

Inducibility of κ Immunoglobulin Enhancer-Binding Protein NF- κ B by a Posttranslational Mechanism

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Summary

NF- κ B is a nuclear protein, found only in cells that transcribe immunoglobulin light chain genes, that interacts with a defined site in the κ immunoglobulin enhancer. This protein can be induced in pre-B cells by stimulation with bacterial lipopolysaccharide (LPS). The induction involves a posttranslational activation, and the combined action of LPS and cycloheximide causes a superinduction. An active phorbol ester also induces this factor, and with kinetics more rapid than those for LPS stimulation. Phorbol-ester-mediated induction of NF- κ B was observed in a T cell line (Jurkat) and a nonlymphoid cell line (HeLa), and is therefore not restricted to B-lymphoid cells. We interpret these results to indicate that factors that control transcription of specific genes in specific cells may be activated by posttranslational modification of precursor factors present more widely.

Introduction

The ability to reintroduce modified genes into cells has allowed the definition of regulatory sequences that specify appropriate gene expression. Such experiments have led to the identification of novel regulatory elements known as enhancers (Serfling et al., 1985). These elements have been defined as *cis*-acting DNA sequences that increase the levels of transcription when placed in either orientation at large distances from a promoter. Although enhancers were originally observed in viral genomes, a large number of tissue-specific (Banerji et al., 1983; Gillies et al., 1983; Queen and Baltimore, 1983; Queen and Stafford, 1984; Picard and Schaffner, 1984) and inducible genes (Karin et al., 1984; Treisman, 1985; Payvar et al., 1983; Goodbourn et al., 1985) have now been shown to be regulated by sequences having similar properties. Many elements originally identified as enhancers of transcription have been shown to be subject to negative regulation as well (Velcich and Ziff, 1985; Borelli et al., 1984; Hen et al., 1985). The many levels of regulation—positive, negative, and inducible—mediated by enhancer sequences make them important elements in the study of gene expression.

The mechanisms by which enhancers act is not known, although various theories have been put forward (for a recent review see Serfling et al. [1985]). There is good evidence that these sequences represent binding sites for *trans*-acting factors that modulate transcription. Thus,

Ephrussi et al. (1985) and Church et al. (1985) have reported *in vivo* footprinting analysis of a putative B-cell-specific enhancer-binding protein that interacts with the immunoglobulin heavy chain enhancer, and *in vitro* (Sassone-Corsi et al., 1985) and *in vivo* experiments (Mercola et al., 1985) have shown that enhancer effects may be competed away by the presence of large excesses of the enhancer sequence.

As a step toward understanding the mechanism of tissue-specific enhancer function, we have recently reported the identification of five factors that interact with the B-cell-specific heavy chain and κ light chain enhancers (Sen and Baltimore, 1986; Staudt et al., 1986; Weinberger et al., 1986; Singh et al., 1986). The most interesting of these factors, NF- κ B, is one that interacts only with the κ enhancer and appears to be stage-specific within the lymphoid lineage, being expressed in mature B cells and plasma cells but not in pre-B cells or T cells (Sen and Baltimore, 1986). This distribution corresponds exactly to those cell types in which κ transcription is normally active, which indicates that this factor may be necessary for κ gene expression.

The cell line 70Z/3 phenotypically represents a pre-B cell because it synthesizes cytoplasmic μ heavy chain but no detectable light chain (Paige et al., 1978; Sakaguchi et al., 1980). However, it does contain a functionally rearranged, though transcriptionally inactive, κ locus (Perry and Kelley, 1979; Maki et al., 1980). 70Z/3 cells may be stimulated by the B cell mitogen bacterial lipopolysaccharide (LPS) to undergo an apparent transition from a pre-B to a B cell, during which κ transcription is initiated, κ protein is synthesized, and the assembled IgM molecule is transported to the cell surface. Activation of κ transcription is postulated to proceed via activation of the κ enhancer because a DNAase I-hypersensitive site associated with the enhancer is also induced upon cell treatment with LPS (Parslow and Granner, 1982). Further evidence for the role of NF- κ B as a critical activator of the κ enhancer would require demonstration of its inducibility in 70Z/3 and other pre-B cell lines after an appropriate stimulus.

We report here that this factor is indeed induced in 70Z/3 cells after the cells are treated with LPS. Furthermore, induction of NF- κ B appears to involve a posttranslational modification mediated either directly or indirectly by protein kinase C. Our results imply that tissue-specific posttranslational modifications may generate factors that regulate gene expression, and thus the data bear on the general problem of activation of tissue-specific genes.

