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Mutual activation of Ets-1 and AML1 DNA binding by direct interaction of their autoinhibitory domains

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The transcription factors Ets-1 and AML1 (the αBl subunit of PEBP2/CBF) play critical roles in hematopoiesis and leukemogenesis, and cooperate in the transactivation of the T cell receptor (TCR) β chain enhancer. The DNA binding capacity of both factors is blocked intramolecularly but can be activated by the removal of negative regulatory domains. These include the exon VII domain for Ets-1 and the negative regulatory domain for DNA binding (NRDB) for αB1. Here we report that the direct interaction between the two factors leads to a reciprocal stimulation of their DNA binding activity and activation of their transactivation function. Detailed mapping revealed two independent contact points involving the exon VII and NRDB regions as well as the two DNA binding domains. Using deletion variants and dominant interfering mutants, we demonstrate that the interaction between exon VII and NRDB is necessary and sufficient for cooperative DNA binding. The exon VII and NRDB motifs are highly conserved in evolution yet deleted in natural variants, suggesting that the mechanism described is of biological relevance. The mutual activation of DNA binding of Ets and AML1 through the intermolecular interaction of autoinhibitory domains may represent a novel principle for the regulation of transcription factor function.

Keywords: AML1/CBF/cooperative DNA binding/Ets-1/PEBP2

Introduction

The transcription factors Ets-1 and AML1 have been shown to cooperate in the regulation of the T cell receptor (TCR) α and β enhancers and in the Moloney leukemia virus enhancer (Wotton *et al.*, 1994; Giese *et al.*, 1995; Sun *et al.*, 1995). AML1 and Ets binding sites are present in close proximity on these enhancers and mutations of either of these sites reduces enhancer activity (Wotton *et al.*, 1994; Sun *et al.*, 1995). Ets-1 and another member of the AML family have also been shown to interact physically (Giese *et al.*, 1995). As is the case for several tissue-specific transcription factors, both Ets-1 and AML1 on their own are essentially unable to bind to DNA unless negative regulatory domains are removed (Hagman *et al.*, 1992; Lim *et al.*, 1992; Nye *et al.*, 1992; Wasylyk *et al.*, 1992; Kanno *et al.*, 1998). The mechanism by which their DNA binding capacity, and thus their function as transcription activators is induced is not known.

AML1 corresponds to the αB1 subunit of PEBP2/CBF. There are two other related mammalian α subunits: Pebp2αA/CbfA1/AML3 and Pebp2αC/CbfA3/AML2 (reviewed in Ito and Bae, 1997). The corresponding genes, together with *Drosophila runt* (Kania *et al.*, 1990) and *lozenge* (Daga *et al.*, 1996), share an evolutionarily conserved region encoding the RUNT DNA binding domain and constitute the RUNT domain family. For simplicity, we will use the terms αA1, αB1 and αC to refer to the products of the major splice forms of these genes. All α gene products are capable of heterodimerization with the β subunit. α*B1/AML1/CBFA2* is the most frequent target of chromosome translocations associated with human leukemias of myeloid and lymphoid lineages (reviewed in Look, 1997). The β subunit, known as PEBP2β or CBFβ, is also involved in a recurrent chromosome anomaly, inv(16), associated with acute myeloid leukemia (Liu *et al.*, 1993). Gene disruption studies in the mouse revealed that αB is required for the formation or function of stem cells for definitive hematopoiesis (Okuda *et al.*, 1996; Wang *et al.*, 1996b). Interestingly, disruption of the gene encoding the β subunit resulted in a nearly identical phenotype (Sasaki *et al.*, 1996; Wang *et al.*, 1996a; Niki *et al.*, 1997), indicating that αB and β function as a heterodimer *in vivo*.

Recently, we found that the DNA binding ability of αB1 is negatively regulated by a region adjacent to the RUNT domain, termed the negative regulatory region of DNA binding (NRDB). Interaction of the β subunit with the RUNT domain relieves the inhibitory activity of NRDB, thus allowing αB1 to bind to DNA (Kanno *et al.*, 1998).

c-Ets-1 was initially identified as the protooncogene corresponding to the v-*ets* oncogene of the E26 leukemia virus (Leprince *et al.*, 1983; Nunn *et al.*, 1983; de Taisne *et al.*, 1984). Ets-1 is a founding member of the Ets family, which is characterized by a strong homology in the ETS DNA binding domain (Karim *et al.*, 1990). Ets-1 is highly expressed in lymphoid cells of adult mice and regulates expression of lymphocyte-specific genes (Chen *et al.*, 1985; Bhat *et al.*, 1989). Gene disruption studies in RAG-2-deficient mice revealed that Ets-1 is essential for the maintenance of resting T- and B-lineage cells as well as for the survival and activation of T cells (Bories *et al.*, 1995; Muthusamy *et al.*, 1995).

The structure of the full length αB1 and Ets-1 and their

natural isoforms is shown in Figure 1. In this study, we have analyzed the molecular mechanism by which αB1 and Ets-1 cooperate in DNA binding and in the transactivation of the TCRβ enhancer. The results revealed that different parts of αB1 and Ets-1 interact with each other and that interaction of the respective negative regulatory domains leads to a derepression of the DNA binding capacities of the two transcription factors.

Results

αB1 and Ets-1 mutually activate their DNA binding activities

To examine the mechanisms of cooperation between PEBP2 and Ets-1, we studied the Tβ3 and Tβ4 core elements of the TCRβ enhancer, which contain partially overlapping PEBP2 and Ets binding sites (designated PBS and EBS, respectively) as well as an individual PBS (Ogawa *et al.*, 1993b; Figure 2A). We mutated each of the PBS and EBS and performed transfection assays in the T cell line BW5147 (Ogawa *et al.*, 1993b) with a CAT reporter plasmid containing the mutant or wild-type

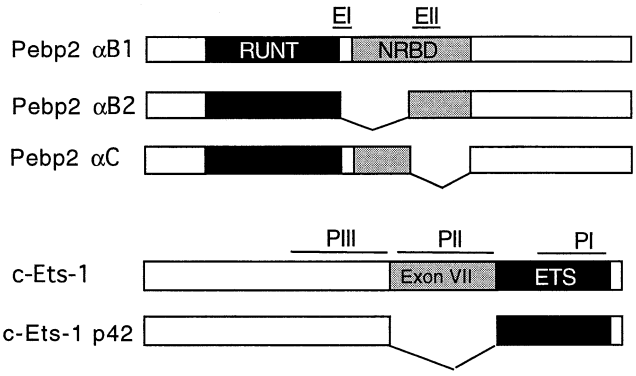

Fig. 1. Diagrammatic representation of wild-type and natural variants of the α subunit of PEBP2 and Ets-1 used in this study. RUNT and ETS: DNA binding domains, NRDB: negative regulatory domain for DNA binding. exon VII: the region encoded by exon VII. EI and EII: regions of αB1 responsible for interacting with Ets-1. PI, PII and PIII: regions of Ets-1 responsible for interacting with αB1.

(wt) enhancer in front of a minimal thymidine kinase (tk) promoter. As shown in Figure 2B, except for the mutation in the 3′ PBS in Tβ4 (M4) which showed little effect, mutations in all the other sites reduced the enhancer activity to ~20% of the level observed with the wild-type enhancer. In addition, a complete loss of the enhancer activity was observed when all three PBS were destroyed. These results indicate that both half sites of composite PBS/EBS elements and, therefore, both PEBP2 and Ets activities are required for enhancer activation.

To examined whether the cooperation between PEBP2 and Ets-1 activities was due to their DNA-binding properties, we performed electrophoretic mobility shift assays (EMSAs). Using a Tβ3 probe containing one PBS and EBS each, Ets-1 bound only very weakly to DNA even at high concentrations (Figure 3A, lanes 2–4, indicated by *). Likewise, αB1 by itself also bound poorly to its cognate site (lane 5, indicated by Δ). Surprisingly, DNA binding of αB1 was strongly stimulated by the addition of Ets-1 even in the absence of the β subunit. Thus, when a constant amount of αB1 was mixed with increasing amounts of Ets-1, there was an increase in the formation of a low mobility complex (lanes 5–8), indicating that Ets-1 and αB1 cooperatively bind to DNA in the absence of the β protein. Addition of the β protein led to the formation of an even more prominent complex (lanes 9–12), suggesting that it can associate with αB1 in the presence of Ets-1.

As shown in Figure 3B, the β subunit by itself could only marginally increase DNA binding of αB1 (lanes 1 and 3), even at ~40-fold molar excess. On the other hand, only a 10-fold molar excess of Ets-1 dramatically stimulated not only DNA binding of αB1 alone (lane 2), but also the formation of an αB1/β complex (lane 4). This indicates that Ets-1 both facilitates αB1 DNA binding (compare lanes 1and 2) and dimerization with the β subunit (compare lanes 3 and 4). The latter point will be discussed in more detail elsewhere (W.-Y.Kim and Y.Ito, in preparation).

