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Multiple Nuclear Factors Interact with the Immunoglobulin Enhancer Sequences

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Summary

To characterize proteins that bind to the immunoglobulin (Ig) heavy chain and the κ light chain enhancers, an electrophoretic mobility shift assay with end-labeled DNA fragments was used. Three binding proteins have been found. One is NF-A, a factor found in all tested cell types that binds to the octamer sequence found upstream of all Ig variable region gene segments and to the same octamer in the heavy chain enhancer. The second, also ubiquitous, protein binds to a sequence in both the heavy chain and the κ enhancers that was previously shown to be protected from methylation in vivo. Other closely related sites do not compete for this binding, implying a restriction enzyme–like binding specificity. The third protein binds to a sequence in the κ enhancer (and to an identical sequence in the SV40 enhancer) and is restricted in its occurrence to B cells.

Introduction

Immunoglobulin (Ig) gene expression is governed by three types of tissue-specific regulatory sequences (Grosschedl and Baltimore, 1985)—the promoter (Falkner and Zachau, 1984; Bergman et al., 1984; Mason et al., 1985; Gopal et al., 1985; Foster et al., 1985; Picard and Schaffner, 1985), the enhancer (Gillies et al., 1983; Banerji et al., 1983; Neuberger, 1983; Mercola et al., 1983; Queen and Baltimore, 1983; Queen and Stafford, 1984; Picard and Schaffner, 1984), and, at least in the case of the μ heavy chain gene, by an intragenic sequence as well (Grosschedl and Baltimore, 1985). Within the upstream promoter region, sequence comparisons (Parslow et al., 1984) followed by deletion analysis have indicated the importance of an octameric motif (ATTTGCAT), located at a characteristic distance upstream of all sequenced variable region genes (Falkner and Zachau, 1984; Bergman et al., 1984; Mason et al., 1985). We have recently reported the detection of a nuclear factor that interacts with this sequence (Singh et al., 1986). Enhancers were defined in viruses as regulatory sequences that can potentiate transcription from a variety of promoters in a distance- and orientation-independent manner, but the Ig enhancers were the first found to be tissue-specific as well. It has been assumed that the action of these *cis* regulatory elements must be mediated by *trans*-acting factors, and there is now some experimental evidence to support this conjecture. Schöler and Gruss (1984) and Mercola et al. (1985) have carried out in vivo competition experiments which imply that the presumed factor can be functionally titrated by cotransfection of increasing amounts of enhancer sequences. Also, Ephrussi et al. (1985) and Church et al. (1985) have obtained footprints of a putative tissue-specific factor on the heavy chain enhancer in living cells and nuclei. Recently, a substantial advance has also been made by the development of enhancer-dependent in vitro transcription systems (Sassone-Corsi et al., 1984; Wildeman et al., 1984; Sergeant et al., 1984; Schöler and Gruss, 1985). Competition experiments carried out in vitro (Schöler and Gruss, 1985; Sassone-Corsi et al., 1985) have further indicated that enhancer function may be mediated by *trans*-acting factors. To understand the mechanisms of enhancer function and their role in the activation of tissue-specific genes, we have searched for the presence of such factors in nuclear extracts.

We report here interaction of factors with Ig μ and κ enhancer sequences as detected by an electrophoretic mobility shift assay (Fried and Crothers, 1981; Garner and Revzin, 1981). This technique, based on the fact that nucleoprotein complexes are resolved from uncomplexed DNA by electrophoresis through polyacrylamide gels in low ionic strength buffers, has been elegantly used for the kinetic and equilibrium analysis of a number of prokaryotic DNA binding proteins (Hendrickson and Schlief, 1984, 1985; Fried and Crothers, 1984a, 1984b; Bushman et al., 1985). More recently it has been used to detect and purify eukaryotic proteins that were believed to recognize specific DNA sequences (Strauss and Varshavsky, 1984; Piette et al., 1985; Carthew et al., 1985; Levinger, 1985; Singh et al., 1986). Because a functional enhancer may consist of multiple protein-binding DNA segments, we have dissected the enhancer into small fragments retaining only one or two binding sites for sequence-specific proteins. In this way, at least three different Ig enhancer–binding proteins have been identified.

Results

The fully functional μ enhancer is included in a 700 bp XbaI–EcoRI fragment from the intron between J_H and Cμ. This fragment can be further subdivided into a 400 bp XbaI–PstI fragment (μ400) and a 300 bp PvuII–EcoRI fragment (μ300). Transient transfection assays have shown that 30%–50% of the tissue-specific enhancer activity is retained in μ300, whereas there is no detectable activity in μ400 (Grosschedl and Baltimore, 1985). We have used an electrophoretic mobility shift assay to investigate protein factors that interact with the μ enhancer. In outline, the assay involves incubating end-labeled, specific DNA fragments plus unlabeled, nonspecific DNA for 30 min at room temperature with nuclear extracts made from tissue culture cells (Dignam et al., 1983). Protein–DNA complexes are then separated from free DNA by electrophoresis through a low ionic strength polyacrylamide gel and

visualized by autoradiography. When the functional 300 bp enhancer fragment (μ300) was used in such an assay, a DNA–protein complex migrating more slowly than free DNA was observed with extracts derived from the human B lymphoma cell line EW (Figure 1B, lanes 1 and 2; the complex is indicated by the arrow). To show that this new band represented a specific complex, we carried out binding reactions in the presence of varying amounts of nonradioactive competitor fragments (Figure 1B, lanes 3–11). When μ300 was added as the competitor fragment (Figure 1B, lanes 3–5), the complex band was completely lost with 200 ng of competitor (lane 5), whereas the adjacent μ400 fragment (lanes 6–8) or the 450 bp fragment containing the κ light chain enhancer (lanes 9–11) yielded, at most, minor competition even at the highest concentrations used. The slight increase of the specific complex caused by the κ enhancer fragment (Figure 1B, compare lanes 9 and 2) could be due to its binding of factors common to both enhancers (described below), thus leaving more of the labeled fragment available to bind to a μ-specific factor.

Localization of Heavy Chain Enhancer Binding

To define the complex detected with μ300 more precisely, we further dissected this fragment by digestion with AluI, HinfI, and DdeI, generating a number of 50–70 bp fragments called μ50, $(\mu 60)_2$ (a mixture of μ60-1 and μ60-2), and μ70 (Figure 2A). Binding reactions were carried out with each of these fragments using EW nuclear extracts in the presence of increasing amounts of the nonspecific competitor poly d(IC) (Figure 2B). Fragment μ50 formed a major complex band (Figure 2B, lanes 2–4) that was barely decreased even in the presence of 3.6 μg of poly d(IC) (lane 4). The mixture of the two 60 bp fragments did not yield a discrete complex band (Figure 2B, lanes 6–8). Finally the μ70 fragment gave three faint, but discrete, nucleoprotein complex bands (Figure 2B, lane 10); the lower one was again barely affected by 3.6 μg of nonspecific carrier poly d(IC) (lane 12). The complex generated with μ50 was specifically competed away by inclusion of 50 ng of μ300 (of which μ50 is a part) (Figure 2C, compare lanes 3 and 2) or a κ promoter fragment (lane 7) in the binding reaction but not by inclusion of corresponding amounts of μ400 (lane 4), of the SV40 enhancer (lane 5), or of a fragment containing the κ enhancer (lane 6). This result implies that the μ50 complex is generated by interaction of the DNA with a previously described factor, NF-A (previously referred to as IgNF-A; Singh et al., 1986), that recognizes a conserved octanucleotide, ATTTGCAT, found both in the promoters of all sequenced immunoglobulin genes and within this subfragment of the heavy chain enhancer. We shall refer to this motif as the O sequence.

