The plasmacytoma J558L lacks constitutively active NF- κ B and is deficient in early response gene activation

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ABSTRACT

In mature B cells the nuclear factor NF- κ B which binds within the kappa enhancer is constitutively present in the nucleus. However, the λ light chain producing myeloma J558L has been found to lack constitutively functional NF- κ B. Deoxycholate released functional NF- κ B from cytoplasmic extracts and functional NF- κ B was present in J558L following cycloheximide but not phorbol ester treatment. J558L was also unable to respond to phorbol ester stimulation with synthesis of mRNA from the early response gene TIS11. J558L differs from S107, another myeloma which was found to be deficient in the synthesis of NF- κ B but not in the activation of TIS11. Somatic cell hybrids were used to further define the defect in J558L; hybrids were made with the myelomas S107 and S194 and the pre-B cell line 70Z/3. In general, complementation of the defect in J558L was observed; however there was not a direct correlation between the levels of TIS11 mRNA and NF- κ B expression in the somatic cell hybrids, suggesting that the pathways of activation of these genes, while possibly sharing common elements, are not identical. The defect in J558L was surprising given that it has frequently been used for the expression of transfected light chain genes.

INTRODUCTION

NF- κ B, a transcription factor related to rel (1,2) can exist in either an active DNA binding form in the nucleus or as an inactive cytoplasmic precursor bound to its inhibitor I κ B (3). Originally NF- κ B was believed to be restricted to B lymphocytes since only cell lines derived from mature B cells had high levels of the constitutively active form (4). However, it was soon discovered that the active form of NF- κ B could be induced in other cell types following stimulation with phorbol ester (5). Therefore, NF- κ B is not B cell specific but is instead present in the majority of cell types in the inducible form. A variety of substances including viruses, viral transactivators, mitogens, protein synthesis inhibitors and cytokines serve to activate NF- κ B (reviewed in 6).

During B cell development there is an ordered expression of immunoglobulin genes. The heavy chain is the first gene to be expressed; only when a functional heavy chain polypeptide has

been expressed does rearrangement and expression of light chains begin. In the vast majority of cases λ light chain rearrangement and expression occurs only if there has been aberrant rearrangement of both κ alleles. After productive rearrangement of both heavy and light chain genes, rearrangement stops. *Cis* acting control regions contribute to this ordered expression of immunoglobulin genes. Tissue specific promoters and enhancers have been identified for both the heavy chain (7,8,9) and light chain loci (10,11,12,13).

Expression of active NF- κ B appears to contribute to this developmental program. In immature B cells activation of transcription of the endogenous κ gene correlates with induction of active NF- κ B; activation of NF- κ B in pre-B cells activates transcription of both the rearranged and germline κ loci (14). In most mature B cells active NF- κ B persists although it appears that κ transcription becomes NF- κ B independent. An exception of this rule is the κ light chain producing plasmacytoma S107 which no longer has functional NF- κ B; however when hybridized to a pre-B cell, S107 can activate the NF- κ B contributed by the pre-B cells. The defect in S107 appears to be in the synthesis of NF- κ B (15,16 and current study), not in the activation pathway.

We now report the characterization of a second myeloma cell line, J558L, lacking constitutively active NF- κ B. J558L differs from S107 however in that the defect appears to be not in the synthesis of NF- κ B, but instead in its activation. Large amounts of inactive NF- κ B are present in the cytoplasm of J558L, but treatment with activators like phorbol ester does not result in dissociation from I κ B and nuclear translocation. The activation defect in J558L also manifests itself in an inability to activate another primary response gene, TIS11. Somatic cell hybridization can complement the activation defect in J558L. J558L also differs from S107 in that it can be used for the expression of transfected light chain genes (17).

MATERIALS AND METHODS

Transient Transfection and CAT Assays of Eucaryotic Cells

The DEAE-dextran method of Grosschedl and Baltimore was used to introduce DNA into the lymphoid cell lines into these studies (18). Cells to be transfected were fed the previous day and seeded to ensure logarithmic growth at the time of

