JBC Papers in Press. Published on June 21, 2002 as Manuscript M204065200

Chromatin Structural Analyses of the Mouse Igκ Gene Locus Reveal New Hypersensitive Sites Specifying a Transcriptional Silencer and Enhancer[1]

Zhi-Mei Liu*, Julia B. George-Raizen*, Shuyu Li*, Katherine C. Meyers*, Mee Young Chang*, and William T. Garrard*[2]

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

Copyright 2002 by The American Society for Biochemistry and Molecular Biology, Inc.

Running Title: A New Silencer and Enhancer in the Mouse Igκ Gene Locus

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

FOOTNOTES

*Department of Molecular Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9148

[1]This investigation was supported by Grants GM29935 from the National Institutes of Health and Grant I-823 from the Robert A. Welch Foundation to WTG.

[2]Address correspondence and reprint requests to Dr. William T. Garrard, Department of Molecular Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9148. Phone: 214-648-1924. FAX: 214-648-1915.

E-mail: william. garrard@utsouthwestern.edu

[3]Abbreviations used in the paper: E3', 3' enhancer; Ed, downstream enhancer; Ei, intronic enhancer; HSs, hypersensitive sites; IS, intervening sequence; Luc, luciferase reporter gene; MAR, matrix association region; PCR, polymerase chain reactions; PVκ, minimal Vκ gene promoter; RPI, ribose-5-phosphate isomerase; Sis, intervening sequence silencer; YAC, yeast artificial chromosome.

[4]The new sequences reported here were scanned against the database and were in part matched with Celera GA x5J8B7W84WR:11000001..11340984 and L10 (GenBank accession number V 01557).

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

ABSTRACT

In order to identify new regulatory elements within the mouse Igκ locus, we have mapped DNase I hypersensitive sites (HSs) in the chromatin of B cell lines arrested at different stages of differentiation. We have focused on two regions encompassing 50 kb suspected to contain new regulatory elements based on our previous high-level expression results with YAC-based mouse Igκ transgenes. This approach has revealed a cluster of HSs within the 18 kb intervening sequence, which we cloned and sequenced in its entirety, between the Vκ gene closest to the Jκ region. These HSs exhibit pro/pre-B cell specific transcriptional silencing of a Vκ gene promoter in transient transfection assays. We also identified a plasmacytoma cell-specific HS in the far downstream region of the locus, which in analogous transient transfection assays proved to be a powerful transcriptional enhancer. Deletional analyses reveal that for each element multiple DNA segments cooperate to achieve either silencing or enhancement. The enhancer sequence is conserved in the human Igκ gene locus, including NF-κB and E-box sites that are important for the activity. In summary, our results pinpoint the locations of presumptive regulatory elements for future knockout studies to define their functional roles in the native locus.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

INTRODUCTION

The mouse immunoglobulin (Ig) κ gene locus has provided a paradigm to investigate many challenging and biologically relevant problems, including: site-specific recombination (1-5), tissue-specific transcriptional regulation (1,6,7), somatic hypermutation (8-11), DNA methylation (12-14), the relationship between chromatin structure and function (15-23), and the evolution of DNA sequence organization (24).

The mouse κ locus is the largest multi-gene-family locus thus far identified with respect to genomic length, spanning more than 3.5 megabases (Mb) (25-30). The locus contains 96 potentially functional Vκ genes that have been grouped into 18 families based on sequence homologies (29,31,32), 4 functional and 1 non-functional Jκ regions, and a single Cκ exon. The Vκ families are semi-clustered but partially interspersed with other Vκ families (25,29). The most 5' Vκ gene is a member of the Vκ24 family, some 3.5 Mb away from the Jκ-Cκ region (29). The most 3' Vκ gene is Vκ21G (29), 18 kb away from Jκ1 gene segment (the present work).

Previous studies have identified several *cis*-acting regulatory elements in the mouse Igκ locus. All of these elements, except for Vκ gene promoter elements, reside in a 16 kb segment

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

near or within the Jκ-Cκ region toward the 3' end of the locus. These include: two germline promoter elements (33,34), KI-KII sequences (35), two non-B cell-specific silencers (36,37), a nuclear matrix association region (MAR) (38), an intronic enhancer (Ei) (39) and a 3' enhancer (E3') (40). In some instances targeted deletions of these elements have been performed in cell lines or mice, permitting their functional significance to be addressed in the native locus. Deletion of a germline promoter, or KI-KII sequences, or both, results in a suppressed recombination phenotype (35, 41,42). Deletion of the MAR in a pre-B cell line results in hyper-recombination (13), whereas its deletion from the mouse germline downregulates somatic hypermutation and mildly stimulates precocious Vκ-Jκ joining (43). Deletion of either Ei or E3' severely reduces but does not abolish Igκ gene rearrangement (44,45), whereas deletion of both enhancers reveals that each has a redundant but critical role in regulating recombination in the locus (46).

While considerable insight has been revealed on the functional significance of the above elements, the results of several investigations strongly suggest that additional crucial regulatory elements within the Igκ locus remain to be discovered. For example, expression of rearranged Igκ transgenes containing both enhancers is influenced by the site integration and fails to exhibit copy-number dependence (16,47,48). In addition, mice harboring human Igκ germline transgenes containing all the corresponding known regulatory elements described above exhibit

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

only poor and erratic expression relative to the endogenous mouse Igκ locus (49-53). However, our recent success of achieving high-level expression of YAC-based mouse Igκ transgenes indicates that regulatory elements are present in these constructs capable of conferring copy number-dependent, position-independent germline transcription, tissue and developmental stage-specific efficient Vκ-Jκ rearrangement and rearranged Igκ gene transcription (54). These constructs contained additional upstream and downstream sequences missing from other poorly expressed transgenes, sequences residing both 5' and 3' of the Jκ-Cκ region.

In an effort to identify new regulatory elements we have therefore focused on the aforementioned previously unstudied regions in the mouse Igκ locus suspected to possess transcriptional regulatory elements based on our transgenic mice studies (54). Our approach takes advantage of the observation that when *cis*-acting elements are functional in a particular cell lineage, they often form nuclease hypersensitive sites (HSs) in chromatin (55). We report here the identification, sequencing and initial functional characterization of several such HSs. Interestingly, one cluster of HSs specifies transcriptional silencing in pro/pre-B cells, while another acts as a powerful B-cell specific transcriptional enhancer.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

EXPERIMENTAL PROCEDURES

Long PCR Amplification of the IS—Template DNA was prepared as total DNA from yeast cells bearing either YAC FAW.A3 or YAC FAW.A3 truncated at Vκ21G by chromosome fragmentation (24,25). To truncate FAW.A3, a genomic Vκ21 fragment was amplified by PCR (Primers Vκ21L: 5'-TGC TGC TGC TCT GGG TTC CAG GTG-3', and Vκcdr2r: 5'-GAT TCT AGG TTG GAT GCA GGA TAG-3'). Amplification conditions were 1 min at 94°C, 2 min at 55°C, 1 min at 72°C for 30 cycles. The amplified sequence was introduced into the acentric fragmentation vector pBP81 (56), linearized and transformed into yeast harboring FAW.A3 using standard lithium acetate transformation (57). Several transformants were analyzed using pulsed-field gel electrophoresis. Long PCR was performed using the Expand Long PCR System (Roche). After equilibration in 1x PCR reaction buffer (excluding enzymes), 25 μl master mix 1 (dNTPs plus primer) was added to gel block slices followed by incubation for 15 min at 65°C, and the subsequent addition of 25 μl of master mix 2 (including enzymes). Amplification conditions were 10 cycles of 94°C for 30 s, 55°C for 45 s and 68°C for 12 min. Twenty additional cycles were performed in which the extension cycle was increased by 10 s per cycle. Primers, Vκ21L: 5'-TGC TGC TGC TCT GGG TTC CAG GTG-3'; Jκ2R: 5'-TTT GAG CTT GAG TAG ACA AAT ATC C-3'. 1-2% of the total products were separated by pulsed-field gel electrophoresis on 1% agarose gels using an auto algorithm for 5 to 50 kb on a CHEF mapper (Bio-Rad).

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

Cell Culture--Cell lines, except for S194, A20 and MPC-11, were maintained in RPMI 1640, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 2 mM L-glutamine. ß-mercaptoethanol (50 μM) was added to pre-B cell cultures. S194 and A20 cells were cultured in Iscove's medium containing 5% FBS, and MPC-11 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% horse serum. The engineered 103/Bcl2 cell lines ΔN1 and ΔN7 (58) were kind gifts of Eugene Oltz (Vanderbilt University). The pre-B cell 103Bcl2 lines were maintained at 34°C with 5% CO_2; S194, A20 and MPC-11cells were maintained at 37°C and 10% CO_2; all other lines were maintained at 37°C and 5% CO_2.

Mapping DNase I Hypersensitive Sites--Cells were permeablized with hen egg white α-lysolecithin (Sigma) and treated with increasing concentrations of DNase I (Worthington Biochemicals) (0.25-8.0 u/ml) (17). After lysis, DNA was purified either using Qiagen genomic columns or by phenol: chloroform extraction (13), and samples were digested to completion with either *Bgl*II, *Hind*III, *Nco*I, or P*st*I as indicated below. For P*st*I digests for mapping with probes A or B (see Fig. 1), 10- to 15-µg samples were electrophoresed in 0.8% agarose (SeaKem GTG, FMC Bioproducts) in 0.5x TBE running buffer overnight at 1.1 V/cm. After blotting using standard neutral transfer to Nytran Plus membranes (59), 0.2 µm pore size (Schleicher and Schuell), prehybridized filters were hybridized overnight in 6xSSC, 10x Denhardt's Solution, 1%

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

SDS and 100 μg/ml Herring Testes DNA with a 1.8 kb or 0.8 kb DNA fragments, corresponding to probes A and B respectively, labeled with ^{32}P-α-dCTP using Rediprime II (AP Biotech). Wash conditions were 1x SSC, 0.5% SDS for three times for 30 min at 65°C, or until sufficient background had been eliminated. For mapping with probes C-E, electrophoresis and blotting was as above, but pre-hybridization, hybridization, and washing were performed according to Church and Gilbert (13, 60). Membranes were exposed to Kodak XAR5 film with intensifying screens at –70ºC or to PhosphorImage screens (Molecular Dynamics). Probe A was a 1.8 kb *Cla*I/*Pst*I fragment gel-isolated from the IS long PCR product. Primers for probe B PCR amplification were: 5'PstL, 5'-TAA AAA TCC TGG TGC CAG GGG TG-3'; 5'PstR, 5'-AGC TTA AGG ACG TCA CAT AGA CT-3'. PCR reactions were performed for 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. *Bgl*II or *Nco*I digests were used for mapping with probe C, P*st*I digests for mapping with probe D, and *Hind*III digests for mapping with probe E (see Fig. 1). Probe C consisted of a 1.3 kb *Bgl*II/*Hind*III fragment isolated from the pRxR-1 recombinant plasmid (61). Probe D consisted of a 1.5 kb *Sac*I fragment isolated from the pRSB recombinant plasmid (61). pRxR-1 and pRSB were the kind gifts of Michael Reth (Max-Plank-Institut für Immunobiologie, Freiburg). Probe E was a 510 bp fragment spanning exon 6 of the mouse ribose-5-phosphate isomerase gene (62), prepared as above using the following PCR primers: 5'-GCT TGC TTG GAC CTG CTG G-3' and 5'-CGG CAG AGA AGA CAA AGG ATC C-3'.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

