

# Human Immunoglobulin $\kappa$ Gene Enhancer: Chromatin Structure Analysis at High Resolution

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**The murine immunoglobulin  $\kappa$  gene enhancer has previously been found to coincide with a region of altered chromatin structure reflected in a DNase I hypersensitivity site detectable on Southern blots of B-cell DNA. We examined the chromatin structure of the homologous region of human DNA using the high-resolution electroblotting method originally developed for genomic sequence analysis by G. Church and W. Gilbert (Proc. Natl. Acad. Sci. USA 81:1991-1995, 1984). Analysis of DNA isolated from cells treated *in vivo* with dimethyl sulfate revealed two B-cell-specific sites of enhanced guanine methylation. Both sites are located within perfect inverted repeats theoretically capable of forming cruciform structures; one of these repeats overlaps an enhancer core sequence. No enhancement or protection of guanine methylation was observed within sequences similar to sites of altered methylation previously described in the immunoglobulin heavy-chain enhancer. Treatment of isolated nuclei with DNase I or a variety of restriction endonucleases defined a B-cell-specific ~0.25-kilobase region of enhanced nuclease susceptibility similar to that observed in the murine  $\kappa$  enhancer. The 130-base-pair DNA segment that shows high sequence conservation between human, mouse, and rabbit DNAs lies at the 5' end of the nuclease-susceptible region.**

Unknown mechanisms allow a eucaryotic DNA sequence to be actively transcribed *in vivo* by one cell type while the identical sequence in another cell is silent. Presumably active and silent genes differ in some structural features other than nucleotide sequence. Such features might include differences in secondary structure (e.g., superhelicity) (16, 24, 55), in covalent modification (methylation) (22), and in associated proteins (4-6, 8, 9, 11, 19, 21, 26, 30, 34, 37, 43, 44, 47, 50, 58-60). A variety of Southern blot strategies have been used to probe such alterations in chromatin structure. These experiments have demonstrated that actively transcribed genes exhibit increased susceptibility to various endonucleases, especially within regulatory sequence elements such as enhancers and promoters (1, 7, 12, 13, 29, 33, 35, 36, 39, 52, 56, 57, 61-63).

Recently, the genomic sequencing technique developed by Church and Gilbert (3) has made it possible to probe chromatin structure with much higher resolution than can be achieved by Southern blot methods. In this procedure genomic DNA modified *in vivo* or *in vitro* by a chemical agent such as dimethyl sulfate (DMS) or by nuclease digestion is isolated and cut to completion with a specific restriction endonuclease; the DNA is then electrophoresed on a high-resolution denaturing acrylamide gel, transferred to a nylon membrane by electroblotting, and hybridized with a single-stranded probe derived from the cloned gene of interest. The resulting pattern of hybridizing bands reflects the position of partial cuts or DNA modifications generated in the initial step. In one of its first applications, Ephrussi and co-workers (2, 11) used this technique to study *in vivo* susceptibility of DNA to methylation by DMS (DMS footprint analysis). Earlier work had shown that susceptibility of prokaryotic regulatory gene segments to DMS could be altered by the binding of purified DNA-binding proteins *in vitro* (14, 18, 31, 42, 46, 48, 49). Ephrussi et al. (11) examined the murine immunoglobulin heavy-chain enhancer and discovered sites within this region that demonstrated B-cell-

specific alterations in DMS reactivity, either protection or enhancement. On the basis of the distribution of these sites, these authors proposed a consensus sequence (CAG GTGGC) for the binding of a hypothetical tissue-specific regulatory factor involved in enhancer function (2, 11). As noted by Church (Ph.D. thesis, Harvard University, Cambridge, Mass., 1984), two close matches to this consensus sequence are found in the murine immunoglobulin  $\kappa$  enhancer.

The  $\kappa$  enhancer has been localized by transfection studies with gene constructs containing various segments from the murine J $\kappa$ -C $\kappa$  intron (38, 40, 41); this enhancer occurs about 0.7 kilobase (kb) 5' to the C $\kappa$  coding block. This position coincides with a region of sequence homology conserved between mouse, rabbit, and human DNAs previously designated the kappa intron conserved region (KICR) (10) and also coincides with the approximate location of a B-cell-specific DNase I hypersensitivity site observed in mouse (35) and human (39) DNAs. It is generally assumed, although it has not yet been formally demonstrated, that the KICR sequences of human and rabbit DNAs also function as enhancers.

In the present work we examined the chromatin structure of the human KICR-enhancer region using the high-resolution genomic sequencing technology of Church and Gilbert (3). DMS footprint analysis was used to look for evidence of B-cell-specific alterations in DMS sensitivity, either in sequences similar to those showing DMS changes in the murine heavy-chain enhancer or elsewhere in the human KICR. We also examined the nuclease sensitivity in this region at high resolution using DNase I and a variety of restriction endonucleases.

## MATERIALS AND METHODS

**Cell culture.** B-cell lines used were BHM-23 (28) and L06 and L22 (23); T-cell lines used were RPMI-8402, CEM, and MOLT4 (23). The characteristics of these cells are given in Table 1. Lymphoid cell lines were maintained in RPMI 1640

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TABLE 1. Human cell lines used in this study

Cell line	Cell type	Kappa gene <sup>a</sup>	Kappa transcription <sup>b</sup>
RPMI-8402	T cell	Germ line	—
CEM	T cell	Germ line	—
MOLT4	T cell	Germ line	—
HeLa	Epitheloid carcinoma	Germ line	—
BHM-23	B cell	1 rearranged; 1 deleted	+
L06	B cell	1 rearranged; 1 germ line	+
L22	B cell	1 rearranged; 1 germ line	+

<sup>a</sup> The status of the kappa gene was determined by Southern blot analysis.

<sup>b</sup> The presence of kappa gene mRNA was determined by Northern blot analysis.

medium (Hazelton) supplemented with 16% fetal calf serum, 1% glutamine, 100 U of penicillin per ml, and 100 U of streptomycin per ml. Cells were grown to a density of  $0.5 \times 10^6$  to  $2 \times 10^6$  cells per ml and harvested by centrifugation at  $350 \times g$  for 5 min at 4°C. HeLa cells were maintained in Dulbecco modified Eagle medium (Hazelton) supplemented with 10% fetal calf serum, 1% glutamine, 100 U of penicillin per ml, and 100 U of streptomycin per ml. Cells were grown to 90% confluency and harvested by mild trypsin digestion and centrifugation.

**DMS treatment of cells.** Cells to be treated with DMS were resuspended in culture medium at  $10^8$  cells per ml and incubated for 2 min at 20°C in the presence of 0.5% DMS. The reaction was terminated by the addition of 10 volumes of ice-cold phosphate-buffered saline.

**Preparation of nuclei.** Cell nuclei were prepared by the technique of Emerson and Felsenfeld (8). Cells were washed in 10 mM Tris (pH 7.6)–10 mM NaCl–3 mM MgCl<sub>2</sub> (RSB) followed by two washes in 0.2% Triton-RSB. The resulting nuclei were washed in RSB and resuspended at an approximate DNA concentration of 1 mg/ml based on the optical density at 260 nm.

