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Inducible DNA–protein interactions of the murine kappa immunoglobulin enhancer in intact cells: comparison with *in vitro* interactions

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ABSTRACT

The large intron of the κ immunoglobulin gene contains a cis-acting enhancer element, which is important in the tissue-specific expression of the gene. We have confirmed the binding activity of a sequence-specific factor present in lymphoid extracts derived from cell lines expressing, or induced to express, the κ gene. We have extended these studies to show the binding activity is present in normal activated splenic B cells as well as λ producing cells, and have demonstrated by DNAse footprint analysis full protection of a sequence containing the 11 bp homology to the SV-40 core enhancer. We have compared these in vitro binding studies with an analysis of protein-DNA interactions in intact murine cell lines using genomic sequencing techniques. We demonstrate significant alterations in DMS reactivity of DNA in the murine 70Z/3 cell line after it is induced to κ expression. These alterations occur at guanine residues which are part of the the 11 bp core sequence, and are identical to those observed in cells constitutively expressing κ. This provides direct evidence for the induced binding of the tissue specific factor to intact chromatin. In intact chromatin we also observed significant alteration in the reactivity of a guanine, 3′ of the core sequence, which is part of a potential secondary DNA structure, and protection of four residues that are part of a region homologous to the heavy chain enhancer.

INTRODUCTION

At least two regions of the κ immunoglobulin locus are important in mediating transcription (1), one located 5′ of the promoter and another within the J-C intron (designated the κ enhancer sequence). The enhancer sequence is cis-acting, functioning independent of position or orientation, and has been implicated in inducible B-lymphoid specific expression of κ genes (1-4). The region is hypersensitive to DNAse and is partially conserved between man and mouse (5, 6). By deletion and transfection studies Picard and Schaffner initially showed the enhancer activity could be localized to 475 bp Alu I-Alu I fragment (7), while Queen and Stafford have further restricted the enhancer location to approximately 200 bp within the same Alu I fragment (8). Enhancer elements have been described for a num-

ber of eukaryotic genes and have in many cases been shown to share short regions of sequence homology.

We and others have shown the existence of multiple factors, present in nuclear extracts, which bind specific sequences in the κ enhancer (9-11). Some of these sequences have been identified which share homology between heavy and light chain genes (designated "E" boxes), and these appear to bind nuclear factors in extracts from both lymphoid and non-lymphoid cells (10, 12, 13). The binding of at least one factor, designated NF-KB, to a region within the κ enhancer has been reported which is restricted to mature B cell extracts and interacts at a sequence which shares homology with the 11 bp sequence GGGGACTTTCC found in many other enhancers, including SV-40, CMV, MSV, adenovirus E1A, and HIV (14-18). Methylation interference experiments indicate that methylation of 3 G residues on the coding strand and 3 G residues on the noncoding strand in this sequence abolish the in vitro binding of NF-KB (10). The in vitro binding of NF-KB has recently been shown to be inducible in the preB cell line, 70Z/3, which expresses κ mRNA and NF-KB binding activity only after treatment of cells with lipopolysaccharide (LPS) (11). Direct evidence for the importance of the 11 bp core sequence in κ expression has been suggested by analyses of CAT expression vectors containing mutations of the 11 bp core sequence (13).

Within the κ intron there are also regions, designated KE1, KE2, KE3, which contain a sequence with partial (KE1, KE3) or complete (KE2) homology to regions within the heavy chain enhancer (12). In vitro binding studies have shown that KE2 and KE3 bind nuclear factors, but do not show tissue specificity (13).

Although the protein-DNA interactions show sequence specificity, the evidence is based on in vitro experiments in which nuclear extracts from transformed cell culture lines, representing different cell types or stages of differentiation, are mixed with naked DNA. In order to probe alterations in intact chromatin structure a variety of Southern blot strategies have been used. The activation of κ transcription, for example, has been correlated to increased susceptibility of the enhancer sequence in intact nuclei to DNAse I and endonucleases (19). Recently, the genomic sequencing technique developed by Church and Gilbert (20) has made it possible to probe chromatin structure at single nucleotide resolution. Intact cells can

be treated with dimethylsulfate (DMS), and changes in methylation of each guanine residue compared to naked DNA can be used to identify sites of altered DNA conformation, presumably due to sites of specific protein-DNA interactions. In the present study we have examined the in vitro interaction of factors derived from mature B cells, uninduced and induced 70Z/3 cells, as well as normal B cells with the κ enhancer. We have compared these results with induced protein-DNA interactions using the genomic sequencing method to detect alterations in DMS reactivity of the κ enhancer in intact cells.

