Assembly and function of a TCR α enhancer complex is dependent on LEF-1-induced DNA bending and multiple protein–protein interactions

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In this study we examine the molecular basis for the synergistic regulation of the minimal TCR α enhancer by multiple proteins. We find that reconstitution of TCR α enhancer function in nonlymphoid cells requires expression of the lymphoid-specific proteins LEF-1, Ets-1 and PEBP2 α (CBF α), and a specific arrangement of their binding sites in the enhancer. We show that Ets-1 cooperates with PEBP2 α to bind adjacent sites at one end of the enhancer, forming a ternary complex that is unstable by itself. Stable occupancy of the Ets-1- and PEBP2 α -binding sites in a DNase I protection assay was found to depend on both a specific helical phasing relationship with a nonadjacent ATF/CREB-binding site at the other end of the enhancer and on LEF-1. The HMG domain of LEF-1 was found previously to bend the DNA helix in the center of the TCR α enhancer. We now show that the HMG domain of the distantly related SRY protein, which also bends DNA, can partially replace LEF-1 in stimulating enhancer function in transfection assays. Taken together with the observation that Ets-1 and members of the ATF/CREB family have the potential to associate *in vitro*, these data suggest that LEF-1 can coordinate the assembly of a specific higher-order enhancer complex by facilitating interactions between proteins bound at nonadjacent sites.

[**Key Words:** LEF-1; PEBP2 α ; Ets-1; TCR α enhancer; DNA bending]

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DNA transactions, including transcription, recombination, and replication, involve the assembly of specific multiprotein complexes at *cis*-acting control regions (Echols 1986). The fidelity of assembly of such complexes, however, is challenged by the existence of protein families that are comprised of multiple members with virtually identical DNA-binding specificities and by large genomes that can sequester DNA-binding proteins nonspecifically. Therefore, recruitment of specific proteins to their genetic target sites *in vivo* has been proposed to require interactions between multiple DNA-binding proteins (Lin and Riggs 1975; Ptashne 1986). Together with the composition of *cis*-acting control regions as arrays of multiple factor-binding sites, such interactions may contribute to the accurate and diverse regulation of DNA transactions that occurs in prokaryotes and eukaryotes. One form of protein–protein interaction involves the dimerization of related or identical DNA-binding proteins that increases the affinity and specificity of sequence recognition (for review, see Johnson and McKnight 1989). Moreover, the juxtaposition of binding

sites for different proteins that interact with each other can mediate cooperative DNA binding (Herskowitz 1990). Finally, contacts between proteins bound at nonadjacent sites involve looping or bending of the intervening DNA and allow for the generation of a higher-order nucleoprotein complex (for review, see Schleif 1992).

Assembly of higher-order nucleoprotein complexes has been studied extensively in site-specific recombination and transcription in prokaryotes. The formation of such complexes was shown to depend on “architectural” proteins that bend the DNA helix to facilitate the spatial alignment of nonadjacent factor-binding sites (for review, see Hoover et al. 1990; Nash 1990). In this context, protein-induced DNA bending is thought to overcome the energetic barrier for the deformation of short and inflexible DNA fragments (Shore and Baldwin 1983; Wang and Gjaever 1988). Recently, several regulatory proteins, including the lymphocyte enhancer-binding factor 1 (LEF-1), the high mobility group (HMG) I(Y) protein, and the protein YY1 have been identified as putative structural components of protein complexes formed at eukaryotic enhancers and promoters (Giese et al. 1992; Thanos and Maniatis 1992; Du et al. 1993; Nathans and Gilman 1993).

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LEF-1 is a pre-B and T lymphocyte-specific DNA-binding protein that is a member of the HMG domain family (Travis et al. 1991; Waterman et al. 1991). These proteins share an 85-amino-acid-region, termed the HMG domain, which recognizes DNA through the minor groove and induces a sharp bend in the DNA helix (for review, see Landsman and Bustin 1993; Grosschedl et al. 1994). In particular, LEF-1 induces a bend of 130°C in the double helix and can functionally replace the bacterial integration host factor (IHF), in the assembly of an higher-order nucleoprotein structure involved in site-specific recombination (Giese et al. 1992). LEF-1 cannot activate transcription by itself but must act in concert with other enhancer-bound proteins (Travis et al. 1991). In addition, LEF-1 contains a unique transcriptional activation domain that, when fused to a heterologous DNA-binding domain, maintains a dependence on a specific arrangement of flanking factor-binding sites for transcriptional stimulation (Carlson et al. 1993; Giese and Grosschedl 1993). One interpretation of this finding is that the transcriptional activation domain of LEF-1 mediates interaction between enhancer-bound transcription factors and, therefore, differs from typical activation domains that interact directly or indirectly with components of the basal transcription machinery (Lin and Green 1991; Hoey et al. 1993).

Studies of enhancer function have been facilitated by the observation that multimerization of individual factor-binding sites yields synthetic enhancers that often mimic the activity of natural enhancers (Herr and Clarke 1986). This seems to imply that the arrangement of individual factor-binding sites within natural enhancers is of relatively little importance. Recent data, however, suggest that the position and arrangement of factor-binding sites are important for function of certain natural enhancers and for the synergistic activation of transcription by enhancer-bound proteins (for review, see Tjian and Maniatis 1994). For example, multimerization of various factor-binding sites from enhancers does not allow for transcriptional activation, and the spacing of nonadjacent factor-binding sites can be important for enhancer function. Moreover, the specificity of the biological response of enhancer function can differ in natural and synthetic enhancers (Thanos and Maniatis 1992; Du et al. 1993).

One example of a natural enhancer that depends on a particular arrangement of factor-binding sites is provided by the minimal T cell receptor (TCR) α -gene enhancer, which functions specifically in T lymphocytes. This enhancer contains a binding site for LEF-1 in the center, and binding sites for the lymphocyte-specific protein Ets-1 and for a cell type-nonspecific protein of the activating transcription factor/cAMP response element-binding (ATF/CREB) family at either end of the enhancer (Ho and Leiden 1990). Changes in the spacing between the ATF/CREB- and the LEF-1-binding sites impaired the activity of this enhancer (Ho and Leiden 1990). Moreover, multimerization of the LEF-1-binding site alone or in combination with the Ets-1-binding site did not generate an active enhancer complex (Waterman and Jones

1990; Travis et al. 1991). Together with the capacity of LEF-1 to induce a sharp bend in the DNA helix, these data were interpreted to suggest a model in which LEF-1 serves as an architectural component in the assembly of a higher-order TCR α enhancer complex by facilitating interactions between ATF/CREB factors and Ets-1 (Giese et al. 1992). Here, we used biochemical and transfection assays to study the assembly and function of a specific multiprotein complex at the TCR α enhancer. We identified an additional T cell-specific protein, termed PEBP2 α (Ogawa et al. 1993a), that participates in the regulation of the TCR α enhancer. We provide evidence that LEF-induced DNA bending and interactions between proteins bound at adjacent and nonadjacent sites increase the specificity and stability of a higher-order nucleoprotein complex that forms at the TCR α enhancer.

