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Nuclear factor NF-κB can interact functionally with its cognate binding site to provide lymphoid-specific promoter function

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Enhancers and promoters, *cis*-acting regulators of mammalian gene expression, are modular units containing multiple short binding sites for specific *trans*-acting transcription factors. To investigate if factors binding to enhancer sequences are functionally different from promoter-binding factors, we asked if a short DNA sequence element in the immunoglobulin kappa (κ) light chain enhancer that binds to the nuclear factor NF-κB could also serve as a functional promoter element. A synthetic oligonucleotide containing this binding site was placed in either orientation upstream of the β-globin TATA-element. In myeloma cells, the NF-κB binding site efficiently directed transcription. The promoter activity was directly correlated with the presence of the nuclear factor NF-κB: there was no transcription in fibroblasts or in unstimulated pre-B cells where the factor was absent. Transcription could be stimulated in pre-B cells by treatments known to activate NF-κB. Thus, the same nuclear factor can act as a positive activator of both enhancer and promoter function, suggesting that the two functions involve similar events in the transcription process.

Key words: nuclear factor NF-κB/promoter/enhancer/activation

Introduction

Genes transcribed by RNA polymerase II in mammalian cells are primarily regulated by two distinct elements, enhancers and promoters. Enhancers are units of DNA sequence that can function to increase transcription in an orientation-independent manner both upstream and downstream from the start site of transcription and at a variety of distances, often kilobase pairs away from the start site (Moreau *et al.*, 1981; Banerji *et al.*, 1981; Fromm and Berg, 1983). Elements functioning as promoters are DNA sequences found in relatively close proximity upstream of the start site of transcription. They function poorly at long distances from the start site, or downstream, but they are also orientation independent with respect to the TATA-box (McKnight and Tjian, 1986; Maniatis *et al.*, 1987).

Both enhancers and promoters consist of multiple short binding sites for *trans*-acting nuclear factors. Many *trans*-acting factors are constitutively active in various cell types (Briggs *et al.*, 1986; Carthew *et al.*, 1985; Sawadogo and Roeder, 1985; Sen and Baltimore, 1986a; Jones *et al.*, 1987), but others are specific for certain cell types (Landolfi *et al.*, 1986; Staudt *et al.*, 1986; Pettersson and Schaffner, 1987; Johnson *et al.*, 1987) or are inducible in response to extracellular stimuli modulating gene expression (Sen and Baltimore, 1986b; Prywes and Roeder, 1986; Angel *et al.*, 1987; Lee *et al.*, 1987).

Mutational analyses and *in vitro* protein-DNA binding experiments have revealed that several distinct short sequence elements within the κ-light chain immunoglobulin enhancer which contribute to its transcriptional activity are recognized by nuclear proteins (Sen and Baltimore, 1986a; Lenardo *et al.*, 1987). Binding of the nuclear factor NF-κB to the B-site in the mouse κ-enhancer (the 'κB site') appears to be the predominant determinant for its tissue- and developmental stage-specific activity. Point mutations within this site completely abolish the activity of the κ-enhancer (Lenardo *et al.*, 1987) and two copies of an oligonucleotide containing the κB site function as a lymphoid-specific enhancer element mimicking the activity of the wild-type κ-enhancer in all of its features (Pierce *et al.*, 1988). The presence of functional κB sites has also been demonstrated for several viral enhancers including those in the SV40 virus, cytomegalovirus and HIV I virus enhancers (Nomiyama *et al.*, 1987; Boshart *et al.*, 1985; Muesing *et al.*, 1987; Nabel and Baltimore, 1987). In addition, a closely related sequence has been found in the enhancer of the H-2K^b MHC gene which binds a distinct factor but also binds to NF-κB (Baldwin and Sharp, 1988).

A specific protein-binding site is generally confined to either enhancer or promoter elements. The octamer motif, however, is present both in several promoters, e.g. the immunoglobulin and histone H2b promoters, and in enhancers found in the SV40 virus and the immunoglobulin heavy chain gene (Falkner and Zachau, 1984; Parslow *et al.*, 1984; Falkner *et al.*, 1986). This raises the question whether other sites might also be bifunctional or if there is a fundamental mechanistic difference between enhancer and promoter sites, with the octamer motif being a rare exception.

To study the potential promoter activity of enhancer elements, we asked whether the κB site, a motif that so far has only been found in enhancers, could also function as a promoter element. Here we show that the κB site functions efficiently as a lymphoid-specific promoter element when placed directly upstream of a solitary TATA-box. Correlative evidence is provided that this promoter activity is due to the interaction of the κB site with NF-κB.