**DNase I digestion of nuclei.** Cell nuclei were digested with pancreatic DNase I (Worthington Diagnostics, Freehold, N.J.) at 0 to 4°C. Nuclei were suspended at 500 µg of DNA per ml in the following digestion buffer: 60 mM KCl, 15 mM NaCl, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 15 mM Tris (pH 7.6) (8). Reactions were initiated by the addition of CaCl<sub>2</sub> (0.1 mM final concentration) and various concentrations of DNase I. Tubes were inverted once and incubated on ice for 10 min. Reactions were terminated by the addition of 0.4% sodium dodecyl sulfate (SDS)–50 mM EDTA–0.3 mg of proteinase K per ml (final concentrations).

**Restriction endonuclease digestion of nuclei.** Cell nuclei were suspended at 500 µg of DNA per ml in 50 mM Tris (pH 7.6)–50 mM NaCl–3 mM MgCl<sub>2</sub>. Enzymes (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were added at the concentrations indicated in the figures. Digestions were performed at 37°C for 60 min. Reactions were terminated by the addition of 0.4% SDS–50 mM EDTA–0.3 mg of proteinase K per ml (final concentrations).

**DNA purification.** Nuclear samples were digested with proteinase K for 3 h at 37°C. Samples were extracted twice (each) with phenol and chloroform. The purified DNA was precipitated from 2.5 volumes of ethanol with ammonium acetate (0.5 M final concentration).

**Southern blots.** Southern blotting was performed with

0.7% agarose gels by the standard technique (51). Blots were hybridized with 5 to 10 µCi of nick-translated probe in a 10% dextran sulfate–40% formamide hybridization solution at 52°C for 16 to 18 h. Blots were washed three times at low stringency (52°C; 0.1% SDS, 150 mM NaCl, 15 mM sodium citrate) for 20 min and once at high stringency (65°C; 0.05% SDS, 15 mM NaCl, 1.5 mM sodium citrate) for 20 min. Blots were exposed for 2 to 10 days at –70°C with an enhancer screen.

**Genomic electroblotting.** Genomic electroblots were performed by the techniques of Church and Gilbert (3). Purified DNA was cut to completion with *EcoRI* or with *PstI* plus *PvuII*, precipitated with ammonium acetate and ethanol, and dried under vacuum. At this point, DNA from DMS-treated cells was reacted for 30 min with 100 µl of 1 M piperidine at 90°C, frozen on dry ice, and dried under vacuum. All DNA samples were subsequently suspended in 5 µl of loading solution (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue), boiled for 1 min, and loaded while still hot onto a 6% acrylamide–7 M urea gel (1.5 mm thick and 30 cm wide). Gels were electrophoresed at 35 mA for 6 to 18 h. The gels were removed to the Scotchbrite surface of an electroblotting apparatus (3), covered with Genescreen (New England Nuclear Corp., Boston, Mass.), and electroblotted for 1 h at 80 V in 35 mM Tris (pH 8.3)–35 mM boric acid–1.4 mM EDTA. The Genescreen blots were removed, covered with polyethylene wrap, and exposed to UV light (254 nm) at 800 µW/cm<sup>2</sup> for 16 min to covalently bind the DNA to the filter. Wet blots could be stored at 4°C sealed in polyethylene wrap before hybridization without any apparent degradation of signal.

**Single-stranded probe preparation.** Probes were prepared with single-stranded DNA from M13 subclones. Approximately 6 µg of M13 DNA in 30 µl was mixed with 2 µl of 100 mM MgCl<sub>2</sub> and 5 µl of 17mer universal primer (Amersham M4511) at 10 ng/µl and incubated for 40 min at 52°C. After cooling on ice, 1 µl of 10-mg/ml bovine serum albumin, 1 µl of 3.3 mM deoxynucleotide triphosphates (C,G,T), 0.1 µl of 0.2 M dithiothreitol, 15 units of Klenow fragment (Amersham), and 25 µl (500 µCi) of [ $\alpha$ -<sup>32</sup>P]dATP (NEG012 Z; New England Nuclear Corp.) were added. The solution was incubated for 40 min at 25°C. The reaction was stopped with the addition of 200 µl of 94% formamide–0.05% xylene cyanol–0.05% bromophenol blue–10 mM EDTA. The tube was boiled for 5 min, loaded directly onto a 6% acrylamide–7 M urea gel, and electrophoresed for 5 min at 90 mA. The gel was removed and wrapped with cellophane. To confirm the location of the radioactive probe as comigrating with the xylene cyanol dye front, this region of the gel was covered for 5 min with a piece of Polaroid film (type 52) which was then developed. The xylene cyanol band, corresponding to a probe size of 100 to 120 base pairs (bp), was excised, crushed into 10 to 20 ml of hybridization solution (500 mM NaHPO<sub>4</sub> [pH 7.2], 7% SDS, 1% bovine serum albumin, 1 mM EDTA), vortexed vigorously, and incubated for 45 min to 1 h at 52°C. Gel fragments were removed by a 1-min centrifugation in a benchtop clinical centrifuge and filtration through a Millex filter (0.22-µm pore size; Millipore Corp., Bedford, Mass.). The radioactivity of the probe was estimated by scintillation counting. Optimal signal-to-noise ratios were obtained with a radioactivity level of 5 to 10 µCi/ml. Higher concentrations consistently resulted in high backgrounds on long (greater than 40 h) exposures. Experiments were performed with probes from the 3' and 5' ends of the 710-bp *EcoRI* fragment containing the human KICR; however, experiments with the 5' probes produced very weak signals, probably because of

the low (28%) guanine-plus-cytosine content of the 5' region, and are not reported here.

**Hybridization.** Genescreen blots were prehybridized for 4 to 16 h at 65°C in a Kapak sealer bag with 20 ml of hybridization solution. Prehybridization solution was drained from the blots and replaced with the solution containing radiolabeled probes. The blots were submerged in a 65°C water bath and hybridized overnight. Bags containing the blots were opened in 1 liter of wash solution (40 mM NaH<sub>2</sub>PO<sub>4</sub> [pH 7.2], 1 mM EDTA, 1% SDS) heated to 65°C. Blots were washed in eight changes of the 65°C wash solution (5 min each) and were then covered with polyethylene wrap and exposed at -70°C with an intensifier screen for 6 h to 12 days.

## RESULTS

**DMS footprint analysis.** A variety of in vitro studies have demonstrated that the binding of purified regulatory proteins to specific sites in DNA may alter the susceptibility of guanine nucleotides within such sites to methylation by DMS; particular guanine residues may show either enhancement or protection from methylation compared with the same sites in naked DNA (14, 18, 31, 42, 46, 48, 49).