The complex observed with μ70 was specifically competed away by only the μ300 fragment (Figure 2D, compare lanes 3 and 2) and to some extent by the κ enhancer (data not shown), but was not at all competed away by either the Moloney murine leukemia virus enhancer (data not shown), the SV40 enhancer (data not shown), or the μ400 fragment (compare lanes 4 and 2). Furthermore,

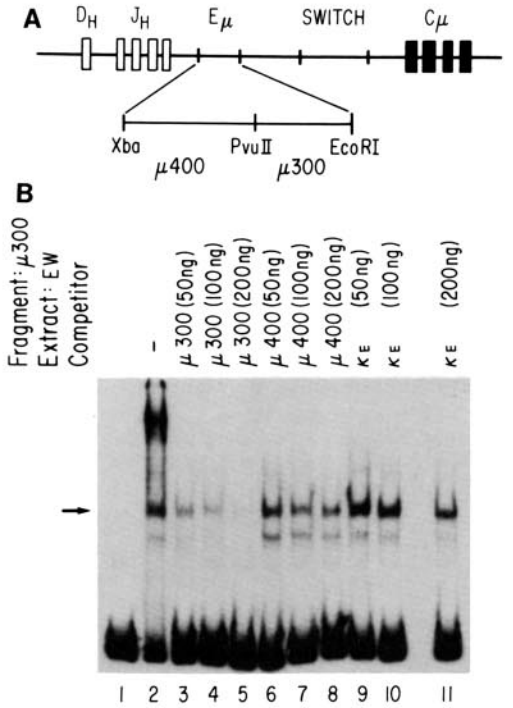


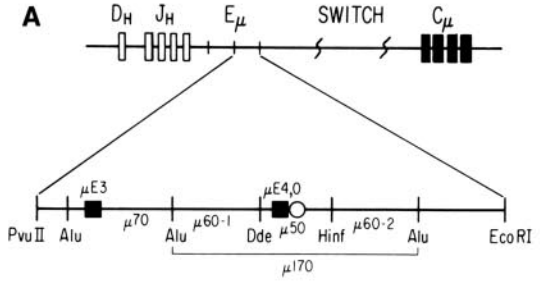
Figure 1. Factor Binding to the μ Enhancer

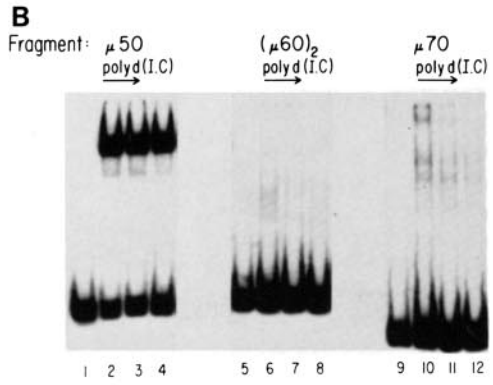
(A) Schematic representation of the germ line immunoglobulin heavy chain locus. The μ enhancer (E_μ) has been localized to a 700 bp XbaI–EcoRI fragment from within the J_H–C_μ intron (Grosschedl and Baltimore, 1985). When further dissected by cutting at the PvuII site, 30%–50% of the enhancer function is recovered in the 300 bp PvuII–EcoRI fragment (μ300), whereas the XbaI–PvuII fragment (μ400) does not carry any detectable enhancer function by transient transfection assay (Grosschedl and Baltimore, 1985).

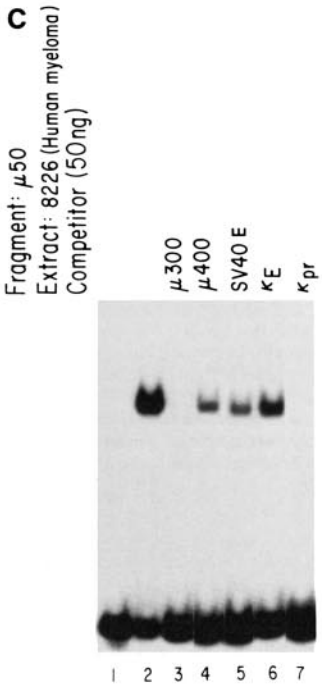
(B) Electrophoretic mobility shift assay of μ300. End-labeled μ300 (10,000 cpm, 0.5 ng) was incubated with 8 μg of a nuclear extract derived from the human B lymphoma EW in the presence of 3.6 μg of poly d(IC) and various amounts of nonradioactive competitor DNA fragments as noted, followed by electrophoresis through a low ionic strength polyacrylamide gel. The specific nucleoprotein complex is indicated by the arrow. Lane 1, free DNA fragment. Lane 2, binding reaction in the absence of competitor DNA. Lanes 3, 4, 5 are 50, 100, and 200 ng of μ300 fragment included during binding. Lanes 6, 7, 8 are 50, 100, and 200 ng of μ400 fragments included during binding. Lanes 9, 10, 11 are 50, 100, and 200 ng of a κ enhancer fragment (κE) included during binding. The κ enhancer fragment was excised from a plasmid that contained the AluI–AluI segment of the J_κ–C_κ intron described by Picard and Schaffner (1984) cloned into the SmaI site of pUC 13 and was a kind gift of N. E. Speck.

competition experiments with subfragments from within μ300 showed that this complex could not be competed away by either μ50 (Figure 2D, lanes 5 and 6), $(\mu 60)_2$ (lanes 7 and 8), or μ170 (lanes 11 and 12), but only by itself (lanes 9 and 10). Thus the dissection of μ300 revealed two distinct and specific regions of binding, one in μ50 (apparently the O sequence) and one in μ70.

Ephrussi et al. (1985) and Church et al. (1985) have used methylation protection experiments to define a set of G residues within the heavy chain enhancer that are specifically resistant to methylation by dimethyl sulfate (DMS) in B cells or B cell nuclei. This result led to the proposal that







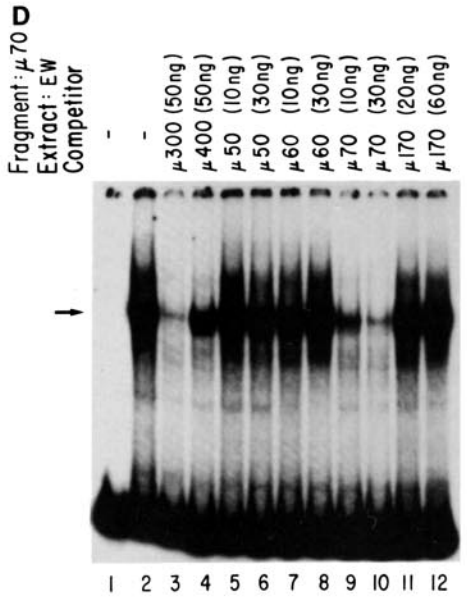


Figure 2. Dissection of the μ300 Fragment to Localize Factor-Binding Sites

(A) Schematic representation. For further dissection, the μ300 segment was cleaved with AluI, DdeI, and HinfI to generate the fragments named μ50, μ70, and μ60 (there are two fragments of approximately 60 bp obtained by restricting the large AluI–AluI piece [μ170] with DdeI and HinfI). The black boxes represent regions of the μ enhancer found to be resistant to methylation by DMS in vivo. Four homologous domains have been identified earlier (Ephrussi et al., 1985), which we refer to as μE1–μE4. Of these, only two, μE3 and μE4, are present in the fragments being analyzed and are located in μ70 and μ50, respectively. The open circle (O) refers to the octamer sequence (ATTTGCAT), which has been found in the promoters of all sequenced V_H and V_L genes and is also found in the μ enhancer. The central G residue in this sequence was also found to be resistant to methylation in whole cells (Ephrussi et al., 1985).

