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Negative Regulation of Immunoglobulin Kappa Light-Chain Gene Transcription by a Short Sequence Homologous to the Murine B1 Repetitive Element

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B-cell-specific expression of the immunoglobulin kappa light-chain (Igκ) gene is in part accomplished by negative regulatory influences. Here we describe a new negatively acting element (termed κNE) immediately upstream of the NF-κB-binding site in the Igκ intronic enhancer. The 27-bp κNE sequence is conserved in the corresponding positions in the rabbit and human Igκ genes, and the human κNE homolog was shown to have a similar negative regulatory activity. Data base searches using the mouse κNE sequence revealed a striking homology to murine B1 repetitive sequences. A sequence homologous to κNE and B1 was also noted in a previously identified silencer element in the murine T-cell receptor α locus. The homologous T-cell receptor α locus sequence, but notably not a corresponding 27-bp B1 consensus sequence, showed a negative regulatory potential similar to that of κNE. The negative effect of κNE by itself was not cell type specific but became so when paired with its 5′-flanking sequence in the Igκ enhancer. A short (30-bp) fragment upstream of κNE (termed κBS) was found to be necessary and sufficient for abolishing the negative effect of κNE in B cells. Point mutations in a T-rich motif within the κBS sequence allowed the transcriptional repression by κNE to be evident in B cells as well as other cells. As suggested by this cell-independent negative activity, proteins binding to the mouse and human κNE sequences were identified in all cell types tested.

The regulation of the immunoglobulin κ light-chain (Igκ) gene during B-cell development involves the Igκ promoter and two distinct enhancer elements (for a review, see reference 11). The better characterized of the two Igκ enhancers resides in the intron between the J and C regions, whereas the more recently identified 3′ enhancer is located several kilobases downstream of the Igκ constant region. Both of these enhancers contain multiple sites for positive and negative regulation.

Some of the B-cell-specific activity of the Igκ intronic enhancer can be attributed to the cell type specificity of the key positive regulator NF-κB (10), which becomes constitutively activated in the nuclei of maturing B cells (2). Although NF-κB can be activated from its inactive cytosolic form in most other cell types, this activation does not lead to an increased Igκ intronic enhancer activity in non-B cells, such as T cells (17). Some of this cell type-specific negative regulation has been mapped to a 200-bp fragment upstream of the NF-κB-binding site (16). Removal of these sequences results in the inducibility of the Igκ enhancer by NF-κB site activators in non-B cells.

Here we show that another component of negative regulation at this locus can be ascribed to a short sequence immediately 5′ of the κB site in the Igκ intronic enhancer. The activity of this element is not cell specific but becomes so when paired with a neighboring short sequence. The minimal negative element, but none of its flanking sequences in the Igκ intronic enhancer, is closely related to the murine B1 repeat elements. Our experiments with a similar sequence from the T-cell receptor α locus (TCRα) enhancer indicate that B1-like sequences in other genetic contexts may be generally important as negative regulatory elements. Finally, we identify specific κNE-binding factors in nuclear extracts from several cells and show that this binding activity correlates with the transcriptional repression by κNE.

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MATERIALS AND METHODS

Plasmid constructs. The pfLUC plasmid was constructed by cloning the mouse *fos* promoter sequences (−56 to +109; from plasmid Δ56-fos-CAT; 6) to plasmid pBL-KS, made by inserting a new cloning site into the pBL vector (kindly provided by David Cobrinik, Whitehead Institute, Cambridge, Mass.) with the *Photinus pyralis* luciferase coding sequence in a pBluescript-based plasmid.

The NF-κB-driven plasmid pBIIX was constructed by inserting a synthetic fragment with two copies of the sequence ACA GAG GGG ACT TTC CGA GAG separated by four nucleotides (ATCT) in front of mouse *fos* promoter in plasmid pfLUC. The Igκ fragments with different 5′ ends were polymerase chain reaction (PCR) amplified from an Igκ J-C region-containing plasmid and cloned into a *Sac*I site immediately upstream of the first κB-binding site in pBIIX. Thus, all the resulting inserts had identical 3′ ends with Igκ sequence extending to position 3934 and had mutated residues at positions 3927 and 3929 (C to G and T to G, respectively) to create the *Sac*I site (see Fig. 3A for sequence in this region). Since the functionally critical region of the Igκ negative element was localized immediately 5′ of the introduced *Sac*I site, we also constructed a plasmid in which the Igκ insert shared its 5′ end with the 192-bp fragment but had a perfect wild-type sequence extending all the way to (and including) the κB motif. No difference in the negative regulatory potential was observed between this construct and the *Sac*I site-containing 192-bp Igκ fragment construct. The plasmids carrying the 192-bp Igκ fragment with point mutations (192L, 192R, and 192M) or deletions (192D) in the B1 homology region were constructed similarly but with longer 3′ PCR primers including the corresponding

base changes. The 27-bp κNE fragment-containing plasmid, as well as the other constructs with short inserted sequences used in the experiments shown in Fig. 2 and 3, were constructed by cloning corresponding synthetic double-stranded oligonucleotides into pBIIX or pfLUC. The mutant 96-bp Igκ fragment (in construct 96B in Fig. 1A) was made by joining two partially overlapping PCR fragments (each having the desired point mutations in their overlapping 3′ and 5′ ends) by a second PCR with the same primers used to amplify the 96-bp wild-type Igκ fragment. All the constructs were verified by sequencing the region of the inserted fragment and the κB motifs.

