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Binding In Vitro of Multiple Cellular Proteins to Immunoglobulin Heavy-Chain Enhancer DNA

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Seven protein-binding sites on the immunoglobulin heavy-chain (IgH) enhancer element have been identified by exonuclease III protection and gel retardation assays. It appears that the seven sites bind a minimum of four separate proteins. Three of these proteins also bind to other enhancers or promoters, but one protein seems to recognize exclusively IgH enhancer sequences. A complex of four binding sites, recognized by different proteins, is located within one 80-base-pair region of IgH enhancer DNA. Close juxtaposition of enhancer proteins may allow protein-protein interactions or be part of a mechanism for modulating enhancer protein activity. All IgH enhancer-binding proteins identified in this study were found in extracts from nonlymphoid as well as lymphoid cells. These data provide the first direct evidence that multiple proteins bind to enhancer elements and that while some of these proteins recognize common elements of many enhancers, others have more limited specificities.

Transcriptional enhancer sequences are *cis*-acting DNA elements which increase the transcription initiation rate from certain viral and cellular promoters in a relatively distance- and orientation-independent fashion (1, 2, 7, 19, 23, 30, 47). Enhancers are associated with many cellular genes, and some appear to act in a tissue-specific or inducible manner (1, 5, 8, 12, 15, 17, 22, 28, 37). Thus, it seems likely that these unique regulatory elements play an important role in regulating cellular gene expression. Since enhancers from different genes have little or no sequence homology, enhancers have been identified by functional analyses.

The mechanism by which enhancers activate transcription remains relatively unclear (30, 47–49). It is known, however, that binding of *trans*-acting cellular factors to enhancer sequences is required for enhancer activity. This was first demonstrated for the simian virus 40 (SV40) enhancer by in vivo competition studies (42). Subsequently, similar studies were carried out by Mercola et al. on the mouse immunoglobulin heavy-chain (IgH) enhancer element (27), a cellular enhancer which had previously been found to function preferentially in B cells (1, 15). In addition to finding a requirement for binding of *trans*-acting factors, Mercola et al. showed that in B cells the SV40 enhancer and the IgH enhancer use at least one factor in common and that B cells contain at least one factor required for IgH enhancer activity which is not functional in fibroblasts. These results are supported by the work of Ephrussi et al., who identified sites on the IgH enhancer which were protected in vivo from dimethyl sulfate modification in B cells but not in fibroblasts (9). The observation that different enhancer elements require particular cellular factors to be active raises the interesting possibility that sets of genes, dependent on similar enhancer elements, could be modulated coordinately by altered levels of an active enhancer factor.

A logical extension of the in vivo studies is to develop in vitro assays for enhancer factors so that estimates of their number and specificity can be made and they can ultimately be purified. Singh et al. (45) recently used gel retardation assays (11) with nuclear extracts to detect a protein which binds to an 8-base-pair (bp) sequence in the immunoglobulin kappa light-chain promoter region and the IgH enhancer region. Wu has recently described an exonuclease III (exoIII) protection assay that is also capable of detecting specific DNA-binding proteins in crude cell extracts. He used this assay to identify and purify proteins that bind a *Drosophila melanogaster* heat shock promoter (50); in addition, Fujimura (11a) and Clos et al. (6) have used exoIII protection to identify a polyomavirus enhancer factor and an rDNA promoter factor, respectively.

Here we report the results of experiments with these two assays, gel retardation and exoIII protection, to study the in vitro binding of cellular proteins to the IgH enhancer. The results show that (i) multiple proteins bind within the heavy-chain enhancer sequence, (ii) three of these proteins also bind to other enhancers and are thus "general" enhancer proteins, (iii) at least one of the proteins binds preferentially to the heavy-chain enhancer region, and (iv) no B-cell-specific enhancer-binding protein was detected by our assays.

