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B lymphocyte-specific protein binding near an immunoglobulin κ-chain gene *J* segment

(DNA binding protein/B cell-specific protein/DNA rearrangement)

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ABSTRACT **Nuclear extracts from pre-B and B cell lines contain a nuclear DNA binding protein (κ locus protein, KLP) that specifically recognizes a DNA sequence in the immunoglobulin κ light chain joining (*J*) segment gene region. KLP is not observed in mature B cells, T cells, or nonlymphoid cell types. Two tandem binding sites for KLP designated KI and KII have been identified by methylation interference analysis to be immediately proximal to the $J_\kappa 1$ nonamer–heptamer recognition sequences and separated by 38 base pairs from each other. Fragments of DNA containing KI and KII sites compete for binding to KLP, and both protein–DNA complexes have the same electrophoretic mobility. Other flanking sequences of immunoglobulin gene fragments do not bind to KLP. The position of KLP–DNA binding and its tissue-specific expression suggest that it may be involved in the regulation of lymphoid gene DNA rearrangements by targeting recombinase to the κ-chain gene region.**

The functional diversity of both Ig and the T-cell receptor (TCR) genes is generated in part by DNA rearrangement events occurring during the maturation of B and T cells (1–3). The six known Ig and TCR gene families undergo assembly of their variable region (*V*) gene portions from *V*, joining region (*J*), and sometimes diversity region (*D*) gene elements. The DNA rearrangements of these six gene families have a lineage specificity, being generally restricted to either the B- or the T-cell compartments. In addition, the individual gene families rearrange at specific points during the differentiation of the lymphoid cell lineages. In spite of these specificities, the gene fragments of these six families have highly conserved flanking nonamer–heptamer DNA sequence elements that are presumed to mediate the site-specific recombination events at their borders (1–5). The only obvious DNA sequence specificity to these joining signals is the spacing between the heptamer and nonamer—in all cases, an element with an approximately 12-base-pair (bp) spacer joins to one with an approximately 23-bp spacer (4). It seems unlikely that a set of unique enzymes, each with the complex strand cleavage and rejoining reactions of the recombinase function, would be synthesized for the independent rearrangement events of each gene family. A more likely possibility is the targeting of a common recombinase function to an Ig or TCR gene-segment site by increased accessibility of a locus by transcription there (6, 7) or by the action of a specific activating protein.

In attempting to understand the molecular mechanisms of activation of these gene families for rearrangement, we speculated that DNA binding proteins that either recognize heptamer–nonamer sequences, enhancers, or other cis-acting elements in the gene regions might be involved. In this paper we characterize a B cell-specific DNA binding protein that binds to two adjacent DNA sequences that flank the J_κ gene segment cluster but are distinct from the heptamer and nonamer elements. The binding of this nuclear protein may direct the rearrangement of the κ-chain gene family in both a lineage- and stage-specific manner.

MATERIALS AND METHODS

Nuclear Extracts. Nuclear extracts were prepared from tissue culture cells by using the protocol of Dignam *et al.* (8), yielding protein concentrations of 6–12 mg/ml.

Gel Binding Analysis and Competition Assays. Restriction fragments from the mouse J_κ genomic region were prepared from plasmid subclones of J_κ segment regions (9). End-labeled restriction fragments were as follows: KI, containing $J_\kappa 1$, was a 130-bp *Hin*dIII–*Hin*fI fragment; KII, 5′ to $J_\kappa 1$ and the KI fragment, was an 83-bp *Dde* I–*Hin*dIII fragment; KIII, containing $J_\kappa 4$, was a 158-bp *Ava* I–*Rsa* I fragment. Fig. 1*A* shows the relative positions of KI, KII, and KIII in the J_κ region.

Protein–DNA binding reactions were conducted in 10 mM Tris chloride, pH 7.5/40 mM NaCl/1 mM EDTA/1 mM 2-mercaptoethanol containing poly[d(I·C)] nonspecific carrier DNA, ^{32}P-labeled restriction fragment (0.1–0.5 ng), nuclear extract, 0.1% Triton X-100, and 4% (vol/vol) glycerol for 15 min at room temperature. Poly[d(I·C)] concentrations and nuclear extract concentrations were titrated against each other; typically, 0.5–5 μg poly[d(I·C)] was used with 5–15 μg of nuclear extract. Low-ionic-strength polyacrylamide gels were prepared and run according to previously published methods (13). For competition assays, various amounts of purified specific and nonspecific restriction fragments were added before the nuclear extract to otherwise complete reaction mixtures described above.