On the basis of homology to the murine immunoglobulin  $\kappa$  enhancer, the corresponding region (KICR) of the human locus is presumed to represent a control region that may include binding sites for tissue-specific regulatory proteins. To probe this region for alterations in chromatin structure, we used an in vivo DMS footprinting strategy similar to the analysis of the murine heavy-chain enhancer reported by Ephrussi and co-workers (2, 11). Cultured human cell lines corresponding to B or T lymphocytes (or other control lines as indicated below) were incubated in the presence of 0.5% DMS for 2 min at 20°C in culture medium; the reaction was terminated by dilution into ice-cold phosphate-buffered saline. Nuclei were prepared, and the DNA was isolated. The DNA was then cut to completion with *Eco*RI and treated with piperidine to cleave at the methylated guanine residues. The resulting genomic DNA fragments were electrophoresed on a thick (1.6-mm) sequencing gel (6% acrylamide-7 M urea) and transferred to a nylon membrane by electroblotting. The membrane was then hybridized to M13-derived single-strand probes specific for the human KICR to reveal the positions of methylation-induced guanine cleavages.

Figure 1 illustrates results of a typical experiment in which electroblots were hybridized with sense and antisense probes from the 3' end of the 710-bp *Eco*RI fragment containing the KICR. The band representing this intact *Eco*RI fragment is observed at the top of each lane, above a pattern of subbands representing guanine residues methylated in vivo by DMS. Control experiments demonstrated that identically prepared DNA from cells not treated with DMS failed to demonstrate any subbands (data not shown). DNA from DMS-treated cells examined without piperidine treatment gave a pattern of subbands similar to those in Fig. 1 but much fainter (data not shown), suggesting that strand scission at methylated guanine residues can occur in the absence of piperidine, either in the cells or during DNA isolation.

Two sites of enhanced guanine methylation were observed in all B-cell lines but not in T cells or HeLa cells (Fig. 1). These sites, indicated by stars in the figure, were observed consistently on the sense strand; no comparable enhanced sites occurred on the antisense strand. The locations of the enhanced sites can be determined approximately from re-

striction fragment markers and the pattern of guanine residues in plasmid DNA; the enhanced sites lie near the 5' end of the KICR.

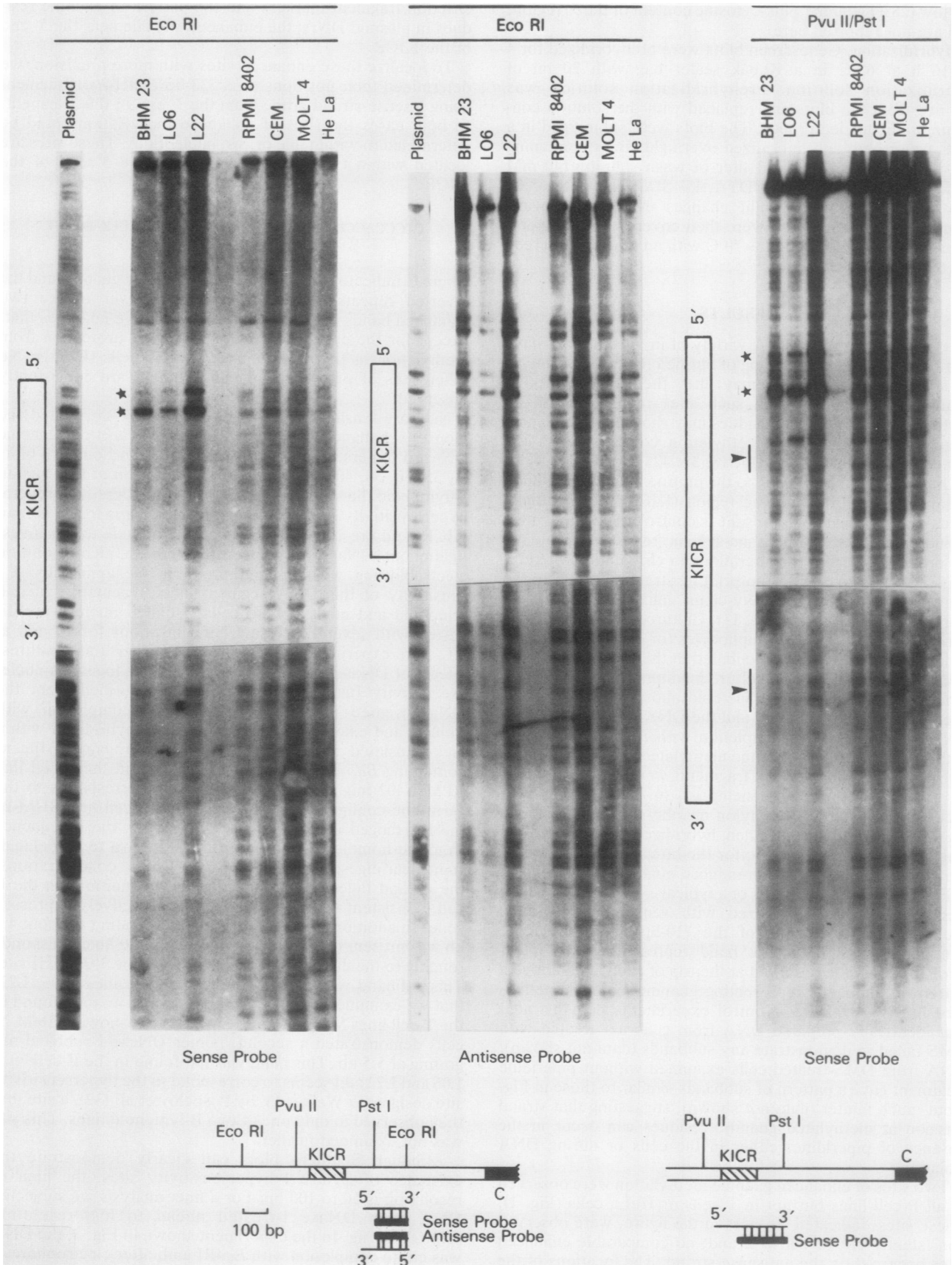
To localize these enhanced sites with more precision, we determined their positions on a 333-bp *Pvu*II-*Pst*I fragment using a sense-strand probe from the 3' end of this fragment. In both cases the sites of enhanced methylation could be determined to within one or two nucleotides. These sites are located within two inverted repeats at the 5' end of the KICR:



where  $\overset{*}{G}$  indicates the methylation-enhanced guanine and the inverted repeats are underlined. The lower of the two enhanced bands occurs within a string of five guanines that, except for the enhanced band, show clear protection from methylation in B cells compared with T cells (Fig. 1). No other sites of protection or enhancement are consistently observed.