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transfection. Cells collected by centrifugation were resuspended at 2×10^7 cells per $0.75 \mu\text{l}$ at room temperature in 25 mM Tris-Cl (pH 7.4), 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl_2 , 0.5 mM MgCl_2 , 0.6 mM Na_2HPO_4 (TBS). $0.75 \mu\text{l}$ of a $1 \mu\text{g}/\text{ml}$ DEAE-dextran (Pharmacia, approximately 500,000 Dalton molecular weight) TBS solution was pipetted into a separate tube, and $10 \mu\text{g}$ of plasmid DNA added. The transfection was initiated by addition of $0.75 \mu\text{l}$ of cells to the $0.75 \mu\text{l}$ of DEAE-dextran-DNA solution and the mixture maintained at room temperature for 30 minutes and finger vortexed at 10 minute intervals. The transfection was terminated by the addition of $8 \mu\text{l}$ of DMEM plus 50 mM Tris (pH 7.5). The cells were pelleted as before and then resuspended with $10 \mu\text{l}$ of DMEM plus 50 mM Tris (pH 7.5). The cells were again pelleted and resuspended in growth medium supplemented with 100 U/ml of nystatin and $0.1 \mu\text{g}/\text{ml}$ gentamycin sulfate.

The cells were harvested 44 to 48 hours post transfection, suspended in $150 \mu\text{l}$ ice cold 50 mM Tris (pH 7.5) and the cell contents released by four cycles of freeze-thawing. The cell supernatant was transferred to a second eppendorf tube and heated at 65°C for 10 minutes to inactivate deacetylases. The cloudy cell extract was clarified by 5 minutes microcentrifugation and the supernatant used for CAT assays.

The bacterial reporter gene chloramphenicol acetyl transferase (CAT) joined to a 230 bp Pst I-Hae III fragment with a λ light chain promoter was used as an assay of gene expression (19). Typically $50 \mu\text{l}$ of extract, $70 \mu\text{l}$ 1 M Tris (pH 7.8), $20 \mu\text{l}$ 4mM acetyl-CoA (Pharmacia), $2 \mu\text{l}$ [^{14}C] chloramphenicol ($200 \mu\text{Ci}/\text{ml}$) and $10 \mu\text{l}$ H_2O were incubated at 37°C for 1 hour. The acetylated products were resolved by thin layer chromatography. The degree of acetylation was determined by counting the excised band in a scintillation counter.

Preparation of Nuclear Extracts

The method of Dignam *et al* (20) was used with slight modifications. In the large scale procedure approximately 1×10^9 logarithmically growing cells were harvested by centrifugation, washed twice with PBS, resuspended in 4 packed cell volumes (PCV) of freshly made Buffer A consisting of 10 mM HEPES-Cl (pH 7.9) 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) $10 \mu\text{g}/\text{ml}$ leupeptin, $10 \mu\text{g}/\text{ml}$ pepstatin, $10 \mu\text{g}/\text{ml}$ Aprotinin, 0.5 mM DTT, and incubated 10 minutes on ice. The cells were pelleted by centrifugation and resuspended in 2 PCV of Buffer A and the cell suspension homogenized with 10 strokes in Dounce homogenizer with a B pestle. The nuclei were pelleted by centrifugation and the supernatant was discarded. The pellet was resuspended in $3 \mu\text{l}$ of Buffer C, containing 20 mM HEPES-Cl (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 5 mM PMSF, $10 \mu\text{g}/\text{ml}$ leupeptin, $10 \mu\text{g}/\text{ml}$ pepstatin, $10 \mu\text{g}/\text{ml}$ Aprotinin, 0.5 mM DTT, and homogenized with 10 strokes of a Dounce homogenizer with a B pestle. The homogenate was transferred to a polypropylene tube and placed on a rotator in the cold room for 30 minutes. The homogenate was then transferred to a $15 \mu\text{l}$ Corex tube and centrifuged for 30 minutes at $50,000 \times g$ at 4°C . The supernatant was dialyzed with stirring for 5 hours against 50 volumes of Buffer D consisting of 20 mM HEPES-Cl (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 5 mM PMSF, 0.5 mM DDT, $1 \mu\text{g}/\text{ml}$ leupeptin $1 \mu\text{g}/\text{ml}$ pepstatin, $1 \mu\text{g}/\text{ml}$ Aprotinin at 4°C . Upon completion of dialysis, the extract was clarified by centrifugation in a $15 \mu\text{l}$ Corex tube at $50,000 \times g$ 4°C for 30 minutes. The clarified

extract was aliquoted and quick frozen in liquid nitrogen. When the extract was prepared using fewer cells, the cells were sheared by 7 passages through a 25 gauge needle, instead of using a Dounce homogenizer. This modified procedure produced extracts indistinguishable in quality from the standard procedure.