To determine whether the cooperative binding to the composite PBS/EBS site required both binding sub-sites,

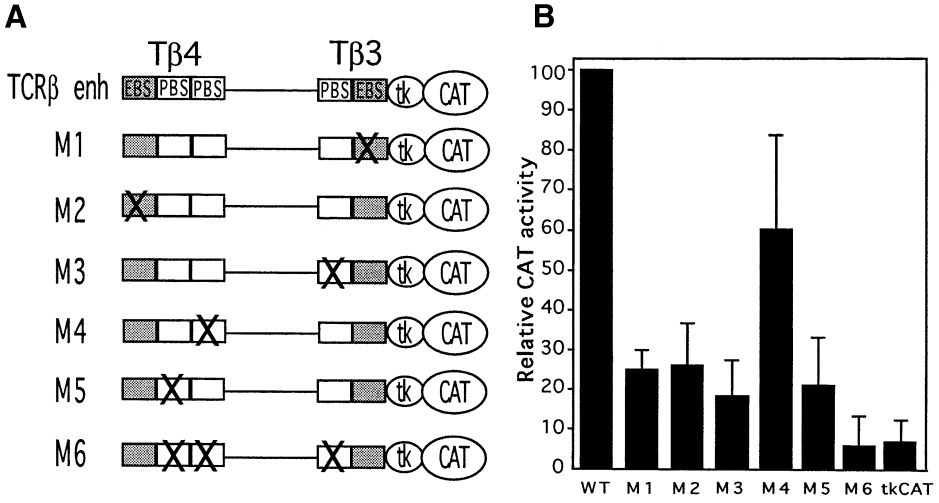

Fig. 2. Mutational analysis of TCRβ. (**A**) Diagrammatic representation of the minimal TCRβ enhancer. Binding sites of PEBP2 (PBS) and Ets (EBS) are indicated by white and grey boxes, respectively. tk, minimal thymidine kinase promoter; CAT, chloramphenicol acetyltransferase reporter. Mutated sites are indicated by crosses. (**B**) Each of the constructs shown in (A) were transfected into the T cell line BW5147 and CAT activity was determined.

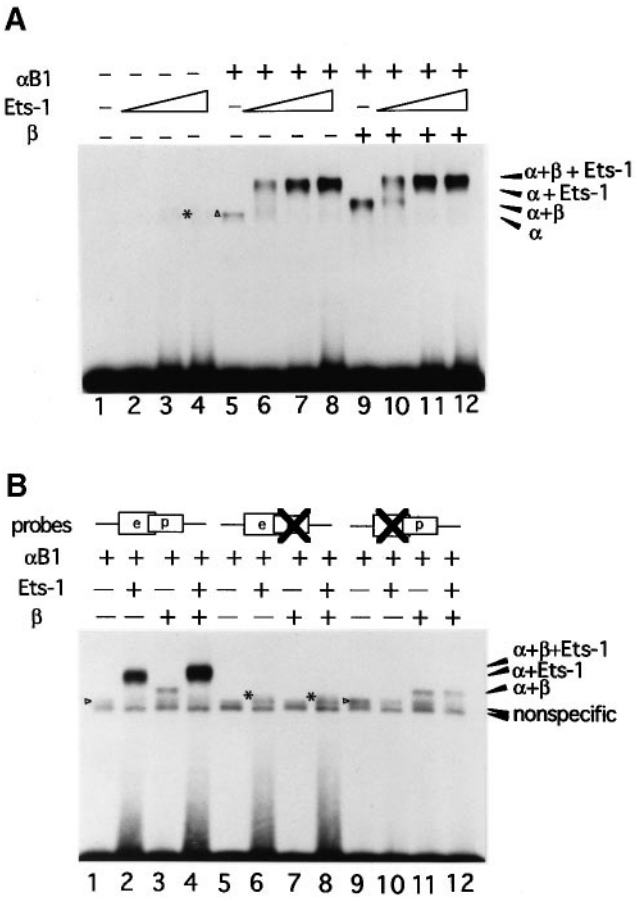

Fig. 3. Cooperation between αB1 and Ets-1 for DNA binding. *In vitro* translated αB1, Ets-1 and purified β were subjected to EMSA in various combinations as indicated above each panel. (**A**) ^{32}P-labeled probe 1 of the Tβ3 element (see Materials and methods) was used. The amounts of proteins used were: αB1,1.4 fmol; β, 100 fmol; Ets-1, 1.7 fmol in lanes 2, 6 and 10, 3.5 fmol in lanes 3, 7 and 11, and 7 fmol in lanes 4, 8 and 12. (**B**) ^{32}P-labeled wild-type and mutant probes used are indicated on top and described in Materials and methods. Amounts of proteins used were: αB1, 1 fmol; Ets-1, 10 fmol; β, 40 fmol. Positions of protein–DNA complexes are indicated on the right. *, Ets-1; Δ, αB1.

EMSAs were performed with probes containing mutations in either the PBS or EBS site and compared with the non-mutated probe. Cooperative binding of αB1 and Ets-1 required that both sites are intact (Figure 3B, lanes 1–4), since mutation of either the PBS (Figure 3B, lanes 5–8) or the EBS (Figure 3B, lanes 9–12) was sufficient to prevent DNA binding of either protein.

These results suggest that PEBP2 and Ets-1 mutually facilitate their binding to their cognate DNA binding sites and that the interaction between PEBP2 and Ets-1 is stabilized through DNA binding.

Ets-1 interacts with regions contained in the NRDB and at the border of the RUNT domain of αB1

To map the domains in αB1 responsible for interacting with Ets-1, a series of *in vitro* translated N- and C-terminally truncated αB1 proteins were subjected to pull-down assays with purified glutathione *S*-transferase (GST)–Ets-1 fusion proteins. A schematic drawing of the constructs is shown in Figure 4A. As shown in Figure 4B, C-terminal deletions of αB1 up to amino acid (aa) 183 moderately reduced the interaction with Ets-1, while a larger deletion up to aa 177 left only residual activity which was completely abolished by a further deletion to aa 173. Therefore, an essential region for interaction with Ets-1 appears to be located between aa 174 and aa 183 (designated Ets binding site I, EI). In a series of N-terminal truncations, deletions up to aa 178 showed full Ets-1 binding activity. Surprisingly, however, deletions extending beyond aa 178 as well as an internal deletion (αB2), all of which completely lacked domain EI, still showed a strong interaction with Ets-1, indicating the presence of a second interaction motif. N-terminal deletion up to aa 292 completely abolished this interaction activity (Figure 4C). Using additional deletion constructs, this second Ets-1 interaction site (EII) was mapped to a region between aa 240 and 261.

The C-terminal last 120 amino acids of αB1 appeared to contribute to the interaction with Ets-1 only by stabilizing the principal contacts regions EI and EII. Although the deletion of this region resulted in a significant decrease of binding (Figure 4B, compare lane 1–451 with lane 1–331), by itself it did not show any binding activity (Figure 4C, lane 292–451).

To confirm that αB1 and Ets-1 not only interact *in vitro* but also *in vivo*, we performed co-immunoprecipitation studies on extracts from COS-7 cells transfected with expression constructs for a FLAG-tagged Ets-1 and myc-tagged versions of full length αB1 or αB1 deletion 292–451, lacking interaction epitopes EI and EII. After immunoprecipitation of Ets-1 complexes with an anti-FLAG antibody and SDS–PAGE, co-precipitated αB1 protein was detected by Western blotting with an anti-myc antibody. As shown in Figure 5, the full length αB1 was co-immunoprecipitated by Ets-1, whereas αB1 deletion 292–451 was not.

Together these results support the conclusion that regions EI, partly overlapping with the RUNT domain, and EII, located within the NRDB domain of αB1, mediate binding of αB1 to Ets-1 *in vitro* and *in vivo*.

The EII domain of αB1 is required for cooperative DNA binding with Ets-1

Since we identified two domains in αB1 that interact with Ets-1, we tested C-terminally truncated αB1 proteins, containing either both EI and EII domains or only domain EI, for cooperative DNA binding with Ets-1. As observed before, αB1 and Ets-1 cooperated for DNA binding on Tβ3 both in the absence and presence of the β protein (Figure 6A, lanes 1–4). Deletion 1–292 of αB1, which contains both domains EI/RUNT and EII/NRDB, bound to DNA only very weakly by itself (Figure 6A, lane 5) but did so strongly in the presence of Ets-1 (lane 6). The DNA binding ability of mutant 1–292 was also strongly activated by the β protein (Figure 6A, compare lanes 5 and 7), as expected from previous studies (Kanno *et al.*, 1998). Mutant αB1 (1–183), which lacks EII/NRDB, bound to DNA on its own (Figure 6A, lane 9) although more weakly than αB1 (50–183) (see Figure 6B, lane 5). However, in contrast to mutant 1–292, mutant 1–183 was defective in forming a complex with Ets-1 both in the absence or presence of the β protein (Figure 6A, lanes 9–12).

