in the ETS domain, lysine 388, alters core DNA-binding specificity of Ets-1 to that of the related transcription factors E74 and Elf1 (19). Taken together these data indicate that the HTH motif, and specially the CRIII region which contains lysine 388 and arginine 391 and 394, plays a key role in ETS domain recognition of the GGAA/T core motif.

Our approach has been slightly different. For a long time, we have been interested in the characterization and biological relevance of the mutations found between *c-ets-1* and its viral activated counterpart, the E26 *v-ets* oncogene (23). In fact, it is generally admitted that these mutations should be important for the oncogenic activation process and thereby should affect crucial regions of the proto-oncogene (24). In addition to its fusion to the *myb* oncogene in the E26 P135$^{gag\text{-}myb\text{-}ets}$ protein, *v-ets* differs from *c-ets-1* by two amino acid substitutions and the replacement of the last 13 C-terminal amino acids present in *c-ets-1* by 16 residues encoded by the opposite strand of the *c-ets-1* C-terminus in *v-ets* (25,26). One of these point mutations, hereafter named mutation B, is located in the ETS DNA-binding domain (Fig. 1). Although it can be considered as chemically conservative, the isoleucine 445 in *c-ets-1* is replaced by a valine in *v-ets*; this mutation affects a residue well conserved in various Ets related proteins with a few noteworthy exceptions such as GABPα or D-Elg and Spi-1 or SpiB with a methionine and glutamic acid, respectively. Furthermore, this isoleucine to valine mutation at residue 445 (I445V) is located two residues upstream of the unique point mutation found in a mutant of E26, E26 ts

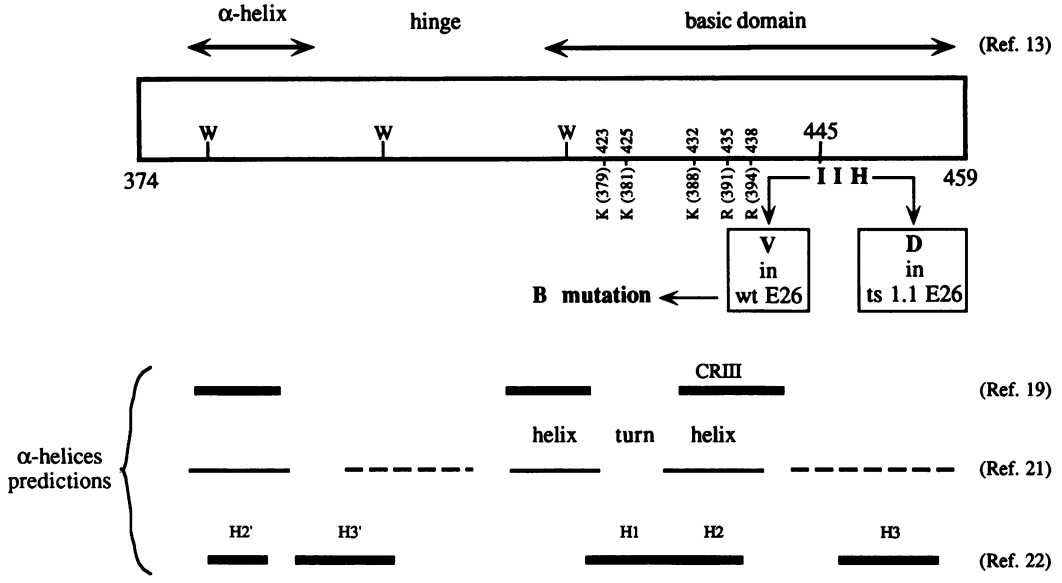


Figure 1. Structural features in the Ets-1 DNA binding domain. The Ets-1 DNA-binding domain (amino acids 374–459 in p68$^{c\text{-}ets\text{-}1}$) is represented as an open box. The three repeated tryptophan are represented. The amino acid sequence of the 445–447 region is highlighted with the mutations found in wt E26 (23) and in ts 1.1 E26 (27). Conserved regions and regions predicted to fold as an α-helix are indicated by arrows at the top and horizontal bars at the bottom of the figure, respectively. References of the original papers are indicated on the right. Localization of p68$^{c\text{-}ets\text{-}1}$ residues corresponding to essential lysine (K) and arginine (R) residues in the human Ets-1 protein (number in parentheses) (see Introduction and refs 19 and 21) is also shown.

1.1, that is temperative sensitive for erythroid cells transformation (27).

In this paper, we addressed the effect of the B mutation on the binding activities of various Ets-1 or Ets-2 proteins. We randomly mutagenized isoleucine 445 of $p68^{c\text{-}ets\text{-}1}$ by PCR and thus demonstrated that mutation of this residue deeply affected the binding to EBS even in the context of a N-terminal truncated polypeptide of 109 amino acids encompassing the ETS domain. Our results shed new light on two important aspects of the Ets-1 proteins, namely the structure of its ETS domain and its oncogenic activation in the E26 virus.