Cells, transfections, and reporter expression assays. Approximately 5×10^6 cells were used for each transfection, which was done as described previously (7). The luciferase assay was performed 38 h after transfection using the Promega Luciferase Assay System. Phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide, and forskolin used to stimulate the cells were purchased from Sigma. The HeLa, Jurkat, EL-4, BW5147, S194, P3X, PD31, 70Z/3, 22D6, and NFS-5 cell lines and references for them can be found from the American Type Culture Collection catalog. The first two are human cell lines, whereas the rest are of murine origin. The efficiency of each transfection was monitored by a cotransfected cytomegalovirus enhancer-driven *lacZ* construct kindly provided by Garry Nolan from our laboratory. The lysates used for luciferase assay were also tested for their β-galactosidase activity by using *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) as a chromogenic substrate, and samples showing more than 25% variation in their optical densities at 420 nm were excluded.

Gel mobility shift assays. Nuclear extracts were prepared as previously described (3). The oligonucleotide probes were synthesized with an Applied Biosystems 392 DNA/RNA Synthesizer and their nucleotide sequences were as follows: mouse κNE, 5′-ACC TCT GTC ACC CAA GAG TTG GGA GCT CAG CTC CCA ACT CTT GGG TGA CAG AGG T-3′; mutant mouse κNE, 5′-ACC TCT GTT GTC CAA GAG TAC TGA GCT CAG CTC AGT ACT CTT GGA CAA CAG AGG T-3′ (mutations in the sense-strand region of the oligonucleotide underlined); and human κNE, 5′-TGC TCT CCC ACC CAA CGG GTG GGA GCT CAG CTC CCA CCC GTT GGG TGG GAG AGC A-3′. The oligonucleotides were purified from an acrylamide gel, 100 ng of each oligonucleotide was end labeled, extracted twice with phenol-chloroform, diluted to 2 ml with TE (Tris-EDTA), boiled for 3 min, and allowed to sit at room temperature to allow self-annealing. The labeled probes were concentrated and separated from unincorporated radioactivity by three rounds of microconcentration using Centricon-10 columns (Amicon). Nuclear extract (10 μg) was incubated for 40 min at room temperature with 0.5 ng of probe (corresponding to 3×10^5 cpm) in a solution containing 10 mM Tris (pH 7.5), 50 mM NaCl, 5% glycerol, 1 mM EDTA, and 1 mM dithiothreitol together with 2 μg of poly(dI · dC) and 200 ng of an unrelated short double-stranded oligonucleotide in a final reaction volume of 20 μl. In some reaction mixtures, 50 ng of a specific competing self-annealed oligonucleotide was also added. The sequences of the TCRα and consensus B1 competitor fragments are given in Fig. 2B. The complexes were electrophoresed in gels containing 4% acrylamide, 0.25× TBE (Tris-borate-EDTA) and 10% glycerol at 4°C for 7 h at 10 V/cm.

RESULTS

Characterization of the Igκ negative element. To analyze the negative regulatory region in the Igκ intronic enhancer, a 192-bp fragment upstream of the κB site was cloned into a luciferase reporter plasmid (pBIIX) in which transcription was stimulated by two copies of the murine Igκ NF-κB-binding site in front of a minimal *fos* promoter (see Materials and Methods). This 192-bp fragment included most of the Igκ intronic sequences used in a previous study (16) but extended closer to the κB site at the 3′ terminus. Other modifications to the previous study included the use of a smaller promoter to amplify the importance of the enhancer, and addition of an upstream polyadenylation site to block any read-through transcription initiated from the vector sequences.

We subjected this 192-bp Igκ fragment to a series of deletions and point mutations (Fig. 1A). Luciferase activity of these constructs was measured after transient transfection into cells representing B-cell and non-B-cell lineages. In all experiments, a cotransfected heterologous reporter construct was used as an internal control for transfection efficiency and cell viability (see Materials and Methods).

After 4-h stimulation with 100 nM PMA or 2 μg of phytohemagglutinin per ml, agents known to induce NF-κB activity, the vector containing two copies of the NF-κB-binding site showed strong activation in T cells (Jurkat and EL-4), and in nonlymphoid cells (HeLa) (Fig. 1A and data not shown). In contrast, the luciferase activity produced by a corresponding construct lacking the κB sites and having only the minimal mouse *fos* promoter (pfLUC) was less than 5% of this activity, indicating that we are indeed measuring transcription stimulated via the NF-κB-binding sites. As expected, high levels of luciferase activity were seen in S194 plasmacytoma cells transfected with pBIIX without the addition of exogenous NF-κB activators.

When the 192-bp Igκ fragment was introduced upstream of the two κB sites in the pBIIX vector, we found that the PMA-stimulated luciferase expression of non-B cells transfected with this construct was reduced to one-third of that of cells transfected with the parental vector pBIIX (shown in Fig. 1A for Jurkat cells). In contrast, in mature B cells (S194 or P3X), the 192-bp Igκ fragment had a marginal or no effect on NF-κB-driven transcription. Also, no negative effect on PMA-stimulated luciferase expression by the 192-bp Igκ fragment was observed in any pre-B-cell lines tested (22D6, PD31, 70Z/3, and NFS-5); instead, slightly higher luciferase activities were observed than in pBIIX-transfected cells (22D6 cells [Fig. 1A]).