Results

Tissue-specific promoter activity of the κB site

The binding site for NF-κB was synthesized as a pair of 24-nt-long complementary oligonucleotides containing the 10-bp κB site (underlined in Figure 1A) flanked by 5 bp

wild-type κB site

```
5' GATCCAGAGGGGACTTTCCGAGAG      3'
3'     GTCTCCCCTGAAAGGCTCTCCATG  5'
```

mut κB site

```
5' GATCCAGAccatggTaTCCGAGAG      3'
3'     GTCTggtaccAtAGGCTCTCCTAG  5'
```

Fig. 1. Wild-type and mutant $\varkappa$B oligonucleotides. The wild-type and mutant sites were synthesized as pairs of complementary oligonucleotides. The binding site for NF-$\varkappa$B identified by methylation interference (Sen and Baltimore, 1986a) is underlined. The 5-bp flanking the binding site on either side are identical to the sequence found in the mouse $\varkappa$-enhancer. Nucleotides changed in the mutant $\varkappa$B site are printed in lower case. The 5′−3′ orientation of the sequence given in the figure represents orientation 'B' with respect to the TATA-box of the β-globin expression construct. In orientation 'A' the shown sequence is inverted.

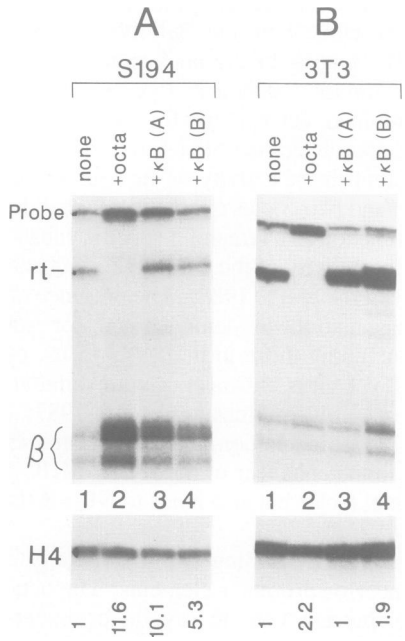

Fig. 2. Activity of the $\varkappa$B promoter construct in S194 and NIH/3T3 cells. S1 nuclease analysis of cytoplasmic RNA from S194 (**A**) and NIH/3T3 cells (**B**) transfected with the indicated plasmids. **Lane 1**, control construct lacking an oligonucleotide insert; **lane 2**, construct containing the octamer insert in the $\varkappa$-orientation (Wirth *et al.*, 1987, see Figure 1 therein); **lane 3**, construct containing the $\varkappa$B site inserted in orientation 'A'; **lane 4**, construct containing the $\varkappa$B site inserted in orientation 'B'. The position of the intact probe, readthrough transcripts (rt), the correctly initiated transcripts (β) and the control transcripts (H4) are indicated at the left side of the figures. The relative level of expression is given in numbers underneath each lane; the number for the construct lacking an oligonucleotide insert was arbitrarily defined as 1.0. As the β-globin probe was derived from the octa-containing plasmid (Wirth *et al.*, 1987), the shorter readthrough bands only occur with the non-octa constructs.

from the $\varkappa$-enhancer and *Bam*HI overhangs for cloning. After initial subcloning into the pUC18 polylinker, the oligonucleotide was introduced into a β-globin expression vector. In this vector, the original mouse β-globin promoter had been truncated upsteam of the TATA-box such that no additional promoter elements were present. It also contained an SV40 enhancer element 3′ of the β-globin cassette (Wirth *et al.*, 1987). In addition, this vector contained the mouse polyoma

