NF-κB and Rel: Participants in a Multiform Transcriptional Regulatory System

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I. Introduction

Molecular regulatory pathways were originally identified as series of covalent enzymatic modifications of substrate molecules during intermediary metabolism. Recent gene regulation research has revealed subtle and complex molecular regulation by information pathways involving noncovalent interactions between macromolecules. High affinity interactions between proteins with DNA sequences play a key role in governing gene transcription. Regulatory interactions also occur between transcription factors and RNA polymerases. In an increasing number of examples, protein–protein associations between various types of transcriptional regulatory factors determine the specificity of gene activation. These noncovalent information pathways are also intimately interlaced with enzymatic control points such as phosphorylation or other forms of post-translational modification. Together these pathways form a network that shuttles information throughout the cell allowing physiological needs to be met by selective gene expression.

Modulation of gene expression by the NF-κB/Rel family of protein complexes illustrates many of the newly emerging themes of intracellular regulation. Associations of the binding and regulatory subunits of NF-κB operate at the center of a variety of different signal pathways. NF-κB can process and integrate instructions that come from the extracellular milieu as well as detect intracellular events originating in either the cytoplasm or the nucleus. NF-κB then transmits this information with astonishing rapidity to the transcriptional machinery by directly binding to a range of different DNA sequences in gene control regions. NF-κB comprises members of a family of dimer-forming proteins with homology to the *rel*

oncogene. Its binding, location in the cell, and transcriptional properties are regulated by a second family of proteins that contain repeats of a domain characteristically found in the erythrocyte protein ankyrin. The DNA sequences which bind NF-κB are also recognized by a third family of structurally distinct proteins bearing the "zinc-finger" DNA interaction domain. This highly evolved system controls a wide diversity of genes and thus provides an interesting paradigm for several areas of molecular and cellular research.

II. NF-κB Function in B Cells

In 1986, Sen and Baltimore first identified NF-κB as an apparently tissue-restricted factor that activated the Ig κ light chain intron enhancer during B-lymphocyte development. They used an electrophoretic mobility shift assay to detect a protein complex with a DNA sequence which was then called the B site (Sen and Baltimore, 1986a) (see Fig. 1). The B site was a 10-nucleotide DNA sequence in a region of the large intron of the κ light chain gene that had been defined by various functional assays as a B-cell-specific enhancer element (Queen and Baltimore, 1983; Bergman *et al.*, 1984). A hint to the importance of this sequence was that it was also present in the enhancer of the simian virus-40 (SV-40) DNA tumor virus (Emorine *et al.*, 1983; Sen and Baltimore, 1986a).

NF-κB had the striking appearance of being constitutively present only in B cells of the "appropriate" stage for Ig κ light chain gene expression (mature B cells and plasma cells, but not pre-B cells)(Sen and Baltimore, 1986a). Even more remarkable was that NF-κB was inducible in pre-B cells. For example, in the pre-B tumor line 70Z/3, NF-κB could be induced by agents such as lipopolysaccharide (LPS), active phorbol esters, cycloheximide, IL-1, tumor necrosis factor-α (TNF-α), and virus infection (Sen and Baltimore, 1986b; Atchison and Perry, 1987; Shirakawa *et al.*, 1989; Lenardo *et al.*, 1989). Because these same agents stimulated transcription of the κ locus, inducible NF-κB in pre-B cells strengthened the correlation with κ light chain gene expression (Nelson *et al.*, 1985). Chromatin changes and occupancy of the NF-κB site were directly demonstrated in intact nuclei from LPS-treated 70Z/3 cells (Gimble and Max, 1987; Hromas *et al.*, 1988). More recent studies have also found poor activation of NF-κB in genetic variants of the 70Z/3 cell line that display defective κ gene expression in response to LPS (Briskin *et al.*, 1990; Rooney *et al.*, 1990a,b).

The observation that cycloheximide induced NF-κB was especially prescient (Sen and Baltimore, 1986b). NF-κB induction apparently did not

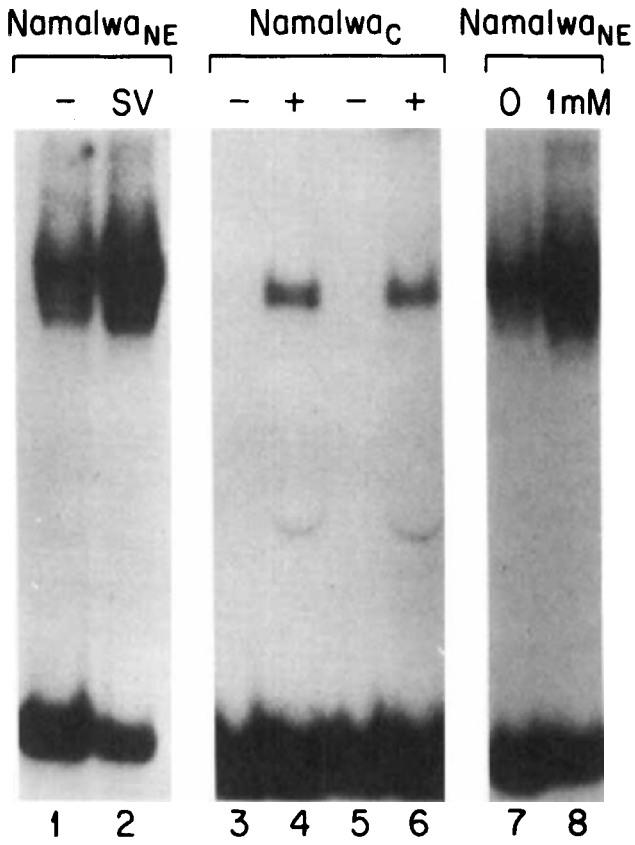


FIG. 1 Mobility shift electrophoresis analysis of extracts from Namalwa human mature B lymphocytes using the κB binding site from the Ig κ gene. Lanes 1 and 7 are nuclear extracts from unstimulated cells and lane 8 is the same with 1 m*M* GTP added to the binding reaction; lane 2 is nuclear extract from Sendai virus-induced cells. Lanes 3 to 6 are cytosolic extracts in which sodium deoxycholate treatment (+, lanes 4 and 6) has been used to free NF-κB from I-κB. These results and the methods for these experiments have been previously described in Lenardo *et al.* (1989).

require new protein synthesis; in fact, cycloheximide and other stimulants caused a superinduction of NF-κB (Sen and Baltimore, 1986b). This correlated with the lack of a requirement for new protein synthesis for κ gene expression (Wall *et al.*, 1986) and led to the proposal that a preexisting form of NF-κB, which was controlled by a labile inhibitory protein, might reside in the cell prior to induction (Baltimore, 1987).

The functional role of NF-κB was demonstrated by mutational analysis of the κ intronic enhancer (Lenardo *et al.*, 1987) (see Fig. 2). Alterations

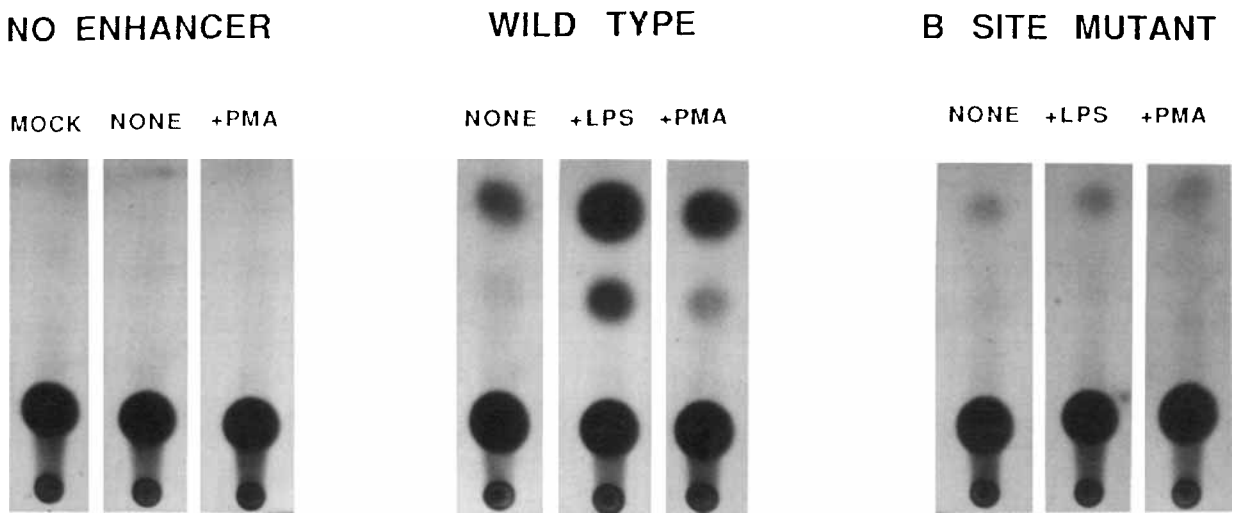


FIG. 2 Analysis of transcriptional activity conferred by the κB binding site in the Ig κ gene enhancer in mouse PD-31 pre-B lymphocytes. The left panel shows the bacterial chloramphenicol acetyltransferase activity for the truncated c-*fos* reporter plasmid with no enhancer. The middle panel shows the bacterial chloramphenicol acetyltransferase activity conferred by the 470-bp version of the Ig κ enhancer in unstimulated cells (none), or in cells stimulated with LPS or phorbol ester (PMA). The right panel shows the Ig κ enhancer with multiple point mutations in the κB binding site that eliminate binding of NF-κB in unstimulated cells (none), or in cells stimulated with LPS or PMA. These results and the methods for these experiments have been previously described in Lenardo *et al.* (1987).

of the B site that interfered with NF-κB binding destroyed both basal enhancer function in mature B cells and inducibility in pre-B cells. The mutated enhancer was completely inactive. Further experiments revealed that the NF-κB binding site on its own could act independently as a strong tissue-specific and inducible enhancer element (Pierce *et al.*, 1988; Wirth and Baltimore, 1988). In fact, two copies of an oligonucleotide containing only the NF-κB site stimulated transcription as well as the entire κ enhancer in transient transfection assays.

The initial conceptualization, based on the correlation to κ light chain gene expression, was that NF-κB was the key regulatory molecule for light chain gene activation. It was even imaginable that NF-κB orchestrated the entire transition from pre-B to mature B phenotype by turning on a set of differentiation genes. Four pieces of evidence have since suggested that the true picture is more complicated and more interesting. First, γ-interferon can activate κ gene expression and not alter the level of NF-κB binding activity in pre-B cells (Briskin *et al.*, 1988; Bomsztyk *et al.*, 1990). Second, the endogenous κ light chain gene has been found to be expressed at high levels in myeloma cell lines that contain little NF-κB and show no κ intronic enhancer activity (see below) (Atchison and Perry, 1987). Third, NF-κB does not seem to regulate the recently delineated enhancer elements in the Ig λ light chain loci (Hagman *et al.*, 1990). Fourth, as described below, elements other than the intron enhancer contribute to κ gene expression and do not involve NF-κB. Taken together, these findings would best portray NF-κB as one part of a transcriptional differentiation program that has multiple, perhaps sequential, but functionally similar elements. Further work will be needed to describe the interplay between the elements of this program and how they coordinate the maturation of pre-B to B cells.

One interesting paradox in Ig gene regulation that illustrates the complexity of eukaryotic gene analysis has been recently resolved. Early studies of the Ig heavy and light chain genes suggested that the intronic enhancer elements were dispensable for high level gene expression (Schaffner, 1988). As mentioned above, a myeloma line with little NF-κB was found to have robust Ig κ gene expression (Atchison and Perry, 1987). This led to the hypothesis that once the Ig genes were triggered, the enhancers and their associated binding proteins might not be needed for the maintenence of transcription (Klein *et al.*, 1985; Atchison and Perry, 1987). Preservation of transcription might be attained by the methylation status of the gene or particular chromatin configurations (Kelley *et al.*, 1988; Atchison and Perry, 1988). Evidence against this idea was that NF-κB was only rarely absent in mature cells of the B lineage (Sen and Baltimore, 1986a). Moreover, a continuous requirement for the enhancer element was found for the Ig heavy chain gene (Grosschedl and Marx,

1988). A coherent picture of Ig gene control subsequently emerged with the discovery of additional enhancer elements for both the Ig heavy chain and κ genes at significant distances downstream of the constant region (Meyer and Neuberger, 1989; Pettersson *et al.*, 1990). In the case of the κ gene, the downstream enhancer was stronger than the intronic enhancer and essential to achieve high enough expression of κ genes in transgenic mice to cause allelic exclusion (Meyer and Neuberger, 1989; Meyer *et al.*,1990). Significantly, the downstream κ enhancer is not activated by NF-κB, indicating that multiple pathways lead to high level κ gene expression (Meyer *et al.*, 1990; M. Atchison, personal communication).

Although constitutively present in the nucleus, NF-κB can be further modulated in B cells. For instance, higher levels of nuclear NF-κB can be induced in human Namalwa cells by virus infection (Lenardo *et al.*, 1989). At least part of the NF-κB that is induced in certain B cells seems to come from the "masked" cytoplasmic form (see below). It also becomes evident in whole cell extracts from Daudi cells with a mild denaturant (Visvanathan and Goodbourn, 1989), or in mouse S194 myeloma cells by HTLV-1 *tax* gene product (Mauxion *et al.*, 1991). However, preexisting but nonbinding NF-κB does not appear to be present in all mature B cells (Baeuerle *et al.*, 1988). Recently, Marcuzzi *et al.* (1989) and Liu *et al.* (1991) reported that in murine splenic B cells, NF-κB, which is already present to some degree constitutively, can be induced further after Ig crosslinking.

NF-κB has been implicated in the transcriptional activation of a variety of genes other than the κ gene in B cells. These include the MHC class I and β2 microglobulin genes (Yano *et al.*, 1987; Baldwin and Sharp, 1988), as well as the genes for MHC class II and its associated invariant chain Ii (Blanar *et al.*, 1989; Pessara and Koch, 1990; Zhu and Jones, 1990), and other early activation genes (Anderson *et al.*,1992). A motif similar to the consensus binding sequence of NF-κB has been found in the promoter of the gene for the murine B-cell tyrosine kinase *hck* (Lock *et al.*, 1990).

As in other cell lineages, NF-κB has to be viewed in B cells as an eclectic mediator of gene transcription that facilitates rapid genetic interpretation of extracellular signals. Two important regulatory issues are highlighted by the study of NF-κB in the B lineage. First, how does NF-κB turn on specific genes in the B lineage despite the fact that it induces transcription of many genes in many lineages? Second, how is NF-κB binding activity maintained constitutively in mature cells of the B lineage? One explanation for the B-cell-restricted response of certain genes to NF-κB has come from a study of the Ig κ intronic enhancer. Pierce *et al.* have shown that NF-κB's effect on the κ enhancer is under the influence of a negative element (1991). They identified a 232-base pair (bp) fragment located 5′ of the NF-κB binding site that makes the enhancer nonresponsive to NF-κB in non-B cells. Strikingly, this silencer element works in a distance- and orientation-independent fashion.

The issue of how B cells maintain a certain fraction of NF-κB in a nuclear binding form has not yet been adequately explained. There is some evidence that NF-κB is constitutively present in some T-cell lines and thymocytes (Baeuerle *et al.*, 1988; Bohnlein *et al.*, 1988; Leung and Nabel, 1988; Cross *et al.*, 1989; Lin *et al.*, 1990; Korner *et al.*, 1991) and possibly in mature macrophages (Griffin *et al.*, 1989). The mechanism for constitutive activity in any cell lineage has not been determined. Evidence from studies with the cloned genes indicates that the mRNAs for the NF-κB binding subunits p50 and p65 are not elevated as cells mature in the B lineage (Ghosh *et al.*, 1990; Nolan *et al.*, 1991). The simplest model would entail modulation of the level of the cytoplasmic inhibitor, I-κB, but the distribution and functional status of this molecule and its relatives have not yet been evaluated in different lineages.

III. NF-κB as an Inducible Transcriptional Activator

The property that gives NF-κB a widespread significance in cellular regulation is its role as a mediator of inducible gene transcription. The key features of NF-κB transcriptional control are that it is fast and versatile and is used in many different gene systems. It also has the important ability to carry signals from the cytoplasm into the nucleus and trans-activate specific genes by binding directly to their promoters.