Methylation Interference Assays. Radiolabeled KI, KII, or KIII fragments (5′-^{32}P-end-labeled on one strand only) were partially methylated with dimethyl sulfate by standard procedures (14) to give an average of one methylated guanine residue per strand. The modified KI, KII, or KIII fragments were then incubated with PD31 nuclear extract, and the protein–DNA complexes were isolated on preparative mobility shift gels. Complexed DNA (complex) and uncomplexed DNA (free) fractions were cut out of the gel, electroeluted onto DEAE-paper (NA45, Schleicher & Schuell), extracted with phenol, precipitated with ethanol, and treated with 1 M piperidine at 90°C for 30 min. These samples were lyophilized to dryness twice, resuspended in 98% formamide with bromophenol blue and xylene cylanol FF dyes, and subsequently fractionated by electrophoresis through a 7% polyacrylamide/8 M urea gel prior to autoradiography.

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Abbreviations: TCR, T-cell receptor; V, variable region; C, constant region; D, diversity region; J, joining region; KLP, κ locus protein; Ab-MuLV, Abelson murine leukemia virus; H, heavy.

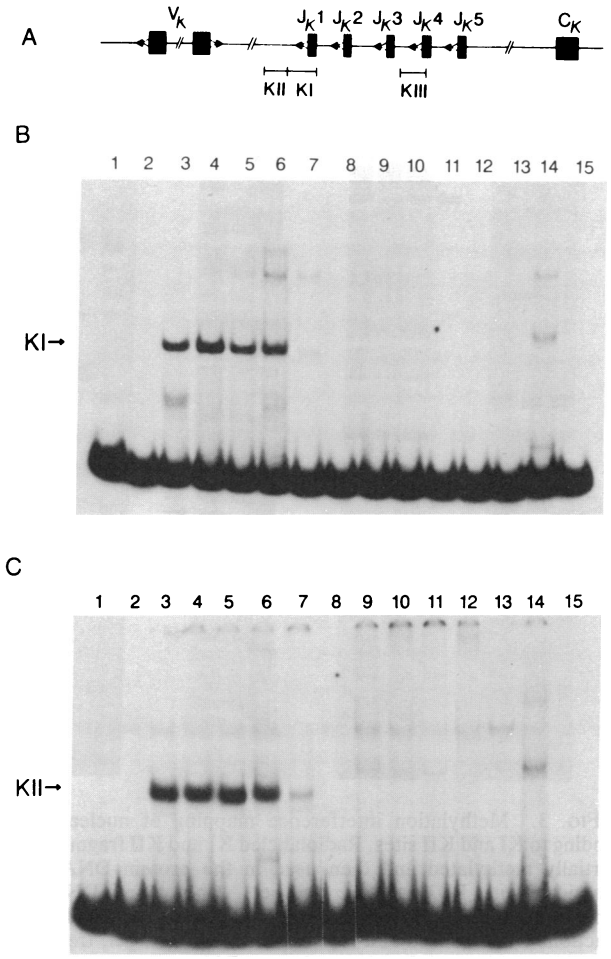

FIG. 1. The κ-chain gene-rearrangement locus and DNA binding proteins. (*A*) The orientation and distribution of the three κ-chain gene elements [V_κ, J_κ, and constant region (C_κ)] are shown with the flanking recombination heptamer (▷) and nonamer (▶) sequences. Both deletional and inversional mechanisms of DNA rearrangements of $V_\kappa + J_\kappa$ are allowed; thus, both orientations of V_κ are shown, even though the V_κ and J_κ regions are unlinked. The positions of restriction fragments KI (130 bp), KII (87 bp), and KIII (158 bp) that form protein–DNA complexes in the electrophoretic mobility-shift assay are shown. (*B* and *C*) Electrophoretic mobility shift assay of nuclear extracts from B-cell, T-cell, and nonlymphoid-cell lines with radiolabeled KI (*B*) or KII (*C*) DNA fragments. Nuclear extracts were prepared as described by Dignam *et al.* (8). In the mobility-shift gel assay, the DNA fragments were analyzed with 10 μg of nuclear extract and 3 μg of poly[d(I·C)] in the binding reaction. Cell lines in lanes: 1, PCC4; 2, BAF3 (10); 3, HAFTL (11); 4, 38B9; 5, PD31; 6, AJ9; 7, EW; 8, SP2/0; 9, EL4; 10, RLM11; 11, W7; 12, BW5147; 13, MEL; 14, COS-7; 15, NIH 3T3. The cell lines in lanes 3–8 are in the B-cell lineage, and the cell lines in lanes 9–12 are in the T-cell lineage; see the text and Staudt *et al.* (12) for further descriptions. The position of the specific protein–DNA complex is designated by an arrow.

