

## Developmental Regulation of the $\kappa$ Locus Involves Both Positive and Negative Sequence Elements in the 3' Enhancer That Affect Synergy with the Intron Enhancer\*

(Received for publication, July 21, 1998, and in revised form, October 12, 1998)

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**Expression of the mouse immunoglobulin  $\kappa$  locus is regulated by the intron and 3' enhancers. Previously, we have reported that these enhancers can synergize at mature B cell stages. Here we present our recent studies on the identification and characterization of the 3' enhancer sequences that play important roles in this synergy. By performing mutational analyses with novel reporter constructs, we find that the 5' region of the cAMP response element (CRE), the PU.1/PIP, and the E2A motifs of the 3' enhancer are critical for the synergy. These motifs are known to contribute to the enhancer activity. However, we also show that mutating other functionally important sequences has no significant effect on the synergy. Those sequences include the 3' region of the CRE motif, the BSAP motif, and the region 3' of the E2A motif. We have further demonstrated that either the 5'-CRE, the PU.1/PIP, or the E2A motif alone is sufficient to synergize with the intron enhancer. Moreover, the PU.1 motif appears to act as a negative element at pre-B cell stages but as a positive element at mature B cell stages. We have also identified a novel negative regulatory sequence within the 3' enhancer that contributes to the regulation of synergy, as well as developmental stage and tissue specificity of expression. While the levels of many of the 3' enhancer binding factors change very little in cell lines representing different B cell stages, the intron enhancer binding factors significantly increase at more mature B cell stages.**

Expression of the immunoglobulin  $\kappa$  (Ig $\kappa$ ) gene is tissue-specific and is developmentally regulated. In addition to tissue-specific variable (V $\kappa$ ) region promoters, at least two enhancers also contribute to this tissue-specific and developmental control (1–5). The intron enhancer ( $\kappa$ Ei) is located between the joining (J $\kappa$ ) segments and the constant (C $\kappa$ ) region, and the 3' enhancer ( $\kappa$ E3') lies approximately 9 kb<sup>1</sup> downstream of the C $\kappa$  region. Both enhancers show tissue specificity and developmental regulation. It has been shown that the transcription activities of these enhancers are modulated through specific

sequence motifs (6). The activity of the  $\kappa$ Ei is contributed by several motifs, including  $\kappa$ A,  $\kappa$ B, E1, E2 and E3; and the activity of the  $\kappa$ E3' is mediated in part through CRE, BSAP, PU.1, PIP, and E2A motifs (7–12). Specific DNA binding proteins for many of these motifs have been identified and characterized (6, 13–16). Although no direct interactions among binding factors of the  $\kappa$ Ei motifs have formally been established, interactions among binding factors of the  $\kappa$ E3' motifs have been described (10–12, 17).

Expression of a functionally rearranged Ig $\kappa$  gene is known to be up-regulated during B cell development, and it reaches maximal activity at mature B and plasma cell stages. Despite numerous reports on these enhancers, it is still not entirely clear how both the enhancers coordinately participate in this developmental up-regulation. While some studies show that the  $\kappa$ Ei is active at a low level in pre-B cells, other studies suggest that it is completely silent in pre-B cells (9, 10, 18). A more recent study, however, suggests that the  $\kappa$ Ei is probably always active but at a relatively low level at early B cell stages, and it is thus considered to play no significant role in the activation of  $\kappa$  locus during pro-B to pre-B transition (19). The approximately 1-kilobase region of the  $\kappa$ E3' is inactive, or active at a low level, at early B cell stages, and its activity increases during B cell maturation and reaches full levels at mature B and plasma cell stages (9, 10, 12, 20). However, a 132-base pair (bp) core within the larger  $\kappa$ E3' has been reported to be active in pre-B cells, and its activity is suppressed by negative sequence elements in its flanking regions (21, 22). Interestingly, like the  $\kappa$ Ei, the  $\kappa$ E3' can also be activated by bacterial lipopolysaccharide (LPS) treatment, and this inducibility is thought to be mediated by some sequences flanking the core as well (22).

While the two enhancers individually contribute to the developmental regulation of the  $\kappa$  locus, we and others have reported that they can synergistically activate  $\kappa$  transcription at mature B cell stages and the combined strength of the two is roughly equivalent to that of a heavy chain  $\mu$  core enhancer (18, 23, 24). Thus, it is important to consider the interactions of the two enhancers in B cell development, and the roles of individual sequence motifs should be examined not only in the context of the individual enhancer, but within the natural context of both enhancers. We previously determined some of the sequence requirements of the  $\kappa$ Ei for synergizing with the  $\kappa$ E3' (24). However, little has been done to characterize the  $\kappa$ E3' with respect to its involvement in the synergistic activation with the  $\kappa$ Ei. Thus, in this current study we sought to identify and characterize the  $\kappa$ E3' sequence elements and the binding factors that are important for the developmentally regulated synergistic  $\kappa$  expression. Using novel reporter constructs, we demonstrate that both positive and negative elements within

\* This work was supported in part by research funds from the University of Minnesota. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: kb, kilobase(s); CRE, cAMP response element; bp, base pair(s); LPS, lipopolysaccharide; Mel, mouse erythroid leukemia; DTT, dithiothreitol;  $\kappa$ E3'NR,  $\kappa$ E3' negative regulator; LSM, linker scan mutation.

the  $\kappa$ E3' contribute to the developmental regulation and the tissue specificity of  $\kappa$  expression.

#### EXPERIMENTAL PROCEDURES

**Plasmid Construction and Mutagenesis**—Standard recombinant DNA procedures were performed as described by Sambrook *et al.* (25). A functionally rearranged  $\kappa$  gene of mouse myeloma MOPC41 was excised as an approximately 7-kilobase *Eco*RI fragment from a vector (pE<sup>tk</sup>.neo.short-MAR<sup>+</sup>ENH<sup>+</sup>) provided by Dr. William Garrard (University of Texas Southwestern Medical Center) (26). The fragment was then inserted into an *Eco*RI restriction site in a multiple cloning region of pGEM1 (Promega) containing a previously inserted neomycin resistance gene.<sup>2</sup> The C $\kappa$  region of the  $\kappa$  gene in this construct was replaced as a *Sac*II-*Bam*HI fragment with a different C $\kappa$  region, a *Sac*II-*Bam*HI fragment from pSP1g.neo-V $\kappa$ 21C<sup>2</sup> in order to generate a unique *Bam*HI restriction site and avoid ambiguity of sequence information at the 3' end of the  $\kappa$  gene. A luciferase gene was amplified by polymerase chain reaction and inserted in-frame into a *Hpa*I site in the C $\kappa$  region. Constructs containing the wild-type larger  $\kappa$ E3' and the  $\kappa$ E3' core were subsequently generated by inserting these enhancer fragments into the *Bam*HI site. Constructs that contain  $\kappa$ E3' mutations were generated by inserting different forms of the  $\kappa$ E3' mutations into the *Bam*HI site. A set of linker scan mutations of the  $\kappa$ E3' core, provided by Dr. Michael Atchison (University of Pennsylvania) (12), was cloned into the *Bam*HI site. Constructs containing individual sequence motifs were generated by inserting double-stranded oligonucleotides of the individual motifs into the *Bam*HI site. Additional mutations of specific sites within the  $\kappa$ E3' core were generated by an overlapping polymerase chain reaction method (27). The construct with the deletion of the  $\kappa$ Ei was made by replacing a part of the  $\kappa$  gene with the one that had a 1042-bp deletion of both the matrix association region and  $\kappa$ Ei from a vector (pE<sup>tk</sup>.neo.MAR<sup>+</sup>ENH<sup>+</sup>) provided by Dr. William Garrard (26). All mutations were confirmed by DNA sequencing with SequiTherm EXCEL DNA Sequencing Kit (Epicentre Technologies).