Results

NF- κ B Can Be Induced in Pre-B Cell Lines by LPS

To examine whether NF- κ B might be inducible in 70Z/3 cells, we stimulated these cells with LPS for 20 hr and assayed nuclear extracts derived from these cells for the presence of NF- κ B using the electrophoretic mobility shift

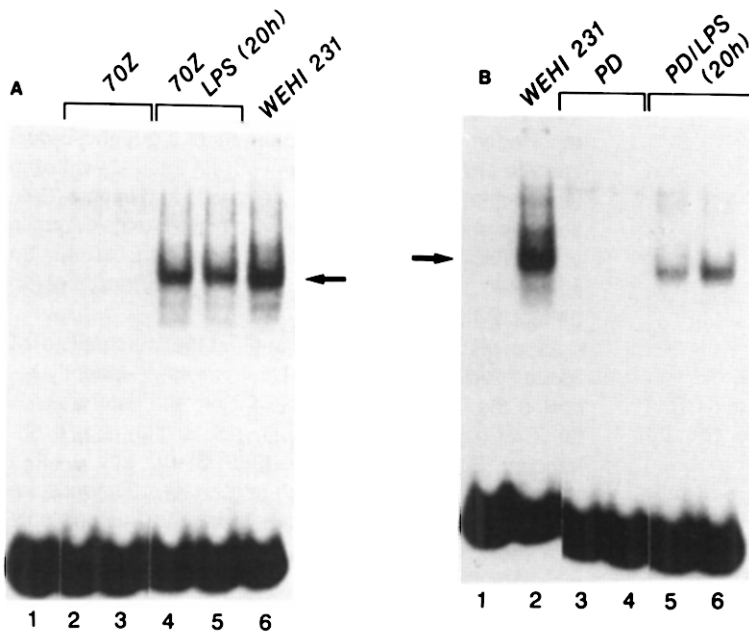


Figure 1. Inducibility of NF- κ B in Pre-B Cell Lines with LPS

(A) Electrophoretic mobility shift analysis of extracts derived from 70Z/3 cells before and after stimulation with LPS. Binding reactions were carried out for 15–30 min at room temperature, in a final volume of 15 μ l containing 9 μ g of total protein, 3.5 μ g (lanes 2 and 4) or 4.5 μ g (lanes 3 and 5) of nonspecific carrier DNA (poly d[IC]), and 0.2–0.5 μ g of probe. Reaction products were fractionated by electrophoresis through low-ionic-strength polyacrylamide gels and were visualized by autoradiography. Lane 1 contains the free DNA fragment; lane 6 contains nucleoprotein complex (arrow) generated by interaction of NF- κ B with the fragment κ 3 in a nuclear extract derived from the B cell line WEHI 231. This complex has been previously characterized by competition experiments and methylation interference experiments (Sen and Baltimore, 1986).

(B) Electrophoretic mobility shift analysis of extracts derived from PD, an Abelson murine leukemia virus-transformed pre-B cell line, before and after stimulation with LPS. Binding reaction conditions were as outlined in (A), with 3.5 μ g and 4.5 μ g of poly d(IC) used with each new extract. Nuclear extracts used were derived from PD before LPS treatment (lanes 3 and 4), PD after LPS treatment (lanes 5 and 6), or from the B cell line WEHI 231 (lane 2). Lane 1 contains free DNA fragment.

assay described previously (Singh et al., 1986). To assay for NF- κ B, a DNA fragment containing its binding site (κ 3 fragment; Sen and Baltimore, 1986) was end-labeled and then incubated with extracts derived from either unstimulated 70Z/3 cells (Figure 1A, lanes 2 and 3) or LPS-stimulated 70Z/3 cells (Figure 1A, lanes 4 and 5) in the presence of increasing amounts of the carrier poly d(IC). Unstimulated 70Z/3 cell extracts lacked a major band evident with B cell extracts (Figure 1A, lane 6; arrow). This nucleoprotein complex band was induced in the 70Z/3 cells after LPS treatment for 20 hr. The band was not competed away even with 4.5 μ g of poly d(IC) (Figure 1A, lane 5). This induction phenomenon was not restricted to the 70Z/3 cell line; another pre-B cell line, PD (Lewis et al., 1982), was weakly positive for the factor prior to induction (Figure 1B, lane 3; Sen and Baltimore, 1986), but the factor was strongly induced by LPS (Figure 1B, lanes 5 and 6). A number of other minor bands could be seen in the binding assay, some of which were inducible and others not. We have not pursued these, but have focused our attention instead on the major inducible band. This band comigrated with the major band produced by B cell and plasma cell extracts (typified by WEHI 231 extracts in Figure 1A, lane 6, and Figure 1B, lane 2). We have earlier characterized this band by competition experiments and have localized the binding site of the factor by methylation interference experiments, defining the band as one produced by interaction of the NF- κ B factor with the B site within the κ enhancer (a site containing the sequence GGGGACTTTC). Thus, two pre-B cell lines, one with a rearranged κ gene (70Z/3) and the other in the process of

undergoing rearrangement (PD), are clearly inducible by LPS for NF- κ B activity.