MATERIALS AND METHODS

DNA manipulations

The pET $p35^{ets\text{-}1}$ clone expressing the carboxy-terminal 311 amino acids of *c-ets-1* has been previously described (28). To randomize the isoleucine 445, we performed PCR with the following oligonucleotides: a 5′ (sense) oligonucleotide; 5′-TGGGAGTTCAAGCTTTCCGAT-3′ corresponding to sequence around a unique *Hin*dIII site (underlined) in the $p68^{c\text{-}ets\text{-}1}$ cDNA clone; a degenerate 3′ (antisense) oligonucleotide containing an equimolar mixture of the four nucleotide precursors at codon 445 of *c-ets-1*; 5′-GCGCTTGCCGGCCGTCTTGT GGATNNNGTTCTTGTCGTAATA-3′. This oligonucleotide includes the 445 codon and a *Xma*III site (underlined) which is the proximal restriction site. However, the pET expression vector contains a *Xma*III site in its back-bone. Thus, we first subcloned the *Hin*dIII−*Eco*RI fragment of the *c-ets-1* cDNA clone (23) into the pUC vector yielding the pUC 3′ *ets-1* clone, which contain unique *Hin*dIII and *Xma*III sites. The PCR product was digested by the *Hin*dIII and *Xma*III restriction enzymes and cloned into the pUC 3′ *ets-1* clone digested by the same enzymes. After transfection in HB101 bacteria, all the obtained colonies were pooled and grown. Plasmid DNA prepared from this mixed population was digested by *Eco*RI and *Hin*dIII. The resulting 150 bp *Eco*RI−*Hin*dIII fragment was cloned into *Eco*RI−*Hin*dIII digested pET $p35^{ets\text{-}1}$ clone. Then, DNA sequencing of plasmids obtained from different clones allows us to select 12 expression vectors carrying a single mutation at the 445 codon. They were named pET p35 I445X, where X represents the newly introduced residue.

Constructs expressing wild-type or mutant *ets-1* DNA-binding domains (residues 377−485) were obtained by PCR using as a template the DNA from the relevant pET p35 I445X clone and the following oligonucleotides: a 5′ (sense); 5′-TTAA-CATATGGGA^{377}CCCATCCAACTG-3′ which creates a *Nde*I restriction site (underlined) and brings an initiation codon just upstream of *c-ets-1* residue 377; a 3′ (antisense): 5′-GGGGAATT CACTCATCAGCATCTGG, which creates an *Eco*RI site (underlined) just upstream of the natural termination codon of $p68^{c\text{-}ets\text{-}1}$. Each PCR product was cut by the restriction enzymes *Nde*I and *Eco*RI and inserted into the *Eco*RI−*Nde*I digested pET5a expression vector to give pET DB I445X (see Fig. 3). To express mutant $p68^{c\text{-}ets\text{-}1}$ in eukaryotic cells, the *Hin*dIII−*Eco*RI of the relevant pET p35 I445X clone was prepared and subcloned into the pSG5 $p68^{c\text{-}ets\text{-}1}$ expression clone (36) partially digested by *Eco*RI and *Hin*dIII.

An Ile to Gly point mutation at the equivalent position in the chicken *c-ets-2* gene was introduced using the 3.1 kbp *c-ets-2* cDNA cloned into M13 and the oligonucleotide directed *in vitro* mutagenesis system (Amersham).

The oligonucleotide used corresponds to nucleotides 1306−1341 in the *c-ets-2* cDNA clone (44). Its sequence is (antisense orientation): 5′-GCGCTTCCCC GA C GTC TTG TGG AT GCC GTT CTT GTC3′. The newly introduced Gly codon (GCC since in antisense orientation) is underlined. We also create, without changing the encoded amino acid sequences, an *Aat*II restriction site (GAC GTC) which was used to screen for mutation prior to sequencing.

Finally, the *Hpa*I−*Bam*HI fragment from the mutated *c-ets-2* M13 clone was inserted into the *Hpa*I−*Bam*HI digested $pSG5^{c\text{-}ets\text{-}2}$ expression clone.

For all the experiments described above, DNA sequencing was used to validate the construction.

Bacterial expression and Western blot

Various pET p35 I445X were expressed into the *Escherichia coli* strain, BL21(DE3) pLysS (29,45). Total protein extracts were prepared (28,46) and subjected to electrophoresis in SDS−PAGE and transferred to Immobilon-P membrane (Millipore). The membrane was treated with the rabbit polyclonal anti *ets*-A serum (30) first adsorbed against BL21 bacterial extracts. After washes in PBS containing 0.1% NP40, the membrane was incubated with 1.5 μCi of [^{125}I] protein A (NEN) for 2 h, washed again and put in autoradiography to reveal Ets proteins.