Transient Transfection Luciferase Assays—For functional tests, various fragments were inserted into the *Nhe*I or the *Spe*I site of the polylinker region of KpLUC or IM.KpLUC, respectively (63), designated here as PVκLuc and EiPVκLuc, respectively. A 3.6 kb fragment encompassing hypersensitive sites HS 3-6 was amplified from the IS long PCR product to add external *Spe*I sites using PCR conditions similar to those described above for probe preparation. The primer pairs for fragments containing HS 3-6, HS 4-6, HS 5-6, HS 6, HS 3-4, HS 3 and HS 4-5 were respectively: 5'-ACG CGT CGA CTA GTG TAC TCT GAA CCT TGT ATG GTG ATG-3' and 5'-ACG CGT CGA CTA GTG CAG GTT ATG GGC CCT CTT CC-3'; 5'-ACT CGT CGA CTA GTC TCT GGG CCT GCA CAG ATT CCA C-3' and 5'-ACG CGT CGA CTA GTG CAG GTT ATG GGC CCT CTT CC-3'; 5'-ACG CGT CGA CTA GTC TGC TAC ATA TGT GCG GGG GAG G-3' and 5'-ACG CGT CGA CTA GTG CAG GTT ATG GGC CCT CTT CC-3'; 5'-ACG CGT CGA CTA GTC CCA CCC TCA AGA CAG GCA CAG-3' and 5'-ACG CGT CGA CTA GTG CAG GTT ATG GGC CCT CTT CC-3'; 5'-ACG CGT CGA CTA GTG TAC TCT GAA CCT TGT ATG GTG ATG-3' and 5'-ACG CGT CGA CTA GTC CTC CCC CGC ACA TAT GTA GCA G-3'; 5'-ACG CGT CGA CTA GTG TAC TCT GAA CCT TGT ATG GTG ATG-3' and 5'-ACG CGT CGA CTA GTG GAA TCT GTG CAG GCC CAG AGA C-3'; 5'-ACG CAC GCG TCG ACT AGT CTC TGG GCC TGC ACA GAT TCC AC-3' and 5'-ACG CAC GCG TCG ACT AGT GCC TGT CTT GAG GGT GGG ACT G-3'. The spacer DNA control was a 2.1 kb rat amylase cDNA fragment. PCR conditions were: 1 min at 94°C, 2 min at 55°C, 2 min at 72°C for 30 cycles. For vector insertions we similarly amplified a

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

1 kb fragment encompassing HS 9 from recombinant plasmid pRxB5 (the kind gift of Michael Reth, Max-Plank-Institut für Immunobiologie, Freiburg)(61), using the PCR primers: L10F1 5'-CCG CCG ACT AGT CGT TAG CCC CTG TCC TTG-3', and L10R1 5'- CCG CCG ACT AGT TGT GCA TAT GTG TGT GTA CAC ATG-3'. For testing smaller segments of the 1 kb sequences, we PCR amplified desired regions again adding external *Spe*I sites for vector insertions as above, using the following primers: L10F2 5'-CCG CCG ATC AGT GAA GCC AGG GAA ATG CCA C-3', L10R2 5'- CCG CCG ATC AGT CTA GCT TTA CAG CTT GTC-3', L10R3 5'- CCG CCG ATC AGT GCT TAA GCA GCA GAC AGT G-3', L10R4 5'- CCG CCG ATC AGT GTG CCC TGC ACC TTC AGG-3', L10R5 5'- CCG CCG ATC AGT GTG GCA TTT CCC TGG CTT C-3'. PCR conditions were: 1 min at 94°C, 30 sec at 58°C, 75 sec at 72°C for 35 cycles. Finally, to replace the Vκ promoter with a 420 bp *Bgl*II/*Hind*III fragment containing the SV40 early enhancer/promoter from the pRL-SV40 vector (Promega), the Vκ promoter was deleted from PVκLuc by excision with *Nhe*I and *Hind*III and from PVκLuc containing the 3.6 kb silencer by *Hind*III digestion. Then the sticky-ends of these two vectors were filled in with Klenow and dephosphorylated. The sticky-ends of the SV40 early enhancer/promoter 420 bp fragment were also filled in with Klenow and ligated to the treated vectors to construct P$_{SV40}$Luc and 3.6kbP$_{SV40}$Luc. Cell lines were transiently transfected, either in triplicate in the same batch or in duplicate in separate batches, using either optimized DEAE dextran concentrations (100 to 250 μg/ml; 500 μg/ml for S194 cells) as previously described

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

(58), Lipofectamine-Plus or Lipofectamine 2000 (GibcoBRL). Typically, 10^6 to 10^7 cells and 1-2 μg of DNA were used per transfection, adjusted for insert sizes to provide equimolar comparisons, along with 20 to 50 ng pRL-CMV Renilla luciferase reporter (Promega Corp). Pre-B and more mature cell lines were harvested 24 and 48 hrs post transfection, respectively. The 24 hr time chosen for pre-B cells allowed for optimum reproducibility of ± 10 μg/ml lipopolysaccharide (LPS) comparisons (data not shown). Cell extracts were assayed for luciferase activity using Dual-Luciferase™ Reporter Assay Systems (Promega) following the manufactures' instructions. The Renilla luciferase activity was used for normalization of transfection efficiencies, except for the pre-B cell samples in Fig. 5A & B where extract protein levels were used. Data from a minimum of triplicate experiments are represented with error bars, while duplicate experiments are represented as means. Data were internally consistent between triplicates with the same batch of cells.

Mutagenesis of Ed--Two-step PCR mutagenesis was used to create mutations of NFκB and E-box sites as described elsewhere (64) in a 600bp fragment amplified with L10F1 and L10R3 primers as described above. Sense and anti-sense primers for NFκB site mutation were: 5'-GAA GTC AAA TTG GTT TCC ACT GTG CCA C-3' and 5'- GAA ACC AAT TTG ACT TCA TTA CCT CAT G-3'; sense and anti-sense primers for first E-box mutation were: 5'-CCT GCA TTT TTG CAG TGC AGA TGG AC-3' and 5'-CAC TGC AAA AAT GCA GGG CTG

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

GAC TC-3'; Sense and anti-sense primers for second E-box mutation were: 5'-CAG TGC ATT TTG ACT TGG CAA AAG AAG-3' and 5'-CAA GTC AAA ATG CAC TGC ACA GGT G-3' (mutated bases are underlined).

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

RESULTS

Overall Experimental Strategy--To locate new candidate regulatory elements within the mouse Igκ gene locus we assayed for the presence of DNase I hypersensitive sites in the chromatin of B cell lines representing different stages of lymphocyte differentiation. Such an approach has been proven to be successful previously and has contributed to the discovery and functional analyses of several enhancer elements in the mouse light and heavy chain Ig gene loci (16,17,19,65-68). As shown in Fig. 1, we selected for study two regions that were present in our highly-expressed YAC Igκ transgenes that have not been previously characterized (54): (i) the intervening sequence (IS) between the closest Vκ gene segment to Jκ1; and (ii) the region extending downstream of E3' up to the next non-Ig related gene, that encoding ribose-5-phosphate isomerase (RPI) (62). By definition, the IS represents a boundary within the locus, separating the array of upstream Vκ gene segments from the downstream Jκ regions. The IS either becomes deleted or translocated far upstream after Vκ-Jκ joining, at least 260 kb based on distance of the closest Vκ gene with a reverse orientation (26). This region therefore is a likely candidate to contain an element(s) that specifies regulation by its physical location or relocation relative to other important components within the Igκ gene locus. By contrast, the downstream region studied is maintained in the locus even after recombination, like the regions harboring Ei and E3'. Fig. 1 summarizes the physical locations of HSs, pertinent restriction endonuclease

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

sites, the various hybridization probes used for indirect end-labeling to map HSs (55), and the newly identified novel DNA sequence motifs, LINES, and SINES.

Isolation and Sequencing of the IS of the Mouse Igκ Gene Locus--For the purpose of generating hybridization probes for our chromatin structure mapping experiments, it was first necessary to clone and physically map the previously uncharacterized mouse Igκ gene IS. The Vκ family residing closest to Jκ1 is Vκ21 (24,26,69,70). Previously we performed chromosome fragmentation of Vκ21 gene segments within the recombinant YAC clone FAW.A3 and found that the length of the IS is about 20 kb and that the closest Vκ21 family member to Jκ1 is Vκ21G (24). These results suggested that it might be possible to PCR amplify the IS, which proved to be the case. As shown in Fig. 2 (arrow), the PCR-amplified IS exhibits a mobility in pulsed-field gels of about 20 kb in length, which is a size also in agreement with the physical mapping studies of Zachau and co-workers (26). Note that prior to truncation of FAW.A3 at Vκ21G, the presence of 5' sequence leads to additional PCR products generated primarily by priming by the Vκ21 primer alone (Fig. 2, compare lanes 2 and 3). For further analyses, the desired PCR product was subcloned and entirely sequenced by bi-directional primer walking in duplicate using an ABI Prism™ 377 DNA Sequencer (≥4-fold coverage). The distance between the RSSs of Jκ1 and Vκ21G proved to be 18,023 bp in length (GenBank accession number AF513926)[4]. Inspection of the sequence for segments with potential for forming alternative DNA structures revealed an interesting region [$(CT)_{26}(GT)_{26}$] with unknown present significance (Fig. 1, T/Z),

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

analogous to a triplex-Z DNA motif found near an origin for replication in the Chinese hamster ovary dhfr gene (71). We also found a number of LINE/L1 and SINE/B2 repeats in the IS (Fig. 1, horizontal arrows and lines). Comparison of the mouse sequence with its 23.4 kb human counterpart (GenBank accession number AF017732) by dot matrix analysis reveals that one upstream LINE sequence is conserved as well as the downstream 4 kb region (Fig. 3), which is known to contain the germline promoters and KI/KII elements (33-35,41,42).