For production of induced extracts, cells were treated with 50 ng/ml of PMA (phorbol 12-myristate 13-acetate) at 37°C for 30 minutes immediately prior to extract preparation.

Gel Retardation Analysis

The method of Landolfi and Tucker was followed (21). The binding reaction contained $3 \mu\text{g}$ of nuclear extract, 20 mM HEPES-Cl (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 5 mM PMSF, 3 mM DTT and nonspecific competitor and probe (typically $4 \mu\text{g}$ of poly dIdC and 10,000 counts of probe) in $20 \mu\text{l}$. The probes used in these assays were the 89 bp Dde I-Mbo I or the 68bp Dde I-Hae III fragments from the α intronic enhancer or a double stranded oligonucleotide probe TCGAGTCA-GAGGGGACTTTCGAG containing the NF- κ B motif of the α intronic enhancer. Probes labeled with ^{32}P using either Klenow or polynucleotide kinase were added last and the reaction incubated at 37°C for 15 minutes. A 4% acrylamide gel buffered with $1 \times \text{TBE}$ was electrophoresed at 10 volts/cm and the samples applied while it was carrying current. Upon the completion of electrophoresis, the gel was dried and placed against film for autoradiography.

Methylation Interference

For methylation interference studies, the gel retardation protocol was modified as follows. The probe was the 68bp Dde I-Hae III fragment of the kappa intronic enhancer inserted into the Sma I site of pUC8. A $25 \mu\text{g}$ aliquot of this construct was cut with Hind III and end labeled with Klenow. Subsequently, the construct was cut with EcoR I releasing the NF- κ B probe flanked by polylinker sequences. The labeled DNA was then extracted with phenol and twice with Sevag before ethanol precipitation. The precipitated DNA was resuspended in $10 \mu\text{l}$ of 50 mM sodium cacodylate, 1 mM EDTA, pH 8.0. The methylation reaction was initiated by addition of $1 \mu\text{l}$ of dimethyl sulfate and allowed to proceed for 5 minutes. The reaction was stopped by the addition of $600 \mu\text{l}$ of 1.5 M sodium acetate, pH 7.0, 1 M β -mercaptoethanol at 4°C and the DNA precipitated with two volumes of DNA. The precipitated DNA was resuspended in $250 \mu\text{l}$ of 0.3 M sodium acetate, 1 mM EDTA, and reprecipitated by the addition of $750 \mu\text{l}$ of 100% ethanol.

The DNA contact points of the NF- κ B complex were assayed in a gel retardation assay as described with the following changes: $3 \mu\text{g}$ of nuclear extract, $1 \mu\text{g}$ of dI-dC and 30,000 counts of probe were used. The wet gel was exposed to X-ray film overnight and the bands corresponding to the NF- κ B complex and free probe excised and eluted in TE plus 0.4M NaCl overnight. The eluted DNA was purified on an elutip-d column and ethanol precipitated. The pelleted DNA was washed once with 75% ethanol before being resuspended in $200 \mu\text{l}$ piperidine and heated at 90°C for 30 minutes. Piperidine was removed by lyophilization. The cleaved DNA was run on an 8% wedge sequencing gel.

RNA Analysis

Total RNA was isolated essentially as described by Queen and Stafford (22). Approximately 2×10^7 cells were washed in PBS. The pellet was resuspended in $3 \mu\text{l}$ of 50 mM sodium acetate,

Table 1. Immunoglobulin Enhancer Activity in Myeloma Cell Lines.

Cell Line	Relative CAT Expression		
	No Enhancer	^H E	^κ E
S194	1	528	132
J558	1	178	3

Cells were transfected (18) with plasmid DNA containing the CAT gene driven by a 230 bp Pst I-Hae III λ immunoglobulin promoter with no enhancer, the 475 bp κ enhancer (κ) or the 660 bp heavy chain enhancer 5' of the promoter. Cells were harvested 44 hours after transfection and CAT activity determined by thin layer chromatography (19). The spots containing acetylated chloramphenicol were cut out and the radioactivity present determined. CAT activity obtained with each enhancer was compared to the activity seen in the absence of an enhancer (arbitrarily assigned the value of 1), and the stimulation calculated. The values represent the average of three independent determinations.