Analysis of the effect of successive 5′ deletions of this 192-bp fragment indicated that its negative effect could be restricted to a 27-bp sequence (referred to as κNE) immediately upstream of the NF-κB-binding site in the Igκ intronic enhancer (Fig. 1A; see also Fig. 3A). When an even shorter 17-bp fragment was tested, the negative effect was weaker, but this construct still showed consistently ~50% lower transcriptional activity than the pBIIX construct. A multimer consisting of three copies of this 17-bp sequence had a negative effect slightly stronger than any of the monomeric Igκ fragments (construct 3×17 in Fig. 1A).

We further examined the κNE sequence requirements by introducing mutations into the 192-bp Igκ fragment within the 17-bp region indicated above (Fig. 1B). We found that the negative effect of the 192-bp fragment could be severely compromised by mutating only four (192L) or three (192R) nucleotides at either end of this 17-bp semipalindromic

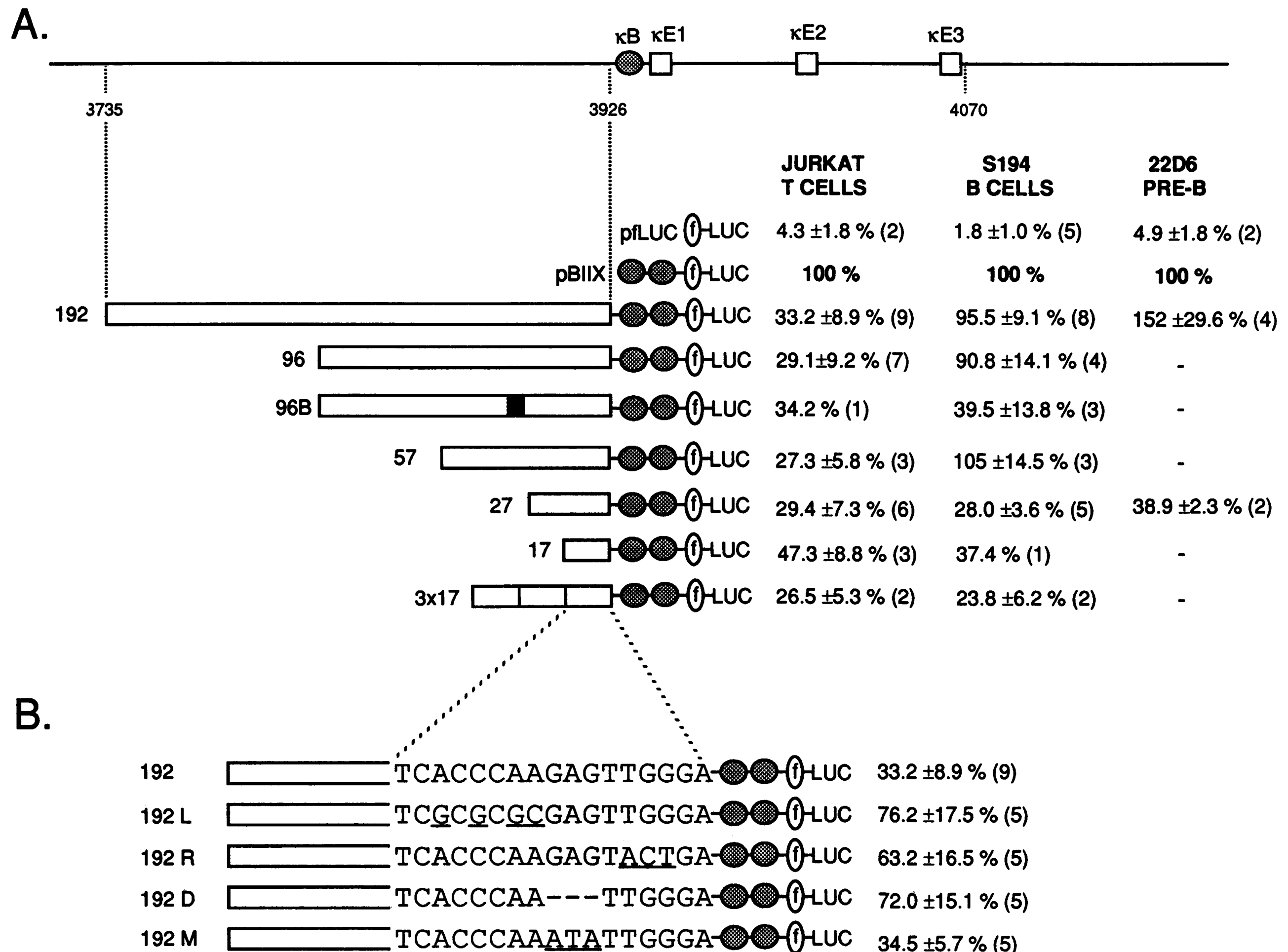

FIG. 1. Mutational analysis of a cell type-specific negative element in the Igκ intronic enhancer. (A) The locations of the subcloned 192-bp fragment upstream of the NF-κB-binding site and the E-boxes within the murine Igκ enhancer are illustrated schematically at the top of the figure. Different Igκ fragments from this region were tested for their ability to downregulate transcription stimulated by two copies of the NF-κB-binding site (shaded ovals) that directed transcription from a minimal (−56 to 109) *fos* promoter (f) in plasmid pBIIX. The 192-bp Igκ fragment and different 5′ truncated versions of this fragment were cloned 5′ of two κB sites in plasmid pBIIX. The lengths of the truncated Igκ fragments that all share a common 3′ end (Igκ residue 3926) are indicated to the left of each vector. Vector 96B is a mutated version of construct 96 and contains the GCGCGC sequence instead of TTCGTT in the position indicated by the dark box. Luciferase expression was then measured in PMA-stimulated T cells (Jurkat) and pre-B cells (22D6) as well as in unstimulated B cells (S194) transiently transfected with these vectors. In all experiments, the luciferase activity produced by the parental pBIIX construct was normalized to 100%, and the activities of the other constructs (± standard deviation) were expressed relative to this. The number of independent experiments performed with each construct is indicated in the parentheses. (B) Effects of point mutations in the 3′ region of the 192-bp Igκ fragment on transcriptional repression in PMA-stimulated Jurkat cells. The nucleotide sequence of the parental 192 construct within the indicated 17-bp region is shown on the top line. The specific nucleotide substitutions in the derived constructs 192L, 192R, and 192M are underlined, and the deleted nucleotides in the 192D construct are indicated by dashes. Luciferase expression of these vectors is indicated relative to that of the pBIIX vector, as described above. LUC, luciferase.