**Nuclease sensitivity.** Several studies have correlated the mouse  $\kappa$  enhancer with the position of a B-cell-specific DNase I hypersensitivity site observable on Southern blots (1, 35, 36, 38, 40, 41, 52). In an examination of two human B-lymphoid lines, Pospelov et al. (39) identified several hypersensitivity sites including one that maps to the human KICR (and presumed enhancer). As a prelude to our investigation of DNase I hypersensitivity with high-resolution electroblotting, we used Southern blots to examine DNase I sensitivity of this DNA segment in representative human lymphoid and nonlymphoid cells. Isolated nuclei were digested with several DNase I concentrations for 10 min at 0°C. (In experiments performed at higher temperatures, effects of DNase I were obscured by an endogenous nuclease activity [data not shown].) DNA purified from the DNase-treated nuclei was digested to completion with *Bam*HI and examined on Southern blots hybridized with a nick-translated probe derived from the 3' end of the  $\kappa$ -containing *Bam*HI fragment. The  $\kappa$  gene in the T-cell line RPMI-8402 migrated at 10.5 kb, which corresponds to the germ line configuration (Fig. 2). In the B-cell line BHM-23 the rearranged  $\kappa$  gene migrated at 6.7 kb; the  $\kappa$  sequence from the homologous chromosome is known to be deleted. In nuclear digestions with a range of DNase concentrations, the  $\kappa$  band faded under mild digestion conditions in the B cell, consistent with expectations for an actively expressed gene. In addition, a DNase I subband migrating at about 4.0 kb was present exclusively in the B cell; 4.0 kb corresponds roughly to the distance of the KICR from the 3' *Bam*HI site. This subband was present in other B-cell lines (L06, L22) that we examined by Southern blotting but was not found in the T-cell lines MOLT4 and CEM (data not shown). BHM-23 cells demonstrated a second, fainter DNase I subband migrating at 5.5 kb. This band was not found in the B-cell lines L06 and L22 and seems to correspond to the hypersensitivity site designated Walker IV by Pospelov et al. (39), a site that they observed in only one of two B-lymphoid lines. This site was not examined further.

Although Southern blots can clearly demonstrate the existence of DNase I hypersensitivity sites, the limit of resolution is 50 to 100 bp. For a finer analysis we subjected DNA from DNase I-treated nuclei to high-resolution electroblotting. In the experiment shown in Fig. 3, the DNA was cut to completion with *Eco*RI and, after electrophoresis and electroblotting, was hybridized to a single-stranded



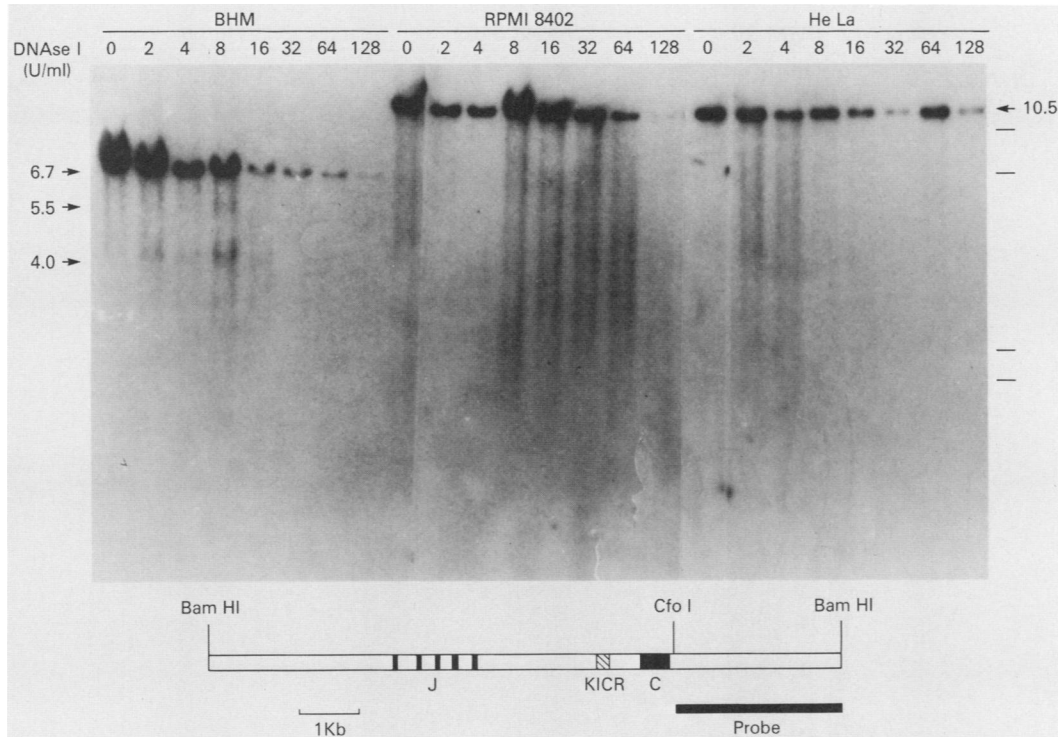


FIG. 2. Southern blot analysis of DNA isolated from DNase I-digested nuclei. Isolated nuclei from BHM-23 (B cell), RPMI-8402 (T cell), and HeLa cells were digested with increasing concentrations of DNase I (units per milliliter as indicated), and the DNA was isolated. Samples of 15  $\mu$ g of genomic DNA were cut to completion with *Bam*HI, electrophoresed on a 0.7% agarose gel, and transferred to nitrocellulose. The Southern blot was hybridized with a 2.7-kb nick-translated probe synthesized from the indicated *Cfo*I-*Bam*HI fragment (see map). Arrows indicate the unrearranged (10.5 kb) and rearranged (6.7 kb) kappa genes as well as the BHM-23-specific DNase I-hypersensitive sites migrating at 4.0 and 5.5 kb, respectively. Lines to the right of the figure indicate molecular weight markers (9.6, 6.6, 2.3, and 2.0 kb). The map indicates the 10.5-kb *Bam*HI fragment of the kappa gene, including the J segments, KICR, and constant region (C).

