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Two transcription factors, NF-κB and H2TF1, interact with a single regulatory sequence in the class I major histocompatibility complex promoter

(DNA-binding proteins/B-cell-specific factor)

ALBERT S. BALDWIN, JR., AND PHILLIP A. SHARP

Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

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ABSTRACT **A sequence centered 166 nucleotides upstream of the mouse H-2K^b class I major histocompatibility gene binds a nuclear factor, H2TF1, found in many cell types. Previous studies have shown that binding of H2TF1 to this sequence stimulates class I gene expression. Furthermore, this factor binds a similar sequence in the 72-base-pair repeat enhancer element of simian virus 40. We show here that NF-κB, an inducible B-cell-specific factor that binds the κ immunoglobulin light chain gene enhancer, also binds the H2TF1 regulatory sequence. Methylation-interference experiments demonstrate that NF-κB closely interacts with six of the eight symmetrically positioned guanines that contact H2TF1. These experiments suggest that NF-κB may play a role in class I major histocompatibility gene expression and that H2TF1 and NF-κB may be related DNA-binding proteins.**

The transcription factor NF-κB is a sequence-specific DNA-binding protein that is believed to regulate tissue-specific gene expression. It is constitutively present only in B cells, where it is thought to be partially responsible for enhanced transcription of the κ immunoglobulin light chain gene (1). The DNA-binding activity of NF-κB is inducible by a posttranslational mechanism when pre-B cells are treated with lipopolysaccharide (LPS) or when non-B cells are treated with phorbol 12-myristate 13-acetate, a tumor-promoting agent (2). Furthermore, NF-κB is activated in T cells by mitogens and likely contributes to transcriptional activation of human immunodeficiency virus (3).

We show here that the NF-κB factor recognizes six guanine residues that are symmetrically distributed within eight guanine residues recognized by another factor, H2TF1. This latter factor stimulates transcription of the mouse H-2K^b class I major histocompatibility complex (MHC) gene and is found in most cell types (4). Consistent with this finding, both the NF-κB and the H2TF1 factors bind a single site in the 72-base-pair (bp) repeat enhancer element of simian virus 40 (SV40) (1, 4). This suggests that a cell-type-specific and inducible factor, NF-κB, could regulate a subset of genes by recognizing sequences contained within the binding site of a constitutive factor.

MATERIALS AND METHODS

Nuclear Extracts. Extracts of 70Z/3 cells (mouse pre-B-cell line) were prepared according to the procedure of Dignam *et al.* (5) and were the gift of L. Staudt and M. Lenardo (Massachusetts Institute of Technology). Nuclear extracts of mouse erythroleukemia (MEL) cells were prepared similarly and were the gift of D. Galson (Massachusetts Institute of Technology).

DNA Binding Assay. The procedure for the electrophoretic mobility-shift assay has been described (4). Approximately 7 μg of nuclear extract protein was used in each binding reaction with 10^4 cpm of probe (0.2 ng). Poly(dI·dC) (Pharmacia) was included as a nonspecific carrier DNA. The κ3 probe was a gift of M. Lenardo. The *Xho* I–*Hin*cII H-2K^b probe was end-labeled using the Klenow fragment of DNA polymerase I. Competitor DNAs were the 460-bp *Xho* I–*Eco*RI fragment of plasmid p190H2KCAT (4); the 450-bp *Hin*dIII–*Eco*RI fragment of pSPKEN, containing the κ enhancer (gift of H. Singh, Massachusetts Institute of Technology); and the 400-bp *Eco*RI–*Eco*RI fragment of pΔE23-CAT (gift of R. Kingston, Massachusetts General Hospital).

Methylation Interference. The end-labeled *Xho* I–*Hin*cII H-2K^b DNA was methylated with dimethyl sulfate (6). Binding reactions and electrophoresis were carried out as described above. Bound and free DNA were eluted, extracted with phenol/chloroform (1:1, vol/vol), and cleaved with piperidine. These DNAs were electrophoresed in 8% polyacrylamide/7.5 M urea gels and exposed for autoradiography.