1% SDS at room temperature. Immediately after lysis, 3 μ l of phenol equilibrated with 50 mM sodium acetate at 60°C was added and the mixture agitated at 60°C for 15 minutes. The solution was cooled on ice for 15 minutes and then centrifuged. The aqueous layer was made 0.3 M in sodium acetate, and precipitated with 2.5 volumes of cold ethanol. The pellet was rinsed with 70% ethanol and the total RNA quantitated by spectrophotometer.

The levels of specific mRNA was determined by applying the RNA to a Gene Screen filter using a slot blot apparatus and hybridizing the immobilized RNA with labeled DNA probes. Two μ g of RNA suspended in 250 μ l of 15% formaldehyde and 250 μ l 20 $\times$ SSC was applied, the filter baked at 80°C under vacuum for 1 hour, and then prehybridized for 1–3 hours at 42°C with 50% formamide, 5 $\times$ Denhardt's, 1% SDS, 5 $\times$ SSPE, 0.1 μ g/ml salmon sperm DNA. The filter were then hybridized overnight with a DNA probe in the prehybridization solution plus 5% dextran sulfate. The filters were washed 4 times in 1 $\times$ SSC, 0.1% SDS at room temperature and for 30 minutes in 0.1 $\times$ SSC, 0.1% SDS at 50°C. The DNA probes were a 1.6kb EcoR I fragment from TIS11 (gift of K. Nakajima) and a 1.8kb BamH I fragment containing a human gamma actin cDNA (gift of L. Kedes).

Cell Culture

Cell culture and hybridization: Cells were maintained in Iscove's Modification of Dulbecco's Medium (Gibco) supplemented with 5% iron supplemented calf serum (Hyclone) except for 70Z/3 and hybrids of 70Z/3 which were supplemented with 5% endotoxin free fetal calf serum. For hybridization 10⁷ of each line to be fused were pelleted together, washed once with Dulbecco's modification of Eagle's medium (DME), and suspended in 0.5 μ l of 41.7% polyethylene glycol 1500 (PEG 1500), 12.5% DMSO, 100 mM Tris, pH 8.0 in DME. After gentle shaking for 1 minute, 0.5 μ l of 50% PEG 1500 in 100 mM Tris, DME, pH 8.0 was added and cells were shaken for an additional 2 minutes. Cells were then diluted with 10 μ l of DME at 37°C, pelleted, resuspended in growth medium with 20% serum (fetal calf or iron supplemented calf) at 10⁶ per μ l and plated into 96 well microtiter dishes, two drops per well. The next day selective medium was added. The S107 cell line was unmarked, J558L was resistant to azaguanine and ouabain, 70Z/3 to thioguanine and S194 to bromodeoxyuridine. Southern blot analysis using immunoglobulin specific probes verified that surviving cells were indeed hybrids.

Figure 1. Gel Shift Assays. Nuclear extracts and gel retardation assays were as described in the materials and methods section. An end-labeled 65 bp Dde I-Hae III κ enhancer fragment containing the NF- κ B site was used as probe. Panel A: LPS (Gibco) stimulation of 70Z/3 was carried out at 10 μ g/ml for 4 hours at 37°C. Panel B: Induction of NF- κ B binding by treatment with an inhibitor of protein synthesis. Cells were treated with cycloheximide (Sigma) at 10 μ g/ml for 4 hours immediately before nuclear extract preparation. Panel C: Release of active NF- κ B by the dissociating agent deoxycholate. Preparation of the postnuclear membrane fraction and deoxycholate treatment were as described by Baeuerle and Baltimore (25). Panel D: Phorbol ester induction of NF- κ B. Cells were treated with 50 μ g/ml of PMA at 37°C for 30 minutes, immediately prior to extract preparation.

The cell lines used in these studies are: 70Z/3, a Ly-1⁺ lymphoma which expresses μ heavy chain and in which transcription of the κ light chain is induced by LPS (originally described by Paige, 23). J558L is a variant of the α , λ producing myeloma J558 selected for synthesis of only the light chain (17). S107, an α , κ myeloma was acquired from Dr. Matthew Scharff. The α , κ myeloma S194 was acquired from the ATCC.