The IS Exhibits Six DNase I Hypersensitive Sites in Chromatin--To locate candidate regulatory regions within the IS we mapped the location of HSs in a variety of cell lines using the gentle technique of permeabilization with α-lysolecithin to introduce DNase I (17). A total of six HSs were found in the IS in two clusters (Fig. 1, HSs 1 & 2 and HSs 3-6). HS 1 & 2 appear to be ubiquitous among the non-B and B-cell lines studied and therefore have not been investigated further (data not shown). Primary data illustrating the detection of HS 3-6 in several cell lines is shown in Fig. 4. HS 3 and 6 also appear to be ubiquitous, being present in P815 mastocytoma, EL-4 T cells, BASC6C2 pro-B cells, 103 Bc12 pre-B cells, and S194 plasmacytoma (Fig. 4, open arrowheads). In contrast, HSs 4 & 5, although weakly detectable in EL-4 T cells and BASC6C2 pro-B cells, are most noticeable in 103Bc12 pre-B cells (Fig. 4, closed arrows) and vary in intensity in other pre-B cell lines, including 3-1, and 1-8, and the pro-B cell line 63-12 established from RAG2 -/- animals (data not shown).

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

We also analyzed the most 3' portion of the IS for HSs. This region includes the potential triplex-Z DNA forming region described above, KI/KII sequences and two germline promoters (Fig. 1, T/Z and horizontal arrows) (33-35,41,42). Initiation of transcription from these germline promoters is induced by LPS in cultured pre-B cell lines and has been correlated with the onset of rearrangement in the Igκ gene locus (33,34,72). However, in our assays we failed to detect HSs in either of these germline promoters, either before or after LPS treatment or heat induction of 103Bc12 cells (data not shown), suggesting that these promoters may only be used transiently so that HSs are not detectable or that the entire region is preferentially sensitive to DNase I in pre-B cells. In summary, our initial analyses of chromatin structure within the IS reveal the presence of HSs 1-6 as new candidates for novel regulatory elements. A computer search of the IS sequence against known transcription factor binding sites within and surrounding HSs 1-6 was not particularly revealing.

Functional Analyses of HSs 3-6 Reveals Transcriptional Silencer Activity--Because HSs 4 and 5 appeared to be enhanced in certain pre-B cell lines, we decided to first perform functional studies on these elements together with their nearby surrounding partners, HSs 3 and 6. To determine if transcriptional enhancer activity might be associated with HSs 3-6, a 3.6 kb fragment spanning the entire region was inserted into a construct containing a luciferase reporter gene, upstream of a minimal Vκ gene promoter (PVκLuc). In contrast to our expectations, the inserted element repressed expression of the reporter gene construct 5- to 10-fold in one pro-B

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

and two pre-B cell lines (Fig. 5A, 38B9, 3-1, 103Bcl2, respectively), but was essentially innocuous in MPC-11 plasmacytoma cells (Fig. 5A). To further examine this silencing activity, the 3.6 kb fragment was inserted upstream of the intronic enhancer in a related reporter gene, EiPVκLuc, in either orientation (Fig. 5B). HSs 3-6 again exhibited pro/pre-B cell specific transcriptional silencing, blocking up to 95% of activity in an orientation independent manner (Fig. 5B). This effect was observed in all six pro/pre-B cell lines tested (Fig. 5B, 63-12, 103Bcl2, and 3-1; data not shown). In contrast, HSs 3-6 again had little effect on reporter gene activities in MPC-11 plasmacytoma cells (Fig. 5B), in S194 plasmacytoma cells and in A20 mature B cells (data not shown). Furthermore, pre-B cell specific silencing could not be overcome in PVκLuc±Ei by exposure to LPS (data not shown). In addition, we have also found that the 3.6 kb silencer fragment was incapable of significantly downregulating transcription driven by the SV40 promoter/enhancer element in P815 mastocytoma cells, but could 4-fold suppress transcription in 103Bcl2 pre-B cells (data not shown). Taken together, these results indicate that silencing is clearly pro/pre-B cell specific. We term this silencer Sis.

To pinpoint the DNA sequences responsible for silencing we created a series of 5' or 3' deletions, or both, in the 3.6 kb Sis, and also performed a spacer DNA control. The test sequences were inserted in the positive orientation upstream of EiPVκ in the luciferase reporter gene. However, this analysis revealed that silencer activity appeared to require all 4 HSs; when

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

HS 3 was deleted, silencing activity was lost, and could not be established by HS 3 by itself, neither could HSs 4 & 5 silence by themselves (Fig. 5C). Furthermore, silencing was not due to a change in the DNA sequence spacing as demonstrated by insertion of a cDNA fragment (Fig. 5C). In summary, these initial functional studies reveal that Sis requires HS 3-6 to reduce transcription from 5- to 20-fold in a pro/pre-B cell-specific manner.

The Downstream Region Exhibits a Plasmacytoma-Cell-Specific HS--To locate additional candidate regulatory regions in the Igκ gene locus using similar techniques to those described above, we mapped the location of HSs in a several pre-B and B cell lines within a 30 kb region downstream of E3'. We took advantage of the existing restriction endonuclease physical map and recombinant plasmids bearing these downstream sequences that were kindly made available to us by Michael Reth (ref. 61 and personal communication). Probes C, D, and E failed to detect HSs in the downstream region in either 63-12 pro-B or 103Bcl2 pre-B or S194 plasmacytoma cells after indirect-end labeling of chromatin DNA digests at *Bgl*II, *Pst*I and *Hind*III sites, respectively (see Fig. 1 for the detection strategy; data not shown). However, using probe C for indirect-end labeling of chromatin DNA digests at an *Nco*I site revealed a new HS in the 30 kb region studied, designated HS 9 (Fig. 1). Primary data illustrating the detection of HS 9 is shown in Fig. 6. Interestingly, HS 9 proved to be present only in terminally differentiated plasmacytoma cells (Fig. 6, MPC-11 and S194), being absent from 3-1 pre-B cells, 103Bcl2 pre-B cells ±LPS

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

(Fig. 6), A20 mature B cells and EL-4 T cells (data not shown). In summary, this analysis revealed HS 9 as a plasmacytoma-cell-specific HS.

The DNA Sequence Encompassing HS 9 Contains Potential NF-κB Binding Sites and E-boxes, is Evolutionary Conserved, and is Distally Flanked by a Novel Sequence Motif--In order to gain insight on potential important or unusual DNA sequence motifs, potential transcription factor binding sites and assess evolutionary conservation, we sequenced a 6 kb region surrounding and encompassing HS 9 (GenBank accession number AF513925)[4]. Inspection of the sequence for segments with potential for forming alternative DNA structures revealed a highly unusual 172 bp polypurine array $[(AAAG)_2(A)_5(G)_2(A)_3(GAAA)_2(GA)_{23}(A)_2(GA)_5(GGGA)_8(GGAA)_{14}]$, exhibiting domains of mirror repeat symmetry, with triplex forming potential and unwinding capabilities (Richard Sinden, personal communication), of unknown present significance (Fig. 1, T). We also found segments of one LINE/L1 and SINE/B2 repeat in this downstream region (Fig. 1, horizontal arrow and line). Significantly, the sequence encompassing HS 9 possesses a potential NF-κB binding site and E-boxes (see below), characteristics shared with Ei (63,64). Comparison of the mouse sequence with its 6 kb human counterpart (GenBank accession number AC096579) by dot matrix analysis reveals that several domains in the downstream region are conserved, including those encompassing HS 9 (Fig. 7A), and its potential transcription factor binding sites (Fig. 7B). However, the mouse triplex-forming motif was not found in the corresponding region

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

of the human sequence, but interestingly nevertheless resides in the IS of the human Igκ gene. In summary, the observed conservation of HS 9 prompted our interest for the functional analyses described below.

Functional Analysis of HS 9 Reveals a B-Cell-Specific Transcriptional Enhancer--To determine if transcriptional enhancer activity might be associated with HS 9, a 1 kb fragment spanning the entire region was inserted upstream of PVκ in the luciferase reporter gene construct. The sequence proved to enhance transcription in an orientation independent fashion in both 103Bcl2 pre-B and S194 plasmacytoma cells (Fig. 8A). We therefore term this enhancer Ed, based on its downstream location in the locus. To determine if such an enhancement might be related to NF-κB activity, we took advantage of a derivative of the 103Bcl2 cell line that was kindly made available to us by Eugene Oltz, termed ΔN1, which has been engineered to express a dominant negative form of IκB (58). As shown in Fig. 8A, enhancement of transcription by the 1 kb fragment was markedly suppressed in the ΔN1 cell line. Similar results were obtained using an independently derived dominant negative expressing clone, ΔN7 (data not shown). We conclude that the enhancement of transcription in pre-B cells requires NF-κB.

In order to explore how the activity of Ed in the 1 kb fragment compares to, or can cooperate with Ei, which is also responsive to NF-κB in pre-B cells (58,63,64), we compared on an absolute scale (normalized to PVκLuc) the activity of luciferase reporter genes containing

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

neither, either, or both enhancers inserted upstream of PVκ, in the absence or presence of LPS. As shown in Fig. 8B (top panel), Ed is a more powerful enhancer than Ei, responds mildly to LPS induction as expected, and cooperates with Ei in an additive fashion. However, in S194 and MPC-11 plasmacytoma cells the two enhancers synergistically activate transcription, leading to activation several fold higher than the multiplication products of expression levels achieved by each individual enhancer alone (Fig. 8B, lower panels).

To investigate further the cell-type and developmental specificity of Ed, we assayed for its activity in several other cell lines. As shown in Fig. 8C, Ed lacks activity in EL-4 T cells, but possesses enhancer activity in 38B9 pro-B cells in PVκLuc or EiPVκLuc reporter genes and in A20 mature B cells only in the PVκLuc reporter gene. We conclude that Ed is a B-cell specific enhancer whose activity emerges early in lymphocyte development when assayed by transient expression. This is in contrast to the plasmacytoma-stage-specific appearance of HS 9 (see Discussion).

Site-Directed Mutagenesis of Ed Reveals that NF-κB and E-box sites are Important for Enhancer Activity--To pinpoint the DNA sequences responsible for enhancement, we created a series constructs bearing different segments of the 1 kb enhancer. Fig. 9A shows that deletion of 5' and 3' sequences together flanking the potential NF-κB binding site and E-boxes significantly reduced, but did not fully erase enhancer activity, either in the absence or presence of Ei, in S194

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

plasmacytoma cells. The majority of Ed activity could be narrowed to a 600 bp fragment bearing a 3' deletion (Fig. 9A); similar results were obtained in 103Bcl2 pre-B cells (data not shown).