Induction of NF- κ B by LPS Does Not Require Protein Synthesis

Recently it has been reported by Nelson et al. (1985) and Wall et al. (1986) that induction of κ transcription in line 70Z/3 does not require new protein synthesis. Thus, induction of κ gene expression was evident in cells pretreated (10 min) with the translation inhibitors cycloheximide or anisomycin and then stimulated with LPS. Further, Wall et al. (1986) reported that κ expression could be induced in the presence of cycloheximide alone—this led them to argue in favor of a labile repressor blocking the activation of κ genes in this cell line. To determine if these characteristics of κ transcriptional activation were accompanied by changes in the levels of NF- κ B, we analyzed extracts derived from 70Z/3 cells that had been treated with LPS alone, with a translation inhibitor alone, or with both together. To be able to make direct correlations with the published reports concerning the effects of translational inhibitors on κ expression in pre-B cells, we examined a 4 hr time point in these experiments, although maximum stimulation of κ expression by LPS takes 14–20 hr. In accord with the results of transcriptional analyses, uninduced 70Z/3 cells were negative for NF- κ B (Figure 2A, lanes 2–4), and treatment with either LPS alone (Figure 2A, lanes 5–7) or cycloheximide alone (Figure 2A, lanes 11–13) for 4 hr induced the factor. Unexpectedly, stimulation of 70Z/3 with LPS in the presence of cycloheximide for 4 hr gave a superinduction of NF- κ B (Figure 2A, lanes

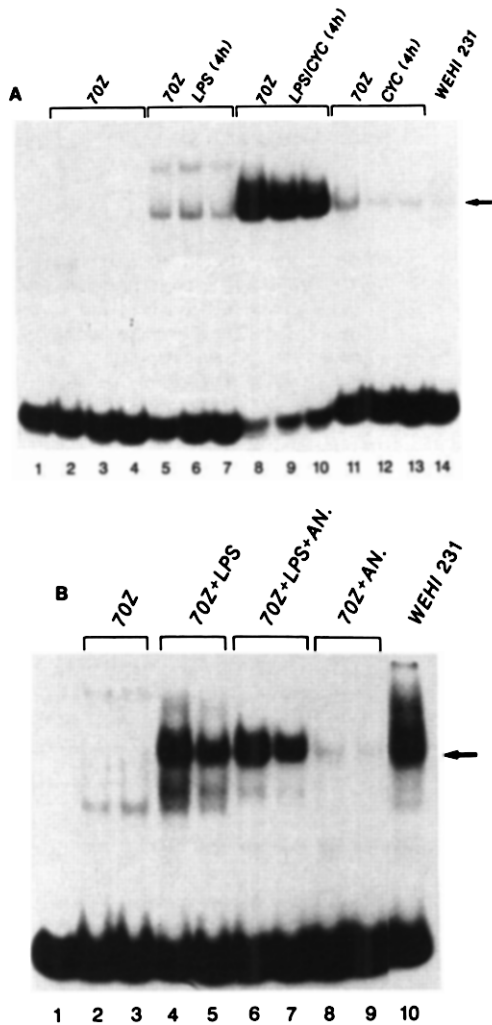


Figure 2. Effect of Translational Inhibitors on the Induction of NF- κ B in 70Z/3 Cells

(A) Effect of cycloheximide on LPS stimulation of 70Z/3 cells. Binding reactions were carried out as detailed in Figure 1A, and contained 2.5, 3.5, or 4.5 μ g poly d(I/C) with each set of extracts. End-labeled κ 3 fragment was the probe (lane 1) and was incubated with 9–11 μ g of protein from extracts derived from the following cells: untreated 70Z/3 cells (lanes 2, 3, and 4); 70Z/3 cells treated for 4 hr with 10 μ g/ml of LPS (lanes 5, 6, and 7); 70Z/3 cells treated for 4 hr with 10 μ g/ml of LPS and 10 μ g/ml cycloheximide (lanes 8, 9, and 10); 70Z/3 cells treated with 10 μ g/ml of cycloheximide alone (lanes 11, 12, and 13); and WEHI 231 cells (lane 14). The characteristic nucleoprotein complex is indicated by the arrow.

(B) Effect of anisomycin on LPS stimulation of 70Z/3 cells. Binding reactions were as detailed in Figure 1A, and contained 2.5 or 3.5 μ g of poly d(I/C). Protein was from extracts of the following: untreated 70Z/3 cells (lanes 2 and 3); 70Z/3 cells after induction with LPS alone (lanes 4 and 5); 70Z/3 cells with LPS induction in the presence of anisomycin (lanes 6 and 7); 70Z/3 cells treated with anisomycin by itself (lanes 8 and 9); and the B cell line WEHI 231 as a positive control (lane 10). The characteristic nucleoprotein complex is indicated by the arrow.