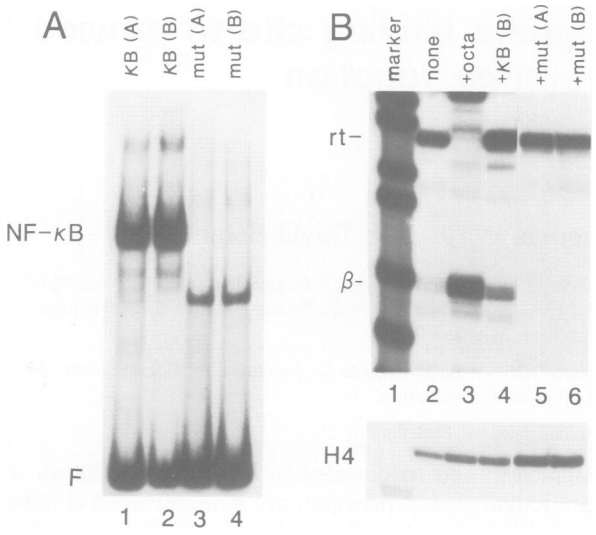

Fig. 3. Clustered mutations within the $\varkappa$B site abolish both NF-$\varkappa$B binding and promoter function. **A.** Electrophoretic mobility shift assays with the wild-type and mutant $\varkappa$B sites present in either orientation in the context of the pUC18 polylinker. Nuclear extract was prepared from TPA-induced mouse 70Z/3 cells (Sen and Baltimore, 1986b). Position of the characteristic complex due to interaction of NF-$\varkappa$B with the $\varkappa$B site is indicated. **B.** S1 nuclease analysis of RNA from S194 cells transfected with the indicated plasmids. Labeling is as in Figure 2.

virus origin of replication and the coding region for polyoma large T-antigen, allowing replication of the constructs in murine cell lines. The presence of the modified mouse histone H4 gene in the test plasmids allowed standardization for differences in transfection efficiencies (Grosschedl and Baltimore, 1985). Introduction of the oligonucleotide in either orientation resulted in constructs where the $\varkappa$B site was localized 26 bp upstream of the TATA-box. Essentially all of the sequences between these elements were linker sequences.

To assay for promoter function, the constructs were transfected into the S194 mouse myeloma cell line. After 48 h, stably accumulated cytoplasmic RNA was analyzed by S1 nuclease analysis. As controls, a plasmid lacking an oligonucleotide insert upstream of the TATA-box and a plasmid containing a synthetic octamer promoter, previously shown to be active only in lymphoid cells (Wirth *et al.*, 1987), were used. Because identical RNAs were synthesized in all cases, differential RNA stability should not have been a factor and levels of stable RNA should be a reliable measure of transcription rates.

Both orientations of the $\varkappa$B site yielded significant promoter activity, with the 'A' orientation almost as effective as the octamer-containing promoter, and the 'B' orientation showing ~50% of the octamer's activity (Figure 2A, lanes 3 and 4). As the actual binding symmetry of NF-$\varkappa$B to its cognate site is not known, it is at present unclear whether this difference in activity reflects an orientation effect or rather an alignment effect. In any case, the $\varkappa$B enhancer site acts as a strong promoter element for β-globin in myeloma cells.

To differentiate between enhancer and promoter function, we moved the $\varkappa$B-site (in both orientations) to a position ~200 bp upstream of the TATA-box. As had been shown

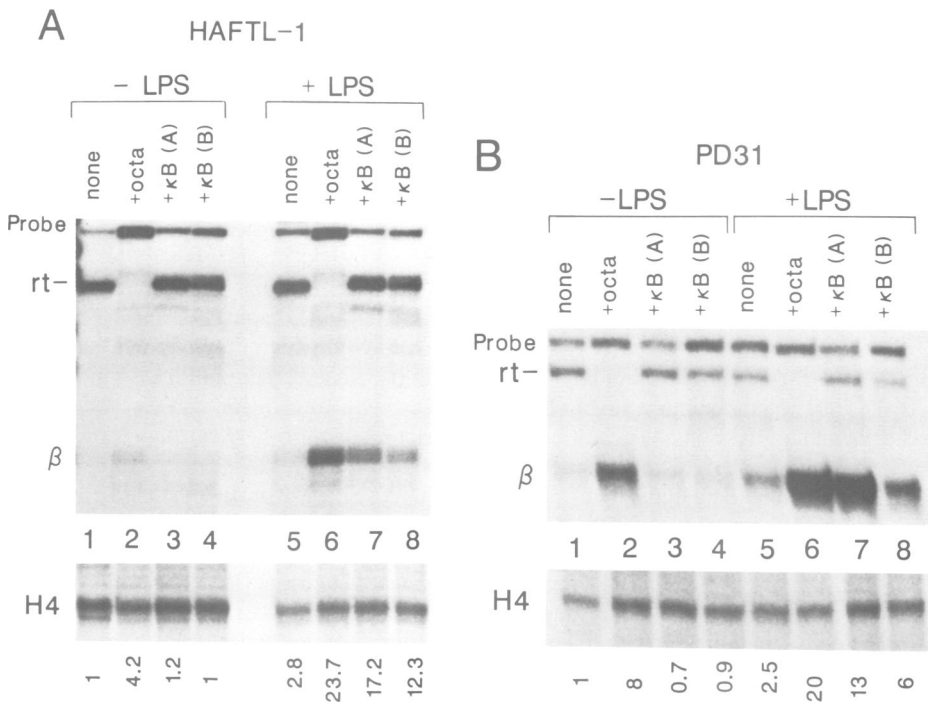

Fig. 4. Inducible promoter activity of the κB site in pre-B cell lines S1 nuclease analysis of RNA from HAFTL-1 (**A**) and PD31 (**B**) cells transfected with the indicated plasmid constructs. Labeling as in Figure 2.