In vitro expression and immunoprecipitation analyses

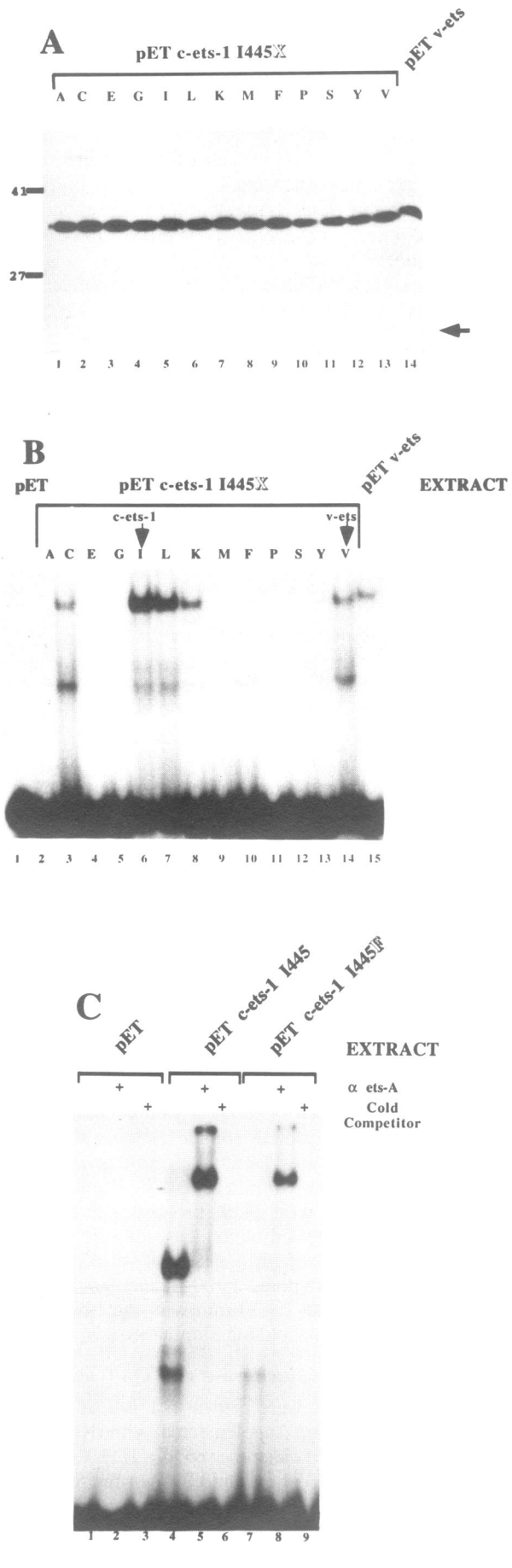
Rabbit reticulocyte lysates system (TNT system from Promega) were programmed with the relevant pET or pSG5 derived expression vector and T7 polymerase in the presence of [^{35}S]-methionine (40 μCi for each reaction) according to the manufacturer's instructions. Samples were then subjected to SDS−PAGE directly or after immunoprecipitation with specific antisera. In that case, they were incubated with anti *ets*-A or anti-DBD serum (Dhordain *et al.*, in preparation) and treated as previously described (30).

Gel mobility shift assays

The EBS oligonucleotide used in DNA-binding assays [TCG-AGCCGGAAGTGACGTCGA] (11,31,32) was end-labeled with T4 polynucleotide kinase using [γ-^{32}P]-ATP. Protein extracts were first incubated with 3 μg of double-stranded poly(dI)-poly(dC) (Pharmacia), 1 mM DTT for 15 min on ice in a final volume of 20 μl of SB buffer [20 mM Hepes pH 7.9; 50 mM NaCl, 0.1 mM EDTA; 20%(v/v) glycerol], then for 20 min on ice with an excess of ^{32}P-labeled double-stranded oligonucleotide. The DNA−protein complexes were then resolved on a 0.2×TBE/6% polyacrylamide gel pre-electrophoresed at room temperature for 60−90 min at 150 V. The gel was dried and autoradiographed. For pDB proteins, in the case of competition with non-labeled oligonucleotide, the proteins and the labeled probe were brought together at various time points and, after 20 min for each point, a 100-fold molar excess of cold probe was added to react 2−30 min with the labeled DNA−protein complexes before loading simultaneously on the gel.

Transient transfections, luciferase and CAT assays

NIH 3T3 and FEP cells were grown in D-MEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal calf serum. One microgram of either the reporter HTLV-1LTR-(6−2 57S + S) plasmid (HTLV-1 LTR) (37,47) or the reporter 3xPy B-tk-CAT (36) plasmid (TORU: TPA Oncogene Response Unit of the polyoma virus enhancer) was cotransfected using DOTAP



(Boehringer Mannheim) with 1 μg of the *ets* expression plasmids and 1 μg of the pSVLuc vector used as an internal control of transfection efficiency. The cells for CAT assays were harvested 48 h after transfection. Preparation of extracts and the enzyme assays were carried out as previously described (48).

RESULTS

DNA-binding properties of the various p35$^{c\text{-}ets\text{-}1}$ I445X proteins

To determine the effects of the B mutation found between *v-ets* and *c-ets-1*, we have randomly mutagenized by PCR the corresponding isoleucine 445 in order to study the binding capacity of the resulting mutant Ets-1 proteins. To obtain these constructs, PCR reactions were performed with an oligonucleotide that encompasses the *c-ets-1* sequence encoding the region of the B mutation and is totally randomized for the 445 codon. The pool of mutated fragments was then subcloned into a pET expression plasmid encoding a 35 kDa Ets-1 polypeptide. This polypeptide represents a *c-ets-1* protein truncated for the first 174 amino-terminal residues and has been shown to bind a probe carrying a GGAA core sequence (28). A similar 35 kDa *v-ets* protein, p35$^{v\text{-}ets}$, containing the A, B and C mutations, has also been described (28). p35$^{c\text{-}ets\text{-}1}$, p35$^{v\text{-}ets}$ and 12 new 35 kDa proteins named p35 I445X (for 'isoleucine 445 substituted by amino acid X') in which isoleucine 445 was substituted by another amino acid were expressed in BL21(DE3) bacteria (29). Total bacterial proteins were extracted and subjected to gel electrophoresis to check for and to calibrate the expression of the expected p35 proteins by Western blot analyses with an *ets*-specific antiserum called 'anti-*ets* A' serum (28,30). This experiment allowed us to determine precisely the amount of Ets-1 polypeptide present in each bacterial extract (data not shown). Calibrated cell extracts were then subjected to the above-described procedure and accuracy of the calibration was verified by Western analysis with the same antibody (Fig. 2A). In addition to the expected 35 kDa protein, we noticed that another smaller protein