RESULTS

In initial experiments to investigate the regulation of light chain expression in myeloma cells producing λ light chains, we examined the transient expression of a CAT reporter gene driven by a λ promoter containing either no enhancer, or the heavy or kappa light chain enhancers from the intervening sequence. We found that J558L effectively activated expression using the heavy chain enhancer, but not the κ light chain intronic enhancer (Table 1). In contrast, as has been reported previously, the κ myeloma S194 activated the CAT gene using both the κ and heavy chain enhancers. These results suggested that the λ myeloma J558L was deficient in a factor required for κ enhancer function; NF- κ B was a likely candidate for the missing factor. The inability of J558L to activate the κ enhancer was surprising since it has been used for the stable expression of κ light chains (17).

To assay for the presence of NF- κ B, nuclear extracts prepared from the myelomas J558L, S107, and S194, as well as from pre-B cell line 70Z/3 plus and minus LPS induction were used in gel

Figure 2. Gel Shift Assays. Postnuclear membrane fraction preparation and deoxycholate treatment were as described by Baeuerle and Baltimore (25) and gel retardation assays were as described in the materials and methods section. Radiolabeled NF- κ B binding motif specific oligonucleotide was used as probe; assays were done either in the absence of competitor or in the presence of a 10 fold molar excess of unlabeled oligonucleotide (+competitor).

shift assays with an end labeled 65 bp DdeI-HaeIII κ enhancer fragment containing the binding site for NF- κ B (Fig. 1A). The extracts from S194 and 70Z/3 induced with LPS retarded an NF- κ B complex. As reported previously (15), the S107 and unstimulated 70Z/3 extracts were much reduced in NF- κ B binding and no NF- κ B binding activity was detected in J558L extracts. The absence of nuclear NF- κ B binding activity in J558L appears to explain the diminished κ enhancer function observed and demonstrates that NF- κ B is not necessary for the continued expression of λ light chain genes in terminally differentiated B cells. Thus far, with the exception of S107 and J558L, functional NF- κ B has been found in all nuclear extracts prepared from mature B cells (5,24).

The absence of NF- κ B binding activity in the myeloma variants could either result from the failure to synthesize NF- κ B or the failure to activate it. Incubation of pre-B cell lines with the protein synthesis inhibitor cycloheximide reveals NF- κ B binding activity (5), presumably due to the spontaneous release of NF- κ B from the NF- κ B/I κ B complex with the released I κ B unable to rebound. Treatment of J558L, 70Z/3 and S107 with cycloheximide yielded an NF- κ B complex (Fig. 1B) consistent with the presence of an inactive NF- κ B precursor; J558L and 70Z/3 contained much more of the precursor than S107.

As a further demonstration of the presence of an inactive NF- κ B precursor within J558L and S107, we took advantage of the finding that the cytosolic NF- κ B-I κ B complex is disrupted by the dissociating agent, deoxycholate, freeing NF- κ B for sequence specific binding (25). Cytosolic fractions prepared from J588L, S107, and 70Z/3 treated with deoxycholate showed NF- κ B binding activity (Fig. 1C) confirming the presence of precursor NF- κ B in J558L and localized at least a portion of this precursor to the cytosolic compartment. Constitutive NF- κ B binding activity in the nuclei in these cell lines appears absent because all of the NF- κ B is bound to its inhibitor in the cytoplasm.

Inducers of protein kinase C (PKC) also lead to activation of NF- κ B; a phosphorylation event is thought to cause either increased dissociation or direct inactivation of I κ B. Data suggest that it is I κ B and not NF- κ B which is altered following PKC activation (26). Therefore, to further understand the reason for

Figure 3. Methylation Interference Footprint of the NF- κ B-DNA Complex. J558L cells were treated with cycloheximide as in Fig. 1 and a methylation interference footprint of the induced NF- κ B complex was obtained essentially by the method of Baldwin and Sharp (27). Free probe (F) was run in the left lane and complexed DNA (B) run on the right.

the absence of active NF- κ B in J558L we determined whether PKC could activate NF- κ B. Treatment of 70Z/3 as well as S107 with PMA (phorbol 12 myristate 13 acetate) resulted in active NF- κ B although the amount present in S107 was much reduced. In contrast, no NF- κ B binding complex was seen in J558L induced with PMA (Fig. 1D). The I κ B in J558L could be unresponsive to signals generated by PKC; alternatively, intermediates in the PKC activation pathway could be missing or the cells may be deficient in a unique isozyme of PKC.