RESULTS

NF-κB Binds a Class I MHC Regulatory Sequence *in Vitro*. The regulatory sequence centered at nucleotide −166 (relative to the transcription initiation site) in the mouse H-2K^b MHC promoter and the binding site for NF-κB in the κ immunoglobulin enhancer share extensive sequence homology (Fig. 1). A gel electrophoresis DNA-binding assay (7–11) was used to determine whether the NF-κB factor could bind to the H-2K^b sequence. In this assay, a labeled DNA probe is incubated with cellular extracts and the mixture is analyzed by electrophoresis on a polyacrylamide gel. Any specific DNA–protein complexes formed during the incubation migrate slower than the free probes in the gel.

Nuclear extracts of uninduced or induced 70Z/3 cells were selected for analysis, since it has been demonstrated that treatment of this pre-B-cell line with LPS and cycloheximide superinduces NF-κB binding activity (2). An end-labeled DNA fragment extending from −190 to −100 in the H-2K^b promoter (see Fig. 1) was used as probe. In the case of the uninduced 70Z/3 extracts, no prominent complex was detected with the H-2K^b probe under these assay conditions (Fig. 2*a*, lane 1). In control experiments with other probes, it was shown that these extracts contain the expected activities of several DNA-binding proteins and, therefore, contain active proteins (A.S.B., unpublished data). In contrast, a prominent DNA–protein complex was formed when the H-2K^b probe was incubated with extracts of 70Z/3 cells that

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Abbreviations: LPS, lipopolysaccharide; MEL, mouse erythroleukemia; MHC, major histocompatibility complex; SV40, simian virus 40.

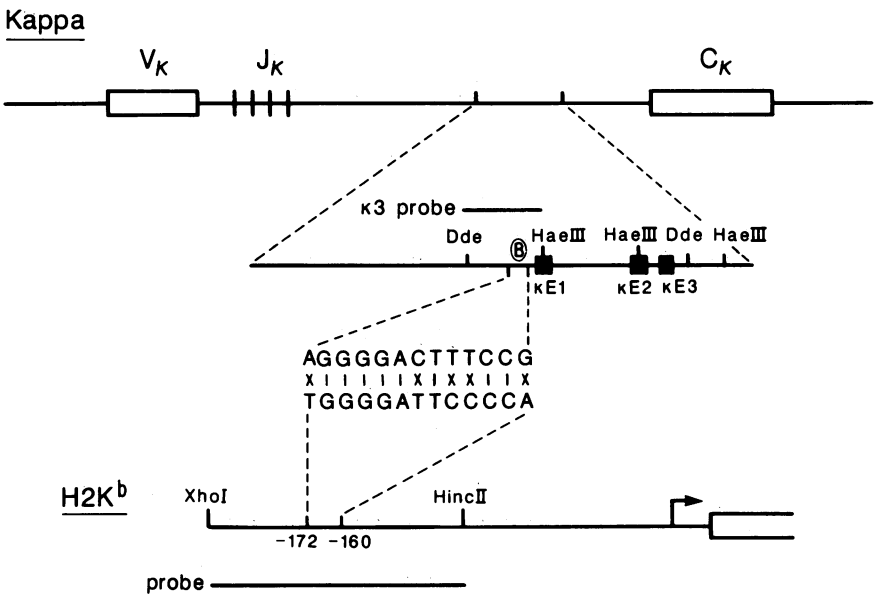

FIG. 1. Schematic representation of the κ enhancer and the 5′ flanking DNA of the H-2K^b gene. The κ immunoglobulin gene is shown in the upper part of the figure and is redrawn from ref. 1. The *Alu* I–*Alu* I restriction fragment containing the κ enhancer is shown expanded. The NF-κB binding site is denoted by Ⓑ and other characterized binding sites (1) are indicated by κE1, κE2, and κE3. The probe used in the binding assays was the *Dde* I–*Hae* III fragment that is shown as κ3. The 5′ flanking DNA of the mouse H-2K^b is shown in the lower part of the figure. The H-2K^b probe used in the binding experiments is shown and extends from an *Xho* I linker at nucleotide −190 to the *Hin*cII site at −100. A sequence comparison of the H2TF1 and NF-κB binding sites is shown.

