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LEF-1 contains an activation domain that stimulates transcription only in a specific context of factor-binding sites

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Lymphoid enhancer factor 1 (LEF-1) is a member of the high mobility group (HMG) family of proteins and participates in the regulation of the T cell receptor (TCR) α enhancer. We have previously shown that DNA binding by the HMG domain of LEF-1 induces a sharp bend in the DNA helix. Together with the dependence of LEF-1 on other factor-binding sites to regulate gene expression, DNA bending induced by the HMG domain suggested an 'architectural' role for LEF-1. In this study, we performed experiments to distinguish between a model in which the HMG domain is the only functional determinant of LEF-1 and a model in which additional domains of LEF-1 are involved in the regulation of gene expression. First, we show that the HMG domain alone is not sufficient to stimulate TCRα enhancer function. Second, we replaced the HMG domain of LEF-1 with the DNA-binding domain of the bacterial repressor LexA, which binds a specific nucleotide sequence without inducing a sharp bend in the DNA helix. The chimeric LEF-LexA protein increased the activity of a TCRα enhancer in which the LEF-1-binding site had been replaced with a LexA recognition sequence. Transcriptional stimulation by LEF-LexA, however, was less efficient than that observed with endogenous LEF-1. The LEF-LexA-mediated activation of gene expression was dependent upon an amino-terminal region of LEF-1 and a specific context of factor-binding sites in the TCRα enhancer. Neither multimerized LexA-binding sites, nor TCRα enhancers with altered spatial arrangements of factor-binding sites, were functional for regulation by LEF-LexA. Together, these data suggest that an amino-terminal region in LEF-1 contributes to the context-dependent regulation of the TCRα enhancer by LEF-1, presumably by interacting with other enhancer-bound proteins.

Key words: HMG domain protein/LEF-1/TCRα enhancer/transcriptional activation

Introduction

The activation of transcription by DNA-binding proteins can be mediated by multiple mechanisms (reviewed in Johnson and McKnight, 1989; Mitchell and Tjian, 1989; Shaw, 1990). One type of transcriptional activator interacts with the basal transcription machinery. For example, the yeast regulatory protein GAL4 contains, in addition to a DNA-binding domain, an acidic activation domain that contacts TFIIB (Ma and Ptashne, 1987; Lin and Green, 1991). Another type of regulatory protein contains an activation domain that stimulates transcription indirectly by facilitating the DNA binding and/or action of other activators (Campbell *et al.*, 1984; LaMarco *et al.*, 1991; Thompson *et al.*, 1991; Dalton and Treisman, 1992; Hill *et al.*, 1993). Thus, an activation domain is operationally defined and does not indicate the mechanism of function. Finally, transcription can be stimulated by proteins that do not contain an identifiable activation domain, but facilitate interaction between distinct factor-binding sites by DNA bending (reviewed in Landy, 1989; Nash, 1990; Pagel *et al.*, 1992). The bacterial integration host factor (IHF) is representative of this class of regulatory proteins. DNA bending by IHF was shown to mediate the interaction of upstream-bound activator proteins with RNA polymerase (Gober and Shapiro, 1990; Hoover *et al.*, 1990). In these experiments, IHF did not appear to interact with any other protein, and therefore can be regarded as a structural component in regulating gene expression and other DNA transactions. This architectural role is further supported by experiments in which the requirement for IHF in the assembly of a higher-order nucleoprotein complex at the bacteriophage λ *att*P locus could be replaced by naturally curved DNA or heterologous DNA-bending proteins (Goodman and Nash, 1989; Giese *et al.*, 1992).

Lymphoid enhancer-binding factor 1 (LEF-1) is a pre-B and T lymphocyte-specific DNA-binding protein that recognizes a specific nucleotide sequence in the T cell receptor (TCR) α chain enhancer (Travis *et al.*, 1991; Waterman *et al.*, 1991). The 112 bp minimal TCRα enhancer is functional in pre-B and T cells and contains at least two additional factor-binding sites that flank the LEF-1 recognition sequence on either side (Ho and Leiden, 1990a). The upstream factor-binding site represents a consensus sequence for members of the CREB family of transcription factors and was found to interact with a nuclear protein that is present in all cell types examined (Winoto and Baltimore, 1989; Ho and Leiden, 1990a). The downstream site was shown to interact with a nuclear protein found in T cells, but not in HeLa cells, and was identified as a target for recombinant Ets-1 protein (Ho *et al.*, 1990). A role of LEF-1 in the regulation of this enhancer has been demonstrated by mutations in the LEF-1-binding site which reduced enhancer function. Moreover, transfection of the LEF-1 cDNA into cell lines lacking endogenous LEF-1 increased the activity of a cotransfected reporter gene containing the TCRα enhancer. The function of LEF-1 in the regulation of gene expression was characterized by two features. First, multimerized binding sites for LEF-1 were unable to stimulate basal activity of a linked promoter (Waterman and Jones, 1990; Travis *et al.*, 1991). Second, changes in the spacing between the LEF-1-binding site and a recognition sequence for a member of the CREB family decreased TCRα enhancer function (Ho and Leiden, 1990a). Together, these data indicated that regulation of gene expression by LEF-1

was dependent upon other factor-binding sites and suggested that a protein recognizing the CREB-binding site interacts with a protein bound at the LEF-1- and/or Ets-binding sites.

LEF-1 contains an 85 amino acid DNA-binding domain which has homology to members of the family of high mobility group (HMG) proteins. This family includes the ubiquitously expressed transcription factors UBF and mtTF, which contain multiple HMG domains and participate in the regulation of transcription by RNA polymerase I and by a mitochondrial RNA polymerase, respectively (Jantzen *et al.*, 1990; McStay *et al.*, 1991; Parisi and Clayton, 1991; Leblanc *et al.*, 1993). The lymphoid-specific proteins LEF-1 and TCF-1, which contain a single and virtually identical HMG domain, regulate TCRα and CD3ϵ enhancer function (Travis *et al.*, 1991; Van de Wetering *et al.*, 1991; Waterman *et al.*, 1991). In contrast to the divergence of the amino acid sequences of LEF-1 and TCF-1 outside the HMG domain, these sequences are highly conserved between murine LEF-1 and human LEF-1, also termed TCF-1α, suggesting a functional role (Travis *et al.*, 1991; Waterman *et al.*, 1991). The HMG domain of LEF-1 was found to mediate binding of the protein as a monomer to the nucleotide sequence 5′-CCTTTGAA (Giese *et al.*, 1991). DNA binding by the HMG domain of LEF-1 was characterized by two rather unusual features. First, methylation interference experiments and replacement of A-T base pairs in the LEF-1-binding site with I-C base pairs indicated that the HMG domain contacts primarily the minor groove of the DNA double helix (Giese *et al.*, 1991, 1992; Van de Wetering and Clevers, 1992). Second, the HMG domain of LEF-1 induced a sharp bend in the DNA (Giese *et al.*, 1992). DNA bending was observed with other HMG domain proteins (Ferrari *et al.*, 1992; Giese *et al.*, 1992) and may be an intrinsic property of HMG-1 which has been shown to interact with cruciform and kinked DNA (Bianchi, 1988; Lilley, 1992; Pil and Lippard, 1992). This mode of DNA binding by LEF-1, together with the dependence on other factors to regulate enhancer function, is reminiscent of the bacterial integration host factor (IHF). Indeed, LEF-1 could in part substitute for the function of IHF to facilitate the assembly of a higher-order nucleoprotein complex at the bacteriophage λ *att*P locus (Giese *et al.*, 1992). These biochemical and functional properties of LEF-1 suggested a model in which this protein plays an 'architectural' role for gene expression by facilitating an interaction between proteins bound at widely separated sites. Therefore, the question arises as to whether LEF-1 functions in a manner analogous to IHF by playing a purely structural role, that is, without contacting other proteins, or whether LEF-1 is dependent upon interactions with other proteins to regulate enhancer activity.