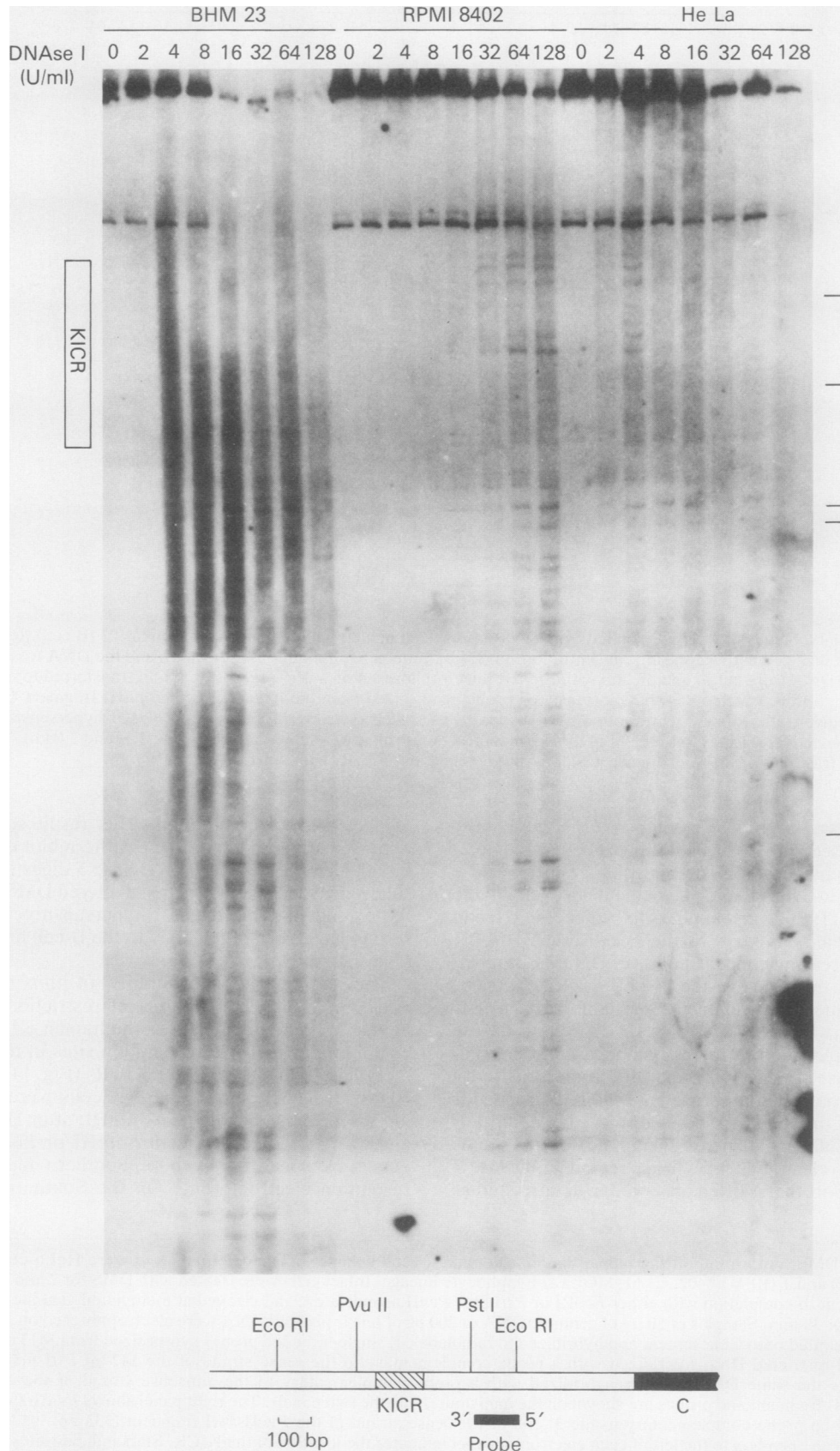
probe from the 3' end of the 710-bp human KICR-containing *Eco*RI fragment (see map in Fig. 3); this probe was complementary to the sense strand. The autoradiogram reveals a 425-bp fragment in DNA from all cell lines, even in the absence of DNase I; this band appears to be generated by an endogenous nuclease activity. In the B-cell line BHM-23, digestion with increasing concentrations of DNase I progressively diminished the signal of the 710-bp *Eco*RI fragment, whereas this fragment was relatively resistant in HeLa cells and the T-cell line RPMI-8402. In BHM-23 cells a diffuse signal from multiple smaller fragments appeared at concentrations of DNase I that produce the hypersensitivity subband on the routine Southern blot. This region extends from the 5' end of the KICR to 100 bp or more beyond the 3' end of the KICR. No single DNase I band in BHM-23 stands out, nor is there clear evidence for a region of DNase I protection of the sort that is often observed in *in vitro* studies

of protein-DNA interaction. This result suggests that the hypersensitivity band on the Southern blot corresponds to a broad region ( $\sim$ 0.25 kb) of DNase I digestion and not to a single cleavage site. The sites of altered DMS reactivity lie in the 5' end of this DNase I-hypersensitive region. Similar results have been obtained with the B-cell lines L06 and L22 (data not shown).

To examine the accessibility of this region to other nucleases, we tested a panel of restriction endonucleases having recognition sites near the human KICR. *Hae*III, for example, has multiple recognition sites in the J $\kappa$ -C $\kappa$  intron, including two within the KICR (Fig. 4). Nuclei from BHM-23, RPMI-8402, and HeLa cells were incubated with increasing concentrations of *Hae*III; after DNA purification and complete digestion with *Bam*HI or *Eco*RI, the *Hae*III cuts were localized by both Southern blotting and high-resolution electroblotting. On the Southern blot shown in

FIG. 1. *In vivo* DMS footprinting of the human immunoglobulin  $\kappa$  gene enhancer. The cell lines used were HeLa cells and cells of B- (BHM-23, L06, L22) and T (RPMI-8402, CEM, MOLT4)-lymphocyte lineage. Intact cells were treated with DMS for 2 min at 20°C. The DNA was then isolated, cut to completion with either *Eco*RI or *Pst*I plus *Pvu*II as indicated, and cleaved at methylated guanine residues with 1 M piperidine at 90°C for 30 min. Samples of 50  $\mu$ g of genomic DNA or 200 pg of linear plasmid DNA were electrophoresed on a 6% acrylamide-7 M urea gel, electroblotted onto Genescreen, and hybridized to radiolabeled, single-stranded probes synthesized from M13 subclones. The left panel shows *Eco*RI-restricted DNA hybridized with a probe complementary to the sense strand of the 147-bp *Pst*I-*Eco*RI fragment. The middle panel shows the same DNA samples hybridized with a probe complementary to the antisense strand of the 147-bp *Pst*I-*Eco*RI fragment. The *Eco*RI fragment and probes are shown on the map underlying the two panels. The right panel shows *Pvu*II-*Pst*I-restricted DNA hybridized to a 120-bp probe complementary to the 3' end of the sense strand of the *Pvu*II-*Pst*I fragment (333 bp), as shown on the map beneath the panel. The open box to the left of each electroblot panel indicates the location of the KICR. Stars indicate sites of enhanced DMS methylation of guanine residues on the sense strands. The arrowheads beside the right panel mark the consensus sequence CAGGTGGC described by Ephrussi et al. (11) in the immunoglobulin heavy-chain enhancer. C, constant region.