(B) Binding analysis of subfragments of μ300. In each set of four lanes, the first shows the free fragment and the next three shows the effect of carrying out a binding analysis in the presence of increasing amounts (1.2, 2.4, and 3.6 μg, respectively) of the nonspecific competitor poly d(IC) and a constant amount (8 μg) of protein derived from the EW cell line. $(\mu 60)_2$ represents a mixture of the two 60 bp fragments. Labeled fragments are as follows: lanes 1–4, μ50; lanes 5–8, $(\mu 60)_2$; lanes 9–12, μ70.

(C) Competition experiments to show that the complex formation on μ50 is sequence-specific. Binding reactions were carried out in a final 15 μl volume with 8 μg of a nuclear extract derived from the human myeloma cell line 8226 with 0.1–0.3 ng of end-labeled probe (~12,000 cpm) and 2.5 μg poly d(IC). Lane 1, free fragment; lane 2, no competitor DNA added; lanes 3–7, binding reactions in the presence of 50 ng of different competitor fragments (as noted above the lanes) added prior to addition of the nuclear extract. κpr is a 300 bp SfaNI–SfaNI fragment derived from the promoter region of the MOPC 41 κ gene. It extends from position −330 to position −30 relative to the cap site (Queen and Baltimore, 1983). The conserved octanucleotide sequence (ATTTGCAT) is located between base pairs −59 and −66. SV40E is a 170 bp fragment containing both 72 bp repeats of SV40 from the HpaII site on the late side. κE is a fragment containing the κ enhancer (see Figure 1B for details).

(D) Competition experiments showing that complex formation on μ70 is sequence-specific. Lane 1, free fragment (0.2–0.3 ng, 10,000 cpm); lane 2, binding reaction in a final volume of 15 μl in the presence of 1.5 μg of poly d(IC) and 12 μg of nuclear extract derived from the cell line EW; lanes 3–12, binding reactions as described for lane 2, but also containing unlabeled competitor DNA derived from the μ enhancer in the amounts shown above each lane.

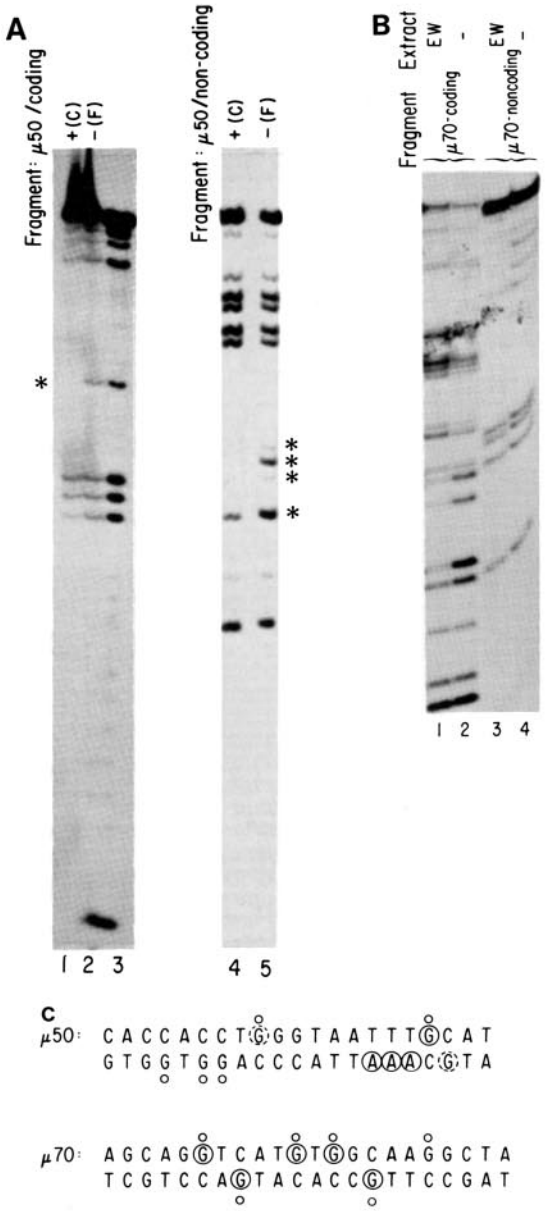


Figure 3. Methylation Interference Experiments to Define the Binding Sites of the Proteins That Interact with μ50 and μ70

The asterisks indicate the location of G residues whose methylation by DMS specifically inhibits the binding of a factor to its cognate sequence.

(A) The μ50 fragment was end-labeled at the DdeI site (0.2–0.3 ng/10,000 cpm) on the coding strand. A typical preparative reaction was done with 80,000–100,000 cpm in an EW nuclear extract. Lane 1, nucleoprotein complex band (C) analyzed after elution from a preparative, low ionic strength polyacrylamide gel and piperidine treatment. Lane 2, free fragment band (F) from the same binding reaction. Lane 3, G ladder generated from end-labeled μ50, not exposed to any proteins. Analysis of the noncoding strand after labeling at the HinfI site. Lane 4, nucleoprotein complex band (C). Lane 5, free fragment band (F) from the same binding reaction.

(B) The AluI–AluI μ70 fragment was subcloned into the SmaI site of pUC 13 (pμ70). The plasmid was restricted with BamHI and end-labeled for coding strand analysis or restricted with EcoRI and end-labeled for noncoding strand analysis. Binding reactions were carried out in EW nuclear extracts. Lanes 1 and 2, analysis of the G residues on the coding strands of the complex band (lane 1) and the free fragment band (lane 2) obtained after a preparative binding and low ionic strength gel electrophoresis. Lanes 3 and 4, analysis of the G residues on the noncoding strand in the nucleoprotein complex band (lane 3) and the free fragment band (lane 4) following preparative binding and low ionic strength gel electrophoresis.

(C) Summary of the methylation interference (in vitro) and the methylation protection (in vivo) experiments used to define protein binding sites within the μ enhancer: the relevant regions of the μ50 and μ70 fragments are shown with the coding strand on top in the orientation in which they appear in the μ enhancer. The circles above the letters show the G residues that were found to be protected against methylation by DMS in vivo (Ephrussi et al., 1985). The encircled Gs are the ones whose methylation interferes with protein–DNA interaction in vitro (dotted circle indicates partial interference).

tissue-specific DNA binding proteins were responsible for this decreased accessibility of the reagent to DNA. The protections fell into four clusters, the DNA sequences of which were sufficiently homologous to derive a consensus sequence for the binding of a putative factor (Church et al., 1985). All four binding sites (μE1–μE4) fall within the 700 bp XbaI–EcoRI fragment; however, μ300 retains only two complete binding domains (μE3 and μE4) along with the O sequence. The μ70 fragment contains the complete μE3 domain and the factor binding in vitro could be the same as that detected in vivo. Therefore it was unexpected that the μ50 fragment containing μE4 (and O) (Figure 2A) should not compete for binding to μ70 (Figure 2D, lanes 5 and 6). In case this was due to the competitor fragment predominantly binding NF-A at the octamer site and thus making it unavailable as a competitor for μ70, we carried out binding reactions and competitions using a partially purified binding protein, generated by chromatography of the crude extract on heparin-Sepharose, that contained μ70 binding activity and was significantly depleted of NF-A. Even with this fraction, μ50 and μ170 failed to compete successfully for the interaction between μ70 and its binding protein (data not shown), thus strongly implying that the binding sites defined in vivo as μE3 and μE4 are not equivalent. Furthermore, when μ50 or μ170 were end-labeled and incubated with column fractions active in μE3 binding, no specific nucleoprotein complexes were seen. Our current level of analysis does not allow us to distinguish whether μE4 has a much lower affinity for the μE3 factor or whether it binds a different factor. Similarly, the μE1 domain (isolated as a HinfI–PstI fragment) does not compete for the factor binding to μ70. However, in this case, the μE1 fragment itself, when end-labeled and analyzed, does generate a discrete nucleoprotein complex that is not effectively competed away by μ70 (Weinberger et al., 1986). The lack of cross-competition implies that μE1 and μE3 are also not equivalent and, furthermore, do not interact with the same nuclear factor. We will refer to the factor binding to the fragment μ70 as NF-μE3 (an abbreviation for nuclear factor interacting with the μE3 sequence).