The versatility of the NF-κB system is evident in the large number of agents able to up-regulate the levels of NF-κB binding in the nucleus. Unlike transcription factors which respond to a limited number of inducers such as the heat shock factor or specific steroid receptors, signals from a number of directions converge on NF-κB to be conveyed into the nucleus. Importantly, signals that pass through NF-κB appear to be launched from different parts of the cell (Lenardo and Baltimore, 1989; Baeuerle, 1991). They may emanate from cell surface molecules such as the receptors for cytokines (IL-1, TNF-α, and TNF-β), lectins, or LPS. They may come from elements of second messenger pathways that are associated with events at the cell membrane such as phorbol esters or calcium ionophores. They may arise in the cytoplasm through kinase induction by dsRNA or by inhibition of protein synthesis. They may also involve triggering by intranuclear proteins such as the Tax trans-activator of the HTLV-1 virus that induces NF-κB. Finally, and perhaps most surprisingly, induction may even be signaled at the DNA level such as by UV light and other agents that cause DNA damage. A list of the known inducing agents for NF-κB is given in Table I.

The kinetics of the NF-κB response to inducing agents indicate that it can be extremely rapid and quite sensitive. For example, early studies

TABLE I
Inducers of NF-κB

Inducer	Cell type	Species	Reference
Lipopolysaccharide	Pre-B (70Z/3; 3-1)	m	Sen and Baltimore, 1986b
	Splenic B	m	Atchison and Perry, 1987
	Fibrosarcoma (WEHI 164)	m h	Liu *et al.*, 1991 Gromkowski *et al.*, 1990
	Monocyte/macrophage (mono-mac-6)		Haas *et al.*, 1990
Protein synthesis inhibitors	Pre-B (70Z/3)	m	Sen and Baltimore, 1986b
	Splenic B	m	Liu *et al.*, 1991
Phorbol ester	Pre-B (70Z/3)	m	Sen and Baltimore, 1986b
	T (Jurkat)	h	Sen and Baltimore, 1986b
	HeLa	h	Sen and Baltimore, 1986b
	Splenic B	m	Liu *et al.*, 1991
	Monocyte/macrophage	h/m	Griffin *et al.*, 1989
Anti-IgM	Splenic B	m m	Marcuzzi *et al.*, 1989 Liu *et al.*, 1991
HTLV-1 *tax*	Plasmacytoma	m	Mauxion *et al.*, 1991
	T (Jurkat)	h	Leung and Nabel, 1988; and others (see text)
dsRNA	Osteosarcoma (MG63) HeLa	h	Visvanathan and Goodbourn, 1989
	Fibrosarcoma (WEHI 164)	h	Visvanathan and Goodbourn, 1989
	Fibroblast L929	m m	Gromkowski *et al.*, 1990 Lenardo *et al.*, 1989
TNF-α	Pro-monocytic (U937)	h	Griffin *et al.*, 1989
	Rat-2 fibroblast	r	Pessara and Koch, 1990
	Myelomonocytic (WEHI 3B)	m	Pessara and Koch, 1990 Osborn *et al.*, 1989
	T (Jurkat)	h	Gromkowski *et al.*, 1990
	Fibrosarcoma (WEHI 164)	m	Banerjee *et al.*, 1989
	Hepatoma (HepG2)	h	
	Erythroleukemia (K562)	h	Meichle *et al.*, 1990
	Macrophages	m	Collart *et al.*, 1990
Lymphotoxin	Myelomonocytic	h	Hohmann *et al.*, 1990a, 1991b
Interleukin-1	T (YT)	h	Freimuth *et al.*, 1989
	T (Jurkat)	h	Osborn *et al.*, 1989
	T (EL-4-E1)	m	Novak *et al.*, 1990
	Foreskin fibroblast	h	Zhang *et al.*, 1990
Interleukin-1	Hepatoma (HepG2)	h	Nonaka and Huang, 1990
Interleukin-2	T (SPB21)	h	Hazan *et al.*, 1990

(*continues*)

TABLE I (*continued*)

Inducer	Cell type	Species	Reference
Phytohemagglutinin	T (Jurkat)	h	Sen and Baltimore, 1986b
	Pro-monocytic (U937)	h	Libermann and Baltimore, 1990
Concanavalin A	T (Jurkat)	h	Rattner *et al.*, 1991
Calcium ionophore	T (EL-4-E1)	m	Novak *et al.*, 1990
Anti-CD2	Monocytes	h	Bressler *et al.*, 1991
Anti-CD3	T (AR-5)	m	Jamieson *et al.*, 1991
	T (SPB21)	h	Hazan *et al.*, 1990
Anti CD28	T (Jurkat)	h	Verweij *et al.*, 1991
Anti-αβ TCR	T (SPB21)	h	Hazan *et al.*, 1990
Antigen	T (SPB21)	h	Hazan *et al.*, 1990
	T (A-E7)	m	Kang *et al.*, 1992a
c-AMP	Pre-B (70Z/3)	m	Shirakawa *et al.*, 1989
UV light	HeLa	h	Stein *et al.*, 1989a; b
4-Nitroquinoline oxide	HeLa	h	Stein *et al.*, 1989a
H_2O_2	T (Jurkat)	h	Schreck *et al.*, 1991
Theilera parva (parasite)	T cells	b	Ivanov *et al.*, 1989
Serum	BALB/c 3T3	m	Baldwin *et al.*, 1991
Okadaic acid	T (Jurkat)	h	Thevenin *et al.*, 1990
Viruses			
CMV	T (Jurkat) or HF (foreskin)	h	Sambucetti *et al.*, 1989
Adenovirus	Fibroblast (3T3)	m	Shurman *et al.*, 1989
	Cervical carcinoma (HeLa)	h	Nielsch *et al.*, 1991
Hepatitis B	Hepatocyte (HepG2)	h	Twu *et al.*, 1989a; Faktor and Shawl, 1990
Sendai	Fibroblast (L929)	m	Lenardo *et al.*, 1989
Herpes simplex, type I	T (Jurkat)	h	Gimble *et al.*, 1988
Human herpes virus, type VI	T (Molt3)	h	Ensoli *et al.*, 1989

indicated that phorbol esters gave maximal induction in 30 min and later studies showed significant induction occurred at much earlier times (Sen and Baltimore, 1986b; vide infra). Also, the kinetics were not identical for different inducers: phorbol stimulation of 70Z/3 cells remained maximal from 0.5 to 6 hr and was gone at 8 hr, whereas LPS induction became maximal at 2 hr and was still apparent at 8 hr (Sen and Baltimore, 1986b).

Careful studies of induction by TNF-α showed that NF-κB was detectable within 2 min and maximal after 10–15 min (Hohmann *et al.*, 1991b). NF-κB can also be detected in nuclear extracts from 70Z/3 cells within 15 min after either LPS or IL-1 treatment (Shirakawa *et al.*, 1989). The sensitivity of NF-κB induction was also demonstrated by the finding that occupancy of only 5–20% of the TNF-α surface receptors was required in HL-60 and HepG2 cells (Hohmann *et al.*, 1991b). The rapidity of the response seems due to the fact that the immediate induction by many stimuli is independent of new protein synthesis (Sen and Baltimore, 1986b; Osborn *et al.*, 1989; Banerjee *et al.*, 1989; Zhang *et al.*, 1990; Liu *et al.*, 1991) and appears to be directly connected to kinase pathways in the cell (see below).

There is also some evidence that NF-κB may respond differently to a given inducing agent depending on the cell type. Phorbol esters alone will strongly induce NF-κB in 70Z/3 pre-B lymphoma cells, but do not induce NF-κB at all in nontransformed CD4$^+$ T-lymphocyte clones (Kang *et al.*, 1992a). Another example is that IL-1 or active phorbol esters will induce NF-κB in 70Z/3 cells or LBRM T cells but neither will induce NF-κB in TH-2 T-lymphoma cells (Osborn *et al.*, 1989). Although many of the pathways that induce NF-κB have been only incompletely mapped, it is clear that NF-κB lies at the intersection of a large number of control routes traversing the cell. NF-κB may therefore serve as an integration node. Consistent with this idea, there are instances where inducers synergize to activate NF-κB. Mimicking T-cell receptor stimulation by phytohemagglutinin (PHA) or phorbol ester (PMA) alone can modestly induce NF-κB in Jurkat T-lymphoma cells, but more significant increases are seen if both are used together (Hoyos *et al.*, 1989). Similarly, PMA + TNF-α induce NF-κB more strongly than either alone, though it is not clear whether the effect is additive or synergistic (Osborn *et al.*, 1989). Though these examples are artificially contrived costimulatory situations, they indicate that multiple signals can be integrated and then transduced by increased levels of NF-κB in the nucleus. The role of protein kinase C and other serine/threonine kinases during the induction of NF-κB will be discussed below.

One area of research for the future will be to more fully explore effects on NF-κB induction pathways by signal transduction pathways such as those mediated by tyrosine kinases, phosphotidyl inositol breakdown products, or prostaglandin and related molecules. One interesting recent development is evidence that protein modifications such as methylation and isoprenylation may be important events in the induction of NF-κB by LPS but not PMA (Law *et al.*, 1991). Proteins with these adducts are typically associated with the membrane during LPS induction and may be among the earliest events in the signaling pathway. It will be interesting to see how these modified proteins connect either to kinases or possibly to the latent form of NF-κB in the cytoplasm.

Once the NF-κB signal has activated a gene, how is it stopped? Some attenuation may be due to the natural turnover of the proteins but the complex of NF-κB and its cognate DNA motif appears to be quite stable (Zabel and Baeuerle, 1990). Another possibility is that IκB or other proteins actively quench NF-κB binding. *In vitro* binding studies indicate that the half-life of an NF-κB complex with DNA can be dramatically reduced by the presence of I-κB, the specific inhibitor of NF-κB binding (see below) (Zabel and Baeuerle, 1990). For this mechanism to be effective, IκB would be required to attack NF-κB complexes in the chromatin and possibly sequester them in the nucleus, ferry them back to the cytoplasm, or facilitate their degradation (for a discussion see Baeuerle, 1991). Another possibility is that there are nuclear inhibitors that can maintain complexes in the nucleus in a nonbinding form. These possibilities have not yet been addressed experimentally.

It is important to note that a second tier of regulation seems to occur at the level of expression of the mRNAs encoding the subunits of NF-κB (Irving *et al.*, 1989; Bours *et al.*, 1990, 1992; Meyer *et al.*, 1991) as will be discussed further below. Perhaps all members of the gene family that includes NF-κB can be induced to some degree at the RNA level by many of the same agents that seem to activate NF-κB. This effect could play a role in sustaining a gene activation signal at times long after the initial release of NF-κB from cytoplasmic stores by a given stimulus. It is interesting to note that genes encoding proteins that inhibit NF-κB binding are also induced soon after certain cell stimuli (Haskill *et al.*, 1991). Complex gene regulatory interactions could therefore result from the long-lived production of certain NF-κB subunits and inhibitors and the exclusion of others.

IV. A Wide Variety of Genes Are Regulated by NF-κB

The target genes of NF-κB are numerous and most share the common feature of being quickly induced in response to an extracellular stimulus. NF-κB is able to signal the occurrence of many extracellular events and its effects are remarkably pleiotropic. Hence, a rise in nuclear NF-κB is not very informative for understanding a program of gene expression in response to a particular inducing agent. Perhaps the major role of NF-κB is simply to send a signal into the nucleus that an acute process that requires a genetic response is under way. A significant body of work has been carried out on genes induced by NF-κB and related binding proteins during T-lymphocyte activation or other aspects of the immune response. Several other genes have no obvious relationship to the immune response.

NF-κB induction of specific genes will be discussed in association with the physiologic role that they play.

A. T-Lymphocyte Activation

An important subset within the wide variety of genes controlled by NF-κB is the collection of genes that are induced during T-cell activation. Over 100 genes appear to be transcribed in T cells after stimulation by antigen in the context of an appropriate major histocompatability complex (MHC) molecule or agents that mimic this interaction (Zipfel *et al.*, 1989; Irving *et al.*, 1989; Crabtree, 1989). Therefore understanding gene control during T-cell activation should provide significant insights into the general molecular regulation of this process (Ullman *et al.*, 1990). NF-κB in cooperation with other transcriptional regulators (activators and repressors) appears to be involved in the expression of many genes especially during the early phases of the response to T-cell receptor stimulation (Jamieson *et al.*, 1991; Lenardo and Baltimore, 1989). Cytokine gene expression provides a good model for understanding NF-κB's role early in T-cell activation. mRNAs for cytokines, or more specifically interleukins, are usually undetectable in T cells unless the appropriate stimuli have been administered (DeMaeyer and DeMaeyer-Guignard, 1988). Though there has been some controversy regarding whether transcriptional or post-transcriptional mechanisms are the prevailing determinant in elevating mRNA levels, careful analysis of cytokine regulation in T cells indicates a major role for transcriptional control (Brorson *et al.*, 1991). The process initiates rapidly since increases in cytokine mRNAs are detected within 2 hr and in some cases peak at 4 hr. NF-κB is well suited for cytokine control because of the speed of its activation and its independence from new protein synthesis.

The best studied examples of T-cell cytokine gene regulation, IL-2 and the IL-2 receptor α chain genes, illustrate how NF-κB plays only one part in an ensemble of regulatory factors. A region of approximately 300 bp located immediately upstream of the transcription start site of the IL-2 gene, variously called the enhancer or the promoter, has been shown to strongly respond to T-cell activation signals (Brunvand *et al.*, 1988; Fujita *et al.*, 1986; Durand *et al.*, 1987; Novak *et al.*,1990). This DNA segment contains binding sites for NF-κB and other nuclear trans-activating proteins including the nuclear factor of activated T cells (NF-AT), octamer factors, AP-1, purine-rich binding factors, and, at least in human cells, a CD28-responsive complex (Lenardo *et al.*, 1988; Durand *et al.*, 1988; Hoyos *et al.*, 1989; Muegge *et al.*, 1989; Randak *et al.*, 1990; Fraser *et al.*, 1991; Granelli-Piperno and Nolan, 1991; Hentsch *et al.*, 1992).

Deletion studies suggest that multiple protein binding sites must be occupied in order to allow full IL-2 enhancer activity (Durand *et al.*, 1988, Serfling *et al.*, 1989; Hoyos *et al.*, 1989). Certain of these are factors are tissue-specific, such as NF-AT; however, others such as AP-1 and NF-κB do not have any obvious tissue restriction but are important in regulatory states such as clonal anergy (Kang *et al.*, 1992b). The molecular switch that activates the IL-2 gene appears to be the constellation of trans-activators rather than any one taking a leading role.

Studies in which point mutations were introduced into the promoter clearly indicated a potentially important role for κB binding motif (IL-2-κB), presumably due to trans-activation by NF-κB or other Rel family members (Ballard *et al.*, 1988; Shibuya *et al.*, 1989; Shibuya and Taniguchi, 1989). Studies have also provided evidence that in certain cell lines the IL-2-κB site may function in a tissue-specific manner (Radler-Pohl *et al.*, 1990; Briegel *et al.*, 1991). Recently, evidence from the study of nontransformed murine T-cell clones indicates that more than one distinct *rel* family member acts at the IL-2-κB sequence (Kang *et al.*, 1992a). A complex, termed NF-κC, that comprises the p50 subunit of NF-κB and is distinct from the NF-κB p50/p65 heterodimer is present in unstimulated T cells but disappears after activation. Activation signals that lead to loss of NF-κC induce IL-2 mRNA. By contrast, conditions that elevate nuclear NF-κB or NF-AT levels but do not lead to loss of NF-κC fail to induce IL-2 mRNA. NF-κC removal appears to be due to its sequestration in the nucleus by a novel inhibitory protein, which we informally refer to as I-κC, whose synthesis is tightly linked to antigen stimulation and sensitive to cyclosporin A. One molecule that may serve an I-κC-like function is the Bcl-3 protein that is located in the nucleus and inhibits the p50 homodimer complex (Wulczyn *et al.*, 1992; G. P. Nolan and D. Baltimore, unpublished results). This pathway illustrates a mechanism by which NF-κB-related complexes can be regulated exclusively within the nucleus during activation of T cells. It also maintains tight regulation of the IL-2 gene, since NF-κB can be activated in response to a variety of signals but the loss of the NF-κC protein is directly linked to antigen stimulation (Kang *et al.*, 1992a). Recent results in human cells suggest that the mechanism described by Kang *et al.* may govern the transcription of HIV-1 and that Bcl-3 serves as an inhibitor of the NF-κC or p50 homodimer complex (Franzoso *et al.*, 1992). In contrast to antigen, stimulation of nontransformed T cells with anti-CD3ε does not stimulate transcription through the IL-2-κB site (Jain *et al.*, 1992). Interestingly, the induction of NF-κB seems to be only weakly blocked by cyclosporin A at physiological doses, but there may be other mitogen-induced complexes binding to κB sites that are sensitive to this immunosuppressant (Randak *et al.*, 1990; Schmidt *et al.*, 1990; Banerjee *et al.*, 1991; Emmel *et al.*, 1989; Granelli-Piperno *et*

al., 1990; Brini *et al.*, 1990). In tumor cells, induction of NF-κB and the transcription activity of κB-binding elements stimulated by PMA appear to be more resistant to effects of cyclosporin A than inducing agents that cause increases in intracellular Ca^{2+} such as ionomycin and lectins (U. Siebenlist, personal communication).