Figure 6B shows the results of similar experiments with αB1 mutants 50–292 and 50–183 that lack the first 49 amino acids. The results were essentially identical to those

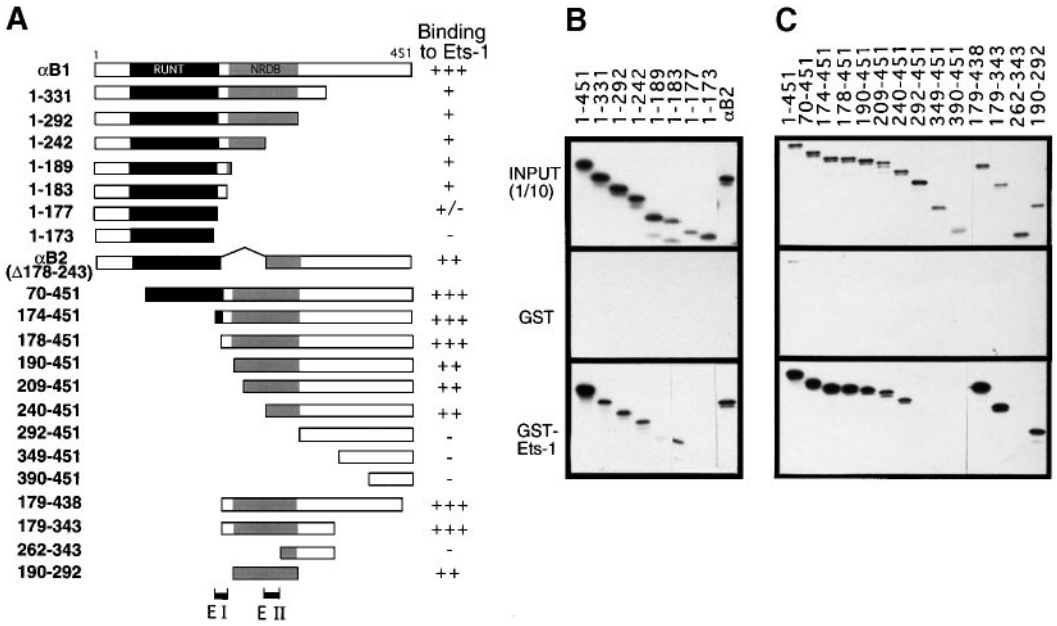

Fig. 4. Identification of αB1 regions essential for interaction with Ets-1. (**A**) Structure of αB1 constructs and of αB2. Amino acid numbers of encoded proteins are indicated for each construct. Degrees of interaction observed in (B) and (C) are indicated on the right (the binding affinity of αB1 to Ets-1 is considered 100%; +, ++, +++ represent 1–20%, 21–70%, >71%, respectively). EI and EII indicate domains that are involved in interaction with Ets-1. (**B** and **C**) GST pull-down assays showing the interaction between αB1 and Ets-1, using the deletion constructs shown in (A). INPUT (1/10), 10% of ^{35}S-methionine-labeled proteins used for the assay were applied. GST, precipitates obtained with GST control. GST–Ets-1, precipitates with GST–Ets-1.

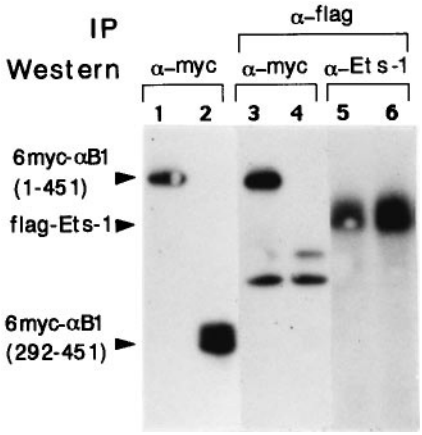

Fig. 5. Co-immunoprecipitation of αB1 and Ets-1. Cos-7 cells were transfected with the expression plasmids for FLAG-Ets-1 and 6myc-αB1(1–451) (lanes 1, 3 and 5) or FLAG-Ets-1 and 6myc-αB1(292–451) (lanes 2, 4 and 6). The untreated cell extracts were subjected to Western blotting with anti-myc antibody (lanes 1 and 2). Ets-1 complexes were precipitated from the same extracts with an anti-FLAG-antibody followed by Western blotting with anti-myc antibody (lanes 3 and 4). The filter was then reprobed by polyclonal anti-Ets-1 antibody (lanes 5 and 6). The additional two lower molecular weight bands in the lanes 3 and 4 represent mouse immunoglobulin heavy chains.

obtained with mutants 1–292 and 1–183 (Figure 6A). However, the increased DNA binding capacity of 50–183 relative to 1–183 suggests that the region located N-terminally to the RUNT domain also contributes to the inhibition of the DNA binding activity of the αB1.

These results indicate that the interaction of Ets-1 with EII of αB1 is required for activation of its DNA binding capacity.

Natural variants of αB1 confirm that domain EII is necessary and sufficient for cooperative DNA binding with Ets-1

We previously described a splice variant of αB1 termed αB2 which has an intact domain EII but lacks 64 aa of αB1 situated immediately C-terminally to the RUNT domain (Bae *et al.*, 1994). This deletion (see also Figure 1) removes a part of domain EI, whose presence is important for strong interaction with Ets-1 (Figure 4). Another variant, αC, lacks an exon equivalent to exon 5 of αA and αB (Bae *et al.*, 1995). As a result, the αC protein lacks the 54 aa region corresponding to the region harboring domain EII of αB1. These two PEBP2α proteins can therefore be regarded as natural variants containing deletions in the two different Ets-1 interaction regions. We therefore investigated the individual contributions of these regions to the cooperativity of αB1 with Ets-1. For this purpose, EMSAs on Tβ3 were performed with the two variants in the presence of increasing amounts of Ets-1. As shown in Figure 7A, there was a stronger cooperativity with αB2 than with αB1 when increasing amounts of Ets-1 were added (compare lanes 2–6 and 8–12 and panel on the right for quantification). The complementary experiment performed with αC (Figure 7B) showed that the DNA binding activity observed with αC was only weakly enhanced by increasing amounts of Ets-1 compared with αB1 (compare lanes 2–6 and 8–12 and panel on the right for quantification).

These results indicate that, although both αB2 and αC can interact with Ets-1, αB2 is much more effective than αB1, whereas αC is significantly less able to cooperate with Ets-1 in DNA binding. This supports the conclusion that domain EII but not EI is primarily responsible for

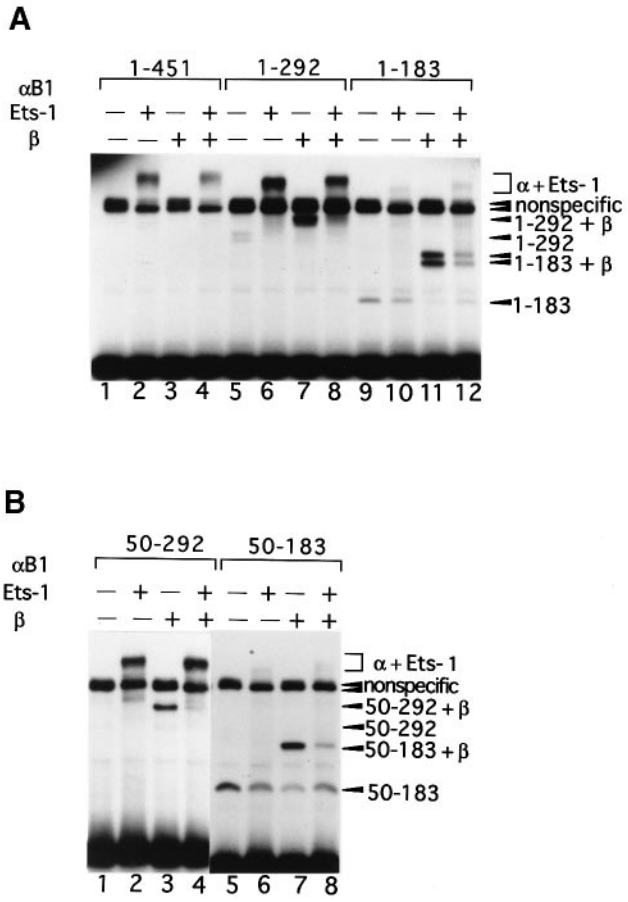

Fig. 6. Cooperative DNA binding of Ets-1 with αB1 deletion mutants. (**A**) EMSA was performed using probe 1 (wild-type tβ3) and 1 fmol of *in vitro*-translated αB1, αB1(1–292) or αB1(1–183) together with 7 fmol of Ets-1 and/or 20 fmol of β. Positions of protein–DNA complexes are indicated on the right. (**B**) *In vitro*-translated αB1(50–292) or αB1(50–183) was subjected to EMSA under the same conditions as in (A).

activating DNA binding capacity through the interaction with Ets-1.