RESULTS

Ig κ light chain *V* regions are assembled by a single joining event in which a V_κ gene fragment is recombined with one of the five J_κ gene elements tandemly positioned in a 1.4-kilobase (kb) cluster proximal to the C_κ exon region (Fig. 1*A*). A nuclear extract was prepared from PD31 cells, an Abelson murine leukemia virus (Ab-MuLV) virus-transformed pre-B-cell line, to test for DNA binding proteins that might recognize sequences within the V_κ region. PD31 cells in culture are able to rearrange their endogenous κ-chain alleles as well as exogenously added substrates for κ-chain gene rearrangement (9, 15). Small restriction fragments from cloned J_κ cluster DNA were analyzed for specific protein–DNA complexes by an electrophoretic mobility-shift assay (13). In this assay, a nuclear extract is mixed with a labeled DNA fragment and a large excess of unlabeled poly[d(I·C)], and DNA–protein complexes are resolved from free DNA by electrophoresis. By using this assay, two DNA fragments (designated KI and KII, Fig. 1*A*), that included the $J_\kappa 1$ gene segment, its nonamer–spacer–heptamer recognition sequences, and its 5′ proximal sequences, were observed to bind to a PD31 nuclear protein that we designate KLP for κ locus protein (bands KI and KII in lanes 5 of Fig. 1 *B* and *C*).

We explored what cell lines contain KLP, concentrating on lines representing different stages of the B-cell and T-cell lineages. The B-cell lineage is usefully separated into several differentiation stages identified by the configuration of their Ig-related DNA. Pre-B-cells of different configuration can be isolated by the transformation of progenitor cells with Ab-MuLV, Harvey murine sarcoma virus, or by growth in the presence of interleukin 3 (10, 11, 16). Certain lymphomas represent B cells (surface Ig-positive), and myelomas or hybridomas represent more mature plasma cells (secreting immunoglobulin). Cells like the BaF3 cell line are presumed to be a progenitor cell of the B-cell lineage because they are able to repopulate the B-cell compartment of irradiated mice and contain no Ig rearrangements (10). BaF3 nuclear extracts were negative for KLP by our assay (Fig. 1 *B* and *C*, lane 2). The Harvey murine sarcoma virus-transformed line, HAFTL, contains primarily germ-line configuration of Ig heavy and light chain genes but frequently undergoes the initial *D*-to-*J* Ig heavy chain rearrangement in culture (ref. 14; A. Alessandrini, J. Pierce, S. Desiderio, and D.B., unpublished data). HAFTL was observed to have the KLP binding activity with both the KI and KII restriction fragments (lane 3), even though κ-chain gene rearrangement has not been demonstrated in this line. Ab-MuLV-transformed later-stage pre-B-cell lines 38B9 and PD31 (Fig. 1 *B* and *C*, lanes 4 and 5) and Ed2-2 and 18-81-2 (data not shown) contained KLP in their nuclear extracts. 38B9 cells did not rearrange endogenous κ-chain genes despite the presence of KLP. All of the nuclear extracts from later nonsecreting stages of the B-cell lineage were positive for KLP by this assay [pre-B- and B-cell stages (Fig. 1, lanes 3–7) and WEHI-231, 70Z, 230-37, Raji, 710C, M1241, and MRL39 nuclear extracts (data not shown)]. Both the KI and KII restriction fragments produced the same pattern (Fig. 1 *B* and *C*). In contrast, nuclear extracts from plasma cell tumor lines [SP2/0 (Fig. 1, lane 8) and MPC-11, KR12, 8826, and S107 (data not shown)] were negative or very weakly positive for KLP. Thus, production of KLP is restricted to the nonsecreting stages of the B-cell compartment. The Epstein–Barr virus-transformed human cell lines EW (Fig. 1 *B* and *C*, lane 7), Raji, and 710C have a reduced level of the DNA binding protein compared with equivalent amounts of nuclear extract protein of several mouse B-cell lines (see the *Discussion*). Minor complexes of KI and KII with other proteins were generally not tissue-specific and were not further characterized (Fig. 1 *B* and *C*).