8–10): NF- κ B reached a level above that seen after a 20 hr induction. The same qualitative result was observed when anisomycin was used as a translation inhibitor (Figure 2B). Thus, the presence of anisomycin (10 μ M) during a 4 hr stimulation with LPS gave a superinduction of NF- κ B

(Figure 2B, lanes 6 and 7) relative to induction with either LPS alone (Figure 2B, lanes 4 and 5) or anisomycin alone (Figure 2B, lanes 8 and 9). Once again, prior to LPS treatment there was no detectable NF- κ B activity in 70Z/3 cells (Figure 2B, lanes 2 and 3). Although treatment of 70Z/3 cells with either cycloheximide alone or LPS alone gave approximately equivalent amounts of NF- κ B (Figure 2A; compare lanes 5–7 with lanes 11–13), the level of NF- κ B induced with anisomycin alone appeared to be much lower (Figure 2B; compare lanes 8 and 9 with lanes 4 and 5). This is probably due to drug toxicity because, even after a short exposure to anisomycin, the cells looked quite unhealthy. Presumably this also accounts for the reduced level of superinduction seen with LPS and anisomycin. Thus, the κ enhancer binding factor NF- κ B appears to be inducible in 70Z/3 cells in the absence of protein synthesis. Furthermore, it appears to be inducible by either of two different translation inhibitors alone and is superinduced when the cells are stimulated with LPS and the inhibitor.

Phorbol Ester Can Induce NF- κ B in 70Z/3

The tumor-promoting phorbol ester phorbol 12-myristate 13-acetate (PMA) has been shown to induce surface immunoglobulin in 70Z/3 cells, presumably via activation of κ transcription and transport of complete immunoglobulin to the cell surface (Rosoff et al., 1984; Rosoff and Cantley, 1985). To determine if this activation is reflected in an increase of NF- κ B, we analyzed extracts derived from 70Z/3 cells after a 4 hr stimulation with PMA at 50 ng/ml. There was a striking induction of NF- κ B activity in these extracts (Figure 3A; compare lanes 3 and 4 with lane 2). Thus, an active phorbol ester by itself is capable of inducing NF- κ B activity in 70Z/3 cells, implicating protein kinase C as a possible intermediate in the posttranslational modification reaction that produces NF- κ B in these cells (Bell, 1986; Nishizuka, 1984). An inactive phorbol ester (phorbol 12,13-didecanoate) did not cause induction of NF- κ B under similar conditions (data not shown).

Time Courses of Activation of NF- κ B by LPS and PMA Are Different

LPS-mediated stimulation of surface Ig expression or mRNA accumulation reaches a maximum after at least one cell cycle, i.e., in 14–18 hr. Recent work has shown that LPS stimulation of κ RNA synthesis, as measured by nuclear run-on assays (Nelson et al., 1985; Wall et al., 1986), can be seen as early as 4 hr after stimulation, and that the DNAase I-hypersensitive site associated with the κ enhancer can be detected as early as 1 hr poststimulation. To examine the time course of NF- κ B induction, we generated 70Z/3 cell extracts after stimulation by either LPS or PMA for varying lengths of time. Analysis for NF- κ B activity using the binding assay showed that the time course of activation of NF- κ B by these two agents was quite different (Figure 3B). With LPS alone, a nucleoprotein complex band reflecting the presence of NF- κ B was seen as early as 0.5 hr after stimulation, but the levels of NF- κ B increased until 2 hr poststimulation, after which a slight decrease occurred and then the level remained constant. By

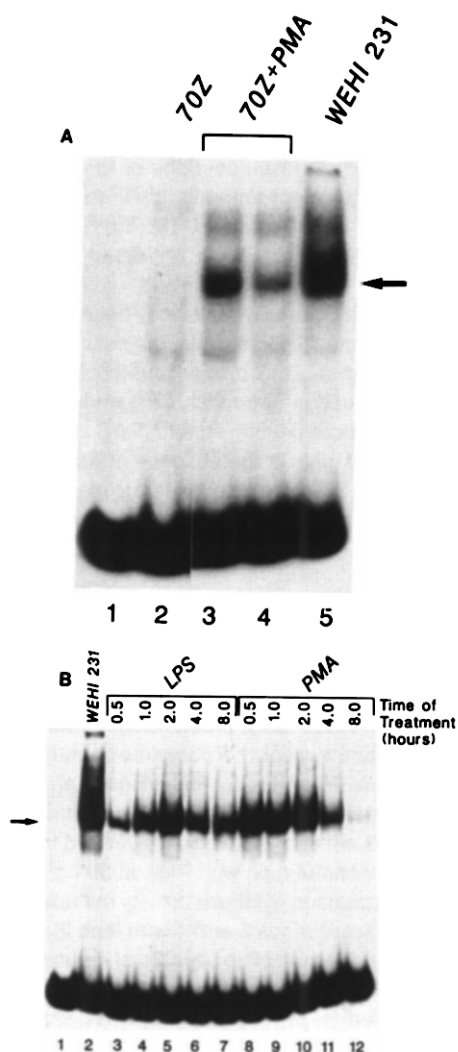


Figure 3. Effect of PMA on NF- κ B in 70Z/3 Cells
(A) Inducibility of NF- κ B by PMA. Binding reactions using κ 3 as a probe (lane 1) were carried out as detailed in Figure 1A, with protein from untreated 70Z/3 cells (lane 2) or 70Z/3 cells that had been treated with PMA at 50 ng/ml for 4 hr (lanes 3 and 4). Lane 5 is the positive control for NF- κ B in extracts from WEHI 231.
(B) Time course of induction of NF- κ B with LPS or PMA. Binding reactions were carried out with extracts derived from 70Z/3 cells that had been treated with LPS at 10 μ g/ml (lanes 3–7) or with PMA at 25 ng/ml (lanes 8–12); the time of treatment is shown above each lane. Lane 2 is a positive control for NF- κ B in WEHI 231 extracts.