αB1 interacts with several regions of Ets-1 including the ETS and exon VII domains

To determine the regions in Ets-1 that interact with αB1, a series of Ets-1 deletion mutants was constructed and fused to GST. These mutants were tested against full-length αB1 and deletions containing only EI or EII. A schematic drawing of the constructs is shown in Figure 8A. As shown in Figure 8B and C, and summarized in Figure 8A, N-terminal deletions of Ets-1 up to aa 238 still interacted with all αB1 proteins tested. Further deletion up to aa 333 abolished interaction with the αB1 mutant 190–451 containing only EII but not EI. By contrast, interaction with αB1 mutants 1–183 and 50–183 containing EI but not EII was still maintained. The reason why Ets 331–441 fails to interact with the full-length αB1 may be that the EI is masked by the NRDB of αB1. The Ets-1 deletions 365–441 and 333–418 but not 420–441 were able to bind αB1 50–183, suggesting that a region in the C-terminal two-thirds of the ETS domain is responsible for the interaction of αB1 with EI. This contact region was designated PEBP2 binding site I, or PI. In addition, Ets construct 238–328 bound only αB1 proteins containing EII (1–451, 190–451), but not αB1 constructs harboring only region EI (1–183, 50–183), suggesting that an epitope in this region interacts with EII but not with EI. This site was designated PII. Finally, we observed a weak interaction of an N-terminal region of Ets-1 (124–236) with all αB1 mutants, which was designated PIII.

These results indicate that αB1 and Ets-1 bind to each other at two independent contact points, via EI/PI binding sites in their DNA binding domains and via EII/PII in their autoinhibitory domains (NRBD and exonVII, respectively). Additional, albeit generally weaker, interactions were also found with the more N-terminal PIII region of Ets-1.

The PII/exon VII domain of Ets-1 is essential for cooperative DNA binding with αB1

To examine whether both PI and PII regions are required to activate αB1 DNA binding, two N-terminally truncated Ets-1 derivatives either containing both PI and PII (238–441) or only PI (333–441) were tested in EMSA assays in combination with αB1(1–292) which contains EI and EII and is thus fully competent to affect cooperative DNA binding with Ets-1 (see above; Figure 9). Ets (333–441) by itself bound to DNA significantly better than Ets 238–441, which contains both the exon VII and C-terminal autoinhibitory domains (data not shown). When a constant amount of αB1 (1–292) was mixed with increasing amounts of mutant Ets proteins, Ets 238–441 (which contains exon VII/PII) stimulated DNA binding of αB1 (1–292) far more efficiently than Ets 333–441, despite the fact that Ets 333–441 (which lacks exon VII/PII) by itself bound to DNA much more strongly than Ets 238–441 (lanes 7 and 13).

These results confirm that the exon VII/PII region is required for autoinhibition of Ets-1 and indicates that it is essential and independent of additional Ets-1 sequences for stimulation of αB1 DNA binding.

Cooperative transactivation of the TCRβ enhancer by αB1 and Ets-1 requires both EI and EII interaction regions

To analyze whether the observed cooperation between αB1 and Ets-1 for DNA binding is also reflected in a synergism for transactivation and to determine the contribution of the different αB1/Ets-1 interaction domains, we performed cotransfection assays with the TCRβ enhancer using P19 embryonal carcinoma cells, which show virtually no basal activity for this reporter. Transfection of increasing amounts of Ets-1 expression plasmid by itself did not show a significant activation of the reporter, whereas in the presence of a fixed amount of full length αB1, reporter activity increased in a dose-dependent manner (Figure 10A). A similar but slightly weaker response was also observed in the presence of αB1(1–292), containing EI and EII, but lacking the C-terminal 160 aa. This suggests that, whereas αB1 (1–292) is sufficient for cooperative DNA binding with Ets-1, the region C-terminal to aa 292 also makes a small contribution to the full transactivation activity of the αB1/Ets-1 complex.

To test the individual contribution of domains EI and EII to cooperative transactivation with Ets-1, we compared the natural αB variants αB2 and αC in the P19 cell

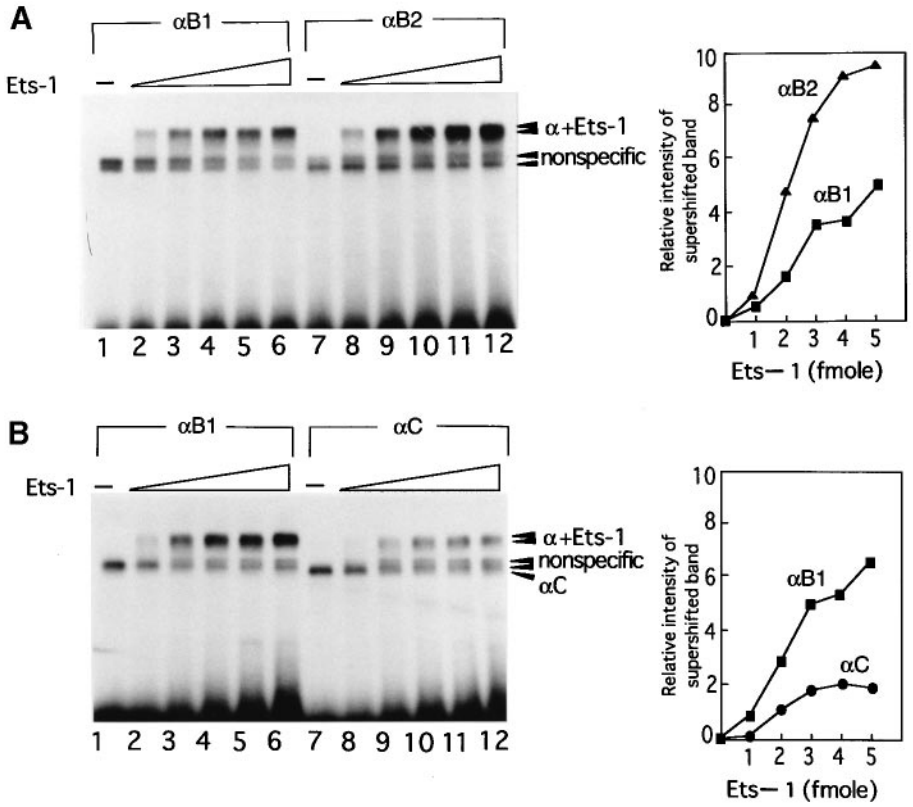

Fig. 7. Cooperative DNA binding of Ets-1 with αB2/αC. (**A**) One femtomole of *in vitro*-translated αB1 (lanes 1–6) or αB2 (lanes 7–12) was mixed with increasing amounts of *in vitro* translated Ets-1 (0, 1, 2, 3, 4, 5 fmol) and were subjected to EMSA using probe 1 (wild-type tβ3). (**B**) One femtomole of *in vitro*-translated αB (lanes 1–6) or αC (lanes 7–12) was mixed with increasing amounts of Ets-1 and assayed as above.

transactivation assay. As shown in Figure 10B, neither of these two proteins exhibited significant activities by themselves and cooperated poorly with Ets-1. Similar results were obtained in transactivation assays in Jurkat T cells (data not shown; Kanno *et al.*, 1998).

The puzzling observation that the lack of EI in αB2 did not prevent cooperative DNA-binding (see Figure 7) but synergistic transactivation suggested that the EI/PI interaction did not play a role in recruitment of Ets-1 but in the activation of its transactivation function. The most plausible explanation would be that the interaction of the DNA binding domains through EI/PI induces a conformational change resulting in the appropriate presentation of the Ets-1 transactivation domain to the basal transcriptional machinery. To pursue this hypothesis further, we fused the transactivation domain of VP16 to the N-terminus of Ets-1, thus generating a molecule with constitutive transactivation function. This construct was transfected in CV-1 cells together with an αB1 or αB2 expression plasmid and a reporter containing the Tβ3 enhancer sequences used in the EMSAs (Figure 7). CV-1 cells were chosen, because in these cells the TCRβ reporter was not activated by wild-type Ets-1 and αB1 proteins (data not shown), ensuring that all observed transactivation activity would be due to the heterologous VP16 transactivation domain. As shown in Figure 10C, both αB1 and αB2 strongly synergized with VP16-Ets-1 in transactivation of the reporter. VP16-Ets-1 by itself showed no activity (data not shown) and a mutation of the PBS in the reporter completely abolished transactivation (Figure 10C), indicating that the VP16 fusion did not enable αB1-independent binding of Ets-1 to the EBS. These data thus confirm the EMSA results (Figure 7) that αB2 can recruit Ets-1 to the Tβ3 enhancer. In addition, they emphasize that the αB1/Ets-1 complex formation not only stimulates DNA binding but also appears to activate the transactivation function of Ets-1 through the PI/EI contacts. The exact molecular detail of this mechanism remains to be determined.

