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Cooperative Binding of Ets-1 and Core Binding Factor to DNA

DAVID WOTTON,[1] JACQUES GHYSDAEL,[2] SHUWEN WANG,[2] NANCY A. SPECK,[3] AND MICHAEL J. OWEN[1]*

Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, United Kingdom[1]; Section de Biologie, Institut Curie, 91405 Orsay, France[2]; and Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03756[3]

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Two phorbol ester-inducible elements (βE2 and βE3) within the human T-cell receptor β gene enhancer each contain consensus binding sites for the Ets and core binding factor (CBF) transcription factor families. Recombinant Ets-1 and purified CBF bound individually to βE2 and βE3, in which the Ets and core sites are directly adjacent. In this report, we show that CBF and Ets-1 bind together to βE2 and βE3 and that Ets-1–CBF–DNA complexes are favored over the binding of either protein alone to βE2. Formation of Ets-1–CBF–DNA complexes increased the affinity of Ets-1–DNA interactions and decreased the rate of dissociation of CBF from DNA. Ets-1–CBF–DNA complexes were not observed when either the Ets or core site was mutated. The spatial requirements for the cooperative interaction of Ets-1 and CBF were analyzed by oligonucleotide mutagenesis and binding site selection experiments. Core and Ets sites were coselected, and there appeared to be little constraint on the relative orientation and spacing of the two sites. These results demonstrate that CBF and Ets-1 form a high-affinity DNA-binding complex when both of their cognate sites are present and that the relative spacing and orientation of the two sites are unimportant. Ets and core sites are found in several T-cell-specific enhancers, suggesting that this interaction is of general importance in T-cell-specific transcription.

The Ets family of proteins represents a novel class of DNA-binding factors (12, 23). The v-*ets* oncogene was identified as part of the genome of the E26 virus (19, 22) and has been shown to be derived from the chicken gene encoding Ets-1. Other members of the Ets family, Ets-2, Erg, Elk-1, and Elf-1 (4, 27, 28, 31, 37), were isolated on the basis of sequence homology to Ets-1. Additional Ets family members, E74, GABPα, and PEA3 (6, 17, 18, 40), have been purified and cloned on the basis of their ability to bind to specific DNA sequences. PU.1, the product of the *Spi-1* (21) gene, and Fli-1 (1) were identified at sites of integration of either spleen focus-forming virus or Moloney murine leukemia virus (MLV). SAP-1 (7) was isolated by its ability to form a ternary complex with serum response factor (SRF) on DNA containing a serum response element (SRE), a property also shared by Elk-1 (13). All Ets proteins share sequence homology over the 85-amino-acid Ets domain, which is responsible for DNA binding (23) and which specifies the family.

The known DNA binding sites for Ets proteins share a common purine-rich central motif, GGAA/T. Binding site selection assays using Ets-1 have defined flanking nucleotides on either side of the core that also form part of the high-affinity binding site (8, 23, 39). Binding sites for Ets proteins have been identified in several viral and cellular promoters and enhancers. Human T-cell leukemia virus type I (HTLV-I), polyomavirus, and murine sarcoma virus all have Ets binding sites within their promoters. Ets-1 and Ets-2 have been shown to bind and transactivate the HTLV-I long terminal repeat (3), PEA3 binds the polyomavirus Ets site (40), and Ets-1 has been shown to bind the murine sarcoma virus enhancer (11). The stromelysin promoter contains an Ets site and is activated by Ets-1 (36). SAP-1 and Elk-1 have been shown to bind to the Ets-like site adjacent to the SRE in the *fos* promoter (7, 13). Elf-1, but not Ets-1, can bind the GGAA motif within the NF-AT site of the interleukin-2 enhancer (34). Ets sites have been identified within the T-cell receptor α chain (TcRα), and subsequently the TcRβ gene enhancers and these sites were shown to bind to Ets-1 (14, 26).

It has been shown that variations in the flanking sequences surrounding the central nucleotides can control the differential binding of Ets family members (23, 34). However, in several instances, the binding of accessory proteins has been shown to confer different binding specificities on Ets proteins. Thus, binding of SAP-1 and Elk-1 to an Ets-like site adjacent to the SRE in the c-*fos* promoter has been shown to be dependent on the presence of SRF bound to the SRE (7, 13). GABPα was copurified as a heterodimer with an ankyrin repeat-containing protein (GABPβ). GABPα binds only weakly to the herpes simplex virus immediate-early promoter, except in the presence of GABPβ (18, 32), which also interacts with DNA. PU.1 binds to a site in the immunoglobulin κ enhancer and recruits a second factor (NF-EM5) to an adjacent binding site (25); NF-EM5 binds DNA only in the presence of PU.1 and its own binding site. Thus, a common feature of Ets proteins may be that they bind to response elements in conjunction with a second protein.

Several Ets-1 binding sites, including the two within the TcRβ enhancer, are located close to a T/G-rich motif which in the murine sarcoma virus enhancer (29) has been called the core site. Recently a protein, called core binding factor (CBF), which binds this core motif has been purified (16, 35). We have shown that the core sites within the TcRβ enhancer bind to purified CBF in vitro (26). The close proximity of the Ets and core sites within the two inducible elements βE2 and βE3 (originally referred to as Tβ3 and Tβ4 in reference 10) of the TcRβ enhancer led us to investigate whether Ets-1 and CBF could bind together to βE2. Here we show that both

* Corresponding author. Mailing address: Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, United Kingdom. Phone: 71-269 3069. Fax: 71-269 3479.

CBF and either Ets-1 or Ets-2 will bind to βE2 together and that this binding is cooperative. We further show that cooperative binding is relatively independent of the orientation and distance between the two sites.

MATERIALS AND METHODS

Proteins. Ets-1 was expressed from a chicken *Ets-1* cDNA clone which encodes the p54 form of Ets-1, the chicken equivalent of the major form of Ets-1 found in human T cells (3). The human c-*ets-2* cDNA was cloned at the *Bam*HI site of the pVL941 transfer vector (Pharmingen). The recombinant *Autographa californica* nuclear polyhedrosis virus–human Ets-2 baculovirus was obtained from supernatant of *Spodoptera frugiperda* Sf9 cells cotransfected with the pVL941-h-c-ets-2 construct and a linearized *A. californica* nuclear polyhedrosis virus DNA lacking the polyhedrin gene, using the Pharmingen baculovirus kit as instructed by the manufacturer. The Ets-1 and Ets-2 proteins used were produced in baculovirus-infected insect Sf9 cells (3). CBF protein was purified from bovine thymus as previously described (35).

Oligonucleotides. All oligonucleotides were synthesized on an Applied Biosystems 380A machine. Labelling was performed by end filling with avian myeloblastosis virus reverse transcriptase in the presence of [^{32}P]dCTP. All radiolabelled double-stranded oligonucleotides were purified from 7.5% acrylamide gels. The sequences of oligonucleotides (upper strand only) were as follows: βE2, GATCCACAACAGG ATGTGGTTTGACATTTA; βE2M1, GATCCACAACATT ATGTGGTTTGACATTTA; βE2M2, GATCCACAACAGG ATGTGAGATCTCATTTA; βE3, GATCTGGGTGCCGGA TGCGGTGAGTGTGGTTCTCATGATGCG; βE3M2A, GA TCTGGGTGCCGGATGCGGTGAGTGTAATTCTCAT GATGCG; and βE3M2B, GATCTGGGTGCCGGATGCAA TGAGTGTGGTTCTCATGATGCG.