Results

The T cell-specific protein PEBP2 α recruits Ets-1 into a ternary complex at the TCR α enhancer

Several lines of evidence suggest that in addition to ATF/CREB, LEF-1, and Ets-1, other factors may participate in the regulation of the TCR α enhancer. First, transfection of LEF-1 and Ets-1 expression plasmids into HeLa cells conferred only modest activity on a cotransfected TCR α enhancer construct (data not shown). Second, DNase I footprint analysis of the TCR α enhancer with T cell nuclear extracts indicated that a region between the LEF-1- and Ets-1-binding sites was protected from nuclease digestion (Ho et al. 1989; Winoto and Baltimore 1989). Finally, mutations in this region decreased the activity of the enhancer (data not shown). Inspection of the nucleotide sequence between the LEF-1- and Ets-1-binding sites identified two putative recognition motifs for a T cell-specific factor, termed core-binding factor (CBF; Wang et al. 1993) or polyoma enhancer-binding factor 2 (PEBP2; Ogawa et al. 1993a). This factor was shown to consist of a T cell-specific α subunit that mediates specific DNA recognition and a ubiquitously expressed β subunit that modestly enhances DNA binding by the α subunit, apparently without contacting DNA (Ogawa et al. 1993a,b; Wang et al. 1993).

We examined binding of purified recombinant PEBP2 α protein to a radiolabeled DNA probe containing nucleotides 60–105 of the minimal TCR α enhancer in an electrophoretic mobility-shift assay (EMSA) (Fig. 1A,B). PEBP2 α formed a specific complex that could be abrogated by mutations in either of the putative PEBP2 α -binding sites. The specificity of DNA recognition was confirmed by the resistance of the complex to competition with nonspecific DNA (data not shown). One of the PEBP2 α -binding sites overlaps with the Ets-1 recognition sequence. Although binding of recombinant Ets-1 to the TCR α enhancer has been reported (Ho et al. 1990), this protein binds only weakly (Fig. 1C, lane 4). To examine whether PEBP2 α facilitates DNA binding by Ets-1, we incubated the 45-bp TCR α wild-type probe with purified

Figure 1. PEBP2α and Ets-1 form a ternary complex at the TCRα enhancer. (A) Nucleotide sequence of the 98-bp minimal human TCRα enhancer (position 12–109) with factor-binding sites indicated. The brackets below indicate the nucleotide sequences protected from DNase I digestion in T-cell nuclear extract (this study; Ho and Leiden 1989). Mutations in the PEBP2α- and Ets-1-binding sites are indicated with lowercase letters. (B) EMSA of ³²P-labeled wild-type TCRα oligonucleotide probes comprising nucleotide sequences 60–105 with 200 ng of purified recombinant PEBP2α protein. (Lane 1) Wild-type TCRα probe; (lanes 2,3) mutant TCRα probes containing nucleotide substitutions in the PEBP2α-binding sites. (C) EMSA of 200 ng of purified PEBP2α protein or 150 ng of purified GST-Ets-1 protein, or combined, with a TCRα wild-type probe (lanes 1–4) or a mutant probe (mut 3) containing base-pair substitutions in the Ets-1-binding site (lanes 5–8). Incubation of ≤2 μg of GST-Ets-1 protein did not result in detectable DNA-binding activity (data not shown). (D) EMSA of PEBP2α and GST-Ets-HA in the absence or presence of antibody against the HA epitope (lanes 2–4) or control antibody against a Myc epitope (lane 5). (E) EMSA of the TCRα wild-type probe with a constant amount of PEBP2α (20 ng) and increasing amounts of GST-Ets-1 protein (lanes 2–4; 45 ng, 150 ng, 450 ng). The positions of the protein–DNA complexes and the free probes (F) in each experiment are indicated.

glutathione *S*-transferase (GST)–Ets-1 protein alone or in combination with recombinant PEBP2α (Fig. 1C). Coincubation of PEBP2α and GST–Ets-1 yielded a complex that migrated with a slower mobility than that formed with PEBP2α alone (lanes 2,3). No detectable complex was formed with GST–Ets-1 protein alone (lane 4) suggesting that PEBP2α augments DNA binding by GST–Ets-1.

To determine whether the formation of the slower migrating complex is dependent on the Ets-binding site, we mutated the trinucleotide 5′-GGA, which was shown previously to constitute the core motif for members of the Ets-family of DNA-binding proteins (Karim et al. 1990). Formation of the slower migrating complex with this mutated DNA probe (mut 3) was abrogated, whereas PEBP2α binding was observed at normal levels (Fig. 1C, lanes 6,7). To confirm the presence of GST–Ets-1 in the slower migrating complex, we performed a gel mobility-shift assay with a hemagglutinin (HA)-tagged GST–Ets-1 protein. Addition of monoclonal antibody against the HA epitope abrogated the formation of the slower migrating complex but did not affect DNA binding by PEBP2α (Fig. 1D, lanes 3,4). No change in the mobility of

the slower migrating ternary complex was observed with control antibody (lane 5). On the basis of the inability of GST–Ets-1 to bind TCRα DNA by itself, we examined whether this protein can bind DNA cooperatively with PEBP2α. To this end, we used a constant but limiting amount of PEBP2α protein and increased the concentration of GST–Ets-1 protein in the EMSA. The abundance of the ternary complex increased 20-fold at the maximal concentration of added GST–Ets-1 protein (Fig. 1E). Together, these data suggest that DNA binding by PEBP2α and GST–Ets-1 is highly cooperative.

PEBP2α protein is a member of the “Runt family” of proteins (Kagoshima et al. 1993). To examine whether the DNA-binding domain of PEBP2α, termed the Runt domain, is sufficient to form a ternary complex with Ets-1, we incubated the 45-bp TCRα probe with purified Runt domain alone or together with GST–Ets-1 (Fig. 2). Addition of GST–Ets-1 protein decreased the rate of migration of the Runt domain–DNA complex and increased its abundance (Fig. 2B, lanes 4,5). This suggests that Ets-1 can cooperate with the Runt domain of PEBP2α to bind DNA.