contrast, in PMA-stimulated cells NF- κ B was detected at maximum levels within 0.5 hr after stimulation, remained at this level for 2–3 hr, and then began to drop off rapidly, such that by 8 hr it was barely detectable. Because prolonged exposure of cells to phorbol esters is known to decrease activity of endogenous protein kinase C (Rodriguez-Pena and Rozengurt, 1984, 1986), a possible explanation for the rapid decline of NF- κ B may be that its maintenance as a binding factor requires continuous activity of protein kinase C. A similar phenomenon has been described recently by Blemis and Erikson (1986), where S6 kinase activity (assayed by phosphorylation of S6 protein)

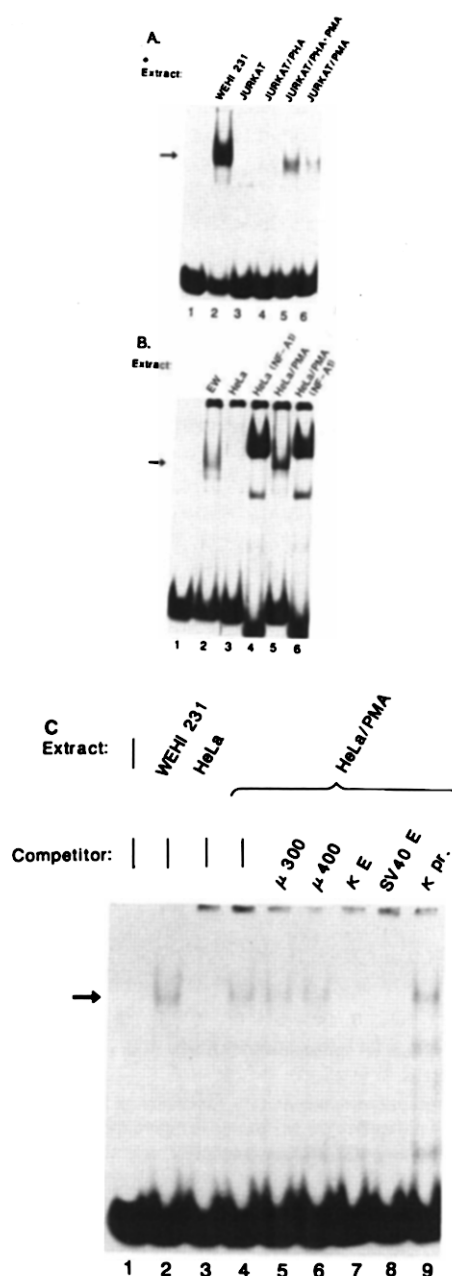


Figure 4. Induction of NF- κ B in Jurkat Cells and HeLa Cells
(A) Analysis of induced and uninduced Jurkat cells for NF- κ B. The human T lymphoma Jurkat was stimulated with PHA or PMA individually or together for 20 hr. Nuclear extracts made after treatment were analyzed by the mobility shift assay using κ 3 fragment as the labeled probe. Binding reactions typically contained 6 μ g of protein, 2.5–3.5 μ g of poly d(I,C), and 0.3–0.5 ng of end-labeled DNA probe. Lane 1, no protein added; lane 2, WEHI 231 extract (positive control); lane 3, extract from uninduced Jurkat cells; lane 4, extract from Jurkat cells stimulated with PHA alone; lane 5, extract from Jurkat cells stimulated with PHA and PMA; lane 6, extract from Jurkat cells stimulated with PMA alone. The arrow shows the position of the expected nucleoprotein complex generated by interaction of NF- κ B with κ 3 fragment.
(B) Analysis of HeLa cells for NF- κ B before and after stimulation with PMA. HeLa cells were treated with PMA (50 ng/ml) for 2 hr, and the extracts derived thereafter were analyzed for induction of NF- κ B. Binding reactions contained 15–18 μ g of protein, 2.5 μ g of poly d(I,C), and 0.3–0.5 μ g of end-labeled DNA probe. Lane 1, κ 3 fragment, no protein added; lane 2, κ 3 fragment incubated with extracts derived from the

first rises and then falls during prolonged exposure of cells to PMA. Although it has been reported that LPS may directly activate protein kinase C (Wightman and Raetz, 1984), the different kinetics of induction of NF- κ B by LPS and PMA imply that these activators feed into a common pathway through distinguishable sites of activation.