To determine the role of NF-κB and E-box sites on Ed activity, two-step PCR mutagenesis was used to create mutations in the NF-κB and E-box sites in the 600 bp Ed fragment. As shown in Fig. 9B, mutations in the NF-κB site caused at least a 3-fold decline in enhancement in either the PVκLuc or EiPVκLuc reporter genes in both 103Bcl2 pre-B cells and S194 plasmacytoma cells. Mutations in the E-box 1 or 2 had little effect on the these reporter genes in 103Bcl2 pre-B cells, but mutated E-box 1 led to at least a 3-fold decline in expression of reporter genes in S194 plasmacytoma cells. Mutations in E-box 2 also were deleterious to expression in S194 plasmacytoma cells (Fig. 9B). In conclusion, the NF-κB site is important for activity regardless of the presence or absence of Ei in either pre-B or plasmacytoma cells, whereas E-box 1 and 2 are most important for maximal expression in the presence of Ei in plasmacytoma cells. These results further suggest that the synergy between Ei and Ed in plasmacytoma cells requires both the NF-κB site and E-boxes 1 and 2.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

DISCUSSION

We have physically mapped, cloned and sequenced the mouse Igκ gene IS. Comparison of this sequence with its human counterpart reveals roughly similar lengths of 18- and 23-kb, respectively. Besides conserved LINE elements in their 5' regions, only the 3' regions share significant extended sequence homology, which correspond to segments containing the germline promoters and KI/KII elements (33-35,41,42). Thus, even though we have identified several HS within the mouse IS in the chromatin of several cell lines, the sequences corresponding to these sites appear not to be heavily conserved. However, in the human IS a cluster of V-regions in closest proximity to Jκ1 possess inverted orientations (73), while in the mouse the corresponding V-regions are in the forward orientation. The consequence of this difference is that V-J joining using these closest V-regions would simply invert the IS in the human Igκ gene locus, but lead to a deletion of the corresponding element in the mouse. The first inverted V-region in the mouse is Vκ19/3, residing some 260 kb upstream from Jκ1 (26). Hence, the mouse IS HS could have evolved a regulatory function(s) that requires their deletion or far removal from the Jκ-Cκ region after V-J joining, and/or their ensured presence near the Jκ-Cκ region in germline but **not** rearranged alleles.

We have demonstrated that the IS of the mouse Igκ locus contains several previously unidentified HS in chromatin and that a subset of these sites, HS 3-6, constituting Sis are

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

associated with a pro/pre-B cell specific transcriptional silencer activity. Although an analogous region in the chicken Igλ gene has been reported to also contain a transcriptional silencer (74-76), the mouse element that we have identified differs in two significant respects from the chicken component. The latter is not cell type-specific, and does not share sequence similarity with the mouse element (74-76). Furthermore, the previously identified non-B cell specific silencers within the mouse Igκ gene locus share no sequence homology with Sis (36,37). We conclude that we have identified a novel regulatory element within the Igκ gene locus. Indeed, Igκ germline transgenes containing the IS in addition to all previously known regulatory elements and Ed discovered here exhibit high level germline transcription, tissue-specific rearrangement and subsequent transcription of rearranged genes, and apparent allelic exclusion (54), whereas other transgenes lacking the IS do not (49-53).

The mechanism of silencing is an interesting subject to consider. Possibly the IS silencer exerts its effects by disrupting the assembly of functional transcription factor complexes on promoter elements. It is also possible that the silencer works by targeting the reporter genes to a heterochromatic nuclear subcompartment, as has been associated with the process of allelic exclusion at the mouse Igκ gene locus (77). Interestingly, the silencer contains LINE element segments, and LINES have been implicated in the process of X-chromosome inactivation (78). Although the silencer activity appears complex requiring a combination of HSs, other DNA

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

regulatory elements such as MARs, insulators and polycomb response elements also appear to be quite large and often difficult to define (79).

We have identified a powerful enhancer associated with HS 9, termed Ed, in the downstream region of the Igκ gene locus. Interestingly, enhancers also exist in related far downstream positions in the IgH locus (68). This location ensures that the element will not be deleted upon normal V(D)J joining and class-switch recombination (1). However, Ed is located 5' of the RS segment in a region that is frequently deleted in Igλ expressing cells. HS 9 is not present in pre- or mature-B cells, yet the associated sequence exhibits enhancer activity in transient transfection experiments in these lines. We suggest that this is because the chromatin structure is more open in the transiently transfected plasmid as compared to the endogenous locus to bind NF-κB and E2A proteins (63,64), which are required for Ed activity in B cells, as demostrated by expression of a dominant negative form of IκB, and by mutations in the corresponding *cis*-elements. Although both Ei and Ed possess binding sites for NF-κB and E2A, Ed is a stronger enhancer than Ei. This suggests that that Ed may interact with additional transcription factors, or other NF-κB subunits, or other combinations of E-proteins, or different post-translational-modified forms of these proteins.

Both NF-κB and E2A have previously been demonstrated to be required for B cell development (reviewed in refs. 7, 80). Knockouts of several NF-κB subunits in a variety of combinations leads to a complete block in B cell commitment. Surprisingly, mice lacking both

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

the p50 and p52 subunits of NF-κB fail to develop mature IgM$^+$ B cells with the earlier stages of B cell development proceeding normally (81). It is possible that NF-κB binding to Ed, most active in later stages of B cell development, is required for full high-level expression of rearranged Igκ alleles and final B cell development.

Our data also indicate a requirement of an intact E-box-1 for full enhancer activity. The requirement for E-box binding proteins in B cell development is well established (reviewed in ref. 80). The alternatively spliced forms of E2A, E12 and E47, are both required for B cell development at multiple stages, including binding to the E boxes in Ei and E3' (82-84). E2A knockout mice form barely detectable levels of B220$^+$ cells (85,86), but E47 knock-in experiments demonstrate that E47 can allow cells to progress through to the mature B220$^+$ IgM$^+$ stage (87). Although roles for E2A have been identified at multiple points during the development of both B and T cells, no role has yet been found at the transition from pre-B to mature B cells. The E boxes in Ed may provide a context to identify yet another role for these transcription factors in lymphocyte development.

Previously we and others have shown that Ei and E3' act synergistically when together in expression constructs in plasmacytoma, but not pre-B cells (16, 63). Here we show that Ei and Ed synergize, again in plasmacytoma, but not pre-B cells, through a process requiring sites for both NF-κB and E2A. Although we have not explored interactions between E3' and Ed, previous

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

studies have shown that dimerization of either Ei or E3' alone also yields to similar levels of synergy (88). Thus, interactions between unique aspects present in different enhancers are not necessary for synergy.

It is interesting to consider the relative timing of appearance and functions of HS 7-9 during B cell development. HS 8 (E3') appears at the earliest stages and persists throughout B cell development, but exhibits differentiation specific changes in its fine structure (17,20). This enhancer is thought to play a negative role during early, followed by positive roles later, in B cell development (17,20,63,89). HS 7 (Ei) is LPS inducible in early B cell lines (19,65), but becomes constitutive in plasmacytoma cells (16). Both HS 7 and HS 8 contribute to the efficiency of rearrangement at the Igκ locus (44-46). As we have suggested above, the latest appearing hypersensitive site, HS 9 (Ed), presumably contributes to high-level transcription in terminally differentiated B cells.

After sequencing the region encompassing HS 9, we found that the segment already existed in the database and had been termed L10 (GenBank accession number V 01557)[4](90). An aberrant rearrangement in MOPC 41 plasmacytoma cells resulted in the linkage of L10 to the RSS of Jκ1 (90), which is now recognized to have occurred by creating an inversion in the Jκ-Cκ and downstream sequences in the locus (26). Besides the recombining sequence (RS) associated

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

with deletion of the Igκ gene locus in Igλ producers (61), L10 is the only rearranging structure downstream of Cκ.

Obviously, targeted deletion of Sis and Ed from the native Igκ locus will be required to establish if they play any essential roles in Igκ gene dynamics. To obtain a definitive answer on this point, we have instituted such an experimental plan using the YAC-based Igκ transgenic system that we have previously developed (54). We have targeted, through reverse genetics in yeast, LoxP sites on either side of HS 3-6, and have established transgenic mouse lines harboring such an engineered Igκ transgene. Once we identify lines in which this transgene is fully functional, we will breed such animals with those that conditionally express Cre recombinase for subsequently determining the functional consequences during or after B cell development of deleting the element. We plan to carry out a similar approach for the elucidation of Ed function.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

ACKNOWLEDGEMENTS

We thank S. Hall and R. Conner for automated sequencing, A. Tizenor for aid in graphics, and Drs. Michael Reth, Brian VanNess and Eugene Oltz for providing plasmids and cell lines.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

REFERENCES

1. Sleckman, B. P., Gorman, J. R., and Alt, F. W. (1996) *Annu. Rev. Immunol.* **14**, 459-481.

2. Lewis, S. M., and Wu, G. E. (1997) *Cell* **88**, 159-162.

3. Papavasiliou, F., Jankovic, M., Gong, S., and Nussenzweig, M. C. (1997) *Curr. Opin. Immunol.* **9**, 233-238.

4. Grawunder, U., West, R. B., and Lieber, M. R., (1998) *Curr. Opin. Immunol.* **10**, 172-180.

5. Cedar, H., and Bergman, Y. (1999) *Curr. Opin. Immunol.* **11**, 64-69.

6. Henderson, A., and Calame, K. (1998) *Annu. Rev. Immunol.* **16**, 163-200.

7. Reya, T. and Grosschedl, R. (1998) *Curr. Opin. Immunol.* **10**, 158-165.

8. Green, N. S., Lin, M. M., and Scharff, M. D. (1998) *BioEssays* **20**, 227-234.

9. Neuberger, M. S., Ehrenstein, M. R., Klix, N., Jolly, C. J., Yelamos, J., Reda, C., and Milstein, C. (1998) *Immunol. Rev.* **162**, 107-116.

10. Storb, U., Peters, A., Klotz, E., Kim N., Shen, H. M., Hackett, J., Rogerson, B., and Martin, T. E. (1998) *Immunol. Rev.* **162**,153-160.

11. Rada, C., and Milstein, C. (2001) *EMBO J.* **20**, 4570-4576.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

12. Lichtenstein, M., Keini, G., Cedar, H., and Bergman, Y. (1994) *Cell* **76**, 913-923.

13. Hale, M. A., and Garrard, W. T. (1998) *Mol. Immunol.* **35**, 609-620.

14. Mostoslavsky, R., Singh, N., Kirillov, A., Pelanda, R., Cedar, H., Chess, A., and Bergman, Y. (1998) *Genes Dev.* **12**, 1801-1811.