with the octamer element (Wirth *et al.*, 1987), the level of transcription dropped to the baseline showing that by this test the κB-site is not supplying enhancer function in these constructs (data not shown).

When the identical set of plasmids was analyzed in the NIH/3T3 mouse fibroblast cell line, none of the constructs showed consistent activity above the baseline level obtained with the construct lacking an oligonucleotide insert (Figure 2B). Therefore, the juxtaposition of the κB site and the TATA-box resulted in the creation of a lymphoid-specific promoter, consistent with the presence of NF-κB in S194 cells, but weakly if at all in NIH/3T3 cells (M.Lenardo and D.Baltimore, unpublished results). Interestingly, as in the case of the octamer-containing promoter (Wirth *et al.*, 1987), no additional promoter elements besides the κB site and the TATA-box were required to create a tissue-specific promoter element.

To exclude the possibility that other sequences within the synthetic oligonucleotide were responsible for the observed tissue-specific promoter activity, we mutated the κB site by introducing a cluster of point mutations (mut-κB in Figure 1). When tested for binding to NF-κB in an electrophoretic mobility shift assay, neither orientation of the mutant oligonucleotide resulted in the typical protein−DNA complex detected with the wild-type sequence (Figure 3A). The faster migrating complex formed with the mutant oligonucleotides (Figure 3A, lanes 3 and 4) was not tissue specific and was also detectable upon longer exposure in the assays using the wild-type probes (data not shown). The weak complexes seen with the wild-type κB-site as a probe were also not tissue specific (data not shown). Thus, the mutations introduced into the κB site interfere specifically with binding of NF-κB to the oligonucleotide.

The mutant oligonucleotides were also tested for promoter activity in S194 cells. The two constructs containing the mutant oligonucleotides in either orientation did not support transcription beyond the baseline level of the construct lacking an added oligonucleotide (Figure 3B). Binding of NF-κB to its cognate binding site therefore appears to be responsible for the lymphoid-specific promoter activity observed with the synthetic promoters containing the wild-type binding site.

NF-κB mediated promoter activity is inducible in pre-B cells

NF-κB binding activity is not detectable in pre-B cells, but can be induced by a post-translational mechanism with the mitogen bacterial lipopolysaccharide (LPS) (Sen and Baltimore, 1986b). The same treatment induces transcription of endogenous κ-genes (Nelson *et al.*, 1985) as well as of transfected genes containing the wild-type κ-enhancer (Lenardo *et al.*, 1987). In addition, an oligonucleotide containing two NF-κB binding sites has been shown to act as a LPS-inducible enhancer element in the pre-B cell line 38B9 (Pierce *et al.*, 1988). Therefore it appears that NF-κB acting through the κB site in the enhancer is the principal transcriptional regulator for the stage-specific expression of the immunoglobulin κ-genes. To determine whether the induction of NF-κB binding activity would also be sufficient for activity of the synthetic κB promoter element, we tested the effect of LPS induction in murine HAFTL-1 and PD31 cell lines.

HAFTL-1 is a Harvey murine sarcoma virus-transformed early pre-B cell line that undergoes frequent rearrangements of the D and J_H segments of immunoglobulin heavy chains as well as occasional rearrangements at the T-cell receptor β-locus (Alessandrini *et al.*, 1987). No κ-light chain rearrangements have been detected in this cell line. PD31 is an Abelson murine leukemia virus-transformed pre-B cell line that has its heavy chain genes already rearranged and undergoes continual κ-light chain gene rearrangements (Lewis *et al.*, 1982). In both cell lines, NF-κB binding

activity can be induced by treatment of the cells with LPS (Sen and Baltimore, 1986b; T.Wirth and D.Baltimore, unpublished results).