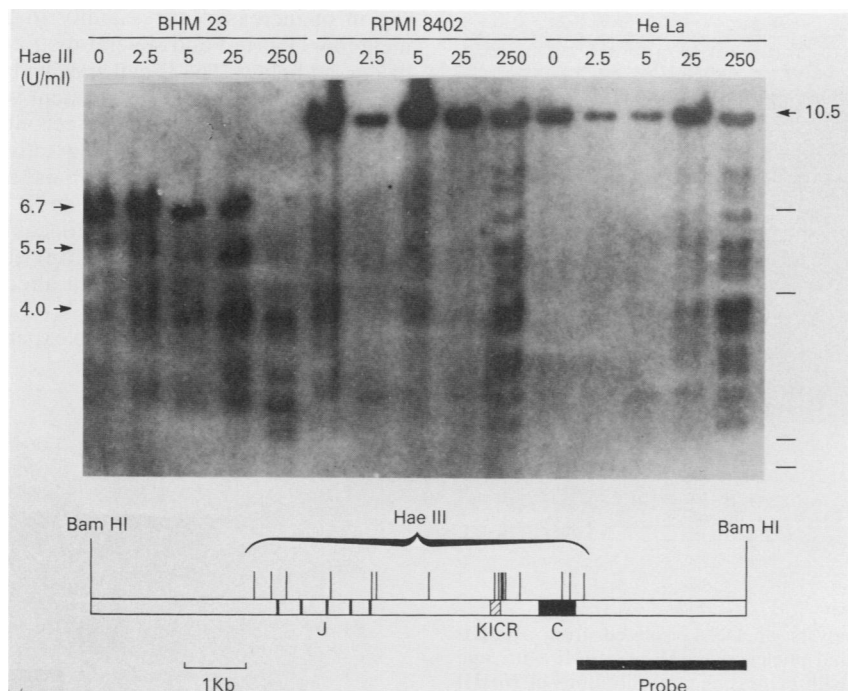


FIG. 4. Southern blot analysis of DNA isolated from *Hae*III-digested nuclei. Nuclei isolated from BHM-23, RPMI-8402, and HeLa cells were digested with increasing concentrations of *Hae*III at 37°C for 1 h. After the DNA was purified, 15- $\mu$ g samples were cut to completion with *Bam*HI, electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose paper, and hybridized to the nick-translated *Cfo*I-*Bam*HI probe used in Fig. 2. Arrows indicate the unrearranged (10.5 kb) and rearranged (6.7 kb)  $\kappa$  genes as well as the BHM-23-specific sites of increased *Hae*III accessibility migrating at 4.0 and 5.5 kb. Lines to the right of the panel show the positions of molecular weight markers (9.6, 6.6, 2.3, and 2.0 kb). The map indicates the known *Hae*III recognition sites in the sequence of the human  $\kappa$  gene. Additional *Hae*III sites may exist in the unsequenced regions near the 3' and 5' ends of the *Bam*HI fragment. C, constant region.

Fig. 4, *Hae*III subbands occur in BHM-23 at 5.5 and 4.0 kb, essentially the same positions of the DNase I subbands of Fig. 2. These sites were cut in RPMI-8402 and HeLa cells only at about 20-fold-higher *Hae*III concentrations. Since a similar pattern of bands occurs in all three cell lines at the highest *Hae*III concentrations, it seems likely that the 5.5- and 4.0-kb bands in BHM-23 cells do not result simply from increased permeability of BHM-23 nuclei to *Hae*III but rather reflect a localized alteration in chromatin structure. *Hae*III-digested DNA samples were analyzed with high-resolution electroblots after digestion with *Eco*RI. These experiments indicated that the five *Hae*III sites in the vicinity of the KICR all showed similar enhanced sensitivity to digestion in BHM-23 nuclei (Fig. 5); these sites were less sensitive in HeLa and RPMI-8402 cells. Other B-cell lines examined (L06 and L22) gave results similar to those with BHM-23 cells; while the T-cell lines CEM and MOLT4 behaved like RPMI-8402 (data not shown). We conclude that the *Hae*III sites near the KICR are 10- to 100-fold more accessible to digestion in B-cell versus non-B-cell nuclei.

In addition to *Hae*III, 13 other restriction enzymes known to have recognition sequences in the vicinity of the human

KICR were tested on B- and T-cell nuclei. Figure 6 summarizes the findings for BHM-23 (B-cell) nuclei: all potential restriction sites falling within a region of about 0.25 kb including the KICR demonstrated cleavage, whereas all the sites outside this region were resistant. The enzymes *Bst*NI, *Fnu*4HI, and *Hinf*I are noteworthy in that, as for *Hae*III, recognition sites for these enzymes exist within both the accessible and inaccessible regions so the sites where cleavage is observed represent internal positive controls that emphasize the dichotomy between the two regions. The region of increased B-cell accessibility to restriction endonucleases coincides with that defined by DNase I hypersensitivity. In RPMI-8402 and HeLa cell nuclei the same restriction endonucleases cleaved the DNA more weakly than in BHM-23 cells or not at all (data not shown).

## DISCUSSION

Enhancers are DNA segments that increase transcription of nearby genes in DNA constructs transfected into eucaryotic cells. The effects of enhancers are independent of their orientation in constructs and can be transmitted to promot-

FIG. 3. Electroblot analysis of DNA isolated from DNase I-digested nuclei. In the experiment shown here, the DNA samples from DNase I-treated nuclei are portions of the same samples studied in Fig. 2. After the DNA samples were isolated, 50  $\mu$ g of genomic DNA was cut to completion with *Eco*RI; the DNA was then electrophoresed on a 6% acrylamide-7 M urea gel and electroblotted onto Genescreen. The membrane was hybridized to a radiolabeled single-stranded probe complementary to the sense strand of the 147-bp *Pst*I-*Eco*RI fragment. This probe is identical to that used in Fig. 1, left panel. The open box to the left of the panel indicates the location of the KICR. Lines to the right of the panel indicate the position of restriction fragments generated by partial *Hae*III digestion of plasmid DNA followed by complete *Eco*RI digestion (sizes 365, 305, 247, 239, and 155 bp; see map in Fig. 5 for locations). C, constant region.

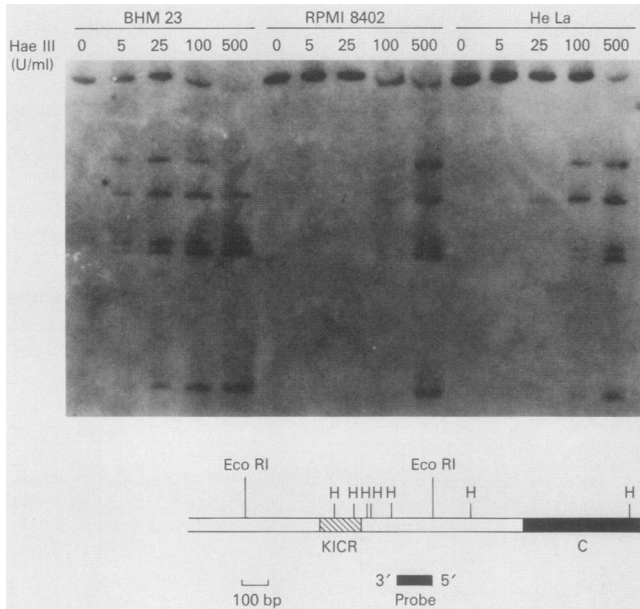


FIG. 5. Electroblot analysis of DNA isolated from *HaeIII*-digested nuclei. The isolated nuclei of BHM-23, RPMI-8402, and HeLa cells were digested with increasing concentrations of *HaeIII* at 37°C for 1 h. After the DNA was purified, 50- $\mu$ g samples were cut to completion with *EcoRI*, electrophoresed on a 6% acrylamide-7 M urea gel, electroblotted to a Genescreen membrane, and hybridized to a single-stranded probe complementary to the sense strand of the 147-bp *PstI-EcoRI* fragment (Fig. 1, left panel). *HaeIII* concentrations are indicated (units per milliliter). The map indicates the location of the *HaeIII* restriction sites close to the KICR.

ers several kilobases distant. The mechanism of enhancer action is under intense investigation with enhancers derived from several different genes (reviewed in reference 45). One common feature of mechanisms hypothesized for enhancers is that they may represent binding sites for specific nuclear regulatory proteins analogous to the well-characterized prokaryotic DNA-binding regulatory proteins, e.g., the *lac* repressor or the lambda repressor. Indeed, several eucaryotic examples of specific proteins binding to regulatory regions of genes have been found including the chicken  $\beta$ -globin promoter (8, 9), the *Drosophila* heat shock promoter (34, 58-60), the *c-myc* gene (19, 47), the insulin gene enhancer (32), and the mouse dihydrofolate reductase promoter (5).

