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NF-κB Binds within a Region Required for B-Cell-Specific Expression of the Major Histocompatibility Complex Class II Gene E_α^d

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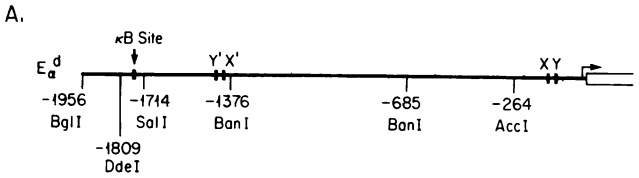
A region upstream of the murine major histocompatibility complex gene, E_α^d, has been shown previously to be required for B-cell expression. Binding of the B-cell-specific factor, NF-κB, to a site within this region is indistinguishable from that observed with the κ enhancer binding site. NF-κB may be responsible for E_α^d B-cell expression.

The class II genes of the murine major histocompatibility complex encode heterodimeric cell-surface glycoproteins that regulate the immune response through their role as restriction elements in antigen presentation (8). These molecules are expressed primarily in B lymphocytes and macrophages. During hematopoiesis, constitutive class II gene expression is observed on developing B cells. Previously, we have shown that deletion of a 580-base-pair region (−1956 to −1376) of an E_α^d transgene resulted in the selective loss of expression of the transgene in B cells (21). Present within the deleted region is a DNA sequence similar to that found at the κ immunoglobulin enhancer B (κB) site.

It has been demonstrated that a protein factor, NF-κB, binds to the κB site (17). Expression of the κ immunoglobulin gene is restricted to B cells and is correlated with the binding activity of NF-κB (17). Pierce et al. (16) have reported that a short sequence encompassing the κB-binding site is sufficient in itself to direct B-cell-specific expression of a heterologous promoter. As a first step in the dissection of the B-cell-specific expression of the E_α^d gene, we have characterized the interaction of the potential binding site with the B-cell-specific regulatory protein NF-κB.

The DNA sequence centered at nucleotide −1748 (relative to the first nucleotide of the initiation codon) of the murine major histocompatibility complex E_α^d gene shares extensive sequence identity to the binding site for NF-κB in the κ immunoglobulin enhancer as well as to various other sites shown previously to bind NF-κB (Fig. 1). A gel electrophoresis DNA-binding assay (4, 9, 10, 20) was used to determine whether the NF-κB factor could bind to the site in E_α^d. Nuclear extracts of either uninduced or induced 70Z/3 cells were selected for analysis, since it has been demonstrated that treatment of this pre-B-cell line with lipopolysaccharide, cycloheximide, or a combination of these two induces NF-κB-binding activity (18). An end-labeled DNA fragment extending from −1714 to −1809 (Fig. 1A) in the E_α^d 5′ region, which includes the putative NF-κB-binding site, was used as a probe. No prominent complex was detected in the case of the uninduced 70Z/3 extracts. However, when incubated with lipopolysaccharide and cycloheximide-induced 70Z/3 extracts, a prominent DNA-protein complex was formed. This complex appeared to be due to the binding of a κ enhancer sequence-specific factor, since formation of the complex was prevented by inclusion of an excess of unlabeled κ enhancer DNA but not by addition of a similar excess of heterologous DNA (data not shown).

A double-stranded oligonucleotide probe, encompassing the proposed E_α^d:NF-κB-binding site, was prepared. This oligonucleotide, which included the sequence from −1741 to −1756 in the E_α^d upstream region, was end labeled and used in DNA-binding experiments (Fig. 2). Consistent with the experimental results described above, one complex was detected in extracts of cells treated either with cycloheximide alone (Fig. 2, lane 8) or with lipopolysaccharide alone (Fig. 2, lane 7) which was not present in extracts of untreated 70Z/3 cells (Fig. 2, lane 6). The induced DNA-binding activity was specific, as shown by competition with an approximately 50-fold molar excess of unlabeled E_α^d:NF-κB DNA (Fig. 2, lane 10) but not by addition of a similar excess



B.