MATERIALS AND METHODS

Cells and Nuclear Extracts

The characteristics and growth of cells used in this study are as previously described (9). MPC-11 and S 194 are murine plasmacytoma cell lines expressing the κ light chain; 315J is a murine plasmacytoma expressing λ light chain; Bal-8 is a murine T cell line: HO-85 is a human large cell lymphoma expressing κ. 3-1 is an Abelson virus derived murine preB cell; 70Z/3 is a transformed preB line which has a single rearranged κ gene which is expressed after addition of 10 ug/ml LPS. Normal, quiescent B cells were prepared from mouse spleen preparations as described (21). Using this procedure greater than 90 % of the cells obtained were B cells, and less than 3% had exited the Go phase of cell cycle as determined by flow cytometry. Preparations of splenic B cells were induced to light chain expression with 50 ug/ml LPS plus 20 ug/ml dextran sulfate in RPMI 1640 with 10% FCS for 72 hours. Nuclear extracts were prepared according to the methods of Dignam, et al. (22).

DNA-protein Binding Assays and DNAse Protection

In vitro binding of factors in nuclear extracts to DNA fragments was assayed by altered electrophoretic mobility of complexes on polyacrylamide gels as previously described (9). All fragments were end-labelled with ^{32}P-ATP and polynucleotide kinase. For DNAse protection experiments, 50-100 ug of nuclear extract protein from induced 70Z/3 cells was mixed with the appropriate DNA fragment (labelled at a single end), and digested with 0.01-0.5 units/ml of RQ1 DNAse (BRL) for 90 sec. Free DNA and DNA in protein complex were separated by gel electrophoresis, and eluted from the gel by diffusion. The DNA was extracted, precipitated, redissolved in

sequencing loading buffer, heated to 90° and run on an 8% polyacrylamide gel containing 8M urea, along with an A/G ladder generated by the Maxam-Gilbert chemical cleavage reaction.

Genomic Sequencing

DMS treatment of whole cells, DNA extraction, gel electrophoresis, blotting, and hybridization were performed essentially as described (20). Genomic DNA isolated after DMS treatment was digested with Hinf 1, which generates a 512 bp fragment containing the kappa enhancer. Probes were generated for coding and non-coding strand by digestion of the 512 bp Hinf 1 fragment with Dde I and cloning the 5' Hinf 1 - Dde I fragment (126 bp) in both orientations into the Sma I site of M13. Probes were labelled with ^{32}P-ATP by primer extension.

RESULTS

In vitro binding of NF-KB

The binding of nuclear factors to specific DNA sequences can be determined in vitro by a sensitive gel electrophoresis assay which is based on the shift in mobility of labelled DNA fragments when complexed with protein. In the presence of nonspecific DNA sequences, and through competition studies, the complex formation can be shown to be sequence specific. In this study, we examined a number of nuclear extracts for binding to a 99 bp fragment within the κ enhancer. This region of DNA contains a sequence which has been previously reported to bind a factor designated NF-KB. The binding of NF-KB appears to be tissue specific, the activity only observed in B lymphoid cell lines. Moreover, the mouse preB cell line, 70Z/3, can be induced to express κ light chains with lipopolysaccharide (LPS), and this correlates with an induction in NF-KB binding by the in vitro assay (10, 11). As shown in Fig. 1, we also demonstrate the binding of NF-KB in B lymphoid cell lines, and observe a significant induction of binding factor in LPS induced 70Z/3 cells. We have extended the analysis to examine whether this same factor can be induced in normal splenic B cells. Normal, quiescent murine B cells were selected as described in Methods, and had levels of κ mRNA which were undetectable by northern blot analysis. Extracts from these cells did not possess significant amounts of NF-KB. The splenic B cell preparation was activated with LPS and dextran sulfate as described in Methods, and was found by northern analysis to express κ