15. Garrard, W. T. (1989) In *Tissue Specific Gene Expression,* R. Renkawitz, ed. VCH Publishers, N. Y., p. 13-29.

16. Blasquez, V. C., Hale, M. A., Trevorrow, K. W., and Garrard, W. T. (1992) *J. Biol. Chem.* **267**, 23888-23893.

17. Roque, M. A., Smith, P. A., and Blasquez ,V. C. (1996) *Mol. Cell. Biol.* **16**, 3138-3155.

18. Stanhope-Baker, P., Hudson, K. M., Shaffer, A. L., Constanitinescu, A., and Schlissel, M. S. (1996) *Cell* **85**, 887-897.

19. O'Brien, D. P., Oltz, E. M., and Van Ness, B. G. (1997) *Mol. Cell. Biol.* **17**, 3477-3787.

20. Shaffer, A. L., Peng, A., and Schlissel, M. S. (1997) *Immunity* **6**, 131-143.

21. Nagawa, F., Ishigur,o K., Tsuboi, A., Yoshida, T., Ishikawa, A., Takemori, T., Otsuka, A. J., and Sakano, H.. (1998) *Mol. Cell. Biol.* **18**, 655-663.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

22. Roth, D. B., and Roth, S. Y. (2000) *Cell* **103**, 699-702.

23. Maës, J., O'Neill, L. P., Cavelier, P., Turner, B. M., Rougeon, F., and Goodhardt, M. (2001) *J. Immunol.* **167**, 866-874.

24. George, J. B., Li, S., and Garrard, W. T. (1997) In *The Antibodies,* Vol. 4, J. D. Capra and M. Zanetti, eds. Gordon and Breach/Harwood Academic Publisher, San Diego, p. 41-62.

25. George, J. B., Li, S., and Garrard ,W. T. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 12421-12425.

26. Kirschbaum, T., Pourrajabi, S., Zocher, I., Schwendinger, J., Heim, V., Roschenthaler, F., Kirschbaum, V., and Zachau, H. G. (1998) *Eur. J. Immunol.* **28**, 1458-1466.

27. Kirschbaum, T., Röschenthaler, F., Bensch, A., Hölscher, B., Lautner-Rieske, A., Ohnrich, M., Pourrajabi, S., Schwendinger, J., Zocher, I., and Zachau, H. G. (1999) *Eur. J. Immunol.* **29**, 2057-2064.

28. Roschenthaler, F., Kirschbaum, T., Heim, V., Kirschbaum, V., Schable, K. F., Schwendinger, J., Zocher, I. and Zachau, H. G. (1999) *Eur. J. Immunol.* **29**, 2065-2071.

29. Thiebe, R., Schable, K. F., Bench, A., Brensing-Kuppers, J., Heim, V., Kirschbaum, T., Mitlohner, H., Ohnrich, M., Pourrajabi, S., Roschenthaler, F., Schwendinger, J., Wichelhaus, D., Zocher, I. and Zachau, H. G. (1999) *Eur. J. Immunol.* **29**, 2072-2081.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

30. Schable, K. F., Thiebe, R., Bench, A., Brensing-Kuppers, J., Heim, V., Kirschbaum, T., Lamm, R., Ohnrich, M., Pourrajabi, S., Roschenthaler, F., Schwendinger, J., Wichelhaus, D., Zocher, I. and Zachau, H. G. (1999) *Eur. J. Immunol.* **29**, 2082-2086.

31. Strohal, R., Helmberg, A., Kroemer, G., and Kofler, R. (1989) *Immunogenetics* **30**, 475-493.

32. D'Hoostelaere, L. A., and Klinman, D. (1990) *J. Immunol.* **145,** 2706-2712.

33. Van Ness, B. G., Weigert, M., Coleclough, C., Mather, E. L., Kelley, D. E., and Perry, R. P. (1981) *Cell* **27**, 593-602.

34. Martin, D. J., and Van Ness, B. G. (1990) *Mol. Cell. Biol.* **10**, 1950-1958.

35. Ferradini, L., Gu, H., Smet, A. D., Rajewsky, K., Reynaud, C. A., and Weill, J. C. (1996) *Science* **271**, 1416-1420.

36. Pierce, J. W., Gifford, A. M., and Baltimore, D. (1991) *Mol. Cell. Biol.* **11**, 1431-1437.

37. Saksela, K., and Baltimore, D. (1993) *Mol. Cell Biol.* **13**, 3698-3705.

38. Cockerill, P. N., and Garrard, W. T. (1986) *Cell* **44**, 273-282.

39. Queen, C., and Baltimore, D. (1983) *Cell* **33**, 741-748.

40. Meyer, K. B., and Neuberger, M. S. (1989) *EMBO J.* **8**, 1959-1964.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

41. Cocea, L., De Smet, A., Saghatchian, M., Fillatreau, S., Ferradini, L., Schurmans, S., Weil, J. –C., and Reynaud, C. –A. (1999) *J. Exp. Med.* **189**, 1443-1450.

42. Liu, X., and Van Ness, B. G. (1999) *Mol. Immunol.* **36**, 461-469.

43. Yi, M., Wu, P., Trevorrow, K. W., Claffin, L., and Garrard, W. T. (1999) *J. Immunol.* **162**, 6029-6039.

44. Xu, Y., Davidson, L., Alt, F. W., and Baltimore, D. (1996) *Immunity* **4**, 377-385.

45. Gorman, J. R., Van der Stoep, N., Monroe, R., Cogne, M., Davidson, L., and Alt, F. W. (1996) *Immunity* **5**, 241-252.

46. Inlay, M., Alt, F. W., Baltimore, D., and Xu, Y. (2002) *Nature Immunol.* **3**, 463-468.

47. Blasquez, V. C., Xu, M., Moses, S. C. and Garrard, W. T. (1989) *J. Biol. Chem.* **264**, 21183-21189.

48. Xu, M., Hammer, R. E., Blasquez, V. C., Jones, S. A., and Garrard, W. T. (1989) *J. Biol. Chem.* **264**, 21190-21195.

49. Green, L. L., Hardy, M. C., Maynard-Currie, C. E., Tsuda, H., Louie, D. M., Mendez, M. J., Abderrahim, H., Noguchi, M., Smith, D. H., Zeng, Y., David, N. E., Sasai, H., Garza, D., Brenner, D. G., Hales, J. F., McGuinness, R. P., Capon, D. J., Klapholz, S., and Jakobovits, A. (1994) *Nature Genetics* **7**,13-21.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

50. Lonberg, N., L. Taylor, D., Harding, F. A., Trounstine, M., Higgins, K. M., Schramm, S. R., Kuo, C. C., Mashayekh, R., Wymore, K., McCabe, J. G., Munoz-O'Regan, D., O'Donnell, S. L., Lapachet, E. S. G., Bengoechea, T., Fishwild, D. M., Carmack, C. E., Kay, R. M., and Huszar, D. (1994) *Nature* **368**, 856-859.

51. Zou, X., Xian, J., Davies, N. P., Popov, A. V., and Bruggemann, M. (1996) *FASEB J*. **10**, 1227-1232.

52. Cavelier, P., Nato, F., Coquilleau, I., Rolink, A., Rougeon, F., and Goodhardt, M. (1997) *Eur. J. Immunol*. **27**, 1626-1631.

53. Xian, J., Zou, X., Popov, A. V., Mundt, C. A., Miller, N., Williams, G. T., Davies, S. L., Neuberger, M. S., and Bruggemann, M. (1998) *Transgenics* **2**, 333-343.

54. Li, S., Hammer, R. E., George-Raizen, J. B., Meyers, K. C., and Garrard, W. T. (2000) *J. Immunol*. **164**, 812-824.

55. Gross, D. S., and Garrard, W. T. (1988) *Ann. Rev. Biochem*. **57**, 157-197.

56. Reeves, R. H., Pavan, W. J., and Hieter, P. (1992) *Meth. Enzymol*. **216**, 584-603.

57. Ito, H., Fukuda Y., Murata K., and Kimura A. (1983) *J. Bacteriol*. **53**, 163-168.

58. Scherrer, D. C., Brockman, J. A., Bendall, H. H., Zhang, G . M., Ballard, D. W., and Oltz, E. M. (1996) *Immunity* **5**, 563-574.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

59. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*.

60. Church, G. M., and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1991-1995.

61. Müller, B., Stappert, H., and Reth, M. (1990) *Eur. J. Immunol.* **20**, 1409-1411.

62. Apel, T. W., Scherer, A., Adachi, T., Auch, D., Ayane, M., and Reth, M. (1995) *Gene* **156**, 191-197.

63. Fulton, R., and Van Ness, B. G. (1993) *Nucl. Acids. Res.* **21**, 4941-4947.

64. Schanke J. T., and Van Ness B. G. (1994) *J. Immunol.* **153**, 4565-4572.

65. Parslow, T. G., and Granner, D. K. (1982) *Nature* **299**, 449-451.

66. Hagman, J., Rudin, C. M., Haasch, D., Chaplin, D., and Storb, U. (1990) *Genes Dev.* **4**, 978-992.

67. Giannini, S. L., Singh, M., Calvo, C.-F., Ding, G., and Birshtein, B. K. (1993) *J. Immunol.* **150**, 1772-1780.

68. Madisen, L., and Groudine, M. (1994) *Genes Dev.* **8**, 2212-2226.

69. Heinrich, G., Traunecker, A., and Tonegawa S. (1984) *J. Exp. Med.* **159**, 417-435.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

70. Alanen, A., and Weiss, S. (1989) *Eur. J. Immunol.* **19**, 1961-1963.

71. Brinton, B. T., Caddle, M. S., and Heintz, N. H. (1991) *J. Biol. Chem.* **266**, 5153-5161.

72. Schlissel, M. S., and Baltimore, D. (1989) *Cell* **58**, 1001-1007.

73. Zachau, H.G. (1996) *The Immunologist* **4**, 49-54.

74. Lauster, R., Reynaud, C.-A., Martensson, I.-L., Peter, A., Buccini, D., Jami, J., and Weill, J.-C. (1993) *EMBO J.* **12**, 4615-4623.

75. Ferradini, L., Reynaud, C.-A., Lauster, R., and Weill, J.-C. (1994) *Sem. in Immunol.* **6**, 165-173.

76. Bulfone-Paus, S., Reiners-Schramm, L., and Lauster, R. (1995) *Nucl. Acids. Res.* **23**, 1997-2005.

77. Skok, J. A., Brown, K. E., Azuara, V., Caparros,,M.-L., Baxter, J., Takacs, K., Dillon, N., Gray, D., Perry, R. P., Merkenschlager, M., and Fisher, A. M. (2001) *Nature Immunol.* **2**, 848-854.

78. Bailey, J. A., Carrel, L., Chakravartl, A., and Eichler, E. E. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 6634-6639.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

79. Scheuermann, R. H., and Garrard, W. T. (1999) In: Critical Reviews in Eukaryotic Gene Expression, vol. 9, pp. 295-310, CRC Press, Inc.