Figure 2. Sequence specific binding of the various I445X 35 kDa polypeptides to the EBS probe. (**A**) Calibration of the Ets-1 protein by Western blot analysis. Total proteins of the various pET *c-ets-1* I445X and *v-ets* containing extracts were fractionated on SDS/PAGE gels, electrophoretically transferred on to nitrocellulose and submitted to immunoblot analysis using the anti-*ets*-A serum to determine the amount of Ets-1 specific polypeptide in each extract as previously described (data not shown) (28). Then, the same analysis was performed with calibrated amounts of total proteins. The pET *v-ets* clone expressing a 35 kDa polypeptide carrying the A, B and C mutations and displaying a lower mobility (lane 14) has been described (28). The amino acid replacing the isoleucine 445 in each clone is indicated above each lane using the single letter code. The arrow indicates the migration of a higher mobility band which is visible in most samples after long exposure (data not shown). This band presumably yields the higher mobility complex in the gel shift assay (see below). (**B**) Electromobility shift assays of the various 35 kDa I445X and *v-ets* protein. A ^{32}P labeled EBS probe was incubated with equivalent amounts of total proteins from control pET extracts (lane 1) or calibrated amounts of p35 I445X and p35v-ets proteins as indicated above each lane. The I445 protein corresponding to *c-ets-1* and the I445V protein corresponding to the substitution found in *v-ets* are highlighted by an arrowhead. (**C**) Competition experiments. A ^{32}P labeled EBS probe was incubated with equivalent amounts of total proteins from control pET extracts (lanes 1–3) or calibrated amounts of p35 I445 (lanes 4–6) and P35 I445 F (lanes 7–9). Competition was performed by adding either 2 μl of anti-*ets*-A serum (28) (lanes 2, 5 and 8) or a 200-fold molar excess of cold oligonucleotide (lanes 3, 6 and 9) as indicated by a + above these lanes.

of about 20 kDa can be observed in each lane on a longer exposure, with the notable exception of the $p35^{v\text{-}ets}$ protein (see legend Fig. 2A; data not shown). This perhaps represents either a protein initiated on an internal AUG codon or a cleavage product, generated in the bacterial context. Sequence-specific DNA-binding of each p35 I445X protein to an 'optimized' Ets-1 binding site (EBS; 11,31,32) was analysed in gel mobility shift assays containing similar amounts of proteins, as defined by the Western blot. In order to reach an optimized binding, each binding reaction described in this work has been performed with buffer conditions re-examined, in comparison with those usually used in our laboratory. In particular, we have shown that, for a given protein, the addition of 5 mM Mg^{2+} always diminishes its specific-binding (33) and that a stronger binding was observed in the presence of 1 mM DTT (data not shown). Nevertheless, in all the conditions tested, we never observed a weaker binding of *c-ets-1* proteins than the corresponding *v-ets* proteins (N.S., unpublished results). As previously shown (28), p35 I445 binds to the purine-rich motif in a sequence-specific manner since the DNA−protein complex can be further shifted following incubation with the *ets*-specific antiserum, anti-*ets*-A, and a 100-fold molar excess of cold probe competes for the binding of the probe to p35 I445 (Fig. 2C and data not shown). Turning now to the p35 I445X, excepted for the I445L which appears quite similar to I445 (Fig. 2B, lanes 6 and 7), they can be classified into three groups according to the residual binding activity they display. The I445C, I445K and I445V mutations fall into a first group exhibiting a residual ~30% DNA-binding activity of the wild-type *c-ets-1* protein, p35 I445 (Fig. 2B, compare lane 6 with lanes 3, 8 and 14 respectively). The I445A, I445G, I445M, I445F, I445S and I445Y substitutions, represent a second group of mutations that show no significant DNA-binding activity. In fact, a very long exposure of the gel is necessary to detect a barely visible signal, characteristic in size of a 35 kDa protein−DNA complex (data not shown) as compared with no signal on the short exposure (Fig. 2B, lanes 2, 5, 9, 10, 12 and 13). Finally, the last group contains the I445E and I445P substitutions where no signal is observed regardless of the exposure time (Fig. 2B, lanes 4 and 11). For comparison, we also show that $p35^{v\text{-}ets}$ that carries the three A, B and C (carboxy-terminal) mutations binds to the EBS probe but in a weaker manner than $p35^{c\text{-}ets\text{-}1}$ (Fig. 2B, lane 15). Indeed, the binding affinity of the $p35^{v\text{-}ets}$ protein is quite similar to the one displayed by the I445V protein carrying only the B mutation (Fig. 2B, compare lanes 14 and 15). A DNA−protein complex of higher mobility is observed in almost all cases where we observed a low mobility complex with the notable exception of the *v-ets* protein (Fig. 2B). This sequence-specific complex (Fig. 2C) presumably contains the smaller Ets-1 protein indicated in the Western blot analysis (Fig. 2A). Another notable exception is the I445F protein where a barely visible high mobility complex is seen in the absence of a low-mobility complex (Fig. 2B, lane 10 and 2C, lane 7). These observations prompted us to investigate the effect of the isoleucine substitution in other forms of *c-ets-1* proteins, in particular in a more truncated form similar to the minimum ETS DNA-binding domain.