Electrophoretic mobility shift assays. Radiolabelled probe (10 fmol) was incubated in a final volume of 12 μl with Ets-1, Ets-2, or CBF in 40 mM KCl–5 mM $MgCl_2$–0.05 mM EDTA, with 2 μg of acetylated bovine serum albumin and 0.1 μg of poly(dI-dC) · poly(dI-dC), for 20 min at 25°C. For Ets-1 and Ets-2, 0.25 μg of insect cell extract was used; for CBF, 0.5 μl of purified protein was used. Where this protocol was varied, the modifications are described in the relevant figure legend. Complexes were separated by electrophoresis on 5% polyacrylamide gels in 0.25× Tris-borate-EDTA.

Binding site selection. A pool of double-stranded 94-mer oligonucleotides with 50 bases of random sequence flanked on either side by 22 bases of fixed sequence was used for binding site selection. Twenty-two-base oligonucleotides complementary to either flanking region were used to amplify selected DNA by PCR. The sequences of oligonucleotides used were as follows: BSSN50, CGTCCATCGATCTG AATTCGAC$(N)_{50}$CTGAAGCTTGACTGCAGTATCG; BSSL, CGTCCATCGATCTGAATTCGAC; and BSSR, CG ATACTGCAGTCAAGCTTCAG.

The binding site selection procedure was essentially as previously described (24, 39). DNA-protein complexes were selected by using an Ets-specific antiserum (3) in the presence of 50 ng of Ets-1-containing insect cell extract and 1 μl of CBF. Complexes were isolated on protein A-Sepharose (Pharmacia), and the DNA was eluted and amplified with *Taq* polymerase (Promega) in the presence of [^{32}P]dCTP. The amplified DNA was gel purified, and 1 pg was used as starting material for the next round of selection. After four rounds of selection for Ets-1–CBF complexes with the Ets-specific antiserum, two further rounds were performed by gel shift. The complexes were allowed to form and then separated by gel electrophoresis, and the band corresponding to Ets-1–CBF was excised and amplified. The gel shift selection was carried out in the presence of 25 ng of competitor oligonucleotide containing an Ets site. After the final round, amplified DNA was digested with *Eco*RI and *Hin*dIII and ligated into pBluescript (Stratagene). Clones were sequenced by the dideoxy-chain termination method, using Sequenase (U.S. Biochemical). Selection with CBF alone was carried out entirely by gel shift. When no shifted band was visible, the area adjacent to an authentic CBF-DNA complex was excised and the DNA was amplified as described above. Three rounds of selection by gel shift were carried out before cloning of the oligonucleotides.

Measurement of dissociation rates. The dissociation rates of Ets-1–CBF together and Ets-1 or CBF alone were determined by electrophoretic mobility shift assay. Scaled-up binding reactions with an excess of labelled βE2 were allowed to reach equilibrium at 25°C for 20 min and then transferred to ice, and the zero-time-point material was removed and loaded onto a running gel at 4°C. A 50-fold excess of unlabelled βE2 was added, and aliquots of the binding reaction mixture were loaded onto the gel at 20, 40, and 80 s and 2, 3, 5, and 10 min. The retarded bands were quantitated directly from the dried gel on an Ambis scanner. The dissociation rate constant was calculated from the slope of the plot of log (percent bound at t − percent bound at t = infinity), where infinity = 10 min.

RESULTS

CBF and Ets-1 or Ets-2 bind together to βE2. To determine whether Ets-1 and CBF can bind to the same βE2 molecule, recombinant chicken Ets-1 and purified CBF were incubated with a radiolabelled βE2 oligonucleotide and complexes were analyzed by electrophoretic mobility shift assays. Incubation of labelled βE2 with Ets-1 generated a retarded complex, the intensity of which increased with increasing amounts of Ets-1 (Fig. 1, lanes 2 to 4). Incubation of βE2 with CBF results in a complex of higher mobility than Ets-1–βE2 complexes (lanes 10 to 12). To demonstrate whether Ets-1 and CBF preferentially bind to the same βE2 oligonucleotide, binding reactions were performed with a fixed amount of CBF and with increasing amounts of Ets-1. When Ets-1 was included in the binding reaction, a complex of lower mobility than that generated with either Ets-1 or CBF alone was observed (lanes 5 to 8). When this experiment was repeated with a fixed amount of Ets-1 and increasing amounts of CBF, an additional complex of the same mobility was observed (lanes 13 to 16). When human Ets-2 was used in place of chicken Ets-1, a ternary complex was also observed (lanes 17 to 19). Taken together, these data demonstrate that Ets-1 or Ets-2 and CBF preferentially bind together to βE2.

To investigate whether both DNA binding sites are necessary for cooperative binding of Ets-1 and CBF, mutational analysis was performed. A mutant Ets site was created by changing the G residues of the central GGA sequence (Fig. 2B, βE2M1) (26). The core site was mutated by altering the bases at the 3′ end of the consensus sequence (Fig. 2B, βE2M2) (26). Mutation of the Ets site did not affect CBF binding (Fig. 2A, lane 1). However, no Ets-1 or Ets-1–CBF ternary complexes were seen when Ets-1 was added to the binding reaction (lanes 2 to 4). When a similar experiment was carried out with the core site mutant, no binding of CBF