**Cell Culture**—The mouse pre-B cell lines 3-1 and 1-8, the mouse nonsecreting mature B cell line A-20, and mouse mature B plasmacytoma cell line S194 have been characterized and referenced previously (18). The cell lines A-20 and S194 were obtained from the American Type Culture Collection. The mouse pre-B cell line 38B9 was obtained from Dr. Eugene Oltz (Vanderbilt University). The mouse erythroid leukemia (Mel) cell line and the human Jurkat T lymphoma cell lines were provided, respectively, by Drs. Jane Little and Lizhen Gui (University of Minnesota). Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 25 units/ml penicillin, and 25  $\mu$ g/ml streptomycin (all from Life Technologies, Inc.). 50  $\mu$ M  $\beta$ -mercaptoethanol was added only to pre-B cell cultures. For LPS induction, cells were treated with LPS (Difco) at 1  $\mu$ g/ml. Transfection analyses were normally performed after 20–24 h LPS induction.

**Transient Transfection**—Mouse B cell lines were transfected by a modified DEAE-dextran method (18). Briefly,  $5 \times 10^6$  cells per transfection were harvested and washed in 25 ml of  $1 \times$  sterile TS solution warmed to 37 °C (137 mM NaCl, 5 mM KCl, 0.3 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 25 mM Tris, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, pH 7.4). The cells were then resuspended in 1.5 ml of warm TS containing 500  $\mu$ g/ml DEAE-dextran (Amersham Pharmacia Biotech) with 1  $\mu$ g of test plasmid and 1  $\mu$ g of a control plasmid, SV40- $\beta$ -gal, a  $\beta$ -galactosidase expression vector, and the mixture was incubated for 20 min at 37 °C. Then the cells were washed with 10 ml of RPMI 1640, pelleted, and resuspended into 10 ml of RPMI 1640 (10% fetal calf serum, 2 mM L-glutamine, 25 units/ml penicillin, 25  $\mu$ g/ml streptomycin, and 50  $\mu$ M  $\beta$ -mercaptoethanol ( $\pm 1$   $\mu$ g/ml LPS if required)), and placed in a 37 °C, 7% CO<sub>2</sub> incubator. Transfected cells were harvested after 20–24 h and used in luciferase and  $\beta$ -galactosidase assays. Transfection efficiency was normalized by cotransfecting with the  $\beta$ -galactosidase expression vector, dividing luciferase activity by  $\beta$ -galactosidase activity. The luminescence ratio was plotted in arbitrary units. Typically, transfection assays for each construct were performed in duplicate with a minimum of three transfections for each construct, and average values were plotted with standard error of the mean indicated.

**Luciferase and  $\beta$ -Galactosidase Assays**—Luciferase assays were performed as follows: at harvest,  $2 \times 10^6$  cells were transferred into 1.5-ml microcentrifuge tubes, washed once with  $1 \times$  phosphate-buffered saline, and pelleted. The cells were then lysed for 10 min at room temperature in 50  $\mu$ l of  $1 \times$  Reporter Lysis Buffer (Promega). Luciferase

Assay Reagent (Promega) (100  $\mu$ l) was added to 20  $\mu$ l of the cell lysate and counted for 15 s in a Lumat 9501 luminometer (EG & G Berthold) according to the manufacturer's instructions. Relative light units are reported.

$\beta$ -Galactosidase activity was measured using a Galacto-Light kit (Tropix, Bedford, MA). Ten  $\mu$ l of the above cell lysate were incubate with 67  $\mu$ l of  $1 \times$  Galacton substrate (diluted with  $1 \times$  Galacto-Light Reaction Buffer Diluent) for 45 min at room temperature in the dark. Light Emission Accelerator reagent (100  $\mu$ l) was injected immediately prior to measurement in the luminometer.

**Nuclear Extract Preparation**—Nuclear extracts were prepared according to the method of Dignam *et al.* with modifications (28). Briefly, cells were washed in cold  $1 \times$  phosphate-buffered saline and resuspended in 5 packed cell volumes of cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.1% Nonidet P-40) and incubated for 2 min on ice to lyse the cells. The nuclei were pelleted by centrifugation at 9,000  $\times g$  for 30 s at 4 °C. The supernatant was removed, and the pellet was then resuspended in 0.5 packed cell volume of protein extraction buffer (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT). Nuclei were rocked for 30 min at 4 °C and then centrifuged at 12,000  $\times g$  for 20 min to remove insoluble debris. The supernatant (nuclear extract) was then dialyzed against 20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M NaCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1.0 mM DTT for over 4 h and stored at -70 °C. Protein concentrations were determined by the Bradford colorimetric assay with Protein Assay Dye Reagent (Bio-Rad) according to the manufacturer's recommendations.

**Electrophoresis Mobility Shift Assays**—Electrophoresis mobility shift assays were carried out with 20,000 cpm of the <sup>32</sup>P-end-labeled oligonucleotide probes, which were incubated for 30 min at room temperature with 10  $\mu$ g of nuclear extracts in a final volume of 20- $\mu$ l binding reactions (50 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, and 5% glycerol, pH 7.9), including 0.5–2  $\mu$ g of poly(dI-dC) (Sigma). Samples were separated on 5% nondenaturing polyacrylamide gels. The specificity of protein-DNA complexes was confirmed in competition experiments with unlabeled specific competitors.<sup>2</sup> The images were acquired with a PhosphorImager (Molecular Dynamics). The sequences of the oligonucleotide probes (upper strand shown) are as follows.

$\kappa$ A: 5'-AAGAACTCTCAGTTTTCGTTTTACTACCTCTG-3'  
 $\kappa$ B: 5'-TCTCAACAGAGGGGACTTTCCGAGAGGCCATCTG-3'  
E2: 5'-TCCTCCAGGCAGGTGGCCAGATTACA-3'  
E3: 5'-CTAAAAATTGTCCCATGTGGTTACAAACC-3'  
5'-CRE: 5'-AGATAGCACTGTCTAGTAA-3'  
PU.1/PIP: 5'-GACCCCTTTGAGGAACGAAACAGAAC-3'  
E2A: 5'-AGGCACATCTGTTGCTTTCGCTCCCATC-3'  
Octamer: 5'-CTGTATCTTGCGATTTGCATATTACATTTTCAG-3'

**Oligonucleotides**—All oligonucleotides were synthesized by the MicroChemical Facility at the University of Minnesota. Sequences of the oligonucleotides used for generating constructs containing the  $\kappa$ E3' individual motifs are as follows.

5'-CRE.UP: 5'-GATCCAGATAGCAACTGTCTAGCTAG-3'  
5'-CRE.LOW: 5'-GATCCTAGCTATGACAGTTGCTATCTG-3'  
PU.1/PIP.UP: 5'-GATCCGACCCCTTTGAGGAACGAAACAGAAC-3'  
PU.1/PIP.LOW: 5'-GATCCTTCTGTTTTAGTTCCTCAAAGGGTCG-3'  
E2A.UP: 5'-GATCCAGGCACATCTGTTGCTTTCGCTCCCATC-3'  
E2A.LOW: 5'-GATCCGATGGGAGCGAAAGCAACAGATGTGCTG-3'

Sequences of the oligonucleotides used for replacing the negative regulator region are as follows.