To determine the location of the binding sites within individual fragments we have used the technique of methylation interference. End-labeled DNA fragments were par-

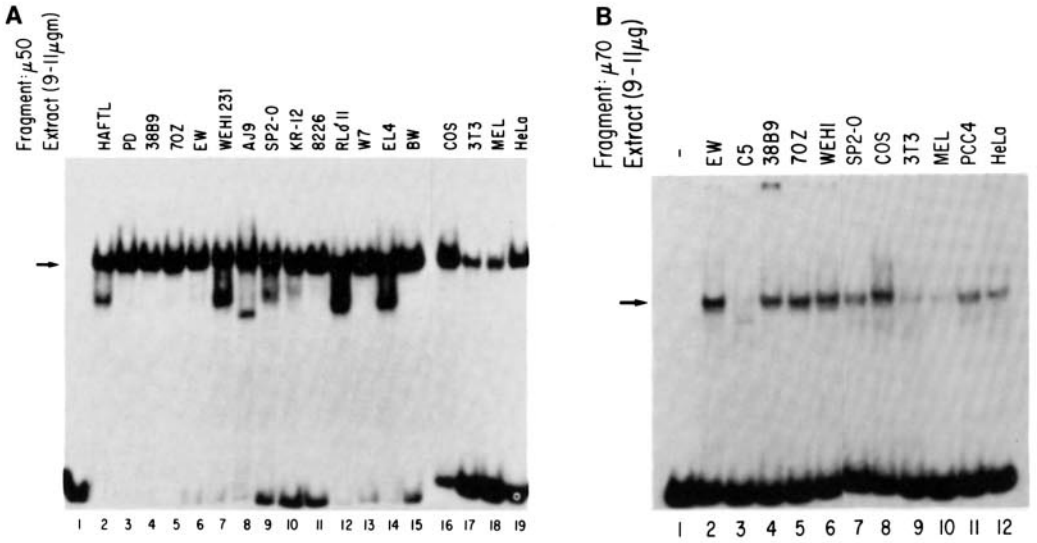


Figure 4. Analysis of μ50 and μ70 Binding in Extracts Generated from a Variety of Lymphoid and Nonlymphoid Cell Lines

(A) End-labeled μ50 was incubated with 9–11 μg of protein from various cell extracts and 2.5 μg of poly d(IC) in a 15 μl binding reaction, followed by electrophoretic analysis. The major complex that has been characterized by competitions (Figure 2C) and methylation interference (Figure 3A) is indicated by the arrow. Lane 1, free fragment; lanes 2–19, binding analysis in various extracts. The cell lines used to derive the extracts are indicated above each lane. Briefly, HAFTL is a very early pre-B mouse cell line; PD, 38B9, and 70Z are mouse pre-B cell lines; EW is a human B cell line; WEHI 231 and AJ9 are mouse B cell lines; SP2-0 is a mouse myeloma; KR12 and 8226 are human myelomas; RL 11, W7, EL4, and BW are various T cell lines; COS is a monkey cell line; 3T3 is a mouse fibroblast line; MEL is a mouse erythroleukemia line; HeLa is a human cervical carcinoma cell line.

(B) Binding of μ70 in various extracts under the same conditions as in (A). The characterized complex is indicated by an arrow. Lane 1, free fragment; lanes 2–12, binding in various extracts.

tially methylated on guanines using DMS. Methylated DNA was then used for a binding reaction with crude extracts, and the complex was resolved from the free fragments by electrophoresis. Piperidine cleavage (Maxam and Gilbert, 1977) of eluted fragments was followed by electrophoresis through 12% polyacrylamide–urea sequencing gels. If any of the methyl groups introduced by reaction with DMS interfered with the binding of a specific protein then that molecule of DNA will be selectively missing in the complex form and subsequently in the corresponding G ladder. The method therefore allows the identification of G residues making intimate contacts with the protein. When the μ50 DNA fragment was used in such an experiment, the free fragment generated a characteristic G ladder (Figure 3A, lanes 2 and 3) and the complex form was specifically depleted of DNA molecules carrying a methyl group at the G residue indicated by the asterisk (lane 1), which lies in the middle of the O sequence. This further implies that the NF-A protein is involved in the binding because the interaction appears to be specifically mediated by its cognate sequence. Presumably, modification of this key G residue seriously impedes the formation of a stable complex between the protein and its cognate sequence. Methylation of a second G residue (Figure 3A, lane 1, lowest of the triplet) also appeared to partially inhibit complex formation. Both of these residues have been shown to be protected against methylation by DMS in vivo (Ephrussi et al., 1985). On the noncoding strand, methylation of the G residue in the middle of the octamer sequence only partially inhibited complex formation (Figure 3A, lane 4, asterisk) whereas modification of any of the three A residues (marked by asterisks) abolished binding completely (lane 4). Interestingly, however, none of the other G residues protected in vivo in this region of the μ enhancer appears to be involved in complex formation as measured by this methylation interference assay (Figure 3C). Therefore, if these protections in vivo are due to the binding of a protein, this factor is different from NF-A and is not binding to the μ50 fragment in vitro.

On the μ70 fragment several G residues were identified as being important in forming intimate contacts with the binding protein (Figure 3B). On the coding strand, bands due to three Gs (Figure 3B, asterisks) were significantly reduced in intensity in the complex as compared with the free DNA fragment (compare lanes 1 and 2); on the noncoding strand two Gs were significantly affected (compare lanes 3 and 4). In Figure 3C, open circles above the sequence indicate the residues identified by Ephrussi et al. (1985) to be protected against methylation in vivo whereas the encircled Gs are the ones identified by us in vitro. The pattern of protection and interference on the μ70 fragment over the consensus sequence is strikingly similar in vivo and in vitro, indicating that the protein identified here may be the one that interacts with this sequence in vivo. Analogous to μ50, however, a second set of protections seen in this region in vivo was not observed in vitro. Interestingly, several G residues in the complex (Figure 3B, lane 1) appear to be more intense than the corresponding residues in free DNA (lane 2). This may mean that some of the modifications allow better interaction between the DNA