MATERIALS AND METHODS

Preparation of nuclear extracts. Nuclear extracts were prepared from tissue culture cells or from the solid tumor line M603 (34) grown subcutaneously in C × D2 F_1 mice. Nuclei were prepared by a modified version of the procedure of Storb et al. (46), and extracts were prepared by a modified version of the procedure of Wu (50). All procedures were performed at 0 to 4°C. Briefly, tissue culture cells were harvested over crushed Dulbecco phosphate-buffered saline (PBS), and cell pellets were washed twice with ice-cold PBS. Solid tumor tissue was isolated from contaminating connective tissue by dissection and dounced with a "B" pestle (loose) in 0.5× TKM buffer (25 mM Tris, pH 7.5, 25 mM KCl, 7.5 mM $MgCl_2$) to produce a cell suspension. These cells were then washed twice with 0.5× TKM. Washed cell pellets were suspended routinely at 1×10^6 to 3×10^6 per ml in a detergent lysis buffer containing 0.5× TKM, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride

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(PMSF), 100 U of aprotinin per ml, 5 μg of leupeptin per ml, and 5 μg of pepstatin A per ml. Cells were gently lysed by tilting the tube several times. Nuclei were harvested and washed several times with RSB (10 mM NaCl, 10 mM Tris, pH 7.5, 10 mM $MgCl_2$).

Nuclei were suspended in approximately 1/4 of the lysis volume in extraction buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 8.0, 1.5 mM $MgCl_2$, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid], gt, 0.5 mM dithiothreitol [DTT], 5% glycerol, 200 mM NaCl, 0.5 mM PMSF, 100 U of aprotinin per ml, 5 μg of leupeptin per ml, 5 μg of pepstatin per ml), and then an equal volume of extraction buffer B (extraction buffer A at 600 mM NaCl) was added. The final NaCl concentration was then adjusted to 400 mM. Nuclei were extracted on ice with gentle stirring. The nuclear extract was clarified at 100,000 × *g* for 60 min, dialyzed against 1,000 volumes of chromatography buffer A (20 mM HEPES, pH 8.0, 0.1 mM EGTA, 100 mM NaCl, 20% glycerol, 0.5 mM DTT, 0.2 mM PMSF, 10 U of aprotinin per ml, 0.5 μg of leupeptin per ml, 0.5 μg of pepstatin A per ml) until the conductivity was equal to that of the original dialysis buffer, quick-frozen in liquid N_2, and stored at −70°C. Protein concentrations were measured by the method of Bradford (3), with bovine serum albumin as a standard.

Fractionation of nuclear extracts. Nuclear extracts were partially purified by chromatography on 1-ml DEAE-Sephacel (Pharmacia) columns. Columns were washed with chromatography buffer A, and specific binding activities were eluted at 0.25 M NaCl (data not shown). The pooled DE.25 fraction was dialyzed and stored as described above.

Alternatively, nuclear extracts were more extensively fractionated through four successive steps. First, undialyzed nuclear extracts were divided into 1-ml portions, heated at 67°C for 10 min in a circulating water bath, cooled on ice, and clarified for 10 min at 4°C. This step removes 90% of the total protein with no observable loss in total specific binding activity for sites A, B, C, and D. The extract was then pooled and chromatographed on DEAE-Sephacel which had been equilibrated with chromatography buffer A at 400 mM NaCl to remove residual nucleic acids. The flowthrough peak was pooled, dialyzed against chromatography buffer B (50 mM Tris, 7.4, 6 mM $MgCl_2$, 100 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol, and proteinase inhibitors as in chromatography buffer A), clarified, and concentrated by chromatography on heparin-Sepharose 4B. The column was equilibrated and washed with chromatography buffer B, and specific binding activity was eluted with 600 mM NaCl (data not shown). The HO.6 fraction was dialyzed into chromatography buffer A and stored as described above. For some analyses the HO.6 fraction was further fractionated by chromatography on quaternary aminoethyl-Sephadex. The HO.6 fraction was dialyzed into chromatography buffer B at 50 mM NaCl and loaded onto quaternary aminoethyl-Sephadex which had been equilibrated in the same buffer. Specific binding activities were eluted at 300 mM NaCl (data not shown) and dialyzed into chromatography buffer A, and the QS.3 fraction was stored as described above.

Restriction fragment labeling and isolation. DNA restriction fragments were isolated from agarose gels by binding and elution from DE81 paper. Fragments were 5'-end labeled by the method of Maxam and Gilbert (26), isolated from acrylamide gels, and purified by DE52 chromatography and ethanol precipitation.