GENE	SEQUENCE
Murine Ig Kappa, SV40, CMV, HIV	GGGGACTTTCC
Human Ig Kappa	GGGGGATTTCC
IL-2 Receptor, Alpha	GGGGAATCTCC
Class I MHC (H-2K^b)	GGGGAATCCCC
β_2-Microglobulin, HIV, CMV	AGGGACTTTCC
Class II MHC (E_α^d)	CGGGACTTCCC
Consensus:	$^{Pu}_{C}$GGG$^{Pu}_{C}$$^{A}_{C}$TPyPyCC

FIG. 1. (A) Schematic representation of the E_α^d upstream region. The region is numbered relative to the first nucleotide of the initiation codon. Conserved class II boxes (X and Y) and upstream boxes (X′ and Y′) are indicated. The κB-like site is centered at position −1748. (B) Sequences of κB-related sites and their associated genes (murine immunoglobulin κ [Ig kappa] [17], simian virus 40 [SV40] [5, 14], cytomegalovirus [CMV] [3], human immunodeficiency virus [HIV] [15], human immunoglobulin κ [Ig kappa] [7], interleukin 2 receptor, alpha [12], class I major histocompatibility complex [MHC] [1, 2], and β_2-microglobulin [11]).

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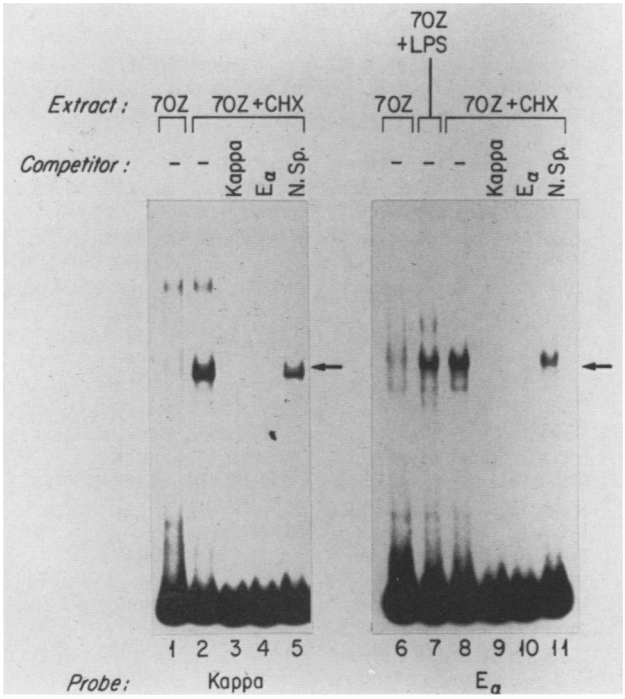

FIG. 2. Binding of NF-κB to the E_α^d:NF-κB sequence. Nuclear extracts were prepared according to the method of Dignam et al. (6), and the gel electrophoresis DNA-binding assay was performed as described previously (4, 9, 10, 20). A double-stranded oligonucleotide containing the κB site derived from the κ enhancer (positions 3930 to 3950) (13), with *Kpn*I-compatible ends, was used as the competitor or was radiolabeled and used as the probe. Similarly, a double-stranded oligonucleotide containing the E_α^d:NF-κB site (position −1756 to −1741) (21), with *Bam*HI-compatible ends, was used both as the competitor and as the radiolabeled probe. End-labeled probe, as noted at the bottom, was incubated with 7 μg of nuclear extract protein either from untreated 70Z/3 cells or from 70Z/3 cells treated with either cycloheximide (CHX) at 10 μg/ml for 4 h (18) or with bacterial lipopolysaccharide (LPS) at 10 μg/ml for 4 h (18) as indicated above the lanes. All binding reactions (10.4 μl) contained 2 μg of poly(dI · dC) and either no competitor (lanes 1, 2, 6, 7, and 8) or an approximately 50-fold excess of κB DNA (lanes 3 and 9), E_α^d:NF-κB DNA (lanes 4 and 10), or a nonspecific, double-stranded oligonucleotide (lanes 5 and 11).

of heterologous DNA (Fig. 2, lane 11). To test whether the sequence-specific binding factor was NF-κB, an excess of a double-stranded oligonucleotide which encompassed the κB site was added to the binding reaction mixture containing the cycloheximide-induced 70Z/3 extract and the E_α^d:NF-κB probe (Fig. 2, lane 9). The κB oligonucleotide competed for the complex formation by the E_α^d:NF-κB probe. These results suggest that an inducible factor, NF-κB, recognized the E_α^d probe.

To demonstrate the presence of the NF-κB factor directly, the double-stranded κB oligonucleotide was end labeled and incubated with the 70Z/3 nuclear extracts. A DNA-protein complex that had a mobility similar to that observed with the E_α^d:NF-κB probe was generated (Fig. 2, lanes 2 and 8). Formation of this complex was effectively inhibited by both the κB oligonucleotide (Fig. 2, lane 3) and the E_α^d:NF-κB oligonucleotide (Fig. 2, lane 4). Heterologous DNA at the same concentration had no effect on the formation of this complex (Fig. 2, lane 5). Thus, the E_α^d:NF-κB oligonucleotide bound NF-κB in a manner similar to that of the κ enhancer-derived binding site.