The capacity of the Runt domain to recruit GST–Ets-1

Figure 2. The Runt domain of PEBP2 α interacts with a defined region of Ets-1. (A) Schematic diagrams of deletion derivatives of PEBP2 α and Ets-1. (B) EMSA with recombinant PEBP2 α and GST-Ets-1 proteins. Wild-type TCR α probe (nucleotides 60–105) was incubated with 200 ng of purified PEBP2 α (lanes 2,3) or with ~200 ng of the Runt domain of PEBP2 α [PEBP2 α (93–226)] (lanes 4,5) in the absence or presence of 150 ng of GST-Ets-1 protein. (Lane 6) Binding reaction with 150 ng of GST-Ets-1. (C) In vitro association assay between GST-Runt domain immobilized on glutathione agarose beads and in vitro 35 S-labeled Ets-1 polypeptides. (Top) Ten percent of radiolabeled full-length and truncated Ets-1 polypeptides. Molecular size markers are shown in kilodaltons. (Middle) In vitro association of Ets-1 proteins with GST-Runt domain. (Bottom) In vitro association of Ets-1 proteins with GST protein. The input and the bound proteins were analyzed by SDS-PAGE and visualized by autoradiography.

into a ternary complex suggested that these proteins may interact directly with each other. To this end, we examined whether 35 S-labeled wild-type or truncated Ets-1 polypeptides can interact with immobilized GST-Runt domain fusion protein (Fig. 2C). We included ethidium bromide in this experiment to disrupt nonspecific interactions caused by contaminating DNA (Lai and Herr 1992). Weak association was observed with full-length Ets-1 protein (lane 1). Significant association was observed with the truncated Ets-1 polypeptides Δ 123, 60/300, and 60/240 (lanes 2,4,5). This association seems to be specific, as no interaction was observed between 35 S-labeled Ets-1 polypeptides and immobilized GST protein. The lack of association between immobilized GST-Runt domain and Δ 167 Ets-1 and 60/180 (lanes 3,6) delineates the interaction domain in Ets-1 to between amino acids 123 and 240.

Synergistic function of factor-binding sites in the TCR α enhancer

To determine the functional role of PEBP2 α and the other TCR α enhancer-binding proteins in vivo, we mutated individual factor-binding sites in the context of the

minimal enhancer. Transfections of the wild-type TCR α enhancer into T cells increased the activity of a linked minimal *fos* promoter 55-fold (Fig. 3A). Mutations in one of the PEBP2 α -binding sites (TCR α M3) decreased enhancer function by a factor of 10. Likewise, mutations in any of the binding sites for Ets-1 (TCR α M4), ATF/CREB (TCR α M1), and LEF-1 (TCR α M2) resulted in a similar 9- to 16-fold reduction in TCR α enhancer function, suggesting that proteins recognizing these four binding sites synergize to activate transcription.

This synergy could reflect interactions between multiple enhancer-bound proteins. To examine whether a specific helical phasing relationship is necessary for TCR α enhancer function, we inserted 4 nucleotides between the ATF/CREB- and LEF-1-binding sites. This mutation (TCR α +4) decreased the activity of the TCR α enhancer in T cells by a factor of three (Fig. 3B). In contrast, insertion of 10 nucleotides (TCR α +10), which maintained the helical phasing relationship, did not alter the activity of the enhancer. These data suggest that an ATF/CREB protein interacts functionally with a factor bound at one of the downstream sites. To further explore the requirement for a specific arrangement of factor-binding sites in the TCR α enhancer, we generated two

Figure 3. Synergistic function of factor-binding sites in the TCRα enhancer. (A) Schematic diagram of wild-type and mutant TCRα enhancer constructs containing nucleotide substitutions in the ATF/CREB-, LEF-1-, PEBP2α-, or Ets-1-binding sites. In these constructs the TCRα enhancer was linked to the minimal *fos* promoter (–56 to +109) (●), and the chloramphenicol acetyltransferase (CAT) gene. BW5147 T cells were transfected with 1 μg of the TCRα enhancer constructs shown in A. CAT levels shown are relative to the level obtained with a *fos*/CAT reporter construct lacking an insert. (B) Schematic diagram of wild-type and mutant TCRα enhancer constructs with 4 or 10 nucleotides inserted between the ATF/CREB- and LEF-1-binding sites. T cells were transfected with 1 μg of the different TCRα reporter constructs. (C) TCRα enhancer constructs with altered spatial arrangement of factor-binding sites. T cells were transfected with 1 μg of the different TCRα reporter constructs. Two hundred nanograms of a pRSV-luciferase plasmid was included in all transfection experiments as an internal standard. CAT activities were determined relative to 100% wild-type activity. Representative CAT assays from a minimum of three experiments are shown. The variability between individual experiments was <15%.

mutations in which the LEF-1-binding site was placed at the 3' end of the enhancer. Transfection of these mutant enhancer constructs in which the ATF/CREB-binding site was juxtaposed with the binding sites for either Ets-1 (TCRα M5) or PEBP2α (TCRα M6) showed a threefold and sixfold decrease in enhancer function, respectively (Fig. 3C). Mutation of the LEF-1-binding site in the TCRα M5 construct did not decrease enhancer activity further (data not shown), indicating that LEF-1 is unable to function in an altered context of factor-binding sites. Together, these observations suggest the importance of a specific arrangement of factor-binding sites in the TCRα enhancer.

Reconstitution of TCRα enhancer function in nonlymphoid cells

Previously, we and others have found that LEF-1 stimulates TCRα enhancer function in B cells that contain Ets-1 but lack LEF-1 and PEBP2α (Travis et al. 1991; Carlson et al. 1993; Giese and Grosschedl 1993). To examine whether the activity of the TCRα enhancer could be increased further by expression of PEBP2α, we transfected into B cells a wild-type TCRα reporter construct together with expression plasmids for LEF-1 or PEBP2α, or both (Fig. 4A). A 15-fold stimulation of enhancer activity was observed with LEF-1 and PEBP2α, a level sig-

nificantly higher than the multiplicative product of the levels observed with LEF-1 and PEBP2α alone (Fig. 4A). With the aim of reconstituting TCRα enhancer function in nonlymphoid cells, we transfected into HeLa cells a TCRα reporter gene construct together with various combinations of expression plasmids for LEF-1, PEBP2α, and Ets-1 (Fig. 4B). Individual expression of any of these proteins did not increase the basal activity of the reporter construct significantly. Pairwise transfections of these expression plasmids indicated that Ets-1 in combination with either LEF-1 or PEBP2α increased enhancer function seven- to eightfold. Coexpression of all three proteins stimulated TCRα enhancer activity 36-fold to a level similar to that observed in T cells.

The presence of multiple Ets family members in lymphoid cells raises questions as to the recruitment of one particular member to the TCRα enhancer. In particular, Ets-1 and Fli-1 are both expressed in T and B cells and have very similar DNA-binding specificities (Wang et al. 1992; Gunther and Graves 1994). Moreover, purified recombinant Fli-1 protein resembles Ets-1 in its inability to bind the TCRα enhancer by itself and in its ability to cooperate with PEBP2α to form a ternary complex in EMSAs (data not shown). To examine whether Fli-1 can participate in the stimulation of TCRα enhancer activity in HeLa cells, we cotransfected expression plasmids for Fli-1, LEF-1, and PEBP2α, together with a TCRα reporter construct (Fig. 4C). In comparison with Ets-1, Fli-1 showed a significantly reduced ability to stimulate

TCR α enhancer function in this context. Immunoblot analysis of lysates of COS cells transfected with expression plasmids encoding HA epitope-tagged proteins confirmed that Fli-1 was expressed in these cells at an even higher level than Ets-1 (Fig. 4D). Assuming that the levels of steady-state expression of Fli-1 and Ets-1 are similar in COS and HeLa cells, these data suggest that specific members of the Ets family of proteins differ in their potential to synergize with other proteins to regulate the TCR α enhancer.