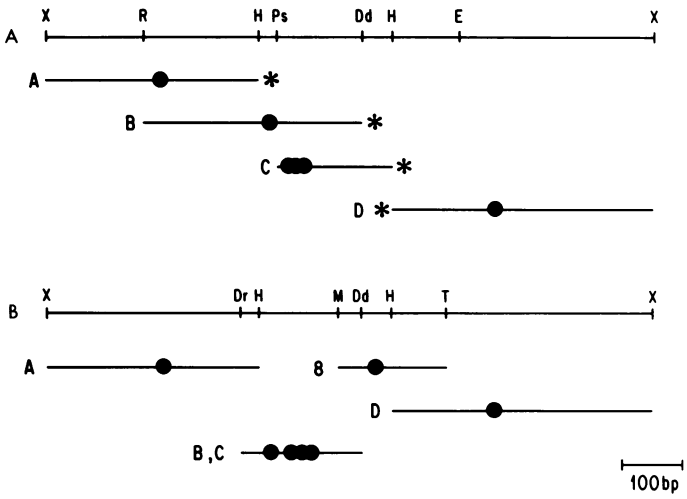
ExoIII and gel retardation assays. ExoIII reactions were performed by a modified version of the procedure of Wu (50). ExoIII was prepared by the method of Rogers and Weiss (36) or obtained from Boehringer Mannheim. Reactions were carried out in 100-μl volumes, and the reaction mixtures contained 10 mM HEPES, pH 8.0, 0.1 mM EGTA, 70 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.3 to 1.0 ng of end-labeled probe DNA, variable amounts of pBR322 DNA digested with *Hpa*II as nonspecific competitor, 5 to 15% glycerol, and protein extract. Reactions with crude nuclear extracts also contained 2 mM Na_2PO_4, pH 7.0, and 15 μg of *Saccharomyces cerevisiae* tRNA as phosphatase inhibitors. Binding reaction mixtures were incubated for 20 min at 24°C, and then 3,000 U of exoIII per ml was added and incubated for 10 min at 30°C. When competitions were performed with unlabeled DNA restriction fragments, the indicated molar ratios (competitor-probe) of unlabeled fragments were preincubated with reaction mixtures that lacked the probe DNA for 10 min at 24°C. In the case of pBR322-*Hpa*II competitions, the molar amount is based on an average fragment size of 170 bp. Probe was then added, and binding was allowed to continue for 10 min at 24°C. ExoIII was then added as above. Reactions were stopped by addition of an equal volume of 20 mM EDTA–1% sodium dodecyl sulfate–50 μg of yeast tRNA per ml. Products were purified by phenol extraction and ethanol precipitation. Samples were then loaded onto 6% sequencing gels.

Gel retardation reactions were performed by a modified version of the method of Singh et al. (45). Binding reactions were carried out in 15-μl volumes, and the reaction mixtures contained 0.5 to 1.0 ng of end-labeled probe DNA, 50 to 70 mM NaCl, variable amounts of poly(dI-dC) · poly(dI-dC) double-stranded polymer (Pharmacia), and protein extract. Binding reaction mixtures were incubated for 15 min at 24°C, 2 μl of dye mix (30% glycerol, 0.1% bromophenyl blue, 0.1% xylene cylenol) was added, and the samples were loaded onto 6% TBE (100 mM Tris, 83 mM borate, 1.3 mM EDTA)–acrylamide gels. Electrophoresis was carried out at 24°C. For each probe a titration of poly(dI-dC) · poly(dI-dC) concentration was performed with extract constant to determine the optimal amount to detect specific binding (data not shown). These amounts of poly(dI-dC) are indicated in the legend to Fig. 3. The competitions with unlabeled pBR322-*Hpa*II and enhancer subfragments were preincubated as described above.

RESULTS

The entire mouse IgH enhancer sequence is contained on a 1.0-kilobase (kb) *Xba*I fragment which occurs in the large intervening sequence between the J_H and Cμ gene segments (15, 27). Figure 1 shows diagrams and the sequence of that region; the restriction fragments which were used as probes in the exoIII protection and gel retardation studies and the locations of the protein-binding sites are also indicated.