sequence, confirming the functional importance of this region, as indicated by our deletion studies. The negative effect of the 192-bp fragment was also affected by deletion of the three nucleotides, GAG, in the middle of this region (192D), but not by changing these residues to ATA (192M), suggesting that not every residue within this 17-bp region is critical as long as a correct nucleotide spacing is conserved. Some residual negative effect remained in all of these 192-bp fragments, as was the case when the whole 17-bp region was changed to an unrelated sequence (not shown), indicating some contribution of the more 5′ sequences, presumably the CT-rich region present in the 27-bp fragment but not in the 17-bp fragment shown in Fig. 1A.

Pierce et al. (16) defined a broadly localized negative effect in the Igκ enhancer region upstream of the κB site. In our constructs, a negative activity is distinctly localized to a region that was truncated and therefore probably not active in the fragment studied by Pierce et al. (16). Conversely, we saw little or no regulatory activity in the Igκ region upstream of the κNE. The negative effect that we have mapped is also position dependent, because this activity was completely abolished by a 260-bp nonspecific pUC-derived fragment inserted between the κNE- and NF-κB-binding sites (not shown). We are uncertain why the activity defined in the earlier experiments was not evident in the present experimental set-up, but we have gone on to define the new activity

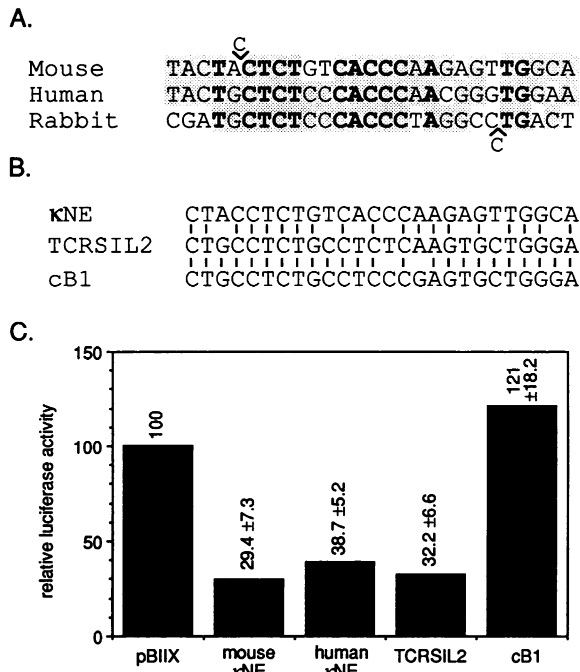

FIG. 2. Comparative analysis of sequences homologous to the murine κNE. (A) Alignment of the corresponding human and rabbit Igκ sequences with the 27-bp murine κNE. The nucleotide residues conserved in all three species are shown in boldface type. (B) Comparison of the murine κNE and TCRSIL2 fragments and a murine B1 element consensus (cB1) sequences. (C) Analysis of transcriptional effects of the mouse κNE and other B1-homologous sequences. Twenty-seven-base-pair fragments corresponding to mouse and human κNE, mouse TCRSIL2, and the consensus B1 element shown in Fig. 2B were introduced upstream of the κB sites in plasmid pBIIX as in Fig. 1A and tested for their effect on NF-κB-stimulated luciferase expression in PMA-stimulated Jurkat cells (human and mouse κNE) or unstimulated S194 cells (TCRSIL2 and cB1).

because it has properties that suggest it has general significance.

To study whether the negative effect of the κNE was restricted to NF-κB, we precisely replaced the κB sites in the pBIIX construct and its derivative carrying the 192-bp Igκ fragment (Fig. 1A) by two copies of the cyclic AMP responsive element consensus sequence (TGACGTCA [19]) and measured the forskolin-stimulated and unstimulated luciferase expression of these constructs in Jurkat cells. In both cases, an approximately threefold-lower luciferase activity was produced by the 192-bp Igκ fragment-containing vector (not shown). Thus, the negative activity of κNE is not restricted to NF-κB, but it can downregulate transcription activated via other binding sites.