The IL-2 receptor α chain (IL-2R) gene also displays an interesting regulatory pattern centered on an NF-κB binding motif in its promoter. Elevated cell surface expression of the IL-2 receptor α chain is a critical step in generating high affinity receptors for IL-2 during the response of T lymphocytes to antigen (Waldmann, 1986). Several groups have shown that the p65/p50 heterodimer of NF-κB or a related complex transduces T-cell activation signals into increased activity of the IL-2R promoter (Leung and Nabel, 1988; Bohnlein *et al.*, 1988, 1989b; Lowenthal *et al.*, 1989a,b; Cross *et al.*, 1989; Lin *et al.*, 1990). Significantly, IL-2R promoter activation by the Tax trans-activator gene of HTLV-1 or TNF-α also works through NF-κB (Fig. 3) (Cross *et al.*, 1987, 1989; Siekevitz *et al.*, 1987a; Ruben *et al.*, 1988; Lowenthal *et al.*, 1989a). By contrast, phorbol ester inducibility of the IL-2R promoter is mediated by a distinct sequence and did not involve the NF-κB binding site (see below) (Ballard *et al.*, 1989; Lin *et al.*, 1990). The κB motif in the IL-2R gene only weakly binds NF-κB and manifests a poorer response to trans-activation than other κB motifs (Shibuya *et al.*, 1989). Recently it has been found that the activity of the κB motif in T cells is due to cooperation between NF-κB and the serum responsive factor (SRF) and other promoter factors such as Sp1 (Pomerantz *et al.*, 1989; Ballard *et al.*, 1989; Toledano *et al.*, 1990; Roman *et al.*, 1990). Serum responsive factor appears to be present at very high levels in both resting and activated T cells and binds adjacent to NF-κB in the promoter region (Kuang *et al.*, 1993). Constitutively higher levels of NF-κB are associated with deregulation of IL-2 and its receptor in certain T-lymphoma lines that depend on IL-2 for autocrine growth (Hemar *et al.*, 1991a). It is notable that a putative transcription regulatory molecule unrelated to NF-κB, but that binds to an IL-2R promoter sequence encompassing the NF-κB site, has been recently cloned (Adams *et al.*, 1991).

A variety of other cytokines that are produced by activated T cells also appear to involve NF-κB in their transcriptional control. These include IL-6, which stimulates B cells to proliferate, differentiate and produce Ig (Shimizu *et al.*, 1990; Libermann and Baltimore, 1990); GM-CSF, which sustains proliferation of bone marrow precursors for macrophages and granulocytes (Sugimoto *et al.*, 1990; Shreck *et al.*, 1990); TNF-α and -β, which are inflammatory mediators (Messer *et al.*, 1990; Paul *et al.*, 1990; Collart *et al.*, 1990; Goldfeld and Maniatis, 1989; Shakhov *et al.*, 1990; Hass *et al.*, 1990; Drouet *et al.*, 1991; Kuprash and Nedospasov, 1992;

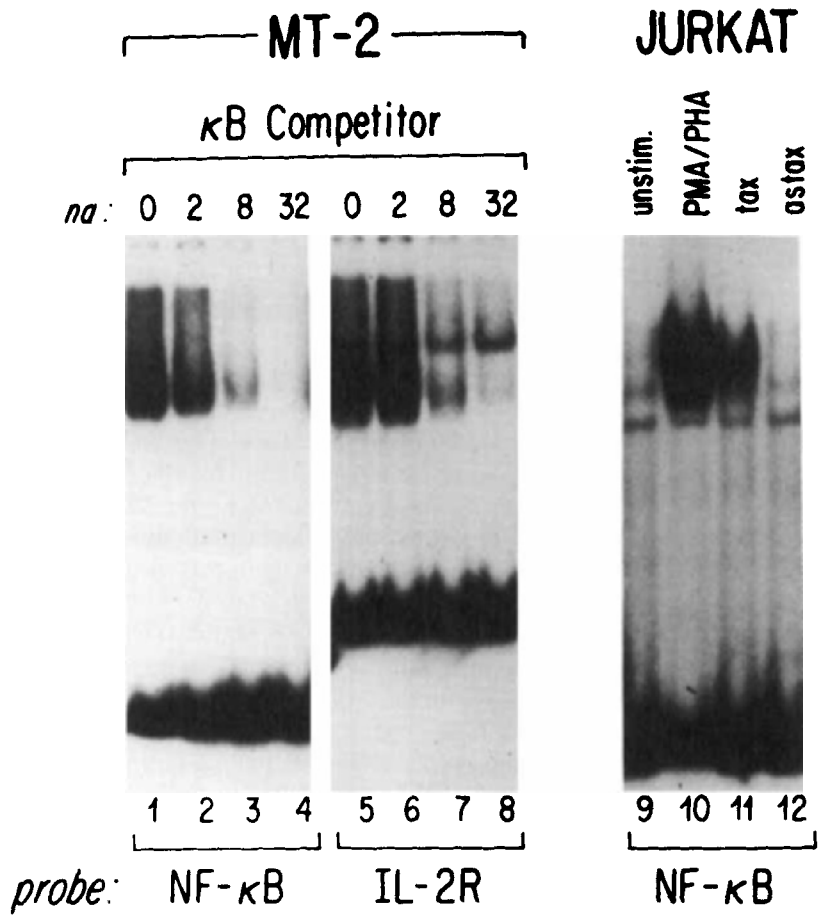


FIG. 3 Mobility shift electrophoresis analysis of nuclear extracts from the HTLV-I infected human T-lymphoid tumor line MT-2 or the human T-lymphoma cell Jurkat. Lanes 1–4 show the complex detected with the κB binding site from the Ig κ gene, competed with increasing amounts of the same sequence to indicate specificity of the nucleoprotein complex. Lanes 5–8 show that a comigrating specific complex is formed with the κB binding site from the IL-2 receptor (IL-2R) α chain promoter. Lanes 9–12 are the complexes detected using the κB binding site from the Ig κ gene in nuclear extracts from Jurkat cells with unstimulated, stimulated with phorbol ester and phytohemagglutinin (PMA/PHA), or stably transfected with expression plasmids containing the HTLV-I Tax coding sequence in the sense (tax) or antisense orientation (astax). These results have been previously described in Cross *et al.* (1989), and Leonardo *et al.* (1988).

Zuckerman and Evans, 1992); and proenkephalin, a precursor for neuropeptides whose mRNA is expressed, for unknown reasons, at high levels in activated T-helper lymphocytes (Rattner *et al.*, 1991). Interestingly, in the case of TNF-α and -β, these same cytokines are potent NF-κB inducers indicating that an autostimulatory loop could be set into motion in the response to antigen (Griffin *et al.*, 1989; Hohmann *et al.*, 1991a). Therefore NF-κB appears to form part of a coordinated program that underlies the immune response.

Precisely how NF-κB connects to signals initiated by the antigen receptor is still obscure. Different answers are obtained in various cellular models that have been studied. In tumor cells, often a relatively simple signal will suffice. For example, in EL-4 murine thymoma cells, PMA or lectin alone will induce NF-κB (Lacoste *et al.*, 1990; Shibuya and Taniguchi, 1989). PMA alone also induces NF-κB in non-T cells (Nelson *et al.*, 1988). In Jurkat both PMA and phytohemagglutinin together give an even greater induction than either agent alone (Hoyos *et al.*, 1989). The fact that induction of NF-κB by PMA and phytohemagglutinin is not blocked by physiologic concentrations of cyclosporin A in this system has been taken to indicate that NF-κB itself is not involved in the immunosuppression caused by this drug (Emmel *et al.*, 1989; Mattila *et al.*, 1990). In evaluating other lines, especially nontransformed T lymphocytes, PMA does not induce NF-κB (Kang *et al.*, 1991). Stimulation of the T-cell antigen receptor by physiologic ligands appears to activate NF-κB (Jamieson *et al.*, 1991; Kang *et al.*, 1992a). Signaling pathways through other T-cell surface markers such as CD28 also activates NF-κB (Gruters *et al.*, 1991; Verweij *et al.*, 1991; Granelli-Piperno and Nolan, 1991).

In many types of cells, including T cells, a single cytokine such as IL-1 or TNF-α will induce NF-κB (Krasnow *et al.*, 1991; Lacoste *et al.*, 1990; Hohmann *et al.*, 1990b). It is unclear, however, how many and what types of different signal transduction pathways are activated following engagement of the receptors for these cytokines. Some evidence suggests that TNF induction does not go through protein kinase C, at least in Jurkat T cells (Meichle *et al.*, 1990). Furthermore, the pathways activated by an individual cytokine may differ between cell types: TNF induction of NF-κB is increased by cycloheximide in Jurkat cells, but blocked by cycloheximide in HepG2 cells (Banerjee *et al.*, 1989). As in the case of NF-κB induction in B cells, other signal transduction pathways in T cells, especially tyrosine phosphorylation, have not been evaluated.

One interesting observation is that a decrease in the level of intracellular thiols can be one of the steps in NF-κB induction (Staal *et al.*, 1990). For example, the induction of NF-κB by TNF-α or phorbol ester can be blocked by *N*-acetylcysteine, an agent that will maintain high levels of

glutathione and other thiol compounds. It has been recently reported that this may be a useful intervention to prevent the activation of HIV transcription (Roederer *et al.*, 1990; Kalebic *et al.*, 1991). Reducing agents have the capacity to release NF-κB from I-κB in cytosolic extracts (Toledano and Leonard, 1991). It has also been found that H_2O_2 can induce NF-κB and the replication of a latent HIV provirus in a human T lymphoma. This presumably occurs by generating reactive oxygen intermediates, and can be blocked with *N*-acetylcysteine, dithiocarbamates, and metal chelators (Schreck *et al.*, 1991, 1992).

B. Other Genes Involved in Immune or Inflammatory Function

Certain cytokines that are primarily derived from leukocytes that are not lymphocytes are also controlled by NF-κB. These include G-CSF, a growth cytokine for granulocytes (Nishizawa *et al.*, 1990; Nishizawa and Nagata, 1990); TNF-α and IL-6, made primarily by monocytes but also by T cells (Collart *et al.*, 1990; Shakhov *et al.*, 1990; Libermann and Baltimore, 1990; Zhang *et al.*, 1990; Shimizu *et al.*, 1990); and IL-8, a chemotactic factor that recruits neutrophils into inflammatory sites (Mukaida *et al.*, 1990). Recent evidence has shown that NF-κB physically interacts with the NF-IL-6 transcription factor which is an important regulator of the IL-6 and IL-8 genes (K. LeClair, personal communication). The involvement of NF-κB in β-interferon (β-IFN) gene induction was first reported by Visvanathan and Goodbourne (1989). They found that double-stranded RNA induced a binding complex with characteristics of NF-κB and pointed out the possibility that multiple kinases, including DI kinase, may release NF-κB from I-κB. Similar work by others showed that virus infection can also induce NF-κB (Lenardo *et al.*, 1989; Fujita *et al.*, 1989b; Hiscott *et al.*, 1989). NF-κB binds to and positively activates a site called PRD-II in the β-interferon promoter. As such it is part of a group of virus-regulated events including activation by two positive effectors and the release of a repressor. Because the β-interferon gene can be induced in almost every cell type, NF-κB is likely involved in gene regulation in perhaps all nucleated cells. Curiously, another factor that binds to the PRD-I motif of the β-interferon promoter, IRF-1, is itself regulated by NF-κB, but its role in β-interferon activation is controversial (Fujita *et al.*, 1989a; Baeuerle, 1991; Pine *et al.*, 1990; Reis *et al.*, 1992). Other investigators have provided evidence that the distinct expression of α- and β-interferons is due to different virally induced transcription factors since the α1-interferon promoter is not regulated by NF-κB (MacDonald *et al.*, 1990).

Key tasks of immune recognition may be carried out by NF-κB. For example, the ability of specialized cells such as B cells, macrophages, and dendritic cells to participate in antigen activation of T cells seems to involve gene control by NF-κB. This regulation embraces the genes for molecules, mostly receptors, that play key roles in the events of antigen processing, presentation, or recognition: MHC class I and II; β_2-microglobulin molecules; MHC class II molecules: the invariant chain, Ii; and the T-cell antigen receptor β chain (Baldwin and Sharp, 1988; Israel *et al.*, 1987; Jamieson *et al.*, 1989; Blanar *et al.*, 1989; Mauxion *et al.*, 1990; Schwemmle *et al.*, 1991; Pessara and Koch, 1990; Blanchet *et al.*, 1992).

Another inflammatory response that involves NF-κB and gene activation is the acute phase response of hepatocytes (Edbrooke *et al.*, 1989, 1991). During this process, various cytokines (IL-1, IL-6, and TNF-α) acting at the liver cell membrane and steroid hormones acting at the nucleus produce an increase in the expression of particular genes and NF-κB is the mediator for elevated expression of the gene for serum amyloids A2β and A1 (Edbrooke *et al.*, 1989; Li and Liao, 1991). SAA2β is the precursor of amyloid A protein subunits of amyloid fibrils in secondary amyloidosis (Edbrooke *et al.*, 1989). Other acute phase genes—angiotensinogen, a precursor for the potent pressor hormone angiotensin II, and α_1-acid glycoprotein, a nonspecific immunosuppressant—are also controlled by NF-κB or a trans-activator of related specificity (Ron *et al.*, 1990a,b,c; Brasier *et al.*, 1990; Won and Baumann, 1990).

Other hepatocyte genes loosely included under the rubric of immune/inflammatory genes which are apparently regulated by NF-κB include the complement factor 4 and factor B genes (Yu *et al.*, 1989; Nonaka and Huang, 1990). The factor B protease is a member of the complement cascade that plays a critical role in the activation of the alternative pathway and its induction by TNF-α seems due to NF-κB (Nonaka and Huang, 1990).

Data supporting a role for NF-κB in the direct regulation of cell growth are emerging. Renan (1989) and Duyao *et al.* (1990) have both suggested that NF-κB takes part in a novel type of growth gene regulation. By binding to a site in the first exon of the c-*myc* mRNA, NF-κB could act to relieve transcriptional blockade which governs the level of full-length mRNA produced. NF-κB would interrupt a stable stem–loop structure in this region of the c-*myc* message that appears to halt transcriptional elongation by RNA polymerase (Duyao *et al.*, 1990). Another set of growth-related genes, the *gro* family, which are transiently induced by serum stimulation and may participate in the growth of melanomas, involve NF-κB when actuated by IL-1 and TNF-α (Anisowicz *et al.*, 1991). Lastly, Baldwin and colleagues have found that serum stimulation of BALB/c 3T3 cells leads to the presence of a binding complex resembling NF-κB (1991). Collectively,

these findings support the notion that NF-κB may be a central regulator of genes involved in cell growth in both lymphocytes and nonlymphoid cells.

Other genes whose expression is influenced by NF-κB or its congeners cannot be characterized as strictly immunological or inflammatory. These diverse targets for NF-κB complete the picture of a very versatile transcription factor. NF-κB or a member of the family of zinc-finger proteins that bind to κB-related motifs may regulate a site in the gene for the α1-crystallin protein that is a constituent of the lens of the eye (Nakamura *et al.*, 1990). The promoter of the urokinase-type plasminogen activator has at least one or possibly multiple κB-like sites that bind complexes of the c-*rel* protein and p65 (Novak *et al.*, 1991; Hansen *et al.*, 1992). Also, NF-κB binding sites have been postulated by sequence homologies within a novel transcription unit in the rat insulin-like growth factor gene II (Matsuguchi *et al.*, 1989), the mouse perforin gene (Youn *et al.*, 1991), and the mouse MHC class II Eβ gene (Shenkar *et al.*, 1991). Finally many viral promoters and enhancers contain functional NF-κB sites which will be discussed further below.

V. Structure of NF-κB Sites: Clues for Specificity?

One of the features of NF-κB binding motifs is that they can differ quite significantly in their primary nucleotide sequence compared to the Ig-κB sequence (originally defined as the 10-nt 5′-GGGACTTTCC-3′). These variant sites can differ in their affinity for NF-κB and may be functionally distinct depending on the structure of the nucleoprotein complex formed (Lenardo and Baltimore, 1989; Baeuerle, 1991; Zabel *et al.*, 1991; Kunsch *et al.*, 1992). These sites have been summarized in Table II. At a glance, it can be noticed that most NF-κB motifs are asymmetric. The first three nucleotides are invariably guanine and the last two are virtually always cytosine. As one moves from 5′ to 3′ through the nucleotide sequence, the complexion of the motif shifts from primarily purines to pyrimidines. The nucleotides in the very middle are quite variable (but they tend not to be G in the alignments depicted) and may contribute very little to binding. For example, in comparing the NF-κB motifs from the Ig κ genes in different species, they appear as versions of the mouse site in which either one (rabbit) or both (human) of the central nucleotides have been lost (see Table II) (Emorine *et al.*, 1983). Nonetheless, the central nucleotides may play a role in the ultimate structure of the nucleoprotein complex (Schreck *et al.*, 1990).