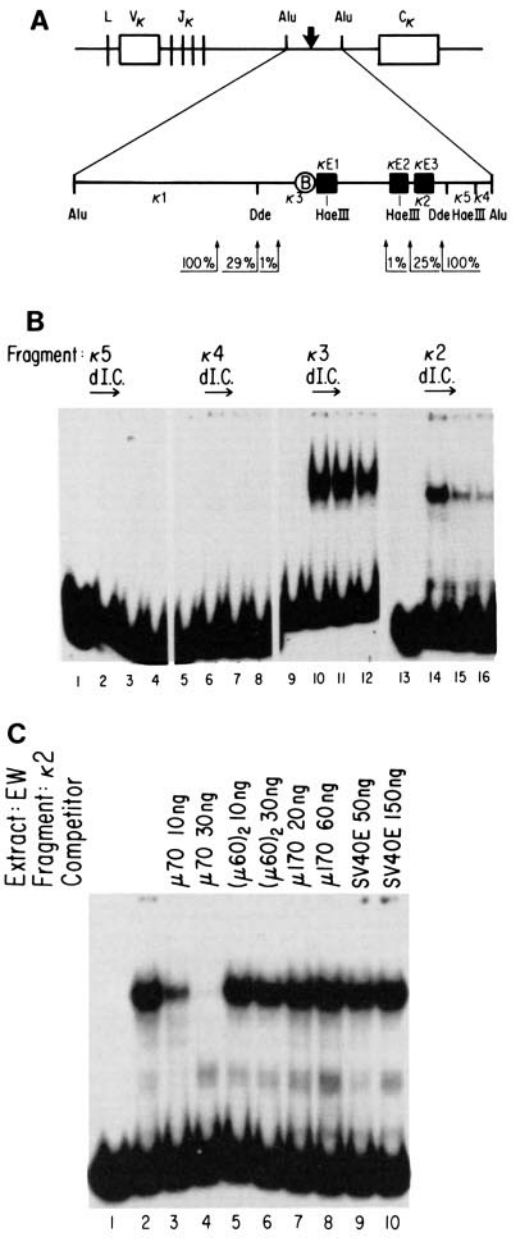


Figure 5. Dissection and Binding Analysis of the κ Enhancer

(A) Schematic representation of the essential 475 bp AluI–AluI fragment containing the κ enhancer as defined by Picard and Schaffner (1984). The vertical arrow represents the approximate location of a DNAase I hypersensitive site in the J_κ–C_κ intron. This enhancer was further dissected by cutting with DdeI and HaeIII to generate the fragments κ1 through κ5, which were then used as probes in binding assays. The black boxes represent sequences homologous to the consensus sequence derived by Church et al. (1985) based on the protections against methylation by DMS observed in vivo on the μ enhancer. The lowest line summarizes the results of Queen and Stafford (1984), who have carried out fine deletion mapping of the κ enhancer. Thus deletions from the 5′ end to about 20 bp past the DdeI site cause a significant loss of enhancer function. Similarly 3′ deletions extending 10–15 bp beyond the second HaeIII site cause a significant loss of enhancer function. B locates the B cell–specific protein binding site described in this paper.

(B) Binding analysis of subfragments of the κ enhancer. In each set of four lanes, the first shows the free fragment followed by binding analyses in the presence of increasing amount (1.2, 2.4, and 3.6 μg) of the nonspecific competitor poly d(IC) and a constant amount (~8 μg) of protein derived from an EW nuclear extract. Labeled fragments are as follows: lanes 1–4, κ5; lanes 5–8, κ4; lanes 9–12, κ3; lanes 13–16, κ2.

(C) Competition analysis to show that κ2 interacts with the same nuclear factor that binds to μ70. Lane 1, free fragment; lane 2, binding of end-labeled κ2 (0.2–0.3 ng, 10,000 cpm) in the absence of competitor DNA. Typical reaction contained 10 μg protein from EW nuclear extracts and 2 μg of poly d(IC) in a 15 μl volume. Lanes 3–10, binding reactions essentially as described above, but additionally containing unlabeled competitor DNA fragments derived from the μ enhancer (refer to Figure 2A) or the SV40 enhancer (Figure 2C) in the amounts shown above each lane.

(D) κ3 binds a specific factor in EW nuclear extracts. Lane 1, free fragment; lane 2, binding of end-labeled κ3 (0.1–0.3 ng, 10,000 cpm) in the absence of competitor DNA. A typical reaction contained 10 μg protein from EW nuclear extracts and 2.5 μg of poly d(IC) in a 15 μl volume. Lanes 3–12, binding reactions in the presence of competitor DNAs added in the amounts shown above each lane. Refer to Figure 2C for derivations of the κpr, κE and SV40E fragments.

and the specific protein presumably by subtly altering the DNA conformation.

Tissue Specificity of the Factors Detected

To ask whether the proteins we have identified are limited to expression only in B cells, we have screened extracts from a large number of cells (Figure 4). Complexes that comigrated with the ones generated and characterized in the B cell line EW were observed on both the fragments (μ50 [Figure 4A] and μ70 [Figure 4B]) in all the cell lines examined. (Comparison of independent extracts indicates that estimates of the abundance of proteins in different cell lines using this assay are not meaningful.) Although the complex generated in each extract has not been further characterized, we interpret this data as indicating that both of these factors are not tissue-specific. A second

complex (having a greater mobility) was observed with the μ50 fragment that appears to be restricted to B and T cells only and will be described fully later (Staudt et al., unpublished results).

Dissection of the κ Enhancer

An enhancer element has also been identified in the major intron of the κ light chain gene. Picard and Schaffner (1984) showed that the enhancement activity can be localized to a 500 bp AluI–AluI fragment, and Queen and Stafford (1984) have shown that deletion of the 5′ AluI–DdeI fragment has a minimal effect on enhancer activity, restricting the enhancer to 275 bp from DdeI to the 3′ AluI site (Figure 5A; the black boxes represent sequences identified by Church et al. [1985] as homologous to the series of E domains detected in the μ enhancer). Fragments were generated by cutting with DdeI and HaeIII (see Figure 5A) and assayed for binding in the presence of increasing amounts of poly d(IC) as a nonspecific competitor. Fragments κ4 and κ5 appeared negative (Figure 5B, lanes 1–8), while κ3 and κ2 formed complexes (lanes 10–12 and 14–16). κ1 is too large a fragment to be reliably assayed and has not been further dissected yet. Preliminary results show that the internal undesignated HaeIII fragment does not contain any specific binding sites either. The competition pattern for κ2 was strikingly similar to what had been observed earlier with the μ70 fragment: relatively large amounts of μ400, the Moloney leukemia virus enhancer, the SV40 enhancer, or the κ promoter (containing the O sequence) did not compete for binding, while μ300 and the κ enhancer did (data not shown). Because κ2 contains a putative E box identified by sequence comparison (as does μ70), we competed away its binding with smaller fragments from μ300 (Figure 5C). The complex is specifically competed away by the addition of unlabeled μ70 during the incubation (Figure 5C, compare lanes 3 and 4 with lane 2), but not by the addition of $(\mu 60)_2$ (lanes 5 and 6), μ170 (lanes 7 and 8), or the SV40 enhancer (lanes 9 and 10). Furthermore, the protein that binds to this sequence cofractionates with NF-μE3, the μ70 binding activity, through two sequential chromatographic steps (heparin agarose and DEAE–Sepharose) (data not shown). We conclude that the same sequence-specific protein (NF-μE3) binds to both fragment μ70 and fragment κ2; therefore, at least one common protein interacts with both the μ and the κ enhancers.

The κ3 complex (Figure 5D, arrowhead) failed to be competed away by μ300 (lanes 3 and 4), μ400 (lanes 5 and 6), or a κ promoter-containing fragment (lanes 7 and 8). The complex, however, was specifically competed away by both the complete κ enhancer (Figure 5D, lanes 9 and 10) and the SV40 enhancer (lanes 11 and 12). The band below the major κ3 complex was seen at variable intensities in different experiments and failed to compete even with the complete κ enhancer in this experiment and has not been further investigated. The observation that the SV40 enhancer specifically competes for binding of this factor suggests that an identical stretch of 11 nucleotides (GGGGACTTTCC) shared with the SV40 enhancer may be responsible for the binding.