A sequence homologous to the murine κNE is also present adjacent to the κB site in the human Igκ intronic enhancer (Fig. 2A), suggesting that it might have a similar negative regulatory role. We tested this directly by introducing the human 27-bp κNE sequence into the pBIIX reporter construct and found that this element could indeed interfere with NF-κB-driven transcription and have a negative effect corresponding to that of the mouse κNE (Fig. 2C). The conservation of κNE between the mouse, human, and rabbit sequences highlights the significance of this element and points to the importance of the specific residues indicated by our mutagenesis studies.

κNE is homologous to the B1 repeat element. When we screened the GenBank data base using the 27-bp murine κNE sequence, a large number of almost perfect alignments was found. This turned out to be due to the homology of κNE to the mouse B1 short repetitive sequences and their primate *Alu* counterparts (Fig. 2B). This homology was shared only by the κNE region and did not extend to its flanking sequences in the Igκ enhancer.

Interestingly, we noted that a B1-homologous sequence corresponding to κNE is also present in a previously characterized silencer element, TCRSIL2 (24), in the mouse T-cell receptor α gene locus (Fig. 2B). We tested this 27-bp TCRα fragment in our κB-driven reporter gene system and found it to have a negative transcriptional effect similar to that of κNE (Fig. 2C). The plasmid constructs used in these experiments had the TCRα sequence inserted in an orientation opposite to that of κNE (which was in the sense orientation). Both TCRα and Igκ 27-bp fragments were, however, able to repress transcription in either orientation.

We then tested a 27-bp B1 consensus fragment (cB1 in Fig. 2B) corresponding to the single most prevalent sequence in this region of B1 elements (on the basis of our GenBank data base searches and references 12 and 18). However, no negative effect on NF-κB-stimulated transcription was observed with this consensus B1 fragment, suggesting that not all B1 repeats have negative regulatory potential. The data shown in Fig. 2C on the transcriptional effects of the human and mouse 27-bp κNE fragments were obtained from PMA-stimulated Jurkat cells, and the data on the 27-bp TCRα or consensus B1 fragments were obtained from S194 cells. However, as shown for the mouse κNE in Fig. 1A, none of these short B1-homologous fragments showed cell type specificity in their action, and the negative effects of the mouse TCRα fragment and human κNE were equally evident in T cells and B cells (data not shown).

Inactivation of κNE in B cells by an upstream element. When the series of constructs containing differentially 5′ deleted versions of the 192-Igκ fragment were tested in S194 cells (Fig. 1A), we found that disruption of the κNE sequence from its upstream flanking region resulted in an activation of its negative potential in B cells (Fig. 1A). In contrast to the 192-bp Igκ fragment, the 27-bp κNE fragment could repress transcription in all cell types tested, including B cells. The presence of only 30 bp of Igκ sequences upstream of the κNE, however, was sufficient to restore the cell type specificity of the κNE, since no negative effect was observed for the 57-bp fragment when tested in S194 cells (Fig. 1A). Therefore, this 30-bp region is a critical determinant of the cell type specificity of κNE. The nucleotide sequence of this 30-bp fragment (referred to as κBS for Igκ B-cell specificity) is shown in Fig. 3A. The κBS sequence contains two copies of the T-rich motif GTTTT separated by a C residue. Disruption of this region of the κBS element in the reporter construct with a 96-bp Igκ fragment by a TTCGTT-to-GCGCGC mutation activated the negative potential of this fragment in S194 cells, rendering it as potent a negative element in B cells as in non-B cells (compare constructs 96 and 96B in Fig. 1A).

To test whether the κBS-mediated B-cell-specific alleviation of the negative regulation by κNE could be due to an independent positive transcriptional effect by κBS, we introduced two copies of the κBS sequence in front of the minimal *fos* promoter in the pfLUC luciferase construct. Luciferase expression of the resulting plasmid pκBS was

FIG. 3. Cell-type-specific enhancement of transcriptional activation by NF-κB via a T-rich sequence (κBS) from the Igκ intronic enhancer. (A) Nucleotide sequence of the murine Igκ gene from positions 3880 to 3952. The bars indicate the exact sequences of the κBS and κB motifs introduced in the *fos* promoter-luciferase reporter constructs. The 27-bp κNE sequence is also indicated to illustrate the close proximity of these elements in the Igκ intronic enhancer. The core Igκ NF-κB-binding site is indicated by boldface type. (B) The vectors in the indicated transfection experiments contained two tandem copies of the κBS sequence (shown in panel A) upstream of a minimal *fos* promoter either alone (pκBS) or in combination with two copies of the Igκ κB motif (pκBS-κB). The luciferase activities in transiently transfected unstimulated B cells (S194) cells or PMA-stimulated T cells (Jurkat) are expressed relative to the activity of a construct containing only the minimal *fos* promoter (pfLUC). Separate scales for the relative transcriptional activity are given for the two cell lines. The mean values from three or more independent experiments and their standard deviations are shown.

then compared with that of pfLUC in B cells and non-B cells. No significant transcriptional stimulation was observed in either cell type, suggesting that the κBS element by itself has no transcriptional activation potential.