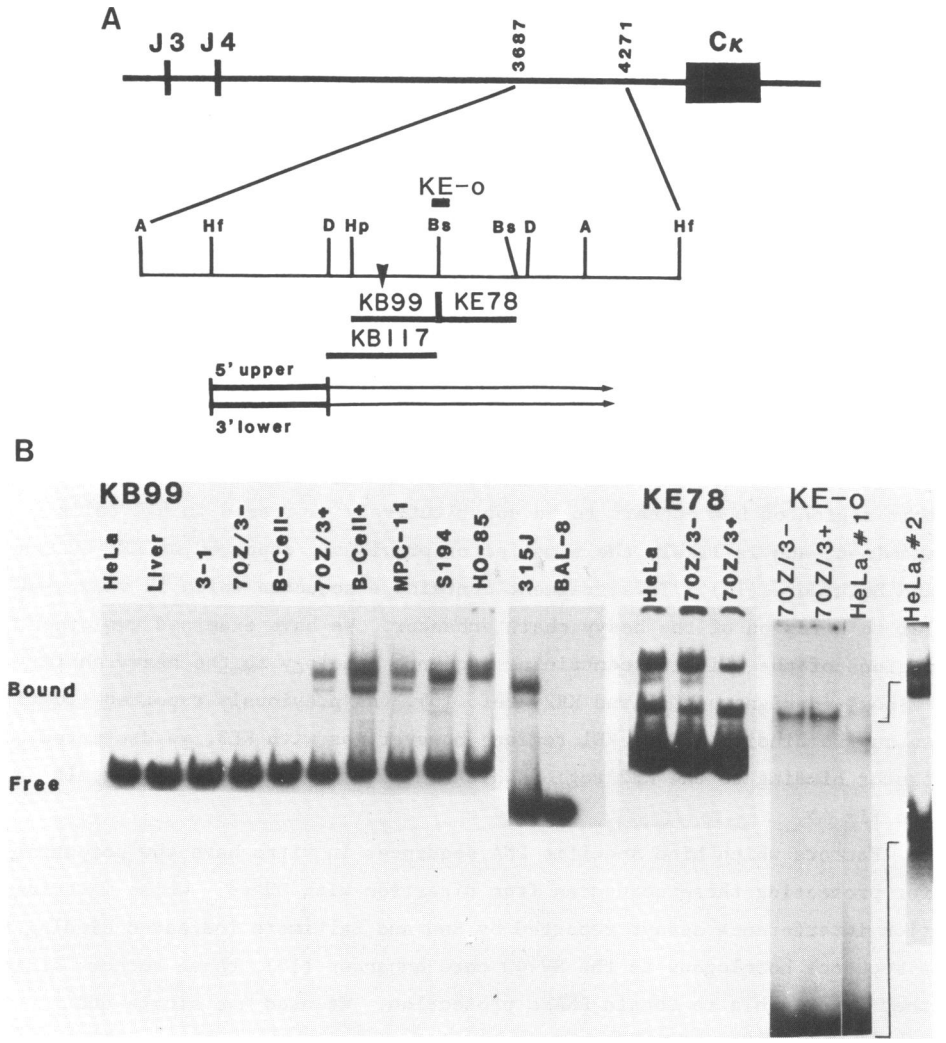

Figure 1. A. Schematic diagram of the κ immunoglobulin intron. Numbering is from a previous sequence report (23). The expanded restriction map contains the enhancer region. DNA fragments used for mobility shift experiments, DNAse protections, and genomic sequencing are indicated and described in Methods. The solid triangle indicates the region of tissue specific nuclear factor (NF-KB) binding. A, Alu I; Hf, Hinf I; D, Dde I; Hp, Hph I; Bs, BstNl. B. Sequence-specific binding of nuclear factors assayed by mobility-shift (see Methods). 70Z/3 cells and normal splenic B cells were either uninduced (-), or induced (+) prior to preparation of nuclear extract. The KB99 fragment contains the SV40 core enhancer sequence; the KE78 fragment contains the "E-box" homology previously designated KE3 (10); the KE-o fragment represents a synthetic duplex oligo containing the "E-box" homology previously designated KE2 (10).

mRNA (not shown). Extracts from these cells are shown to contain significant NF-KB binding activity (Fig. 1). This result demonstrates that NF-KB binding activity is present in normal cells, and eliminates any possibility that its presence is an artifact of transformed cells. Further, we have observed that the binding activity to the κ enhancer is not limited to κ expressing cells. The cell line 315J does not express κ, but does express lambda light chains. NF-KB binding activity is present in these cells (Fig. 1). NF-KB binding activity is, however, apparently limited to the B lineage of lymphoid cells, as the T cell lymphoma line, Bal 8, has no detectable NF-KB binding activity.