had been induced by treatment with LPS and cycloheximide (Fig. 2*a*, lane 2). This complex was due to the binding of a sequence-specific factor, since formation of the labeled complex was prevented by inclusion of an excess of unlabeled H-2K^b DNA in the binding reaction mixture (Fig. 2*a*, lane 3) but not by addition of a similar excess of heterologous DNA (lane 5). To test whether the sequence-specific binding factor was NF-κB, an excess of κ immunoglobulin enhancer

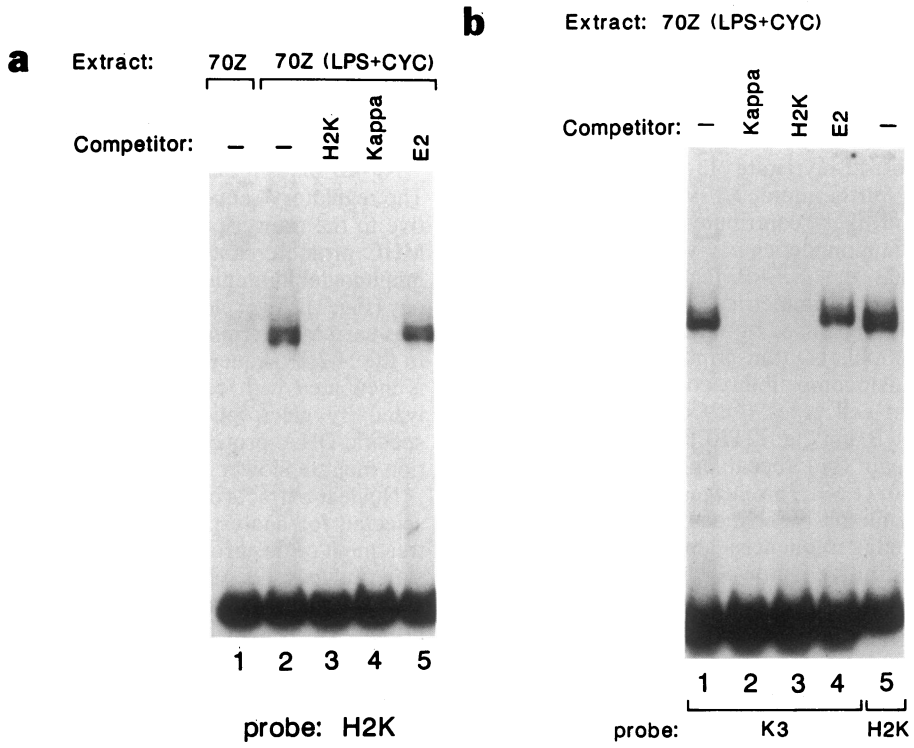

FIG. 2. Binding of NF-κB to an H-2K^b regulatory sequence. (*a*) Analysis of binding, using an electrophoretic mobility-shift assay (7–11). End-labeled *Xho* I–*Hin*cII H-2K^b DNA was incubated with 7 μg of nuclear extract protein of either untreated 70Z/3 cells or 70Z/3 cells treated with LPS and cycloheximide (CYC), as indicated at the top. All binding reaction mixtures contained poly(dI·dC) and either no competitor DNA (lanes 1 and 2) or an ≈40-fold excess of H-2K^b DNA (lane 3), κ enhancer DNA (lane 4), or adenovirus early region 2 (E2) promoter DNA (lane 5). Electrophoresis was in low-ionic-strength 4% polyacrylamide gels. (*b*) Analysis of binding to the κ3 fragment. Nuclear extracts of LPS- and cycloheximide-induced 70Z/3 cells were incubated with κ3 probe and electrophoresed as described for *a*. Competitors (as described for *a*) were as follows: no competitor (lane 1), κ enhancer DNA (lane 2), H-2K^b DNA (lane 3), and adenovirus E2 promoter DNA (lane 4). Lane 5: H-2K^b probe with no competitor.