However, when the two copies of the κBS sequence were cloned 5′ of the NF-κB binding sites in plasmid pBIIX (resulting in plasmid pκBS-κB), a marked enhancement in transcription was observed in B cells (Fig. 3B). The strong transcriptional activation by two murine Igκ κB sites in S194 cells was further increased two- to threefold by the additional tandem κBS element. In contrast, no such potentiation of the NF-κB-activated transcription by κBS was seen in PMA-stimulated Jurkat cells; instead, less luciferase was expressed in these cells by the pκBS-κB plasmid than by pBIIX (Fig. 3B). A sequence homologous to the mouse κBS element is also present adjacent to the κNE element in the human kappa enhancer. A similar B-cell-specific enhancement of transcriptional activation via the Igκ κB site was also observed with the human κBS sequence (data not shown).

These data suggest a role for the κBS element as a cell type-specific modulator of transcriptional activation via the NF-κB-binding site within the Igκ intronic enhancer. Thus, combined influences by these three closely positioned binding sites (κBS, κNE, and κB) appears to create a composite enhancer element whose activity is regulated by NF-κB but is mainly restricted to the B-cell lineage.

Sequence-specific nuclear factor binding to κNE correlates with its negative regulatory potential. To characterize factors mediating the negative transcriptional effect of the κNE, we prepared nuclear extracts from various B-cell and non-B-cell lines and tested them for specific binding to the κNE sequence in gel retardation assays. In agreement with the ubiquitous activity of κNE, a specific complex binding to the mouse κNE probe was detected in mouse (S194 and 22D6) and human (Jurkat and HeLa) cell lines regardless of their lineage of differentiation (complex A in the left panel in Fig. 4; also data not shown). The specificity of DNA binding by complex A was strongly suggested by the experiments utilizing different unlabeled competitor binding sites and a mutated version of the κNE probe (discussed below). The binding of complex A was not very strong; this binding became evident only when glycerol was added to the mobility shift gels to stabilize the binding and increased when electrophoresis was carried out at 4°C. Under these conditions, an additional slower migrating specific complex was also observed in HeLa cells (A′ in Fig. 4, left panel). This complex was not detected in nuclear extracts from other human or mouse cell lines. Complex A′ could therefore be

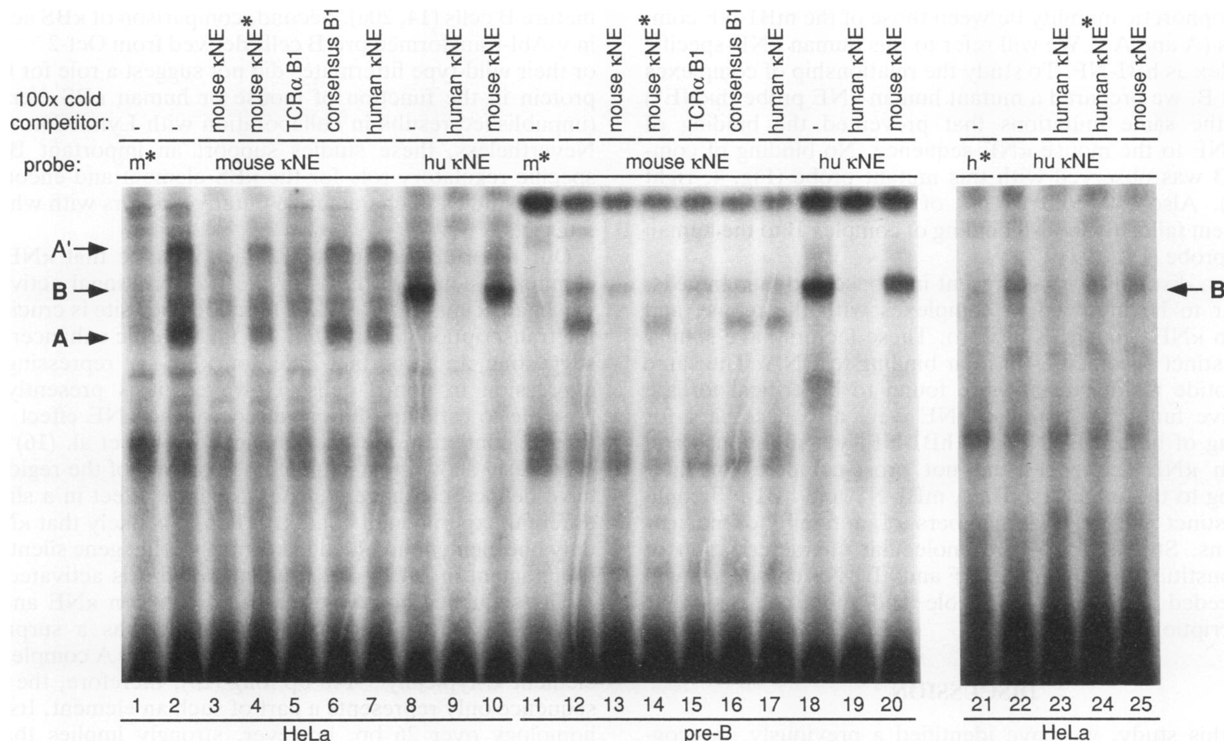

FIG. 4. Specific binding of nuclear factors to the mouse and human κNE sequences. Nuclear extracts from cells representing non-B (HeLa [human carcinoma cell line]) and B-cell lineages (22D6; v-Abl-transformed mouse pre-B-cell line) were tested for factors that could specifically bind to mouse and human (hu) κNE probes, as well as a mutant mouse and human κNE probes (*) with changes in nucleotide residues critical for transcriptional repression by the murine κNE sequence. In some lanes, a 100-fold excess of the indicated unlabeled competitor was also included in the binding reaction mixture. In lanes labeled −, no specific competitor was used. The details of the assay and the sequences of the oligonucleotide probes used are specified in the Materials and Methods. Complexes specifically interacting with the mouse (A and A′) and human (B) κNE sequences are indicated by arrows.