LEF-induced DNA bending contributes to the regulation of TCR α enhancer function

The helical phasing experiment strongly suggested that an ATF/CREB protein interacts functionally with proteins bound at a nonadjacent site. Such an interaction would require the deformation of the intervening DNA helix. DNA binding by LEF-1 has been shown previously to induce a sharp bend in the DNA helix (Giese et al. 1992). To examine whether LEF-induced DNA bending contributes to TCR α enhancer function, we used the HMG domain of LEF-1 in *trans*-activation experiments. To this end, we transfected into HeLa cells the wild-type TCR α enhancer construct, together with expression plasmids encoding PEBP2 α , Ets-1, and the HMG domain of LEF-1. The HMG domain of LEF-1 increased the activity of the enhancer two- to threefold relative to the level observed with PEBP2 α and Ets-1 alone (Fig. 5A).

This stimulation of TCR α enhancer function by the LEF-HMG domain was two times less efficient than that obtained with intact LEF-1 in a parallel experiment (data not shown). To obtain further support for a role of protein-induced DNA bending rather than protein-protein interaction, we used the HMG domain of the distantly related testis-determining factor SRY in a transfection experiment. The HMG domains of LEF-1 and SRY both bend the DNA helix but share only 24% amino acid identity (Ferrari et al. 1992; Giese et al. 1992, 1994). The SRY-HMG domain also increased, by a factor of two to three, the activity of a TCR α enhancer in which the LEF-1-binding site had been replaced with the SRY-binding site (Fig. 5B). In contrast, the activity of a TCR α enhancer containing a LexA-binding site, instead of the LEF-1-binding site, cannot be increased by LexA in transfection assays (Giese and Grosschedl 1993). Together with the analysis of mutations in the TCR α enhancer, these data support a role for DNA bending in the regulation of the TCR α enhancer.

Association between Ets-1 and members of the ATF/CREB family

The ATF/CREB-binding site in the TCR α enhancer strongly resembles a cAMP response element, although the enhancer is not responsive to cAMP (Waterman and Jones 1990). A virtually identical sequence motif in the T

Figure 5. HMG domain-induced DNA bending participates in the regulation of TCRα enhancer activity. (A) *Trans*-activation of a TCRα enhancer reporter construct by the HMG domain of LEF-1. HeLa cells were transfected with 1 μg of expression plasmids for the HMG domain of LEF-1, PEBP2α, and Ets-1 in various combinations together with 0.3 μg of a wild-type TCRα enhancer reporter plasmid. (B) *Trans*-activation of a modified TCRα enhancer construct, in which the LEF-1-binding site had been replaced with the SRY-binding site, by the HMG domain of the testis-determining factor SRY. HeLa cells were

transfected with 1 μg of expression plasmids for the SRY-HMG domain, PEBP2α, and Ets-1 in different combinations together with 0.3 μg of the modified TCRα enhancer construct. Two hundred nanograms of a pRSV-luciferase plasmid was included in all transfection experiments as an internal standard. The variability between individual experiments was <10%. Representative CAT assays from multiple transfections are shown for each experiment.

cell-specific CD3δ enhancer was shown previously to be recognized in thymocytes by various isoforms of the CRE-binding protein (CRE-BP) (Georgopoulos et al. 1992), which is identical to ATF-2 (Maekawa et al. 1989; Hai et al. 1990). In EMSAs, the ATF/CREB-binding site in the TCRα enhancer can be recognized by recombinant CREB and ATF-2 proteins and by proteins in T cells that react with monoclonal antibodies directed against ATF-1, ATF-2, and CREB (C. Kingsley and R. Grosschedl, unpubl.). To examine whether either CREB or ATF-2 can interact directly with one of the three proteins that recognize the 3' half of the TCRα enhancer, we performed a protein affinity blot analysis. In this assay, total bacterial lysates containing various TCRα enhancer-binding proteins were immobilized on a nitrocellulose membrane and incubated with ³⁵S-labeled Ets-1 polypeptide in the presence of ethidium bromide (Fig. 6A,B). In preliminary experiments we detected weak associations of Ets-1 with both CREB and the ATF-2₁₉₅ isoform, which is the predominant activating form of ATF-2 (Du and Maniatis 1994), but not with other TCRα enhancer-binding proteins (data not shown). Significant association, however, was detected between Ets-1 and the ATF-2₁₉₄ isoform, which lacks part of the dimerization domain (see Materials and methods). The interaction between Ets-1 and ATF-2₁₉₄ was also detected in an association assay in which nondenatured GST-ATF-2₁₉₄ protein was immobilized on agarose beads (Fig. 6C). This association was dependent on an amino-terminal region in Ets-1 and the presumably monomeric nature of GST-ATF-2₁₉₄, because no significant association was detected between Ets-1 and GST-ATF-2₁₉₅ or CREB in this assay.

LEF-1 and an ATF/CREB protein collaborate in the stabilization of DNA binding by PEBP2α and Ets-1

The contribution of DNA bending to TCRα enhancer function and the potential of ATF/CREB proteins to associate with Ets-1 raised the interesting possibility that LEF-1 facilitates the assembly of a higher-order nucleoprotein complex by juxtaposing the widely separated

ATF/CREB- and Ets-1-binding sites. Although PEBP2α and Ets-1 cooperate to bind the TCRα enhancer, the ternary complex is very unstable. Determination of the resistance of the PEBP2α-Ets-1-DNA complex to excess unlabeled TCRα probe in EMSAs as a function of time indicated a half-life of <5 sec for the complex at 20°C (data not shown). Consistent with the short half-life of this complex, purified PEBP2α and Ets-1 were unable to protect their binding sites in a DNase I footprint assay even with PEBP2β included in the reaction (data not shown). Attempts to examine the potential of bacterially expressed ATF-2 and LEF-1 to augment DNA binding by PEBP2α and Ets-1 failed, possibly because of a requirement for a different member of the ATF/CREB family, post-translational modification of the protein, or association with an additional yet unidentified cofactor.