To further confirm that the factor released by deoxycholate treatment is NF- κ B the gel retardation assays were repeated using the NF- κ B binding motif oligonucleotide. Binding to the oligonucleotide was seen and could be competed by unlabeled oligonucleotide (Fig. 2). A non-specific oligonucleotide failed to compete (data not shown). Methylation interference also confirmed that the DNA contact points of the DNA binding complex induced by cycloheximide were identical to those expected for NF- κ B (Fig. 3). As anticipated the cycloheximide induced DNA binding protein protected guanine residues in the κ enhancer characteristic of NF- κ B binding (27). Therefore, J558L is not deficient in the synthesis of NF- κ B and when it is released from its inhibitor by treatment with detergent or protein synthesis inhibitors the NF- κ B produced by J558L can bind DNA. Instead J558L appears deficient in the ability to activate NF- κ B, both constitutively and in response to activation signals.

To further analyze the defect in the NF- κ B activation pathway, somatic cell hybrids were produced between J558L and S107, 70Z/3, or S194. In the somatic cell hybrid between S194 and J588L, NF- κ B binding complexes were seen indicating that J558L cannot repress constitutively activated NF- κ B. In these

Figure 4. Expression of NF- κ B in a S194XJ558L Somatic Cell Hybrid. Nuclear extract preparation, gel retardation and cycloheximide (CHX) and PMA induction were as in Fig. 1.

somatic cell hybrids neither phorbol ester nor cycloheximide treatment increased the availability of functional NF- κ B. One example is shown in Fig. 4.

When two different 70Z/3 X J558L hybrids were examined two different phenotypes were observed (Fig. 5). In the hybrid HAE6, functional NF- κ B was present constitutively and its presence correlated with protein production: λ , κ , and μ chain synthesis was seen in untreated cells. In the hybrid HAE8 functional NF- κ B was observed only after PMA treatment and there was a lack of κ chain synthesis in the untreated cells. These results suggest that 70Z/3 and J558L lack two different components of the NF- κ B activation pathway: in one hybrid (HAE6) both are present resulting in constitutive NF- κ B activation and κ light chain expression. In the second hybrid, the factor contributed by J558L has been lost, presumably through chromosomal segregation. However, the ability to activate NF- κ B following PMA treatment, a characteristic of 70Z/3 lacking in J558L, is present in the hybrid.

When two hybrids between S107 and J558L were examined, complementation was observed (Fig. 6). The two hybrids had slightly different phenotypes. In HAB12 there was constitutive activation of NF- κ B which was further augmented by PMA. In HAB19 no constitutively active NF- κ B was seen and PMA induced only low levels of functional NF- κ B. Therefore in the hybrids J558L can contribute the ability to synthesize large quantities of NF- κ B, an ability that S107 lacks; in turn S107 contributes the ability to activate NF- κ B following PMA treatment. However, in neither hybrid was complete constitutive activation like that in the J558L X70Z/3 hybrid HAE6 seen.

Phorbol ester is a potent inducer of the transcription of the early response genes. To determine whether the block in phorbol

Figure 5. Immunoglobulin and NF- κ B Expression in 70Z/3 X J558L Somatic Cell Hybrids. HAE6 and HAE8 are two somatic cell hybrids between 70Z/3 and J558L. Panel A: NF- κ B expression in nuclear extracts prepared from untreated cells and cells induced with PMA. Extracts, gels and induction are in Fig. 1. Panel B: Immunoglobulin production was analyzed by incubating the cells for 1 hour at 37°C in 35 S-methionine in the absence of PMA, lysing the pelleted cells with 0.5% NP-40 in isotonic buffer, and immunoprecipitating the labeled immunoglobulins with rabbit anti-sera specific for μ , κ and λ . The immunoprecipitates were analyzed in 5% SDS-PAGE gels and the position of the labeled immunoglobulins determined by radio autography. The position of free κ and λ light chains, HL half-molecules and fully assembled H_2L_2 molecules are indicated. Marker immunoglobulins run for comparison are not shown.

Figure 6. Expression of NF- κ B in Somatic Cell Hybrids Between S107 and J558L. HAB12 and HAB19 are two somatic cell hybrids between S107 and J558L. S194 is shown as an example of a cell line with constitutively active NF- κ B; 70Z/3 is an example of an inducible cell line.

ester activation of NF- κ B seen in J558L was common to other cellular responses and whether there was a direct correlation between the ability to activate NF- κ B and the early response genes, we assayed the mRNA levels of the early response gene TIS11 (28) in induced and uninduced cells (Fig. 7).