due to a cell-type-restricted κNE-binding factor but more likely reflects a higher quality of the nuclear extracts prepared from HeLa cells. Although there is no direct evidence that the constituents of complexes A and A′ are related, we will refer to both of them as mB1-NF (for murine B1-binding nuclear factor).

To test whether binding of mB1-NF to the 27-bp κNE element correlated with its transcriptional repression potential, we prepared a mutant probe (mouse κNE* [lanes 1 and 11 in Fig. 4]) in which residues CAC and TGG were changed to TGT and ACT, respectively (for details, see Materials and Methods). On the basis of our mutagenesis studies (Fig. 1B), these changes should abolish transcriptional repression by mouse κNE and, therefore, presumably disrupt binding of any functionally relevant negative factors. No mB1-NF binding was observed with this mutant version of the κNE sequence, indicated by the complete absence of complexes A and A′ in Fig. 4 (compare lanes 1 and 2 and lanes 11 and 12). Identical arrays of complexes binding nonspecifically to both the mutant and wild-type probe highlighted the specificity of the loss of mB1-NF interaction with the mutant κNE probe. In agreement with these results, a 100-fold excess of the mutant κNE fragment was unable to abolish B1-NF binding to the wild-type κNE probe. In contrast, under the same conditions, a 100-fold excess of the wild-type κNE competitor completely abolished the B1-NF complex (Fig. 4). These data confirm that the mB1-NF complex binds specifically to the mouse κNE sequence and show that this interaction is dependent on the nucleotide residues critical for the negative regulatory potential of κNE.

These results prompted us to use other fragments homologous to κNE and B1 and previously tested for their transcriptional regulatory potential as specific competitors for B1-NF binding to the mouse κNE probe. It is striking that the κNE-like fragment from the TCRα silencer, which had a negative regulatory potential comparable to that of κNE, was also able to efficiently abolish mB1-NF binding to the κNE probe (Fig. 4, lanes 5 and 15). In contrast, despite its close sequence similarity, the corresponding 27-bp mouse B1 repeat consensus fragment failed to abolish mB1-NF binding (lanes 6 and 16), which correlated with its lack of negative regulatory potential in reporter gene assays (Fig. 2C). These results substantiate the significance of the mB1-NF as a mediator of the negative regulation by κNE and suggest a role for it in negative regulation by a subset of other B1-homologous sequences dispersed in the mouse genome, such as the B1 homology region in the TCRα silencer.

The negatively acting human κNE homolog, however, did not abolish binding of mB1-NF to the mouse κNE probe in nuclear extracts either from mouse or human cell lines (Fig. 4, lanes 7 and 17). In order to understand this finding, we prepared a similar mobility shift probe corresponding to the human κNE sequence. A single specific binding complex was observed by using the human κNE probe in mouse and human cell lines (complex B in Fig. 4), which had an

electrophoretic mobility between those of the mB1-NF complexes (A and A′). We will refer to this human κNE-specific complex as hB1-NF. To study the relationship of complexes A and B, we prepared a mutant human κNE probe (hκNE*) with the same mutations that prevented the binding of mB1-NF to the mouse κNE sequence. No binding of complex B was observed with this mutant probe (Fig. 4, right panel). Also, 100-fold excess of unlabeled human κNE* fragment failed to abolish binding of complex B to the human κNE probe.

Thus, distinct factors, present in mouse and human cells, appear to be involved in complexes with the mouse and human κNE sequences in vitro. These factors have similar but distinct specificities in their binding to DNA. The same nucleotide residues that were found to be critical for the negative function of mouse κNE were also necessary for binding of both mB1-NF and hB1-NF, yet the mouse and human κNE sequences did not cross-compete for their binding to these factors. Thus, mB1-NF and hB1-NF could be distinct but related members of a family of nuclear proteins. Studies examining molecular characterization of the constituents of the mB1-NF and hB1-NF complexes will be needed to clarify their role and mode of action in transcriptional repression.

DISCUSSION

In this study, we have identified a previously unrecognized short negative element (κNE) close to the NF-κB-binding site in the Igκ intronic enhancer. Computer analysis of the κNE sequence revealed a striking homology to the murine B1 repetitive element of the short interspersed repetitive element superfamily (see below). Nuclear factors specifically binding to κNE were identified and termed B1-NF. Functional significance of the B1-NF complex was supported by mutagenesis and binding site competition studies which established a strong correlation between B1-NF binding to κNE in vitro and transcriptional repression in vivo. Further characterization of B1-NF will be necessary to uncover the molecular mechanisms involved in the transcriptional repression by κNE and other similar elements.