Results

The HMG domain of LEF-1 is not sufficient to regulate TCRα enhancer function

If bending of the DNA helix was the sole functional determinant of LEF-1, the HMG domain should be sufficient to stimulate the activity of the TCRα enhancer. To examine the requirement for amino acid sequences in LEF-1 to regulate gene expression, we generated a set of progressive amino-terminal truncations (Figure 1A). LEF-1 cDNA clones carrying deletions of 5′ terminal sequences were inserted into an expression vector containing the cytomegalovirus enhancer/promoter and the translation initiation region of the thymidine kinase gene (Matthias *et al.*, 1989). In addition, the LEF-1 cDNA sequences were extended at the 3′ end to include sequences encoding 16 amino acids of the HA1 influenza hemagglutinin polypeptide which can serve as an epitope tag (Wilson *et al.*, 1984). To determine the relative stabilities of the wild-type or amino-terminal truncated LEF-1 proteins, we transfected the various LEF-1 gene constructs into COS-7 cells which allow for amplification of the plasmid DNAs. Nuclear extracts were prepared from the transfected cells and the expressed proteins were detected by immunoblot analysis using a monoclonal antibody directed against the HA1 tag (Figure 1B). Full-length protein and the various truncated LEF-1 proteins,

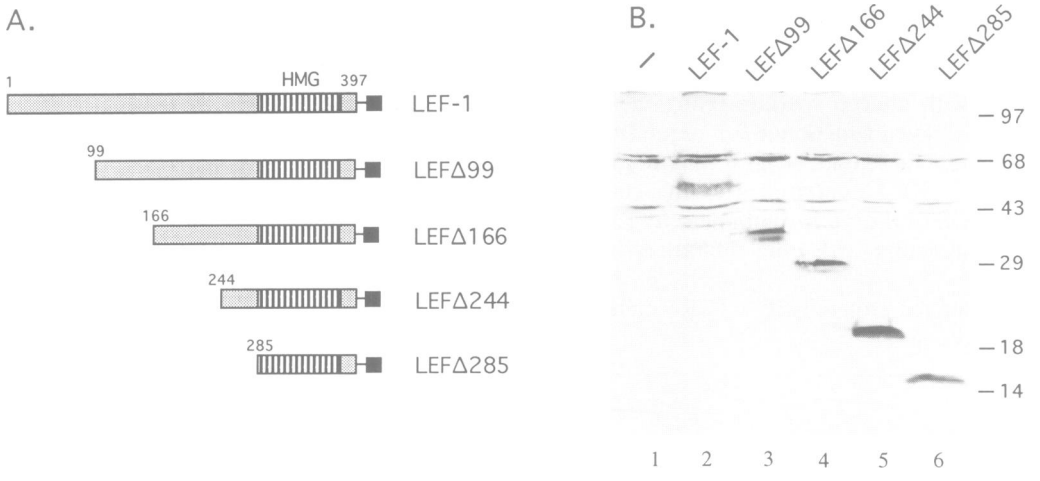

Fig. 1. Expression of full-length and truncated LEF-1 proteins in transfected cells. (**A**) Schematic structure of full-length LEF-1 and various amino-terminal truncations. The HMG domain of LEF-1, representing the DNA-binding domain, is indicated by the hatched box. The numbers indicate the amino acid positions in the LEF-1 protein. The HA1 influenza hemagglutinin epitope, fused to the carboxy-terminus of the LEF-1 proteins, is indicated by a black box. (**B**) Expression of LEF-1 and various amino-terminal truncations. Plasmids expressing various LEF-1 proteins were transfected into COS-7 cells. Nuclear extracts were prepared and the proteins were separated by SDS−PAGE using a 12−15% step gradient. The various LEF-1 proteins were detected by immunoblot analysis using monoclonal antibody 12CA5 directed against the HA1 epitope tag. Molecular size markers are shown in kilodaltons.

which migrated at rates expected from their relative molecular masses, were detected at similar levels indicating an equivalent stability of the proteins.

The truncated LEF-1 proteins were examined for their ability to stimulate TCRα enhancer function by cotransfection of the various LEF-1 expression plasmids together with a TCRα reporter plasmid into the B cell line M12, which lacks endogenous LEF-1. For quantification of the transfection efficiency, we included a plasmid expressing the luciferase gene (De Wet *et al.*, 1987). The reporter plasmid contained the minimal wild-type TCRα enhancer linked to a minimal *fos* promoter and the chloramphenicol acetyltransferase (*CAT*) gene (Figure 2). In the absence of exogenous LEF-1 protein, the TCRα enhancer increased the level of CAT expression 3-fold relative to that of the *fosCAT* reporter gene. As anticipated from our previous experiments, expression of the full-length LEF-1 protein further increased the activity of the TCRα enhancer by a factor of 3.5. By contrast, none of the truncated LEF-1 proteins augmented enhancer activity, indicating that amino-terminal sequences of LEF-1 are important for the ability of this protein to regulate gene expression.

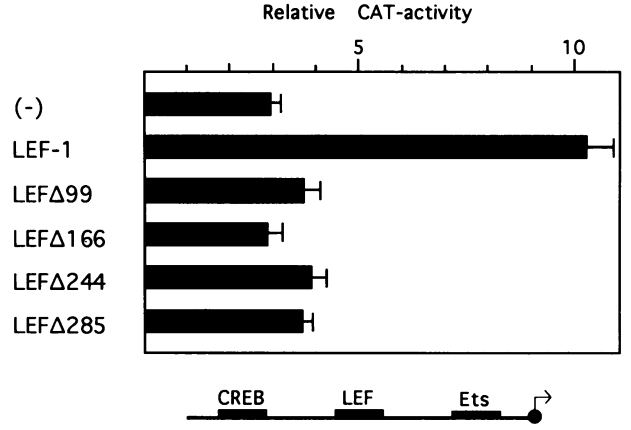

Fig. 2. Regulation of TCRα enhancer function by full-length and truncated LEF-1 proteins. Mature B cells (M12) were transfected with 3 μg effector plasmid, expressing either full-length LEF-1 or amino-terminal truncated proteins, together with 0.3 μg of a CAT reporter plasmid containing the 112 bp minimal human TCRα enhancer fragment (Ho and Leiden, 1990a; Travis *et al.*, 1991). This enhancer fragment comprises factor-binding sites for CREB (Jones *et al.*, 1988), LEF-1 (Travis *et al.*, 1991; Waterman *et al.*, 1991) and recombinant Ets-1 protein (Ho *et al.*, 1990) which are indicated by black boxes. The circle with the arrow represents the minimal *fos* promoter. For the control of transfection efficiency, 0.2 μg of an RSV-luciferase gene was included in each experiment. CAT activity was normalized to the activity obtained with a *fosCAT* reporter gene lacking the TCRα enhancer. Each column represents the mean of three independent experiments after normalization to values obtained from a cotransfected plasmid containing the luciferase gene. Error bars represent standard errors from multiple transfections. (−), no effector plasmid added.

Substitution of the HMG domain of LEF-1 with a heterologous DNA-binding domain

The inability of amino-terminally truncated LEF-1 proteins to stimulate TCRα enhancer function can be explained in two different ways. First, amino-terminal sequences may be involved in DNA binding by LEF-1, possibly by interaction with another protein. We have shown previously that the equilibrium dissociation constant of specific DNA binding by the monomeric LEF-1 protein is only 20- to 50-fold lower than that of nonspecific DNA-binding (Giese *et al.*, 1991). Therefore, interaction with another DNA-binding protein could augment the modest specificity of sequence recognition by LEF-1. Second, amino-terminal residues in LEF-1 could interact with other proteins to aid them in binding to DNA, and/or regulating gene expression. To distinguish between these possibilities, we replaced the HMG domain of LEF-1 with a heterologous DNA-binding domain that recognizes DNA with higher affinity and specificity. Moreover, the inability of the heterologous DNA-binding domain to bend DNA should provide some insight into the role of HMG domain-induced DNA bending for TCRα enhancer activity.