Effects of the same mutation introduced into an ETS-domain protein

The ETS family is characterized by a conserved domain of 85 amino acids: the ETS-domain, located in the carboxy-terminal part of Ets-1 proteins (10,11). In connection with that, a carboxy-terminal chicken or murine Ets-1 polypeptide of 109 and 105 amino acids respectively, has been shown to be sufficient for the binding to EBS in gel mobility assays. Furthermore, such a polypeptide binds to DNA with a much higher binding affinity than the full-length protein (12,32,34). The observation of a higher mobility complex in the presence of a lower mobility complex (Fig. 2B, lanes 3, 6−8, and 14) or even in its absence as for the I445F protein (Fig. 2B, lane 10) suggested that the effect of some I445X mutations could be somehow compensated by the higher binding affinity of a truncated protein. To address this question, we introduced some of the isoleucine substitutions that induce the highest decrease of binding of p35 I445X, into pET plasmids expressing the 109 carboxy-terminal amino acids of chicken *c-ets-1* to induce proteins named pDB I445X (Fig. 3A). In fact, thanks to its T7 responsive promoter, pET plasmids can direct the synthesis of proteins both in bacteria and in rabbit reticulocyte lysates. Thus, rabbit reticulocytes (Promega) were programmed with the pDB I445, I445E, I445G, I445P and I445V pET clones in presence of [^{35}S]-methionine and immunoprecipitated with an ETS-domain specific antiserum named anti-DBD (Ph.D. *et al.*, in preparation) (Fig. 3A). Briefly, this serum

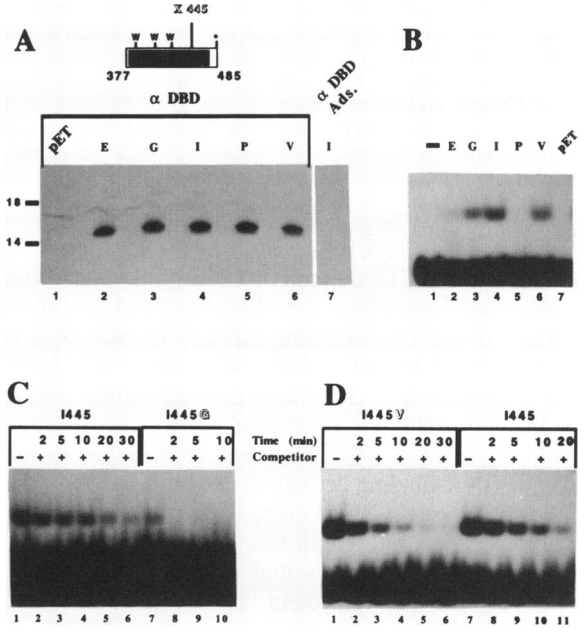


Figure 3. DNA-binding capacity of the pDB I445X proteins. (**A**) Immunoprecipitation analyses. Rabbit reticulocytes lysates programmed with the pET vector as a control (lane 1), pET plasmids expressing the DB I445X proteins (see the schematic drawing at the top of the figure) with the corresponding amino acid indicated above were immunoprecipitated with an anti-DNA-binding domain serum, α DBD (lanes 1−6). As a control the DB I445 programmed lysate was immunoprecipitated with the α DBD serum adsorbed against an excess of the immunogen used to immunize the rabbit (α DBD ads., lane 7). (**B**) Electrophoretic mobility shift. The EBS probe was incubated with no extract (lane 1) or with pDB I445X, carrying the above indicated substitution (lanes 2−6). As a further control the probe was also incubated with total proteins from control pET extracts (lane 7). (**C**) Dissociation rate of the I445 and I445G−EBS complexes. Calibrated amounts of pDB I445 and I445G proteins were incubated for 20 min on ice with the ^{32}P labeled and incubated with a 100-fold excess of cold competitor for the above indicated time. All the samples were time scheduled so that they can be loaded simultaneously on the gel. (**D**) Dissociation rate of the I445 and I445V−EBS complexes. Same experiment as above with the I445 and I445V proteins.

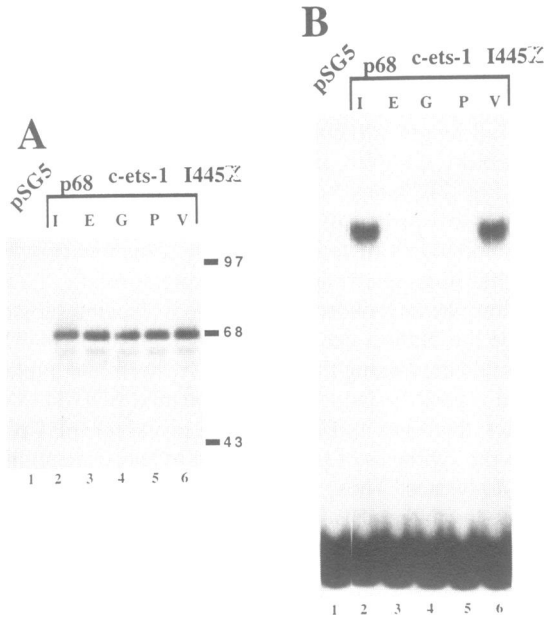


Figure 4. Synthesis of p68 I445X proteins *in vitro* and analysis of their DNA binding activity. (**A**) Expression in rabbit reticulocyte. Lysates were programmed with the pSG5 vector as a control (lane 1) or with the various pSG5 p68 I445X expressing vectors where X is glutamic acid (E; lane 3); glycine (G, lane 4); proline (P, lane 5) and valine (V, lane 6). The non-mutated p68c-ets-1 where X is isoleucine corresponds to lane 2. (**B**) DNA-binding activity of p68 I445X proteins. Calibrated amounts of proteins as defined by the above described experiment were incubated with the EBS probe and EMSA were performed as described in Materials and Methods. The nomenclature for the various p68 I445X proteins is exactly the same as in panel A.