The cell type specificity of κNE was found to be determined by a short element, termed κBS, immediately upstream of κNE in the Igκ enhancer. Disruption of the κBS sequence allowed transcriptional repression by κNE to be evident in B cells. When analyzed separately from κNE, the κBS sequence was able to cause a B-cell-specific enhancement of transcriptional stimulation via adjacent NF-κB-binding sites but by itself could not mediate transcriptional activation. In these properties, κBS is reminiscent of the sequence that binds the high-mobility-group protein-related lymphoid enhancer factor 1, a protein that bends DNA and potentiates the activity of other factors while being inactive on its own (5).

Nelms and collaborators (14, 15) have previously studied nuclear factors binding to the human and mouse Igκ enhancers, using probes including the κBS sequences described here. They concluded that the human sequence is a functional octamer-binding site, whereas the corresponding mouse sequence instead binds another nuclear factor referred to as κBF-A. Our gel shift experiments using mouse and human κBS probes (20a) are consistent with their findings. However, neither the predominantly B-cell-specific Oct-2 protein nor κBF-A seems likely to be responsible for the in vivo effect of the κBS element. First, κBF-A can be detected only in stimulated pre-B cells, but not in more mature B cells (14, 20a). Second, comparison of κBS activity in v-Abl-transformed pre-B cells derived from Oct-$2^{-/-}$ mice or their wild-type littermates did not suggest a role for Oct-2 protein in the function of mouse or human κBS elements (unpublished results in collaboration with Lynn Corcoran). Nevertheless, these studies support an important B-cell-specific regulatory role for the κBS element and encourage further studies to characterize putative factors with which it interacts.

Our reporter transfection studies indicate that κNE can eliminate about two-thirds of the transcriptional activation via an adjacent Igκ κB site. A functional κB site is crucial for the transcriptional activity of the Igκ intronic enhancer (10), suggesting an important role for κNE in repressing Igκ expression in non-B cells. However, it is presently not possible to estimate the magnitude of the κNE effect in its natural context in the Igκ enhancer. Pierce et al. (16) have previously shown that sequences upstream of the region we have defined can have a strong negative effect in a slightly different experimental context. Thus, it is likely that κNE is only one element involved in keeping the Igκ gene silent until the moment in B-cell development when it is activated.

The sequence homology revealed between κNE and the B1 family of genomic repeat elements was a surprising finding and may have important implications. A complete B1 element is typically ~140 bp long (23); therefore, the κNE sequence only represents a part of such an element. Its high homology over 26 bp, however, strongly implies that its evolutionary origin is from a B1-like repeat. The presence of a homologous κNE sequence in the corresponding positions in the human and rabbit Igκ J-C intronic enhancers (Fig. 2A) supports the functional significance of κNE and suggests that this sequence may have evolved from an archaic B1 or *Alu*-like element and then suffered a deletion some time before these species diverged in evolution.

The idea that B1 elements may have a widespread role in the negative control of gene expression was strongly supported by the finding that a sequence homologous to κNE and B1 is also shared by another previously identified negative element, namely, TCRSIL2, which is involved in down-regulation of TCRα expression in cells other than αβ-lineage T cells (24). The B1-like sequence in TCRSIL2 is a more typical B1 element than κNE is, which suggests a more recent evolutionary origin. Therefore, the ability of the 27-bp κNE- or B1-like TCRSIL2 fragment to negatively regulate reporter gene transcription and to abolish binding of mB1-NF to the κNE sequence demonstrates that the B1 homology of the κNE sequence is functionally relevant. Another 27-bp fragment, corresponding to the most prevalent (consensus) sequence in this region of the B1 elements, however, did not show any negative regulatory activity in our test system and it did not compete with the κNE probe for mB1-NF binding. Thus, it appears that only certain variants of the B1-like sequences may acquire a negative regulatory function.

A role for some other highly repeated sequences in the negative control of transcription has been suggested by previous studies. The silencer element in the rat insulin 1 locus was identified as a member of a rat long interspersed repetitive sequence family (9). One of the three identified chicken lysozyme silencer elements was found to consist of sequences homologous to the chicken middle repetitive sequence element CR1 (1). Also, although the *Alu* sequences have not been indicated in negative regulation of any specific genes, their potential for transcriptional repression has been suggested (20, 21).

B1 sequences, as well as many of the other highly repeated genomic sequences, are thought to represent retrotransposons, DNA elements which can be copied into new positions in the genome through RNA intermediates. Evolutionary characterization, as well as rare studies in real time (4), has confirmed that some of repeated sequences, including the *Alu* elements (13, 22), continue to retrotranspose. The notion that these sequences, after perhaps a small number of mutations, can play a physiological role has been an attractive one for many years, but has received little support (recently reviewed by Howard and Sakamoto [8]). Involvement of a mobile DNA element in transcriptional regulation would allow alterations in gene expression to occur with great flexibility, ease, and speed over evolutionary time.

It is tempting to speculate that in addition to Igκ and TCRα enhancers, B1-like sequences could be widely used as versatile building blocks that confer a negative transcriptional effect onto a variety of different cell type- and differentiation-specific enhancers. A large number of the murine and primate genes so far sequenced and characterized contain B1- and *Alu*-like sequences in positions which could imply regulatory significance (20a). Further studies addressing the detailed sequence and spatial requirements functionally critical for B1-like negative elements should help predicting which ones are likely to be involved in the control of gene expression.

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