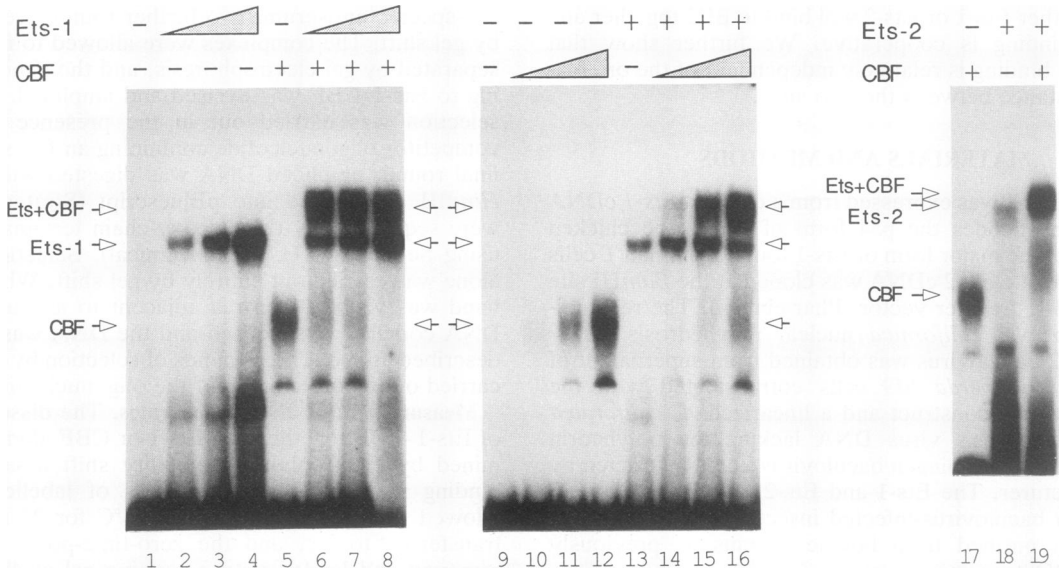

FIG. 1. Cooperative binding of Ets-1 or Ets-2 and CBF to βE2. Radiolabelled βE2 oligonucleotide was incubated in the presence of chicken Ets-1 (lanes 2 to 4 and 13), CBF (lanes 5 and 10 to 12), or Ets-1 plus CBF (lanes 6 to 8 and 14 to 16). In lanes 2 to 4 and 6 to 8, increasing amounts of Ets-1 were used (0.05, 0.1, and 0.2 μl). In lanes 10 to 12 and 14 to 16, increasing amounts of CBF were used (0.1, 0.2, and 0.4 μl). In lanes 1 and 9, no protein was added; in lanes marked +, 0.2 μl of CBF or 0.1 μl of Ets-1 was added, as indicated. In lanes 17 to 19, 0.4 μl of human Ets-2, 0.2 μl of CBF, and 0.4 μl of Ets-2 plus 0.2 μl of CBF, respectively, were added. Complexes were resolved by electrophoresis through a 5% polyacrylamide gel. The specific complexes formed are indicated.

alone or ternary complex formation was observed (lanes 5 to 8). When the same amounts of Ets-1 and CBF as used in lanes 2 and 6 were added to unmutated βE2, the major band observed corresponded to the ternary complex (lane 9). Thus, the integrity of both DNA binding sites is required for cooperative binding of Ets-1 and CBF to βE2 to generate a ternary complex.

CBF binding to βE2 increases Ets-1 binding. A 50-fold excess of unlabelled βE2 or βE2M1 oligonucleotide, but not βE2M2, fully competed for the observed CBF binding to labelled βE2 (Fig. 3A, lanes 1 to 4). When Ets-1 alone was used in place of CBF, virtually all specific complexes were abolished by a 50-fold excess of βE2 or βE2M2 but not βE2M1 (lanes 5 to 8). These results demonstrate that mutation at one site of βE2 failed to affect cognate binding to the second site when either Ets-1 or CBF alone was used. To determine whether either Ets-1 or CBF binding was increased by ternary complex formation, competition experiments were performed with a range of concentrations of βE2 and the mutants βE2M1 (Fig. 3C, Ets site mutant) and βE2M2 (Fig. 3C, core site mutant). The ternary complex formed on βE2 (Fig. 3B, lane 1) was competed for efficiently by a 50-fold excess of βE2 (lane 2), although some residual Ets-1 complex was seen. The same excess of βE2M1 (Ets site mutant) competed for almost all of the ternary complex (lane 6), and at 100-fold excess, the ternary complex was completely absent. Therefore, there was little difference between the competition with a core site for CBF when bound to βE2 alone or as part of the ternary complex. As expected, this competitor did not interfere with Ets-1 binding, as a strong Ets-1 complex was detected even with a 400-fold excess (lane 9). In contrast, competition with a 50-fold excess of βE2M2 had little effect on the ternary complex (lane 10), and only a low level of competition was seen with up to a 400-fold excess (lanes 11 to 13), as evidenced both by the intensity of the ternary complex band and by the small amount of CBF-βE2 complex seen with increasing competitor. This lack of competition by βE2M2 for Ets-1 within the ternary complex was not due to mutation of the core site affecting the Ets-1 binding site (Fig. 3A). Thus, we conclude that formation of the ternary complex greatly increases the binding of Ets-1 to βE2.

Binding of Ets-1 decreases the rate of dissociation of CBF from DNA. The ternary complex formed on βE2 by CBF and Ets-1 was more resistant to competition by either an Ets site or a core site alone than when only one of the proteins was bound to DNA. One possible explanation for this observation is that the rate of dissociation of the ternary complex is slower than that of either protein alone. To investigate this possibility, binding reaction mixtures containing labelled βE2 together with either Ets-1 or CBF alone or with both Ets-1 and CBF were prepared. Binding was allowed to reach equilibrium at 25°C for 20 min and then transferred to ice, and an aliquot of each reaction mixture was loaded onto a gel. A 50-fold excess of unlabelled βE2 was then added, and samples were removed from each reaction mixture and loaded onto a running gel at specific times after addition of competitor. The amount of retarded complex at each time point was then quantitated from the dried gel. The decrease in bound βE2 probe over the time course of the competition for each reaction is shown in Fig. 4. The half-life of the CBF-βE2 complex was significantly less than that for either Ets-1–βE2 or the ternary complex. The dissociation rate constants for each of the three complexes are $1.4 \times 10^{-2}\ s^{-1}$ for CBF alone, $0.45 \times 10^{-2}\ s^{-1}$ for Ets-1, and $0.35 \times 10^{-2}\ s^{-1}$ for Ets-1 plus CBF. Thus, binding of Ets-1 decreases the rate of dissociation of CBF from βE2, whereas only a small difference between the dissociation rate constants for Ets-1 alone and the ternary complex was observed.

Cooperative binding of Ets-1 and CBF occurs on βE3. βE3 constitutes a second element within the TcRβ enhancer that has also been shown to bind to both Ets-1 and Ets-2 as well