To this end, the LEF-1 cDNA sequences encoding amino acids 1−258 were fused to nucleotide sequences encoding

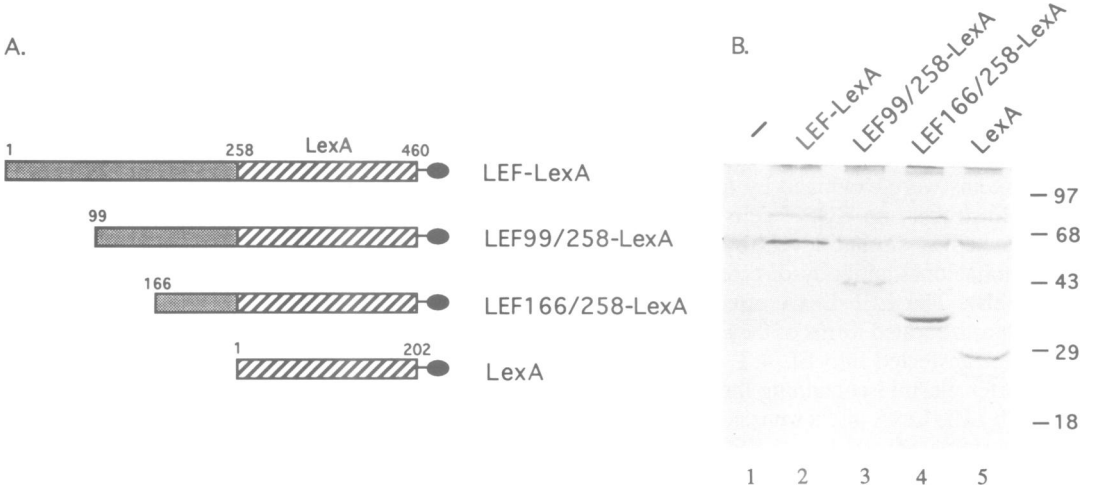

Fig. 3. Expression of chimeric LEF-LexA proteins in transfected cells. (**A**) Schematic structure of chimeric LEF-LexA protein and various amino-terminal truncations. Different lengths of the coding region of LEF-1, represented by a shaded box, were fused to the the DNA-binding and dimerization domain of the bacterial repressor LexA, shown as a hatched box. Numbers above indicate the amino acid positions in the proteins. The KT-3 epitope, fused to the carboxy-terminal ends of LEF-LexA proteins, is indicated by a black oval. (**B**) Expression of full-length and various amino-terminal truncations of the chimeric LEF-LexA protein. Plasmids expressing the LexA and the various LEF-LexA proteins were transfected into COS-7 cells. Nuclear extracts were prepared and proteins were separated by 12% SDS−PAGE. The LexA and the chimeric LEF-LexA proteins were detected by immunoblot analysis using a monoclonal antibody directed against the KT-3 epitope. Molecular size markers are shown in kilodaltons.

the DNA-binding and dimerization domain of the bacterial repressor LexA which binds DNA with high specificity and affinity (Brent and Ptashne, 1984; Oertel-Buchheit *et al.*, 1992). Moreover, the LexA protein was shown to interact with this particular binding site without inducing a detectable bend in the DNA helix (Lloubes *et al.*, 1988). The fusion of LEF-1 and LexA coding sequences generated a chimeric protein in which the carboxy-terminal location of the DNA-binding domain was maintained relative to LEF-1. For detection of expressed proteins in transfected cells, we linked an oligonucleotide encoding eight amino acids of the KT-3 epitope (MacArthur and Walter, 1984) to the LEF-LexA gene construct (Figure 3). We first examined the function of the chimeric protein by transfecting M12 B cells with increasing amounts of the LEF-LexA effector plasmid together with a modified TCRα reporter plasmid. In this reporter plasmid, we replaced 17 nucleotides containing the LEF-1-binding site in the enhancer with 17 nucleotides comprising a high-affinity recognition sequence for LexA (Materials and methods). Expression of the chimeric LEF-LexA protein stimulated the activity of the modified TCRα enhancer by a factor of three relative to the activity of the enhancer in the absence of LEF-LexA (Figure 4). The replacement of the HMG domain of LEF-1 with the LexA DNA-binding domain also allowed us to examine the potential of this chimeric protein to stimulate TCRα enhancer function in T cells containing endogenous LEF-1 protein. Transfection of the effector and reporter plasmids into EL-4 T cells increased the activity of the modified TCRα enhancer by a factor of seven. By contrast, transfection of the effector and reporter plasmids into the non-lymphoid cell line NIH3T3 did not increase enhancer function at any detectable level. Together, these data indicate that replacement of the HMG domain of LEF-1 with the DNA-binding domain of LexA allows the stimulation of TCRα enhancer activity in a cell-type specific manner. Thus, these experiments identified a region in LEF-1 that is involved in the regulation of gene expression, but does not overlap with the HMG domain.

LEF-1 contains an activation domain

To examine the requirement for amino-terminal sequences in activation by the chimeric LEF-LexA protein, we generated a set of truncations similar to those of the full-length LEF-1 protein (Figure 3A). The relative stabilities of the chimeric proteins were examined by transfection of the various gene constructs into COS-7 cells. The protein products were detected at similar levels by immunoblot analysis using a monoclonal antibody directed against the KT-3 tag (Figure 3B). The LEF-LexA effector plasmids encoding full-length or truncated forms of the chimeric LEF-LexA protein were transfected into EL-4 T cells together with a TCRα reporter plasmid containing the Lex-binding site. Both full-length LEF-LexA and a truncated polypeptide lacking the amino-terminal 99 residues (LEF99/258-LexA) stimulated TCRα enhancer activity by a factor of seven (Figure 5). Likewise, both chimeric proteins increased enhancer activity in transfected M12 B cells by a factor of three (Figure 4 and data not shown). By contrast, deletion of an additional 67 amino-terminal amino acids (LEF166/258-LexA) abrogated the ability of this protein to stimulate TCRα enhancer function (Figure 5). To determine a carboxy-terminal boundary for the activation domain of

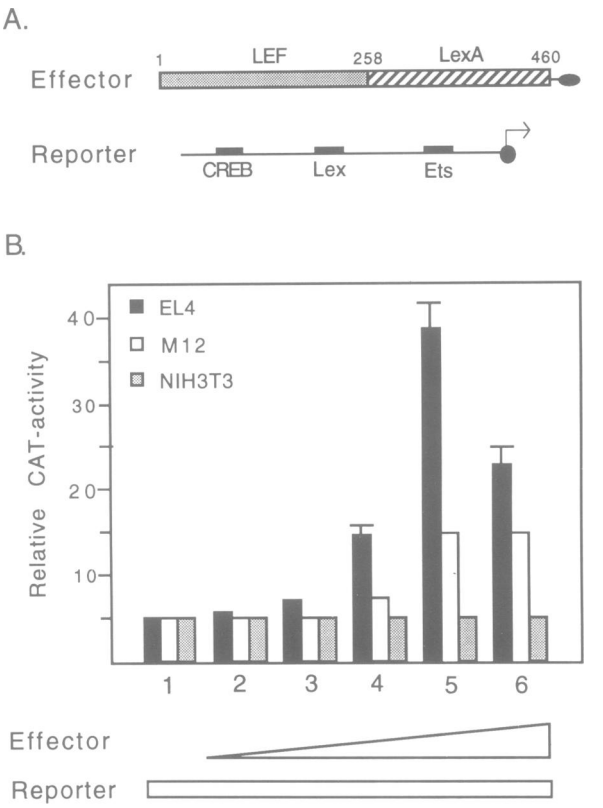

Fig. 4. The chimeric LEF-LexA protein stimulates TCRα enhancer activity in a cell-type specific manner. (**A**) Schematic representation of the LEF-LexA effector and the TCRα reporter plasmid. The representation of the various domains in the chimeric LEF-LexA protein are the same as in Figure 3. In the TCRα enhancer of the reporter plasmid, 17 nucleotides containing the LEF-1-binding site were replaced with 17 nucleotides encompassing a LexA recognition sequence. The filled circle and arrow represent the *fos* promoter linked to the *CAT* gene. (**B**) Increasing amounts of the LEF-LexA effector plasmid were transfected together with 1 μg of the TCRα reporter plasmid into various cell lines. The total DNA concentration in each experiment was kept constant by adding appropriate amounts of plasmid DNA lacking the coding region for the chimeric protein. Relative CAT activity in EL-4 T cells (filled columns), M12 B cells (open columns) and NIH3T3 cells (dotted columns) is shown as the mean of three independent experiments. 1, no effector plasmid; 2, 0.03 μg; 3, 0.1 μg; 4, 0.3 μg; 5, 1 μg and 6, 3 μg effector plasmid.