TABLE II

NF-κB Target Genes

	Gene	Sequence[a]	Reference
Well defined			
a. Surface molecules involved in immune function	Ig κ light chain (mouse)	GGGACTTTCC	Sen and Baltimore, 1986a
	Ig κ light chain (human)	GGGGATTTCC	Emorine *et al.*, 1983
	Ig κ light chain (rabbit)	GGGGTTTCCC	
	IL-2Rα chain	GGGAATCTCC	Ballard *et al.*, 1988 Leung and Nabel, 1988
	TCR β chain	GGGAGATTCC	Jamieson *et al.*, 1989
	MHC class I (H-2K^b)	GGGGATTCCC GGGGAAGCCC	Baldwin and Sharp, 1988 Israel *et al.*, 1989
	MHC class II (Eα^d) (Eβ)	GGGACTTCCC	Blanar *et al.*, 1989
	Invariant chain Ii	GGGAATTTCC	Pessara and Koch, 1990
	β2 microglobulin	GGGACTTTCC	Israel *et al.*, 1989
	ELAM-1	GGGGATTTCC	Whelan *et al.*, 1991
b. Soluble molecules involved in immune function	IL-2	GGGATTTCAC	Lenardo *et al.*, 1988
	IL-6	GGGATTTTCC	Shimizu *et al.*, 1990 Lieberman and Baltimore, 1990
	IL-8 (human)	GGAATTTCCT	Mukaida *et al.*, 1990 Mahe *et al.*, 1991
	GM-CSF (mouse)	GGGAACTACC	Sugimoto *et al.*, 1990 Schreck *et al.*, 1990
	G-CSF	GGGGAATCTC GGG(AA)AT(CAC)	Nishizawa *et al.*, 1990
	TNF-α (cachectin)	GGGGCTTTCC GGGAATTCAC GGGGAATCCT GGGGCTGCCC GGGAATTTCC	Shakhov *et al.*, 1990 Collart *et al.*, 1990 Kuprash and Nedospasov, 1992
	TNF-β (lymphotoxin)	GGGGCTTCCC	Paul *et al.*, 1990 Messer *et al.*, 1990
	β-interferon	GGGAAATTCC	Visvanathan and Goodbourn, 1989 Fujita *et al.*, 1989b Lenardo *et al.*, 1989 Hiscott *et al.*, 1989
	Proenkephalin (rat)	GGGACGTCCC	Rattner *et al.*, 1991
c. Acute phase response genes	Serum amyloid A (SAA)	GGGACTTTCC	Edbrooke *et al.*, 1989
	Angiotensinogen (rat)	GGGATTTCCC	Ron *et al.*, 1990a
	Complement factor B (mouse)	GGGAATCCCC	Nonaka and Huang, 1990 Visvanathan and Goodbourn, 1989
	Complement C4 protein	GGGAAGTCCC	Yu *et al.*, 1989
d. Oncogenes	c-*myc*	GGGAAAACCC	Duyao *et al.*, 1990
e. Viruses	HIV	GGGACTTTCC	Nabel and Baltimore, 1987
	SV-40	GGGACTTTCC GGGAAGTACC	Kanno *et al.*, 1989 Macchi *et al.*, 1989
	Cytomegalovirus	GGGACTTTCC GGGGATTTCC	Sambucetti *et al.*, 1989
	Adenovirus	GGGACTTTCC	Williams *et al.*, 1990

(*continues*)

TABLE II (*continued*)

	Gene	Sequence[a]	Reference
f. Others	Invariant chain	GGGAATTTCC	Pessara and Koch, 1990
	Vimentin	GGGGCTTTCC	Lilienbaum *et al.*, 1990
	Gro		Anisowicz *et al.*, 1991
	Interferon regulatory factor	GGGGAATCCC	Baeuerle, 1991
	Consensus:	GGGRNNYYCC	

Nucleotide distribution

Position:	1	2	3	4	5	6	7	8	9	10	
	40	40	40	13	1	3	2	0	0	0	G
	0	0	1	27	21	8	1	2	3	0	A
	0	0	0	0	5	28	35	20	1	2	T
	0	0	0	0	13	1	2	18	36	38	C

Undefined (genes containing potential NF-κB sites)			
	Perforin	GGGCCTTTCA	Youn *et al.*, 1991
	Insulin-like growth factor II	TGGGGAGCCCCC AGGGGCTCCCCAG	Matsuguchi *et al.*, 1989
	1731 LTR (retrotransposon)	AGGCAATTTCCA	Ziarczyk *et al.*, 1989
	TGF β2	GGGCATTGAC	Malipiero *et al.*, 1990
	Apolipoprotein CIII	GGGAAATCCC	Reue *et al.*, 1988
	Urokinase		Novak *et al.*, 1991; Hansen *et al.*, 1992
	ICAM-1	GGAGGATTCC GGGGAAGTCC	Voraberger *et al.*, 1991 Stade *et al.*, 1990
	CD7	GGGAGTCCCT GGGACGCCCT	Schanberg *et al.*, 1991

[a] Note that additional variant 10-bp binding sequences can be obtained by shifting the site one or two nucleotides upstream or downstream or by the sequence encoded by the opposite strand in many genes.

As will be recounted in more detail below, NF-κB comprises two subunits, p50 and p65, which can bind individually as homodimers or as a p50/p65 heterodimer. A careful comparison of the binding of either p50 or p65 homodimers to the p50/p65 heterodimer indicates that p50 apparently binds to the 5′ (more conserved) half site and p65 binds to the 3′ (less conserved) half site (Urban *et al.*, 1991). Does this mean that different versions of the κB motif prefer p50 in combination with different partners from the Rel family? Direct evidence is just emerging that this will likely be an important theme in the future (Perkins *et al.*, 1992). As might be expected, the binding of homodimers of Rel proteins is strongest to sites that are palindromic or otherwise highly symmetric (Urban *et al.*, 1991). In any case, it is clear that variations in the nucleotide sequence of the κB motif have an important influence on function.

How could specificity be encoded in the various versions of the κB motif? One obvious way is simple affinity—if a site was of high affinity, it would respond to lower levels of NF-κB, whereas a weaker site would

be relatively insensitive to NF-κB. This type of functional distinction has been pointed out (Cross *et al.*, 1989; Kuang *et al.*, 1992). In fact, weaker binding may lead to a greater dependence of the κB motif on other factors within a given control element (Kuang *et al.*, 1992). A second means to achieve specificity would be the ability of a given site to discriminate between different members of the κB binding protein family (Mauxion and Sen, 1989; Perkins *et al.*, 1992). A third mechanism could be an alteration of the structure of the DNA binding site caused by factor binding. Evidence has been obtained that bending of the κB DNA sequence is induced by interactions with various Rel proteins (Schreck *et al.*, 1990; Kuprash and Nedospasov, 1992). Moreover, the angle of the bent site differs depending on whether NF-κB or other *rel* complexes are bound (Schreck *et al.*, 1990; Kuprash and Nedospasov, 1992). Fourth, the sequences surrounding an NF-κB site could have effects on both binding and function of NF-κB.

Finally, a more subtle and perhaps more powerful mechanism would involve the DNA site acting as a ligand that causes an allosteric change in the Rel protein complex. Depending on the primary sequence of the κB binding motif, it could be envisioned that the bound factor could adopt different structures that have different functional consequences for transcription. Evidence supporting such a mechanism has been recently obtained in studies using purified NF-κB subunits in transcription and binding assays *in vitro* (Fujita *et al.*, 1992). They find that p50 homodimers activate transcription through the κB site from the MHC class I gene but not that from the β-interferon gene even though binding affinity for both is equivalent. The NF-κB p50/p65 heterodimer did not show differential activation of the two sites. This type of "induced fit" may also be determined to some extent by structural variations for *rel* proteins in solution: p50 appears to adopt a globular shape in solution though p65 or p50/p65 heterodimers seem to be more elongated (Schreck *et al.*, 1990).

VI. The Biochemistry of NF-κB

The cardinal feature of NF-κB regulation is that at least two different cellular forms of NF-κB exist: (*a*) a form that is competent to bind DNA that is constitutively present in mature B lymphocytes and some T lymphocytes and monocytes (Sen and Baltimore, 1986a; Cross *et al.*, 1989; Griffin *et al.*, 1989) and (*b*) a non-DNA-binding form that is present in the cytoplasm of many, or perhaps all, cell types. The cytoplasmic form can be induced to translocate to the nucleus and bind DNA following a great variety of cellular stimuli (Sen and Baltimore, 1986b; Baeuerle and Baltimore, 1988a). A key question, therefore, was how these forms of NF-κB

differ biochemically. Considerable light was shed on this problem by the description of a highly specific and reversible protein inhibitor of NF-κB (Baeuerle and Baltimore, 1988a, b; Baeuerle *et al.*, 1988). This was first revealed by using dissociating treatments such as formamide or sodium deoxycholate to separate NF-κB from its inhibitor, I-κB, in cytosolic extracts (Baeuerle and Baltimore, 1988a; Harel-Bellan *et al.*, 1989). I-κB appeared to be noncovalently associated with NF-κB. The presence of a metastable protein inhibitor of NF-κB fits well with the fact that protein synthesis inhibitors cause superinduction of NF-κB (presumably by preventing new synthesis of the inhibitor) (Sen and Baltimore, 1986b). Also, the finding that phorbol ester induction reduced the amount of cytosolic NF-κB and increased the nuclear form gave rise to the idea that preexisting NF-κB in the cytoplasm was freed from an inhibitor and shuttled into the nucleus (Baeuerle and Baltimore, 1988b). Because NF-κB and I-κB do not freely exist in the cytosolic extracts, it is likely that each is present in stoichiometrical amounts (Baeuerle and Baltimore, 1988b). Though the true range of regulatory interactions is much more complex than this simple formulation, it represents a central theme in the inducible control of NF-κB.

Original purifications of NF-κB indicated that the binding activity was contained in proteins in the 40,000–60,000 molecular weight (MW) range (Kawakami *et al.*, 1988; Lenardo *et al.*, 1988). These analyses also showed that NF-κB was a positive regulator of transcription *in vitro* (Kawakami *et al.*, 1988) and revealed the interesting property that NF-κB binding could be dramatically stimulated by the addition of nucleoside triphosphates (Lenardo *et al.*, 1988). Other studies showed that several agents will increase binding including spermine, spermidine, and other multivalent cations (Schreck *et al.*, 1990). Subsequent biochemical analysis revealed that the NF-κB mobility shift complex comprised two distinct subunits. These components of 50,000 and 65,000 MW have been referred to as p50 and p65, respectively (Baeuerle and Baltimore, 1989). Initially, it was proposed that p50 but not p65 was a DNA binding subunit and that together they formed a heterotetrameric complex in which two p50 subunits contacted each half of the NF-κB binding site (Baeuerle and Baltimore, 1989). However, subsequent studies have determined that p65 does interact with DNA and, in fact, may dictate the binding strength of the p50/p65 heterodimer to NF-κB motifs of certain sequence (see below; Nolan *et al.*, 1991; Urban *et al.*, 1991). Importantly, p65 and p50 each interact with different forms of I-κB introducing an important mode of regulation that has the capacity to distinguish κB site binding complexes of divergent subunit compositions (Kerr *et al.*, 1991; Inoue *et al.*, 1992a).

The p50/p65 complex obtained from the nuclei of stimulated cells was biochemically indistinguishable from that obtained from the cytosol of unstimulated cells (Baeuerle and Baltimore, 1988c; Baeuerle *et al.*, 1988).

Complexes from either source could be inhibited by partially purified I-κB. Thus, the induction process appeared to result from a change in I-κB rather than NF-κB. Evidence for biochemical modulation of I-κB came from studies in which treatment of cytosolic extracts with a variety of kinases including protein kinase A or C (PKA or PKC), or heme-regulated eIF-2 kinase (HRI) released NF-κB from its inhibition by I-κB (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990). Moreover, whereas purified and unmodified I-κB had potent NF-κB inhibitory activity, I-κB that had been phosphorylated by either PKC or HRI kinases lost all of its NF-κB inhibitory activity (Ghosh and Baltimore, 1990). However, PKA did not phosphorylate or abrogate the inhibitory activity of purified I-κB. This suggested that PKA might release NF-κB from a complex with I-κB through an indirect mechanism (Ghosh and Baltimore, 1990). Interestingly, a 65-kDa protein that binds to κB sites has been shown to be physically associated with a serine/threonine kinase (Ostrowski *et al.*, 1991). In summary, there appears to be no single kinase or underlying mechanism that governs all NF-κB induction events (see below). For example, though PMA induces NF-κB in many cell types, NF-κB induction by IL-1 or LPS seems not to be mediated by PKC (Bomsztyk *et al.*, 1991). Also, dephosphorylation of I-κB may also accentuate its release from NF-κB (Hohmann *et al.*, 1992; Link *et al.*, 1992). Nonetheless, the release of NF-κB via I-κB phosphorylation provides an elegant explanation for the missing link between NF-κB regulation of nuclear genes and signal transduction pathways from the cell surface.

Biochemical analysis of I-κB purified from cytosolic extracts of human placenta revealed that it exists in two isoforms that can be separated chromatographically: I-κBα, a 35- to 37-kDa polypeptide (Ghosh and Baltimore, 1990; Zabel and Baeuerle, 1990), and a minor form (~20–30% of the placental inhibitory activity), I-κBβ, a 43-kDa polypeptide (Zabel and Baeuerle, 1990). Interestingly, an early report of an I-κB activity from the cytosol of unstimulated 70Z/3 cells that had a molecular size of ~60 kDa by gel filtration was likely a distinct form of I-κB (I-κBγ) that was recently found to be specific for lymphoid cells (Baeuerle and Baltimore, 1988b; Inoue *et al.*, 1992a). I-κBα and I-κBβ appear to interact with p65 but not the p50 subunit. Other studies showed that I-κBβ also bound to and inhibited c-*rel* DNA binding (Kerr *et al.*, 1991). Importantly, p50 heterodimers with either p65 as well as c-*rel* and p65 or c-*rel* homodimers, but not p50 homodimers, are targets for inhibition (Urban and Baeuerle, 1990; Kerr *et al.*, 1991; G. Nolan and D. Baltimore, personal communication). Therefore p65 or Rel appear to be "receptors" for I-κBα and I-κBβ. By contrast, I-κBγ interacts with p50 homodimers, p50/p65 heterodimers, or Rel (Inoue *et al.*, 1992a). The presence or absence of various I-κBs may determine the expression of complexes that comprise only p50 or p50 paired with other *rel* proteins.

Though both the α and the β isoforms of I-κB are modulated by phosphorylation, interesting differences have been found. Apparently the inhibitory activity of I-κBβ can be abrogated by phosphorylation with PKA and PKC, whereas I-κBα is only affected by PKC (Kerr *et al.*, 1991). Furthermore, phosphatase treatment is able to extinguish the NF-κB inhibitory activity of I-κBβ but does not affect I-κBα (Baeuerle, 1991). *In vitro* experiments have shown that either isoform of I-κB can dissociate a preformed complex between NF-κB and its DNA binding site (Zabel and Baeuerle, 1990). It is possible that I-κB could actively reverse NF-κB-mediated gene regulation, but it is not clear whether I-κB molecules have nuclear localization signals that would allow them to reach NF-κB bound in the chromatin (Zabel and Baeuerle, 1990; Haskill *et al.*, 1991). Further experiments will be required to elucidate the various cellular roles of I-κB isoforms. Possible mechanisms for controlling the nuclear transport of NF-κB and other gene regulatory proteins have been recently discussed (Silver, 1991; Schmitz *et al.*, 1991).