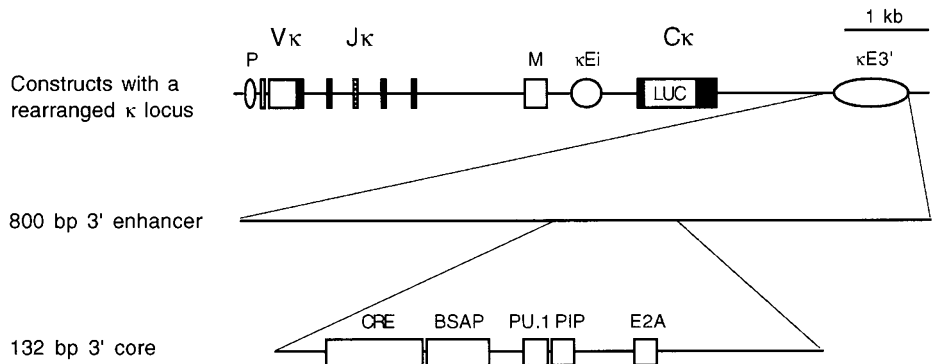
LSM(H).UP: 5'-GAGGAAGTGAAGacagcttcgAGGCACATCTGT-3'  
LSM(H).LOW: 5'-ACAGATGTGCTCGaagctgtctTTTCAGTTCCTC-3'

Sequences of the oligonucleotides used for polymerase chain reaction amplification of the linker scan mutations are as follows.

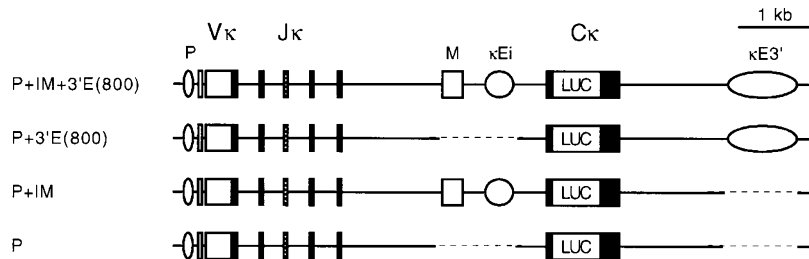
5'-Bam $\kappa$ E-5: 5'-CGGGATCCGACCAAGATAGCAACTGTCTAT-3'  
3'-Bam $\kappa$ E: 5'-GCGGGATCCACCCAGGCTGTTGGAGG-3'  
5'-LSM.3'E: 5'-CGGGCCGGATCCTCTAGAGTCGA-3'  
3'-LSM.3'E: 5'-CGGCCGGATCAAGCTTGCATGCCTGCAGG-3'

<sup>2</sup> X. Liu and B. Van Ness, unpublished data.

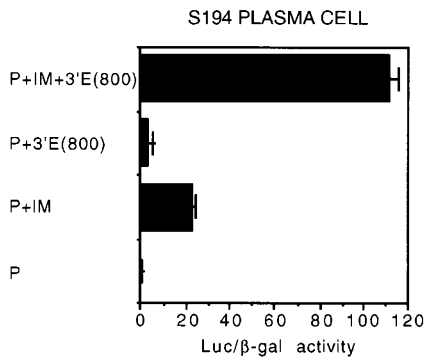
**FIG. 1. A schematic diagram of the rearranged  $\kappa$  gene and construct design.** At the top, the rearranged  $\kappa$  gene with an integrated luciferase gene used as a backbone in the generation of all constructs is shown. The  $\kappa$ E3' core and its sequence motifs are also indicated below. P, M,  $\kappa$ Ei,  $\kappa$ E3', V $\kappa$ , J $\kappa$ , C $\kappa$ , and LUC represent variable region promoter, nuclear matrix association region, intron enhancer, 3' enhancer, variable region, joining segments, constant region, and a luciferase gene, respectively.



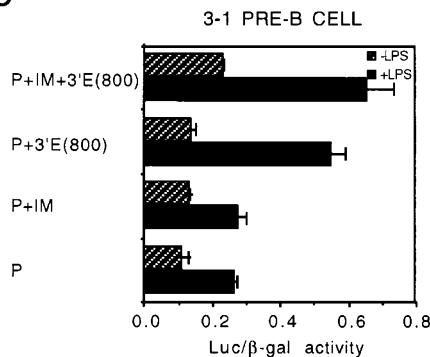
A



B



C



**FIG. 2. Synergistic activation of expression by the intron and 3' enhancers in mature B cells but not in pre-B cells and developmental up-regulation of the enhancer activities.** A, constructs containing different combinations of a V $\kappa$  region promoter with the two enhancers. IM represents matrix association region and intron enhancer. 3'E(800) represents the 800-bp 3' enhancer. B, transient transfection and synergistic activation in S194 plasmacytoma cells. C, transient transfection and transcription activities in untreated and LPS-treated 3-1 pre-B cells.