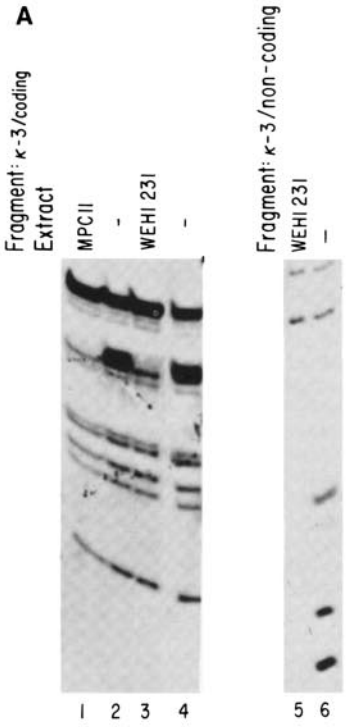


Figure 6. Methylation Interference Analysis of the Nucleoprotein Complex Generated on κ3

(A) Preparative binding reactions were carried out with partially methylated κ3 end-labeled at the DdeI site in two different extracts: MPC11 (mouse myeloma) and WEHI 231 (mouse B cell line). The complex and free fragment bands were eluted from a low ionic strength polyacrylamide gel, treated with piperidine, and analyzed by electrophoresis through a 12% sequencing gel. Lanes 1 and 3, G ladder corresponding to the nucleoprotein complex bands generated in MPC11 extracts and WEHI 231 extracts, respectively. Lanes 2 and 4, G ladder corresponding to the free fragment isolated after binding in MPC11 and WEHI 231 extracts, respectively. Lanes 5 and 6, analysis of the G residues on the noncoding strand in the nucleoprotein complex band (lane 5) and the free fragment (lane 6) following preparative binding and low ionic strength gel electrophoresis. G residues whose methylation interferes with nucleoprotein complex formation are indicated by the asterisks.

(B) Summary of the methylation interference (in vitro) experiments used to define the B site within the κ enhancer. The relevant region of the κ3 fragment is shown with the coding strand on top. The encircled Gs are the ones whose methylation interferes with protein DNA interaction.

We have localized the binding site of this factor on the κ3 fragment by carrying out methylation interference experiments. In two different extracts, methylation at three of a stretch of four G residues on the coding strand within the sequence shared with SV40 completely abolished binding (Figure 6A, compare the complexed fragments in lanes 1 and 3 with the free fragments in lanes 2 and 4; asterisks indicate the position of Gs whose methylation seriously interferes with binding). On the noncoding strand, methyla-

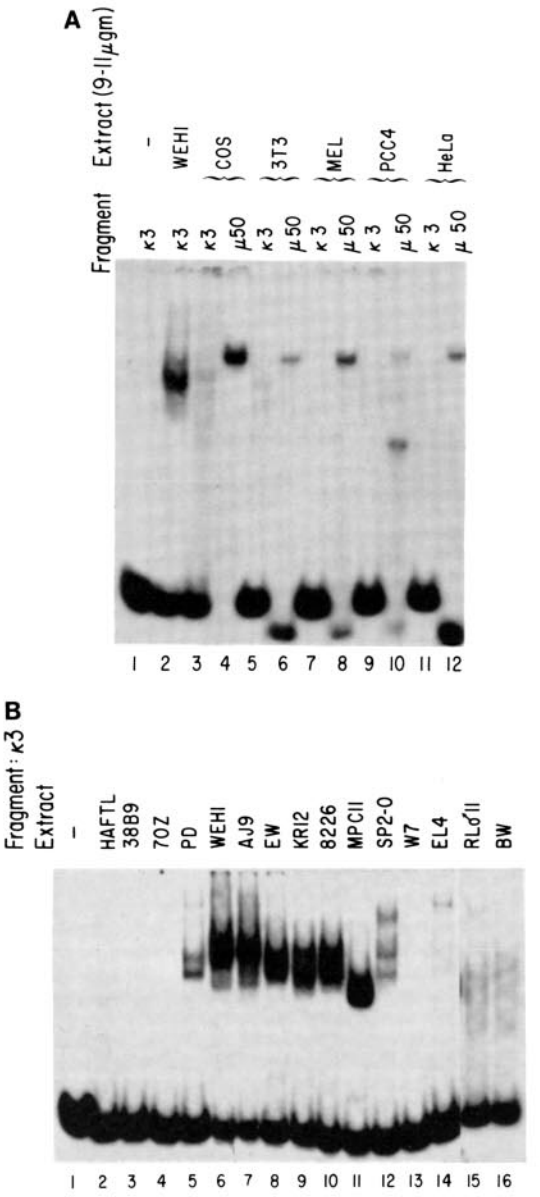


Figure 7. Analysis of κ3 Binding in a Variety of Lymphoid and Nonlymphoid Cell Extracts

(A) κ3 binding reactions carried out in five nonlymphoid lines. Lane 1, free fragment; lane 2, binding in a cell line positive for this factor (WEHI 231). A typical reaction had 0.1–0.3 ng (10,000 cpm) of labeled fragment together with 2.5 μg of poly d(IC) and 9–11 μg of nuclear extract. Odd numbered lanes 3–11, binding reactions in the same conditions as above in a variety of extracts generated from nonlymphoid lines as noted above each lane (refer to Experimental Procedures for a description of these lines). Even numbered lanes 4–12, binding of μ50 (which detects a ubiquitous factor) under the same conditions, serving as a positive control for each extract.

(B) κ3 binding reactions in lymphoid cell extracts. Lane 1, free fragment. Binding reactions were performed, as detailed above, in extracts derived from pre-B cell lines, (HAFTL, 38B9, 70Z, and PD) (lanes 2–5); B cell lines (WEHI 231, AJ9, and EW) (lanes 6–8); plasma cell lines (KR12, 8226, SP2-0, and MPC11) (lanes 9–12); and T cell lines (lanes 13–16). Note that the mobility of the complex formed differs slightly between extracts derived from mouse cells (e.g., AJ9, WEHI 231) or human cells (EW, KR12, and 8226). The significantly altered mobility in the MPC11 extracts is probably due to proteolysis, since many other binding sites also show higher mobility complexes in this extract.

tion of three G residues (Figure 6A, lane 5, asterisks) inhibited complex formation. Thus the binding site was (shown in Figure 6B; circled G residues are those determined by methylation interference experiments to be important for complex formation) localized toward one end of the κ3 fragment (Figure 5A, represented by B). This result also served to explain the specific competition observed earlier with the SV40 enhancer. Interestingly, deletion mapping of the κ enhancer was shown that sequences within the κ3 fragment are extremely important for enhancer function (Queen and Stafford, 1984).

The tissue range of this factor was examined by carrying out binding analysis with κ3 using extracts from a variety of cell lines. Nucleoprotein complex formation with κ3 was detected in a mouse B cell line (Figure 7A, lane 2) but not in 5 other non–B cell lines (odd numbered lanes from 3–11). Even numbered lanes in Figure 7A show that the ubiquitous factor detected by μ50 is present in all of these cell lines and serves as a positive control for the experiment. The factor therefore appears to be restricted in expression to B lymphoid cells. We then examined extracts made from cells at various stages of B cell differentiation (Figure 7B). Interestingly, κ3 binding protein was detected in the pre–B cell line PD (Figure 7B, lane 5), which spontaneously rearranges its κ genes (Lewis et al., 1982); two mouse B cell lines (WEHI 231 and AJ9, lanes 6 and 7); one human B cell line (EW, lane 8); two mouse myeloma cell lines (MPC11, SP2-0, lanes 11 and 12); and two human myelomas (KR12 and 8226, lanes 9 and 10). However, it was not apparent in a very early pre–B cell line (HAFTL, Figure 7B, lane 2) and two standard mouse pre–B cell lines (38B9 and 70Z, lanes 3 and 4). Thus this factor appears to be not only tissue-specific and limited to cells of the B lymphoid lineage, but also stage-specific within that lineage. For these reasons, we refer to the binding site for this factor as the B site and refer to the factor as NF-κB.