The murine gene for immunoglobulin  $\kappa$  is known to possess an enhancer upstream of the constant-region sequence (38, 40, 41); the corresponding region of the human gene, the KICR, shows high sequence conservation with the murine gene and is assumed to also function as an enhancer (10). We examined the human KICR region by analyses of *in vivo* DMS footprinting and nuclease hypersensitivity. When cells were treated *in vivo* with DMS, two B-cell-specific sites of enhanced guanine methylation were found near the 5' end of the KICR (Fig. 1). These guanine residues were located within separate regions of dyad symmetry (Fig. 7). One of these dyad symmetries overlaps the simian virus 40 (SV40) core sequence TGG<sub>TTA</sub> which has been found in several viral enhancers and which, by mutation analysis, appears to be critical for the function of the SV40 enhancer (24, 54). The associated enhanced guanine occurs within a cluster of four other guanines that show B-cell-specific protection from methylation. We also detected around the KICR a broad

region of increased accessibility to a variety of nucleases including DNase I and several restriction endonucleases, as discussed below. The B-cell specificity of these features and their occurrence in a DNA segment with probable enhancer function suggest that they reflect alterations in chromatin structure that are related to the activation of the  $\kappa$  gene in these cells. The exact nature of these alterations is unknown, but possible explanations include (i) modifications of DNA secondary structure, possibly (ii) stabilized by the binding of sequence-specific proteins, or (iii) effects of DNA-binding proteins involving no significant alteration in DNA secondary structure.

Data from *in vivo* and *in vitro* experiments have suggested

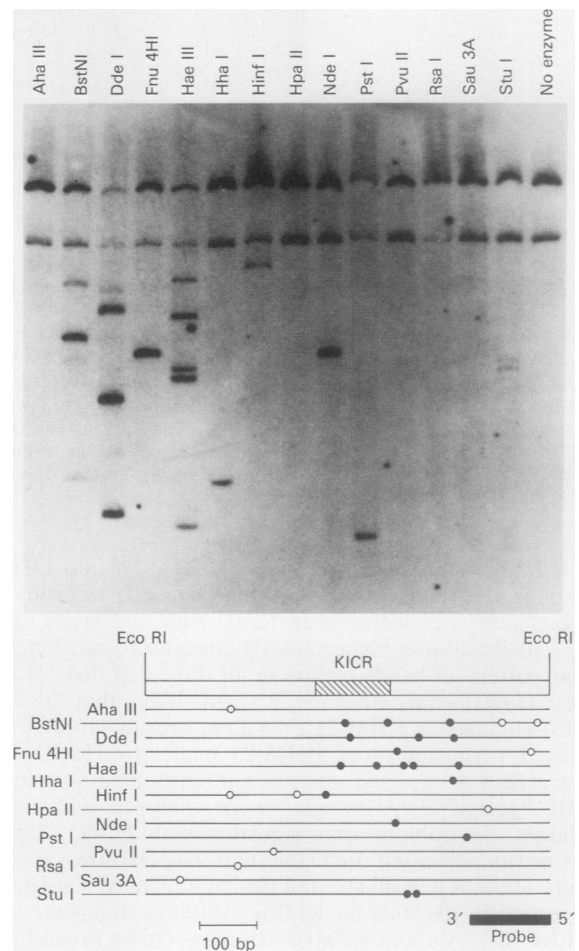


FIG. 6. Electroblot analysis of DNA isolated from nuclei digested with a panel of restriction endonucleases. Nuclei isolated from BHM-23 cells were suspended at 1-mg/ml DNA and digested with 50 U of the indicated restriction enzyme per ml for 1 h at 37°C. After the DNA was isolated, 50- $\mu$ g samples were cut to completion with *EcoRI*, electrophoresed on a 6% acrylamide-7 M urea gel, electroblotted onto Genescreen, and hybridized with a radiolabeled single-stranded probe complementary to the sense strand of the 147-bp *PstI-EcoRI* fragment (Fig. 1, left panel). The map shows restriction endonuclease sites predicted from the nucleotide sequence within the 710-bp *EcoRI* fragment. Closed circles indicate those sites which exhibit increased accessibility to enzyme digestion in the B-cell nuclei; open circles represent those sites which are relatively resistant to enzyme digestion. The resistance of the *HpaII* site to digestion in intact BHM-23 nuclei was not due to methylation of the recognition sequence since naked DNA isolated from BHM-23 is cut to completion at this site by *HpaII* (data not shown).



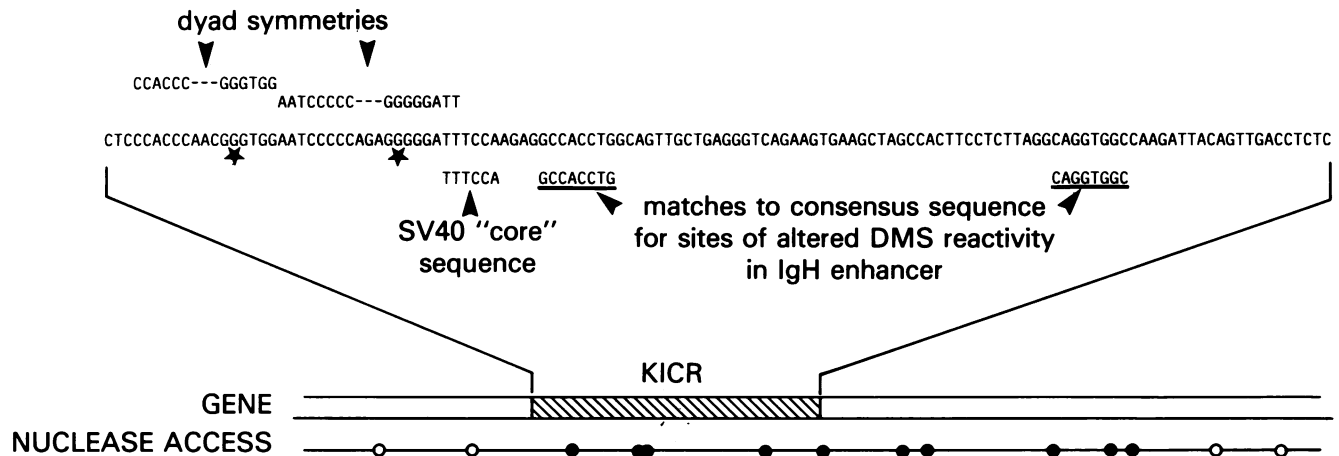


FIG. 7. Summary of footprinting data. The complete sequence of the human KICR is given. The locations of the guanine residues exhibiting B-cell-specific enhanced DMS methylation are indicated by stars. The figure indicates the relationship of the regions of dyad symmetry, the SV40 enhancer core sequence, and the immunoglobulin enhancer consensus sequence homology. The nuclease-accessible region is indicated relative to the KICR by the position of selected restriction sites that are accessible (filled circles) or inaccessible (open circles) in B-cell nuclei.

that DNA secondary structure plays a role in the creation of DNase I hypersensitivity sites. Larsen, Weintraub, and Groudine (16, 25, 55) have shown that hypersensitivity sites for both S1 nuclease and DNase I occur at the same position of the chicken  $\beta$ -globin promoter *in vivo* in cells expressing the gene. They showed that supercoiled plasmids containing the  $\beta$ -globin promoter demonstrate the same S1 nuclease-hypersensitive sites in the absence of tissue-specific *trans*-acting factors, suggesting that features of the primary DNA sequence induce an S1-accessible conformation in the presence of negative supercoiling. The topoisomerase inhibitor novobiocin has been shown to decrease DNase I hypersensitivity of the  $\beta$ -globin (53) and *Drosophila* heat shock (17) promoters *in vivo*, presumably by decreasing supercoiling.