Non-Pre-B Cell Lines Can Also Be Activated to Produce NF- κ B

In our previous analysis we have shown that NF- κ B is present only in cell lines representing the B cell or plasma cell stages of B-lymphoid differentiation, but was undetectable in a variety of non-B cells, pre-B cells, and T cells (Sen and Baltimore, 1986). However, as shown above, this factor may be induced to high levels in pre-B cells stimulated with LPS. To check if this inducibility was restricted to cells having a pre-B phenotype only or was a general characteristic of the other constitutively "negative" cell lines, we have taken representative examples of other cell types (T cells and nonlymphoid cells) and examined them for induction of NF- κ B after appropriate stimulation.

The human T leukemia cell line Jurkat can be stimulated to produce interleukin 2 (IL-2) by the combined influence of phytohemagglutinin (PHA) and phorbol ester (PMA) (Gillis and Watson, 1980; Weiss et al., 1984). Nuclear extracts were prepared from Jurkat cells that had been stimulated with PHA alone, PMA alone, or both together and were analyzed for the presence of NF- κ B (Figure 4A). As originally observed, extracts derived from uninduced Jurkat cells were negative for NF- κ B activity (Figure 4A, lane 3). However, extracts made from Jurkat cells stimulated with either PHA or PMA contained detectable levels of NF- κ B (Figure 4A, lanes 4 and 6), and the extracts from the costimulated cells showed higher levels of the factor (Figure 4A, lane 5). Thus, a factor with the properties of NF- κ B can be induced in a T cell line after appropriate activation.

As an example of a nonlymphoid line we used the human HeLa cell line, which is constitutively negative for NF- κ B (Sen and Baltimore, 1986). These cells were induced with PMA for 2 hr, and extracts derived from treated

and untreated cells were analyzed for NF- κ B activity (Figure 4B). Extracts from the untreated HeLa cells (Figure 4B, lane 3) did not show a nucleoprotein complex comigrating with the complex generated in B cell extracts. However, treatment with PMA induced a factor that generated the characteristic DNA-protein complex produced by NF- κ B (Figure 4B, lane 5). As a control, extracts from both the uninduced and induced cells showed equivalent levels of the ubiquitous NF-A1 DNA-binding protein when analyzed using a probe containing the sequence ATTTGCAT (Figure 4B, lanes 4 and 6; Singh et al., 1986). Therefore, treatment of HeLa cells with PMA induces a factor that can form a nucleoprotein complex with the κ 3 fragment.

To characterize further the DNA-protein complex formed in extracts from the PMA-treated HeLa cells, we carried out competition experiments. The complex generated in extracts from PMA-induced HeLa cells (Figure 4C, lane 4) was specifically competed away by the inclusion of 50 ng of unlabeled DNA in binding reactions containing either the κ enhancer (Figure 4C, lane 7) or the SV40 enhancer (Figure 4C, lane 8), but was unaffected by two DNA fragments that together span the μ enhancer (Figure 4C, lanes 5 and 6) or by a 250 bp fragment containing the κ promoter (Figure 4C, lane 9). This pattern of competition exactly parallels the pattern observed earlier using the κ 3 fragment in binding experiments with B-cell-derived extracts (Sen and Baltimore, 1986). These results further strengthen the conclusion that NF- κ B factor can be induced to nonlymphoid cells as well as lymphoid cells following appropriate stimulation.

Discussion

We have studied the induction of a nuclear factor (NF- κ B) that can be detected in crude nuclear extracts by means of an electrophoretic mobility shift assay. NF- κ B interacts with a site (which we refer to as the B site) in the κ immunoglobulin gene enhancer (Sen and Baltimore, 1986), and its presence in nuclear extracts had previously been evident only in B cells and plasma cells. Our current analysis reveals several aspects of NF- κ B induction in cells where it is not constitutively present. First, this factor can be induced by the mitogen LPS in two cell lines representing a pre-B stage of B cell differentiation. Second, induction of this factor involves a posttranslational modification of a preexisting protein, because the induction takes place even in the presence of translation inhibitors like cycloheximide and anisomycin. Third, these translational inhibitors by themselves can at least partially induce NF- κ B, and act in synergism with LPS to produce a superinduction. Fourth, an active phorbol ester like PMA can induce NF- κ B by itself, and the time course of this activation is more rapid than that with LPS alone. Fifth, it is also possible to induce this factor in cell lines other than those having a pre-B phenotype by means of an appropriate stimulus (e.g., in the human T cell line Jurkat, by PHA and/or PMA; in HeLa cells, by PMA). Thus, B cells and plasma cells appear to support constitutive presence of this factor, whereas in other cell types the factor can be induced transiently by an appropriate stimulus.

human B lymphoma EW; lane 3, κ 3 fragment incubated with nuclear extract from uninduced HeLa cells; lane 4, μ 50 fragment (derived from the μ heavy chain enhancer and containing a copy of the conserved octamer sequence ATTTGCAT) incubated with extract from uninduced HeLa cells; lane 5, κ 3 fragment incubated with extract from induced HeLa cells; lane 6, μ 50 fragment incubated with extract from induced HeLa cells.