In the series of extracts examined, the presence of the NF-κB factor is strikingly correlated with κ gene expression, but there is one apparent discrepancy concerning its stage specificity. Cell line PD, which was derived by Abelson murine leukemia virus transformation of adult bone marrow cells (Rosenberg and Baltimore, 1976) and undergoes κ light chain rearrangement in culture, had the factor. Cell line 70Z, which is apparently further along the B cell differentiation pathway relative to PD, having already completed rearranging its κ light chain genes (Maki et al., 1980), had no detectable factor. We believe that these may not be contradictory because 70Z cells do not actively transcribe the κ locus and do not have the DNAase I hypersensitive site (Parslow and Granner, 1982) in the J_κ–C_κ intron that has been correlated with κ gene expression. For PD, however, the κ enhancer is active after transfection (Speck and Baltimore, unpublished results); and the DNAase I hypersensitive site associated with the κ enhancer can be detected (Sen and Baltimore, unpublished results).

Table 1. Summary of Immunoglobulin Enhancer–Binding Factors

Factor	Binding Site(s)	Tissue Distribution
NF-A	Octamer sequence (ATTTGCAT) in V(H), V(L) promoters and μ enhancer	Ubiquitous (B cell specific component)
NF-μE3	E3 site in μ enhancer	Ubiquitous
NF-μE1	E1 site in μ enhancer	Ubiquitous
NF-κB	B site in κ enhancer	κ-Producing B cells only

E1, E2, E3, etc., refer to the E homology identified by Ephrussi et al. (1985). NF-μE1 has been identified by Weinberger et al. (1986).

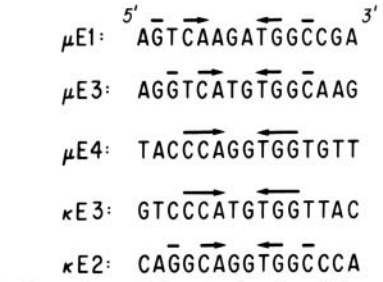


Figure 8. Comparison of E Domains from the μ Enhancer and the κ Enhancer

μE1–μE4 were defined by Ephrussi and Church in the μ enhancer on the basis of methylation protection experiments in vivo (Ephrussi et al., 1985). κE1–κE3 were identified within the κ enhancer as being homologous to the consensus sequence derived by comparing μE1–μE4 (Church et al., 1985) (see Figure 5A). The arrows over the sequence point out a mini dyad axis of symmetry within each domain. (These also include in every case the most conserved residues in the consensus sequence [Church et al., 1985].) In the fragments used by us to dissect the enhancers, μE3 is completely present within the μ70 fragment, μE4 within the μ50 and μ170 fragments, and κE3 within the κ2 fragment.

Discussion

We have detected interaction of multiple factors (summarized in Table 1) with Ig μ and κ enhancer sequences using an electrophoretic mobility shift assay. Within the 300 bp PstI–EcoRI fragment of the μ enhancer, two sites have been localized. One is an octamer (O) sequence (ATTTGCAT) that is also conserved upstream of all heavy and κ chain variable region genes and appears to bind the ubiquitous NF-A factor. The second sequence coincides with one of the motifs (μE3) shown by Ephrussi et al. (1985) and by Church et al. (1985) to be bound to a factor in B cells. Both of the factors were detected in a broad range of cells and therefore did not appear tissue-specific. There appears also to be a tissue-specific factor that can bind to the O sequence (Staudt et al., unpublished results). The μE1 and μE4 sequences that have close homology to the μE3 sequence competed poorly, if at all, for binding to the μE3 sequence, implying that the sequence specificity of the μE3-binding protein is quite exquisite. A different protein has been shown to bind to μE1 (Weinberger et al., 1986).

Our dissection of the κ enhancer has revealed two binding sites. One of these, κE3, corresponds closely in sequence to one of the μ sites, μE3 (see Figure 8), and its binding protein appears to be NF-μE3, the factor that binds to μE3. Thus there is at least one protein that interacts with both the μ enhancer and the κ enhancer. The second site, B, is the most unique one we have found because NF-κB, its binding protein, is restricted in appearance to cells that ordinarily express κ chains. It is not present in the μ enhancer, but is found in the SV40 enhancer.

Although all of the data presented here involved crude nuclear extracts as the source of protein, preliminary results of chromatographic fractionation are consistent with the proposal that at least three separable factors interact with the immunoglobulin enhancers.

The enhancers are defined partly by their ability to enhance transcription when present in either orientation relative to a promoter. It might therefore be thought that their binding sites should have dyad symmetry. The E series of sequences (those suggested by Ephrussi et al. [1985] to be homologous) do contain an element of dyad symmetry. As illustrated in Figure 8, there is a motif of CA/TG in all of these sequences with a separation of two or three bases. One of the next outer two bases also has a symmetric counterpart giving a dyad of three out of four bases in each sequence. The two sequences that appear to bind the same factor (μE3 and κE2) also have identical internal two base spacers of TG. Of those that do not compete for binding with μE3 and κE2, μE1 has a three base spacer and μE4 has a spacer of GG. Another related sequence (κE2) within the κ enhancer also has a GG dinucleotide within its inverted repeat but has yet to be analyzed. We have been unable to detect any binding to μE4 thus far, but μE1 appears to have a specific binding factor (Weinberger et al., 1986). The inability of such closely homologous sequences (particularly μE4) to compete for binding to μE3 suggests that these nuclear binding proteins may have restriction enzyme–like specificity in their binding. Perhaps some of the enhancer-binding proteins belong to families of related proteins with slightly different binding specificities.

Two of the identified binding sites have no homology either to the E series, or to each other. One, the O sequence from the μ enhancer (ATTTGCAT), is bound by NF-A, a non-tissue-specific factor, but also is bound by a factor found only in cells of the B lymphoid series (Staudt et al., unpublished results). The other, the B sequence from the κ enhancer (found around the sequence GGGGACTTTCC), binds to NF-κB, a factor specific to cells that express κ chains. Ignoring the nonspecific factor that binds to the O sequence, it might appear that each enhancer has E-related elements that bind to nonspecific factors and unique elements that bind to specific factors and that could be responsible for the tissue specificity of the individual enhancers. As discussed elsewhere (Singh et al., 1986), the existence of a nonspecific factor that binds to the O sequence might relate to the use of this site for

regulating transcription by promoters such as those for the U1 and U2 RNAs.

There is an apparent discrepancy between the in vivo binding data (Mercola et al., 1985; Ephrussi et al., 1985) and the in vitro data we have accumulated. Ephrussi et al. (1985) and Church et al. (1985) find that the E sites and the O site are protected against methylation in B cells but not in fibroblasts. We find that NF-μE3 and NF-A are present in fibroblastic and other nonlymphoid cells. It would therefore appear that the mere presence of a factor is not sufficient for it to bind in such a way as to generate protection of the G residues at the site in vivo; in all likelihood, the protein is not bound to a site except in specific cells. This implies that for binding to occur, a given segment of DNA may have to be "activated," a process that may involve making chromatin accessible to the binding factors. It is possible that tissue-specific binding proteins play the role of activation and therefore open the DNA to interaction with nonspecific transcription enhancing proteins. In vitro, where naked DNA is used for assay, such interactions would not be evident and the factors would all appear to be equivalent DNA binding proteins.