LEF-1, we removed from the LEF-LexA protein amino acids between 244 and 217 (LEF99/217-LexA) or between 244 and 185 (LEF99/185-LexA). Both of these fusion protein constructs retained amino acids 245−258 of LEF-1 which were found to be important for DNA binding by the chimeric LEF-LexA protein (see below and Materials and methods). The stability of these fusion proteins lacking carboxy-terminal LEF-1 sequences was examined by immunoblot analysis and found to be similar to that of the LEF-LexA fusion protein (data not shown). Expression of the LEF99/217-LexA protein in EL-4 T cells stimulated a modified TCRα enhancer to a level similar to that of the LEF-LexA protein, whereas stimulation by LEF99/185-LexA protein was halved (Figure 5). As a control, expression of the DNA binding and dimerization domain of LexA alone did not result in any increase in the activity of the TCRα enhancer. To confirm that the stimulatory effect of the chimeric LEF-LexA proteins was dependent upon DNA binding, we transfected the LEF-LexA effector plasmid together with a TCRα reporter plasmid in which the

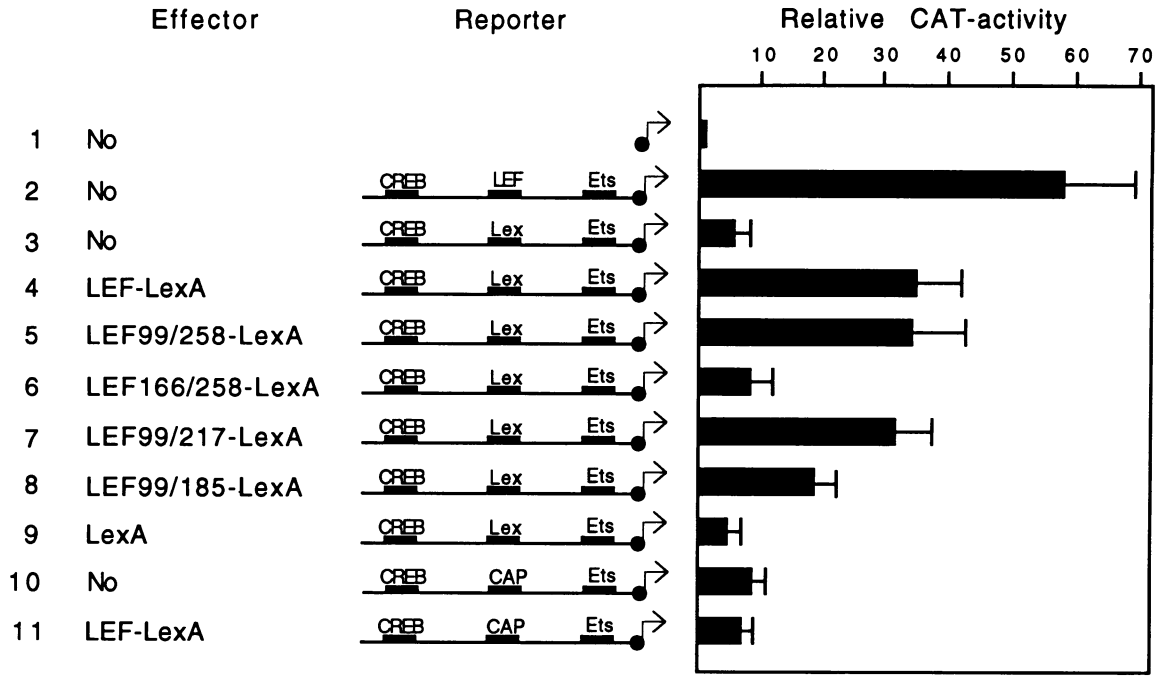

Fig. 5. LEF-1 contains an activation domain. T lymphoid cells (EL-4) were transfected with either 1 μg reporter plasmid alone (2, 3 and 10) or together with 1 μg effector plasmids expressing full-length or amino- and carboxy-terminal truncations of the chimeric LEF-LexA protein (4–8 and 11) or LexA protein (9). The structures of the effector and the reporter plasmids are indicated in the left-hand and middle panel, respectively. Columns show relative CAT activity and represent the mean of six independent experiments. The TCRα enhancer in the reporter plasmids contains a LEF-1-binding site (2), the LexA-binding site (3–9) and the recognition site for catabolic activator protein (CAP) (10 and 11). The filled circle and arrow represent the *fos* promoter linked to the *CAT* gene.

LEF-1-binding site was replaced with the recognition site for the bacterial catabolic activator protein (CAP). No significant increase in the activity of the TCRα enhancer was detected suggesting that the function of the chimeric protein is dependent upon DNA binding. For comparison, we included in these experiments the wild-type TCRα reporter plasmid containing the LEF-1-binding site, which was stimulated by the endogenous LEF-1 protein at a level twice as high as that observed with the modified TCRα enhancer and the chimeric LEF-LexA protein. Together, these data show that the region of LEF-1 encompassing amino acids 99–217 is capable of regulating TCRα enhancer function in the context of a heterologous DNA-binding domain and identified this region of LEF-1 operationally as an activation domain.

DNA binding by chimeric LEF-LexA proteins

The observed difference in the activation potential of the chimeric protein and LEF-1 could be explained if the chimeric protein had a lower DNA-binding affinity than LEF-1. Alternatively, other differences in the DNA-binding properties such as bending of the DNA helix or the stoichiometry of LEF-1 and LEF-LexA polypeptides bound to DNA could account for the observed difference. To examine DNA binding, we prepared nuclear extracts from COS-7 cells transfected with plasmids expressing either the chimeric LEF-LexA or the LexA protein. Incubation of these nuclear extracts with a radiolabeled duplex oligonucleotide comprising the LexA-binding site resulted in the formation of a LEF-LexA–DNA complex at a level similar to that of the LexA–DNA complex (Figure 6A). By contrast, a chimeric LEF-LexA protein in which amino acid 244 was directly fused to the LexA DNA-binding domain did not allow DNA binding at a high level (data not shown). Amino acids between 245 and 258 of LEF-1 may be important for a functional juxtaposition of the activation domain and the LexA DNA-binding domain which is normally located at the amino-terminus of the LexA protein. The specificity of DNA binding by the LEF-LexA protein was confirmed by addition of unlabeled oligonucleotide comprising either the Lex or the LEF-1 recognition sequence (Figure 6A). Binding of both proteins to DNA was sensitive to competition with an unlabeled oligonucleotide comprising the Lex-binding site but not with an oligonucleotide comprising the LEF recognition sequence. To quantify the DNA-binding affinity for the LEF-LexA protein, we determined the dissociation constants at equilibrium for this protein relative to the LexA protein. For these experiments we used limiting amounts of nuclear extract from transfected COS-7 cells, radiolabeled DNA at concentrations well below the expected K_D and increasing amounts of unlabeled DNA as competitor (Ingraham *et al.*, 1990). The binding saturation curves are graphically summarized in Figure 6B and C. The calculated dissociation constant of the LEF-LexA was only twice as high as that of the LexA protein, indicating that the chimeric protein binds DNA with high affinity and specificity. Thus, the high DNA-binding affinity of the chimeric LEF-LexA protein does not suffice to mediate transcriptional activation at the same level observed with endogenous LEF-1 protein.