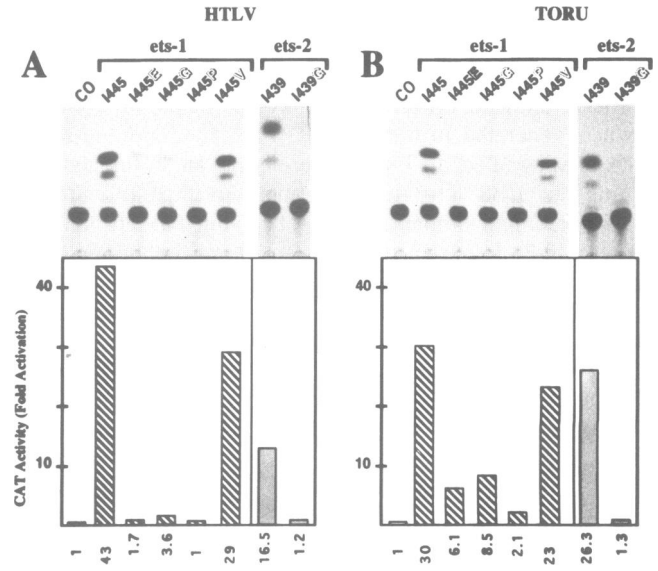


Figure 5. Transcriptional activation by p68c-ets-1, p58-64c-ets-2 and by point mutants derived thereof. Chicken embryo fibroblasts were transfected with the HTLV-1 LTR (6−2 57S+S) vector (37) in panel A or with the 3X Py-b-tk-CAT (TORU) vector (36) in panel B and with the pSG5 expression vector alone (Co); the p68c-ets-1 expression vector (I445), the above indicated I445X point mutants, the p58-64c-ets-2 expression vector (I439) and the I439G point mutant. Results of a typical experiment are presented. The histogram represents the average of two experiments.

directed against the ETS domain of the Erg proteins (35) cross-reacts with its close relatives, the Fli-1, Ets-1 and Ets-2 proteins. Comparison of the DNA-binding capacity of each pDB I445X protein performed by gel mobility shift assay with calibrated amounts of proteins (Fig. 3B) was in good agreement with results obtained with the p35 I445X proteins. Similar classifications are obtained when each mutation is ranked according to its induced decrease in DNA-binding activity either in the p35 I445X or in the pDB I445X proteins. However, as already demonstrated for the truncated versus full-length Ets-1 proteins (12,32,34), each pDB I445X protein displays a higher binding affinity than the corresponding p35 I445X protein. This is particularly obvious for the pDB I445G, and to a lesser extent, pDB I445E proteins which bind to the EBS probe (Fig. 3B, lanes 2 and 3) in contrast to the p35 I445E and I445G proteins that do not bind it (Fig. 2B, lanes 4 and 5). A notable exception is the I445P mutation which abolishes the DNA-binding properties of the p35 I445P (Fig. 2B, lane 11) and of the 'high affinity' truncated protein, pDB I445P (Fig. 3B, lane 5).

A further indication of complex stability as a function of residue 445 was obtained by estimating the dissociation rate of complexes between the EBS oligonucleotide and various pDB Ets-1 polypeptides (Fig. 3C and D). The complex formed between pDB I445G and the EBS probe is much more rapidly competed with a 100-fold molar excess of cold probe than the complex formed between pDB I445 and the same probe. In this latter case, a residual labeled complex is still clearly visible after 30 min (Fig. 3C, lanes 1−6), whereas the complex formed between EBS and pDB I445G is totally dissociated after 2 min in the presence of competitor (Fig. 3C, lanes 7−10). A similar experiment performed with the pDB I445V shows that again its complex with the EBS probe is more rapidly competed than the complex formed by I445. However, in that case the difference between the two dissociation rates is smaller albeit significant (Fig. 3D). These experiments demonstrate that wild-type pDB I445 dissociates more slowly from the EBS probe than pDB I445V and at a greater extent than pDB I445G suggesting that these two proteins adopted a different conformation.

Correlation between binding and transactivating activities of mutated p68 I445X

We then analysed whether the effects of mutations also held true for full-length $p68^{c\text{-}ets\text{-}1}$ proteins and if this effect on the DNA-binding properties reflected the ability of these proteins to activate transcription in transient transfection assays. We thus cloned into the pSG5 eukaryotic expression plasmid, $p68^{c\text{-}ets\text{-}1}$ sequences, either with isoleucine 445 (wild type) or with I445X mutations that had been shown to decrease DNA-binding activity of p35 and pDB I445X peptides to EBS, i.e. the I445E, I445G and I445P substitutions, or with the substitution of isoleucine to valine corresponding to the B mutation in *v-ets*, the I445V. To analyse their effect on the DNA-binding activity, these mutated full-length p68 I445X proteins were expressed in rabbit reticulocyte lysates and subjected to SDS-PAGE (Fig. 4A). In perfect agreement with the above described results, $p68^{c\text{-}ets\text{-}1}$ I445 and p68 I445V were able to bind to the EBS probe (Fig. 4B, lane 2 and 6), in contrast to p68 I445G (Fig. 4B, lane 4), for which a very weak signal was detected after long exposure (data not shown) and to p68 I445E and p68 I445P (Fig. 4B, lanes 3 and 5) which did not form any detectable complex even on a long exposure (data not