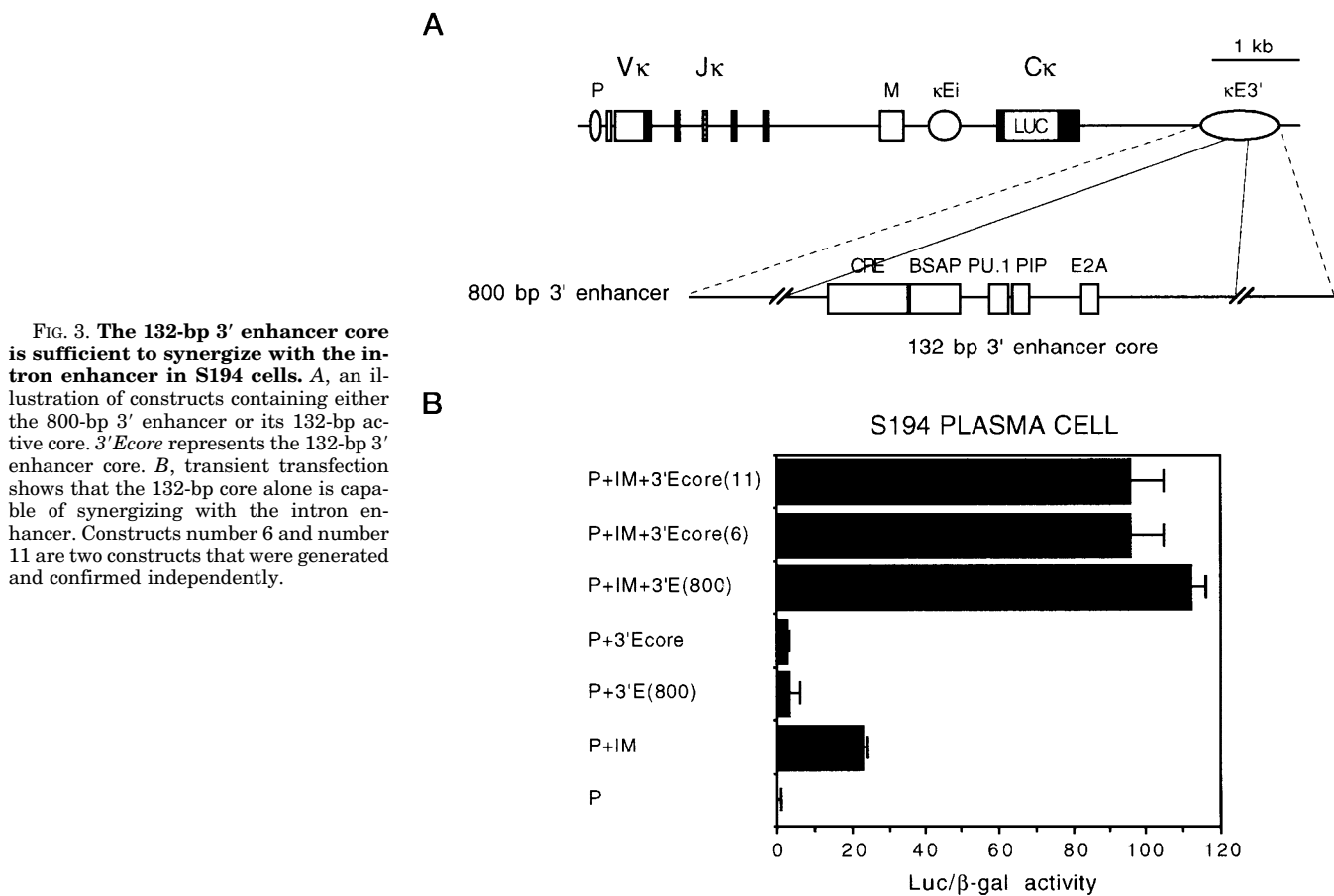
## RESULTS

Previous reporter constructs that have been developed introduced enhancer elements and heterologous promoters into artificially arranged vectors (9, 10, 12, 18). In this study we designed reporter constructs that preserve the natural context of enhancer elements. The base construct (Fig. 1) contains a functionally rearranged V $\kappa$ J $\kappa$ 1 with the  $\kappa$ Ei and  $\kappa$ E3' in a more natural context than previous constructs (18, 24, 26), and a luciferase gene fused in-frame within the C $\kappa$  region. To identify sequence elements important for the developmental regulation, a series of modifications, including small deletions, linker scanner mutations, and substitution mutations, were designed and are presented in each subsequent figure. In this study our particular focus is on modifications to the  $\kappa$ E3'. We used these constructs to transiently transfect a number of cell lines representing different stages of B cell development. The advantage of this approach is the ability to examine clonal responsiveness of early and late B cells without the selective expansions of heterogeneous B cell populations in transgenic or embryonic stem cell-generated mice.

**The Intron and 3' Enhancers Synergistically Activate  $\kappa$  Transcription at Mature B Cell Stages but Not at Pre-B Cell Stages, and the Activities of These Enhancers Are Up-regulated from Pre-B to Mature B Cell Stages**—In previous studies we designed constructs in which the  $\kappa$ Ei and  $\kappa$ E3' were placed adjacent to each other upstream of a V $\kappa$  region promoter, driving a

luciferase reporter (18, 24). We showed that in this context the intron and 3' enhancers together synergistically activate transcription at mature B and plasma cell stages, but not at pre-B cell stages (24). To be sure that the newly generated constructs possess the same synergistic property and to retest the previous observation with the  $\kappa$  regulatory elements in a more natural context, we performed transient transfection analysis with new constructs that had different combinations of a V $\kappa$  region promoter with the two enhancers (Fig. 2A). In S194 mature B cells, the addition of the  $\kappa$ Ei alone increased transcription level by 25–35-fold above the promoter alone, and the addition of the  $\kappa$ E3' increased transcription level by 5–7-fold. The two enhancers together increased transcription level by more than 100-fold compared with the promoter alone (Fig. 2B). In 3-1 pre-B cells, however, either enhancer alone showed minimal activity, and the two enhancers together only slightly increased transcription level compared with the promoter alone (Fig. 2C). Because the activities of these enhancers reportedly can be induced by LPS stimulation at pre-B cell stages, we then treated transfected 3-1 cells with LPS and found that even in LPS-stimulated 3-1 cells, the up-regulation of transcription was rather modest by these constructs (Fig. 2C). Similar effects were also observed in other mature and pre-B cell lines (data not shown). These results suggest that the activities of both enhancers are significantly up-regulated from early to late B cell stages, and with these constructs the syn-





**FIG. 3. The 132-bp 3' enhancer core is sufficient to synergize with the intron enhancer in S194 cells.** *A*, an illustration of constructs containing either the 800-bp 3' enhancer or its 132-bp active core. 3'Ecore represents the 132-bp 3' enhancer core. *B*, transient transfection shows that the 132-bp core alone is capable of synergizing with the intron enhancer. Constructs number 6 and number 11 are two constructs that were generated and confirmed independently.

ergistic activation is still a developmentally regulated phenomenon that is evident only at mature B cell stages but not at pre-B cell stages.

**The 132-bp 3' Enhancer Core Alone Is Sufficient to Synergize with the Intron Enhancer**—The synergistic activation of  $\kappa$  expression is conferred by the intron and 3' enhancers together. Previously, we have carried out detailed studies on the sequence requirements of the  $\kappa$ Ei for the synergy (24). In this current study, we sought to determine specific  $\kappa$ E3' sequences that are functionally important for the synergy and therefore contribute to the developmental regulation of  $\kappa$  expression. In all previous studies, including experiments shown in Fig. 2, we used the 800 bp  $\kappa$ E3' (Ref. 24, and see "Experimental Procedures" for details). However, several reports have suggested that the 132-bp  $\kappa$ E3' core accounts for most of the activity that the 800-bp enhancer has in regulating  $\kappa$  transcription (9, 10). To test whether the core is sufficient to confer synergy we carried out transfection analysis with constructs in which the 800-bp enhancer was replaced by the 132-bp core (Fig. 3A). We find that the core by itself is sufficient to synergize with the  $\kappa$ Ei to achieve high level transcription in S194 cells (Fig. 3B).

**Linker Scanner Analysis of Enhancer Synergy**—Because the  $\kappa$ E3' core is sufficient for the synergistic activation, we then investigated what specific core sequences are required for the synergy by incorporating linker scanner mutations of the  $\kappa$ E3' into the reporter vector (Fig. 4A). Each linker scan mutation has a 10-bp sequence substitution across the  $\kappa$ E3' core sequence (12). As shown in Fig. 4B, three regions were identified that, when mutated, significantly decreased the enhancer synergy. These three regions overlap with the 5' region of the CRE, the PU.1/PIP, and the E2A motifs. All of these motifs have been shown previously to be important for independent enhancer activity in the context of heterologous promoters (9, 10, 12).

However, other motifs that contribute to the independent enhancer activity had no significant effects on the synergistic activity, including the 3' region of the CRE motif, the BSAP motif, and the region 3' of the E2A motif (12). Our results suggest that while some motifs are dispensable for the synergy, the 5'-CRE, PU.1/PIP, and E2A motifs are required for the full level synergistic activity.

As is evident in Fig. 4B, we also identified a region that when mutated significantly increased the overall transcription activity by 2–3-fold compared with the wild-type  $\kappa$ E3' or the core (LSM(H) in Fig. 4B). This region covers a 10-bp sequence (ACAGAACCTT) located between the PU.1/PIP and the E2A motifs, and it defines a novel negative activity for reporter expression. We therefore refer to this region as the  $\kappa$ E3' negative regulator ( $\kappa$ E3'NR). To rule out the possibility that the linker scanner mutation H artifactually caused the increase in reporter activity, we independently introduced a completely different 10-bp sequence at position H, and observed the same 2–3-fold increase in activity (data not shown).

