Purified Bovine NF-κB Recognizes Regulatory Sequences in Multiple Genes Expressed During Activation of T- and B-Lymphocytes

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A. Introduction

A crucial event in the differentiation of B-lymphocytes is the transcription of the immunoglobulin light-chain gene which leads to expression of immunoglobulin antigen receptor on the surface of the cell. In an apparently separately regulated arm of the immune response, antigenic stimulation causes proliferation of T-lymphocytes by transcriptional activation of the IL-2 gene and the IL-2 receptor gene. Previous studies have shown that a lymphoid-specific enhancer element plays an important role in achieving high-level transcription of the kappa light-chain gene [1–4]. Molecular genetic dissection of this enhancer has revealed that a DNA sequence which binds to a nuclear factor, NF-κB, is essential for its function [5–8]. NF-κB binding activity is constitutively present only in mature B-lymphocytes and exceptional T-lymphocyte lines [5, 13]. Its binding may be induced in cells early in the B-lymphoid lineage, T-lymphocytes, and in nonlymphoid cells by various treatments such as bacterial lipopolysaccharide, cycloheximide, lectins, and phorbol esters [9]. Previously we have shown that NF-κB binding is critical for the kappa enhancer activity that is constitutively present in mature B-lymphocytes and inducible by lipopolysaccharide or phorbol esters in pre-B cells [7]. Recently, NF-κB has been found to act through an enhancer element in the LTR of human immunodeficiency virus I [10–12]. Evidence has also accumulated that NF-κB is important for the expression of the IL-2 receptor α chain gene (Tac antigen) [16, 19]. We have purified a protein which corresponds to this binding activity and describe its molecular characteristics and binding specificities. Our results suggest that a single protein is sufficient to recognize regulatory sequences in multiple genes that are expressed during the activation of B- and T-lymphocytes.

B. Purification of NF-κB from Bovine Spleen Tissue

To identify a source from which a large quantity of binding activity could be purified, numerous cultured cell lines and various tissues of human, murine, or bovine origin were tested. High levels of NF-κB binding activity were found in spleen tissue of human, murine, or bovine origin which corresponds to its constitutive expression in mature B-lymphocytes. The bovine extracts produced specific complexes with NF-κB binding sites from the kappa enhancer that had a methylation interference pattern identical to that described for the murine binding activity [5, 13]. Because of its ready availability, NF-κB was purified from bovine spleen.

We designed a series of chromatographic steps including S300-Sephacryl, phosphocellulose, hydroxylapatite, *Escherichia coli* DNA Sepharose, and DNA site-specific chromatography to purify the activity over 50000-fold with an approximately 8% recovery. This procedure yielded a 42000-dalton protein spe-