The cell lines varied widely in their phenotype. The two plasmacytomas S107 and S194 and the two S107 X J558L hybrids showed high levels of TIS11 mRNA following PMA induction. S107 is noteworthy because it has both a high basal level of TIS11 and a high level following PMA induction. The S107 X J558L hybrids demonstrated that S107 can complement the defect in ability to activate TIS11 observed in J558L; however since the

Figure 7. TIS11 Expression in Cells Without PMA Induction. Total RNA was prepared, blotted on to nylon filters, and hybridized with a TIS11 probe. The resulting autoradiogram was scanned with a densitometer. Shown are the scan with the auto radiogram beneath.

hybrids show both a decreased basal level of TIS11 and a decreased level following PMA induction compared to S107, J558L must exert a negative influence on the induction pathway.

J558L also had a negative influence on the TIS11 induction level in its hybrids with S194 and 70Z/3. The J558L X S194 hybrid HAC6 had approximately the same basal level of TIS11 expression as S194; however, PMA treatment led to negligible induction. HAC1 had barely detectable TIS11 expression prior to induction which was increased by PMA treatment to approximately the level seen in HAC6. Both J558L X 70Z/3 hybrids showed only very modest levels of TIS11 expression following PMA treatment; HAE6 showed a low level of TIS11 expression prior to induction.

These data therefore indicate that the defect in the ability to activate NF- κ B seen in J558L is also reflected in a decreased ability to activate TIS11 expression. Although there sometimes a correlation between NF- κ B activity and TIS11 mRNA levels, this correlation is not absolute.

DISCUSSION

Identification of J558L with its defect in the ability to activate NF- κ B and the analysis of this defect using somatic cells hybrids points to the complexity of the activation pathways to inducible and constitutive gene expression.

Precedent exists for cell lines synthesizing NF- κ B but with defects in the ability to activate it. Mutants of the pre-B cell lines 70Z/3 have been isolated which fail to activate NF- κ B following either LPS or PMA treatment (29) but which contain a precursor that can be mobilized by detergent treatment. It was suggested that these lines have a defect in a late step in the activation pathway common to several different activators, possibly a protein kinase. One of these cell lines was shown to also be defective in OTF-2 induction (30) suggesting shared elements in the OTF-2 and NF- κ B activation pathways. J558L differs from 70Z/3 in that it represents the mature stage of B cell development and is

Table 2. Inducible and Constitutive TIS11 and NF- κ B Expression

Cell line	PMA	Relative* TIS11 Expression	NF- κ B**
70Z/3	—	0	—
S107	+	91	+++
S194	—	157	—
	+	1000	+
J558	—	27	++++
	+	446	nd ⁺
HAB12	—	0	—
	+	0	—
HAB19	—	21	++
	+	475	++++
HAC1	—	72	—
	+	266	+
HAC6	—	0	+++
	+	24	nd
HAE6	—	16	+++
	+	43	nd
HAE8	—	5	+++
	+	16	+++
	—	0	—
	+	8	+++

*The amount of TIS11 specific probe hybridizing was quantitated by scanning the slot blot with a densitometer (see Fig. 7). Two independent blots were analyzed and the results averaged. The values were normalized for the amount of mRNA applied by hybridization with an actin specific cDNA. PMA induced S107 was arbitrarily assigned a value of 1000 and all others are expressed relative to this. **Values are from Figs. 1,3–5 except for HAC6 where the NF- κ B expression is not shown.

⁺nd=not determined.

producing a λ light chain. However the absence of functional NF- κ B observed in the J558L myeloma does not appear to be obligatory for λ expression, since we have found normal κ enhancer function in LPS stimulated λ subclones of the pre-B cell line NFS-5 and constitutively activated NF- κ B in the HOPC1 and OMM λ myelomas (data not shown).

S107, the other mature B cell lacking functional NF- κ B differs from J558L in that it appears deficient in NF- κ B synthesis. Additionally, the lack of constitutive NF- κ B in J558L contrasts with that seen in pre-B cell and in non-B cells which activate NF- κ B following phorbol ester treatment.

The failure of J558L to activate NF- κ B following PMA stimulation extends to an inability to activate the early response gene TIS11. This failure to activate is complemented in the S107 X J558L hybrids and would not appear to be a consequence of the lack of NF- κ B since TIS11 can be activated to high levels in S107 cells containing very low levels of NF- κ B. The transcription of the early response genes is induced by external stimuli and occurs in the absence of protein synthesis presumably by activating transcription factors (31). Cell lines differ in their ability to activate early response genes and early response genes show tissue specific expression patterns (32). Therefore a developmentally determined process may operate in specific cells lineages to extinguish the capacity for expression of certain early response genes.