In contrast to the inducible binding seen in lymphoid extracts to the KB99 fragment, we see evidence for factor binding to the KE78 fragment which is not tissue specific (Fig. 1B). Binding of a nuclear factor to this region of DNA appears to be constitutively expressed in all cells examined, and is likely the same factor previously reported as KE3 by Sen and Baltimore (10). This fragment contains a sequence which is very similar to a region of the heavy chain enhancer. We have examined two other regions of the κ intron containing sequence homology to the heavy chain "E-box" (designated KE1 and KE2, ref. 10). As previously reported (10,13), we saw no binding to the KE1 region; however, as with KE3, we dectected factor binding to the KE2 region which is not tissue specific (Fig. 1B and see Fig.4).

Factors which bind specific DNA sequences in vitro have the potential for protecting these sequences from digestion with DNAse. While methylation interference assays reported by Sen and Baltimore indicated binding to a sequence homologous to the SV-40 core enhancer (10), these authors state they were unable to obtain DNAse protection. We used two single-end labelled fragments for DNAse footprinting studies. These two complementary fragments contain the SV-40 enhancer homology and two inverted repeat sequences adjacent to the core enhancer sequence. Extracts from induced 70Z/3 cells were incubated with each of the two labelled DNA fragments and complexes were treated with various concentrations of DNAse and run on a polyacrylamide gel. Labelled DNA fragments which ran as a complex were eluted and run on a denaturing sequencing gel, along with DNAse-treated DNA which was uncomplexed and a Maxam-Gilbert A/G ladder of the same fragment. Figures 2A and B show significant protection of the DNA from DNAse diges-

tion at the sequence 5'-CTCGGAAAGTCCC-3' (non-coding strand) and 5'-GGGGACTTTCCGA-3' (coding strand). The 11 bp homology to the SV-40 core sequence is fully protected in our assay. Figure 2C shows the sequence 5'-CACATGG-3' (non-coding) is also protected from DNAse digestion. This sequence is homologous to the E-boxes (KE3) found in the heavy chain enhancer (12). Because of relatively limited in vitro binding of KE2, we were unable to obtain a DNAse footprint of this region.

Evidence for inducible DNA-protein interactions in intact cells

While there is significant evidence for specific protein-DNA interactions at the κ enhancer, it has all derived from in vitro assays using extracts and naked DNA. It is important to confirm that within the context of intact chromatin the same interactions can take place, and they correlate with the induction of κ expression. By treating whole cells with DMS and examining the methylation of specific G residues it is possible to observe alterations in reactivity due to changes in protein-DNA interactions in intact cells. Single-stranded probes were generated as described in Methods (see Fig. 1) and used for genomic sequencing across both the coding and non-coding strand of the κ enhancer. In every case the genomic sequencing was repeated a minimum of three times on independently treated and isolated DNA. The results presented were consistently reproducible. As shown in Fig. 3A, there is evidence for protein interaction within the 11 bp core sequence in the DNA obtained from cells expressing κ. We were able to make a direct comparison of the reactivity of DNA from intact 70Z/3 cells before and after LPS induction of κ expression (see Fig. 3A and B). The DNA examined from induced 70Z/3 cells shows that the cleavage of G residues at positions 3938 and 3939 are significantly enhanced, while it appears the G residue at position 3940 may be protected. These reactivities were also observed in DNA from S194 and MPC-11 cells which constitutively express κ, indicating that the protein-DNA interactions induced in 70Z/3 cells are maintained in fully differentiated cells which express κ. These results are in contrast to those seen in naked DNA, DNA from MEL cells, or uninduced 70Z/3 cells (Fig. 3A).