**The Individual 5'-CRE, PU.1/PIP, and E2A Motifs Are Sufficient to Synergize with the Intron Enhancer, and the Activities of These Motifs Are Developmentally Regulated**—To further examine the importance of the 5'-CRE, PU.1/PIP, and E2A motifs in the developmentally regulated synergistic activation, we tested whether each of these motifs alone was sufficient to synergize with the  $\kappa$ Ei. We performed transfection analysis with constructs in which the wild-type  $\kappa$ E3' was replaced by either the 5'-CRE, PU.1/PIP, or E2A motif alone (Fig. 5A). We found that in S194 mature B cells each motif alone is sufficient to synergize with the  $\kappa$ Ei (Fig. 5B). In fact, each motif conferred an activity even higher than the wild-type  $\kappa$ E3' or the 132-bp core. Notably, the individual  $\kappa$ E3' motifs did not contain the complete  $\kappa$ E3'NR sequence; thus, the result further supports

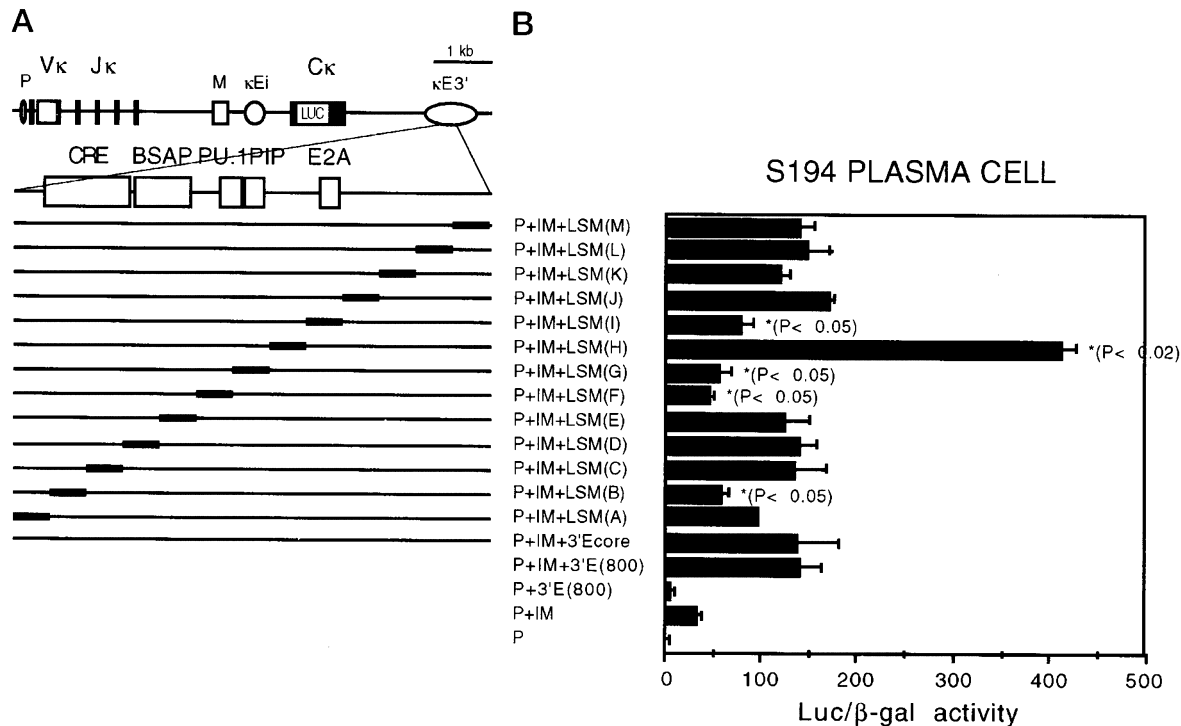


FIG. 4. **Distinct involvement of different 3' enhancer core sequences in the synergy in S194 cells.** A, a diagram of the constructs containing a set of linker scan mutations of the core. LSM stands for linker scan mutation. 13 linker scan mutations are designated as A–M. B, effects of the linker scan mutations on the synergy in S194 cells. *p* values are indicated only for those that have shown significant changes in transcription activities.

the apparent negative effect of the  $\kappa$ E3'NR. In contrast, the  $\kappa$ E3' core, or the individual motifs that showed synergy in S194 cells, showed minimal increases in induced pre-B cells (Fig. 5C), again indicating the synergistic activity is developmentally regulated.

The role of individual linker scanner mutations of the  $\kappa$ E3' was also assessed in induced pre-B cells. Synergy is not observed by the inclusion of both enhancers in pre-B cells (shown in Fig. 2). Indeed, when the intron enhancer was paired with LSMs B (5'-CRE motif), G (PIP motif), and I (E2A motif), there was very little effect on total reporter activity (Fig. 6). However, we noted that in contrast to the negative effect mutation of PU.1 motif had in mature B cells (Fig. 4, LSM(F)), this same mutation served to increase activity in 3-1 pre-B cells (Fig. 6). Similar effects were seen in 38B9 and 1-8 pre-B cells (data not shown). The results we obtained with the PU.1 mutation are also consistent with previous reports, suggesting that the PU.1 motif may serve as a negative regulator in early B cell development (29, 30).

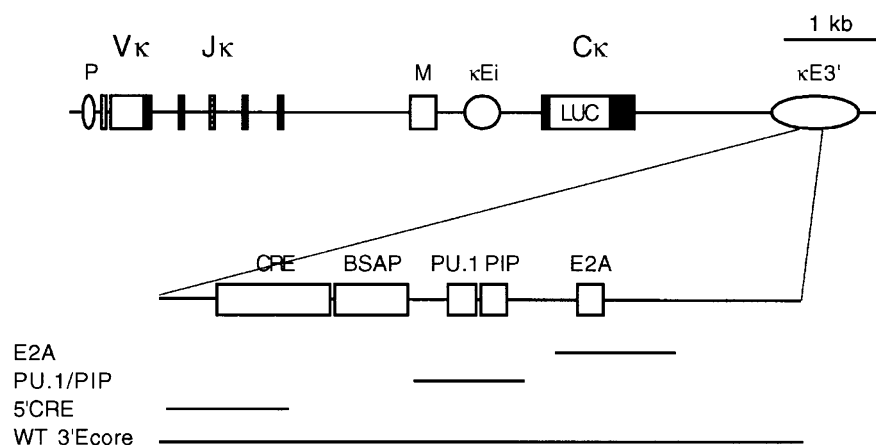
**The Negative Regulator Participates in the Developmental and Tissue-specific Regulation of  $\kappa$  Expression**—While the positive regulators defined by the 5'-CRE, PU.1/PIP, and E2A motifs play significant roles in the developmental synergistic regulation of  $\kappa$  expression, we also identified a novel negative transcription regulator in the  $\kappa$ E3' core (Fig. 4). Considering that  $\kappa$  expression is up-regulated during B cell maturation, we examined whether this region might participate in the developmental regulation. We conducted transfection analysis with the wild-type  $\kappa$ E3' core and the  $\kappa$ E3'NR mutation (LSM H) in the B cell lines representing different developmental stages. The cell lines included the 3-1 and 1-8 pre-B cell lines, the A-20 B cell line, and the S194 plasma cell line. We then compared transcription activities conferred by the two constructs in the same cell lines. We found that the  $\kappa$ E3'NR mutation caused a significantly more dramatic increase (>12-fold) in transcription activity in 3-1 pre-B cells, and the magnitudes decreased

progressively from 3-1 cells to S194 cells where the increase of transcription activity was around 2–3-fold (Figs. 4B and 7A). These results suggest that the negative regulator plays a more prominent role in suppressing transcription at early B cell stages, thus participating in the developmental regulation of the locus.