VII. Molecular Cloning of NF-κB and I-κB

The recent molecular cloning of cDNAs encoding p50, p65, and I-κB-like molecules has substantially enhanced our overall understanding of this transcription regulatory system (Ghosh *et al.*,1990; Kieran *et al.*, 1990; Bours *et al.*, 1990, 1992; Nolan *et al.*, 1991; Meyer *et al.*, 1991; Ruben *et al.*, 1991; Haskill *et al.*, 1991; Davis *et al.*, 1991; Inoue *et al.*, 1992b). Most significantly, the binding subunits of NF-κB are members of a family of proteins that are related to the *rel* oncogene (for an overview see Gilmore, 1990) (Figs. 4 and 5). These genes are related to the *Drosophila dorsal* gene product as well as a recently identified *Xenopus* gene, demonstrating that the Rel DNA binding protein structure arose early in evolution (Steward, 1987; Kao and Hopwood, 1991). Molecular cloning studies have now identified a number of Rel family members including p49 or p50B, p50, p65, *rel*B, and c-*rel* (Schmid *et al.*, 1991; Ghosh *et al.*, 1990; Nolan *et al.*, 1991; Kieran *et al.*, 1990; Meyer *et al.*, 1991; Bours *et al.*, 1990, 1992; Ryseck *et al.*, 1992; Wilhelmsen *et al.*, 1984). Significantly, proteins that have an I-κB-like inhibitory activity for NF-κB share a structural domain known as the "cell cycle" or "ankyrin" repeat. The binding subunits of the Rel family can form both homodimeric and heterodimeric complexes that differ in both their binding and their transcriptional properties. Heterodimeric regulatory systems allow for a greater combinatorial array of distinct gene regulatory molecules (Murre *et al.*, 1989; Bohmann *et al.*, 1988). Also, the Rel protein sequence does not match previously identified DNA binding or dimerization structures. Thus, the protein struc-

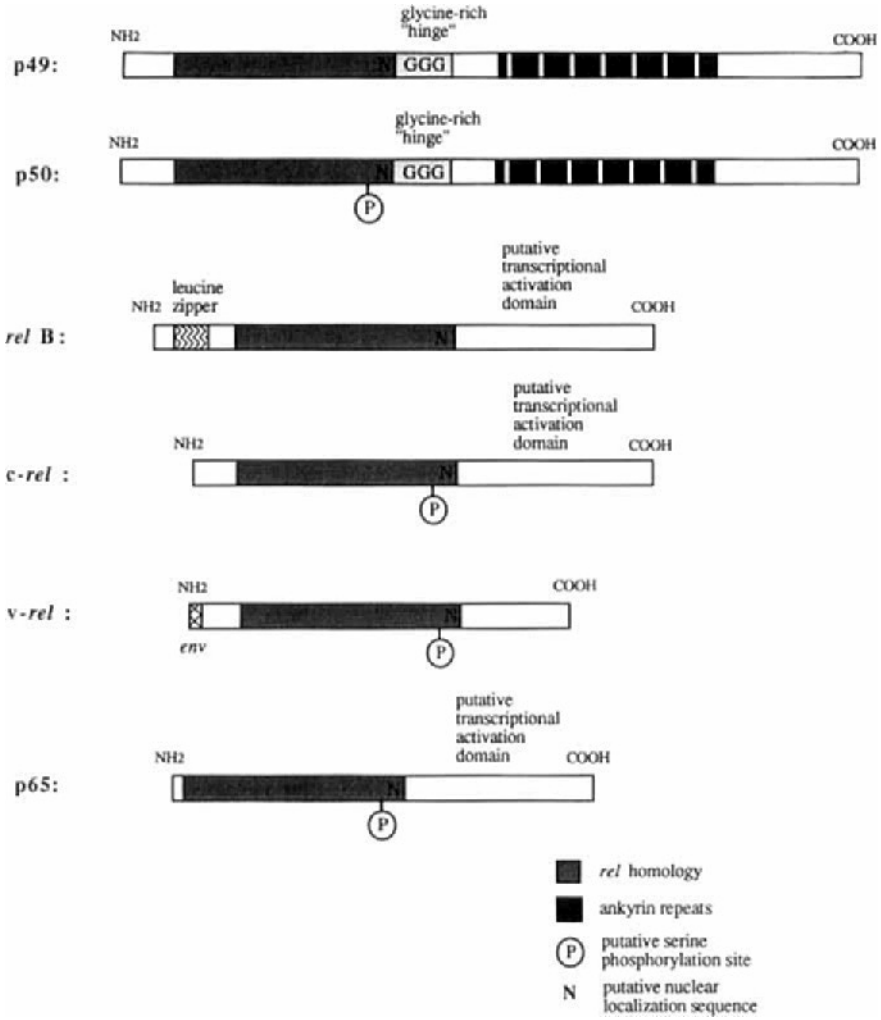


FIG. 4 Shown schematically are the primary structures of the molecularly defined members of the Rel gene family. Only two (p50 and p49/p50B) have bipartite structures that include Rel and ankyrin repeat domains.

ture that governs the interactions among Rel subunits, the ankyrin repeat proteins, and DNA will be of great interest. Finally, it is notable that a chromosomal translocation juxtaposing the gene encoding p49/p50B, called lyt-10, with the immunoglobulin Cα-1 locus has been described in a patient with B-cell lymphoma (Neri *et al.*, 1991). The fusion gene created by the translocation encodes a *rel* DNA binding domain lacking the ankyrin

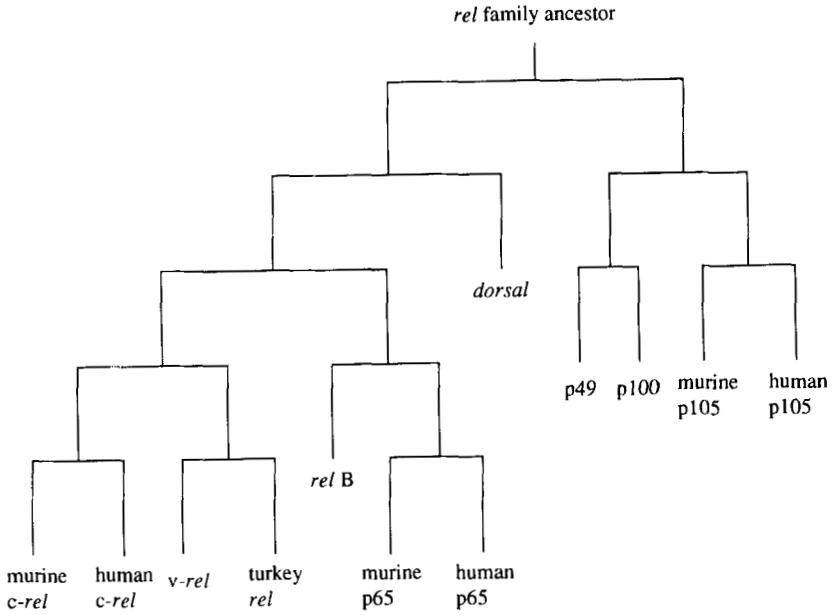


FIG. 5 Phylogenetic relationships between members of the Rel gene family in different species. This figure was adapted from Schmid *et al.* (1991).

repeat domain (see below), suggesting that the protein may be inappropriately regulated and play a role in oncogenesis. Chromosomal localization of the p49-p50B/p97 gene to 10q24 and p50/105 gene to 4q21.1-q24 by other workers places each of these genes at genomic positions associated with acute lymphatic leukemia (Liptay *et al.*, 1992; Ten *et al.*, 1992). Also, the c-*rel* gene has been mapped to the chromosomal region 2p12.2-13, which is rearranged in some large cell lymphoma (Brownell *et al.*, 1988; Lu *et al.*, 1991).

A. Rel: The Cellular Counterpart of a Viral Oncogene

Determination of the primary sequence of the p50 cDNA first established the connection between NF-κB, the proteins encoded by the v-*rel* oncogene, its cellular counterpart c-*rel*, and the *Drosophila* maternal effect gene *dorsal* (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Bours *et al.*, 1990; Meyer *et al.*, 1991). v-*rel* is the oncogene carried by Rev-T, a highly transforming defective avian retrovirus that causes a rapid and fatal lymphomatosis in young birds (Sylla and Temin, 1986; Rice and Gilden, 1988).

The viral genome encodes a single protein product, a 59-kDa phosphoprotein called $p59^{v\text{-}rel}$ (Gilmore and Temin, 1986; Herzog and Bose, 1986; Rice *et al.*, 1986; Walro *et al.*, 1987; Garson and Kang, 1990; Garson *et al.*, 1990). This protein is located mostly in the cytoplasm but also is present in small amounts (10–15%) in the nucleus.

Early studies suggested that *rel* proteins were involved in transcriptional control (Gelinas and Temin, 1988; Hannink and Temin, 1989; Bull *et al.*, 1990; Kamens *et al.*, 1990). Recent work shows that $p59^{v\text{-}rel}$ encodes a protein that binds to κB sites but appears to be incapable of stimulating transcription from reporter genes that depend on transactivation through κB sites (Inoue *et al.*, 1991; Ballard *et al.*, 1990; Kabrun *et al.*, 1991; Richardson and Gilmore, 1991). By contrast, the protein encoded by the c-*rel* oncogene, $p85^{c\text{-}rel}$, also binds with high affinity to κB sites but works as a transcriptional activator (Inoue *et al.*, 1991). In fact, v-*rel*, but not nontransforming mutants of v-*rel*, blocked transcriptional trans-activation by NF-κB (Ballard *et al.*, 1990). This effect could be due to either competition of the two factors at the level of DNA-binding or a "trans-dominant" effect in which $p59^{v\text{-}rel}$ inhibited NF-κB by a protein–protein interaction that sequesters one of its subunits (Ballard *et al.*, 1990; Inoue *et al.*, 1991). The mechanism of malignant transformation by Rel has been recently discussed more completely elsewhere (Gilmore, 1991).

v-*rel* and its cellular counterpart of c-*rel* have been cloned in birds (Chen *et al.*, 1983; Wilhelmsen *et al.*, 1984; Hannick and Temin, 1989) and in mammals (Grumont and Gerondakis, 1989, 1990a,b; Brownell *et al.*, 1985) (Fig. 5). For c-*rel*, the murine cDNA (Bull *et al.*, 1990), the human cDNA (Brownell *et al.*, 1989), and the chicken cDNA (Capobianco *et al.*, 1990) have all been cloned (Fig. 5). The avian tissues that have the highest c-*rel* expression contain the population of target cells for Rev-T. Indeed, although constitutive expression of c-*rel* is seen in many cell types, high levels are observed only in lymphoid cells (Herzog and Bose, 1986; Brownell *et al.*, 1987, 1988; Moore and Bose, 1989; Grumont and Gerondakis, 1990a,b; Zhang *et al.*, 1991). Expression of the c-*rel* gene is induced by serum or phorbol ester in fibroblasts or by phorbol ester and concanavalin A in T cells—conditions very similar to those that induce the mRNA for the p50 and p65 NF-κB subunits (see below; Grumont and Gerondakis, 1990a,b; Bull *et al.*, 1989). Recent characterization of the c-*rel* promoter has shown that it contains a consensus NF-κB site and that it can be directly repressed by v-*rel* but not other viral oncoproteins (Hannink and Temin, 1990; Capobianco and Gilmore, 1991). Interestingly, repression of the c-*rel* promoter can still take place even if the κB site is mutated (Capobianco and Gilmore, 1991).

Cloning of c-*rel* has revealed that v-*rel* encodes a truncated version of the c-*rel* protein product, $p85^{c\text{-}rel}$ (Stephens *et al.*, 1983; Wilhelmsen *et al.*, 1984; Hannink and Temin, 1989; Capobianco *et al.*, 1990, 1991; Grumont

and Gerondakis, 1989; Brownell *et al.*, 1989). $p59^{v\text{-}rel}$ replaces two N-terminal amino acids of c-*rel* with a stretch of amino acids derived from the viral *env* gene. It also lacks 118 carboxyl-terminal amino acids that may include the transcription activating domain for $p85^{c\text{-}rel}$ (Bull *et al.*, 1990; Inoue *et al.*, 1991). The *rel* homology region appears to be critical for transformation (Kamens *et al.*, 1990; Garson and Kang, 1990). It is further notable that a protein kinase A site in the v-*rel* coding sequence also appears to be critical for both the transforming and the transcriptional repression functions of the $p59^{v\text{-}rel}$ protein (Mosialos *et al.*, 1991).

Intriguingly, $p59^{v\text{-}rel}$ isolated from cellular extracts is associated with at least five different proteins in a complex that has an apparent molecular mass of over 400 kDa (Lim-Tung *et al.*, 1988, 1990; Simek and Rice, 1989; Morrison *et al.*, 1989; Davis *et al.*, 1990; Kochel *et al.*, 1991). This is true whether $p59^{v\text{-}rel}$ is isolated from transformed B-cell lines, or overexpressed in other avian cell types (Kochel *et al.*, 1991). In view of the regulatory features known for complexes between NF-κB and I-κB, the so-called *rel*-associated proteins take on potential importance for understanding the biochemical mechanism of *rel* transformation. In the cytosol of transformed cells, $p59^{v\text{-}rel}$ is mainly associated with a 40-kDa phosphoprotein termed pp40; in smaller amounts with the constitutive avian heat shock protein hsp70; and with three other proteins, p75, p115, and p124; the nuclear p59 associates with pp40 (Davis *et al.*, 1990). pp40 appears to be the β form of I-κB and will be discussed below (Kerr *et al.*, 1991; R. Taub, personal communication). Strikingly, p75 is the product of the protooncogene c-*rel* (Simek and Rice, 1988). Moreover, $p85^{c\text{-}rel}$ has been found complexed with the very same proteins as v-*rel* especially the ~40-kDa and the 115-kDa proteins (Morrison *et al.*, 1989; Davis *et al.*, 1990; Kochel *et al.*, 1991). The identities of p115 and p124 are unknown. One hypothesis to emerge from these analyses is that the transforming effect of v-*rel* may be due to its ability to bind to NF-κB or related DNA binding complexes and prevent their transcriptional function. This might explain why v-*rel* oncoproteins can apparently transform cells whether they are located in the nucleus or the cytoplasm (Gilmore and Temin, 1988; Hannink and Temin, 1989). For instance, it is striking that v-*rel* seems to preferentially transform the same cell lineage that exhibits constitutive NF-κB binding activity. This connection leads one to imagine that NF-κB could regulate something analogous to a tumor-suppressor gene that would inhibit the growth of mature B cells.

B. Cloning of the p50 and p49/p50B cDNAs

Though not initially recognized, the NF-κB p50 and p49/p50B subunits were first cloned in a collection of cDNAs isolated from a library made

from mitogen-induced human peripheral blood mononuclear cells (Irving *et al.*, 1989; Zipfel *et al.*, 1989). The identity of these coding sequences was then conclusively established through independent cloning efforts based on protein microsequence from purified protein and binding analyses (Kieran *et al.*, 1990; Ghosh *et al.*, 1990; Bours *et al.*, 1990, 1992; Meyer *et al.*, 1991; Schmid *et al.*, 1991). Evidence indicates that p50 could be the same as KBF-1, the constitutively active nuclear factor already known to bind NF-κB sites in the H2 gene (Kimura *et al.*, 1986; Israel *et al.*, 1987, 1989; Baldwin and Sharp, 1987; Kieran *et al.*, 1990). The p50 cDNA encodes for a 105-kDa protein, p105, that contains an N-terminal domain of 360 amino acids homologous to *rel*. The full-length protein product is unable to bind DNA. It needs to be proteolytically processed, near a stretch of glycine amino acids in the middle of the protein, to a mature form of approximately 50 kDa that includes the Rel domain. This has been found to occur by an ATP-dependent process that leads to the production of mature p50 and the rapid degradation of the C-terminal portion of the precursor protein (Fan and Maniatis, 1991). The C-terminal domain of 55 kDa that typically is cleaved off contains eight "ankyrin repeat" protein domains that can serve to keep the p50 moiety in a nonbinding form and prevent its nuclear localization (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Bours *et al.*, 1990; Meyer *et al.*, 1991; Henkel *et al.*, 1992) (see Figs. 4 and 6). This may be a crucial regulatory interaction because in a B-cell lymphoma-associated translocation, a fusion gene has been created in which the Rel DNA binding domain of the p49/p50B gene, lyt-10, is preserved but the ankyrin repeat domain has been lost (Neri *et al.*, 1991). When independently expressed in bacteria, this portion of p105 is a potent inhibitor of DNA binding of p50 homodimers but not NF-κB or p65 homodimers (Liou *et al.*, 1992). In addition to specifying a previously undescribed DNA binding motif, the *rel* homology domain promotes dimerization. Thus, p50 forms homodimers or participates in heterodimer formation with p65 or other *rel* homology proteins (Kieran *et al.*, 1990).