Therefore, we performed DNase I protection assays with nuclear extracts from HeLa cells complemented with various combinations of purified lymphocyte-specific proteins. Incubation of a radiolabeled TCRα probe with nuclear extract from HeLa cells that had been infected with a recombinant LEF-1-vaccinia virus resulted in protection of the ATF/CREB- and LEF-1-binding sites (Fig. 7A, lanes 2–6). Addition of a constant amount of GST-Ets-1 and increasing amounts of PEBP2α proteins generated a DNase I footprint that included both the PEBP2α- and Ets-1-binding sites (lanes 5,6). The extent of this footprint was similar to that observed previously with T cell nuclear extract (Ho et al. 1989; Winoto and Baltimore 1989). To examine the LEF-1 dependence of this DNase I footprint, we used an extract from HeLa cells infected with a HA-vaccinia virus (lanes 10–13). In this extract, addition of purified PEBP2α and GST-Ets-1 did not result in a footprint suggesting that LEF-1 is essential for the stabilization of the PEBP2α-Ets-1 complex (lane 11). Indeed, addition of either purified LEF-1 or the HMG domain of LEF-1 generated a DNase I footprint at the PEBP2α- and Ets-1-binding sites (lanes 12,13).

ATF/CREB proteins can associate with Ets-1 in the absence of DNA and the function of the TCRα enhancer requires a particular helical phase between the ATF/CREB- and the downstream factor-binding sites. To-

Figure 6. Association between ATF-2₁₉₄ and Ets-1. (A) TCRα enhancer-binding proteins were expressed in *Escherichia coli* and total lysates were analyzed by SDS-PAGE. Proteins were visualized by Coomassie Blue staining, and in parallel transferred onto nitrocellulose membranes. Molecular size markers are shown in kilodaltons. (B) The immobilized proteins were assayed for protein interaction by incubation of the nitrocellulose membrane with in vitro-translated ³⁵S-labeled Ets-1. The largest detected polypeptide corresponds to the expected molecular weight of the GST-ATF-2₁₉₄ fusion protein. The additional proteins may represent degradation products of the fusion protein. (C) Association of Ets-1 with GST-ATF-2₁₉₄ immobilized on glutathione-agarose beads. (Lanes 1–3) 10% input of radio-labeled full-length and truncated Ets-1 polypeptides. In vitro associations of Ets-1 proteins with immobilized GST-ATF-2₁₉₄ protein (lanes 4–6) or immobilized GST-p85 protein (lanes 7–9). The input and the bound proteins were analyzed by SDS-PAGE and visualized by autoradiography.

gether these observations can be interpreted to suggest that an ATF/CREB protein also contributes to stabilization of DNA binding by PEBP2α and Ets-1. To test this possibility, we performed the DNase I footprint analysis with the mutant TCRα +4 enhancer in which 4 nucleotides had been inserted between the ATF/CREB- and LEF-1-binding sites (Fig. 7B). In contrast to the wild-type TCRα fragment, no footprint at the PEBP2α- and Ets-1-binding sites was observed despite the presence of LEF-1 (lanes 6,7). This result strongly suggests that the stabi-

lization of the PEBP2α-Ets-1 complex at the TCRα enhancer is dependent on both the HMG domain of LEF-1 and a protein bound at the ATF/CREB-binding site. Taken together, these data support a model in which a LEF-induced DNA bend facilitates an interaction between an ATF/CREB protein and Ets-1 to generate a higher-order nucleoprotein complex (Fig. 8).

Discussion

In this report we provide evidence that various protein-protein and protein-DNA interactions contribute to the coordinate assembly and synergistic function of multiple factors at the minimal TCRα enhancer: (1) Interactions between two sequence-specific proteins that bind adjacent sites in the enhancer mediate cooperative DNA binding; (2) interactions between proteins that bind nonadjacent sites in the enhancer stabilize DNA-bound proteins; and (3) protein-induced DNA bending facilitates the interaction between proteins bound at nonadjacent sites and contributes to the assembly of a higher-order nucleoprotein structure.

Ternary complex formation of factors bound at adjacent sites

In the TCRα enhancer, we observed cooperative DNA binding by the lymphocyte-specific proteins PEBP2α and Ets-1 that recognize adjacent sites. Similar to the ternary complex formed at the *c-fos* promoter between the serum response factor (SRF) and the Ets proteins Elk-1 or SAP-1 (Dalton and Treisman 1992; Janknecht and Nordheim 1992; Hill et al. 1993), we found that binding of PEBP2α and Ets-1 to the TCRα enhancer is augmented ~20-fold. This cooperativity of DNA binding presumably involves protein-protein contacts between the Runt domain of PEBP2α and Ets-1. Such interactions may antagonize an inhibitory domain between amino acids 207 and 280 of Ets-1 that was found previously to impair DNA binding by Ets-1 (Lim et al. 1992). Consistent with this view, removal of these amino acid residues allows Ets-1 to bind the TCRα enhancer by itself, although the level of DNA binding could be increased further by interaction with PEBP2α (data not shown). Moreover, the nucleotide sequence of the Ets-1-binding site may influence the relative dependence of Ets-1 on PEBP2α for DNA recognition. For example, the TCRβ enhancer, which contains an arrangement of factor-binding sites similar to that in the TCRα enhancer but differs in the sequence of the Ets-1-binding site, is recognized by Ets-1 in the absence of partially purified CBF/PEBP protein (Wotton et al. 1994). The half-life of the ternary complex, however, is twofold longer than that of the Ets-1-DNA complex (Wotton et al. 1994).

In contrast to the stability of SRF- and SRF-Elk-1-DNA complexes, which can be detected readily in DNase I protection assays, we found that the stability of the PEBP2α-DNA complex and that of the PEBP2α-Ets-1-DNA ternary complex appears to be low. The half-life of this ternary complex, as measured by competition of

Figure 7. Stabilization of the PEBP2α–Ets-1–DNA ternary complex by LEF-1 and an ATF/CREB protein. (A) DNase I footprint analysis of the 5′-end-labeled wild-type TCRα DNA fragment (12–109) using HeLa cell nuclear extract infected with a recombinant vaccinia virus expressing either LEF-1 (lanes 1–7) or HA protein (lanes 9–14). Nuclear extracts were complemented with purified lymphocyte-specific proteins. The amount of each protein added is indicated at top (in μg). Brackets indicate DNase I-protected regions. Lane M contains a G + A sequence ladder of the same DNA fragment. (B) DNase I footprint analysis of the mutant TCRα +4 DNA probe in HeLa cell nuclear extract infected with a vaccinia virus expressing LEF-1 protein. The position of the inserted nucleotides is indicated at left.

binding in EMSAs, is very short (<5 sec). Moreover, we did not observe protection of the PEBP2α- and Ets-1-binding sites in a DNase I footprint assay, even in the presence of PEBP2β. Occupancy of these binding sites in DNase I protection assays was shown to require LEF-1 and a specifically positioned upstream ATF/CREB-binding site, suggesting that the PEBP2α–Ets-1–DNA ternary complex is stabilized by interaction with other proteins (see below).