Protection of four residues from the DMS was consistently observed (in three independent experiments) at positions 4008, 4009, 4011, and 4012 in the intact chromatin of induced 70Z/3 cells compared to uninduced cells (two gels shown in Fig. 3A and B). These guanine residues are within the

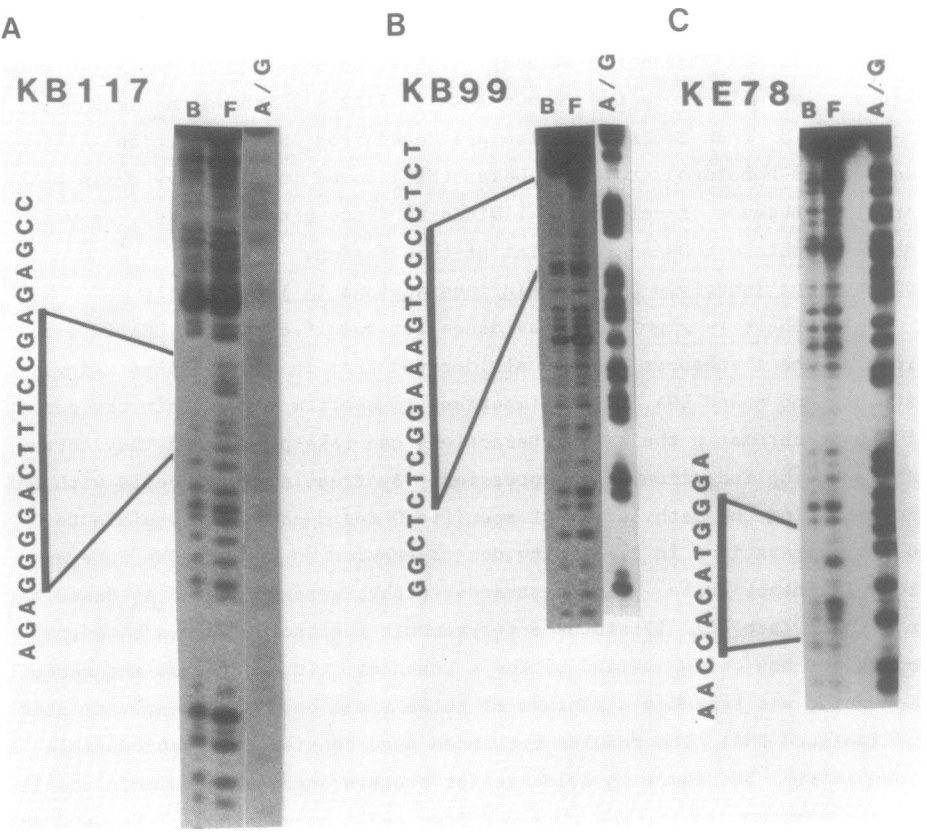

Figure 2. DNAse protection of DNA-protein complexes. DNA fragments (shown in Fig. 1) were labelled at a single end followed by DNAse digestion of protein-DNA complexes and free DNA, as described in Methods. Both free and bound DNA were then run on a 10% denaturing sequencing gel along with an A/G ladder of the same fragment generated by Maxam-Gilbert chemical cleavage reaction. The sequence protected by DNAse digestion is indicated.

KE2 region, containing the sequence homologous to a portion of the heavy chain enhancer (12). We did not observe any significant alterations in the DMS reactivity of guanine residues within the KE3 region of the DNA. Thus, while the in vitro data indicated all cells contain factors which bind KE2 and KE3, in intact chromatin alterations in DMS reactivity were only detected in the KE2 region in cells induced to express kappa.

Another significant alteration in reactivity to DMS was observed at position 3976 on the non-coding strand (Fig. 3C). Enhanced cleavage was consistently seen at this position in DNA treated with DMS in all intact