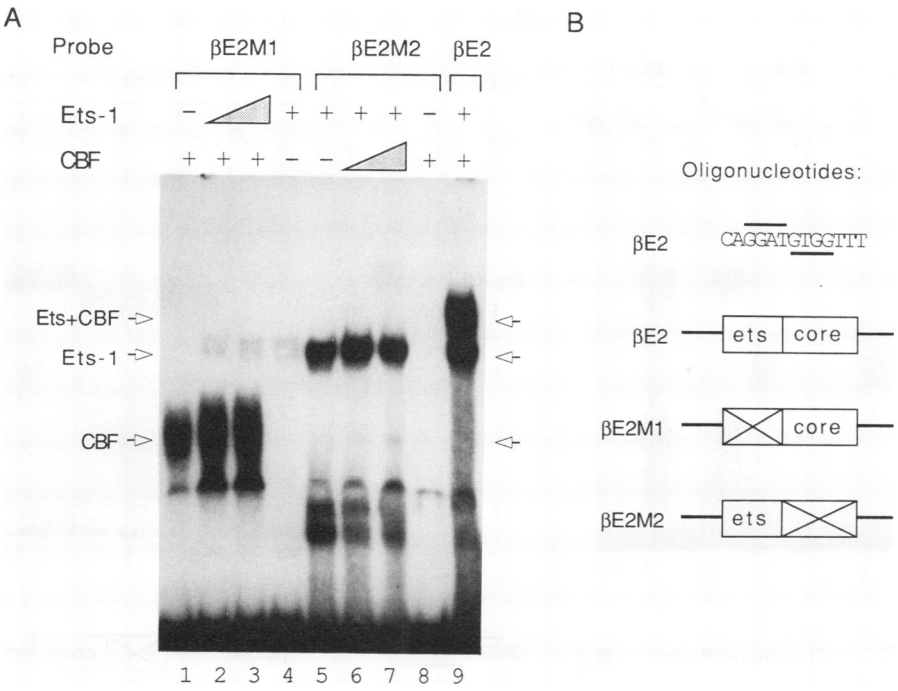

FIG. 2. Cooperative binding of Ets-1 and CBF is dependent on the presence of both the Ets and core sites. (A) Radiolabelled mutant oligonucleotides were incubated with Ets-1, CBF, or both proteins together. The Ets site mutant (βE2M1) was incubated with 0.4 μl of CBF (lanes 1 to 3) in the absence of Ets-1 (lane 1) and with 0.1 μl (lane 2) or 0.2 μl (lane 3) of Ets-1. Lane 4 shows radiolabelled βE2M1 incubated with 0.2 μl of Ets-1 in the absence of CBF. The core site mutant (βE2M2) was incubated with 0.2 μl of Ets-1 in the absence of CBF (lane 5) and with 0.2 μl (lane 6) or 0.4 μl (lane 7) of CBF. Radiolabelled βE2M2 incubated with 0.4 μl of CBF alone is shown in lane 8. Formation of Ets-1–CBF complexes with radiolabelled unmutated βE2 oligonucleotide is shown in lane 9 (0.2 μl of CBF plus 0.1 μl of Ets-1). Complexes were separated on 0.5% polyacrylamide gels, and specific complexes are indicated. (B) Schematic representation of the oligonucleotides used. The mutations to the Ets and core sites are described in reference 26.

as CBF (26). However, βE3 contains two core sites as well as a single Ets site. One core site is in the same position relative to the Ets site as in βE2, where the two sites are directly adjacent. The other is in the same orientation, although the central GTGG is separated from the GGAT of the Ets site by 9 bp (Fig. 5B). CBF or Ets-1 alone bound to βE3 (Fig. 5A, lanes 1 and 2). Comparison of the mobility of the complexes formed by CBF on βE3 with those formed on βE3M2A and βE3M2B (mutations in each core site) failed to reveal an additional complex, corresponding to the binding of two CBF molecules to βE3 (lanes 1, 4, and 7). This result indicates a lack of cooperativity of binding between CBF molecules, in marked contrast to the situation with Ets-1 and CBF binding. When both Ets-1 and CBF were included in the binding reaction, a complex of slower mobility was seen (lane 3), indicating cooperativity of binding of Ets-1 and CBF. A similar pattern of binding was seen when each of the core sites was mutated individually (lanes 4 to 6 and 7 to 9). Both sites bound CBF (lanes 4 and 7), and both formed ternary complexes with Ets-1 (lanes 6 and 9). Therefore, the interaction of Ets-1 and CBF on DNA can occur not only when their cognate sites are adjacent (as in βE2 and βE3M2A) but also when they are separated by 9 bp (βE3M2B).

Coselection of Ets and core sites by binding site selection. The observed cooperativity of binding of Ets-1 and CBF to both of the βE3 core site mutants (βE2M2A and βE3M2B; Fig. 5) suggests that there was some flexibility in the configuration of binding sites permissive for cooperative binding. Therefore, the technique of binding site selection was used to investigate the distance and orientation requirements for cooperative binding of these two factors. For these experiments, an oligonucleotide containing a central region of 50 bases of random sequence flanked on either side by 22 bases of fixed sequence was used. Four successive rounds of selection were carried out in the presence of both Ets-1 and CBF (to select for ternary complexes), using an Ets-1-specific antiserum, followed by two rounds of selection by electrophoretic mobility shift. As shown in Fig. 6A, successive rounds of selection using the Ets-specific antiserum resulted in the formation of a progressively stronger CBF-DNA complex, indicating that core sites were selected using this strategy.

Sequences selected by this technique were analyzed for the presence of the central bases of Ets (GGAA/T) and core (GC/TGG) sites. Ets sites preceded by a G and core sites surrounded by G residues were excluded, as these sites failed to bind Ets-1 or CBF (data not shown). All 115 sequences obtained contained at least one Ets site, and 86 contained at least one core site. The number of Ets and core sites within each of these 86 sequences is shown in Fig. 6B. Interestingly, 26 sequences contained adjacent Ets and core sites of the configuration found in βE2 and βE3. Of the remaining 60 sequences with nonadjacent Ets and core sites, 50% contained a single Ets site plus a single core site (Fig. 6B). The sequences were separated into several groups for detailed analysis of Ets and core binding sites.

βE2-like Ets and core sites. Those sequences containing a βE2-like sequence were analyzed separately because the overlapping Ets and core sites might be expected to bias the