shown). The expression plasmids for the p68 I445X proteins were co-transfected, either in murine NIH 3T3 cells or in chicken embryo fibroblasts (CEF), together with a pSVLuc vector used as an internal control of transfection efficiency and with reporter plasmids carrying sequences derived either from the TPA Oncogene Response Unit of the polyoma virus enhancer (TORU) (36) or from the LTR of the HTLV-1 virus (HTLV-1 LTR) (37) in front of the CAT gene. Results demonstrated that the wild-type $p68^{c\text{-}ets\text{-}1}$ I445 functioned as a transcriptional activator both of the HTLV-1 LTR in NIH 3T3 (sevenfold activation, data not shown) and in CEF (about 43-fold; Fig. 5A) and of the TORU in NIH 3T3 (fourfold; data not shown) and in CEF (30-fold, Fig. 5B). On the other hand, point mutations in $p68^{c\text{-}ets\text{-}1}$ decreased slightly (p68 I445V) or dramatically (p68 I445G and particularly p68 I445E) or even abrogated (p68 I445P) the ability to transactivate via the same reporter plasmids (Fig. 5A and B). We also noticed the perfect correlation obtained between the *in vitro* DNA-binding activity and the *in vivo* transactivating experiments.

Effect of the mutation of this well-conserved amino acid in ck-Ets-2 protein

The isoleucine 445 of $p68^{c\text{-}ets\text{-}1}$ is rather well-conserved into the ETS family, notably in ck-$p58\text{-}64^{c\text{-}ets\text{-}2}$ proteins (11). As the amino acid change from Ile to Gly in $p68^{c\text{-}ets\text{-}1}$ strongly decreases binding and transactivating properties, we wanted to determine the effect of the same mutation into the Ets-2 protein that shares close structural and functional similarities with Ets-1 protein (Albagli *et al.*, submitted). For this purpose, Ile to Gly substitution was created in the ck-Ets-2 protein at the residue equivalent to isoleucine 445 in $p68^{c\text{-}ets\text{-}1}$, namely isoleucine 439, using the oligonucleotide-directed *in vitro* mutagenesis system (Amersham). Immunoprecipitation with the specific 'anti-*ets* A' serum performed as a control, revealed similar levels of expression of the wild-type and mutant proteins in transfected COS-1 cells (data not shown). As expected from the results obtained with the *c-ets-1* protein, gel mobility shift assays demonstrated that $p58\text{-}64^{c\text{-}ets\text{-}2}$ but not $p58\text{-}64^{c\text{-}ets\text{-}2}$ I439G is able to bind the EBS probe (data not shown). Moreover, the wild-type $p58-64^{c\text{-}ets\text{-}2}$ protein transactivates reporter plasmids carrying either TORU (26-fold activation) or HTLV-1 LTR (16-fold activation) in CEF, whereas the Ile to Gly substitution (I439G) abolishes the transactivating properties of the protein (Fig. 5A and B). These data demonstrate that this isoleucine residue plays an essential role in both Ets-1 and Ets-2 proteins.