Endogenous PEBP2 and Ets proteins cooperate on the TCRβ enhancer in T cells

To investigate whether endogenous PEBP2 and Ets proteins cooperate on the TCRβ enhancer *in vivo*, we examined the effects of dominant interfering molecules of αB1 and Ets-1. Overexpression of the Runt domain of αB1, which lacks a transactivation domain but is able to bind to PBS, strongly inhibited the TCRβ3,4 activity in Jurkat T cells (Figure 11A). Likewise, an N-terminally truncated Ets-1, containing only the ETS DNA binding domain and no transactivation domain, also strongly inhibited the activity of the TCRβ3,4 reporter (Figure 11B). Both these molecules bind strongly to DNA by themselves but do not cooperate with Ets-1 or αB1, respectively (Figures 6 and 9). Therefore, our results strongly suggested that endogenously expressed PEBP2 and Ets proteins cooperatively supported the TCRβ3,4 activity.

To examine the significance of the PII/EII interaction between the autoinhibitory exon VII and NRDB domains for the cooperation of the endogenous proteins, we overexpressed the exon VII domain in Jurkat T cells together with the Tβ3,4 reporter. The domain was tagged with a GAL4 DNA binding domain to assure nuclear localization.

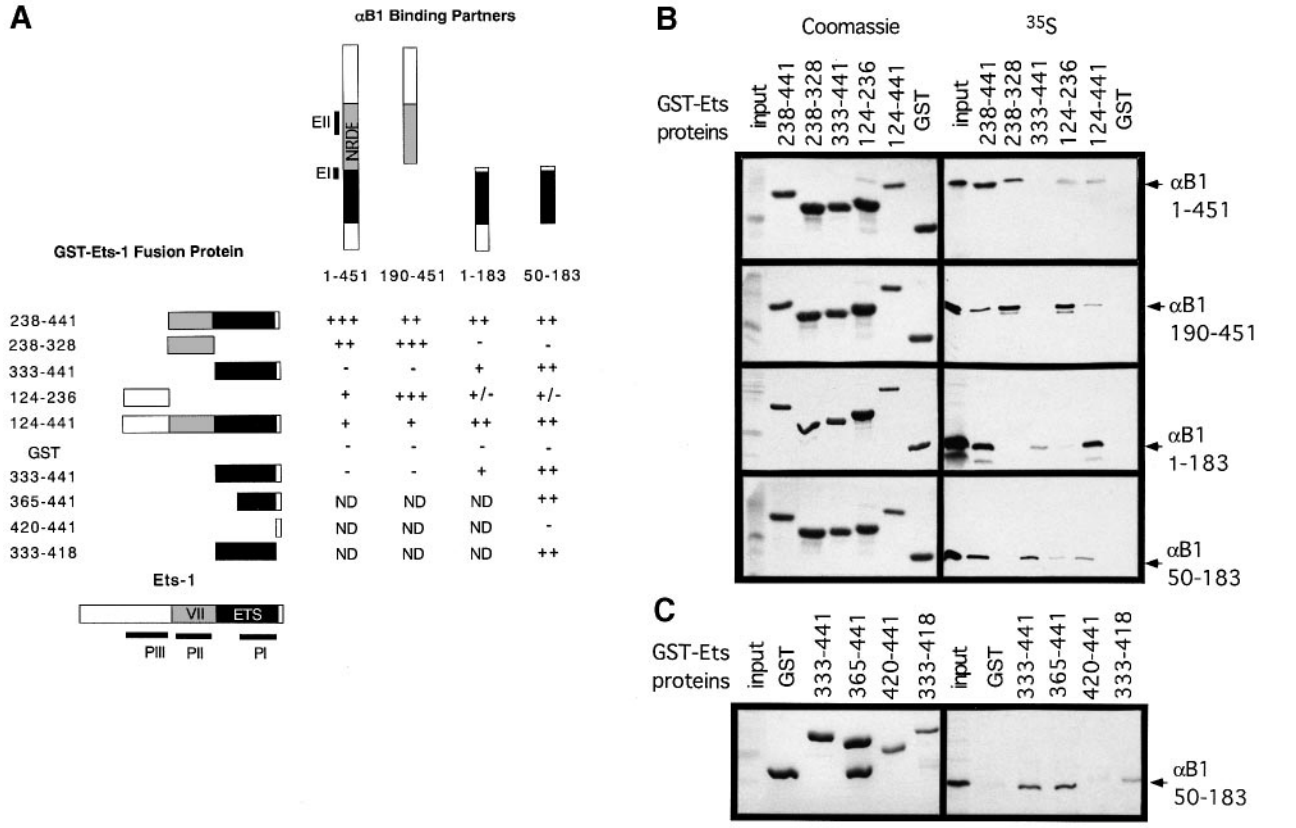

Fig. 8. Mapping of regions in Ets-1 involved in interaction with αB1. (**A**) Diagram of Ets and αB1 deletion derivatives, indicating the first and last amino acids. A summary of the results from (B) and (C) is shown in the table in (A). Degrees of interaction are indicated as described in the legend of Figure 4. (**B** and **C**) Deletion analysis of Ets-1. *In vitro*-translated ^{35}S-methionine-labeled full-length αB1 (1–451) or deletion mutants containing only domain EI (1–183 and 50–183) or domain EII (190–451) were incubated with affinity matrix-bound GST–Ets proteins (indicated on top), washed, resuspended in SDS sample buffer and analyzed by SDS–PAGE. Coomassie Blue staining (left) and autoradiography (right) of the same SDS–PAGE gels are shown.

As shown in Figure 11C, this fusion construct but not the GAL4 DNA binding domain alone had a strong repressive effect on the Tβ3,4 enhancer. This indicates that the exon VII domain can act as a dominant interfering molecule by preventing the EII/PII contacts and thus cooperative DNA binding of αB1 and Ets-1. Similar observations have been made previously with an analogous αB1 construct (aa 209–453) which lacked the DNA binding domain but contained EII of the NRDB (Figure 9D and E in Zhang *et al.*, 1997). This construct but not one lacking the EII region (aa 283–453) strongly interfered with endogenous PEBPα/Ets activity on a TCRβ3,4 reporter in T cells (Figure 9D and E in Zhang *et al.*, 1997).

Together, these results provide *in vivo* evidence that the direct interaction between the autoinhibitory domains of Ets-1 and αB1 is critical for cooperative DNA binding.

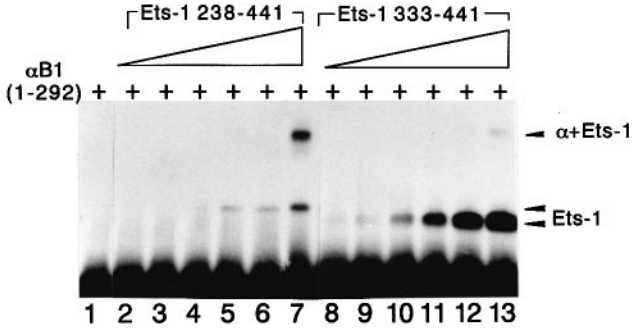

Fig. 9. Effects of N-terminal truncation of Ets-1 on cooperation with αB1. Three femtomoles of *in vitro*-translated αB1(1–292) were incubated with increasing amounts of purified Ets 238–441 or Ets 333–441 and EMSA was performed. The highest protein amount corresponds to ~1 pmol; 2-fold dilutions were used in the subsequent samples.

Discussion

The transcription factors PEBP2αB1 (AML1/CBFA2) and Ets-1 are both autorepressed for DNA binding in a remarkably similar fashion. As schematically shown in Figure 12A and B, in both cases the molecules are kept in a conformation disfavoring DNA binding by inhibitory domains (NRDB and the N-terminus for αB1; exonVII and the C-terminus for Ets-1) which are flanking the respective DNA-binding domains (RUNT and ETS). Here we show that the direct interaction of the two proteins mutually activates DNA binding of a composite PBS/EBS element. Our finding that this critically depends on *inter*molecular contacts between autoinhibitory domains of Ets-1 (exonVII) and αB1 (NRDB) suggests a model in which complex formation induces a conformational change that relieves their repressive effect (Figure 12C). This represents a novel mechanism of transcription factor regulation in cell type-specific gene expression that links activation of DNA binding to the generation of new combinatorial DNA binding specificities.