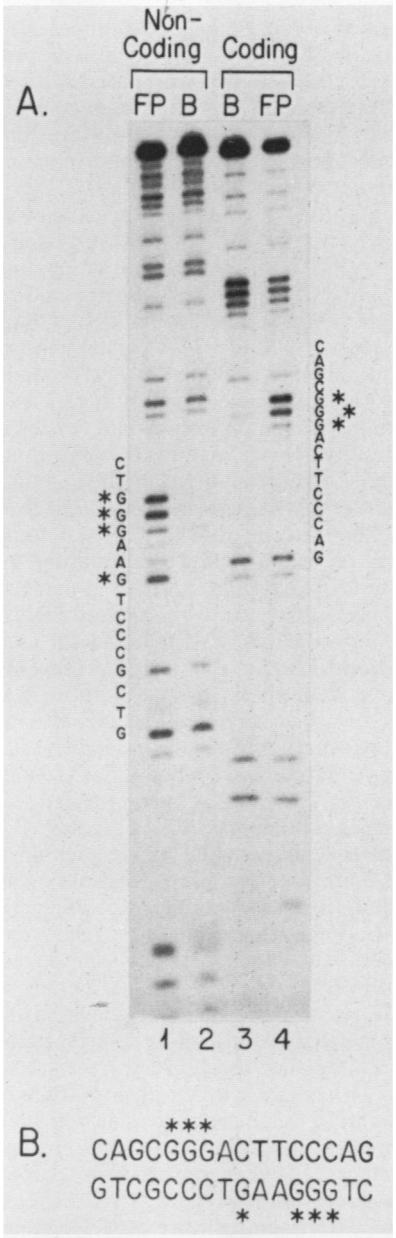

FIG. 3. DNA contacts made by NF-κB on the E_α^d:NF-κB binding site. Methylation interference was used to determine guanine residue contacts. (A) The E_α^d:NF-κB double-stranded oligonucleotide (described in Fig. 2) was radiolabeled on either the noncoding or the coding strand and then used as a probe. The sequence of the binding site DNA sequence is shown, with the methylated guanine residues that interfere with NF-κB binding indicated by asterisks. FP, Cleavage products of unbound, free probe DNA; B, cleavage products of the specific protein-DNA complex. (B) The double-stranded E_α^d:NF-κB sequence; guanosine contacts are indicated.

A methylation interference assay (19) was used to determine the sequence specificity of binding of the 70Z/3 cycloheximide-induced binding activity to the E_α^d:NF-κB oligonucleotide probe. This assay identifies those guanine residues which, when methylated, prevent binding of the relevant factor to DNA. If methylation of a particular guanine residue interfered with the binding of the induced factor, end-labeled fragments modified at this site would be excluded from the specific DNA-protein complex. To deter-

mine the position of these guanine residues, DNA was eluted from the specific complex and cleaved with piperidine, and the pattern of cleavage was compared with that of free, unbound probe obtained from the same gel.

When the E_α^d:NF-κB oligonucleotide was end labeled on the noncoding strand, methylated, and used as a probe in the methylation interference assay with a 70Z/3 cycloheximide-induced extract, four guanine cleavage sites were markedly reduced in the specific (bound) complex compared with in the free probe (Fig. 3A). Methylation of guanine residues at positions −1743, −1744, and −1748 appeared to completely inhibit formation of a stable protein-DNA complex, and the guanine residue at position −1745 partially interfered. Methylation of the adenine residues at −1746 and −1747 also appeared to interfere with the binding. This data corresponds entirely with the published data concerning the close contacts made by NF-κB at the κB site and at the H-2K^b-binding site (2, 17). When labeled on the coding strand, three guanine cleavage sites, at positions −1750 to −1752, were markedly reduced in the specific (bound) complex. Again, this data was entirely consistent with the interaction of NF-κB to the binding sites in the κ enhancer and in the H-2K^b promoter. Note that this is the first NF-κB-binding site that has a pyrimidine (C) residue at the first position on the coding strand instead of a purine residue (Fig. 1B).