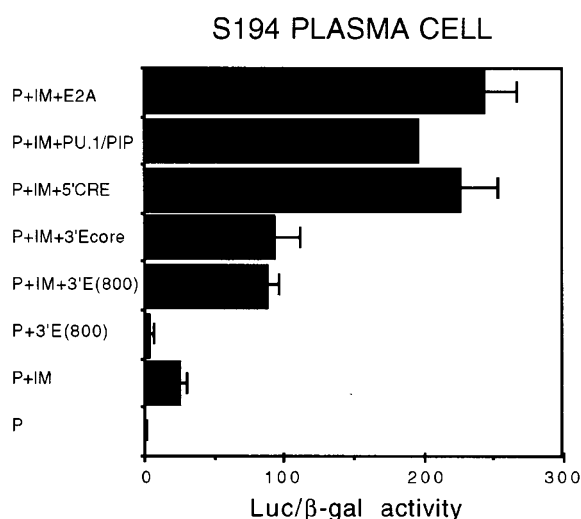
It is known that  $\kappa$  expression is restricted to B cells. Some cis-acting elements including  $\kappa$ E3' sequences have previously been implicated in this cell type-specific control (29, 31, 32). To determine whether the identified negative regulator also participates in the determination of the tissue specificity, we conducted similar transfection studies in several non-B cell lines. The results from the Mel cell line and human Jurkat T lymphoma cell line are presented in Fig. 8, A and B. They show that mutating the negative regulator significantly activates reporter expression in both cell lines, suggesting that the negative regulator may also be involved in determining the tissue-specificity of  $\kappa$  expression.

**Correlation of Transcription Factor Binding Activities with  $\kappa$  Transcription Activity during B Cell Development**—We demonstrated that the activities of both enhancers and the individual  $\kappa$ E3' motifs are up-regulated from early to late B cell stages. This result is consistent with previous reports on  $\kappa$  expression pattern during B cell development (6). To further investigate whether there is any correlation between our reporter expression and transcription factor binding activities of the enhancer sequence motifs, we performed electrophoresis mobility shift assays to examine the protein complexes formed at individual enhancer sequence motifs at different B cell stages. These sequence motifs included the  $\kappa$ A,  $\kappa$ B, E2, and E3 motifs of the  $\kappa$ Ei and the 5'-CRE, PU.1/PIP, and E2A motifs of the  $\kappa$ E3' (Fig. 9). Radiolabeled double-stranded oligonucleotide probes containing individual motifs and nuclear extracts from untreated and LPS-treated 3-1 pre-B cells, and S194 mature B cells were used. To quantitatively measure the differences of the complex formation between different B cell stages, we quantified the

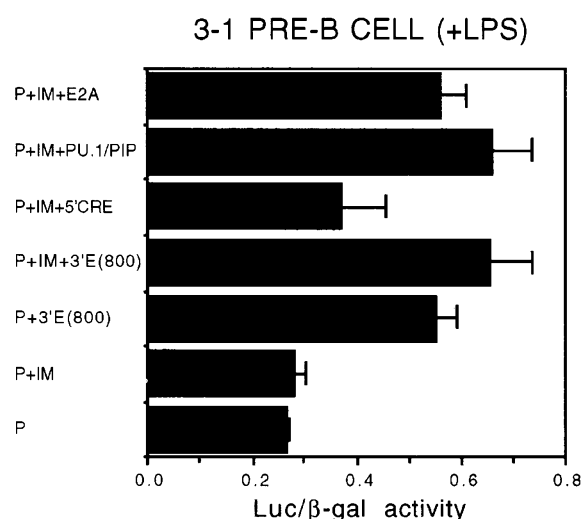
A



B

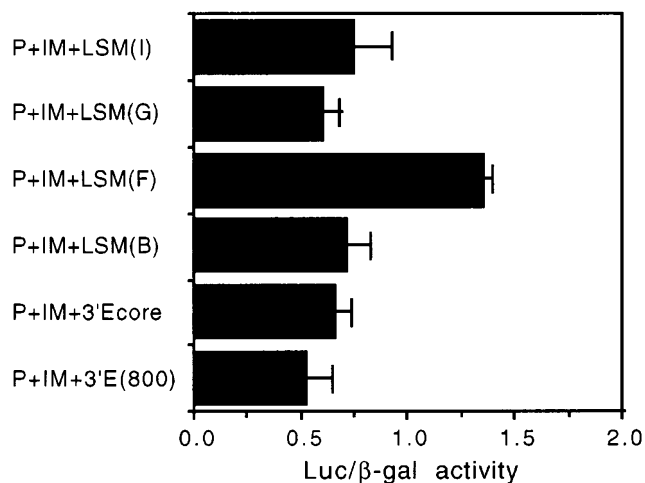


C



**FIG. 5. The individual 5'-CRE, PU.1/PIP, and E2A motifs are sufficient to synergize with the intron enhancer in S194 cells, and their activities are developmentally up-regulated from pre-B to mature B cell stages.** A, a diagram of the constructs containing either the 5'-CRE, PU.1/PIP, or E2A motif of the 3' enhancer core. B, transfection results in S194 cells. C, transfection results in LPS-stimulated 3-1 cells.

### 3-1 PRE-B CELL (+LPS)



**FIG. 6. Linker scanner mutations of the 3' enhancer have different effects in pre-B cells.** Transient transfections were done for linker scanner mutations B, F, G, and I (see legend to Fig. 4A).

shifts of protein-DNA complexes (normalized to Oct-1 binding) (plots shown in Fig. 9). Interestingly, when comparing factor binding activities of uninduced pre-B, induced pre-B, and ma-

ture B cell extracts, we consistently found that there was a modest increase in factor binding to the individual motifs of the  $\kappa$ E3' (less than 2-fold) and a significant increase in most of the factors binding to the  $\kappa$ Ei (up to 20-fold). We have made repeated attempts to identify binding to the  $\kappa$ E3'NR and have been unable to identify specific binding to the region encompassing the  $\kappa$ E3'NR defined by the linker scanner mutations.

### DISCUSSION

In most previous *in vitro* studies of  $\kappa$  enhancer function, the activities of each enhancer have been examined independently. The linker scanner mutations used in this study were originally used to identify sequence motifs that impact on the independent activity of the  $\kappa$ E3' driven by a heterologous herpesvirus thymidine kinase promoter (12). There are a number of conflicting reports on the role of each enhancer at different stages of B cell development. We reported that the  $\kappa$ Ei appeared to be solely responsible for early expression, particularly germ line transcription at the pre-B cell stage (18); however, other reports have indicated the  $\kappa$ E3' may be active at very early stages of B cell development as well (19, 20). In addition, the roles of the enhancers in regulating tissue specificity of transcription as well as the developmental regulation of  $V\kappa J\kappa$  rearrangements have also been addressed (29, 33-36). In this current study we attempted to re-address the roles of individual motifs of the  $\kappa$ E3' in coordinating expression with