In the context of DNA secondary structure, our observation that sites of methylation enhancement and protection occur within palindromic sequences in the human KICR raises the possibility that these sequences exist *in vivo* as cruciforms, which are known to be favored by DNA supercoiling; such secondary structure could conceivably induce the altered DMS reactivity and might have a role in the function of the KICR in gene regulation. Palindromic sequences have been found in the regulatory sequences of several genes including the  $\beta$ -globin (9) and *Drosophila* heat shock gene (32) promoters and the insulin (60) and murine  $\kappa$  immunoglobulin (36) enhancers. Although dyad symmetries have the theoretical potential of forming cruciform structures, it is doubtful whether the KICR palindromes under discussion are long enough (6 and 8 bp) for such structures to be energetically favored *in vivo*, unless facilitated by specific proteins or a very high degree of supercoiling. In the murine KICR a cruciform structure was postulated by Parslow and Granner (36) on the basis of the failure of *Sau3A* to cleave a recognition site between two elements of a dyad symmetry, a site that would lie in the single-stranded terminal loop of a cruciform. In the human system, the fact that a *Hin*I site (GAATC) spanning the two dyad symmetries (Fig. 7) is cleaved when B-cell nuclei are treated with *Hin*I (Fig. 6) suggests that the three-dimensional structure of this region is not drastically different from that of the typical double helix.

While DNA secondary structure may play a role in enhancer function and may partially explain the observed

alterations in chromatin structure, an alternative explanation involves the interaction of regulatory sequences of DNA with specific *trans*-acting factors, probably proteins. Clearly a role for such *trans* factors does not exclude important effects of secondary structure. It is known, for example, that the *E. coli* DNA G protein associates with its specific DNA-binding site only when the DNA assumes a specific secondary structure, which is thus stabilized by the protein (50). Moreover, a recently proposed model for lambda repressor action on the *lac* promoter involves bending of DNA mediated by protein binding (20).

Although evidence for the combined effects of secondary structure and protein binding is furthest advanced in prokaryotic systems, good evidence for binding of specific proteins to regulatory sequences already exists in several eucaryotic systems (glucocorticoid receptor,  $\beta$ -globin promoter, *c-myc* gene, *Drosophila* heat shock gene, insulin enhancer, dihydrofolate reductase promoter, adenovirus and SV40 enhancers) (4, 5, 9, 32, 34, 43, 44, 47, 60). For the immunoglobulin heavy-chain gene enhancer, suggestive evidence for *trans*-acting factors is available from several lines of investigation. By enhancer competition assays Mercola et al. (30) have demonstrated that the heavy-chain enhancer must interact with saturable cell components to effect transcriptional stimulation. Church and Ephrussi and co-workers (2, 11) have documented B-cell-specific alterations in DMS reactivity of the heavy-chain enhancer consistent with the binding of regulatory proteins to the sequence. Maeda et al. (27) have shown that nuclear proteins from B-lymphoid cells can stimulate the transcription of a heavy-chain gene transfected into L cells by a mechanism dependent on the presence of the enhancer in the transfected gene. The demonstration of altered DMS reactivity of the  $\kappa$  light-chain enhancer in the present investigations provides suggestive evidence that this enhancer also interacts with sequence-specific regulatory proteins. In this context, the palindromic sequences surrounding the sites of altered DMS reactivity can be viewed as binding sites for a (possibly dimeric) protein with rotational symmetry; this interpretation, rather than the potential for cruciform structure, has been favored for palindromic sequences found in other regulatory regions of DNA (9, 15, 32, 60).

that showed B-cell-specific alterations in DMS reactivity were clustered within four segments of DNA having a six of eight or seven of eight match to a consensus sequence CAGGTGGC (2, 11). The human KICR includes two eight of eight matches to this consensus sequence (one in the reverse orientation [Fig. 7]). If the alterations in DMS susceptibility in the heavy-chain enhancer region are due to binding of B-cell-specific regulatory proteins to this consensus sequence, such proteins might also regulate the  $\kappa$  gene by binding to the same sequences; therefore, it would be of interest to determine whether the occurrences of the consensus sequence in the  $\kappa$  enhancer show similar alterations of DMS reactivity in B cells. We were unable to detect any consistent alterations in DMS susceptibility at the two CAGGTGGC sites in the human KICR (Fig. 1), although it should be noted that our gels show somewhat lower resolution in these regions than the gels of Ephrussi and Church (2, 11).

Although our observations from DMS footprint analysis revealed a specific feature of chromatin structure confined to a small segment at the 5' end of the KICR, the nuclease accessibility analysis suggested a broader region of B-cell-specific chromatin alteration. We initially confirmed by conventional Southern blot analysis the earlier observations by Pospelov et al. (39) that a DNase I hypersensitivity site exists in human B-cell DNA 5' of the constant-region gene at a position that coincides with the KICR. The same samples of DNase I-digested DNA were then examined by the high-resolution electroblotting technique and revealed a region of DNase I cuts that extends, with minimal fine structure, from near the 5' end of the KICR to beyond its 3' end. The DNase I-hypersensitive region coincides with a region of increased accessibility of several restriction endonucleases with recognition sites in the KICR (Fig. 6 and 7). Similar accessibility to restriction endonucleases was observed for the murine KICR region in a lower-resolution Southern blot analysis by Parslow and Granner (36). Thus, the human and murine KICR share not only sequence homology but also similar tissue-specific features of nuclease accessibility.

Several lines of evidence support a model for the SV40 enhancer in which independent sequence elements within the enhancer may function as clustered binding sites for specific *trans*-acting elements (45, 64). In this perspective, the fact that the altered DMS reactivity in the human KICR is found in only a small part of the region of increased nuclease accessibility may reflect different properties of the *trans*-acting elements bound at different regions within the enhancer. The view that the  $\kappa$  enhancer function results from the combined action of individual sequence motifs is consistent with the findings of Queen and Stafford (41) that incremental loss of enhancer function is observed with progressive deletions over about 200 bp spanning the murine KICR. Ultimately, a full understanding of the relationship between enhancer function and the features of B-cell-specific chromatin alterations observed in the KICR will require direct analysis of the *trans*-acting elements.

#### ADDENDUM IN PROOF

The 0.7-kb *EcoRI* fragment bearing the human KICR has recently been found to activate transcription when placed in either orientation on a plasmid containing the chloramphenicol acetyl transferase gene driven by an SV40 promoter (E. E. Max, unpublished results). This finding is consistent with enhancer function of the human KICR.

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