DNA was added to the binding reaction mixture containing the induced extracts and the H-2K^b probe (Fig. 2*a*, lane 4). The κ fragment competed for complex formation by the H-2K^b probe. Titration of competitor DNA levels showed that the H-2K^b and κ enhancer DNAs competed equally well for binding of the induced factor to the H-2K^b probe (data not shown). The above results suggested that an inducible factor, almost certainly NF-κB, recognized the H-2K^b probe.

To directly demonstrate the presence of the NF-κB factor, a fragment encompassing the NF-κB binding site of the κ enhancer (see Fig. 1) was end-labeled and incubated with the extract of induced 70Z/3 cells. A DNA–protein complex was generated that had mobility similar to that observed with the H-2K^b probe (Fig. 2*b*, lanes 1 and 5). Formation of this labeled complex was also effectively inhibited by both the κ enhancer (lane 2) and H-2K^b (lane 3) DNAs. Heterologous DNA at the same concentration had no effect on the binding activity (lane 4). Thus, the H-2K^b fragment binds the NF-κB factor as effectively as the κ fragment.

H2TF1 Has Low Affinity for the κ Enhancer. Previous experiments have shown that NF-κB activity is B-cell-specific (1). This contrasted with the presence of the H2TF1 factor in most cell types (4) and indicated that these two factors must be distinguishable in terms of their sequence specificity for binding. To be consistent with previous results, the κ element should not bind the H2TF1 factor. Therefore, we tested the κ enhancer fragment for competition with the binding of the H2TF1 factor to the H-2K^b probe in extracts of a non-B cell. We have shown previously that extracts of MEL cells contain the H2TF1 factor that binds specifically to the −190 to −100 H-2K^b probe (4). With a MEL extract, binding of H2TF1 to the H-2K^b probe was inhibited by an excess of H-2K^b promoter DNA (Fig. 3, lane 2) but not by a similar excess of κ enhancer DNA (lane 3). The κ enhancer did compete for binding of H2TF1 to the H-2K^b probe, but only at relatively high molar excesses

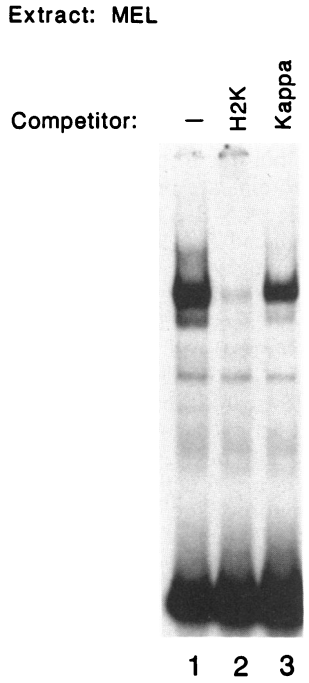

FIG. 3. Specificity of binding of H2TF1 to H-2K^b DNA. The end-labeled *Xho* I–*Hin*cII H-2K^b fragment was incubated with 10 μg of MEL nuclear extracts in the presence or absence of competitor DNA and then was electrophoresed in a low-ionic-strength 4% polyacrylamide gel. Lanes: 1, no competitor DNA; 2, a 40-fold excess of H-2K^b competitor; 3, a 40-fold excess of κ enhancer DNA.

(data not shown). The H-2K^b −166 region has a 10- to 20-fold higher affinity for H2TF1 than does the κ enhancer DNA. The DNA–protein complex formed in the MEL extracts (the H2TF1–DNA complex) had a slower mobility than that formed in extracts of induced 70Z/3 cells (the NF-κB–DNA complex), suggesting that these two factors differ either in molecular weights or in conformation.

NF-κB Makes Close Contacts with a Subset of Nucleotides that Contact H2TF1. A methylation-interference assay (12) was used to compare the sequence specificity of binding of the NF-κB and H2TF1 factors. This assay identifies guanine residues that, when methylated at the N-7 position in the major groove, prevent binding of the particular factor. The end-labeled DNA probe is randomly methylated to an average density of one guanine per molecule and is used as a substrate in the electrophoretic mobility-shift assay. If methylation at a particular guanine interferes with binding, fragments modified at this site are excluded from the specific DNA–protein complex. To determine these sites, the bands containing the complex and free probe are eluted from the gel and extracted with phenol/chloroform, and the sites of modification are cleaved with piperidine.