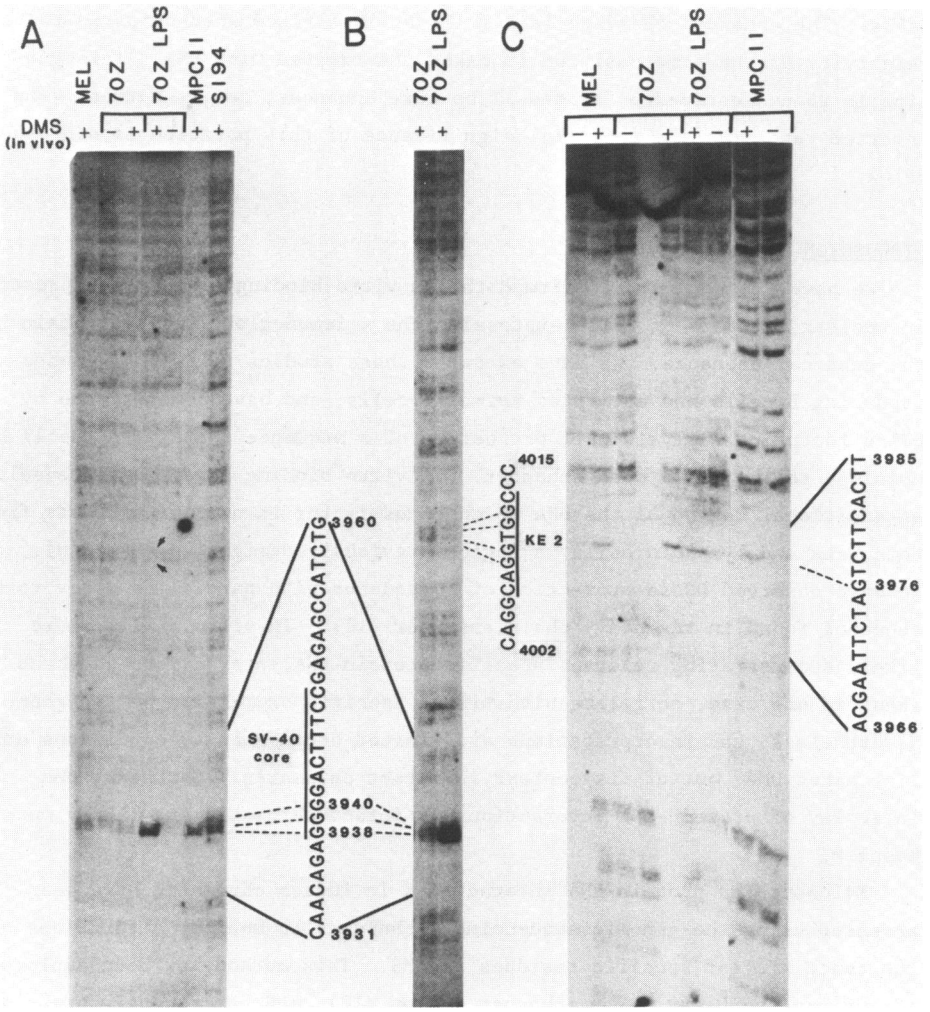

Figure 3. Genomic guanine sequence ladder across the κ enhancer. DNA was treated with DMS in intact cells (+) or after deproteinization (-), restricted with Hinf I, and subjected to electrophoresis in a 6% sequencing gel (20). The Hinf I - Dde I fragment shown in Fig. 1 was cloned in both orientations into an M13 vector and radiolabelled primer extended DNA was used as a 5'-upper (A,B) or 3'-lower (C) probe. (A) The sequence of the region (coding strand) is shown; the SV40 core sequence homology is indicated. Altered reactivity of the G residues was observed only in cells expressing κ (note in particular altered reactivity as a result of LPS induction of κ expression in 70Z/3 cells). (B) A repeat of the 70Z/3 induction, showing alterations in DMS reactivity, with better resolution of the four G residues. (C) The sequence shown is of the non-coding strand. The enhanced reactivity of the G residue at position 3976 was observed only in DNA treated with DMS in vivo. Numbering according to Max, et al. (16).

cells. The enhanced cleavage is significantly altered when compared to the reactivity of the same position in naked DNA treated with DMS. This guanine is 28 nucleotides 3' of the 11 bp core enhancer, and is between 7 bp inverted repeats. The potential significance of this position is discussed below.

DISCUSSION

We have repeated and confirmed the in vitro binding of a factor present in nuclear extracts of cells expressing the κ immunoglobulin light chain to the enhancer sequence. We have extended these studies to include lambda-producing B cells and activated normal B cells, and have demonstrated by DNAse footprint analysis full protection of a sequence containing the 11 bp homology to the SV-40 core enhancer. In vitro binding assays have revealed an additional region of the DNA which binds factor in nuclear extracts from both HeLa and lymphoid cells, a region previously designated KE-3 (10). We have observed DNAse protection of a sequence with partial homology to a sequence found in the heavy chain enhancer (12). In vitro analyses are likely demonstrating relevant specific protein-DNA interactions which at least in one case, correlate with tissue specific expression of the gene. Nevertheless, the interpretations are limited because assay conditions utilize naked DNA, out of its context in intact chromatin. Certainly the interplay of protein-DNA interactions in intact chromatin are likely more complex.