Although one might expect that at least some of the sequences to which factors bind in the tissue-specific Ig enhancers would be unique to these structures, most of the sequences have close relatives in other regulatory elements. The two sequences most likely to be involved in tissue specificity—the O sequence and the B sequence—are both found in the SV40 enhancer, and that viral sequence will compete for binding of factors to O and B in vitro. Transcriptional potentiation by the SV40 enhancer can be competed away by the μ enhancer both in vivo and in vitro, suggesting that a common factor may interact with both of these sequences (Mercola et al., 1985; Sassone-Corsi et al., 1985). A possible explanation for this could be that when the SV40 enhancer is activated in a non–B cell, it uses some other sequences or binding sites for its activity, whereas when it is activated in a B cell, it uses, for instance, its homolog of the NF-κB binding site. In this model, the SV40 enhancer could be a mosaic of different sequence motifs recognizing a number of different factors, only a subset of which is needed in any one cell to generate function.

In previous studies using the mobility shift assay, we have determined the site of binding using a variant of the DNAase I footprinting method (Singh et al., 1986). In the present study we have used a methylation interference assay both because it allows a higher resolution analysis of the binding site and because we found that many complexes cannot be assayed by the footprint method. For those complexes that do not yield a footprint, there appears to be too rapid an equilibration between complexed and free DNA to allow complexes to be treated with DNAase and then resolved by electrophoresis. For instance, the half-life of the nucleoprotein complex with the μ70 fragment is less than a minute (Sen, unpublished observation). In the methylation interference protocol, DNA that is methylated will not rebind eluted proteins, and therefore the bound complexes never contain DNA methylated at a residue critical to the binding reaction.

Experimental Procedures

Extracts and Cell Lines

Nuclear extracts were made from the following tissue culture cell lines exactly according to the protocol of Dignam et al. (1983) and usually contained 6–12 mg/ml of protein: HAFTL, Harvey sarcoma virus transformant (Pierce and Aaronson, 1982), which presumably represents an early stage in B cell differentiation because it is still in the process of carrying out $D_H \rightarrow J_H$ rearrangements at the immunoglobulin heavy chain locus (Desiderio and Baltimore, unpublished results); 38B9 and PD, Abelson murine leukemia virus transformants, which are pre-B-like because they either contain a rearranged (VDJ) heavy chain locus (PD, Lewis et al., 1982) or are in the process of assembling their heavy chain genes (38B9, Yancopoulos et al., 1984); 70Z, mouse pre-B cell line; WEHI 231 and AJ9, mouse B cell lines containing functionally rearranged heavy and light chain genes; EW 36, human EBV-negative Burkitt lymphoma; KR12 and 8226, human myelomas (gift from Dr. C. M. Croce); SP2-0 and MPC 11, mouse myelomas; BW5147, W7, EL4, and RLσ11, mouse T cell lines; COS, monkey cell line; 3T3, mouse fibroblast cell line; MEL, mouse erythroleukemia cell line; PCC4, mouse embryonic carcinoma line; HeLa, human cervical carcinoma cell line.

Plasmids were constructed as follows. The 300 bp PvuII–EcoRI fragment of the μ enhancer was digested with AluI, and the fragments were subcloned into pUC13 cut with SmaI to yield pμ70 (containing the 70 bp AluI–AluI insert) and pμ170 (containing the 170 bp AluI–AluI insert). See Figure 2A for a restriction map of the relevant region.

Competitor DNA corresponding to the various μE boxes were prepared as follows: μE1, by BamHI–PvuII digestion of pμEc (a plasmid containing the 220 bp HinfI–HinfI fragment of the μ enhancer, subcloned into SmaI-cut pμC13, which was a gift from Dr. J. Weinberger); μE3, by EcoRI and BamHI digestion of pμ70; μE4, by HinfI–DdeI digestion of the insert obtained by cleaving pμ170 with EcoRI and BamHI.

The 475 bp AluI–AluI fragment containing the κ enhancer (κE) was subcloned into pUC13 cut with SmaI. Competitor DNA was prepared by cutting at flanking sites within the polylinker. DdeI and HaeIII were used to generate the various smaller fragments as shown in Figure 5A. The κ promoter (κpr) was obtained from a plasmid that contained approximately 300 bp (spanning positions −35 to −330 relative to the cap site of the MOPC 41 κ gene) of an SfaNI fragment that was subcloned into SmaI-cut pSP64 (a gift of Dr. N. E. Speck). Large competitor fragments (greater than 150 bp) were isolated from low melting point agarose gels by four extractions with phenol and one extraction with chloroform, followed by precipitation with ethanol. Smaller competitor fragments were isolated from 8%–12% native polyacrylamide gels by soaking the minced gel slice in elution buffer containing 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS. After a 6–8 hr incubation at 37°C, the supernatant was extracted once with phenol and once with chloroform and the DNA was precipitated by adding 2.5–3 volumes of ethanol. Competitor DNA was quantified by comparison to standard weights of DNA either after electrophoresis through agarose or after spotting onto an agarose plate. Radioactive probe was obtained by end-labeling dephosphorylated DNA with [γ-^{32}P]ATP in the presence of polynucleotide kinase (Boehringer Mannheim Biochemicals). Typical specific activities ranged from 30,000–70,000 cpm/ng of DNA.

Gel Binding Analysis

Binding reactions were carried out in 10 mM Tris (pH 7.5), 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 4% glycerol for 20–30 min at room temperature. Poly d(IC) was added as a nonspecific carrier, and a typical reaction contained 10,000 cpm (0.2–0.5 ng) of end-labeled DNA with 9–11 μg of extract (which was added last). Following binding, the mixture was electrophoresed through a native 4% polyacrylamide gel (acrylamide: bisacrylamide ratio 30:1) containing 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA. The gel was preelectrophoresed for 2 hr at 11 V/cm. Electrophoresis was carried out at the same voltage for 2 hr at room temperature with buffer recirculation. The gel was then dried and autoradiographed with a screen at −70°C. For competition experiments the conditions were exactly as above, except that specific and nonspecific competitor DNAs were included in the mixture (in amounts as detailed in the figure legends) prior to addition of the protein.

Methylation Interference Experiments

End-labeled DNA fragments were partially methylated at the guanine residues, as detailed by Maxam and Gilbert (1977) with the following modification. The reaction was quenched with 1.5 M sodium acetate (pH 7.0), 1 M β-mercaptoethanol, and 100 μg/ml of poly d(IC). Methylated DNA was precipitated twice, rinsed with 70% ethanol, dried, and taken up in TE (10 mM Tris [pH 8.0] and 1 mM EDTA). For a typical preparative binding reaction, the usual conditions were scaled up 5 to 10 fold. Binding and gel electrophoresis were as above. After electrophoresis the gel was wrapped with Saran wrap and exposed wet for 4–6 hr at room temperature. The complex and free fragment bands were then excised and electroeluted for 1–2 hr (tRNA was added to prevent adsorption of labeled DNA to the membrane) to recover the DNA. Prior to ethanol precipitation, the solution was extracted sequentially with phenol and chloroform. The pellet was rinsed thoroughly with cold 70% ethanol, dried, then redissolved in 100 μl of 1 M piperidine. Base cleavage reactions were carried out for 45 min at 90°C followed by removal of the piperidine by lyophilization. After two additional rounds of lyophilization from water, the products were analyzed by separation by electrophoresis through a 12% polyacrylamide gel in the presence of 8 M urea followed by autoradiography at −70°C with a screen.

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