After transfection of the constructs into the pre-B cell, half of the cells were treated with LPS for 48 h before total cytoplasmic RNA was prepared. In both cell lines, the activity of the two κB promoter-containing plasmids increased from the baseline level in uninduced cells to a level almost identical to that of the octamer-containing promoter (Figure 4A and B). Again, the construct containing the κB site in orientation 'A' showed ~2-fold higher activity than that with the reverse orientation. This promoter activity was attributable to binding of NF-κB to its cognate binding site because the mutant κB sites did not show inducible binding activity in LPS-treated cells and expression constructs containing the mutant κB sites did not give transcriptional activity before or after LPS treatment (data not shown). The fact that functional NF-κB activity can even be induced in HAFTL-1 cells, which show no evidence of endogenous κ-gene rearrangement, is consistent with an earlier report (Nelson *et al.*, 1985) of transcription from the unrearranged κ-loci of LPS-treated pre-B cells. In both cell lines, the two control plasmids, the one containing the octamer promoter and the one lacking any oligonucleotide insert, showed some induction after LPS treatment (Figure 4A and B, compare lanes 1 and 2 with lanes 5 and 6). The reason for this general increase in RNA levels in cells is at present unclear. Non-specific activation of the transcriptional machinery or a change in mRNA turnover might be possible explanations. Interestingly, induction of the octamer-containing construct was higher than the background induction in HAFTL-1 cells. This phenomenon is currently under investigation.

Previous data had suggested that NF-κB can also be induced in non-B cells by treatment of the cells with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sen and Baltimore, 1986b). In addition, it had been shown that the HIV-1 enhancer, which contains two κB sites, can be activated in the human Jurkat T-cell line by treatment with phytohemagglutinin (PHA) and TPA and that this induction is eliminated by mutations in the κB sites (Nabel and Baltimore, 1987). Furthermore, an oligonucleotide containing two κB sites has been shown to act as TPA-inducible enhancer element in Jurkat cells (Pierce *et al.*, 1988). We transfected the κB promoter plasmids into Jurkat cells and subsequent treatment of these cells for 20 h with PHA and TPA resulted in a specific induction of transcription as compared to the constructs containing no added oligonucleotide or the octamer insert (data not shown). Thus, the κB site independently acts as a TPA-inducible promoter element in Jurkat cells.

Discussion

We have shown that the nuclear factor, NF-κB, previously known to interact with several different enhancer elements, can also interact functionally with its binding site in a promoter configuration to stimulate transcription specifically. We have defined promoter action as stimulation of transcription from a site near to the start site in the presence of strong enhancer. As a control, we have shown that the immunoglobulin octamer sequence, a recognized promoter element, provides an otherwise limiting activity to the construct. Several results provide clear evidence that the same factor is responsible for both κB promoter and enhancer activity. (i) NF-κB was the only factor detectable in the electrophoretic mobility shift experiments that interacted strongly with a site within the oligonucleotide used to generate promoter activity. (ii) Activity of the synthetic promoter correlated exactly with the presence of detectable NF-κB factor. (iii) A mutation within the NF-κB binding site that abolished the specific protein−DNA interaction also abolished promoter activity.

Identification and analysis of protein-binding sites in promoters and enhancers and to some extent the protein factors with which they interact, have yielded new insights into our understanding of transcriptional regulation over the past few years. In some cases, there appears to be a fairly strict distinction between those regulatory sites present in promoters and sites found exclusively within enhancers. Binding sites for nuclear factors EBP20/AP3, NF-κB, GT IIB and GT IIC and the 'E'-sites in the immunoglobulin enhancers have only been detected in cellular or viral enhancer elements (Johnson *et al.*, 1987; Chiu *et al.*, 1987; Xiao *et al.*, 1987; Sen and Baltimore, 1986a). Similarly, the binding site for the nuclear factors MLTF/USF has been found exclusively in regions considered to be promoters (Carthew *et al.*, 1985, 1987; Sawadogo and Roeder, 1985). Some protein-binding sites, however, are not restricted to only enhancers or promoters. The octamer motif is present in several enhancer elements as well as in the immunoglobulin and other promoters (Falkner *et al.*, 1986). Similarly, a sequence motif closely related to the CCAAT sequence element that is most consistently found in many viral or cellular promoter elements was also described in the immunoglobulin heavy chain and the other enhancer elements (Peterson and Calame, 1987; Jones *et al.*, 1987). In addition, binding sites for the transcription factor AP1, originally identified as an enhancer-binding protein, have been detected in some promoter elements (Angel *et al.*, 1987; Lee *et al.*, 1987). In these cases, however, it is not clear whether the same protein is active in the enhancer and promoter context, because more than one nuclear protein might specifically interact with the same sequence elements (Landolfi *et al.*, 1986; Staudt *et al.*, 1986; Dorn *et al.*, 1987; Bohmann *et al.*, 1987). The transcription factor Sp1, however, seems to interact not only with a variety of promoter elements (Briggs *et al.*, 1986), but also binds to its cognate binding site in the human U2 snRNA gene enhancer (Ares *et al.*, 1987). It has seemed likely that there are mechanistic differences between the functions of factors interacting with sites within enhancers and those interacting with sites in promoters. Those mechanistic differences might explain the observed differences between enhancers and promoters. We, however, have shown here that the κB site efficiently functions as both an enhancer and a promoter element. Furthermore, mutational, electrophoretic mobility shift and induction experiments all indicate that NF-κB is mediating this activity. These results do not support the idea of fundamental mechanistic differences between enhancers and promoters. If a single nuclear protein can interact functionally with its cognate binding site and provide either enhancer or promoter activity, then it is likely that the two activities reflect similar if not identical events. Although we cannot generalize this finding to all nuclear factors involved in enhancer and promoter activity, it clearly suggests that enhancers and promoters may carry out quite similar actions