When nuclear extracts were prepared from T-lymphoid-lineage cells or nonlymphoid cells, no KLP complex was detected similar to that produced by PD31 extracts. Nuclear extracts from the T-cell lineage (BW5147, RLM11, EL4, or W7), embryonic precursor cell types (PCC4 or C5), or nonlymphoid differentiated cells (NIH 3T3, HeLa, COS-7, B104 retinoblastoma, or MEL cells) did not form the characteristic specific DNA–protein complex with either the KI or KII restriction fragment (Fig. 1 *B* and *C* and data not shown).

We suspected that the KI and KII restriction fragments were binding to the same protein because of the identical tissue specificity of both protein–DNA complexes. A com-

petition assay between the KI and KII fragments for binding to KLP was conducted by using the mobility shift assay (12, 17). Radiolabeled KII restriction fragment (0.1 ng) was mixed with increasing quantities of unlabeled KII fragment, KI fragment, or an unrelated restriction fragment from pBR322 of similar size, and the retention of the protein–DNA complex was assessed by the mobility-shift assay. The KI and KII fragments (5–50 ng) were both able to compete for the protein binding as demonstrated by the decrease in specific ^{32}P-labeled KII–protein complex with the addition of increasing quantities of these restriction fragments (Fig. 2*A*). In contrast, a nonspecific restriction fragment from pBR322 (5–50 ng) was unable to compete with the specific fragment for complex formation (Fig. 2*A*). With radiolabeled KI, a similar competition result was observed with either KI or KII fragment (data not shown). After adjustment for molar amounts of KI and KII fragments, it appeared that KII competed about 2 times better than KI at a similar concentration for binding of end-labeled KII fragment. In the reciprocal competition for radiolabeled KI binding, KII competed slightly better than the KI fragment. The experience of our laboratory indicates that the specific mobility of the protein–DNA complex in this assay is mostly determined by the protein when small DNA restriction fragments are used. A comparison of adjacent lanes showed virtual comigration of the KLP complexes formed with the KI and KII restriction fragments in spite of the DNA size difference of 43 bp (Fig. 2*B*). The competition assay and the electrophoretic mobility of the complexes thus suggest that KI and KII bind the same nuclear protein, KLP.

We analyzed the guanine contact residues of the specific KLP–DNA complexes by a methylation interference assay. Fragments KI or KII, 5′-end-labeled on one strand, were partially methylated with dimethyl sulfate and subsequently incubated with PD31 nuclear extract. The protein–DNA complexes with KI and KII and the unbound fragments were isolated from a preparative mobility-shift gel, cleaved with piperidine, and fractionated on a denaturing DNA sequencing gel (12, 14, 17). The complexed DNA from the two strands of the KII fragment (Fig. 3 *A* and *B*, lanes labeled Complex) and

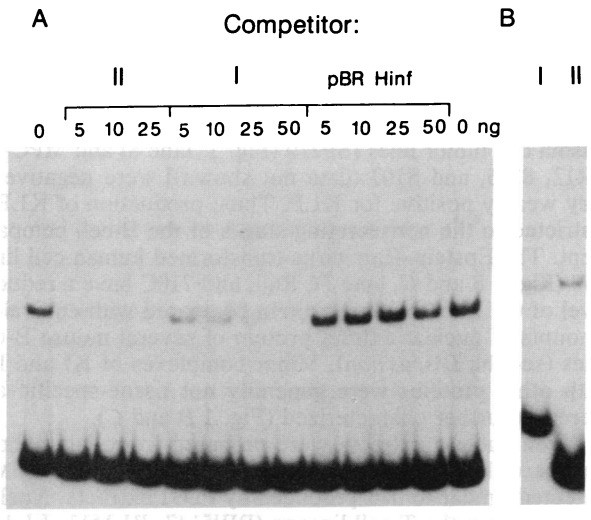

FIG. 2. Competition assay of KI and KII sites for nuclear protein binding. (*A*) Radiolabeled KII fragment (0.1 ng; 5000 cpm) was put in competition with increasing amounts (5–50 ng) of unlabeled 87-bp KII fragment (KII), 130-bp KI fragment (KI), or 142-bp pBR322 *Hin*fI restriction fragment (pBR *Hinf*) for binding to PD31 nuclear extract protein (10 μg) in the presence of 3 μg of poly[d(I·C)] as described. A mobility-shift gel assay was conducted to determine the stability of the complexes in the presence of a DNA competitor. (*B*) Mobility-shift assay of radiolabeled KI or KII fragments complexed with PD31 nuclear extract and fractionated in adjacent lanes.