Examination of the somatic cell hybrids shows a very complex pattern for NF- κ B and TIS11 expression and activation. Phenotypic variation between different representatives of the same cross presumably results from selective chromosome loss.

Although J558L is deficient in expression and induction of both TIS11 and NF- κ B, in general the induction of these two early response genes seems to be independently regulated, although certainly there could be shared elements in their induction

pathways (Table II). TIS11 is highly induced by PMA in both S107 and S194 and in the J558L X S107 hybrids. Effective induction of NF- κ B occurs only in the J558L X S107 hybrid HAB12. The pre-B cell line 70Z/3 exhibits inducible expression of both TIS11 and NF- κ B. The J558L X 70Z/3 hybrids show reduced TIS11 induction compared to 70Z/3 and only one of the hybrids retains inducible expression of NF- κ B. In the other hybrid there is a high level of constitutively active NF- κ B which results in a high level of κ light chain synthesis, consistent with the proposed role of NF- κ B in controlling light chain transcription.

Constitutive high level expression of TIS11 is seen in both the plasmactomas S107 and S194 but not in J558L. This correlated with high levels of NF- κ B in S194 but not in S107 which is deficient in its ability to synthesize NF- κ B. Of the somatic hybrids, only the J558L X S107 hybrid HAB19 shows constitutive high level TIS11 expression. In contrast high level constitutive NF- κ B expression is seen in both S194 X J558L hybrids and in one 70Z/3 X J558L hybrid. Therefore there is independent control of the constitutive expression of TIS11 and NF- κ B.

We have not resolved the mechanism of the block in phorbol ester activation of NF- κ B and the induction of TIS11 although it does not seem to be primarily through a negative regulatory factor since it is positively complemented in somatic hybrids. However the decreased level of constitutive NF- κ B and the decreased activation of TIS11 in the J558L X S194 hybrids suggests that negative regulation may make some contribution. Possible mediators of negative regulation may be membrane protein phosphatases like CD45 and LAR which have been implicated in signal transduction in T lymphocytes (33).

Direct binding of phorbol ester to protein kinase C activates protein kinase C enzymatic activity (34). Possibly only one or few of the protein kinase C isoenzymes are involved in the activation of NF- κ B *in vivo* and cell lines may vary either in expression, localization, or activity of the kinase subspecies. It has been previously reported that differences in isoenzyme expression occur in clonal lymphoid cell lines (35). Another possibility is that J558L might lack the target for PKC phosphorylation and this target is common to both TIS11 and NF- κ B activation.

Activation and nuclear localization of NF- κ B appears to result when it is released from its inhibitor I κ B. Activation of protein kinase C activates NF- κ B presumably because a protein kinase C catalyzed phosphorylation event results in the release of NF- κ B from I κ B. Unlike non-B cells, J558L is unable to activate NF- κ B via this pathway presumably because the lack of the appropriate kinase or because its target is missing or unresponsive. In some cell lines such as the Jurkat T cell line, phorbol ester treatment activates little or not NF- κ B (5). By an as yet unknown mechanism, addition of a second stimulus strongly potentiates NF- κ B binding. The question remains as to whether phorbol esters plus a second signal would activate NF- κ B in J558L and if a second signal is required, what would it be.

One of the puzzling aspects of the J558 phenotype is that it was one of the first cell lines used for the successful expression of transfected κ light chain genes (17). It is difficult to explain this in the light of the absence of active NF- κ B since NF- κ B has been hypothesized to be necessary for activation of κ transcription. Possibly sufficient transient activation and nuclear translocation of NF- κ B occurs to permit activation of these exogenous genes. Alternatively, an NF- κ B independent light chain activation pathway may be operative in J558L; such a pathway has been described for γ -interferon treated 70Z/3 cells (36).

Significant gaps exist in our understanding of the integration of developmental and inducible signals regulating nuclear factors such as NF- κ B. The identification of a variant defective in these pathways facilitates their genetic dissection.

ACKNOWLEDGEMENTS

Dedicated to Paul Berg on the occasion of his sixty-fifth birthday. Work in the laboratory was supported by grant CA16858 from the National Institute of Health and IM-550 from the American Cancer Society.

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