(C) Competition experiments to characterize the major nucleoprotein complex seen in extracts from PMA-induced HeLa cells. Binding reactions were carried out using end-labeled κ 3 fragment, 3.5 μ g of poly d(IC), and 15–18 μ g of nuclear extract in the presence of 50 ng of unlabeled competitor DNA derived from various immunoglobulin and viral regulatory sequences (lanes 5–9). Lane 1, no protein added; lane 2, WEHI 231 nuclear extracts (positive control); lane 3, extracts from uninduced HeLa cells; lane 4, nuclear extracts from PMA-induced HeLa cells. Lanes 5–9 show results of binding reactions in extracts from induced HeLa cells, with 50 ng of competitor DNA added as follows (see Experimental Procedures for the exact derivation of these fragments): lane 5, μ 300 (active fragment of the μ enhancer); lane 6, μ 400 (inactive fragment of the μ enhancer); lane 7, κ enhancer fragment; lane 8, SV40 enhancer fragment; lane 9, κ promoter fragment.

We have previously argued that the restricted tissue distribution of NF- κ B—compared with the ubiquitous distribution of other factors that bind to immunoglobulin gene enhancers (Sen and Baltimore, 1986)—makes it a possible candidate for being the critical *trans*-acting factor that mediates B-cell-specific expression of κ genes (Sen and Baltimore, 1986). Our current analysis makes this identification even stronger, because various stimuli that activate κ transcription in pre-B cell lines show parallel effects on NF- κ B activity. The results of LPS treatment provide the strongest argument because LPS both induces κ gene transcription and induces NF- κ B. This parallelism can be carried further because LPS induction of κ transcription and NF- κ B occurs in the presence of protein synthesis inhibitors (Nelson et al., 1985; Wall et al., 1986), and the inhibitors themselves partially induce both events (Wall et al., 1986). One previous study is discrepant, because anisomycin was found not to induce κ transcription in a pre-B cell (Nelson et al., 1985). We attribute that result to long-term treatment with a high concentration of a relatively toxic drug, although perhaps cells are heterogeneous in their response to inhibitors of protein synthesis.

The superinduction phenomenon we have observed has not been evident in transcription studies, possibly because lower amounts of NF- κ B may already be saturating. Although superinduction of transcription has been observed in a number of systems (e.g., *c-myc* and *c-fos*) (Kelly et al., 1983; Cochran et al., 1984), to the best of our knowledge this is the first example of superinduction of a factor that can interact with a regulatory element of DNA transcription. That a putative *trans*-acting factor may be superinduced raises the interesting possibility that the transcriptional superinduction of genes such as *c-fos* and *c-myc* may be mediated by positive-acting factors similar to NF- κ B (see Note Added in Proof).

The final association of NF- κ B with κ transcription comes from studies with PMA. This compound induces surface immunoglobulin on 70Z/3 cells—an indication that it induces κ transcription—and rapidly induces NF- κ B. This and the other correlations of NF- κ B activity and κ transcription argue strongly in favor of this enhancer-binding factor being critical for κ gene transcription, but other studies will be needed to determine unequivocally that the relationship is causal.

The rapidity of PMA action deserves comment. We examined this protein kinase C activator because of evidence that LPS-induced B cell mitogenesis involves protein kinase C activation directly (Wightman and Raetz, 1984; Bijsterbosch et al., 1985). The much faster response of cells to PMA than to LPS is consistent with the notion that LPS binds to a receptor that then activates phospholipase C, which in turn generates inositol trisphosphate and diacylglycerol, the latter being a physiological activator of protein kinase C (Berridge, 1984). PMA can act directly on protein kinase C, thus bypassing the earlier steps and generating a faster response.

An important aspect of our analysis is the demonstration that a cell-specific enhancer-binding protein may be induced by the posttranslational modification of a preexisting protein. Although it has been widely conjectured

that the activity of tissue-specific genes must be regulated by tissue-specific *trans*-acting factors (Blau et al., 1983; Chin and Blau, 1984, 1985; Enoch et al., 1986; Baron and Maniatis, 1986), such hypotheses reformulate the regulatory problem around the issue of regulating the tissue-specific expression of the *trans*-acting factor. A solution to this regulatory regression problem would be a preexisting factor that itself is regulated not by transcription but by interactions with an inducer. For κ gene expression, the preexistence of an NF- κ B precursor brings the regulatory regression chain to an immediate halt. The precursor to NF- κ B is present in pre-B cells (and in many other cells), and our data suggest that it can be activated to constitutive expression when a cell responds to either internal or external signals during ontogeny. During physiological differentiation, induction of gene expression has to become permanent and independent of the inducer. We have no evidence on how that might be achieved for NF- κ B, but perhaps when it is understood how LPS induces NF- κ B (presumably through protein kinase C), we will be in a better position to consider how the induction might be made permanent.