When an H-2K^b fragment extending from −190 to −100 was end-labeled on the noncoding strand, methylated, and used as a probe in the methylation-interference assay with an MEL nuclear extract, four cleavage sites were markedly reduced in the specific-complex material as compared with the free probe (Fig. 4*a*). The four sites correspond to guanines at positions −164 to −161, and these results agree with our previous analysis of H2TF1 binding in a HeLa cell extract (4). In contrast, a parallel analysis of complex material formed with an extract from the induced 70Z/3 cells gave a related but different profile of cleavages. Methylation of the guanine at position −161 did not interfere with the NF-κB binding activity (Fig. 4*a*). However, a complete and a partial interference with NF-κB binding was observed when the two internal guanines and the guanine at −164, respectively, were methylated. Very similar results were obtained from analysis of the coding strand. All four guanines from −171 to −168 are in close contact with the H2TF1 factor in the MEL extracts (Fig. 4*b*). Again, the binding of NF-κB can be distinguished from that of H2TF1, since methylation at the terminal guanine at −171 did not inhibit the binding of the former factor. As expected, the binding of NF-κB factor was blocked by methylation of the internal guanines.

The identification of an NF-κB binding site within an H2TF1 binding site confirms previous observations regarding binding of these factors to the SV40 72-bp repeat (1, 4). The sequence TGGAAAGTCCCCA in the 72-bp repeat competes for binding of NF-κB to κ enhancer DNA (1) and competes with lower affinity for binding of H2TF1 to H-2K^b DNA (4). The NF-κB binding site in the κ enhancer element is identical to the SV40 sequence with the exception of changes in the symmetrically positioned thymines (1). Methylation-interference experiments showed that all guanines in the symmetric SV40 sequence are in close contact with H2TF1 (A.S.B., unpublished work).

DISCUSSION

The two factors that bind the H-2K^b regulatory sequence centered at nucleotide −166 are distinguishable by several criteria. First, they can be resolved on the basis of their affinities for H-2K^b and κ immunoglobulin enhancer DNAs. H2TF1 has high affinity for H-2K^b site but low affinity for the NF-κB binding site in the κ enhancer (Fig. 3). However, NF-κB has relatively high affinity for both κ enhancer and H-2K^b sites. In fact, binding experiments that titrated competitor DNA demonstrated that NF-κB has similar affinities