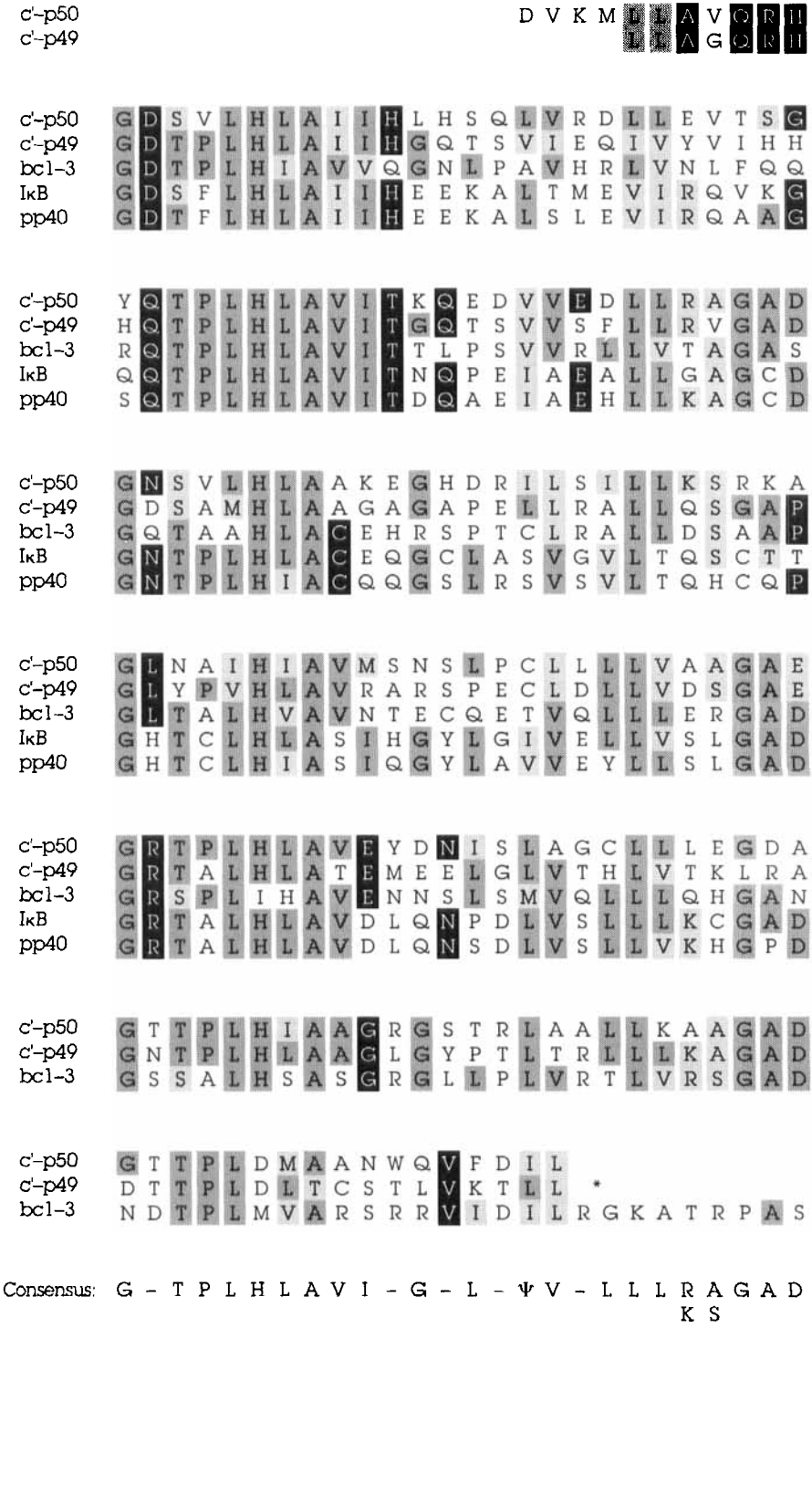
The 4.0-kb mRNA for p105 is present in many tissues but can be upregulated by many of the same agents that promote the dissociation of NF-κB from I-κB (Bours *et al.*, 1990; Meyer *et al.*, 1991). An additional 1.9-kb mRNA encoding I-κBγ is also present in certain cells (Inoue *et al.*, 1992a). Phorbol ester stimulation of HL60 or HeLa cells or TNF-α stimulation of HL60 cells strongly induced the p105 mRNA. In human peripheral blood T cells, mitogenic stimuli such as PMA/PHA and calcium ionophore also elevate the p105 mRNA (Bours *et al.*, 1990). The mRNA is superinduced by inhibitors of protein synthesis. Interestingly, the immunosuppressive agent cyclosporin A strongly inhibited p105 mRNA induction by calcium ionophore but had only a modest effect on induction by PMA or PMA/PHA (Bours *et al.*, 1990). These findings give rise to the

idea that sustained gene expression after various stimuli may be primarily due to increased synthesis of p105 mRNA and p50 protein (Hohmann *et al.*, 1991a). It has been recently shown that the p105 gene promoter is itself activated through an NF-κB binding site (Ten *et al.*, 1992).

Analysis of subcellular localization by both immunofluorescence and immunoprecipitation reveals that the p105 precursor is restricted to the cytoplasm, whereas processed p50 is both nuclear and cytoplasmic (Blank *et al.*, 1991). The sixth ankyrin repeat and adjacent acidic region of p105 were found to be important for cytoplasmic localization (see Fig. 6). Moreover, excision of the seventh ankyrin repeat or the acidic region between ankyrin repeats 6 and 7 allows DNA binding of p105. Thus, each ankyrin repeat in p105 may not function equivalently in causing subcellular localization or inhibition of DNA binding. Recent evidence indicates that p105, which is situated extranuclearly, can bind and thereby moor p65 in the cytoplasm (Rice *et al.*, 1992).

The function of the p50 subunit has been analyzed by cotransfection assays both on its own and as a partner to other subunits. It appears that p50 has little or no ability to cause transcriptional trans-activation through certain DNA sites that it binds (Kieran *et al.*, 1990), though when tested by *in vitro* transcription assays, p50 can function as an activator (T. Fujita and D. Baltimore, personal communication). In fact, p50 seems to suppress the activation of certain binding sites by p50/65 heterodimers (Schmitz and Baeuerle, 1991; Kang *et al.*, 1992a). KBF-1 has features of a transcriptional antagonist at κB sites (Israel *et al.*, 1989; Hemar *et al.*, 1991b). By contrast, p65 likely contains a trans-activating domain and may make p50/p65 heterodimers much stronger transcriptional activators (see below) (Nolan *et al.*, 1991; Ballard *et al.*, 1992). Interestingly, coexpression of the p65 and p50 subunits in Jurkat cells led to synergistic activation of κB-dependent transcription, but coexpression of p65 and the v-*rel* oncogene inhibited transcription (Ballard *et al.*, 1992).

Recently a cDNA has been cloned for an alternative DNA binding subunit of NF-κB that is very similar to p50 and which has been called variously p49 or p50B (Schmid *et al.*, 1991; Bours *et al.*, 1992). The full-length protein encoded is 97–100 kDa and, like p105, contains an N-terminal Rel domain and a C-terminal ankyrin repeat domain. The mRNA for p97/p100 is approximately 3.3 kb. The amino acid similarity between p97/p100 and p105 in the Rel region is ~60% identity but then drops off in the C-terminus to give an overall amino acid identity of ~40%. Schmid and co-workers have suggested that a cluster of histidine and cysteine residues in p49/p50B may chelate zinc in a functionally important way (1991). Just as for p105, the p97/p100 protein must be cleaved to an approximately 50-kDa form in order to specifically bind DNA. The mature form, p49/p50B, can heterodimerize with p65 and other Rel proteins and


c'-p50
c'-p49
bcl-3
IκB
pp40
Consensus:

binds κB sites with high affinity. When cotransfected with p65, p49/p50B can drive HIV-1 long terminal repeat (LTR) transcription but seems to have different functional effects depending on the exact sequence of the κB site with which it interacts (Schmid *et al.*, 1991; Perkins *et al.*, 1992).

C. The p65 Subunit

The cDNA isolated for the p65 NF-κB subunit reveals homology to the *rel* protein family (Nolan *et al.*, 1991; Ruben *et al.*, 1991) (Fig. 4). The cDNA clone corresponds to an mRNA of 2.6 kb that was detectable in all tissues tested and encoded a protein of 549 amino acids. The p65 mRNA does not seem to be increased very significantly by mitogenic agents that potently induce p50 (Ito *et al.*, 1992; U. Siebenlist, personal communication). As expected, the full-length protein from the cDNA clone could heterodimerize with p50 and together these subunits form a tight nucleoprotein complex with κB sequences. Thus, in contrast to p50, the p65 protein appears not to require cleavage from a larger precursor.

Studies aimed at the characterization of the different subunits have been focused mainly on the p50 subunit because of its strong DNA binding activity (Kawakami *et al.*, 1988; Baeuerle and Baltimore, 1989). However, p65, which originally appeared to be unable to bind DNA in mobility shift assays (Baeuerle and Baltimore, 1989) or by UV crosslinking (Kawakami *et al.*, 1988; Collart *et al.*, 1990), has now been shown to be a DNA binding subunit with important properties (Nolan *et al.*, 1991; Urban and Baeuerle, 1991). In addition to the ability of p65 to change the affinity of p50 for NF-κB binding sites, it may have potentially important transcriptional properties that differ from p50 (Baeuerle, 1991). A naturally occurring spliced variant of p65 has been shown to cause cellular transformation (Narayanan *et al.*, 1992).

Binding of the p65 homodimer can be completely inhibited by purified I-κBβ, indicating that p65 alone could directly interact with I-κBβ. These results were taken to suggest that a domain in the Rel homology region of p65 (which is missing in the corresponding part of p50) is responsible for interaction of I-κB with NF-κB complex. Interestingly, p65 is most highly related to the c-*rel* protein in sequence, especially the *rel*B variant (Ryseck *et al.*, 1992) (Fig. 5), and seems to contain a C-terminal region that is

FIG. 6 Comparison of protein sequences of the ankyrin repeats in proteins that have been demonstrated to inhibit the DNA binding of Rel/NF-κB family members. Dark grey shading indicates amino acids that are conserved in all repeats. Light grey shading indicates conservative substitutions in otherwise conserved positions. The black shading indicates amino acids that are the same within a given repeat as the protein sequences are aligned. A consensus for ankyrin repeats is shown at the bottom.

believed to be a transcriptional trans-activating domain of c-*rel* (Nolan *et al.*, 1991; Schmid *et al.*, 1991; Ballard *et al.*, 1992; Schmitz and Baeuerle, 1991; Ruben *et al.*, 1991, 1992). A region of the protein from amino acids 435 to 459 appears to be functionally important for activation. Notable features of this domain include negatively charged amino acids and a motif resembling a partial leucine zipper (Ruben *et al.*, 1992). p65 can form homodimers that can function as transcriptional trans-activators (Nolan *et al.*, 1991; Schmitz *et al.*, 1991). A spliced variant of p65 that has lost a protein region important for dimerization has been described in certain cell types (Ruben *et al.*, 1992).

D. Other Modes of Regulation

A *rel* homology protein that heterodimerizes but does not allow recognition of certain κB motifs would likely interfere with transcriptional activation of those sites. A precedent for this form of gene control comes from the protein named Id, which is a helix–loop–helix-type transcriptional regulator and inhibits gene activation by these proteins without binding to DNA itself (Benezra *et al.*, 1990; Sun *et al.*, 1991). This type of dominant negative interference has been demonstrated for a mutant version of the p50 protein that conserves dimerization ability but is unable to bind DNA (Logeat *et al.*, 1991). This mutant can block binding of other members of the Rel family *in vitro* and can act as a transcriptional dominant negative *in vivo*. Such a dominant negative inhibitor of p50, termed I-rel, has been reported recently (Ruben *et al.*, 1992).

In summary, patterns of protein–protein association have been clearly defined using polypeptides generated from cloned Rel/NF-κB subunit genes. These would seem to indicate multiple levels of interactive control to finely tune transcriptional responses (see Fig. 7). Regulation may result from the different combinatorial possibilities of p50 or p49/p50B when coupled to p65 or other *rel* homology proteins. All of the DNA binding subunits of NF-κB as well as Rel proteins may be controlled by release from cytoplasmic stores, though they seem to appear in the nucleus with different kinetics (Ballard *et al.*, 1990; Molitor *et al.*, 1990). Another level may emerge from the transcriptional effects that a given subunit combination generates at differing DNA sites in the repertoire of κB motifs (Sica *et al.*, 1992). A third level may result from interactions between Rel family members that do not involve DNA binding. There appears to be synergistic transcriptional effects of multiple binding sites for NF-κB that are not a function of cooperative binding (Pettersson and Schaffner, 1990).

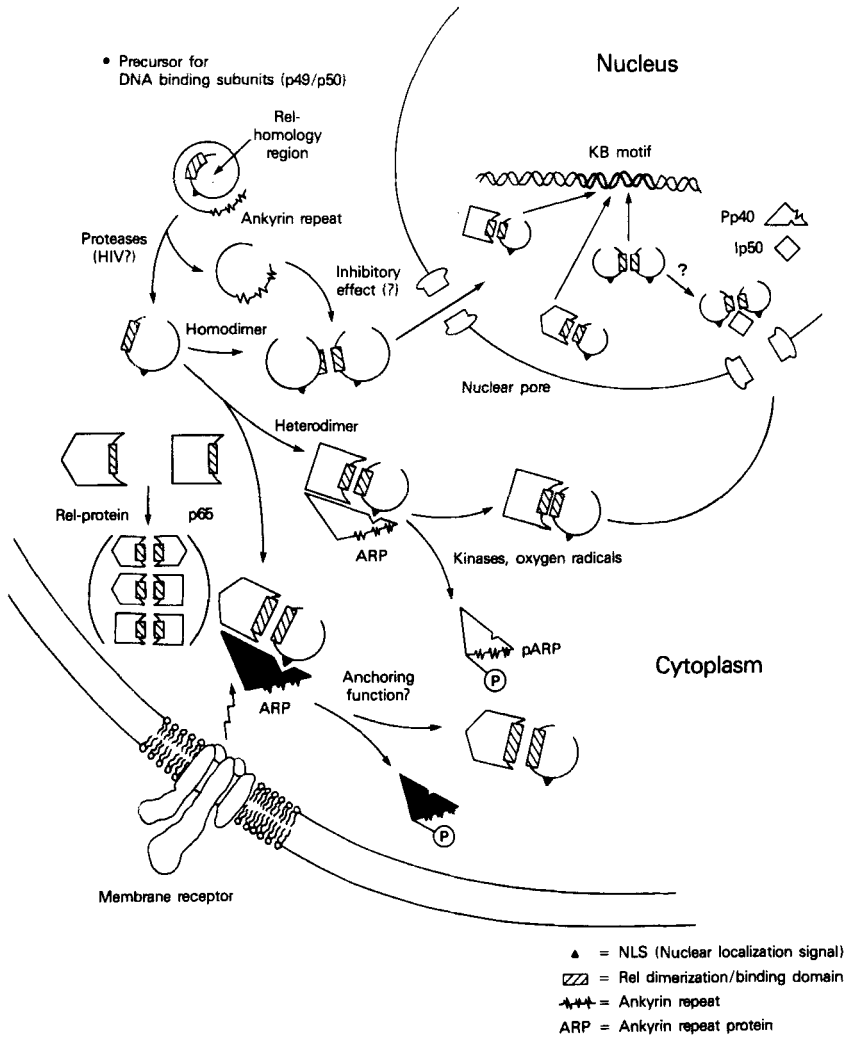


FIG. 7 A hypothetical scheme depicting several pathways of NF-κB and Rel regulation.

E. The Rel Homology Family Extends to *Drosophila*

v-*rel* and c-*rel* share a strong homology (47%) with the amino-terminal half of the *Drosophila dorsal* protein (Steward, 1987) (Fig. 5). The protein encoded by this maternal effect gene appears to govern the establishment of dorsal–ventral axis in the developing fly embryo (Ip *et al.*, 1991).

Importantly, the *dorsal* protein is regulated by partitioning between the cytoplasm and nucleus; this was an early clue that *dorsal* and NF-κB could be related (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Hunt, 1989). Relocalization of the dorsal protein from cytoplasm to the nucleus is crucial for its morphogenetic function (Steward, 1989). The protein region that is conserved between *dorsal* and the Rel family members is responsible for DNA binding and dimerization (Steward, 1987). Recently it has been shown that *dorsal* binds to κB motifs with high affinity (Ip *et al.*, 1991). The *dorsal* gene can exert a negative transcriptional effect on genes such as zerknüllt (*zen*) and decepentaplegic (*dpp*) but seems to activate other genes such as *twist* (Jiang *et al.*, 1991; Pan *et al.*, 1991). Also, the nuclear translocation and possibly DNA binding of the *dorsal* gene product may be inhibited by the protein product of the *cactus* gene much the same way that NF-κB and I-κB interact (see Hunt, 1989).