Although it would appear that NF- κ B is an important regulator of κ gene expression, it is surprisingly not a protein restricted to B-lymphoid cells. HeLa cells treated with PMA contain a protein with the binding specificity of NF- κ B and that gives the same-sized complex with specific DNA fragments as NF- κ B; it appears, by all criteria we have, to be NF- κ B itself. Jurkat cells also contain an indistinguishable protein. The site for NF- κ B binding is also not restricted to the κ enhancer; although NF- κ B is not found in the μ enhancer or in other B-cell-related genetic regions, it is present in many viral enhancers (SV40; cytomegalovirus [Boshart et al., 1985]); human immunodeficiency virus [our unpublished observation]), and we would not be surprised if it were found in other cellular enhancers when a sufficient number are investigated. We are thus driven to the notion that although the NF- κ B protein and the B site are used for a specific purpose during B lymphocyte differentiation—namely, activation of κ transcription—this regulatory loop is not dedicated to this single event but is used more broadly in transcriptional regulatory circuits.

Based on their demonstration that cycloheximide alone can induce κ transcription in 70Z/3 cells, Wall et al., (1986) proposed that a labile inhibitor blocks transcription in the uninduced line. More specifically, they argued that this factor might bind to the κ locus, thus preventing transcription of the gene. We would argue from its constitutive tissue distribution, and from our present results, that NF- κ B is a positive activator of κ transcription. Because NF- κ B activity is induced by cycloheximide alone, the labile repressor (if there is one) probably interacts with NF- κ B itself rather than the chromosomal DNA. It is also quite possible that there is no labile repressor, but rather that the inhibition of protein synthesis has consequences (such as protein kinase C activation) that lead to activation of NF- κ B. Because NF- κ B is superinducible, there presumably exists a direct activation pathway, one that might act in synergism with the effect of a putative labile repressor.

An obvious candidate for the posttranslational activation event, particularly given the rapid involvement of protein kinase C, is a phosphorylation of pre-NF- κ B. In such a model, a labile repressor could be either a phosphatase or a molecule that blocks the site of phosphorylation by direct interaction with pre-NF- κ B. Alternatively, protein kinase C could phosphorylate the inhibitor, releasing it from interaction in the NF- κ B. It would appear that such an inhibitor is not B-cell-specific, which suggests that stabilization of NF- κ B in B cells is a consequence of inaccessibility of the inhibitor to NF- κ B, perhaps because of some form of permanent inactivation.

Experimental Procedures

Cell Lines and Extracts

70Z/3 and PD cells were grown in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum, 50 μ M β -mercaptoethanol, and penicillin and streptomycin sulfate (pen-strep). LPS (GIBCO) stimulation was carried out at a concentration of 10–15 μ g/ml. For experiments with protein synthesis inhibitors and LPS, cell cultures were treated with inhibitors approximately 20 min prior to addition of LPS. Cycloheximide (Sigma) was used at 10 μ g/ml, which causes more than 95% inhibition of protein synthesis in 70Z/3 cells (Wall et al., 1986). Anisomycin (Sigma) was used at 10 μ M, which causes approximately 99% inhibition of protein synthesis in HeLa cells (Grollman, 1967). Phorbol ester activation of 70Z/3 cells was carried out using the active ester PMA or the inactive ester phorbol 12,13-didecanoate at a concentration of 25 ng/ml for the times indicated in the text. All treatments were carried out at cell densities varying between 5×10^5 – 10^6 /ml. Jurkat cells were grown in RPMI 1640 medium with 10% inactivated fetal calf serum and pen-strep. PHA treatment was done at 5 μ g/ml, and PMA treatment was at 50 ng/ml. HeLa cells were grown in MEM medium with 5% horse serum and pen-strep. Phorbol ester (PMA) treatment was at 50 ng/ml with a cell density of 7×10^5 – 10^6 /ml.

Nuclear extracts were generated essentially according to the protocol of Dignam et al. (1983), and protein concentrations were determined using a Bradford assay with serum albumin standards.

Gel Binding Analysis

Gel binding analyses were carried out as described earlier using a radioactive Ddel–HaeIII fragment (κ 3) derived from the κ enhancer (Sen and Baltimore, 1986). Levels of NF- κ B induced by various stimuli were normalized to total protein present in the extracts. Furthermore, analysis with a fragment that contains a binding site for the ubiquitous factor NF-A shows that this nuclear protein remains at approximately constant levels in all of the extracts reported here. Thus, the modulation of NF- κ B activity is not a reflection of variability of nuclear factors in general under these conditions. For competition experiments, the specific and nonspecific competitor DNAs were included in the mixture (in amounts described in Figure 4C) prior to addition of the protein. The competitor fragments μ 300, μ 400, κ E, and SV40E, which have been described earlier (Sen and Baltimore, 1986), were isolated from low-melting-point agarose gels and were quantitated by spotting onto ethidium-bromide-containing agarose plates.

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Note Added in Proof

Recently, Prywes and Roeder (*Cell* 47, 777-784, 1986) and Hayes et al. (*Proc. Natl. Acad. Sci. USA*, in press) have demonstrated binding of an inducible factor to the *c-fos* regulatory region.