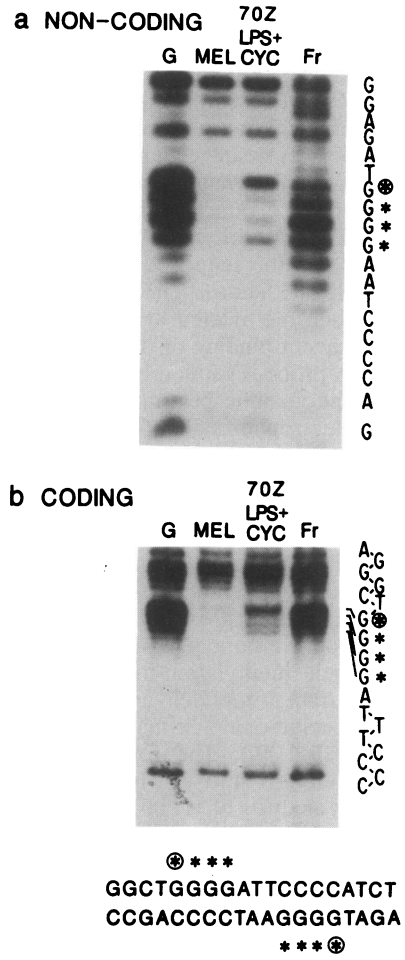

FIG. 4. Comparison of DNA contacts made by H2TF1 and NF-κB on H-2K^b DNA. Methylation interference (12) was used to determine guanine contacts. The −190 to −100 *Xho* I–*Hin*cII H-2K^b probe was methylated and used as a probe in binding reactions with MEL and induced 70Z/3 extracts. (*a*) The H-2K^b probe was end-labeled on the noncoding strand and used as a probe. The sequence of the DNA is presented at right. Methylated guanines that interfere with MEL (H2TF1) and NF-κB binding in this assay are indicated by asterisks. The guanine in this group that does not interfere with NF-κB binding is indicated by a circled asterisk. (*b*) The H-2K^b DNA was labeled on the coding strand and used as a probe. Methylated guanines that interfere are indicated as described for *a*. The double-stranded H-2K^b sequence with contacts is shown at the bottom. Lanes G: cleavage products of unreacted probe. Lanes Fr: cleavage products of free DNA.

for the class I promoter and κ enhancer sites (Fig. 2 and data not shown). A second difference between H2TF1 and NF-κB is that the two binding activities make slightly different contacts on the H-2K^b DNA (Fig. 4). H2TF1 makes close contacts with four symmetric guanines on each strand of the DNA at the binding site in the MHC promoter. In contrast, NF-κB makes contacts with a subset of these sequences, interacting with the internal three guanines on each strand and not the external guanine. Consistent with this observation, an NF-κB binding site in the long terminal repeat of human immunodeficiency virus has an adenine in place of one of these external guanines (3). Finally, H2TF1 and NF-κB are distinguishable by their cell-type distribution. We have detected the H2TF1 binding activity in extracts of several cell types, including human epithelial (HeLa), mouse erythroid (MEL), and mouse fibroblast (BALB/c 3T3) (4). In contrast, the NF-κB binding activity is apparently found only in B cells expressing κ immunoglobulin light chains and in non-B cells after induction by a phorbol ester (2).

What is suggested by the finding that two distinct DNA-binding activities interact with the sequence TGGGGATTCCCCA centered 166 nucleotides upstream of the transcription initiation site of the H-2K^b MHC gene? This sequence has been shown to contribute some 5- to 10-fold to class I transcription in fibroblasts (4, 13) and to be required for induction of the H-2K^b gene by interferon (14). A similar sequence is conserved in most class I MHC genes, including the human class I HLA genes (13–16). We propose that in most cell types, the binding of factor H2TF1 to this sequence probably mediates the 5- to 10-fold increase in class I gene expression. Particularly interesting is the possibility that the highly regulated factor NF-κB could bind and stimulate transcription of class I genes by recognizing the central core of the binding site for the ubiquitous H2TF1 factor. The binding sites of these two factors share six symmetrically distributed guanines, suggesting that most, if not all, H2TF1 binding sites might also be recognized by NF-κB.

NF-κB activity is transiently inducible by phorbol esters in most cell types (2) and is induced during T-cell activation (3). Phorbol esters are thought to exert their effects in cells by activation of protein kinase C (17). The natural intracellular signal for this activation is diacylglycerol, which is generated by degradation of phosphatidylinositol 4,5-bisphosphate by phospholipase C (18, 19). Upon binding of hormones, many receptors activate phospholipase C and thus protein kinase C. It is not unlikely that lymphokines and hormones, which are released during inflammation and other immunological reactions, increase the level of MHC class I gene expression in surrounding tissues by induction of the intracellular factor NF-κB. This may enhance the presentation of antigens in the context of MHC class I protein on the surface of these cells, thus assuring that the cells possessing foreign antigens would be killed by appropriate cytotoxic T lymphocytes (20). In this case, the binding of the regulatory factor NF-κB to a subset of the sequences in the constitutive factor H2TF1 site would be important in the physiology of the organism.