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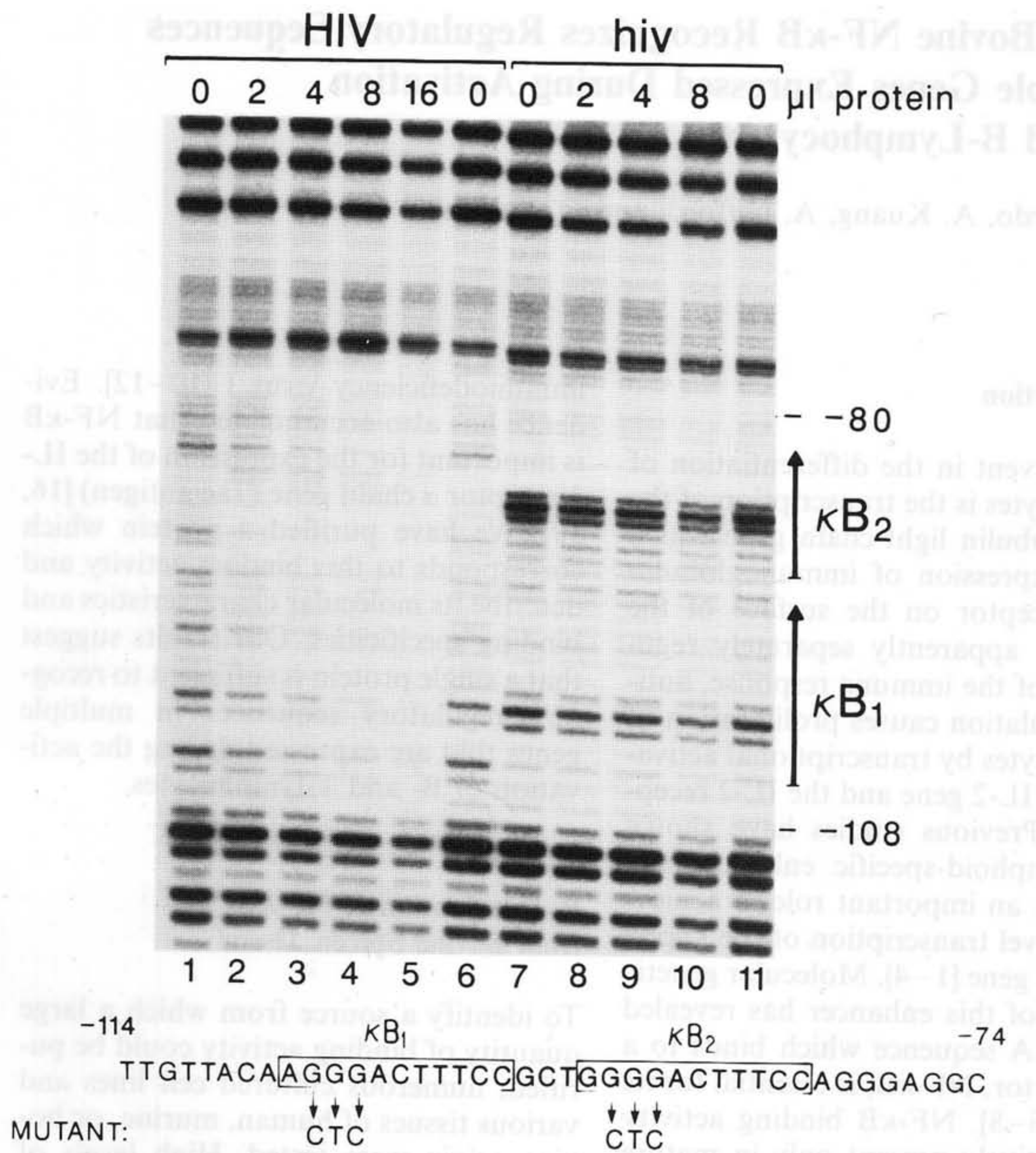

Fig. 1. Binding of purified bovine NF-κB to the HIV-1 enhancer. *Top:* Shown is the DNAse-I cleavage pattern of either the wild type (HIV) or the mutated (hiv) enhancer region following binding with the indicated amounts of affinity-purified bovine protein. The positions of the NF-κB binding motifs and the extent of the footprint (−80 to −108) are indicated. *Bottom:* The DNA sequence of the region containing the enhancer is given. The NF-κB binding motifs are boxed. Base substitutions which abrogate binding in the mutant version are indicated by arrows [10]

cies on an SDS polyacrylamide gel from which binding activity could be recovered by a denaturation-renaturation protocol [13]. Glycerol gradient sedimentation indicated that the protein may exist in solution as a dimer.