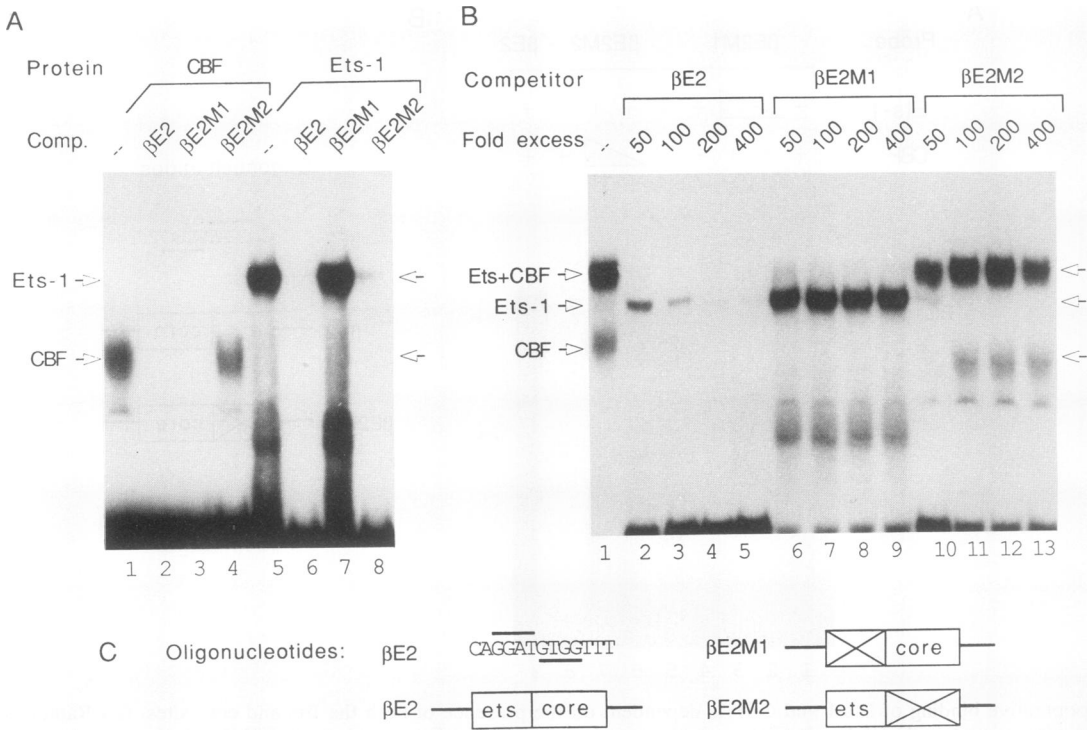

FIG. 3. Competition of ternary complex formation by mutant βE2 oligonucleotides. (A) Radiolabelled βE2 was incubated with CBF (0.4 μl) alone (lanes 1 to 4) or Ets-1 (0.2 μl) alone (lanes 5 to 8) in the presence of a 50-fold excess of the competitors indicated. (B) Radiolabelled βE2 was incubated with CBF (0.4 μl) and Ets-1 (0.2 μl) together in the absence of specific competitor (lane 1) or with a 50- to 400-fold excess the competitors indicated. All competitors were incubated with the protein for 5 min prior to the addition of probe. Complexes were resolved on 5% polyacrylamide gels, and specific complexes are indicated. (C) Schematic representation of the oligonucleotides used as competitors.

consensus derived for each site. When a consensus site was derived from this group (Fig. 7A), there was no apparent difference from the consensus Ets site derived previously (8, 23, 33, 39) or from the single Ets sites analyzed in this study (Fig. 7B). In contrast, there was a subsite selection for core sites within this group. As shown in Fig. 7A, there was a clear preference for a T nucleotide within the GC/TGG in core sites from βE2-like sequences, whereas a slight preference for C was observed in all other core sites selected (Fig. 7C and D). This strong preference for a T residue may reflect the use of this residue in βE2-like sequences by both Ets-1 and CBF, although we cannot rule out that this preference arose as a result of the selection procedure. The high frequency of βE2-like sequences suggests that there is a strong selection for this arrangement of Ets and core sites when sequences are selected with Ets-1 and CBF.

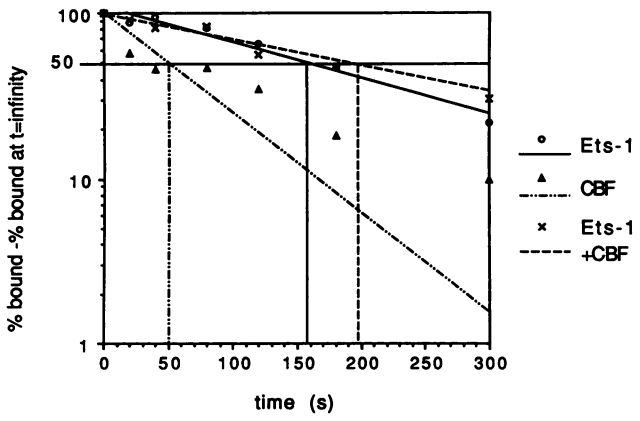

FIG. 4. Analysis of dissociation rates of Ets-1–βE2, CBF-βE2, and ternary complexes. Binding reactions were transferred to ice, a 50-fold excess of competitor βE2 (unlabelled) was added, and aliquots of each reaction were loaded onto a running gel at defined time points. Complexes were resolved on a 5% polyacrylamide gel electrophoresed at 4°C. The amount of retarded probe in each complex was quantitated and plotted against time.

Core sites selected with Ets-1 and CBF. The 49 clones which contained single core sites that were not adjacent to Ets sites were analyzed to derive a consensus core site. As shown in Fig. 7C, there was strong selection for an A or a T in the position preceding the first G of the core site and T then A in each of the two positions after the four central bases of the core site. The frequencies of both the most common bases as well as the second-choice bases at each of these positions were significant at >99.5% by chi-square analysis. There was little statistically significant selection of specific bases flanking this central region, although at position +3, there seemed to be a bias against C. The major difference between these sequences and the core site consensus from the βE2-like group of sequences is the strong (3:1) preference for GTGG cores in the βE2-like group compared with a slight preference for GCGG among the others. These results demonstrate that core sites were selected with an Ets-specific antiserum from random sequence in the presence of CBF and Ets-1, further supporting the idea that CBF stabilizes Ets-1 binding.

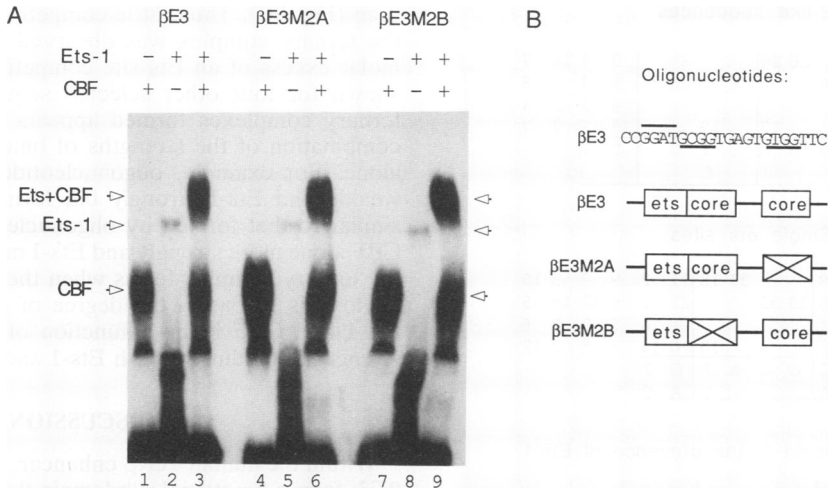

FIG. 5. Ternary complex formation with βE3. (A) Binding reactions were as for Fig. 1 (0.2 μl of Ets-1 and 0.4 μl of CBF), with either Ets-1 or CBF alone or both together, as indicated. βE3 (lanes 1 to 3) and the two core site mutants βE3M2A (lanes 4 to 6) and βE3M2B (lanes 7 to 9) were used as probes. The specific complexes are indicated. (B) Schematic representation of the oligonucleotide probes used.

Determination of the sequence of the core site in the absence of Ets-1. To determine the range of sequences to which purified CBF can bind in the absence of Ets-1, the random oligonucleotide was used with purified CBF alone. Binding reactions were fractionated by electrophoresis, and bound DNA was excised from a polyacrylamide gel. In the first round, where no retarded band was visible, the region of the gel where complexes would be expected to migrate (as determined by reference to an adjacent CBF-DNA band) was excised, and DNA was eluted and amplified. In subsequent rounds, more intense CBF-DNA complexes were observed. We analyzed 69 sequences for the central four nucleotides of the core site, GC/TGG. Seven did not contain recognizable core sites, 41 contained single core sites, and the remaining 21 contained two or more. The 41 oligonucleotides with a single core site were used to generate consensus sequences for CBF binding (Fig. 7D). The selected consensus was cTGC/TGGTT/CA/t, where nucleotides in uppercase were significant at >99.5% and those in lowercase were significant at >90%. There was a slight preference for a C rather than a T in the center of the core site. This consensus was in broad agreement with the core site consensus recently defined by Melnikova et al. (20). Compared with the core site consensus obtained from the Ets-1–CBF selection, the ratios of GCGG to GTGG were very similar (with the exception of the βE2-like sequences).