The function of the activation domain of LEF-1 is dependent upon a specific context of factor-binding sites

Previous experiments indicated that LEF-1 was unable to stimulate transcription from a minimal promoter containing multimerized LEF-1-binding sites. LEF-1 was found to

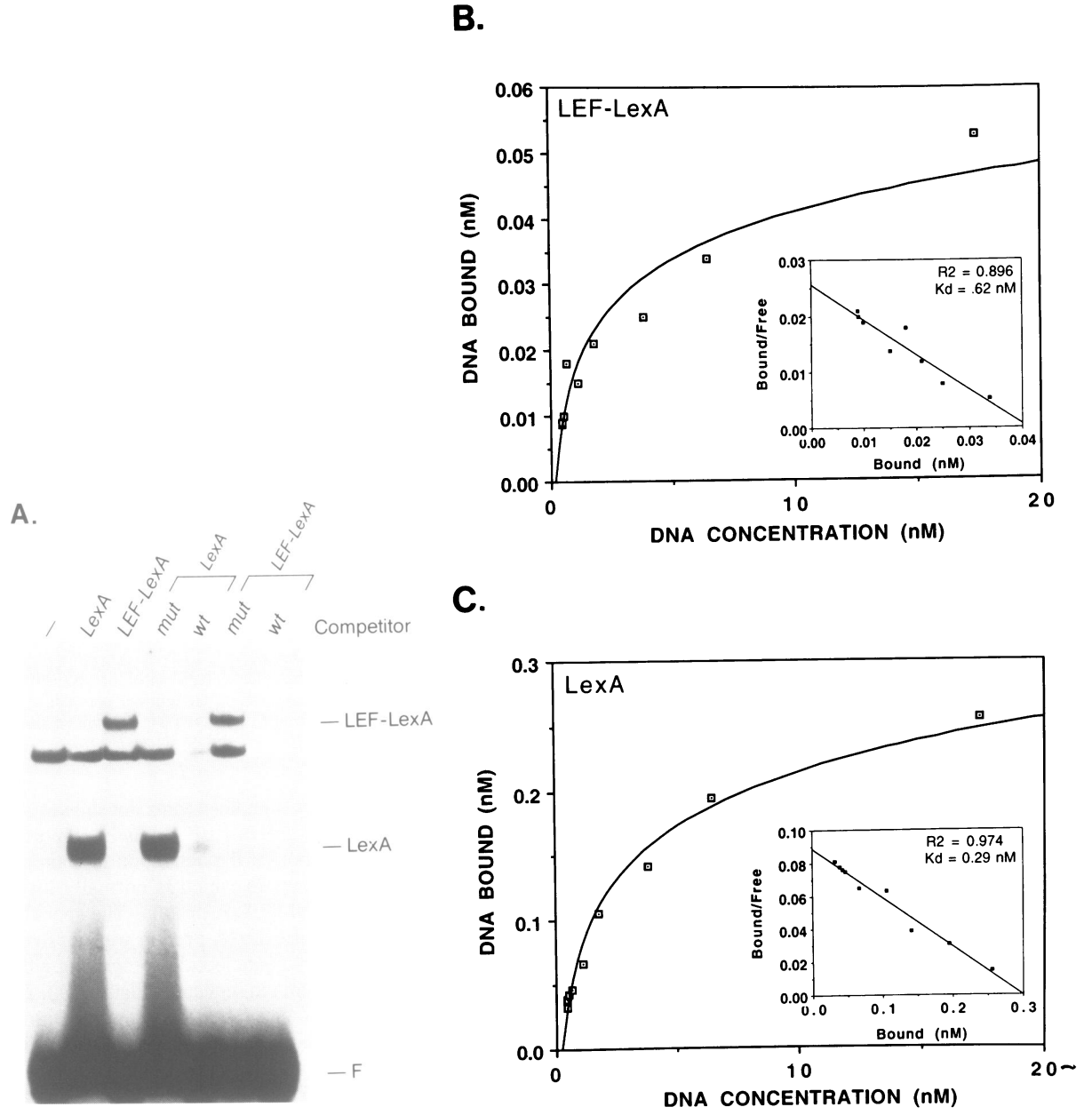

Fig. 6. DNA binding by the LexA and chimeric LEF-LexA proteins. (**A**) Electrophoretic mobility shift assay of nuclear extracts from transfected COS-7 cells with an end-labeled oligonucleotide containing the LexA-binding site. Cells were transfected with no effector plasmid (−) or transfected with effector plasmids expressing LexA protein or the chimeric LEF-LexA protein. The positions of protein−DNA complexes and the free probe (F) are indicated. A complex containing an endogenous DNA-binding activity migrates between the LexA− and the LEF-LexA−DNA complexes. The specificity of DNA binding was confirmed by showing a differential sensitivity of the protein−DNA complexes to competition with an excess of unlabeled oligonucleotides comprising the LexA (wt) or an unrelated binding site (mut). (**B** and **C**) Determination of the relative dissociation constants for the LexA and the LEF-LexA proteins. The DNA-binding affinities for LEF-LexA (B) and LexA (C) were determined by electrophoretic mobility shift assays under conditions of limiting protein with increasing concentrations of a labeled oligonucleotide probe comprising the LexA-binding site. Radioactivity in the protein−DNA complexes and in the free probe was determined by exposure of the dried gels to a PhosphorImager (Molecular Dynamics). Each saturation binding curve is representative of two independent sets of experiments. A linear representation of the data and the calculated dissociation constant is presented as an inset in the lower right corner of each graph.

regulate gene expression only in the context of other factor-binding sites in the TCRα enhancer (Travis *et al.*, 1991; Waterman *et al.*, 1991). These observations suggested that either DNA binding or action by LEF-1 is dependent upon other nuclear factors. To distinguish between these possibilities, we first examined the potential of the chimeric LEF-LexA protein to stimulate a minimal promoter from multimerized Lex-binding sites. Transfection of EL-4 T cells with the LEF-LexA effector plasmid together with a reporter gene containing six LexA-binding sites did not stimulate *CAT* gene expression (Figure 7). This result suggested that, similar to the intact LEF-1 protein, the stimulation of transcription by the activation domain in the chimeric LEF-LexA protein was dependent upon other factors. To examine the context dependence of the LEF-1 activation domain in more detail, we generated a set of reporter genes in which we altered the spacing or arrangement of factor-binding sites in the TCRα enhancer. Insertion of 15 or 19 nucleotides