F. I-κB and Other Ankyrin Repeat Proteins

An important mechanism for controlling NF-κB binding activity is provided by protein–protein interactions that involve I-κB-like molecules. A cDNA clone has been recently isolated that encodes a 36-kDa protein (318 amino acids), called MAD3, that appears by several criteria to be identical to I-κBβ (Haskill *et al.*, 1991; I. Verma, personal communication). The MAD3 protein is the appropriate molecular weight and is a highly specific inhibitor of NF-κB DNA binding but not KBF-1. In addition, the sequences of two peptides derived from purified I-κBα are found in the coding sequence of the MAD3 clone (Davis *et al.*, 1991). Also, the MAD3 protein and NF-κB could be coimmunoprecipitated with antibody against NF-κB, indicating a direct interaction between the two proteins (Haskill *et al.*, 1991). Interestingly, the mRNA for MAD3 is rapidly induced during the process of adherence of monocytes to plastic. A homologous rat protein is rapidly induced during liver regulation following partial hepatectomy (Tewari *et al.*, 1992a,b). Together these results suggest that I-κBβ might be part of a negative feedback mechanism to limit NF-κB activation. As might be expected for I-κB, the MAD3 protein also exhibits a putative PKC phosphorylation site near its C-terminus (Haskill *et al.*, 1991).

One of the most significant features of the MAD3 protein is that it contains five tandem repeats of the ankyrin motif (see Fig. 6). As mentioned, this repeat had also been observed in multiple copies in the C-terminal region of the p105 protein precursor to p50 and the p97 precursor to p50B (Kieran *et al.*, 1990; Ghosh *et al.*, 1990; Bours *et al.*, 1990, 1992; Meyer *et al.*, 1991). The ankyrin repeat had been identified as a motif that was found in human erythrocyte protein ankyrin and then also observed

in proteins required in cell cycle control and tissue differentiation (for a review see Lux *et al.*, 1990). Because both MAD3 and the C-terminal portions of p105 and p97 had the ability to inhibit the binding of p50 to DNA, these ankyrin repeats were thus implicated in the interaction between NF-κB and I-κB. A comparison of several ankyrin repeat proteins (ARPs) revealed that the ankyrin repeat seems to be the sole domain in common (G. Nolan, personal communication) (see Fig. 6).

The realization that NF-κB is a member of the *rel* homology family prompted an investigation of the functional properties of the pp40 *rel*-associated protein (Kerr *et al.*, 1991). pp40 is a phosphoprotein that is complexed with Rel proteins (Tung *et al.*, 1988; Simek and Rice, 1988). It can be detected in both the cytoplasm and the nucleus of v-*rel*-transformed cells (Simek and Rice, 1989; Davis *et al.*, 1990, 1991) and the B-lymphoma lines WEHI 231 and 70Z/3 (Kerr *et al.*, 1991). pp40 associates with the amino terminus of v- and c-*rel* (Simek and Rice 1988; Davis *et al.*, 1990, 1991); it is reminiscent of I-κB because of both its molecular size and the ability of phosphorylation or detergent to diminish its binding to *rel* proteins. Biochemical studies determined that purified pp40 would inhibit DNA binding of *rel* homology proteins. Intriguingly, pp40 and I-κBβ inhibit DNA binding of p50/p65, p50/c-*rel* complexes but not p50 homodimers. (Kerr *et al.*, 1991; Davis *et al.*, 1991). Tryptic peptide mapping indicates that pp40 may, in fact, be identical to I-κBβ. The distribution of pp40 would suggest that the activation loops for complexes involving *rel* proteins and ARPs have the potential to be taking place in both the nucleus and the cytoplasm.

The sequence of a cDNA for pp40 has been recently reported by Davis and colleagues (1991). The pp40 coding sequence contains five ankyrin repeats just as for other proteins that inhibit the binding of Rel family members (see Fig. 6). This strengthened the idea of a gene family of ARPs that have properties akin to those of I-κB. pp40 also contains potential casein kinase II and tyrosine kinase sites (Davis *et al.*, 1991).

Recent work has led to the molecular characterization of a third form of I-κB, termed I-κBγ (Inoue *et al.*, 1992a). This form is derived from a 2.5-kb mRNA that is an apparent splice product of the p50 gene that appears in lymphoid cells. The encoded 607-amino acid protein (70 kDa) corresponds to the C-terminus of the p105 precursor protein that contains ankyrin repeats. I-κBγ prevents NF-κB, p50 homodimer, and c-*rel* from binding to DNA. Furthermore, it has been shown to prevent transit to the nucleus and gene activation by c-*rel*. The protein sequence also contains a consensus protein kinase A phosphorylation site (RKLS), indicating the possibility that it too is regulated by phosphorylation.

A putative leukemia oncogene, Bcl-3, also appears to have I-κB-like properties (Hatada *et al.*, 1992; Wulczyn *et al.*, 1992). Bcl-3 is an ARP

encoded at the locus of the chromosomal translocation t(14:19)(q32;q13.1) that occurs in cases of chronic lymphocytic leukemia and has seven copies of the ankyrin repeat (Ohno *et al.*, 1990). In contrast to I-κBα or β, Bcl-3 may preferentially inhibit p50 homodimers and NF-κB, but not p65 homodimers. In an important link to previous studies that have implicated various *rel* proteins in cell proliferation, the expression of Bcl-3 increases in peripheral blood mononuclear cells following mitogenic stimulation (Ohno *et al.*, 1990; Franzoso *et al.*, 1992). The ankyrin repeats in this protein bear a striking resemblance to those found in the C-terminal region of the p105 protein. Ankyrin repeats are likely to differ in their function depending on their precise amino acid sequence, thus affording another venue for specifity in the interactions between the *rel* and the ARP families.

The function of the ankyrin repeat is not completely understood. Usually proteins, unless very small, cannot pass freely in the nucleus and need to carry specific basic sequences called nuclear localization signals (NLS) exposed on the protein surface (for review see Dingwall and Laskey, 1986; Nigg *et al.*, 1991). Such a sequence has been shown to be important for the nuclear accumulation of processed p50 (Blank *et al.*, 1991). One hypothesis was that the ankyrin repeat could either mask the NLS or anchor in some way the *rel* protein to cytoskeletal or cellular membrane components such that the NLS would not function (Ghosh *et al.*, 1990). I-κB not only prevents DNA binding activity when associated to NF-κB in the cytosol but also apparently blocks nuclear translocation (Baeuerle and Baltimore, 1988a,b; Henkel *et al.*, 1992; Beg *et al.*, 1992). This has also been seen clearly in the cytological localization of the *dorsal* protein (Hunt, 1989). An alternative view is that the ankyrin repeat is really a protein–protein interaction domain that yokes the *rel* homology proteins to ARPs (Thompson *et al.*, 1991) and allows cytoplasmic localization secondarily, possibly through other domains. The latter conjecture is given some credence by the fact that the pp40 : *rel* complexes are found in the nucleus as well as the cytoplasm (Davis *et al.*, 1990, 1991).

The model of cytoplasmic localization by the union of NF-κB and I-κB is very similar to the regulation of the glucocorticoid receptor (Picard and Yamamoto, 1987). The glucocorticoid receptor is cytoplasmic in the absence of glucocorticoids, and its NLS is presumably covered by the binding of heat shock protein 90 (hsp90; Picard and Yamamoto, 1987). Steroid binding to the receptor could overcome inhibition by dissociating the receptor and hsp90. Recent evidence suggests that the regulation is more complicated since the receptor bound to hsp90 is not necessarily excluded from the nucleus—a feature also observed in complexes between *rel* and pp40 (Sanchez *et al.*, 1990). The major difference between the glucocorticoid system and NF-κB is that in the case of the glucocorticoids the allosteric modification that results in nuclear translocation affects the transcription factor itself and not the "anchoring" protein.

Finally, it is intriguing that the precursors for p50 and p49/p50B couple both a *rel* homology domain and an ARP in a single bifunctional polypeptide (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Bours *et al.*, 1990, 1992; Meyer *et al.*, 1991; Schmid *et al.*, 1991). p50 and p49/p50B are, so far, the only members of the Rel homology family that exist in the form of precursors. Many roles can be envisioned for the ankyrin repeats that are located in the C-terminal portion of these proteins. Most intriguing is the possibility that cleavage of the mature form of the binding protein from the precursor is a critical step in the regulation of NF-κB binding. The process requires ATP, and the ankyrin repeats are not required for processing (Fan and Maniatis, 1991). Because of the strong DNA binding ability of p50, this proteolytic event may represent a mechanism to govern the preponderance of p50 in homodimeric versus heterodimeric form. This could be crucial since p50 homodimers appear to be unaffected by I-κBβ that would result in cytoplasmic localization of NF-κB through its interaction with p65.

VIII. NF-κB and Viruses

Aside from the obvious role of the Rel protein as the transforming gene product of the Rev-T virus, NF-κB and potentially other Rel proteins have been found to play a role in transcriptional regulation of many other viruses. Most work to date has been done on retroviruses that infect human T lymphocytes—the human immunodeficiency viruses and the human T lymphotropic virus, type I. Several other viruses have also been studied and will be discussed: adenovirus; SV-40; hepatitis B virus; herpesvirus types I and VI and their relative, cytomegalovirus. The characteristic rapidity of NF-κB induction can be utilized by viruses as a strategic tool to initiate its reproduction or to place its life cycle under the control of the host cell.

A. Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) is a retrovirus that infects $CD4^+$ T lymphocytes and some monocytes causing acquired immunodeficiency syndrome (AIDS) (Cullen and Greene, 1989). The role of NF-κB in controlling the HIV long terminal repeat (LTR) was first established by Nabel and Baltimore, who demonstrated a direct correlation between increases in NF-κB binding and the transcriptional activity of the LTR following activation of T cells (1987). Deletion analysis of the HIV LTR had established that a strong enhancer element within the LTR was centered over a nucleotide sequence

that contained two homologies to the Ig-κ gene NF-κB site (Muesing *et al.*, 1987). Most isolates of HIV-1 and simian immunodeficiency virus (SIV) have two functional NF-κB sites in the LTR, but in HIV-2 there is conservation of only one of the NF-κB binding sites (Dewhurst *et al.*, 1990; Anderson and Clements, 1991; Novembre *et al.*, 1991).

The possibility of a strong association between T-cell activation and expression of the HIV LTR has come to dominate thinking about the regulation of the life cycle of HIV. A characteristic feature of HIV infection is that fulminant disease often occurs many months or even years after the initial infection (Haseltine, 1988; Cullen and Greene, 1989). This delay in viral pathogenesis, called the latent period, could be due to the residence of a provirus in a small population of T lymphocytes in a quiescent state (reviewed in Garcia-Blanco and Cullen, 1991). Activation of those T lymphocytes by antigen stimulation could increase NF-κB and lead to transcriptional expression of the provirus and active production of the virus (Rosen *et al.*, 1985; Zagury *et al.*, 1986; Nabel and Baltimore, 1987; Harada *et al.*, 1988; Tong-Starksen *et al.*, 1989). Other inducers of NF-κB, such as TNF-α and IL-1 also could increase virus production (Folks *et al.*, 1989; Osborn *et al.*, 1989). This reawakening of the virus would presumably cause decreased numbers of the $CD4^+$ cells leading to the immunodeficiency syndrome.

A correlation between the induction of NF-κB and LTR transcription has received considerable experimental support. A variety of studies show that induction of NF-κB in either T lymphocytes or monocytes causes increased transcriptional activity of the HIV LTR (Nabel and Baltimore, 1987; Garcia *et al.*, 1987; Franza *et al.*, 1987; Kaufman *et al.*, 1987; Bielinska *et al.*, 1989; Griffin *et al.*, 1989, 1991; Zeichner *et al.*, 1991; Berkhout and Jeang, 1992; Hillman *et al.*, 1992). Moreover, LTR activity does not seem to be restricted to cells such as $CD4^+$ T cells and monocytes that normally manifest productive infection but may depend more on the availability of NF-κB. If the LTR is tested in B cells, NF-κB stimulates transcription strongly and B cells that artificially express the CD4 surface molecule permit HIV replication quite well (Calman *et al.*, 1988). T cells that do not express the p50/p65 heterodimer have restricted HIV expression (Raziuddin *et al.*, 1991). Methylation of cytosine nucleotides can inhibit NF-κB binding, suggesting that a methylated HIV provirus may be resistant to activation (Bednarik *et al.*, 1991). Other viruses that can activate NF-κB such as herpesvirus and cytomegalovirus and the c-Ha-*ras* oncogene can cause transactivation of the HIV LTR through the NF-κB binding site and lead to viral activation (Ensoli *et al.*, 1989; Horvat *et al.*, 1991; Arenzana-Seisdedos *et al.*, 1989; Geng *et al.*, 1992). Curiously, one study found that antisense oligonucleotides complementary to the NF-κB binding motif interfere with trans-activation of the LTR (Laurence *et al.*, 1991).

Most studies on the transcriptional control of HIV have been carried out by transiently transfecting cells with plasmids containing the HIV LTR linked to various reporter genes (usually bacterial chloramphenicol acetyltransferase). These artificial gene constructs may only partly reflect the normal transcriptional control of HIV replication. Thus it became important to also test the infectivity of HIV when various *cis*-acting elements in the LTR have been mutated to gain an independent assessment of the role of these sequences in the virus life cycle. Studies of HIV replication in response to cytokines found an association between induction of NF-κB binding and transcriptional activation of the virus (Hemar *et al.*, 1991a; Israel *et al.*, 1989; Rosenberg and Fauci, 1990). One study found that viruses containing deletions of both NF-κB sites were still infective in MT-4 cells (Leonard *et al.*, 1989). Further analysis revealed that the two NF-κB sites and three Sp1 sites were functionally redundant and not all required for infectivity (Ross *et al.*, 1991). Various alterations of these sites seemed to change the cell type that permitted virus production as well as the replicative and cytopathic properties of HIV-1 (Ross *et al.*, 1991; Englund *et al.*, 1991; Parrott *et al.*, 1991). This contrasts with the complete loss of viral activity seen when the TAR region was altered (Leonard *et al.*, 1989). Moreover, specific NF-κB inducers can stimulate virus replication but the effect varies with cell type—TNF-α can induce virion production in certain T-cell lines but not others and LPS will apparently stimulate HIV production in monocytic/macrophage lines but not T cells (Matsuyama *et al.*, 1989; Pomerantz *et al.*, 1990). Thus viral replication seems to involve multiple levels of control of which NF-κB is only a part.

Recent work has more fully assessed the transcriptional control of the HIV LTR and has found transcriptional induction mechanisms that are independent of NF-κB. One early report identified an 86-kDa protein (HIVEN 86A) that interacted strongly with the HIV enhancer, which is now believed to be the c-Rel protein (Franza *et al.*, 1987; Ballard *et al.*, 1990,1992). Other biochemical studies have described interactions of proteins with the LTR enhancer elements that were the same size as NF-κB but had some distinctive features (Wu *et al.*, 1988a,b). Moreover, LTR transcription seems to also depend on factors that bind sites outside of the enhancer comprised of NF-κB sites, such as factor Sp1, which has three binding sites, potentially the factor NF-AT as well as other factors (Jones *et al.*, 1986; Lu *et al.*, 1991). Finally, interesting evidence has emerged that enhancer-independent inducibility of the HIV LTR may involve other proteins binding in close proximity to the TATA motif (Jones, 1989; Sakaguchi *et al.*, 1991; D. Margolis and S. Strauss, personal communication). The participation of factors other than NF-κB has been recently demonstrated in nontransformed T lymphocytes—phorbol ester or antigen

were able to induce both NF-κB and LTR transcription but TNF-α and antibodies against CD3 chains induced NF-κB and not LTR transcription (Hazan *et al.*, 1990). Also, c-*rel* may be an activator of the LTR (Muchardt *et al.*, 1992).

Other new findings introduce further considerations into the model that assumes NF-κB primarily controls whether HIV becomes transcriptionally "latent" or productively infects a target cell (Nabel, 1991; Doppler *et al.*, 1992). Recent work has shown that HIV infection causes induction of NF-κB in monocytic cell lines that typically only have very low levels of this factor (Bachelerie *et al.*, 1991; Neuvent *et al.*, 1991; Suzan *et al.*, 1991; Roulston *et al.*, 1992). This suggests that NF-κB is involved in an amplification loop that contributes to mantaining productive infection. Other work suggests that a protease encoded by the virus can cleave the p105 precursor to activate binding of the p50 subunit of NF-κB (Riviere *et al.*, 1991). Therefore, infection by the virus alters the host cell by inducing NF-κB, thereby making the environment more hospitable for its own replication. To adhere to the original model of transcriptional latency, a means of silencing NF-κB driven LTR transcription following infection must also be contemplated.