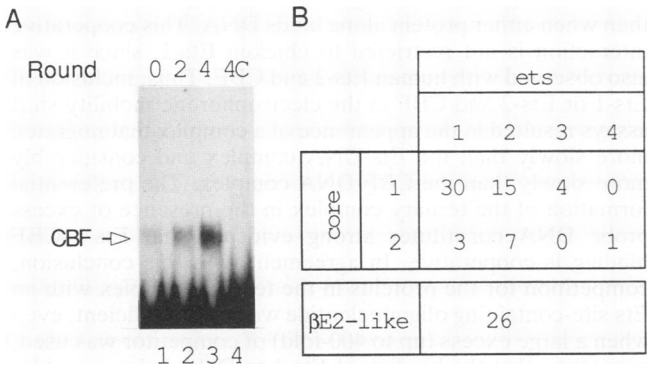

FIG. 6. Selection of Ets-1 and CBF binding sites. (A) Core sites were selected from random DNA sequence, using an Ets-specific antiserum in the presence of both Ets-1 and CBF. CBF (0.4 μl) was incubated with radiolabelled oligonucleotide pools, either unselected (lane 1, round 0) or after two (lane 2) or four (lanes 3 and 4) rounds of selection with an Ets-specific antiserum, Ets-1, and CBF. The complex retarded by CBF is indicated. This complex was competed for (lane 4) by the core site-containing oligonucleotide (βE2M1). (B) The distribution of Ets and core sites in the selected sequences after four rounds of antibody selection and two rounds of selection by gel shift is summarized. The number of Ets sites per oligonucleotide is indicated at the top, and the number of core sites is indicated at the left. The number of oligonucleotides with each combination of Ets and core sites is shown. Those oligonucleotides with a βE2-like combination of Ets and core sites are excluded from this analysis. A further 29 sequences which contained no obvious core-like sequence were obtained. All of this group contained multiple Ets sites.

Some differences were, however, noted in the positions flanking the core. Most obviously, A residues were observed at the −1 and +1 positions with increased frequency when selected in the presence of Ets-1. A residues at these positions have been shown to result in poor CBF binding (see Fig. 9). It is possible that these differences reflect preferences for CBF-DNA interactions when binding cooperatively with Ets-1; alternatively, these differences may be a consequence of the binding site selection strategy used; for example, weak core sites may have been selected from among the Ets binding sites enriched by the Ets-specific antiserum.

Electrophoretic mobility shift analysis of nine of the selected oligonucleotides derived from the CBF alone selection demonstrated that GC/TGG sites bind CBF unless surrounded on both sides by G residues. Those with sequences closer to the consensus bound CBF more efficiently (see Fig. 9; also data not shown).

Taken together, the results from the two selection procedures provide strong support for the conclusion that CBF binding enhances Ets-1 binding.

The relative orientation and spacing of Ets and core sites are flexible. When sequences with a single copy of each site were analyzed, all four relative orientations of the two sites were observed (Fig. 8). The distances separating the four central

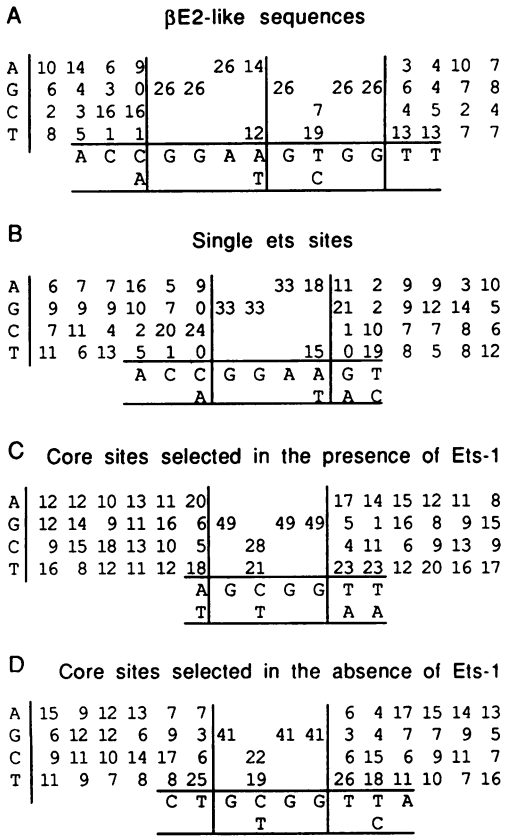

FIG. 7. Analysis of the sequences obtained by binding site selection. (A) Consensus sequence from the 26 oligonucleotides containing Ets and core sites with a βE2-like spacing from the binding site selection with Ets-1 and CBF. (B) Consensus Ets binding sequence derived from all 33 clones with a single Ets site not overlapped by a core site from the binding site selection with Ets-1 and CBF. (C) Analysis of 49 core sites from the selection with Ets-1 and CBF. Only oligonucleotides with a single core site were used. (D) Selection was performed in the presence of CBF alone, complexes were selected by gel shift, and 69 were sequenced after three rounds of selection. Of those sequences, 41 contained single core sites. The consensus derived from oligonucleotides with a single core site is shown.

nucleotides of each site varied from 1 to 33 bp. These results suggest that there are only very weak spatial constraints on the interaction of these two proteins when bound to DNA. Consistent with this conclusion are the results of electrophoretic mobility shift assays carried out on a selection of these oligonucleotides. Cooperative binding was assessed by the differential ability of an Ets site competitor (βE2M2) and a core site competitor (βE2M1) to compete for binding of Ets-1 or CBF alone and together in the ternary complex. Cooperative binding was observed on all oligonucleotides tested that contained both Ets and core sites. As shown in Fig. 9, Ets-1–CBF cooperative binding occurred when the two sites are separated by up to 33 bases. Thus, for oligonucleotide 30, the Ets-1 complex (Fig. 9A) was competed for by the Ets site but not by the core site, and the CBF complex was competed for by the core but not by the Ets site (Fig. 9B). When both Ets-1 and CBF were included in the binding reaction, Ets-1–DNA complexes were observed in the presence of the core site competitor, whereas in the presence of the Ets site competitor, a complex of slower mobility was seen (Fig. 9C). Thus, little competition for the Ets-1 within the ternary complex was observed with use of a 100-fold molar excess of an Ets site competitor. Similar results are shown for four other selected sequences: the amount of ternary complexes formed appears to be dependent on a combination of the strengths of binding of Ets-1 and CBF alone. For example, oligonucleotide 53 bound CBF very weakly and Ets-1 strongly but formed a ternary complex similar to that formed by oligonucleotide 30, which bound CBF alone more strongly and Ets-1 more weakly. Therefore, the ternary complex forms when the sites are separated by up to tens of bases, the degree of cooperative binding of Ets-1 and CBF being a function of a combination of the strength of binding of both Ets-1 and CBF alone.