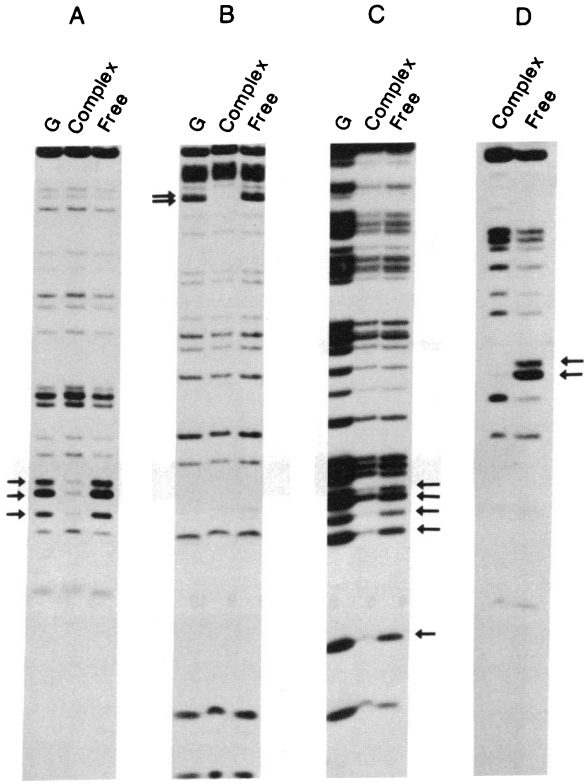

FIG. 3. Methylation interference mapping of nuclear protein binding to KI and KII sites. Radiolabeled KI and KII fragments were partially methylated and then used in the protein–DNA binding reactions with PD31 nuclear extracts. Complexed DNA (lanes labeled Complex) and uncomplexed DNA (lanes labeled Free) fractions were excised and electroeluted from the gel, treated with piperidine (14), and fractionated on a DNA sequencing gel. The partially methylated KI or KII fragment without treatment with nuclear extract (lanes G) was also fractionated. (*A*) KII coding strand. (*B*) KII noncoding strand. (*C*) KI coding strand. (*D*) KI noncoding strand. Arrows designate guanine residues that are of reduced intensity or absent in complexed DNA relative to unbound DNA.

KI fragment (Fig. 3 *C* and *D*, lanes labeled Complex) was deficient in DNA modified at the specific guanine residues indicated by arrows. In contrast, the partial methylation pattern of the uncomplexed fragment (Fig. 3, lanes labeled Free) was the same as the methylated fragment not treated with a nuclear extract (Fig. 3, lanes G). The same binding region was localized by using other pre-B-cell nuclear extracts from 38B9 and 18-81-2 cells (data not shown).

The methylation interference assay delineated two binding sites that were 5′ to the J_κ1 nonamer–heptamer elements and separated by 38 nucleotides (Fig. 4*A*). Site KI has five guanine residue contacts on the coding strand and two guanine residue contacts on the noncoding strand, forming a binding site of 16 bp with a 10-bp inverted repeat symmetry. Similarly, the site KII consists of two guanine residue contacts on the coding strand and three guanine residue contacts on the noncoding strand, forming a 16-bp binding site with a 14-bp imperfect inverted repeat symmetry. Methylation of a guanine residue 2 nucleotides further 5′ to KI partially interfered with binding but was not included in the postulated binding site because it fell outside the inverted repeat. Two guanine residues internal to KI (noncoding strand) and four guanine residues internal to KII (coding and noncoding strands) did not interfere with binding when methylated. Thus, both KI and KII have a similar asymmetric KLP binding motif in the same orientation in the J_κ locus on both the coding and noncoding strands (Fig. 4*A*). KI and