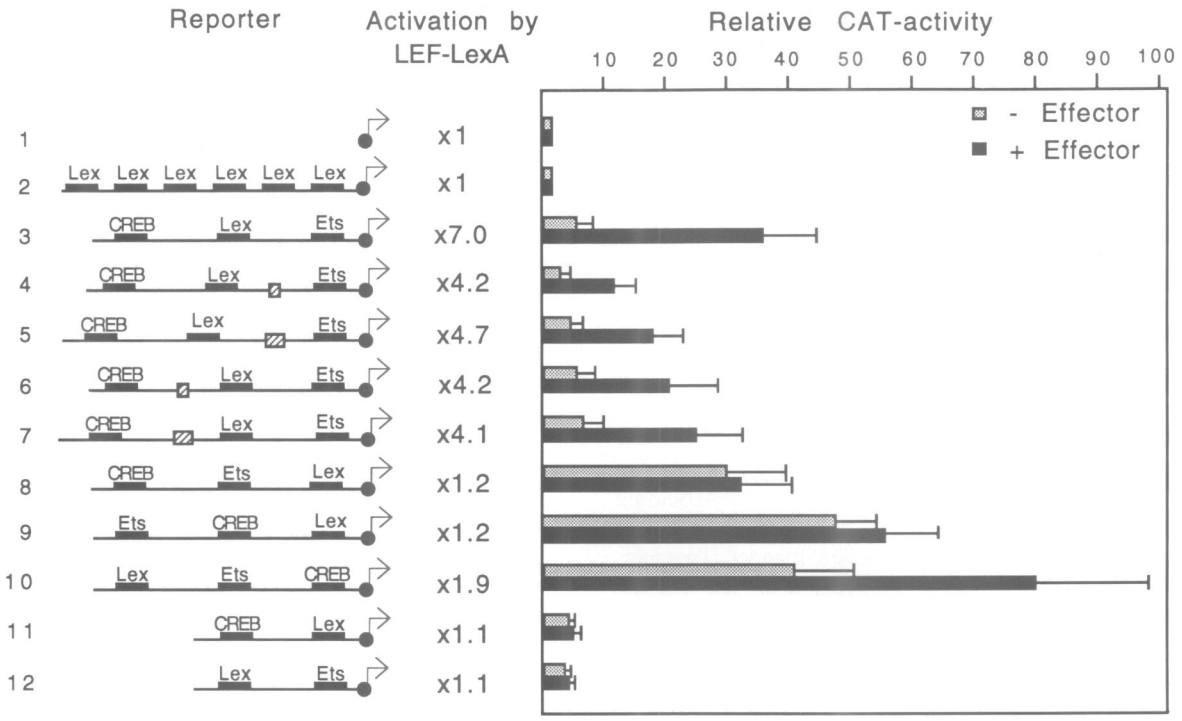

Fig. 7. The activation of LEF-1 stimulates TCRα enhancer function in a specific context of factor-binding sites. One microgram of various reporter plasmids was transfected alone or together with 1 μg of effector plasmid expressing the chimeric LEF-LexA protein. The structures of the TCRα enhancers in the various reporter gene constructs is indicated on the left-hand side and the corresponding nucleotide sequences are presented in Materials and methods. The filled circle and arrow represent the *fos* promoter linked to the *CAT* gene. Construct 2 contains six LexA-binding sites. In the reporter constructs 4,6 and 5,7 a hatched box represents a 15 and 19 oligonucleotide insertion, respectively. In constructs 11 and 12, one of the factor-binding sites in the TCRα enhancer is deleted. Relative CAT activities from three independent transfection experiments were determined in the absence (hatched columns) or presence (filled columns) of the LEF-LexA protein. The relative stimulation of CAT activity by LEF-LexA is shown in the middle panel.

between the Lex- and Ets-binding sites decreased the extent of LEF-LexA-mediated activation of gene expression. Likewise, insertion of these oligonucleotides between the CREB- and Lex-binding sites slightly, but reproducibly, decreased the level of activation by LEF-LexA protein. In contrast, changes in the spatial configuration of factor-binding sites in the TCRα enhancer resulted in more pronounced effects on gene expression. Reversal of the order of the Lex- and Ets-binding sites in the enhancer, which juxtaposed the CREB- and Ets-binding sites, increased gene expression 6-fold, even in the absence of LEF-LexA (Figure 7, gene construct 8). Likewise, two additional constructs in which the Ets- and the CREB-binding sites were juxtaposed increased the activity of the enhancer 8- to 10-fold in the absence of a cotransfected LEF-LexA effector plasmid (Figure 7, gene constructs 9 and 10). Interestingly, in the context of these three gene constructs, the LEF-LexA chimeric protein stimulated gene expression only by a factor of less than two. In particular, in constructs 8 and 9 in which the LexA-binding site was placed at the 3′ end of the enhancer no significant transcriptional stimulation by LEF-LexA was observed. Finally, the LexA-binding site in combination with only one of the other factor-binding sites did not allow activation of gene expression by the chimeric LEF-LexA protein. Together, these data indicate that the activation domain in LEF-1 is dependent upon a specific context of multiple factor-binding sites, suggesting that this domain mediates interaction with other enhancer-bound proteins.

Discussion

LEF-1 participates in the regulation of the TCRα enhancer function, but is unable to stimulate transcription by itself (Travis *et al.*, 1991; Waterman *et al.*, 1991). LEF-1 contains an HMG domain that binds DNA in the minor groove and induces a sharp bend in the DNA helix (Giese *et al.*, 1992). In this report, we provide evidence that the function of LEF-1 in regulating gene expression is dependent not solely upon the HMG domain but also upon additional protein regions. The deletion of the 99 amino-terminal residues from LEF-1 identified a region that is required for the regulation of TCRα enhancer activity in cotransfection assays in B lymphocytes. Interestingly, these amino-terminal residues are dispensable for the regulation of TCRα enhancer activity by the chimeric LEF-LexA protein in transfected B and T cells. This difference between LEF-1 and the chimeric LEF-LexA protein could, in principle, be accounted for by the distinct DNA-binding properties of the two proteins. The HMG domain of LEF-1 was shown to recognize DNA with modest sequence specificity. The equilibrium dissociation constant for the interaction of the monomeric HMG domain of LEF-1 with its binding site in the TCRα enhancer was only 20- to 50-fold lower than those determined with unrelated nucleotide sequences (Giese *et al.*, 1991). Thus, the amino-terminal residues in LEF-1 may serve to increase the specificity of DNA binding by the HMG domain. Because the DNA-binding properties of purified full-length LEF-1 protein and the HMG domain are very similar *in vitro*

(unpublished observations), we favor the view that the amino-terminal residues in LEF-1 mediate the interaction with another protein to increase the specificity of DNA binding by the HMG domain. Such a mechanism has been reported for the yeast homeodomain protein Matα2, which alone binds DNA with low specificity but interacts with another DNA-binding protein, termed MCM1, resulting in a marked increase in the specificity and affinity of DNA recognition (Keleher *et al.*, 1988). By contrast, the dimeric DNA-binding domain of LexA recognizes its binding site with high affinity and high specificity (Brent and Ptashne, 1984; Oertel-Buchheit *et al.*, 1992). Therefore, the LEF-LexA fusion protein may recognize its target site *in vivo* independently of the 99 amino-terminal amino acids.

Another functional region in LEF-1 was identified by fusing amino acids 99−217 of LEF-1 to the LexA protein. This region in LEF-1 conferred upon the DNA-binding domain of LexA the ability to regulate TCRα enhancer function in a cell-type and context-specific manner. Together, these data operationally define this region in LEF-1 as an activation domain. The cell-type and context-specific function of this protein domain, however, suggests that it is not the functional equivalent of an activation domain that interacts with components of the basal transcription machinery. Instead, the activation domain of LEF-1 is likely to be involved in interaction with other enhancer-bound proteins. This interpretation is supported by two observations.

First, the function of the LEF-1 activation domain in the chimeric protein was dependent upon the cell type used for the analysis. In T cells containing endogenous LEF-1, the activity of the TCRα enhancer was stimulated 7-fold by the chimeric LEF-LexA protein, whereas no stimulation was observed in fibroblastic cells. This cell-type specific function of the chimeric LEF-LexA protein parallels the cell-type specific binding of other nuclear proteins to the TCRα enhancer. DNase I footprint analysis of the human and murine TCRα enhancers using nuclear extracts indicated that a factor interacted with the Ets-binding site in T cells, but not in HeLa cells (Ho *et al.*, 1989; Winoto and Baltimore, 1989). Therefore, the protein generating this footprint specifically in T cells is a likely candidate for an interaction with LEF-1. The identity of this cell-type specific protein, however, is unclear to date. Although recombinant Ets-1 was shown to bind the site located 3′ of the LEF-1-binding site (Ho *et al.*, 1990), no experimental evidence could be obtained for an interaction between LEF-1 and this particular Ets protein (unpublished data). Moreover, the DNase I footprint analysis of the human TCRα enhancer incubated with T cell nuclear extract indicated an extended and contiguous protected region that not only includes both the LEF-1- and Ets-binding sites but also some 10 nucleotides in between. This contiguous DNase I footprint suggests the possibility of the presence of a binding site for another, as yet unidentified, protein (Ho and Leiden, 1990a). Consistent with this scheme, mutations of nucleotides between the LEF-1- and the Ets-binding sites reduced enhancer function in T cells (data not shown).