DISCUSSION

Within the human TcRβ enhancer, two elements, βE2 and βE3, form a functional subdomain that constitutes the major determinant of both basal enhancer activity and phorbol ester inducibility (26). βE2 and βE3 each contain a consensus Ets binding site, and sequences that share homology with the core site of MLV and other lymphotrophic viruses. Inducibility has been shown to be dependent on the Ets sites alone, whereas both Ets and core sites are required for basal activity, with the core site playing the major role. Both Ets-1 and Ets-2 have been shown to bind specifically to βE2 and βE3 in vitro, and both elements will bind purified CBF (26). In βE2, the Ets and core sites are adjacent and may be partially overlapping, whereas in βE3, in addition to the region homologous to the adjacent Ets and core sites of βE2, there is also a second core site separated from the Ets site by nine nucleotides.

The experiments presented in this report demonstrate that the interaction of CBF and Ets-1 together with DNA facilitates formation of a ternary complex, which is more stable than when either protein alone binds DNA. This cooperative interaction is not restricted to chicken Ets-1, since it was also observed with human Ets-2 and CBF. Thus, inclusion of Ets-1 or Ets-2 and CBF in the electrophoretic mobility shift assays resulted in the appearance of a complex that migrated more slowly than the Ets-DNA complex and considerably more slowly than the CBF-DNA complex. The preferential formation of the ternary complex in the presence of excess probe DNA constitutes strong evidence that Ets-1–CBF binding is cooperative. In agreement with this conclusion, competition for the proteins in the ternary complex with an Ets site-containing oligonucleotide was very inefficient, even when a large excess (up to 400-fold) of competitor was used, suggesting that the binding of Ets-1 to DNA is increased by cooperative interactions with CBF. The stabilizing effect of ternary complex formation on CBF binding as assessed by competition with a core site-containing oligonucleotide was much less striking. Specific DNA sequences appear to be necessary for interaction of CBF and Ets-1 because destruction of either binding site abolishes cooperative binding.

Ternary complex formation affected the DNA binding characteristics of CBF by decreasing the dissociation rate of CBF from DNA by approximately fourfold. Therefore, formation of the ternary complex appears to have different effects on Ets-1 and CBF. The dissociation rate of CBF from DNA is decreased; in contrast, the binding of Ets-1 to its site is increased without greatly decreasing its off rate. Thus, the increased Ets-1 binding in the presence of CBF may be explained by an increase in the rate of association of Ets-1 and DNA in the presence of CBF. A plausible mechanism

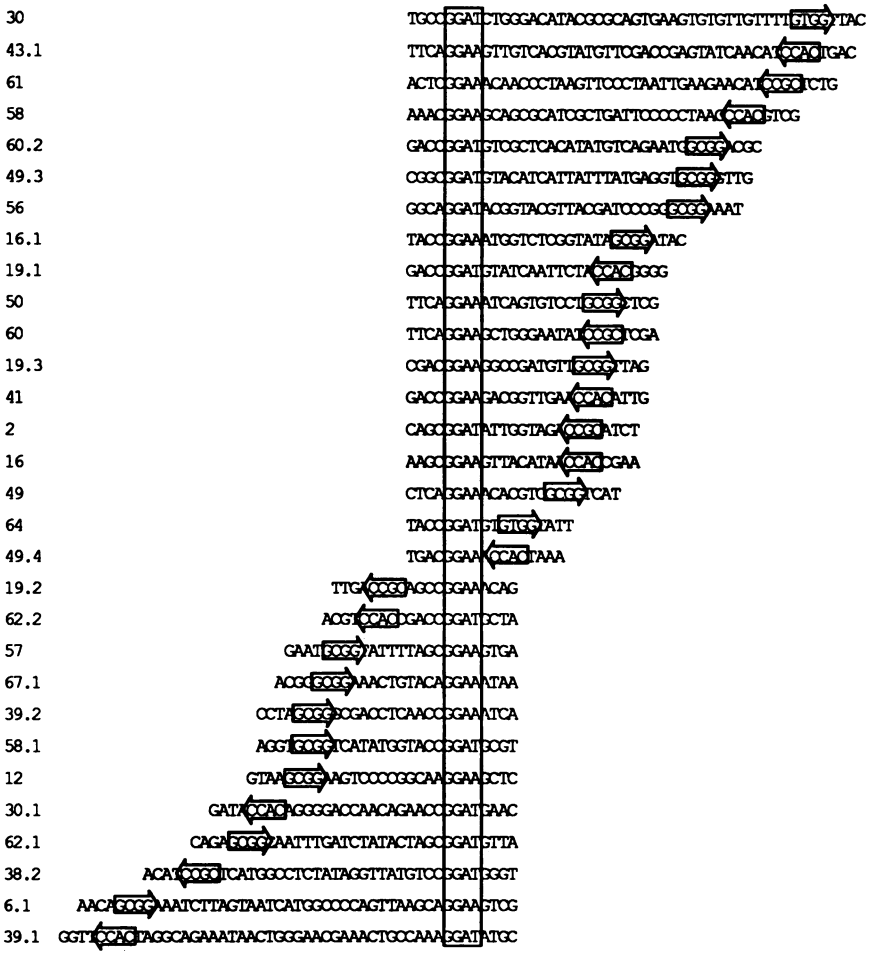

FIG. 8. Alignment of oligonucleotides containing one core and one Ets site from the Ets-1-plus-CBF selection. The Ets sites are boxed, and the core sites indicated by arrows, which represent their orientation relative to the Ets site. The sequence between the two sites, plus four bases on either side, is shown.

for this effect would be prior binding of CBF to its binding site, followed by recruitment of Ets-1. Attempts to measure association kinetics of Ets-1 and CBF to βE2 have failed, since maximal binding occurred within 15 s (data not shown).

Cooperative binding of Ets-1 and CBF was also seen on βE3, and mutation of each of the two core sites individually demonstrated that both could take part in ternary complex formation. This result demonstrated that the Ets and core sites are not required to be adjacent, as they are in βE2, for Ets-1 and CBF to bind cooperatively. In contrast, no evidence was obtained for cooperative binding of two CBF molecules to βE3.

This result led us to investigate further the spatial constraints on cooperative binding of Ets-1 and CBF, using the technique of binding site selection to select for both Ets and core sites in a pool of random sequences. Coselection of core and Ets sites at a high frequency with an Ets-specific antiserum, in the presence of Ets-1 and CBF, provided further evidence that ternary complex formation is preferred over binding of either Ets-1 or CBF alone. Analysis of the relative orientation and spacing of the Ets and core sites in oligonucleotides containing only one copy of each site suggests that the interaction of Ets-1 and CBF is flexible. The degree of ternary complex formation is dependent on a combination of the strengths of binding of Ets-1 and CBF when they bind alone. Thus, for example, ternary complexes could efficiently form on a low-affinity core site and a high-affinity Ets site. Similar results were recently obtained for the interaction of SRF and Elk-1 on the SRE and an Ets site (33). In this case, the position of the SRE was fixed and complexes were selected by excising them from a gel. The Ets sites selected were spaced over the 27 bases of variable sequence adjacent to the SRE and were found in both orientations relative to it. In the case of Ets and core sites, which are not palindromic, there are four possible relative orientations. All four were observed, with no obvious bias toward any one.