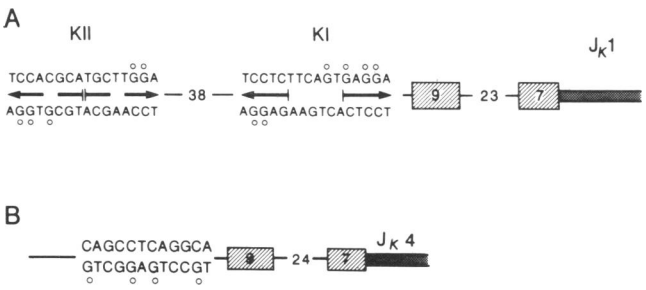

FIG. 4. KI, KII, and KIII binding sites for nuclear proteins in PD31 extract. (*A*) The KLP binding sites KI and KII. Using the methylation interference methodology (Fig. 3), two binding sites of KLP were identified. The KI and KII sites were separated by 38 bp and immediately flanked the $J_\kappa 1$ nonamer–heptamer sequence in the κ-chain gene region. Those guanine residues whose methylation interfered with binding are marked with a superior or inferior circle. Both sites have inverted repeat symmetry designated by bold arrows. The top strand is the coding strand (Fig. 3 *A* and *C*); the bottom strand is the noncoding strand (Fig. 3 *B* and *D*). (*B*) Methylation interference analysis of a ubiquitous nuclear protein binding to the site KIII in the κ-chain gene region. A preparative mobility shift gel was used to isolate a complex of KIII ($J_\kappa 4$) with a different nuclear protein from KLP, as in Fig. 2 and the text.

KII shared a conserved DNA sequence at the external arms of both inverted repeats and a conserved spacer length of 10 intervening nucleotides separating them (TCC-N_{10}GGA). The conservation of nucleotides and spacing of residues in KI and KII also suggests that they bind the same B-cell nuclear protein. The structure of the KLP binding site is reminiscent of the structure of the nuclear factor I DNA binding site, which consists of an inverted repeat and conserved spacing (TGGN$_7$CCA) (18).

A third complex, KIII, was observed by testing a 158-bp *Rsa* I–*Ava* I restriction fragment containing 105 bp of 5′ flanking sequences, nonamer–23-bp spacer–heptamer, and 13 bp of $J_\kappa 4$ DNA (Fig. 1*A*). The KIII complex was detected in every nuclear extract tested, including B-, T-, and non-lymphoid-cell types and was readily distinguishable from KI and KII complexes because it had a lower mobility. The methylation interference assay on the noncoding strand indicated that four guanine residues are significant to the binding site for KIII (Fig. 4*B*). Although the KIII motif was mapped to the same relative position of $J_\kappa 4$ as KI was for $J_\kappa 1$, this may not have any functional relevance for the κ-chain gene region. The KIII DNA sequence is not repeated upstream of $J_\kappa 4$ nor is it conserved between mouse and human. Other small restriction fragments from the J_κ cluster formed protein–DNA complexes in the assay, but only at a reduced intensity, and have not been considered further in this study.

We considered the possibility that other rearrangement sites such as the Ig *D* regions, heavy (H) chain V_H, V_κ, or other *J* gene elements would similarly have recognition sites for binding proteins flanking the nonamer–heptamer sequences. A fourth complex, DI, was observed with a 107-bp *Hin*fI–*Sac* I restriction fragment containing the *DQ52* element and the two sets of flanking nonamer–12-bp spacer–heptamer DNA sequences on either border. However, like KIII, DI was observed in all nuclear extracts tested (data not shown). Whereas KI and KII complexes had the same mobility in the electrophoretic assay, complexes with KIII and DI had different mobilities from each other and from complexes with KI or KII. There was no significant protein–DNA complex formation for a restriction fragment containing the $V_\kappa 21$ variable gene element, heptamer, nonamer, and 100 bp of flanking sequence (data not shown). We tested $J_H 1$, $J_\kappa 2$, $J_\kappa 3$, and V_H restriction fragments containing 50–100 bp of flanking sequences on the border of their nonamer–23-bp spacer–heptamer motifs with PD31 nuclear extract and found no stable protein–DNA complexes under our conditions (data not shown). Thus, we consistently failed to observe DNA binding protein complexes in association directly with nonamer–spacer–heptamer sequences, the proposed site for recombinase action.