Second, the function of the LEF-1 activation domain in the chimeric protein was dependent upon a specific context of factor-binding sites in the TCRα enhancer. Similar to the full-length LEF-1 protein, the chimeric LEF-LexA protein was unable to activate gene expression from multimerized binding sites. Thus, LEF-1 contains an activation domain distinct from the type found in regulatory proteins such as GAL4. Moreover, changes in the spacing or relative position of any of the factor-binding sites in the TCRα enhancer diminished activation by LEF-LexA. The presence of a putative factor-binding site between the LexA- and the Ets-binding sites makes it difficult to distinguish whether the modest decrease of enhancer function observed with constructs 3 and 4 in Figure 7 is due to a disruption of this putative factor-binding site and/or to changes in spacing. Significant activation of TCRα enhancer function by the chimeric protein was observed only with reporter constructs in which the LexA-binding site is located between the CREB- and Ets-binding sites. By contrast, expression of TCRα gene constructs was stimulated by LEF-LexA only by a factor of two or less when the Lex-binding site was switched with either the CREB- or the Ets-binding site and placed at the ends of the TCRα enhancer fragment. Interestingly, juxtaposition of the CREB- and Ets-binding sites resulted in a 6- to 10-fold stimulation of TCRα enhancer function which was independent of the LEF-LexA fusion protein. It is possible that these mutations allow a functional interaction of proteins bound at these sites in the absence of LEF-LexA protein. Together, these data indicate that the regulation of the TCRα enhancer by LEF-LexA is dependent upon functional interaction between factors occupying the CREB-, LexA- and Ets-binding sites, arranged in a specific configuration. The observed dependence on a specific context of multiple factor-binding sites for the regulation of gene expression by LEF-LexA appears to be distinct from the simpler context dependence of the activation domain of Elk-1. Stimulation of gene expression by Elk-1 requires only interaction with SRF bound at an adjacent site in the serum response element of the c-*fos* promoter (Hill *et al.*, 1993). By contrast, juxtaposition of the LexA-binding site with either the CREB- or the Ets-binding sites was not sufficient for stimulation of gene expression by LEF-LexA. This dependence of LEF-LexA protein on multiple factor-binding sites is consistent with previous observations indicating that deletion of the CREB-binding site from the minimal TCRα enhancer virtually abrogates enhancer function and generates a T cell-specific silencer of gene expression (Ho and Leiden, 1990b).

The functional replacement of the HMG domain of LEF-1 with the DNA binding domain of LexA raises the question as to the role for DNA bending in the regulation of the TCRα enhancer function. Consistent with a putative role for DNA bending, the level of transcriptional stimulation by the LEF-LexA protein in T lymphocytes was half that of the endogenous LEF-1 protein, despite the higher affinity and specificity of DNA binding by the LEF-LexA protein. Moreover, our data suggest some functional interaction between proteins bound at the CREB- and Ets-binding sites. In the normal minimal TCRα enhancer, these sites are separated by 60 nucleotides and the LEF-1-binding site between them is required for full enhancer function. In contrast, juxtaposition of the CREB- and Ets-binding sites increases the activity of the TCRα enhancer in the absence of LEF-LexA protein. Therefore, we favor the view that both DNA bending and interactions between the activation domain of LEF-1 and other enhancer-bound proteins may be important for the regulation of gene expression.

Regulation of gene expression by LEF-1 is likely to be distinct from other types of DNA-bending proteins that

facilitate the binding and/or action of other proteins. LEF-1 does not appear to be the functional equivalent of IHF, which regulates gene expression and site-specific recombination in *Escherichia coli*. IHF plays a purely structural role by inducing a sharp bend in the DNA helix to align widely-separated factor-binding sites. These processes do not require direct interaction of other proteins with IHF because the IHF-binding site can be replaced by naturally curved DNA or other binding sites for DNA-bending proteins (Goodman and Nash, 1989; Nash, 1990; Giese *et al.*, 1992). LEF-1 also displays some differences from HMG-I(Y), a member of a distinct family of HMG proteins (Bustin *et al.*, 1990), which binds the minor groove of DNA, induces a bend into the DNA helix and is unable to activate transcription on its own (Solomon *et al.*, 1986; Thanos and Maniatis, 1992). HMG-I(Y) was found to facilitate binding of the transcription factor NF-κB to a sequence in the interferon β gene promoter by binding to the opposite side of the DNA helix and forming a ternary complex that can stimulate gene expression from multimerized NF-κB binding sites (Thanos and Maniatis, 1992). By contrast, LEF-1 is unable to augment gene expression from multimerized LEF-1 binding sites and may facilitate the functional interaction between factors bound at widely separated sites by both contacting other proteins and bending the DNA helix. Without further evidence, however, other models in which DNA bending reflects only a particular mode of DNA recognition by the HMG domain cannot be ruled out.

Materials and methods

Cell culture

COS-7 cells and NIH3T3 cells were grown in Dulbecco's modified Eagle's medium. Lymphoid cell lines were grown in RPMI medium. The media were supplemented with 10% fetal calf serum and 50 μg/ml each of penicillin and streptomycin. In addition, the RPMI medium contained 50 μM 2-mercaptoethanol.

Plasmid constructions

The full-length LEF-1 protein and the various amino-terminal truncations used in this study are shown in Figure 1. The tagged full-length LEF-1 protein was generated by the polymerase chain reaction (PCR) using the LEF-1 cDNA (Travis *et al.* 1991) as template, and forward primer Nde-LEF (5'-GCGCATATGCCCCAACTTTCCGGAGGAGG-3'), introducing an *Nde*I restriction site at the amino-terminus, and LEF-1 reverse primer (5' TGTTCCTTGGGGTCAGCCGG 3'). The DNA fragment thus generated was cleaved with *Nde*I (amino acid 1) and *Pvu*II (amino acid 394) and cloned into plasmid pCG containing the sequence for the HA1 epitope tag (SYPYDVPDYASLGGPS; Wilson *et al.*, 1984) which was followed by a stop codon. Plasmid pCG is a derivative of pEVRF (Matthias *et al.*, 1989) and contains the human CMV enhancer/promoter region and the translation initiation region of the HSV thymidine kinase (*tk*) gene. This construction generated a translational fusion of the tagged LEF-1 coding region with the first three amino acids encoded by the HSV *tk* gene. Amino-terminal deletion mutants of LEF-1 were generated by restriction digestion with *Stu*I (LEFΔ99), *Bam*HI (LEFΔ166) or *Nde*I (LEFΔ244) and fused in-frame to the translation initiation region of the *tk* gene. Deletion mutant LEFΔ285 was generated by cleavage of the DNA fragment containing the HMG domain of LEF-1 (Giese *et al.*, 1991) with *Nde*I and *Sal*I and ligation into *Nde*I- and *Sal*I-cleaved expression vector containing deletion mutant LEFΔ244. Nucleotide sequences of the coding regions for the various expression plasmids were verified by DNA sequencing.