Consistent with the cooperative DNA binding by

PEBP2α and Ets-1, we observed synergy of activation of the TCRα enhancer by these proteins in transfection assays. A similar synergy was also observed between Ets-1 and LEF-1 but not between PEBP2α and LEF-1. Moreover, we found that Ets-1 could synergize with the HMG domains of either LEF-1 or SRY. This functional synergy may be indirect and involve the interaction between Ets-1 and an ATF/CREB protein, because we were unable to detect specific interactions between Ets-1 and the HMG domain of LEF-1 in vitro. One interpretation of this finding is that the HMG domain synergizes with Ets-1 by bending the DNA helix and juxtaposing the nonadjacent ATF/CREB- and Ets-1-binding sites.

Figure 8. Model of the multiprotein complex formed at the minimal TCRα enhancer. The LEF-induced DNA bend at a specifically positioned site in the enhancer augments binding of a PEBP2α–Ets-1 complex through protein–protein interactions with an ATF/CREB protein, which results in the assembly of a higher-order nucleoprotein complex.

LEF-induced DNA bending in assembly and function of the TCRα enhancer complex

We have shown previously by circular permutation analysis and by a prokaryotic site-specific recombination assay in vitro that LEF-1 induces a sharp bend in the DNA helix (Giese et al. 1992). However, it is difficult to distinguish whether protein-induced DNA bending has a functional role in regulating gene expression or whether it reflects a particular mode of sequence recognition. For example, LEF-1 and HMG-I(Y), along with other DNA-bending proteins like IHF and TATA-binding protein (TBP) recognize the minor groove of the DNA helix (Yang and Nash 1989; Starr and Hawley 1991; Giese et al. 1992; Thanos and Maniatis 1992). Therefore, the distortion of the DNA helix induced with binding of these proteins could be necessary solely to widen the minor groove for optimal sequence recognition as detected in

the TBP–DNA complex (J. Kim et al. 1993; Y. Kim et al. 1993). However, various observations support the view that DNA bending by LEF-1 contributes to normal function of the minimal TCR α enhancer.

The helical phasing relationship between the ATF/CREB- and the LEF-1-binding sites in the TCR α enhancer was found to be important both for the assembly of a stable nucleoprotein complex in vitro and for enhancer function in transfection assays. The activity of the enhancer was decreased threefold by rotating the ATF/CREB-binding site half a helical turn relative to the LEF-1-binding site but was restored to wild-type levels by an additional half-helical rotation. A similar requirement for a specific helical phase between factor-binding sites was observed in the *c-fos* promoter in which changes in the relative positions of binding sites for CREB and YY1 were shown to influence transcription by a factor of four to five (Natesan and Gilman 1993). In this context, YY1 is bound between the CREB site and the TATA box and is thought to juxtapose these nonadjacent sites by DNA bending. A structural role for YY1 in organizing promoter topology was supported further by demonstrating that YY1 in the *c-fos* promoter could be replaced functionally with a binding site for the HMG domain of SRY that bends the DNA helix (Natesan and Gilman 1993). Likewise, the HMG domain of SRY can partially replace LEF-1 in stimulating the TCR α enhancer activity in transfection assays. The HMG domains of LEF-1 and SRY are related only distantly and recognize different nucleotide sequences (Giese et al. 1992, 1994). Therefore, we consider it unlikely that the HMG domain of SRY mediates interactions with proteins that specifically recognize the TCR α enhancer, although we cannot rule out protein–protein contacts that involve a common feature of these structural motifs. We also attempted to replace the LEF-1-binding site in the TCR α enhancer with a 90-bp A-tract sequence, constituting an intrinsic bend of a similar magnitude to the LEF-induced DNA bend. The activity of this modified TCR α enhancer, however, was lower than that of an enhancer lacking a functional LEF-1-binding site, presumably because insertion of such a long A-tract disrupted the overall geometry of the TCR α enhancer (data not shown).

LEF-1 was shown previously to contain a context-dependent activation domain in the amino-terminal half of the protein, which is thought to mediate interactions with other enhancer-bound proteins (Carlson et al. 1993; Giese and Grosschedl 1993). Although these putative protein–protein interactions may contribute to transcriptional stimulation in vivo, they do not participate in the assembly of the TCR α enhancer complex in vitro. The HMG domain of LEF-1, which bends the DNA helix, is sufficient for the formation of a stable multiprotein complex in vitro and can partially stimulate enhancer function in vivo. However, the activation domain is required for full stimulation of enhancer function by LEF-1. Thus, the architectural role of LEF-1 in regulating the TCR α enhancer may consist of both juxtaposing nonadjacent binding sites through DNA bending and position-

ing the activation domain for interaction with other enhancer-bound proteins.

The formation of a stable nucleoprotein complex at the TCR α enhancer in a DNase I protection assay shows a marked dependence on the HMG domain of LEF-1 and on a specific helical phasing relationship of the nonadjacent LEF-1- and ATF/CREB-binding sites. In contrast, the transcriptional effects of mutations in the TCR α enhancer that alter this helical phasing relationship are relatively modest. This can be interpreted to suggest that the architecture of proteins at the TCR α enhancer in vivo may be somewhat flexible. The dependence of TCR α enhancer function on LEF-1 in vivo may be influenced by the presence of abundant nonspecific DNA-binding proteins and by modifications of proteins that alter the strength of protein–protein interactions. For example, the formation of a ternary complex between the Ets-protein Pu.1 and the nuclear factor NF-EM5 is increased markedly by phosphorylation of Pu.1 (Ponguballa et al. 1993). Strong interactions between proteins bound at nonadjacent sites may, in principle, diminish the requirements for a directed DNA bend induced by a sequence-specific protein and thus allow for functional substitution by nonspecific proteins that increase the flexibility of DNA. This view is supported by recent experiments indicating that the sequence-specific DNA-binding protein IHF can be replaced, in part, by the nonspecific DNA-binding proteins HU and HMG-1 in the assembly of a higher-order nucleoprotein complex involved in site-specific recombination (Segall et al. 1994). Moreover, the formation of the specific multiprotein complex at the TCR α enhancer, which appears to involve one architectural component, may be more flexible than the nucleoprotein complexes assembled at the bacteriophage λ *attP* site and the β -interferon enhancer, which both use an architectural element at three distinct sites (Nash 1990; Thanos and Maniatis 1992; Du et al. 1993).