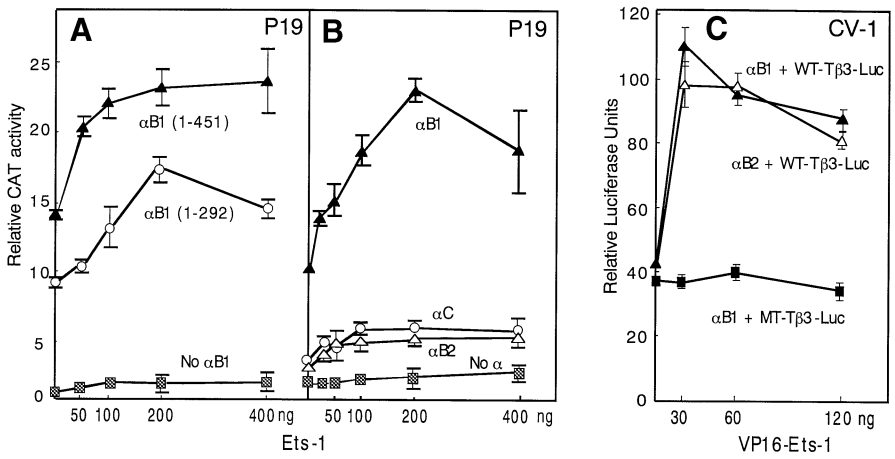

Fig. 10. Functional cooperation between Ets-1 and various αB1 constructs. (**A**) 0.5 μg of αB1 or αB1(1–292) expression plasmid was cotransfected into P19 cells with increasing amounts of Ets-1 expression plasmid as indicated, together with 1 μg of CAT reporter plasmid and the CAT activity was determined. The results shown are the mean ± SD of three independent experiments. (**B**) 1 μg of αB1, αB2 or αC expression plasmid was cotransfected with increasing amounts of Ets-1 expression plasmid as indicated together with 1 μg of the CAT reporter plasmid into P19 cells as above. (**C**) 0.4 μg of the plasmid TCRβ3Luciferase [wild-type (WT-Tβ3-Luc) or PEBP2 site mutant (MT-Tβ3-Luc)] were transfected into CV-1 cells with the indicated expression plasmids for αB1 or αB2 and increasing amounts of VP16-Ets-1 expression plasmid and a constant amount of pEFPEBP2β. Luciferase units relative to an internal control, pRLEF, were calculated. Data are the mean ± SD of three independent experiments.

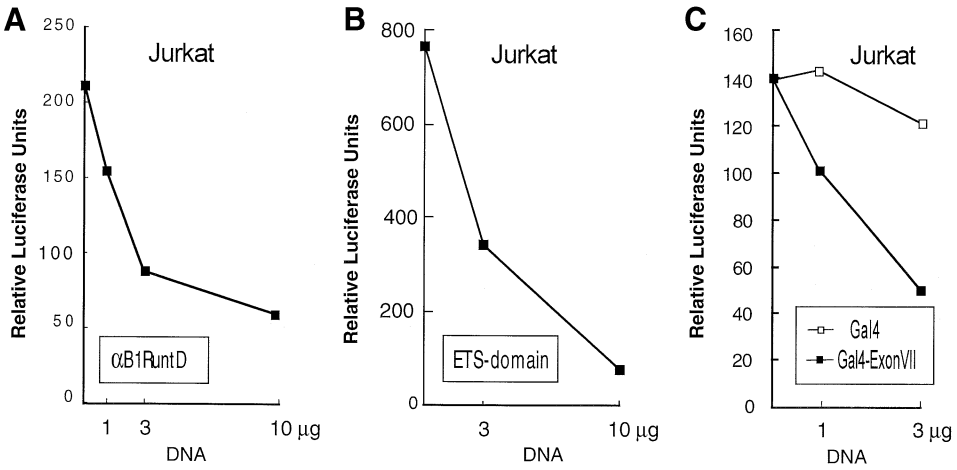

Fig. 11. Inhibition of TCRβ enhancer activity in Jurkat T cells by interference with the interaction of endogenous αB1 and Ets-1 proteins. Jurkat cells were transfected with 4 μg of TCRβLuc and increasing amounts of either the Runt domain of αB1 (αB1 Runt D) by electroporation (**A**) or the ETS domain of Ets-1 (Ets-1 333–441) by lipofection (**B**). (**C**) Jurkat cells were transfected with 4 μg of TCRβLuc and increasing amounts of either GAL4-exonVII or GAL4 by electroporation. Luciferase units were calculated relative to internal controls (pRLEF or pRSVβgal).

Mechanism of mutual activation of Ets-1 and PEBP2αB1

It has been proposed that autoinhibition of Ets-1 DNA binding is regulated by an allosteric mechanism (Jonsen *et al.*, 1996) which involves conformational changes in its autoinhibitory domains (Petersen *et al.*, 1995). The molecule is assumed to be in an equilibrium between a favored unbound state and a disfavored DNA-bound state. Juxtaposition of the autoinhibitory exon VII and C-terminal helix domains with the ETS domain is thought to maintain the molecule in a stressed conformation (Skalicky *et al.*, 1996) which has to be relieved for DNA binding. This can be artificially achieved by deletion or mutation of either the N- or C-terminal inhibitory regions (Hagman *et al.*, 1992; Lim *et al.*, 1992; Nye *et al.*, 1992; Wasylyk *et al.*, 1992). However, how the derepressed state can be stabilized under normal physiological conditions of Ets-1 activation is completely unresolved so far.

Here we have identified αB1 as a partner protein that appears to serve exactly this function. Our data suggest that the direct binding of αB1 to the exon VII domain of Ets-1 activates DNA binding. The simplest explanation in the context of the allosteric model is that this protein–protein interaction can stabilize the relaxed DNA-bound conformation of Ets-1 as shown in the model in Figure 12. Indirect support for such a model also comes from structural studies of the related ETS family protein GABPα. The C-terminal helix of GABPα (which has homology to the inhibitory C-terminal helix of Ets-1) makes contacts with the heterotypic partner molecule GABPβ in a DNA-bound ternary complex (Batchelor *et al.*, 1998). In this conformation, the C-terminal helix does not pack against the ETS domain (Batchelor *et al.*, 1998) as is the case for the C-terminal helix of Ets-1 in the unbound conformation (Donaldson *et al.*, 1996; Skalicky *et al.*, 1996). This then suggests that a DNA-bound conformation of Ets proteins can be stabilized through protein interactions with inhibitory regions, either

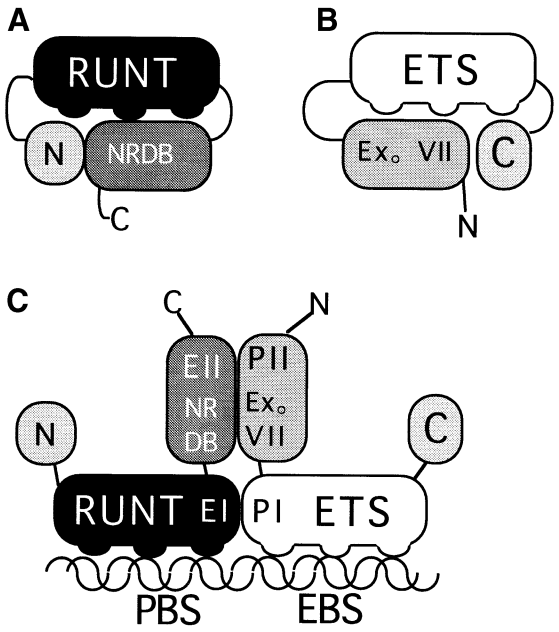

Fig. 12. Model showing cooperation between αB1 and Ets-1 in DNA binding. RUNT and ETS: DNA-binding domains. EI and EII, Ets-1 interaction domains of αB1; PI and PII, αB1 interaction domains of Ets-1; NRDB, negative regulatory domain for DNA binding of αB1; Ex VII, the region of Ets-1 encoded by exon VII; N and C, N- and C-terminal inhibitory domains of the proteins; PBS, PEBP2 binding site; EBS, Ets binding site. For simplicity, the PIII region of Ets-1 has been omitted.

with the C-terminus in the case of GABPα or with the exon VII domain in the case of Ets-1.