Evidence for protein-DNA interactions in intact chromatin have been assessed using the genomic sequencing techniques to monitor alterations of the reactivity of specific residues to DMS. This method has been employed to analyze the heavy chain enhancer region (12), and has recently been applied to a region of the human κ intron (24). In the latter study enhanced cleavage of one guanine residue was reported in a region which is conserved, though not identical, between man and mouse. In the present study we have demonstrated alterations in DMS reactivity of DNA in a cell line after it is induced to κ expression. These alterations occur at guanine residues which are part of the 11 bp SV-40 core enhancer homology, and are identical to those observed in cells constitutively expressing the κ gene. This provides direct evidence for the binding of a factor to intact chromatin, likely NF-KB, which correlates with induced

expression. It is interesting to note that the interaction is maintained in mature B cells, although a recent study by Atchison and Perry (25) suggests the enhancer binding activity may not be required to maintain expression.

In vitro assays, aimed at detecting protein binding to naked DNA, clearly demonstrate that nuclear extracts from all the cells we examined contain factors which bind the KE2 and KE3 regions. Despite significant in vitro binding of factor to the KE3 region, we did not detect any alterations in DMS reactivity within this region in intact chromatin. Either this interaction does not take place within the κ intron in intact cells, or our method of analysis was not sensitive enough to detect it. We did, however, see a consistent protection of four guanine residues in the KE2 region of induced 70Z/3 cells, but not in any nonexpressing cell types. Our results suggest caution in interpreting in vitro binding data. The mere presence of factors which bind specific sequences may not be sufficient for them to interact in vivo. The accessibility of regulatory regions to potentially ubiqutous factors is likely an important consideration in specific gene expression. Similar differences between in vitro and in vivo interactions have been recently reported for other genes (26). It may be significant that KE2 is the only "E-region" which contains a sequence completely homologous to the heavy chain E-Box consensus. Alterations in DMS reactivity of the heavy chain E-regions in intact chromatin have recently been observed (12). The exact significance of this region is not known; however, mutational analyses have indicated this region may be important for enhancer activity (13). The sequence of the κ enhancer, with residues implicated in factor binding by in vitro analyses and our current study of intact chromatin, is presented in Fig.4A.

It has been demonstrated that HeLa cells contain a constitutive nuclear factor which binds to the 11 bp SV-40 enhancer core sequence, and that NF-KB binds to the homologous sequence present in the context of the SV-40 DNA (10, 15). The question obviously raised by these reports is what allows discrimination of the same 11 bp core sequence by NF-KB in lymphoid cells at different stages of differentiation? Tissue-specific, posttranslational modifications have been proposed to explain the induction of NF-KB binding to the κ enhancer (11). Whether NF-KB is directly modified has not yet been reported. In addition, recent reports suggest phorbol