Recently, we examined the biological role of LEF-1 for lymphoid gene expression and mouse development by targeted inactivation of the *LEF-1* gene in the mouse germ line (van Genderen et al. 1994). The LEF-1-deficient mice were found to die shortly after birth and to display severe defects in the formation of various nonlymphoid organs. However, no obvious deficiencies were detected in lymphoid cell populations of the mutant mice at birth. Moreover, expression of the TCR α gene in the thymus of newborn *LEF-1* (–/–) mice appeared to be normal (R. Okamura and R. Grosschedl, unpubl.). This lack of an obvious phenotype in T lymphocytes could be accounted for, in principle, by a redundancy of LEF-1 with the closely related T cell factor-1 (TCF-1), which is also expressed in T cells and contains a DNA-binding domain virtually identical to that found in LEF-1 (van de Wetering et al. 1991). TCF-1-deficient mice also express the TCR α gene but have a decreased number of thymocytes with an increased percentage of an immature cell population (Verbeek et al. 1995). Consistent with a redundant function of LEF-1 and TCF-1 in T cells, preliminary analysis of mice homozygous for a mutation in both genes

revealed a more severe deficiency in the generation of mature T lymphocytes (R. Okamura, H. Clevers, R. Grosschedl, and S. Verbeek, unpubl.).

Coordinate assembly and synergistic function of regulatory proteins in multiprotein complexes

The assembly of proteins into a higher-order nucleoprotein complex may have important implications for the accuracy and diversity of transcriptional regulation. The selection of individual protein family members that share specificity of DNA recognition and protein–protein interactions may be augmented by a multiplicity of protein–protein contacts and by the cooperative nature of assembly of a multiprotein–DNA complex. For example, our data indicate that Ets-1, but not the closely related Fli-1 protein, participates in the regulation of the TCR α enhancer, although both proteins have virtually identical DNA-binding specificities (Wang et al. 1992). Moreover, both Ets family members can interact with PEBP2 α to form a ternary complex at the TCR α enhancer. Although Ets-1 can associate with ATF-2₁₉₄, we were unable to detect such interaction with Fli-1 (data not shown). This result seems to imply that the PEBP2 α –Ets-1–DNA complex but not the PEBP2 α –Fli-1–DNA complex can be stabilized by association with an ATF/CREB protein. Thus, multiple protein–protein contacts may be necessary to allow for the recruitment of a particular protein into a functional enhancer complex.

The formation of a higher-order multiprotein complex may also have an important role in stabilization of protein–DNA interactions. Stable occupancy of the PEBP2 α - and Ets-binding sites in a DNase I protection assay was only detected in the presence of LEF-1 and a specifically positioned nonadjacent ATF/CREB-binding site. In particular, the dependence on a specific helical phase of these factor-binding sites strongly suggests a functional interaction between an ATF/CREB protein and the PEBP2 α –Ets-1–DNA ternary complex. Although a stable TCR α enhancer complex was formed in HeLa cell nuclear extracts complemented with the lymphoid-specific proteins LEF-1, PEBP2 α , and Ets-1, we were unable to generate such a nucleoprotein complex with purified ATF-2₁₉₅ or ATF-2₁₉₄ and these lymphoid-specific proteins. In association assays, we detect specific interaction between Ets-1 and the ATF-2₁₉₄ isoform, which lacks part of the dimerization domain and is presumably a monomer, but not between Ets-1 and the dimeric ATF-2₁₉₅ or CREB proteins (data not shown). This difference in the association potential of the ATF-2₁₉₅ and ATF-2₁₉₄ proteins raises the interesting possibility that a change in the conformation of an ATF/CREB protein occurs within the context of the enhancer complex in nuclear extracts and exposes a surface for efficient association with Ets-1. According to this view, this interaction surface would be obscured in the recombinant ATF-2₁₉₅ protein in solution. For example, phosphorylation is thought to alter the conformation of ATF-2 and increase its potential to interact with DNA and with other pro-

teins (Abdel-Hafiz et al. 1992; Thanos et al. 1993). Alternatively, a cofactor in nuclear extracts may facilitate the association between ATF-2 and Ets-1. Consistent with this scheme, interactions between ATF-2 and the p50 subunit of NF κ B in the β -interferon enhancer were found to be augmented by HMG-I(Y) (Du et al. 1993). HMG-I(Y) bends DNA and, by inducing conformational changes in ATF-2, NF κ B, and DNA, it may provide a structural component that facilitates DNA–protein and protein–protein interactions (Thanos and Maniatis 1992; Du et al. 1993; Du and Maniatis 1994).

Whatever additional factors contribute to the formation of a TCR α enhancer complex, our data suggest that LEF-induced DNA bending and interactions between multiple proteins provide a mechanism to allow for specific recruitment of proteins to the enhancer and for stability of the final higher-order nucleoprotein complex. However, the stereospecific assembly of nucleoprotein complexes, such as those formed at the TCR α and β -interferon enhancers, may be restricted to small functional enhancer units. Regulatory regions that govern intricate developmental patterns of gene expression may consist of multiple such units that are flexible in their relationship to one another and to the promoter.

Materials and methods

Cell culture, viral infections, and nuclear extract preparations

COS-7 cells and HeLa cells were cultured at 37°C in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum (FCS), and T cells (BW5147) and B cells (Namalwa) were grown in RPMI medium, supplemented with 10% FCS and 50 μ M 2-mercaptoethanol. Infections of HeLa cells with recombinant vaccinia virus were performed as described in Travis et al. (1991). Nuclear extracts were prepared according to Dignam et al. (1983).

Plasmid construction

The expression plasmids for full-length LEF-1 protein and the LEF–HMG domain peptide are described in Giese and Grosschedl (1993). The coding region for the SRY–HMG domain (Giese et al. 1992) was ligated into plasmid pCG, a pEVRF derivative (Matthias et al. 1989). The pCG plasmid contains the human cytomegalovirus (CMV) enhancer/promoter region, the translation initiation region of the herpes simplex virus (HSV) thymidine kinase (*tk*) gene, and the sequence for the HA1 epitope tag (SYPYDVPDYASLGGPS; Wilson et al. 1984) that was followed by a translation stop codon. The coding region for PEBP2 α was isolated from the PEBP2 α cDNA (Ogawa et al. 1993a) by restriction with *Fsp*I (amino acid 35) and *Eco*RI (amino acid 501) and ligated into the pCG plasmid. The coding region for the Runt domain of PEBP2 α was isolated from the PEBP2 α cDNA with *Nco*I (amino acid 95) and *Hind*III (amino acid 226), blunt-ended, and ligated into pGEX-2T cleaved with *Sma*I. The coding region of the PEBP2 β protein was obtained by PCR (forward *Nco* primer 5'-CCATGGCGCGCGTCGTCGG-GACC, reverse *Xho* primer 5'-CTCGAGTCTTGCTGTCT-TCTTGC) using the PEBP2 β cDNA (Ogawa et al. 1993b) as template. The amplified product was ligated into a *Nco*I–*Xho*I-