80. Quong, M. W., Romanow, W. J., and Murre, C. (2002) *Annu. Rev. Immunol.* **20,** 301-322.

81. Franzoso, G., Carlson, L., Xing, L., Poljak, L., Shores, E. W., Brown, K. D., Leonardi, A., Tran, T., Boyce, B. F., and Siebenlist, U. (1997) *Genes Dev.* **11,** 3482-3496.

82. Murre, C., Voronova, A., and Baltimore, D. (1991) *Mol. Cell. Biol* **11,** 1156-1160.

83. Pongubala, J., and Atchison, M. (1991) *Mol. Cell. Biol.* **11,** 1040-1047.

84. Shen, C. P., and Kadesch, T. (1995) *Mol. Cell. Biol.* **15,** 4518-4524.

85. Bain, G., Robanus Maandag, E. C., Izon, D. J., Amsen, D., Kruisbeek, A. M., Weintraub, B. C., Krop, I., Schlissel, M. S., Feeney, A. J., van Roon, M., van der Valk, M., te Riele, H. P. J., Berns, A., and Murre, C. (1994) *Cell* **79,** 885-892.

86. Zhuang, Y., Soriano, P., and Weintraub, H. (1994) *Cell* **79,** 875-884.

87. Bain, G., Robanus Maandag, E. C., te Riele, H. P. J., Feeney, A. J., Sheehy, A., Schlissel, M., Shinton, S. A., Hardy, R. R., Murre, C. (1997) *Immunity* **6,** 145-154.

88. Fulton, R., and Van Ness, B. G. (1994) *Nucl. Acids. Res.* **22**, 4216-4223.

89. Liu, X., Prabhu, A., and Van Ness, B. (1999*) J. Biol. Chem.* **274**, 3285-3293.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

90. Höchtl, J., and Zachau, H. G. (1983) *Nature* **302**, 260-263.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

FIGURE LEGENDS

FIG. 1. **Positions of HSs and other key elements in the Igκ gene locus.** Bg, H, N, and P and designate the positions of relevant *Bgl*II, *Hind*III, *Nco*I and *Pst*I sites. Also indicated are the hybridization probes for indirect end-labeling (bold bars), a potential triplex-Z DNA forming sequence (T/Z), germline promoters (horizontal arrows), Vκ-, Jκ-, and Cκ-exons, enhancers Ei and E3', a MAR, a potential triplex forming sequence (T), and the recombining sequence (RS) associated with deletion of the Igκ gene locus in Igλ producers (61). Repeat sequences were identified with a program available on the web (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker). LINE and SINE sequences are depicted as horizontal arrows and lines, respectively.

FIG. 2. **Long PCR amplification of the Igκ gene IS.** PCR amplification products were assayed by pulsed-field agarose gel electrophoresis with ethidium bromide DNA staining. Lanes 1-3, YAC FAW.A3 as template (25); lanes 4-6, YAC FAW.A3 truncated at Vκ21G as template (24). The solid arrow represents the IS amplified product.

FIG. 3. **Dot matrix sequence comparisons of the sequences of the mouse and human ISs.** The stringency used a window with 15 of 21 matches.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

FIG. 4. **Analysis of the chromatin structure of the Igκ gene IS.** Solid arrows indicate pre-B cell enhanced HSs. HSs 3-6 were mapped by Southern analysis after DNase I digestion of the chromatin of various indicated cell lines, after *Pst*I digestion and labeling with probe B (see Fig. 1).

FIG. 5. **DNA sequences encompassing HSs 3-6 in the IS exhibit pro/pre-B cell specific transcriptional silencing.** A. A 3.6 kb fragment spanning the entire region was inserted into a construct containing a luciferase reporter gene, upstream of a minimal Vκ gene promoter (PVκLuc) and assayed in the pro-B cell line 38B9, pre-B cell lines 3-1 and 103Bcl2, and MPC-11 plasmacytoma cells. B. The 3.6 kb fragment was inserted upstream of the intronic enhancer in a related reporter gene, EiPVκLuc, in either orientation. The activities of the luciferase reporter genes were assayed in the pro-B cell line 63-12 derived from RAG2 -/- animals, pre-B cell lines 103Bcl2 and 3-1, and MPC-11 plasmacytoma cells. C. The luciferase activities of a series constructs harboring 5' or 3' deletions, or both, in the 3.6 kb Sis, and a spacer DNA length control assayed in 103Bcl2 cells. The test sequences were inserted upstream of EiPVκ in the luciferase reporter gene. The activity of each construct was measured after transient transfection of the indicated cell lines. The level of activity of each construct is expressed as a percent of the activity of the indicated reporter gene without an insert at the upstream test site, after correction for extract yields by protein assays (A and B panels), or relative transfection efficiencies by

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

monitoring the activity of a co-transfected pRL-CMV Renilla luciferase reporter gene (panel C). Insertion in the native orientation with respect to the promoter is indicated as HS3-6 → (cross-hatched bars), while insertion in the reverse orientation is indicated as HS3-6 ← (open bars). Spacer, cDNA length spacing control.

FIG. 6. **Analysis of the chromatin structure of the Igκ gene downstream region.** Solid arrow indicates the plasmacytoma cell specific HS 9. Mapping by Southern analysis after DNase I digestion of the chromatin of various indicated cell lines, after *Nco*I digestion and labeling with probe C (see Fig. 1).

FIG. 7. **Comparison of the mouse Ed sequence with its human counterpart.** A. Dot matrix sequence comparisons of the sequences of the mouse and human Igκ gene downstream region, using a window with 13 of 21 matches. B. The sequences encompassing HS 9 possess one potential NF-κB binding site [GGG(A/G)NN(T/C)(T/C)CC] and two E-boxes [CANNTG], which are conserved between mouse and human.

FIG. 8. **DNA sequences encompassing HS 9 specify a transcriptional enhancer.** A. A 1 kb fragment spanning the entire region was inserted upstream of PVκ in the luciferase reporter gene construct in both orientations and assayed in S194 plasmacytoma cells, pre-B cell lines 103Bcl2

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

and a 103Bcl2 derivative ΔN1 that possesses a dominantly inhibited NF-κB. B. The activity of luciferase reporter genes containing neither, either, or both Ei and Ed enhancers inserted upstream of PVκ in 103Bcl2 pre-B cell line (in the absence or presence of LPS), S194 and MPC-11 plasmacytoma cells. Panel B represents data normalized to PVκLuc. C. The 1 kb Ed fragment assayed in EL-4 T cells, 38B9 pro-B cells and A20 mature B cells. Except for the upper diagram in panel B, all data are represented as the activity of each construct relative to the activity of the indicated reporter gene without an insert at the upstream test site, after correction for relative transfection efficiencies by monitoring the activity of a co-transfected pRL-CMV Renilla luciferase reporter gene. Insertion in the native orientation with respect to the promoter is indicated as Ed → (cross-hatched bars), while insertion in the reverse orientation is indicated as Ed ← (open bars).

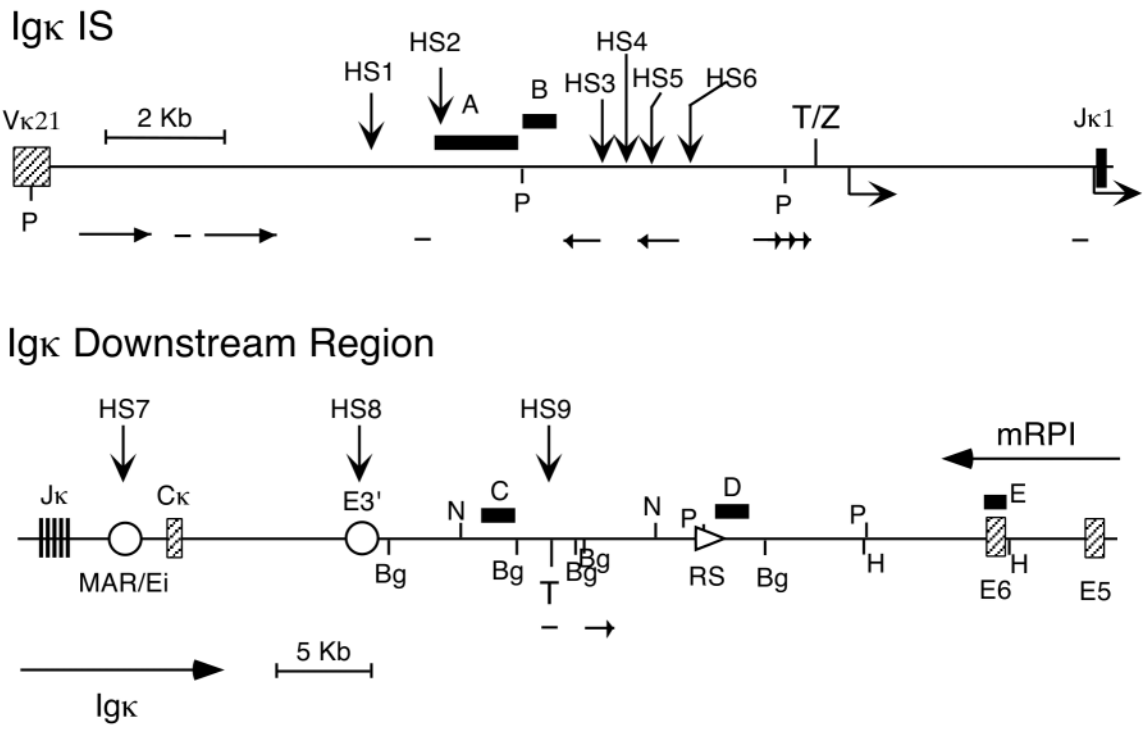
FIG. 9. **The NFκB site and E-box are important for the Ed enhancer activity.** A. The luciferase activity of a series constructs bearing different segments of the 1 kb enhancer with or without Ei in S194 plasmacytoma cells. Data represented in panels A were normalized as described in Fig. 8. B. Functional analysis of the 600 bp Ed fragment with and without targeted mutations in the NF-κB site (ΔκB), the first E-box (ΔE1) or the second E-box (ΔE2), inserted upstream of PVκ or EiPVκ luciferase reporter gene constructs, assayed in S194 plasmacytoma and 103Bcl2 pre-B cell lines. Insertion in the native orientation with respect to the promoter is