Interestingly, Ets-1 does not appear to serve merely a passive role in this partnership, but conversely also activates αB1 DNA binding in a completely analogous fashion. Also, in the case of αB1, DNA binding of the full-length molecule is inhibited, but can be activated by deletion of the autoinhibitory NRDB domain (Kanno *et al.*, 1998; this study). Whether this is based on a steric or allosteric mechanism is not resolved. Regardless of this, interaction with Ets-1 appears to stabilize a conformational change that relieves the repression. Again it is the interaction of the PII region in the exon VII domain of Ets-1 with the EII region in NRDB of αB1 that is necessary for this activation: PEBP2α molecules lacking EII are defective in forming a complex with Ets-1 and an Ets-1 mutant lacking exon VII/PII equally fails to recruit αB1 into a complex, despite additional contact points between the two proteins (see below).

The two partners appear to form an intimate complex which involves additional interaction surfaces besides the critical exonVII/NRDB contacts. Thus, we also found a strong interaction between EI bordering the RUNT and PI in the ETS DNA binding domains, which was, however, neither sufficient nor necessary for cooperative DNA binding. In contrast, deletion of the EI contact region in αB1 abolished synergistic transactivation with Ets-1. The mechanistic basis for this is not clear, but it is possible that a conformational change stabilized by the EI/PI interaction is transmitted to the transactivation domains. Alternatively, this region might be an important binding site for coactivators. That cooperative DNA binding and synergistic transactivation can be functionally separated is also supported by results from other interaction partners of αB1. Thus, αB1 interacts and synergizes with the bZip transcription factor C/EBPα and the Ets family member PU.1 in transactivation of the macrophage colony stimulating factor receptor promoter (Hohaus *et al.*, 1995; Zhang *et al.*, 1996; Kanno *et al.*, 1998) but does not display mutual cooperative DNA binding with these proteins (T.Kanno and Y.Ito, unpublished observation). This is consistent with the fact that the interaction with C/EBPα involves different domains (T.Kanno and Y.Ito, unpublished observation) and that the Ets protein PU.1 lacks a region that is equivalent to the exon VII domain in Ets-1 (Klemsz *et al.*, 1990) which we found to be critical for cooperative binding with αB1.

In addition, we also detected binding of the N-terminal Ets-1 region PIII to both the NRDB and Runt domains, which was, however, significantly weaker than the other contacts reported here. This is consistent with previous results obtained with the close runt family relative, PEBP2αA (Giese *et al.*, 1995). That no EI/PI interaction between the DNA-binding domains was observed in that study could be due to the differences in experimental design.

In summary, multiple contacts appear to contribute to the formation of an intimate complex between αB1 and Ets-1, among which, however, the interaction between the exonVII/PII and NRDB/EII domains is essential for mutual activation of DNA binding, probably by inducing a conformational change of these autoinhibitory domains that relieves their repressive effect (Figure 12).

Other transcription factor activation mechanisms

The activity of transcription factors involved in tissue-specific gene regulation is generally tightly regulated and multiple signaling mechanisms have developed to stimulate an inactive factor, either by enhancing DNA binding or by inducing the transactivation potential. Activation of DNA binding can be achieved by a variety of mechanisms including the destruction of inhibitory proteins which keep the transcription factors in the cytoplasm [such as for NF-κB (Baeuerle *et al.*, 1988) and NFAT (Ruff and Leach, 1995)], or the modification of the DNA-binding domain (p53; Wang and Prives, 1995). Furthermore, several transcription factors besides Ets-1 and αB1 have been found to have negative regulatory domains which repress DNA binding, for example c-Myb. In this case, a broadly expressed protein named p100 can bind to the same surface of the Myb DNA binding domain as the negative regulatory region, suggesting that it may be involved in the regulation of DNA binding (Dash *et al.*, 1996). Compared with these examples as well as with the interaction of αB1 with its β subunit, the αB1/Ets-1 interaction is unique in that it involves the direct intermolecular interaction between the negative regulatory domains of two sequence-specific DNA binding proteins. This suggests direct consequences for their biological function.

Biological consequences of αB1/Ets-1 complex formation

Ets-1, αB1 and its β subunit are all highly expressed in T cells. The coexpression of these proteins could constitute a simple mechanism to show that the activation of DNA binding is linked to the recognition of new composite DNA-

binding elements that are characteristic of T cell-specific enhancers. Indeed, clustered EBS and PBS binding sites are found in the T cell-specific TCRα, TCRβ and MuLV enhancers. The requirement of specific composite DNA elements for DNA binding of the complex appears to be very strict. Even though a ternary complex between Ets-1, αB1 and the β subunit can be detected in the absence of DNA (unpublished results) mutations of either the PBS or EBS half sites in the TCRβ enhancer abolish DNA binding by both Ets-1 and αB1. This suggests that either an activated complex can only bind to a composite element or that specific contacts of the complex with both DNA elements directly contributes to the activation mechanism itself. In either case, the clustering of PBS and EBS must be an important mechanism that contributes to T cell-specific gene expression. The knockout of Ets-1 shows a phenotype in T cells in chimeric RAG-2-deficient mice (Bories *et al.*, 1995; Muthusamy *et al.*, 1995). Since both αB1 and β knockout mice completely lack definitive hematopoiesis (Okuda *et al.*, 1996; Sasaki *et al.*, 1996; Wang *et al.*, 1996a,b; Niki *et al.*, 1997), the analysis of an αB1 function in the T cell compartment is not possible. This has to await similar studies with chimeric RAG-2-deficient mice to determine whether their phenotype further supports the functional cooperation of Ets-1 and αB1 in T cells.

Both αB1 and Ets-1 could have additional as yet unidentified activation partners with different DNA binding specificities. This would then result in the recognition of new composite elements which may be defining for enhancers of other lineages. The possibility for such exchange of partner molecules may be a significant determinant of changing enhancer activities during differentiation (Sieweke and Graf, 1998).

Evolutionary conservation of interaction epitopes and natural variants of Ets-1 and αB1

The significance of the EII and PII interaction regions, contained in the NRDB and exon VII negative regulatory domains of αB1 and Ets-1, respectively, is underscored by the high conservation of these domains during evolution and between different isoforms, as well as by the existence of natural variants that lack these regions.

Thus, the amino acid sequence of domain EII is highly conserved between mouse αB (Bae *et al.*, 1993) and mouse αA (Ogawa *et al.*, 1993b), chicken αB (Castagnola *et al.*, 1996) and sea urchin Runt –1 (Coffman *et al.*, 1996; data not shown). Furthermore, a region that is significantly homologous to the EII domain is also found in the N-terminal region of the *Drosophila lozenge* gene (Daga *et al.*, 1996; data not shown). Interestingly, the *Drosophila* Ets family protein Pointed P2 has been shown to have a region inhibitory for DNA binding activity located on the N-terminal side of the Ets domain (Wasylyk *et al.*, 1997). Hence, a similar intimate relationship may exist between *pointed* and *lozenge*, a suggestion supported by the fact that both genes are involved in complex eye development (Klambt, 1993; Brunner *et al.*, 1994; Daga *et al.*, 1996).

Several natural isoforms exist for both αB1 and Ets-1 which have modifications in the domains involved in the interaction between the two proteins. Thus αB2, a splice variant of αB1, lacks Ets-1 interaction domain EI but contains domain EII. Even though it forms a cooperative DNA binding complex with Ets-1 more efficiently than αB1, the Ets-1/αB2 complex does not stimulate transcription. This suggests that αB2 can act as an effective negative regulator of αB1 function. On the other hand, the product of the αC gene lacks the EII region in the NRDB and thus does not cooperate with Ets-1 in DNA binding. It may, however, act as a sink for other partner molecules of PEBP2α which then would not be available for αB1 interaction and thus favor the formation of αB1/Ets-1 complexes. It could also be involved in the activation of different sets of genes containing PBS elements not clustered with EBS sites.

A natural splice variant of Ets-1, ΔexonVII, which lacks the αB1 interaction site PII, binds DNA with much higher affinity than full length Ets-1 (Wasylyk *et al.*, 1992) and loses mutual cooperation with αB1 on the composite PBS/EBS in the Tβ3 enhancer (data not shown). By preventing access of full length Ets-1 and thus cooperation with αB1 on composite elements, exonVII-less Ets-1 may therefore serve as an effective negative regulator of αB1/Ets-1 complexes.

The expression profiles of the isoforms are partially overlapping and the exact balance of their ratios would determine the read-out from composite PBS/EBS as well as from individual PBS or EBS. The existence of these isoforms thus diversifies the regulatory mechanisms of gene expression through these elements.