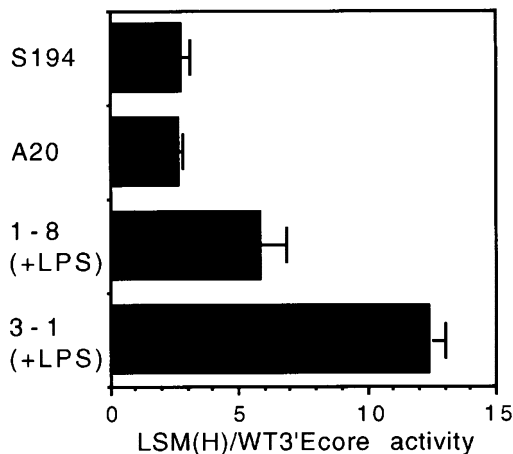
the  $\kappa$ Ei and a representative V $\kappa$  promoter. The use of heterologous promoters and artificial spatial organizations was avoided, and relevant interactions of the locus were preserved. The fusion of the luciferase gene in-frame within the C $\kappa$  region provided a convenient reporter that reflected reasonable tissue specificity and developmental regulation of the locus in B cell lines. While the use of transformed lines has limitations, the clonal responsiveness and enhancer regulation we observe certainly reflect elements that are important for coordinated enhancer regulation of expression. Indeed, transgenic studies of the endogenous  $\kappa$  locus confirm the developmental regulation and synergistic characteristics we are observing (23). Although targeted knockout of either enhancer in mice has only a modest effect on B cell development and overall  $\kappa$  expression, direct comparison of cellular expression levels from an intact and mutated allele has not been done. Moreover, selective pressures in the developing immune response of the mouse can obscure even major effects on expression levels (34, 35).

It is not surprising that sequence motifs that are important

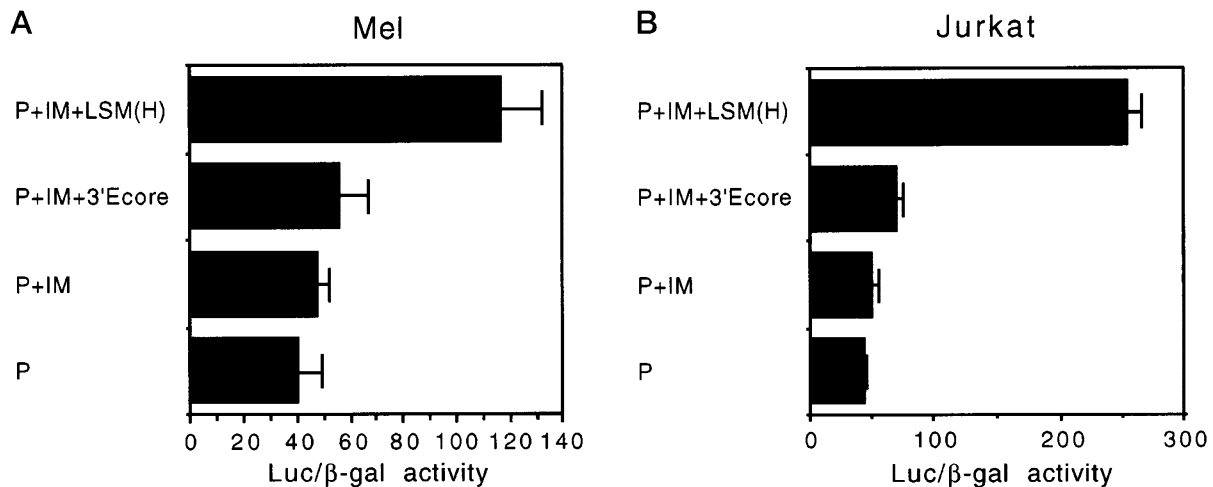
for the independent activity of the  $\kappa$ E3' are important in synergistic activation with the intron enhancer. However, based on the linker scanner mutation analysis, not every sequence motif previously identified to contribute to the enhancer activity is required for the synergistic activity. In addition, we identified a sequence that appears to serve as a negative regulator of  $\kappa$  expression. The increase in activity we observed when using LSM H is a little surprising, since this same linker scanner mutation caused a significant decrease in activity of the enhancer alone when paired with a thymidine kinase promoter (12). In addition, we see no effect of BSAP motif (LSMs D and E) on the synergistic activity; whereas mutating this site had a significant negative effect with the previously reported TK- $\kappa$ E3' construct (12). In comparing these results it is notable that our approach differs in that it is examining the mutations in the context of intron-3' enhancer synergistic activation of the  $\kappa$  locus.

We observed that increases in enhancer activity associated with stages of B cell maturation correlate to increases in relative binding activity of key transcription factors, particularly those within the intron enhancer. Changes in factor binding to the  $\kappa$ E3' motifs have been previously noted during the pro-B to pre-B transition (19). Consistent with our previous results with artificially assembled constructs, synergy between the enhancers was only observed in mature B cells. Somewhat surprisingly, we found that even individual sequence motifs within the  $\kappa$ E3' (5'-CRE, PU.1/PIP, E2A) are capable of synergizing with the  $\kappa$ Ei. Because a number of factor interactions have been reported within complexes forming at the  $\kappa$ E3' (6, 9, 10, 12), it is certainly possible that factor interactions can occur between the enhancer elements that result in different effects of sequence mutations.

The individual enhancer activities appear to be significantly low at early B cell stages. As a result, the impact of the  $\kappa$ E3'NR may be more significant at these stages of development. Mutation of the  $\kappa$ E3'NR has a much more significant effect in pre-B cells than mature B cells (12-fold *versus* 2–3-fold). Thus, as shown in Fig. 10, the net expression of the locus is developmentally regulated by both positive and negative elements, and activity in mature B cells is less sensitive to the effect of the negative regulator. With low enhancer activity in early B cells, the net activity of the locus may be tempered by the NR sequence, whereas the NR sequence has less impact in mature B cells where enhancer activity has increased and synergistic effect is apparent. It appears that the  $\kappa$ E3'NR can also contribute to tissue specificity, as mutation of this region resulted in



**FIG. 7. The negative regulator participates in developmental regulation of  $\kappa$  expression.** Constructs containing either a negative regulator-mutated 3' enhancer core or a wild-type core were transfected into model B cell lines presenting different developmental stages: the 3-1 and 1-8 pre-B cell lines, the A-20 B lymphoma cell line, and the S194 plasmacytoma cell line. Transcription activities conferred by the two constructs were compared (the mutant *versus* the wild-type) in the same cell lines. The average luciferase/ $\beta$ -galactosidase values for each wild-type construct expressed in each cell line are:  $0.698 \pm 0.071$  (3-1),  $0.652 \pm 0.120$  (1-8),  $44.984 \pm 9.960$  (A20), and  $44.601 \pm 8.100$  (S194). The 3-1 and 1-8 cells were treated with LPS for 24 h before luciferase and  $\beta$ -galactosidase assays.



**FIG. 8. The negative regulator is involved in the determination of the tissue specificity of  $\kappa$  expression.** Transfection analysis with the indicated constructs in Mel and human Jurkat T lymphoma cell lines.