DISCUSSION

A B cell-specific nuclear protein (KLP) binds to DNA in a κ-chain gene region proximal to the $J_\kappa 1$ element. Two DNA binding sites, each composed of imperfect inverted repeats and separated by 38 bp, form a common binding motif. These data, in conjunction with competition assays and the identical mobility of the protein–DNA complexes, suggest that the same DNA binding protein is capable of interacting with either site KI or KII.

It is intriguing that KLP is found exclusively in cells of the B-cell type and specifically in the immature stages represented by pre-B- and B-cell lines. κ-Chain gene rearrangement occurs at the pre-B-cell stage and has been observed in tissue culture in several pre-B-cell lines transformed by Ab-MuLV (9). KLP may be directly involved in the κ-chain gene rearrangement process, possibly by targeting a common recombinase enzyme to the site proximal to $J_\kappa 1$. The presence of KLP alone is insufficient to activate κ-chain gene rearrangement because the HAFTL and 38B9 cell lines, which rearrange Ig heavy chain genes but not endogenous κ-chain genes, contained KLP. However, the process of DNA rearrangement is likely to be a complex event involving multiple nuclear proteins; thus, it would not be surprising if the presence of a single nuclear protein does not exclusively determine the appropriate regulation of rearrangement. Alternatively, KLP may be a regulatory protein that prevents κ-chain gene rearrangement until μ-chain gene rearrangement has been successfully completed. A transcription model for the specificity of gene rearrangement has also been proposed whereby the accessibility of DNA sequences to recombination is stimulated by local transcription events (6, 7). Possibly KLP functions as a transcription factor whose activity identifies the κ locus for rearrangement. It is unlikely that KLP functions as a transcription factor for expression of κ-chain protein because the KI and KII sites are usually deleted in κ-chain gene rearrangements, and KLP is absent in mature B-cell stages, where κ-chain transcription is maximal. Also, we have not found an induction of KLP in 70Z cells treated with lipopolysaccharide or cycloheximide, conditions that activate κ-chain genes for transcription and increase the abundance of two κ-chain gene enhancer or promoter binding proteins, NF-κB and NF-A2 (12, 17). Possibly other Ig or T-cell gene families have similar proteins that may direct recombinase to a different region, activating an alternative timing and stage-specific rearrangement or generating other functions such as Ig H chain gene isotype switching.

Of the five J_κ elements, $J_\kappa 1$ is the most frequently utilized in rearrangement events to V_κ, as demonstrated in primary B cells by DNA and nuclear RNA mapping (19, 20). The proximity of the KI and KII sites to the nonamer–spacer–heptamer of the $J_\kappa 1$ gene element and thus to the site of KLP binding may explain why $J_\kappa 1$ is the most frequently rearranged.

There is a significant DNA sequence homology flanking the $J_\kappa 1$ gene elements of the mouse, rat, and human for approximately 100 nucleotides upstream. Although the rat KI and KII DNA sequences are identical to those of the mouse, the human KI and KII sequences differ from them by one residue (human DNA KI site = TCCTCTTCAGTGAG$\overset{*}{A}$A, with asterisk over the distinctive residue). This may explain why human B-cell extracts apparently have lower levels of the KLP complex than do mouse nuclear extracts with the mouse

KI and KII DNA sequences. In addition, the conservation of the DNA sequence in front of the $J_\kappa 1$ element of mouse and human is greater than conservation between the species of the analogous regions in front of the other most similar J_κ elements.

We did not observe a DNA binding protein in association with the $J_H 1$ region in the analogous position to $J_\kappa 1$. Possibly other parameters such as the number of rearrangement steps or the distance between V and J elements also may be relevant to the regulation of the rearrangement process. Because every Ig and TCR gene family contains its own unique gene element structural organization, they also may utilize unique mechanisms for activation of each family for gene rearrangement.

Recombination enhancers have been observed for the site-specific inversion of genes encoding bacteriophage or *Salmonella* surface antigens (21) or yeast mating-type switching. Site-specific inversion of *Salmonella* surface antigen genes requires the binding of a host protein to a sequence flanking the inverted repeat elements where the breakage and rejoining of the phase-variation inversion occurs (22). This cis-acting regulatory sequence is defined as a recombinational enhancer element in that it functions in an orientation- and distance-independent manner. KLP may serve a similar function for aligning the Ig and TCR recombinase.

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