Since most cell types have H2FT1 binding activity, we were surprised to detect reduced binding activity in the uninduced pre-B-cell (70Z/3) extracts. The inability to detect expected levels of NF-κB and H2TF1 binding activities in 70Z/3 cells is not due to protein degradation, since other DNA-binding proteins are present at normal levels in these extracts (ref. 2; A.S.B., unpublished data). Increased amounts of uninduced 70Z/3 protein in the binding reaction and longer autoradiographic exposure times revealed a binding pattern on the H-2K^b probe that is identical to that observed with extracts of BALB/c 3T3 fibroblasts. This suggests that this pre-B-cell line does express low levels of H2TF1. High levels of H2TF1 binding activity are found in extracts of LPS-treated, but not cycloheximide-treated, pre-B cells and in extracts of mature B cells (data not shown). Cytoplasmic mRNA complementary to the H-2K^b gene is present in pre-B cells, although at a 50–70% lower level than in mature B cells (A.S.B., unpublished data). Therefore, reduced levels of H2TF1 in 70Z/3 cells does not block class I gene expression. Regulatory sequences in addition to the −166 region are likely to contribute to class I gene expression in pre-B cells. In fact, deletion analysis has revealed that several sets of sequences control class I gene expression in fibroblasts (4, 13).

What is the relationship between NF-κB and H2TF1? One possibility is that these DNA-binding activities reflect proteins encoded by different genes. H2TF1 and NF-κB may have similar DNA-binding domains, encoded by evolutionarily related genomic sequences, that interact with nearly identical DNA sequences. In particular, NF-κB would rec-

ognize the core sequence of a slightly larger site recognized by H2TF1. H2TF1 and NF-κB would therefore be members of a gene family encoding DNA-binding proteins similar to the gene family identified for the steroid receptors (21). Alternatively, H2TF1 and NF-κB may be different binding activities encoded by a single gene. In this case, a modification of the protein would alter the binding specificities. This modification might involve the interaction of a second factor or possibly some enzymatic activity like phosphorylation or proteolysis. Since phorbol ester treatment can induce NF-κB, protein kinase C may play a role in the activation (17). In this hypothesis H2TF1 would be viewed as a constitutive form of the activity, whereas NF-κB would be generated by a cell-specific modification. Purification of these activities and subsequent biochemical characterization are required to distinguish these possibilities.

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1. Sen, R. & Baltimore, D. (1986) *Cell* **46**, 705–716.
2. Sen, R. & Baltimore, D. (1986) *Cell* **47**, 921–928.
3. Nabel, G. & Baltimore, D. (1987) *Nature (London)* **326**, 711–713.
4. Baldwin, A. & Sharp, P. A. (1987) *Mol. Cell. Biol.* **7**, 305–313.
5. Dignam, J., Lebowitz, R. & Roeder, R. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
6. Maxam, A. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
7. Fried, M. & Crothers, D. (1981) *Nucleic Acids Res.* **9**, 6505–6525.
8. Garner, M. & Revzin, A. (1981) *Nucleic Acids Res.* **9**, 3047–3060.
9. Singh, H., Sen, R., Baltimore, D. & Sharp, P. A. (1986) *Nature (London)* **319**, 154–158.
10. Strauss, F. & Varshavsky, A. (1984) *Cell* **37**, 889–901.
11. Carthew, R., Chodosh, L. & Sharp, P. A. (1985) *Cell* **43**, 439–448.
12. Siebenlist, U., Simpson, R. & Gilbert, W. (1980) *Cell* **20**, 269–281.
13. Kimura, A., Israel, A., LeBail, B. & Kourilsky, P. (1986) *Cell* **44**, 261–272.
14. Israel, A., Kimura, A., Fournier, A., Fellous, M. & Kourilsky, P. (1986) *Nature (London)* **322**, 743–746.
15. Kvist, S., Roberts, L. & Dobberstein, B. (1983) *EMBO J.* **2**, 245–254.
16. Strachan, T., Sodoyer, R., Damotte, M. & Jordan, B. (1984) *EMBO J.* **3**, 887–894.
17. Parker, P., Stabel, S. & Waterfield, M. (1984) *EMBO J.* **3**, 953–959.
18. Berridge, M. (1984) *Biochem. J.* **220**, 345–360.
19. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. & Nishizuka, Y. (1980) *J. Biol. Chem.* **255**, 2273–2276.
20. Zinkernagel, R. & Doherty, P. (1979) *Adv. Immunol.* **27**, 51–77.
21. Weinberger, C., Thompson, C., Ong, E., Lebo, R., Gruol, D. & Evans, R. (1986) *Nature (London)* **324**, 641–646.