The chimeric LEF-LexA proteins are schematically shown in Figure 3. The HMG domain of LEF-1 was replaced with the bacterial repressor LexA as follows: the LexA coding region, consisting of the DNA binding and dimerization domain (amino acids 1–202), was isolated from plasmid pEG202 (gift from R.Brent) by digestion with *Hin*dIII and *Eco*RI and cloned into expression vector pCG. The coding region was extended by a sequence encoding the 8 amino acid KT-3 epitope (TPPPEPET; MacArthur and Walter, 1984) followed by a stop codon. The chimeric LEF-LexA protein was generated by fusing the blunt-ended *Nde*I site at amino acid position 244 of the LEF-1 cDNA to the blunt-ended *Hin*dIII site of the LexA coding region. (This fusion reconstituted the *Hin*dIII site.) Because the chimeric protein bound DNA only with low affinity, oligonucleotides encoding a 'flexible' amino acid linker (KLGGGAPAVGGGPK) and an oligonucleotide encoding additional residues of LEF-1 (amino acids 245–258), were inserted into the unique *Hin*dIII site between the LEF-1 and LexA coding regions. The DNA-binding affinity of the chimeric protein containing additional LEF-1 amino acids was nearly as high as that of the LexA protein. For subsequent studies the chimeric LEF-LexA protein consisting of amino acids 1–258 of LEF-1 and amino acids 1–202 of LexA was used. Amino-terminal truncations of the chimeric LEF-LexA protein were generated by restriction digestion of the DNA with *Stu*I (LEF99/258-LexA) or *Bam*HI (LEF166/258-LexA) and fused in-frame to the coding region of LexA and the HSV *tk* leader. Fusion proteins containing carboxy-terminal truncations from amino acids 217 to 244 (LEF99/217-LexA) and 185 to 244 (LEF99/185-LexA) were introduced by PCR. Both fusion protein constructs consist of amino acids 245–258 of LEF-1 (see above). The different constructs were verified by DNA sequencing.

The different *fosCAT* reporter gene constructs used in this study were generated by inserting either the wild-type minimal TCRα enhancer DNA fragment (Ho and Leiden, 1990a) or various mutants into a plasmid containing the minimal *fos* promoter linked to the *CAT* gene (Berkowitz *et al.*, 1989). The nucleotide sequences of the different TCRα enhancer reporter gene constructs, as shown in Figure 7, are presented with the binding sites for CREB (TGACGTCA, Jones *et al.*, 1988), LexA (CTGTATATACATACAGT, Golemis and Brent, 1992), Ets (CACATC CTC, Ho *et al.*, 1990) and CAP (TAATGTGAGTTAGCTCACTCA, Gartenberg and Crothers, 1988) underlined. Gene construct #2: (TCGA GCTGTATATACATACAGTGCTCGA)$_6$; gene construct #3: GGCGG TCCCCTCCCATTTCCATGACGTCATGGTTACCAAGAGGGGCAA-CTGTATATACATACAGTGCTCTCCCGCAGAAGCCACATCCTC-TGGAAAGA; gene construct #4: GGCGGTCCCCTCCCATTTCCA-TGACGTCATGGTTACCAAGAGGGGCAACTGTATATACATACAGT-GCTCTCCATCATGCGGCCGCATCGCAGAAGCCACATCCTCTGG-AAAGA; gene construct #5: GGCGGTCCCCTCCCATTTCCATGAC-GTCATGGTTACCAAGAGGGGCAACTGTATATACATACAGTGC-TCTCCATCATGCGGCCGGCCGCATCGCAGAAGCCACATCCTC-TGGAAAGA; gene construct #6: GGCGGTCCCCTCCCATTTCCA-TGACGTCATGGTTAATGCGGCCGCATGCACCAAGAGGGGCAA-CTGTATATACATACAGTGCTCTCCCGCAGAAGCCACATCCTC-TGGAAAGA; gene construct #7: GGCGGTCCCCTCCCATTTCCA-TGACGTCATGGTTAATGCGGCCGGCCGATGCACCAAGAGGGGC-AACTGTATATACATACAGTGCTCTCCCGCAGAAGCCACATCCTC-TGGAAAGA; gene construct #8: GGCGGTCCCCTCCCATTTCCA-TGACGTCATGGTTACCAAGAGGGGCAACAGAAGCCACATCCTC-TGGAAAGACTGTATATACATACAGTGCTCTCCCG; gene construct #9: GCTCTCCCGCAGAAGCCACATCCTCTGGAAAGAAGCTTG-GCGGTCCCCTCCCATTTCCATGACGTCATGGTTACCAAGAGG-GGCAACTGTATATACATACAGTGCT; gene construct #10: ACCAA-GAGGGGCAACTGTATATACATACAGTGCTCTCCCGCAGAAGC-CACATCCTCTGGAAAGAGTCGACTCCCATTTCCATGACGTCA-TGGTTA. Gene construct #11 (shown in Figure 5), in which the LEF-1-binding site was replaced with the recognition sequence for the bacterial catabolic activator protein (CAP), 1:1 GGCGGTCCCCTCCCATTTCCA-TGACGTCATGGTTACCAAGAGGGGCAATAATGTGAGTTAGCTC-ACTCATCCGCAGAAGCCACATCCTCTGGAAAGA.

DNA transfections and CAT assays

Transient DNA transfections into lymphocytes were performed as described in Grosschedl and Baltimore (1985) using a DEAE–dextran/chloroquine procedure with various amounts of effector and reporter DNA and 200–400 ng of the Rous sarcoma virus (RSV)-luciferase plasmid (De Wet *et al.*, 1987) as a measure of transfection efficiency. NIH3T3 cells were transfected with lipofectin (BRL) according to the manufacturer's instructions. After 48 h cells were harvested to determine the luciferase activity as described by Mangalam *et al.* (1989). CAT assays were performed as described by Gorman *et al.* (1982). Following incubation for 2–3 h at 37°C, acetylated [^{14}C]chloramphenicol was separated by TLC and autoradiographed. For quantification of acetylated [^{14}C]chloramphenicol, the chromatogram was exposed to a PhosphorImager (Molecular Dynamics) for direct determination of the radioactivity in each spot.

Nuclear extracts, immunoblotting and electrophoretic mobility shift assay

Nuclear extracts of COS-7 cells were prepared essentially as described in Dignam *et al.* (1983) and subsequently diluted with 1 vol of a buffer

containing 20 mM HEPES (pH 7.5), 10% glycerol, 2 mM PMSF (phenylmethylsulfonyl fluoride), 10 μg/ml aprotinin and 20 μM leupeptin. Lysates were cleared by centrifugation at 14 000 *g* for 10 min and aliquots stored at −80°C.

For immunoblotting, extracts were boiled in sample buffer (Laemmli, 1970), separated by SDS−PAGE and transferred to nitrocellulose filters (Schleicher & Schuell). Filters were blocked in TBST buffer (10 mM Tris−HCl pH 7.5, 0.9% NaCl, 0.05% Tween 20, 0.2% sodium azide) containing 3% non-fat dried milk. The indicated monoclonal antibodies were added in TBST at appropriate dilutions. Bound antibodies were detected with an alkaline phosphatase conjugated anti-mouse antibody (Promega) in TBST, washed and developed with nitroblue tetrazolium and 5-bromo 4-chloro 3-indolyl phosphate (Promega).

Electrophoretic mobility shift assays were performed according to Travis *et al.* (1991). Briefly, ~5 fmol of duplex oligonucleotides containing the minimal LexA-binding site were end-labeled using T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP and incubated with nuclear extract in the presence of 400 ng sonicated salmon sperm DNA and 200 ng dI/dC. The protein−DNA complexes were resolved by electrophoresis in 6% non-denaturing polyacrylamide gels in TE buffer (10 mM Tris−HCl pH 8.0, 0.1 mM EDTA). To determine the K_D for the interaction of the LEF-LexA protein and the LexA protein with DNA by Scatchard analysis (Ingraham *et al.*, 1990), equal amounts of nuclear extracts of transfected COS-7 cells were incubated with an oligonucleotide containing the LexA-binding site. After gel mobility shift assay, the radioactivity in the free probe and in the protein−DNA complex was determined on a PhosphorImager (Molecular Dynamics).

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