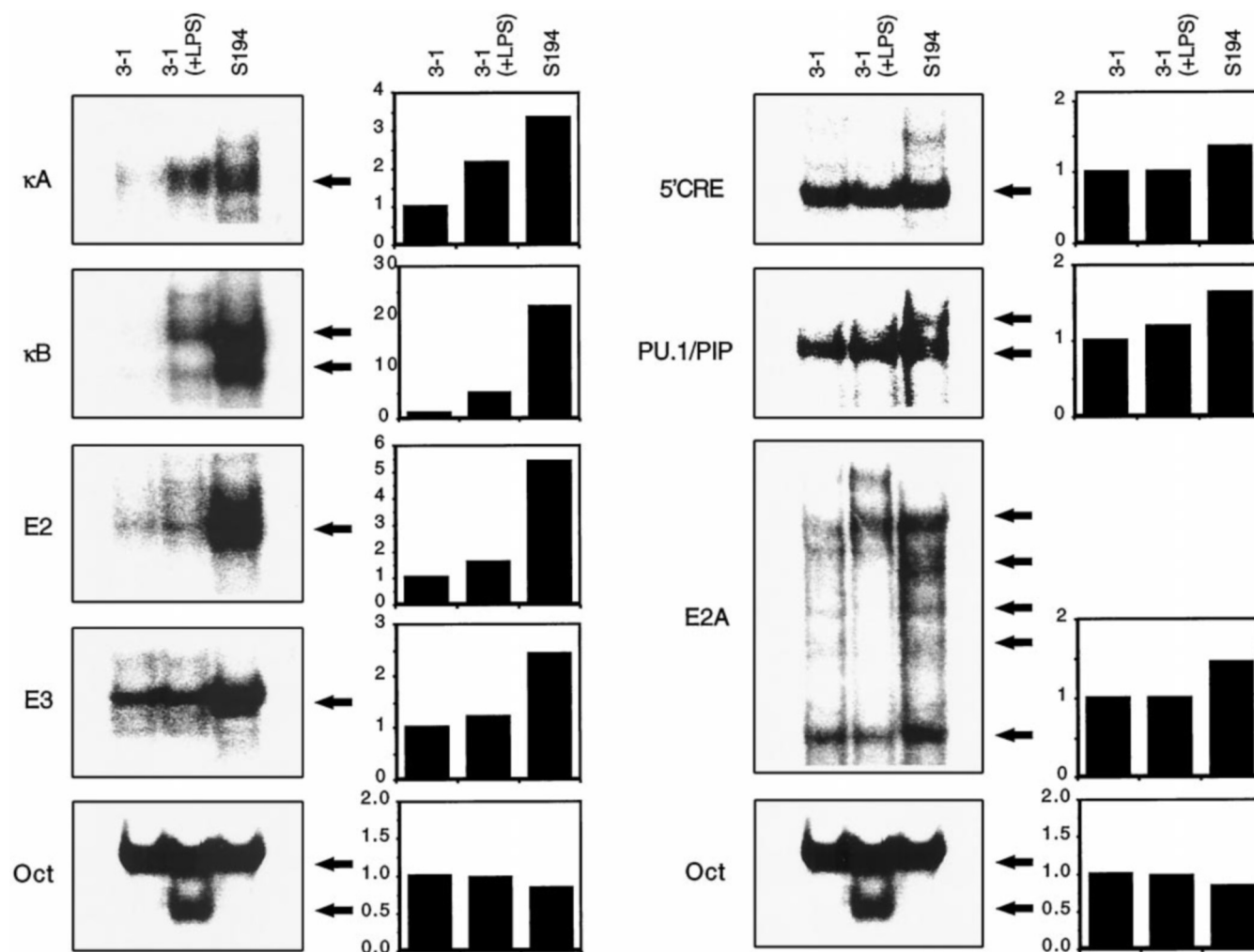


FIG. 9. Transcription factor binding patterns of the individual enhancer sequence motifs at different B cell stages. Electrophoretic mobility shift assays were performed with radiolabeled oligonucleotide probes of the indicated sequence motifs and nuclear extracts of untreated and LPS-treated 3-1 cells and S194 cells. The major protein-DNA complexes were indicated by arrowheads for all tested motifs. The OCTA-1 shifts (upper band) were used as a loading control. The intensities of the major complexes were quantified and normalized to the octamer shifts and plotted for each cell line. When more than one band is indicated, the graphs represent the sum of the signals from all the bands. The value (1) of the shifts at all tested motifs in untreated 3-1 cells is an arbitrary value.

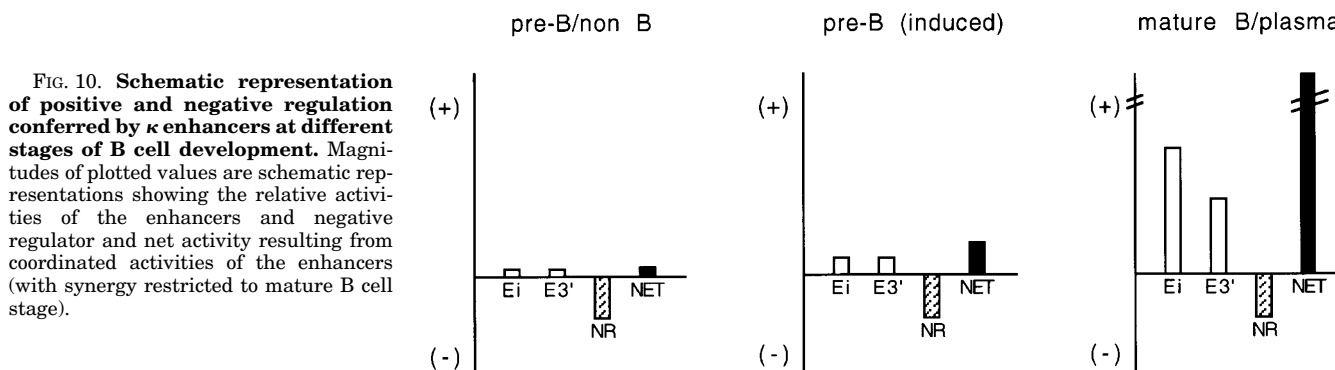


FIG. 10. Schematic representation of positive and negative regulation conferred by  $\kappa$  enhancers at different stages of B cell development. Magnitudes of plotted values are schematic representations showing the relative activities of the enhancers and negative regulator and net activity resulting from coordinated activities of the enhancers (with synergy restricted to mature B cell stage).

reporter activation in non-B cells. Significantly, the 10-bp sequence encompassed by the  $\kappa$ E3'/NR linker scanner mutation is completely conserved between mouse and human (9, 37).

Previous reports suggest the PU.1 motif may act as a negative regulator and may contribute to tissue specificity and developmental timing of V $\kappa$ -J $\kappa$  rearrangements (29, 30). In our study, we find that mutation of the PU.1 motif significantly reduces transcription conferred by both enhancers in mature B cells (Fig. 2), suggesting its role as a positive regulator at this stage of development. However, the same mutation appears to

increase expression in pre-B cells, suggesting its role as a negative element early in development. Therefore, it appears that there is a correlation between regulation of  $\kappa$  rearrangement and transcription by the PU.1 motif. With respect to the  $\kappa$ E3'/NR we have not yet determined its impact on rearrangement events. In addition, we have been unable to identify specific protein-DNA complexes at this region. We also have compared DNA footprints of CRE, PU.1/PIP, and E2A within the context of wild-type and mutant  $\kappa$ E3'/NR sequences and not found any differences (data not shown). Thus, the mechanism



for the negative effect has not been resolved.

As demonstrated in this study, the developmental regulation of the  $\kappa$  expression must be examined in the context of the  $\kappa$  locus, because apparently it is the combined effect of enhancer sequence motifs that coordinately affect expression. Moreover, since there is strong evidence that both enhancers affect  $V\kappa J\kappa$  rearrangement, the coordinate regulation conferred by both enhancers is important to consider.

**Acknowledgments**—We are grateful to Dr. Michael Achitson for providing us the linker scan mutations of the 3' enhancer core and to Dr. William Garrard for constructs with the rearranged kappa locus. We also like to thank members of the Van Ness laboratory for their useful comments.

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