Others have demonstrated that a short NF-κB-binding sequence can act as a B-cell-specific enhancer element independent of other motifs in the κ enhancer (16). Although we have not yet directly identified those elements responsible for selective B-cell expression of the murine major histocompatibility complex class II gene, E_α^d, we have demonstrated that a sequence located within an upstream region of E_α^d required for B-cell expression is capable of forming a stable complex with the B-cell-specific expression factor NF-κB. It is reasonable to suggest, therefore, that B-cell-specific expression of E_α^d may be dependent on the action of NF-κB or an NF-κB-like factor. As has been suggested (12), analysis of cellular genes has revealed that κB-like sites are associated with cell surface molecules (Fig. 1B). It is possible that the NF-κB system has evolved to control the synthesis of surface glycoproteins important to cellular activation and proliferation. Of course, B-cell-specific expression may be dependent on the presence of multiple elements. Experiments are in progress to determine the functional relevance of the E_α^d:NF-κB element to E_α^d expression in a transgenic mouse model system. The possible involvement of additional elements in B-cell-specific expression is also being tested.

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LITERATURE CITED

1. **Baldwin, A. S., Jr., and P. A. Sharp.** 1987. Binding of a nuclear factor to a regulatory sequence in the promoter of the mouse *H-2K^b* class I major histocompatibility gene. Mol. Cell. Biol. **7**:305–313.
2. **Baldwin, A. S., Jr., and P. A. Sharp.** 1988. Two transcription factors, NF-κB and H2TF1, interact with a single regulatory sequence in the class I major histocompatibility complex promoter. Proc. Natl. Acad. Sci. USA **85**:723–727.
3. **Boshart, M., F. Weber, G. Jahn, K. Dorsch-Häsler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of the human cytomegalovirus. Cell **41**:521–530.
4. **Carthew, R., L. Chodosh, and P. A. Sharp.** 1985. An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. Cell **43**:439–448.
5. **Chiu, R., M. Imagawa, R. J. Imbra, J. R. Bockoven, and M. Karin.** 1987. Multiple cis- and trans-acting elements mediate the transcriptional response to phorbol esters. Nature (London) **329**:648–651.
6. **Dignam, J. D., R. M. Lebowitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
7. **Emorine, L., M. Kuehl, L. Weir, P. Leder, and E. E. Max.** 1983. A conserved sequence in the immunoglobulin J_κ-C_κ intron: possible enhancer element. Nature (London) **304**:447–449.
8. **Flavell, R. A., H. Allen, L. C. Burkly, D. H. Sherman, G. L. Waneck, and G. Widera.** 1986. Molecular biology of the H-2 histocompatibility complex. Science **233**:437–443.
9. **Fried, M., and D. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6505–6525.
10. **Garner, M., and A. Revzin.** 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. Nucleic Acids Res. **9**:3047–3060.
11. **Israël, A., A. Kimura, M. Kieran, O. Yano, J. Kanellopoulos, O. LeBail, and P. Kourilsky.** 1987. A common positive trans-acting factor binds to enhancer sequences in the promoters of mouse H-2 and β_2-microglobulin genes. Proc. Natl. Acad. Sci. USA **84**:2653–2657.
12. **Leung, K., and G. J. Nabel.** 1988. HTLV-1 transactivator induces interleukin-2 receptor expression through an NF-κB-like factor. Nature (London) **333**:776–778.
13. **Max, E. E., J. V. Maizel, Jr., and P. Leder.** 1981. The nucleotide sequence of a 5.5-kilobase DNA segment containing the mouse κ immunoglobulin J and C region genes. J. Biol. Chem. **256**: 5116–5120.
14. **Mitchell, P. J., C. Wang, and R. Tjian.** 1987. Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. Cell **50**:847–861.
15. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326**:711–713.
16. **Pierce, J. W., M. Lenardo, and D. Baltimore.** 1988. Oligonucleotide that binds nuclear factor NF-κB acts as a lymphoid-specific and inducible enhancer element. Proc. Natl. Acad. Sci. USA **85**:1482–1486.
17. **Sen, R., and D. Baltimore.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46**: 705–716.
18. **Sen, R., and D. Baltimore.** 1986. Inducibility of a κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell **47**:921–928.
19. **Siebenlist, U., R. Simpson, and W. Gilbert.** 1980. E. coli RNA polymerase interacts homologously with two different promoters. Cell **20**:269–281.
20. **Strauss, F., and A. Varshavsky.** 1984. A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome. Cell **37**:889–901.
21. **Widera, G., L. C. Burkly, C. A. Pinkert, E. C. Böttger, C. Cowing, R. Palmiter, R. L. Brinster, and R. A. Flavell.** 1987. Transgenic mice selectively lacking MHC class II (I-E) antigen expression on B cells: an in vivo approach to investigate Ia gene function. Cell **51**:175–187.