cleaved pET23d vector, in-frame with a hexa-histidine tag. The Ets-1-coding region was generated by PCR (forward *Nco* primer 5'-CATGCCATGGAGGCGGCGCTCGATCTCAAG, reverse *Bam* primer 5'-GGATCCCCGACCTTCGATGTCAGCATCCG-GCTTTACATCC) using the Ets-1 cDNA (Chen 1990) as template and ligated into plasmid pEVF. GST-Ets-1 and GST-Ets-HA were generated by fusion of the Ets-1 and Ets-HA-coding regions in-frame with the coding region of the GST gene in plasmid pGEX-3X (Pharmacia). The coding region of Fli-1 was generated by PCR (*Xba* primer 5'-TCTAGAATGTCGAC-CATGGACGGGACTATTAAGGAG, reverse *Xho* primer 5'-CTCGAGGTAGTAGCTGCCTAAGTGTGA) using the Fli-1 cDNA (Ben-David et al. 1991) and ligated into plasmid pCG. The coding region for CREB was isolated by restriction digest of the CREB cDNA (Berkowitz and Gilman 1990) with *Nde*I and *Bam*HI and ligated into plasmid pHB-40P, a pET derivative (Studier et al. 1990). GST-p85 is described in Klippel et al. (1993). ATF-2₁₉₅ is described in Du and Maniatis (1994). GST-ATF-2₁₉₄ is an ATF-2 isoform that lacks the DNA-binding and the dimerization domain. In this isoform, the nucleotide sequence CAGCTGCAG at the third leucine of the leucine zipper is fused to the sequence CTGCTGATA, 106 nucleotides downstream, which results in a frameshift and the addition of 40 amino acids. *Escherichia coli* K-12 strains DH5 α (Bethesda Research Laboratories) and BL21 (DE3)plyS (Studier et al. 1990) were used for plasmid propagation and for expression of proteins that are under the control of the *lac* or T7 promoter. The wild-type minimal TCR α enhancer construct, which contains the minimal *fos* promoter (-56 to +109) linked to the chloramphenicol acetyltransferase (CAT) gene (Berkowitz et al. 1989), has been described previously (Travis et al. 1991). The sequences of mutated factor-binding sites in the M1-M4 TCR α enhancer constructs, represented in lowercase letters, are shown together with the wild-type sequences. Construct M1, ATF/CREB [TtACcAT; TGACGTCA (wild type; Jones et al. 1988)]; construct M2, LEF-1 [aaTTTcAA; CCTTTGAA (wild type; Travis et al. 1991; Giese et al. 1991; 1992)]; construct M3, PEPB2a [aaGgt; CCGCA (wild type; Thornell et al. 1992)]; construct M4, Ets-1 [CACATggTg; CACATCCTC (wild type; Ho et al. 1990)]. In construct TCR α SRY, the following nucleotides in the LEF-1-binding site were substituted to generate the SRY-binding site (CCaTTGtgc, CCTTTGAAG; Giese et al. 1992, 1994). Construct TCR α +4 and TCR α +10 contain insertions of 4 (CTTA) or 10 (CTAGCTAGCT) nucleotides at position 61 between the ATF/CREB- and LEF-1-binding sites in the minimal TCR α enhancer. Only the upper strand is given for the mutant TCR α enhancer constructs in which the order of the factor-binding sites (underlined) had been changed. Construct M5, PEPB2 α /ETS-1/ATF/CREB/LEF-1, 5'-TCCCGCAGAAG-CCACATCCTCTGGAAAGAGGCGGTCCTCCCATTTCCATGACGTCACTGGTTACCAAGAGGGGCAATCCCGCAGAAGCCAC-ATCCTCTGGAAAGAGTAGGGCACCTTTGAAGCTC. The mutant TCR α enhancer constructs M7 and M8 contain nucleotide substitutions in the LEF-1-binding site as indicated in Figure 5A.

Ets-1 deletion constructs encoding amino acids 60-180, 60-240, and 60-300 were PCR amplified (forward primer 5'-CGACTGGGAATCCCCAAAGAC, reverse primers 5'-CTAGAACGTGGGCTCTGAGAACTC, 5'-TCATCTCCCC-AGGACATGTTGTC, 5'-TCAGGGCTTGTTGGTGGCA-GGGC, respectively), and ligated into modified Bluescript vectors. Ets-1 deletion constructs lacking 123, 167, or 230 amino-terminal residues were generated by digestion of the

Ets-1 cDNA with *Eco*RV, *Sph*I, or *Hpa*I, respectively, and ligation of the Ets-1 DNA fragments into a modified Bluescript vector.

Transient expression of recombinant proteins and CAT assays

Transient DNA transfections into mammalian cells were performed as described in Grosschedl and Baltimore (1985) using the DEAE-dextran/chloroquine procedure with various amounts of effector and reporter DNA and a Rous sarcoma virus (RSV)-luciferase reporter plasmid (De Wet et al. 1987) as a measure of transfection efficiency. Cells were harvested and CAT activity analyzed as described in Giese and Grosschedl (1993).

Immunoblotting, EMSA, and DNase I footprint analysis

Immunoblotting was performed as described in Giese and Grosschedl (1993). DNase I footprint analysis was performed essentially as described in Travis et al. (1991). Briefly, the DNA probes were incubated in binding buffer [10 mM HEPES (pH 7.9), 50 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM spermidine, 10% glycerol] including 750 ng of salmon sperm DNA and 750 ng of poly[d(I-C)] in the absence or presence of 100 μ g of nuclear extract from HeLa cells. Samples were treated with DNase I at 15 μ g/ml for 90 sec, and the reactions were stopped by adding phenol. DNA samples were phenol extracted, ethanol precipitated, and analyzed by denaturing polyacrylamide gel electrophoresis. EMSAs were performed with 50 ng of salmon sperm DNA and the indicated amounts of purified proteins according to Travis et al. (1991).

Protein-protein interactions

Bacterial cell lysates containing various overexpressed proteins were separated by 12% SDS-PAGE, blotted onto nitrocellulose membranes (Schleicher & Schuell), and blocked for several hours at 4°C in TBST buffer [10 mM Tris-HCl (pH 7.5), 130 mM sodium chloride, 0.5% Tween 20, 0.2% sodium azide] containing 3% bovine serum albumin (BSA), 0.1 mM methionine, and 50 μ g/ml ethidium bromide (EtBr). The nitrocellulose filters were incubated with in vitro-translated (reticulocyte lysates; Promega) ³⁵S-labeled proteins in TBST buffer containing cold methionine and EtBr for 12-16 hr at 4°C, and washed in TBST buffer; bound proteins were visualized by autoradiography. For protein association experiments on glutathione-agarose beads, GST-fusion proteins were overexpressed in bacteria and purified. Immobilized proteins were incubated with ³⁵S-labeled proteins in 200 μ l of TBST buffer containing 0.2% BSA and EtBr (50 μ g/ml) at room temperature for 30 min. The agarose beads were washed with 3 ml of TBST buffer, and associated proteins eluted by the addition of sample buffer [0.125 M Tris-HCl (pH 6.8), 8% SDS, 20% β -mercaptoethanol, and 40% glycerol]. Bound proteins were visualized after SDS-PAGE by autoradiography.

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