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

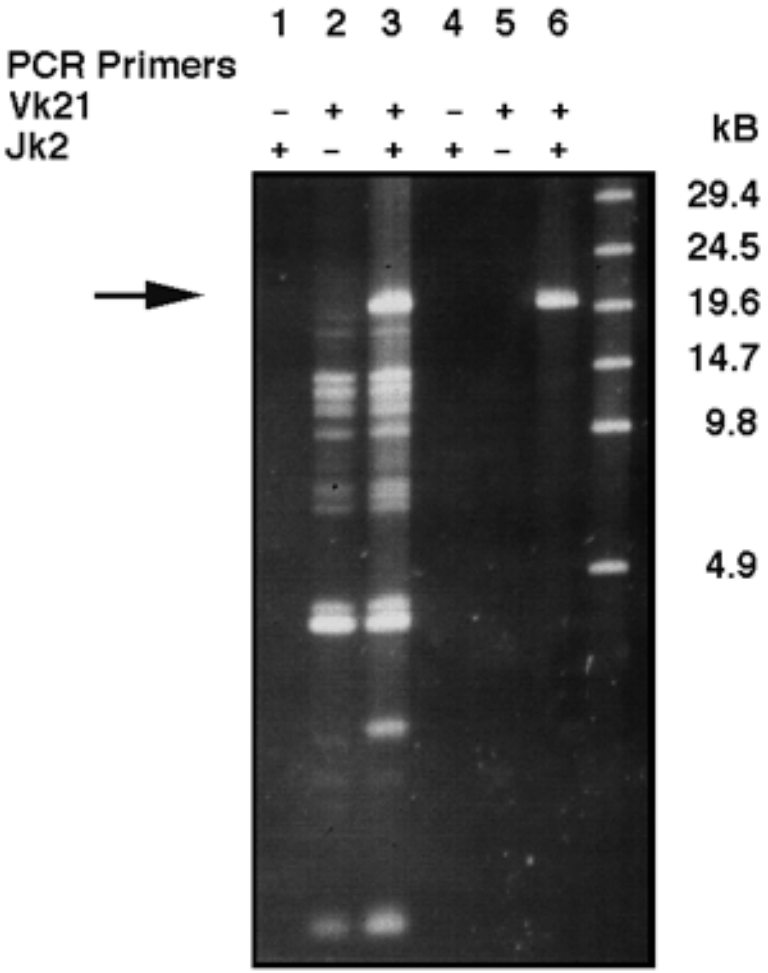
indicated as → ori (cross-hatched bars), while insertion in the reverse orientation is indicated as ← ori (open bars). 

represent a mutated Ed NF-κB site (ΔκB), a mutated Ed first E-box (ΔE1) or a mutated Ed second E-box (ΔE2), respectively.

Downloaded from http://www.jbc.org/ by guest on September 1, 2017



Downloaded from http://www.jbc.org/ by guest on September 1, 2017



Downloaded from http://www.jbc.org/ by guest on September 1, 2017

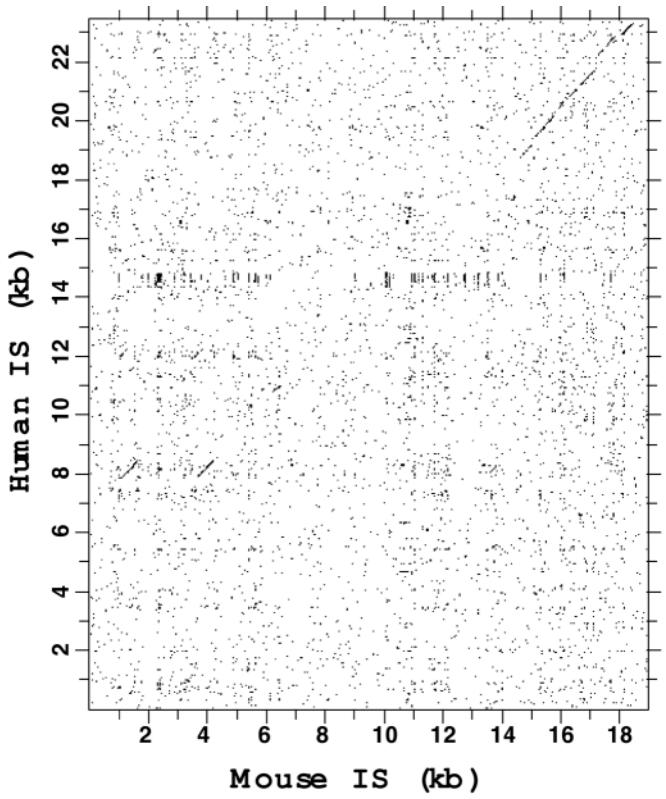
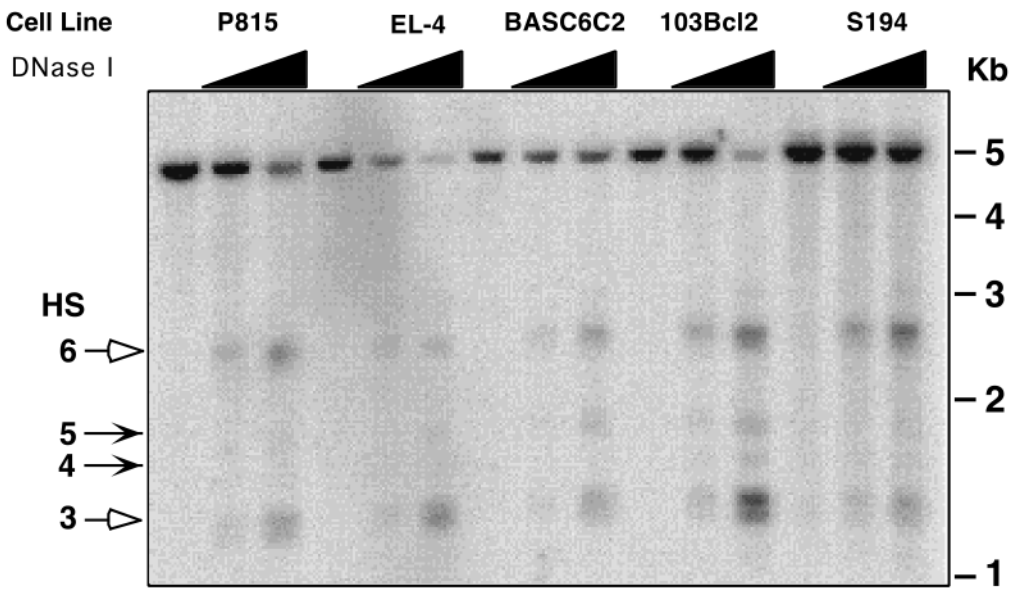
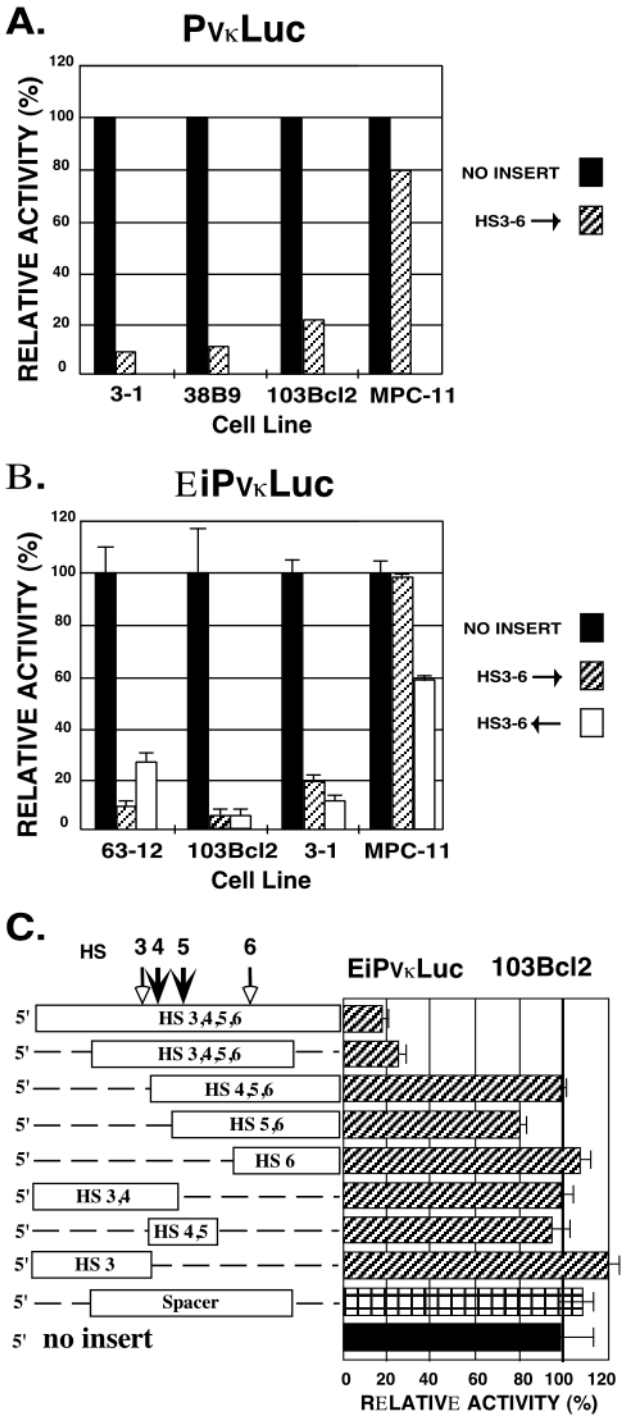