in their stimulation of initiation of transcription. If they are functionally equivalent structures, this leaves open the question of why certain elements appear to function well at long distances while others need to be close. It also leaves open whether some enhancer or promoter elements might have mechanistic activities quite separate from those of NF-κB or the octamer-binding proteins.

Materials and methods

Plasmid constructions

The construction of the plasmids used for the expression studies was performed essentially as described (Wirth *et al.*, 1987). Briefly, the oligonucleotides were initially inserted into the *Bam*HI cloning site of the pUC18 plasmid polylinker. The *SalI*–*SmaI* fragment from these intermediates, containing the inserted oligonucleotides, was subsequently transferred into the β-globin expression vector. For the constructs containing the oligonucleotide insert 200 bp upstream of the TATA-box, the *SalI*–*PvuII* fragments were used. The two control plasmids containing either no oligonucleotide insert or the octamer insert within the polylinker piece were described previously (Wirth *et al.*, 1987).

Cell lines, transfection procedure, drug stimulations

NIH/3T3 fibroblasts and S194 myeloma cells were kept in DME medium supplemented with either 10% calf serum or 5% calf serum plus 5% fetal calf serum, respectively. All the other cell lines were kept in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μM 2-mercaptoethanol. $1-2 \times 10^7$ cells were transfected with 3–5 μg plasmid DNA using DEAE–dextran according to previously described methods (Grosschedl and Baltimore, 1985). For LPS treatment, 10 μg/ml Bacto lipopolysaccharide, *S.typhosa* 0901 (Difco), was added immediately after transfection for 48 h. For PHA plus TPA induction of Jurkat cells, 2 μg/ml PHA and 50 ng/ml TPA were added 20 h prior to RNA extraction.

RNA analysis

Total cytoplasmic RNA was isolated as follows (procedures kindly provided by P.Sarnow and K.Kirkegaard). Cells were harvested and washed twice with 1 ml of phosphate-buffered saline (PBS) in microcentrifuge tubes (10-s spins). After suspension of the cells in 270 μl cold TE buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA) 30 μl of 5% Nonidet P-40 was added and the cells were lysed for 5 min on ice. Nuclei were removed by a 1 min centrifugation, the cytoplasmic supernatant was adjusted to 1% SDS, phenol extracted and then ethanol precipitated. Conditions for nuclease S1 analysis were as described (Grosschedl and Baltimore, 1985). 5–10 μg of RNA were used with the H4 probe and 20–100 μg were used with the β-globin probe. Hybridization was performed at 48°C for 15 h and hybrids were digested with 125 U S1 nuclease (Sigma) for 1 h at room temperature. S1-resistant fragments were resolved on 8% denaturing polyacrylamide gels.

Electrophoretic mobility shift assays

Nuclear extract was prepared from TPA-induced mouse 70Z/3 cells which contain NF-κB binding activity (Sen and Baltimore, 1986b) essentially as described (Dignam *et al.*, 1983). The DNA probes were derived from the pUC18 cloning intermediates containing the indicated oligonucleotide inserted in either orientation into the *Bam*HI site. As probes, the *Eco*RI–*Hin*dIII fragments, end-labeled by filling in the ends with the Klenow fragment of DNA polymerase I, were used. Incubation of the labeled fragments with the nuclear extract and subsequent analysis of the formed complexes on native polyacrylamide gels was performed as described (Staudt *et al.*, 1986).

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