I. The Same Polypeptide Recognizes the Kappa Light Chain Gene and Human Immunodeficiency Virus Enhancers

The bovine protein was purified from spleen tissue and is likely to be derived predominantly from B-lymphocytes. In addition, the purified protein was affinity selected using a column containing kappa enhancer sequences and identified by its interaction with the kappa enhancer as NF-κB. Activation of T-lymphocytes greatly increases the levels of a factor that has similar DNA binding specificity to NF-κB [10]. This binding activity has been implicated in HIV-1 transcription through an enhancer comprised of two sequences related to the kappa enhancer binding motif [10–12]

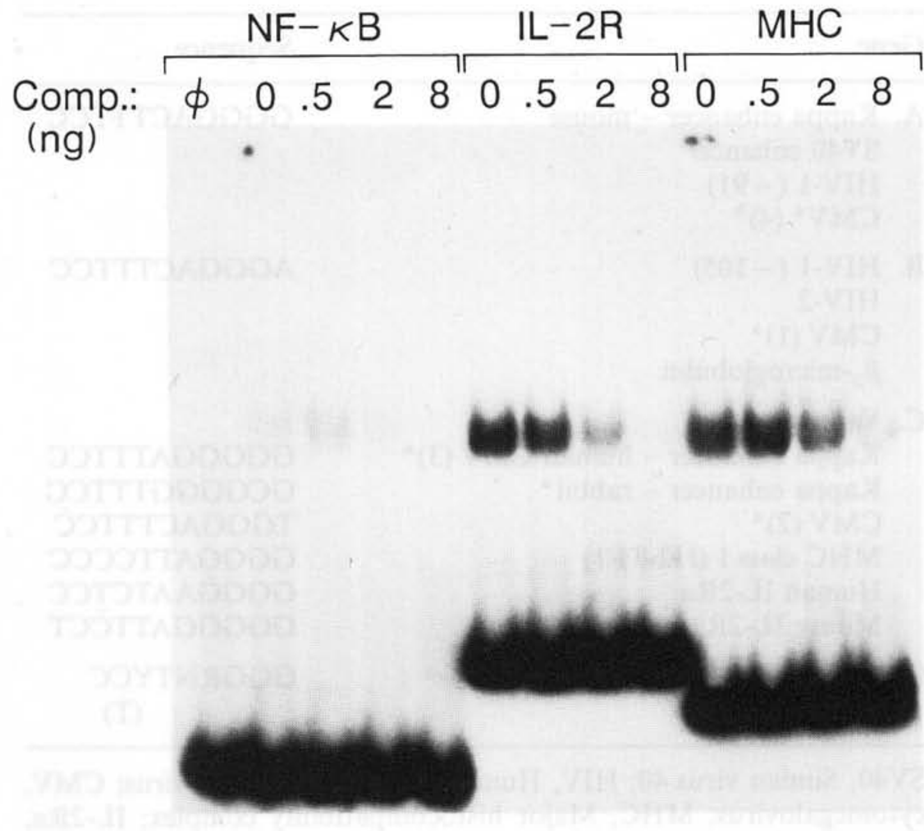

Fig. 2. Competition binding analysis of three NF-κB recognition sites. Shown is a mobility shift electrophoresis assay in which purified bovine protein has been added to all lanes except the first. As indicated at the top, radioactively labeled probes were: lanes 1-5 – kappa enhancer binding fragment; lanes 6-9 – IL-2 receptor α promoter fragment (–298 to –228); and lanes 10-13 – MHC class-I promoter oligonucleotide [15]. Oligonucleotides containing the NF-κB binding motif from the kappa enhancer were added to the binding assays as unlabeled competitor in the nanogram amounts shown. The probe for lanes 1–5 had a specific activity approximately four times lower than the others used

(see Fig. 2). We therefore asked if the same purified polypeptide could interact with the HIV sequence in the absence of any other factors present in T cells. By DNase-I footprint analysis the entire long-terminal repeat enhancer sequence is protected in a pattern virtually identical to that observed with T cell extracts (Fig. 1) [11, 12]. These results support the hypothesis that the same protein may regulate both the HIV and kappa enhancers in T- and B-lymphocytes respectively.