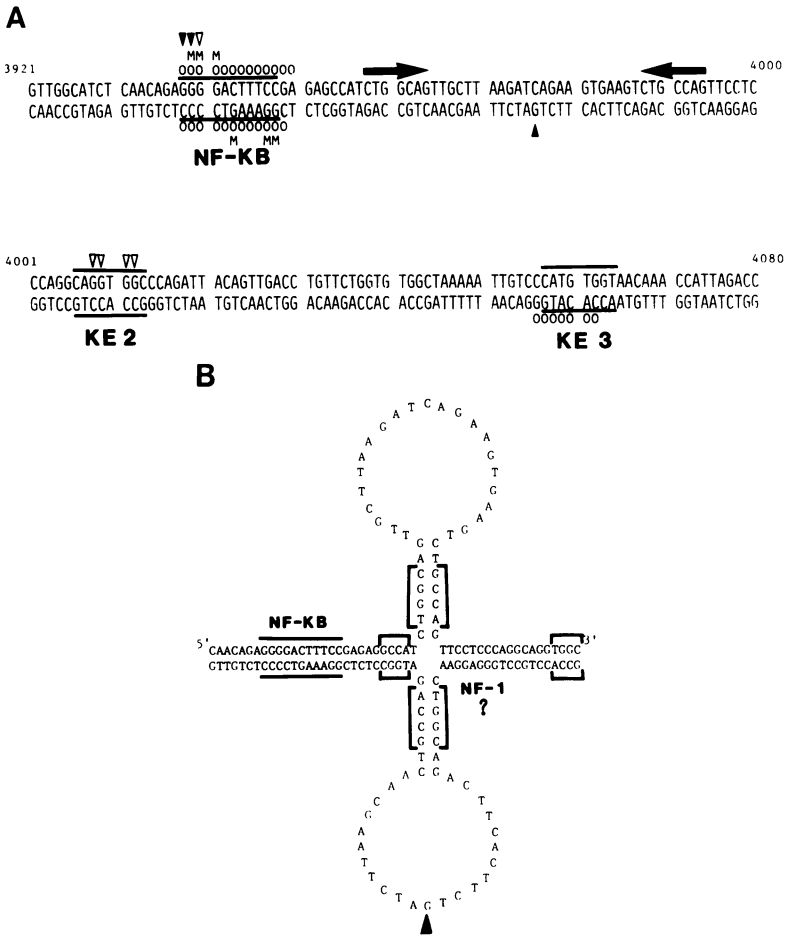

Figure 4. (A) Sequence of the κ enhancer region showing comparison of results of vitro and in vivo binding studies. Residues protected from DNAse digestion in protein-DNA complexes are designated with circles. Residues which when methylated interfere with complex formation are indicated with an 'M' (from ref. 10). Residues which show altered DMS reactivity in vivo in cells expressing κ are indicated with solid triangles (enhanced cleavage) or open triangles (reduced cleavage). Arrows indicate inverted repeat sequence adjacent to the NF-KB binding site. (B) Potential secondary structure adjacent to NF-KB binding site (from ref.19). Boxed sequences represent symmetrical sequences which have been shown in other genes to bind the nuclear factor NF-1 (27). Potential loop could bring these sequences into closer proximity. Guanine residue which shows enhanced DMS reactivity in vivo is shown (solid triangle) to be within one potential loop.

ester treatment of HeLa cells can induce NF-KB binding activity, detected by in vitro assays (11). These results, together with the fact that the 11 bp core sequence is found in a number of different enhancers (14-18), suggests there may be tissue-specific modulations of common transcription enhancing factors. One possibility is that the context of the core sequence is functionally important. We and others have found multiple factors which interact with specific sequences within the κ intron, both 3' (10) and 5' (R.H. and A.M., unpublished) of the NF-KB binding site. Within the intron are multiple inverted repeats with potential for altering DNA structure. Parslow and Granner suggested that the inverted repeats adjacent to the 11 bp core sequence could potentially form a stem loop structure (19). In this context it is interesting to note that the guanine at position 3976, which shows increased DMS reactivity in intact chromatin, lies in the middle of such a proposed loop (see Fig. 4B). We do note, however, that the increased reactivity of this residue appeared in all cell types we examined; thus, it does not show tissue specificity. Nevertheless, the increased reactivity is apparently dependent on intact chromatin structure.

The potential for altering DNA structure in the enhancer may be important for specific factor recognition. Interestingly, the inverted repeats contain the consensus NF-1 binding sites TGGC/A...GCCA (27). If a stem structure could form, it would bring the consensus sequences into closer proximity (Fig. 4B). A recent report indicates that NF-1 binds within the intron of the human heavy chain mu locus at the TGGA...GCCA consensus sequence (27). The NF-1 binding site is also commonly found in regions of DNAse hypersensitivity; the symmetry noted here in the κ locus is within a DNAse hypersenitive site as well (5). While NF-1 has been implicated in viral replication it has been suggested that it plays a role in mediating transcription, possibly by interacting with specific regulatory factors at adjacent sites, or by establishing altered chromatin structure to maintain an "open" region for factor binding (27). This speculation is obviously premature, until a specific factor can be identified to bind this region of the κ locus; but describes a common theme by which the activity of specific trans factors may require multiple protein-DNA interactions which function as a network to mediate transcription.

The interaction of multiple factors, at multiple sites within the κ

intron may be important to mediate transcriptional controls. However, we have observed that a relatively short fragment of DNA (ie. a 32 bp synthetic duplex oligodeoxyribonucleotide), containing the NF-KB binding site, can still demonstrate the differential binding of factor from induced versus uninduced cells (data not shown). This would strongly suggest that binding of NF-KB per se is not dependent on additional protein-DNA interactions. Yet, in a careful deletional analysis Queen and Stafford showed very little transcriptional activity of κ after transfecting with vectors containing NF-KB binding sequence, but lacking flanking sequences (8). Certainly alterations of intact chromatin structure by multiple factors may play an important role in modulating enhancer activity.

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