Fig .3

Downloaded from http://www.jbc.org/ by guest on September 1, 2017

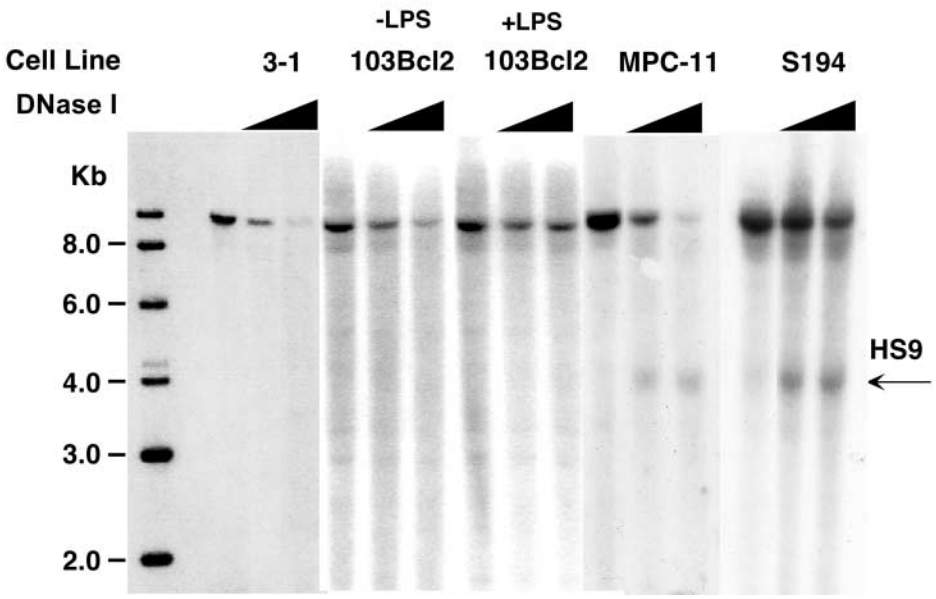


Downloaded from http://www.jbc.org/ by guest on September 1, 2017



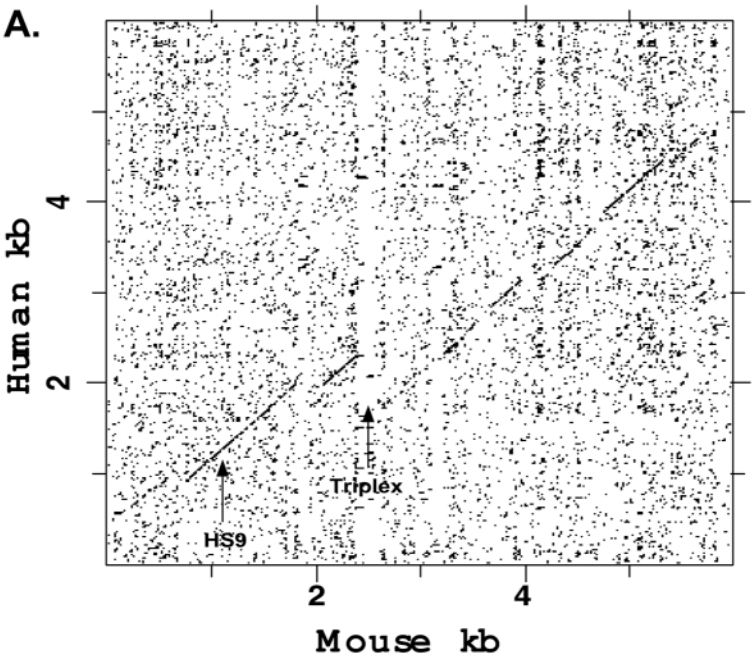
Downloaded from http://www.jbc.org/ by guest on September 1, 2017

Fig.6



Downloaded from http://www.jbc.org/ by guest on September 1, 2017

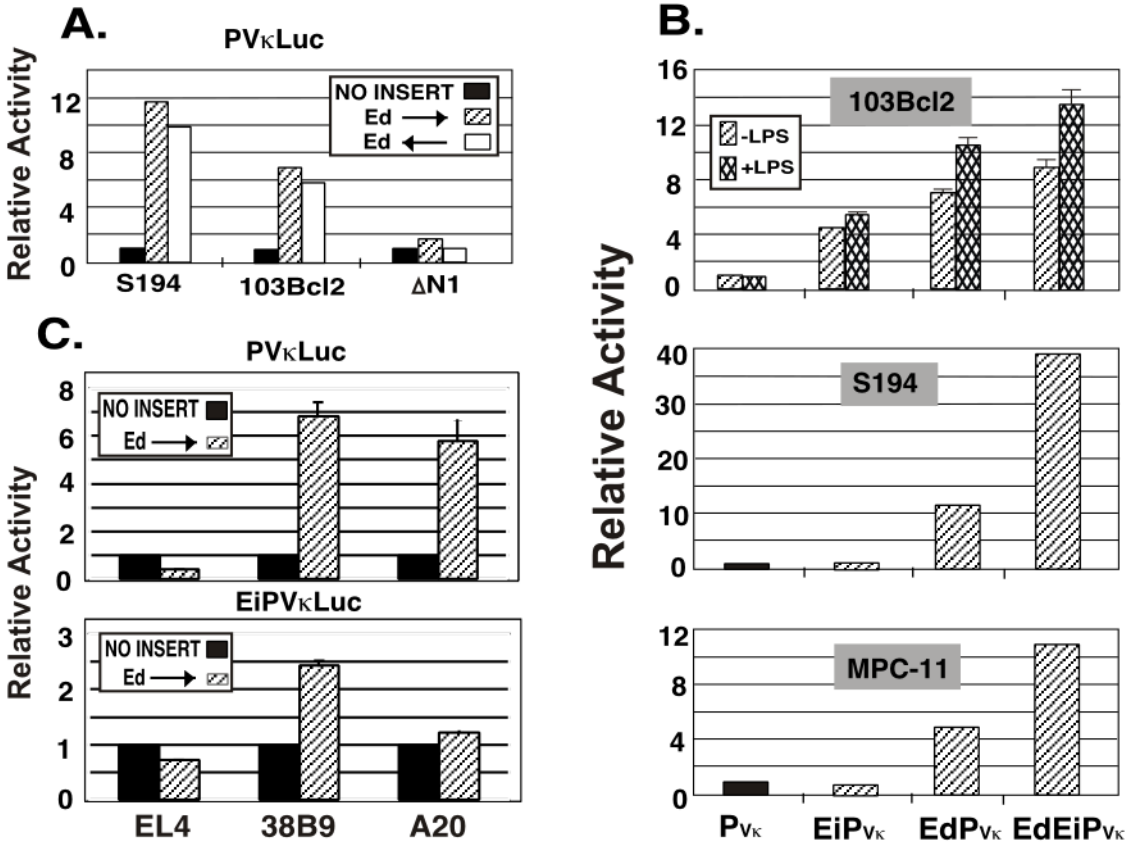
Fig.7



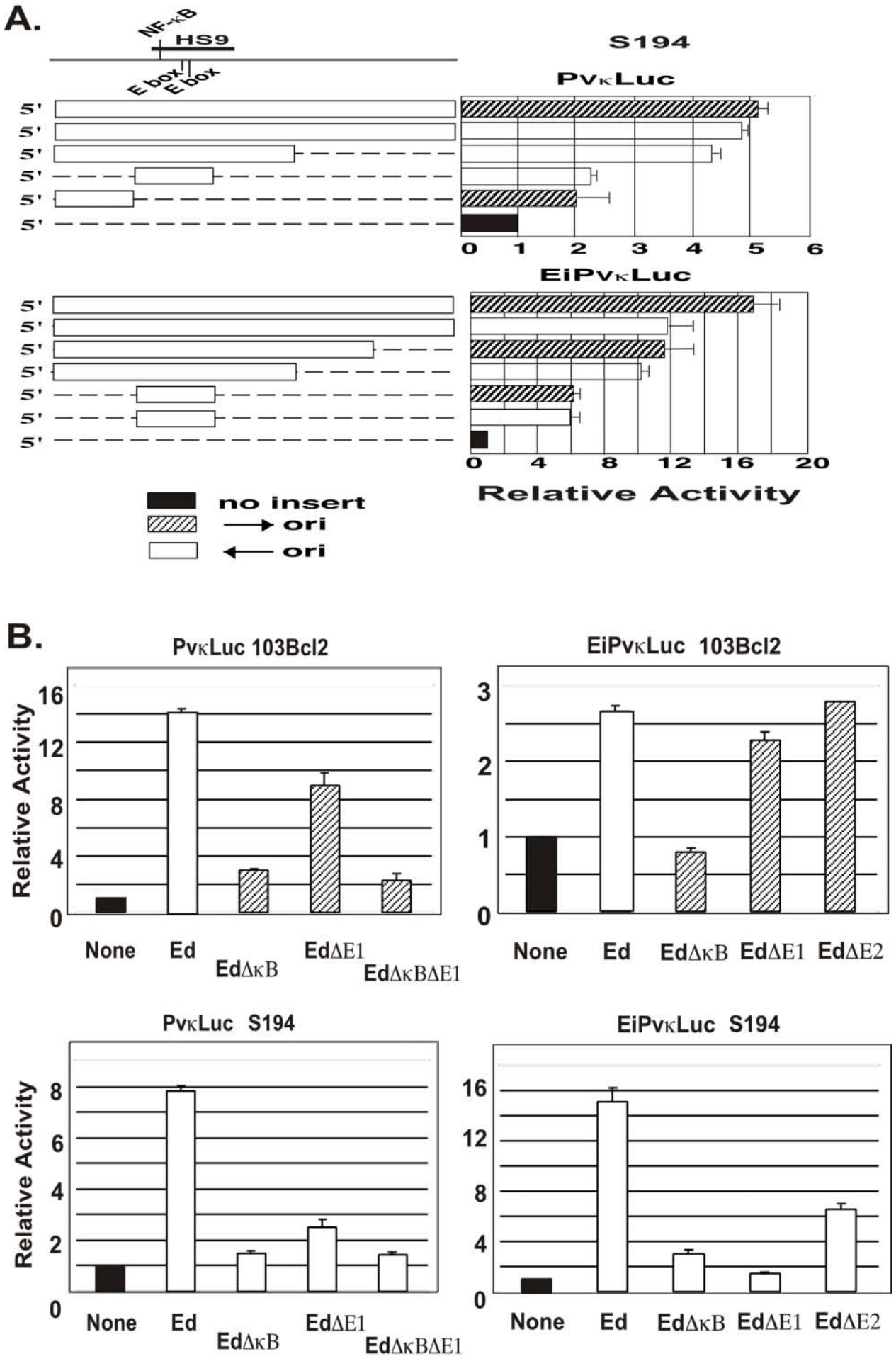
B.

```
                                                                  NFκB
mouse  TCACCATTTCGTTAATCTGAACATGAGGTAATGAAGTCAGGGGGGTTTCC
human  ---T------A---------T--AC---C--G---- .--A---A------

mouse  ACTGTGCCACATCACACAGAACAAAG .TTTCTATTTTAAGAGTCCAGCCC
human  --C-------G-----TGA-----G-GC--------------C-T-----

            E box          E box
mouse  TGCACCTGTGCAGTGCAGATGGACTTGGCAAAAGAAGAAATGAAA .CAGA
human  --------CT-TA-C-------G-----TG---------------A---G

mouse  TTTTTCTCACT
human  C----------
```

Downloaded from http://www.jbc.org/ by guest on September 1, 2017



Downloaded from http://www.jbc.org/ by guest on September 1, 2017



Downloaded from http://www.jbc.org/ by guest on September 1, 2017

Chromatin structural analyses of the mouse Igk gene locus reveal new hypersensitive sites specifying a transcriptional silencer and enhancer

Zhi-Mei Liu, Julia B. George-Raizen, Shuyu Li, Katherine C. Meyers, Mee Young Chang and William T. Garrard

J. Biol. Chem. published online June 21, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204065200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2002/06/21/jbc.M204065200.citation.full.html#ref-list-1

Downloaded from http://www.jbc.org/ by guest on September 1, 2017