II. The Purified Protein Recognizes a Regulatory Sequence in the IL-2 Receptor α Chain gene

Recent evidence also implicates NF-κB in the expression of the IL-2 receptor α chain (IL-2Rα) in activated T-lymphocytes ([16–19] S. Cross, M. Lenardo, D. Baltimore, and W. Leonard, unpublished observations). Since NF-κB is typically found in activated but not resting T cells, it might have an important role in the program of genes expressed during antigenic stimulation. A sequence closely resembling the NF-κB cognate motif was found in a functionally important region of the IL-2Rα promoter [16]. We found that this sequence was able to bind the purified protein, and that the kappa enhancer site cross-competed equivalently with either itself, the MHC class-I promoter binding site, or the IL-2Rα binding site (Fig. 2).

III. Variation in the DNA Sequences Required for Binding NF-κB

It is now clear that a variety of gene regulatory regions appear to interact pro-

Gene	Sequence
A. Kappa enhancer – mouse	GGGGACTTTCC
SV40 enhancer	
HIV-1 (−91)	
CMV[a] (4)[b]	
B. HIV-1 (−105)	AGGGACTTTCC
HIV-2	
CMV (1)[a]	
β_2-microglobulin	
C. Variants:	
Kappa enhancer – human CMV (3)[a]	GGGGGATTTCC
Kappa enhancer – rabbit[a]	GCGGGGTTTCC
CMV (2)[a]	TGGGACTTTCC
MHC class I (H2-TF1)	GGGGATTCCCC
Human IL-2Rα	GGGGAATCTCC
Mouse IL-2Rα	GGGGGATTCCT
Consensus:	GGGRNTYCC (T)

Table 1. Consensus sequence for NF-κB recognition

SV40, Simian virus 40; HIV, Human immunodeficiency virus; CMV, cytomegalovirus; MHC, Major histocompatibility complex; IL-2Rα, Interleukin-2 receptor alpha chain.

[a] This sequence has not been tested in a binding assay.

[b] Since there are four putative NF-κB recognition sites in the cytomegalovirus enhancer, these have been numbered 1–4 as they are found from 5′ to 3′ on the coding strand.

ductively with NF-κB. Alignment of the interaction sites reveals significant microheterogeneity in their DNA sequence (see Table 1). This establishes a surprisingly flexible consensus interaction site, derivatives of which can be expected to bind tightly to NF-κB. The essential features of this consensus site are:

1. The motif is composed to two four-nucleotide half-sites separated by a position that can be occupied by any nucleotide. The 5′ half-site is composed of purines and the 3′ half-site is composed of pyrimidines.
2. The first three nucleotides are G residues and the final two nucleotides are C residues. These G:C base pairs at the beginning and end of the motif make important contacts with the protein which can be interrupted by N-7 methylation in all cases examined.
3. The pyrimidine half-site always takes the form of one or two T residues preceding one to three C residues.

These rules can only provide a basis for recognizing putative interaction sites and do not offer insights into how the DNA-protein interactions come about. From this consensus sequence we have predicted putative NF-κB binding sites in the IL-2 gene promoter in both human and murine genes. Preliminary results indicate that these sequences efficiently bind the pruified protein, suggesting a pivotal role for NF-κB in the expansion of T cells during the immune response [13].

C. Summary

To characterize the NF-κB binding factor in molecular terms and to facilitate the cloning of its gene, we have purified this protein from bovine spleen tissue. We have found it is a 42000-dalton protein that exists in solution as a dimer. We were able to use the purified protein to show that the same polypeptide is able to recognize sites important for activa-

tion of genes in either B- or T-lymphocytes. Moreover, we were able to define a consensus sequence which allows ascertainment of a wider variety of sequences that are capable of interacting with this protein. The implication of the same protein in gene regulation in two different lineages of lymphoid cells reveals an unexpected unity in the mechanism of gene expression during B- and T-lymphocyte activation. This also suggests that other regulatory events must participate with NF-κB activation in determining B- or T-cell-specific expression.

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