Taken together, these two studies demonstrate that the distance between an Ets site (binding Ets-1 or Elk-1) and the binding site for an accessory protein (CBF or SRF) with which it interacts cooperatively can vary extensively. In this context, it has been shown that the interaction of Elk-1 with SRF is via a region (B box) that is outside the DNA binding domain. It has been proposed that this separation of the DNA binding and protein interaction domains allows the flexibility in the interaction of Elk-1 and SRF (33). It will be interesting to determine the region of Ets-1 that interacts

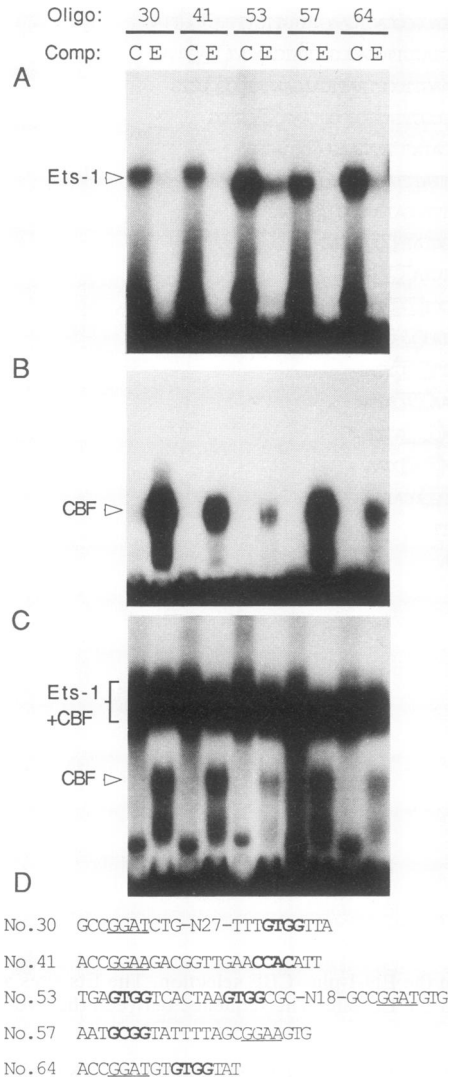

FIG. 9. Electrophoretic mobility shift analysis of oligonucleotides selected in the presence of Ets-1 and CBF. Oligonucleotides were incubated with 0.2 μl of Ets-1 alone (A), 0.4 μl of CBF alone (B), or 0.2 μl of Ets-1 and 0.4 μl of CBF together (C). Complexes were resolved by electrophoresis, and specific complexes are indicated. In panel C, Ets-1–DNA complexes have not been clearly resolved from Ets-1–CBF–DNA complexes. The competitors used were βE2M1 (C[core site containing]) and βE2M2 (E[Ets site containing]), at 100-fold molar excess. Competitors are the same for all three panels. (D) The oligonucleotides used. The Ets sites are underlined, and the core sites are shown in boldface.

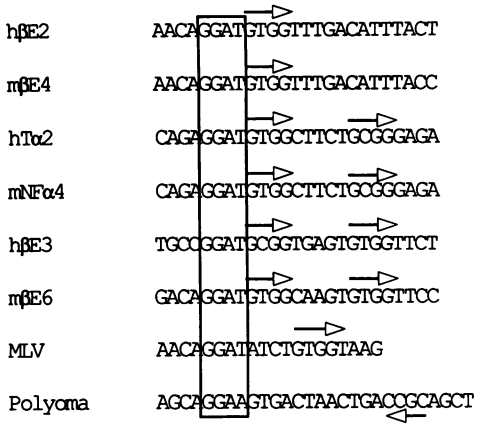

FIG. 10. Alignment of eight naturally occurring sequences that contain consensus Ets and core sites. The sequences have been aligned to the Ets sites (boxed). Core sites are represented by arrows. All eight sites have been shown to be important for the activity of the enhancers in which they are found (see text for discussion). Human βE2 and βE3 (hβE2 and hβE3) are the βE2 and βE3 elements analyzed in the study (referred to as Tβ3 and Tβ4 in reference 10); mβE4 and mβE6 are their murine homologs; hTα2 is the region in the human TcRα enhancer that is homologous to βE2 and mNFα4, its murine homolog. The final two sequences are from the MLV long terminal repeat and polyomavirus enhancer region, respectively.

with CBF and its relationship to the functional differences between c-Ets-1 and v-Ets. Moreover, flexibility in the spacing between DNA binding sites for interacting transcription factors may turn out to be a more general property of transcriptional regulation.

Several Ets binding sites in viral and cellular enhancers are situated close to consensus core sites. As shown in Fig. 10, there appear to be two groups of Ets and core sites within the enhancers of the mouse and human TcRα and -β genes. The human βE2 (10, 26) element and its murine homolog, mβE4 (30), contain one Ets and one core site, and the βE3 (10, 26) and Tα2 (15) elements, along with their murine counterparts (30, 38), contain the same abutting Ets and core sites as βE2 together with a second core site. Both core sites appear to have been specifically conserved in each member of this last group, and where they vary in sequence, the core site is not disrupted. Thus, the Ets adjacent core site in human βE3 is GCGG where the other three have GTGG, and of the second core sites, two are GCGG and two are GTGG. The MLV enhancer also contains binding sites for Ets-1 and CBF (29), and in the polyomavirus enhancer, the Ets (PEA3) binding site is situated close to a core site that binds the PEA2 transcription factor (2); however, neither of these enhancers has spacings homologous to those of the TcRα and -β enhancer elements. We propose, therefore, that cooperative Ets-CBF binding also occurs in the TcRα, MLV, and polyomavirus enhancers. Thus, it appears that the Ets-core site combination constitutes a specific functional unit, which may be particularly important in the regulation of transcription of T-cell-specific genes.

Several Ets-related proteins have recently been shown to bind DNA in association with other proteins. These include interactions of SAP-1 and Elk-1 with SRF (7, 13, 33) and the Ets-related protein GAPBα with another polypeptide, GAPBβ (32), as well as interactions between Ets-1 and SP1. This latter interaction generates a complex which synergistically transactivates the HTLV-I long terminal repeat via adjacent Ets and SP1 sites (9). In each case, high-affinity binding of the Ets-related protein to DNA was observed only in the presence of the other polypeptide. Thus, a general property of Ets-related proteins may be that they require interactions with other proteins for high-affinity binding to DNA, and it has been proposed that specific protein-protein interactions between members of the Ets family and differing second proteins regulate target selection by Ets family members (5). The results presented here extend this idea by demonstrating that the same Ets protein, namely, Ets-1, can

be directed to bind with high affinity to different enhancers, dependent on interactions with different accessory proteins.

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