B. HTLV-1 and NF-κB

HTLV-1 is the etiological agent of the adult T-cell leukemia (ATL) (Yoshida and Seiki, 1987; Wong-Staal *et al.*, 1983, reviewed in Broder and Gallo, 1985). The cellular and molecular basis of HTLV-1 transformation is not completely understood but one protein, Tax, of the three proteins encoded by the pX region of the HTLV-1 genome has been strongly implicated in oncogenesis (Nerenberg *et al.*, 1987; Pozzati *et al.*, 1990). Tax is a transcriptional trans-activator but does not bind to DNA or stimulate the activity of *cis*-acting control elements directly. Its effects are independent of new protein synthesis (Jeang *et al.*, 1988). It appears to transmit activation signals through other transcription factors including NF-κB (Ballard *et al.*, 1988; Leung and Nabel, 1988; Ruben *et al.*, 1988; Cross *et al.*, 1989; Ruben and Rosen, 1990; Mauxion *et al.*, 1991). The Tax protein appears to be the only component of HTLV-1 that is necessary and sufficient to induce NF-κB, but this is not completely clear (Arima *et al.*, 1991; LaCoste *et al.*, 1991). By using NF-κB and other transcription factors to trans-activate a set of genes that could foster T-cell proliferation, infected cells could be predisposed to additional mutations that would cause outright transformation (Yoshida and Seiki, 1987).

Consistent with this hypothesis, the Tax protein of HTLV-1 and the HTLV-2 trans-activator protein activate genes involved in T-cell growth (Greene *et al.*, 1986; Wano *et al.*, 1988). The genes for both IL-2 and the IL-2 receptor α chain are stimulated by Tax-induced NF-κB (Inoue *et al.*, 1986; Cross *et al.*, 1987; Maruyama *et al.*, 1987; Siekevitz *et al.*, 1987a; Ballard *et al.*, 1988; Leung and Nabel, 1988; Ruben *et al.*, 1988; Bohnlein *et al.*, 1989b; Hoyos *et al.*, 1989). Other genes characteristically expressed following antigen stimulation of T cells are also up-regulated by Tax-induced NF-κB, including c-*fos* (Fujii *et al.*, 1988), GM-CSF (Green *et al.*, 1989; Schreck and Baeuerle, 1990), IL3 (Miyatake *et al.*, 1988), the β-interferon gene (LeBlanc *et al.*, 1990), and lymphotoxin (Paul *et al.*, 1990). In addition to contributing to the process of neoplasia, these NF-κB-stimulated genes may cause other sequellae in HTLV-1-infected individuals. One life-threatening complication of ATL is hypercalcemia, which may be the result of bone resorption due to the osteoclast-activating properties of lymphotoxin (Paul *et al.*, 1990). Tax also activates other NF-κB-responsive enhancer elements such as the PRD II motif in the β-interferon gene (LeBlanc *et al.*, 1990). Tax deregulation of NF-κB therefore sets in motion a genetic program that causes malignancy and other pathological consequences.

Tax is also a potent trans-activator of the HTLV-1 LTR (Sodroski *et al.*, 1984; Felber *et al.*, 1985; Seiki *et al.*, 1986; Fujisawa *et al.*, 1985) as well as the viral enhancers from HIV-1 and SV-40 (Siekevitz *et al.*, 1987b; Bohnlein *et al.*, 1988, 1989a; Marriott *et al.*, 1990; Miyatake *et al.*, 1988; Nakamura *et al.*, 1989; Zimmerman *et al.*, 1991). Interestingly, Tax transactivation of its own LTR depends on a promoter region that binds constitutively expressed proteins like CREB and Jun as well as NF-κB (Yoshimura *et al.*, 1990; Xu *et al.*, 1990; Numata *et al.*, 1991). The ability of Tax to transactivate the HIV retrovirus most likely occurs through NF-κB. HTLV-1 could potentially exacerbate the progression of AIDS.

The mechanism by which Tax regulates NF-κB is unclear. Tax expression in the cell leads to greater NF-κB binding activity and may require other cellular proteins (Ballard *et al.*, 1988; Leung and Nabel, 1988; Ruben *et al.*, 1988, 1989; Cross *et al.*, 1987). Tax has been found to increase the level of mRNA for both c-*rel* and the p50 subunit of NF-κB (Arima *et al.*, 1991). It is also likely that Tax releases NF-κB from the cytoplasmic complex with IκB, but this is difficult to reconcile with the fact that Tax is typically a nuclear protein (Goh *et al.*, 1985; Kiyokawa *et al.*, 1985). Strikingly, soluble Tax protein can be taken up by tissue culture cells and activate NF-κB (Lindholm *et al.*, 1990, 1992). In this situation, Tax is found in both the nucleus and the cytoplasm. Because NF-κB is not the only transcription factor induced by Tax, it is interesting to know whether

different parts of the protein stimulate distinct transcription factors. In fact, by placing missense mutations in the Tax coding regions, Smith and Greene have shown that different domains are utilized to activate genes through the NF-κB or CREB/ATF pathways (1990).

C. Other viruses using NF-κB

Several studies have shown that cytomegalovirus uses NF-κB to stimulate its own transcription. Five potential NF-κB binding sites can be found in the defined enhancer region of the virus (Henninghausen and Fleckenstein, 1986). The NF-κB sites in the cytomegalovirus enhancer were found to be important for the transcription of the viral immediate early 1 gene (ie1) (Nelson and Groudine, 1986; Ghazal *et al.*, 1988; Cherrington and Mocarski, 1989). Moreover, the protein product of the ie1 gene was found to activate the binding of NF-κB and thereby autoregulate the expression of its own gene (Sambucetti *et al.*, 1989). Similar to the paradox of Tax activation of NF-κB, ie1 is a nuclear phosphoprotein and therefore it is unclear whether it could directly release NF-κB from its cytoplasmic complex with IκB.

Other DNA viruses such as herpes simplex virus and human herpes virus 6 appear capable of inducing NF-κB through virally encoded trans-activating proteins and may themselves be regulated by NF-κB (Gimble *et al.*, 1988; Ensoli *et al.*, 1989; Martin *et al.*, 1991). These viruses along with cytomegalovirus are frequently carried by HIV-infected individuals and could provoke the progression of AIDS by their ability to stimulate HIV expression through NF-κB (Ensoli *et al.*, 1989; Lusso *et al.*, 1989). Also potentially important for the pathogenesis of AIDS is the fact that a trans-activator from the hepatitis B virus, the X gene, uses NF-κB to stimulate transcription of the HIV LTR, cellular genes such as MHC class I, and other viral control regions (such as the SV-40 enhancer) (Twu *et al.*, 1989a,b; Twu and Robinson, 1989; Siddiqui *et al.*, 1989; Faktor and Shaul, 1990; Zhou *et al.*, 1990; Luber *et al.*, 1991; Lucito and Schneider, 1992; Meyer *et al.*, 1992). Adenovirus-5-transformed cells have high levels of nuclear NF-κB and strongly express the β-IFN and MHC class I genes (Nielsch *et al.*, 1991). NF-κB has also been implicated in the lymphoid-specific transcription of the E3 promoter of adenovirus (Williams *et al.*, 1990) and the core enhancer of SV-40 (Kanno *et al.*, 1989; Macchi *et al.*, 1989; Phares and Herr, 1991). The organization of viral control elements, such as for SV-40, is extremely compact and appears to contain binding sites for a great variety of cellular transcription factors, perhaps to allow expression in many cell types (Fromental *et al.*, 1988).

IX. Other Proteins That Bind κB Motifs

The widespread use of NF-κB in gene regulation has engendered a plethora of studies that describe a large number of κB site–protein complexes. One unexpected turn in our emerging understanding of NF-κB was the finding that two different protein structures are capable of high affinity recognition of κB motifs—the Rel domain and the zinc-finger domain. The Rel domain appears to contain distinct subdomains that are involved in DNA binding and dimerization.

Molecular cloning studies have identified multiple genes that contain each domain. Other studies have been completely descriptive and essentially survey the size of proteins that form high affinity interactions with a particular κB DNA sequence using either electrophoretic mobility shift or UV crosslinking assays. For example, Shakov *et al.* (1990) and Xanthoudakis and Hiscott (1990) describe sets of proteins that bind to the κB binding sequences in the TNF-α and the β-interferon genes, respectively. After detailed characterization by molecular cloning or biochemical analysis, some proteins have been found to have properties of special interest. These include MBP-1/PRDII BFI/HIV-EBP-1; beta; and H2-TF1.

A. MBP-1/PRDII/HIV-EBP-1: A Large Zinc-Finger Protein

MBP-1/PRDII BF1/HIV-EBP-1 is a protein that bears a zinc-finger DNA binding motif that has been cloned and analyzed by several different groups. It was originally identified by screening a bacteriophage expression library using the mouse MHC class I ($H\text{-}2K^b$) enhancer site as a probe (Singh *et al.*, 1988). Because of some combination of its affinity, molecular structure, and abundance, it has been cloned from bacteriophage expression libraries also with the PRDII motif from the β-interferon gene and a κB motif from the HIV enhancer (Fan and Maniatis, 1990; Nomura *et al.*, 1991). Since it displayed high affinity for κB sites, it was initially believed to be a clone for NF-κB itself. The gene for MBP-1/PRDII-BFI/HIV-EBP-1 has been mapped to human chromosome 6p22.3-p24 (Gaynor *et al.*, 1991). Several other κB binding proteins bearing a zinc-finger DNA binding domain that are homologues to MBP-1/PRDII-BFI/HIV-EBP-1 have also been cloned. These are HIV-EBP-2 (Nomura *et al.*, 1991), AT-BP1 and AT-BP2 (Mitchelmore *et al.*, 1990), MBP-2 and KBP-1 (Rustgi *et al.*, 1990), AGIE-BP-1 (Ron *et al.*, 1991), and αA-CRYBP1 (Nakamura *et al.*, 1990). Recent evidence suggests this is a large gene family (Ron *et al.*, 1991). Some of these have been postulated to play roles in specific genes such as αA-CRYBP1 in regulating expression of the α1-crystallin gene (Nakamura *et al.*, 1990).

A key feature of certain members of this family of zinc-finger domain genes is that they specify very long mRNAs (9–10 kb) and encode proteins of immense size (200–300 kDa) (Maekawa *et al.*, 1989; Fan and Maniatis, 1990; Nomura *et al.*, 1991). These are probably the largest DNA binding transcription regulatory proteins known. One of the remarkable features of these proteins is that they contain zinc-finger domains that are separated by a stretch of 50 kDa of intervening protein sequence (Fan and Maniatis, 1990; Baldwin *et al.*, 1990). The zinc-finger domains, though differing in primary sequence, display very similar, if not identical, binding specificities (Baldwin *et al.*, 1990). Also interesting is that the mRNA for these genes is induced by mitogens in T-lymphoma cells or virus and serum in fibroblasts (Baldwin *et al.*, 1990; Fan and Maniatis, 1990). Some members of this gene family appear to be highly expressed in brain (Nomura *et al.*, 1991).

B. Beta: A Brain-Specific Regulator

Analysis of nuclear protein extracts from various regions of the rat brain for binding to the Ig κB binding site revealed a novel mobility shift complex that migrates more slowly than NF-κB (Korner *et al.*, 1989). The complex was not found in extracts from a variety of visceral organs and was detectable in the gray but not the white matter of the brain. It is notable that the proenkephalin gene is highly expressed in these tissues and a binding site for the beta protein is found in the promoter of this gene. Moreover, it was shown that κB site-dependent reporter constructs were active when transfected into primary cultures of cerebeller neurons or astrocytes. Because of its restriction to certain areas of the brain and large apparent size on mobility shift, it may be derived from one of the zinc-finger protein genes whose mRNA is highly expressed in the brain (Nakamura *et al.*, 1990).

C. H2-TF1: A Putative MHC Class I Regulator

Another factor that has been characterized biochemically but has remained elusive in its molecular identity is H2-TF1. Originally identified by Baldwin and Sharp (1987), this factor bound to the palindromic κB motif (5′-TGGGGATTCCCCA-3′) located in the H-2K^b gene and made base contacts that were distinct from those made by NF-κB. Though it has been suggested that H2-TF1 and p50 are identical (Baeuerle, 1991), UV cross-linking experiments indicate that the H2-TF1 complex is due to a 125-kDa protein (R. Scheinman and A. Baldwin, unpublished results). The DNA

site bound by H2-TF1 contributed to the basal activity and α-interferon inducibility of this class I promoter and was found to be conserved in a number of other class I genes (Kimura *et al.*, 1986; Baldwin and Sharp, 1987; Hakem *et al.*, 1989; Ackrill and Blair, 1989). Interestingly, the level of an H2-TF1-like binding protein and the activity of the MHC class I gene enhancer are suppressed by N-*myc* in rat neuroblastoma tumor cells. This seems to be due to suppression of a particular isoform of protein kinase C by N-*myc* (Lenardo *et al.*, 1990; Henseling *et al.*, 1990; Bernards, 1991).

Other proteins that have some binding specificity related to NF-κB include a binding protein to the MHC class II promoter (Gravallese *et al.*, 1989), a protein called KBF-2 that is detected in undifferentiated teratocarcinoma cells (Israel *et al.*, 1989); a protein binding to the HIV enhancer (named EBP-1, but of unknown relationship to the cloned zinc-finger protein named HIV-EBP1) (Clark *et al.*, 1988, 1990; Clark and Hay, 1989); and NF-GMa, a regulator of the expression of the GM-CSF gene (Shannon *et al.*, 1988). One recent report suggests the NF-AT can bind to certain NF-κB sites (McCaffrey *et al.*, 1992).

X. Conclusions

In addition to giving the overall picture of NF-κB in gene regulation, we hope we have also illuminated the weave of this rich tapestry of discovery by a large number of investigators. Knowledge of the various DNA binding proteins in this system has emerged from two long-standing parallel lines of investigation on NF-κB and the *rel* oncogene. Gene cloning has recently brought these fields together to the enrichment of both. Amino acid sequence similarities suggest that the NF-κB/Rel/*dorsal* system arose early in evolution to assume a variety of roles in gene regulation among both vertebrates, invertebrates, and possibly in yeast (Toyama *et al.*, 1992). Importantly, the wide utilization of NF-κB and its congeners in gene control indicates that work in this area will significantly influence many areas of molecular biology in the future.

In closing, it is important to note that studies on NF-κB have the ability to provide new and intriguing answers to old questions in gene regulation. An important paradox in the notion that proteins regulate genes is that the expression of the gene-regulatory proteins would seem to require an infinite regression of gene control proteins. NF-κB illustrates a trans-activation mechanism that does not require new protein synthesis and thus can be directly induced independently of the transcription of its own gene. Another question has been how signal pathways connect the cell membrane to the nucleus. NF-κB itself can convey a signal from the

cytoplasm directly to gene control elements in the nuclear DNA. By transmitting signals across the cell, NF-κB acts as a unique type of second messenger specifically designed for gene regulation. Certain other regulatory questions remain unexplained. We do not understand how NF-κB binding sites and transcriptional initiation sites that are separated by widely varying lengths of DNA communicate with each other within chromatin. Another mystery yet unsolved is how the same regulatory factors can uniquely activate various sets of genes in response to selected stimuli. This phenomenon hints at an information system for controlling RNA synthesis that will be far more complex and flexible than the genetic code that governs protein synthesis.

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NOTE ADDED IN PROOF: Table III is based on discussions by several principal investigators in the NF-κB/Rel/IκB field at a recent meeting sponsored by the Howard Hughes Medical Institutes (November 1992; Bethesda, MD). The following recommendations were made to simplify and clarify the nomenclature for NF-κB/Rel/IκB proteins and genes.

TABLE III

Recommended Nomenclature for NF-κB/Rel/IκB Proteins and Genes[a]

Recommended names	Former/alternative names
NF-κB/Rel proteins	
p50 (NFKB1[b])-binding protein	KBF-1
p105 (NFKB1[b])-precursor protein	p100, p97, Lyt 10, p98/p55, p52, p50B, p49
p52 (NFKB2[b])-binding protein	
p100 (NFKB2[b])-precursor protein	
v-*rel*[b]	
c-*rel*[b]	
RelA (p65)	NF-κB p65[b]
RelB[b]	I-rel
dorsal[b]	
IκB proteins	
IκB α	37 kDa; MAD-3[b]; pp40, RL/IF-1
IκB β	43 kDa, gene unknown
IκB γ	70 kDa (C-terminal portion of p105 as a separately spliced mRNA from NFKB1 gene
bcl 3[b]	46–56 kDa
Δbcl 3	37 kDa
cactus[b]	

[a] G. Nabel, and I. Verma, personal communication. To be published in *Genes and Development,* January, 1993.

[b] Designation of the gene encoding the protein. For example, for IκBα, MAD3 is the name of the gene and IκBα is the name of the protein complex, so that in certain circumstances it may be appropriate to use the designation IκBα/MAD3.