The significance of the interaction regions for protein function is further underscored by the fact that the leukemogenic versions of αB1 (AML1) and Ets-1 bear mutations that should affect the regulation of DNA binding and protein complex formation. Several translocations of the αB1 (AML1) gene generating chimeric proteins, such as AML1/ETO(MTG8) and AML1/EVI-1, have been found in human myeloid leukemia, all of which cause a deletion of the NRDB and the EII interaction surface (Ito and Bae, 1997). Similarly, the viral version of Ets-1 contained in the leukemogenic chicken virus E26 bears a mutation in the inhibitory C-terminal helix. It is also constitutively activated and does not require a cofactor for DNA binding (Hagman *et al.*, 1992; Lim *et al.*, 1992). The escape from the regulatory mechanisms that have evolved to tightly control the DNA binding activity of these transcription factors may thus be a major factor contributing to the leukemogenic potential of their mutant versions.

Materials and methods

Plasmids

A series of point mutations in the PEBP2 and Ets-1 binding sites of the TCRβ enhancer 3 and 4 (Gottschalk and Leiden, 1990) were made using standard PCR methods and the resulting constructs were inserted into the *Bam*HI site of pBLCAT2 (Luckow and Schüts, 1987). pTβ3W4WtkCAT was described previously (Ogawa *et al.*, 1993b). The mutants of the constructs, M1 to M6 shown in Figure 2, have mutations in either the PEBP2 or Ets site, or both as indicated for the EMSA probes 2 and 3 (see below). Luciferase expression reporter plasmids TCRβ3WTLuc and TCRβ3MTLuc were made by inserting the wild-type and PEBP2 site mutated TCRβ3 fragment into the *Bam*HI site of pBLTKLuc (Forman *et al.*, 1995). TCRβLuc was described elswhere (Zhang *et al.*, 1997). As an internal control vector, we used pRLEF, generated by an insertion of the promoter region of pEFBos into the *Renilla* Luciferase expression vector pRL (Promega).

pEFαB1 (Bae *et al.*, 1994), pEFαB2 (Bae *et al.*, 1994) and pEFαC1 (Bae *et al.*, 1995) were described. pEFαB1(1–183), pEFαB1(1–292), pBSKαB1(1–173), pBSKαB1(1–177), pBSKαB1(1–183), pBSKαB1

(1–189), pBSKαB1(1–292) and pBSKαB1(70–451) were constructed by introducing large deletions using site-directed mutagenesis to create the coding regions indicated by the amino acid numbers in parentheses. pCITEαB1(174–451), pCITEαB1(178–451), pCITEαB1(190–451), pCITEαB1(209–451), pCITEαB1(240–451), pCITEαB1(292–451), pCITEαB1(349–451), pCITEαB1(390–451), pCITEαB1(262–343) and pCITEαB1(190–292), pCITEαB1(179–438), pCITEαB1(179–343) were constructed by PCR or restriction enzyme digestion and inserted into pCITE (Novagene) to give the coding regions indicated by the amino acid numbers in parentheses. All constructs made by using PCR were verified by DNA sequencing. pSG-ets1 carries the 1.4 kb fragment including the whole coding region of human c-ets1 at the *Bam*HI site of pSG5 (Stratagene). *Bam*HI fragments of pGEX-ets-1 (Giese *et al.*, 1995) which have the mouse ets-1 coding region were transferred to pCITE to make pCITEets-1. The complete coding sequence of mouse Ets-1 was inserted into pCMXVP16 (Willy *et al.*, 1995) to make pCMX-VP16-ets-1. The exon7 region of Ets-1 was amplified from pGEX-ets-1 by PCR and inserted into pCMX-Gal4 (Willy *et al.*, 1995). pCDNA6myc-αB1(1–451), pCDNA6myc-αB1(292–451) and pCDNAflag-Ets-1 were constructed by inserting the tagging sequences and coding sequences into pCDNA3.1 (Invitrogen).

Chicken Ets proteins were cloned into the GST fusion vector pGEX-2T (Pharmacia) using standard protocols. The amino acid junctions of the GST vector with the Ets-1 sequences (in bold type) from chicken $p54^{Ets\text{-}1}$ are: construct 124–236, GSPHMLSGSPGI**ILW**; construct 238–441, GSPH**MGR**; construct 238–328, GSPHKF**SRG**; construct 333–441, GSPHMLSGSM**GPI**; construct 365–441, GSPH**KLS**; construct 420–441, GSPHMLSGS**SLL**; construct 333–418, GSPHMLSGSM**GPI**. Construct 124–441 contains sequences from mouse Ets-1 cDNA in pGEX-3X (Pharmacia) with the following junction: IEGRGIP**ILW**. The first and last amino acids of Ets-1 present in the constructs are shown in Figure 8. The dominant-negative forms of Ets-1 (Ets-1 333–441; equivalent to His_6/Ets-DBD) and PEBP2α were described earlier (Kanno *et al.*, 1998; Sieweke *et al.*, 1998).

Protein–protein interaction and EMSA

β2, GST-ets-1 and GST-ets-1 deletions were expressed and purified as described previously (Ogawa *et al.*, 1993a, Giese *et al.*, 1995, Sieweke *et al.*, 1996, respectively). N-terminally truncated, His-tagged chicken $p54^{ets}$, Ets-1(238–441) and Ets-1(333–441) were synthesized and purified similarly (Sieweke *et al.*, 1998). *In vitro* transcription and translation was done using the TNT system (Promega) in the presence of ^{35}S-methionine. The product was analyzed by SDS–polyacryamide gel electrophoresis (PAGE) followed by autoradiography. The quantity of synthesized proteins was calculated using a Phospho-Imaging analyzer (Fuji) and a scintillation counter. GST pull-down assays were performed using GST-ets-1 and GST-bound glutathion agarose (Pharmacia). GST-ets-1 (5 μg) or GST (10 μg) bound to glutathion agarose was mixed in 200 μl of Tris-buffered-saline pH 7.4 containing 0.3% NP-40 for 1 h and washed vigorously four times with 1 ml of the same buffer. After boiling in SDS loading buffer, they were analyzed by SDS–PAGE followed by autoradiography. The binding efficiency was calculated with a Phospho-Imaging analyzer. EMSA was performed as described previously (Bae *et al.*, 1994). For the *in vivo* interaction assays, Cos-7 cells were lysed after 48 h of transfection by freezing and thawing in phospate buffer (pH 7.2) containing proteinase inhibitors and 400 mM NaCl. A portion of the lysate was mixed with anti-flag antibody conjugated agarose (Kodak), and agarose was pelleted by centrifugation. After washing six times with 1 ml of phosphate-buffered saline, samples were separated by SDS–PAGE in nonreducing conditions followed by Western blotting using anti-myc antibody (9E10, Calbiochem) or anti-Ets-1 antibody (Santa Cruz) and the ECL detection system (Amersham).

The Tβ3 core of the TCRβ enhancer containing one PBS and one EBS or their mutated derivatives were used as probes. Their sequences are as follows: Probe 1 (wild-type): gatctaacAGGATGTGGTttgacattta, Probe 2 (PBS mutant): gatctaacAGGATGTAGAttgacattta, Probe 3 (EBS mutant): gatctaacATTATGTGGTttgacattta. Oligonucleotides were synthesized, annealed and end-labeled using T4 kinase and [γ-^{32}P]ATP. The reaction was performed in 8 μl and incubated at 25°C for 15 min. After electrophoresis in a 5 or 6% polyacrylamide gel (acrylamide:bisacrylamide, 60:1) in 0.5× TBE at 200 V for 40 min, autoradiograms were made using two layers of X-ray films. The second film shows only ^{32}P signals. The exposure time ranged from 6 to 24 h.

Cells, transfection, reporter assay

P19 cells were maintained in a cocktail of DMEM and HAM-F12(1:1) containing 10% fetal calf serum (FCS). CV-1 and Cos-7 cells were maintained in DMEM with 10% FCS. BW5147 and Jurkat cells were grown in RPMI containing 10% fetal calf serum. Transfection for BW5147 and P19 cells was performed as previously described (Ogawa *et al.*, 1993a; Bae *et al.*, 1994). Rous Sarcoma Virus (RSV)-β-galactosidase or pRL(*Renilla* Luciferase)EF were used as a internal control of transfection effeciency. Transfection of CV-1 and Cos-7 cells were performed with Fugene-6 (Boehringer Mannheim) as described in the manufacturer's instructions and transfection into Jurkat cells was carried out as described previously (Kanno *et al.*, 1998) or with Lipofectamin (Gibco-BRL) following the instructions of the manufacturer. Chloramphenicol acetyltransferase (CAT) activity and β-galactosidase activities were measured according to the standard protocol (Sambrook *et al.*, 1989). The CAT activity was calculated by a Fuji Phospho-Imaging Analyser. The Luciferase activity was analyzed as described previously (Kanno *et al.*, 1998). The Dual Luciferase System (Promega) was used according to the manufacturer's instructions to normalize for transfection efficiency.

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