DISCUSSION

In this paper, we demonstrated that the isoleucine 445 in the $p68^{c\text{-}ets\text{-}1}$ DNA binding domain is essential for the *in vitro* DNA-binding and as a consequence the *in vivo* transactivating properties of the *c-ets-1* and *c-ets-2* proteins. In fact, we observed a good correlation between *in vitro* DNA-binding to an EBS site and *in vivo* transactivating properties, indicating that the only function affected by I445X mutations is DNA-binding affinity. This residue is not included in one of the three regions of strong homology between known ETS domains, namely the N-terminal leucine rich region and the two regions which overlap with the two helices of the HTH motif (19–22). Nevertheless, isoleucine 445 is located seven residues downstream of the 3′ border of this HTH motif. In fact, with the exception the more distantly related Spi-1 and Spi-b members, the HTH motif is followed by a conserved 10 amino acid region characterized by a striking alternation of hydrophobic and charged residues among which five are consensus sequences for the ETS domain (8,11). These residues are three perfectly conserved hydrophobic tyrosine residues which according to the 'mpred' prediction method contribute to the 3′ helix of the HTH motif (19); three variable although often charged residues; two hydrophobic residues including the well conserved I445; a variable residue and finally an invariant lysine residue. Mutagenesis analyses and structure predictions have stressed the role of the α helix in the CRIII region as the 'recognition helix', which would be docked in the DNA major groove (12,19). It is highly unlikely, although not totally ruled out, that the 10 amino acid region following the CRIII motif and including I445 would be also in direct contact with DNA. Rather, we propose, based on previously published observations and our results, that mutations in this region alter the structure of the ETS domain leading to a decreased DNA binding affinity. In particular, a four amino acid region centered around the I445 residue (444–447) appears crucial. Indeed, in their random mutagenesis of the human Ets-1 protein, Mavrothalassitis *et al.* (21) demonstrated that mutations affecting the DNA-binding affinity were scattered all along the domain. A notable exception was the region corresponding to residue 444–446 in $p68^{c\text{-}ets\text{-}1}$ where a mutant affecting each of these three amino acids was isolated. Strikingly, the mutation affecting the I445-corresponding residue is a threonine which decreases the DNA-binding to 10–40% of its original activity. Taken together with our data, 14 point mutations encompassing most of the amino acid categories (i.e. acidic, hydrophobic) have thus been obtained for the I445 residue. The less detrimental mutations are the substitution of the I445 residue by amino acids with other aliphatic side chains, such as leucine or valine. It would be interesting to know if mutations of others conserved hydrophobic non-aromatic residues in this region would also affect the structure of the ETS domain and hence, its DNA binding affinity. Furthermore, the I446T substitution decreases the DNA-binding activity of the protein to 10% of that of the wild-type protein (21). This region, 444–447, can be considered as a 'hot spot' for mutations affecting the DNA-binding affinity presumably by inducing local conformational changes. Maybe the strongest argument in favor of this hypothesis is the point mutation affecting the residue 447. The screening for E26 mutants able to transform erythroid colonies at 37°C which could mature into erythrocyte-like cells upon temperature shift for 2 days at 42°C, led to the isolation of the E26 ts 1.1 mutant (27). This mutant contains a single histidine to aspartic acid substitution in the DNA-binding domain affecting the residue equivalent to 447 in $p68^{c\text{-}ets\text{-}1}$ (27). Recently, a ts 1.1 DNA-binding domain protein (amino acids 377–488) was shown to exhibit strong binding to a PEA3 probe at 22°C as compared with no detectable binding at 42°C (34,38). For comparison, similar *c-ets-1* or wt E26 DNA-binding domain proteins were not affected by such temperature shifts in the gel retardation assay incubation conditions. However, the inactivation of ts 1.1 *v-ets* DNA-binding at 42°C can be reversed if the binding reaction is back-shifted for at least 4 h at 22°C (38). These results strongly suggest that the 447 mutation induces a conformational change resulting in a loss of DNA-binding properties. Alternatively, this mutation could result into a change in DNA-binding specificity, although this hypothesis appears less likely. Furthermore, introduction of a similar point mutation into the ETS domain of Elk-1 renders the corresponding protein Elk D74 not only unable to interact efficiently to its cognate sequence

(39) but, at elevated temperatures, also with a binary complex of the c-*fos* SRE and SRF (40).

In addition to its contribution to the general problem of interaction between ETS domains and DNA, isoleucine 445 raises an interesting question specific to *c-ets-1*, since it is mutated in the viral *v-ets* oncogene of the E26 virus. An emerging model for the oncogenic activation of the *c-ets-1* gene is to consider that *v-ets* is more promiscuous for its binding that *c-ets-1*. Indeed, the C-terminal change of *v-ets* allowed it not only to bind to sequences recognized by *c-ets-1* but also to bind to sequences not or weakly bound by *c-ets-1* (41). This implies that *v-ets* could induce transformation by transactivating promoters of genes with which *c-ets-1* could not interact. However, the participation in this transforming process of genes containing *c-ets-1* high affinity sites in their promoter cannot be totally ruled out.

The B mutation is important for the binding and transactivating properties of Ets-1 and Ets-2 proteins. Whereas numerous substitutions of the isoleucine 445 deeply impede the DNA-binding activity, the I445V B mutation found in v-*ets* is one among the few having the mildest effect since it preserves most of the ability to bind the EBS probe (Fig. 2). Perhaps, the B mutation could slightly decrease the binding of *v-ets* to optimized high affinity, as examplified by the EBS site (Fig. 2) whereas the C-terminal change would increase the binding to low affinity sites (41) thus cooperatively broadening the spectrum of *v-ets* recognized sequences. In fact, the crucial mutations that the *v-erb-A* DNA-binding domain has undergone render it more promiscuous than *c-erb-A* for its binding while reducing its affinity for the high affinity 'TRE' sequence (24,42).

However, turning back to *ets*, it must be stressed that our results as well as those concerning the C mutation (34,41,43), have been obtained with isolated Ets proteins and not in the context of the P135$^{gag\text{-}myb\text{-}ets}$ fusion protein. In fact, this protein, when expressed in reticulocytes lysates fails to recognize conventional Ets binding sites and does so only if it is preincubated with excess Myb binding sites (F.Lim, A.Marknell, N.Kraut, I.Sieweke and T.Graf, manuscript submitted). Clearly, the next challenge will be the characterization of Myb-Ets regulated genes important for immature erythroid cell (MEP) transformation since the promoter of such gene(s) should contain consensus Myb and Ets binding sites for the fusion protein, optimally arranged in terms of relative orientation and spacing. The analysis of the DNA-binding and transactivating properties of this promoter by various P135$^{gag\text{-}myb\text{-}ets}$ mutated proteins should clearly indicate the exact role of each mutation in the oncogenic activation of *c-ets-1*.

ACKNOWLEDGEMENTS

We thank Drs J.Brady and A.Gégonne for the generous gift of the HLTV-1 reporter plasmid. We also thank Drs S.L.Hahn and B.Wasylyk and Dr T.Graf for communicating results prior to publication and for comments on this manuscript. The authors also thank C.Lagrou for his precious help in cell culture, Drs J.H.Chen and Y.de Launoit for critical reading of the manuscript, S.Ung for photographic work and N.Devassine for patient and excellent typing. This work is supported by funds from the Centre National de la Recherche Scientifique (CNRS), Association pour la Recherche contre le Cancer (ARC) and the Pasteur Institute of Lille.

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