Protein binding at four regions within IgH enhancer. ExoIII is a progressive 3' exonuclease whose migration along a DNA strand can be inhibited by specific high-affinity protein-DNA interactions. If protein bound to a 5'-end-labeled fragment halts exoIII digestion, the approximate position of the binding site can be determined by sizing the protected fragment via gel electrophoresis and autoradiography (50). In our experiments, pBR322 plasmid DNA was always present in excess to compete for nonspecific DNA-binding proteins in the crude extracts. In addition, each exoIII protection assay was controlled in two ways (Fig. 2). The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A
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C
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8
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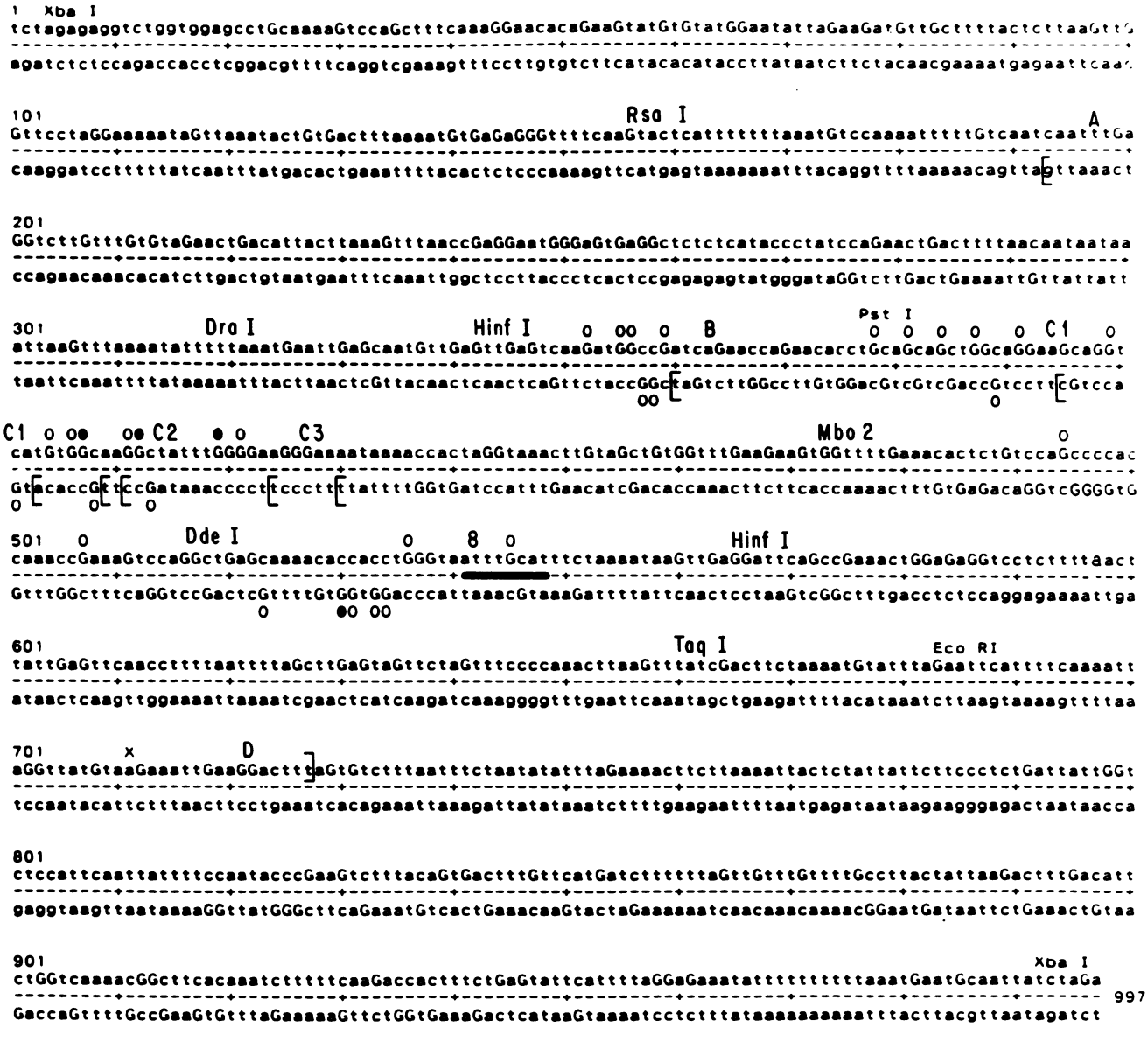
100bp

C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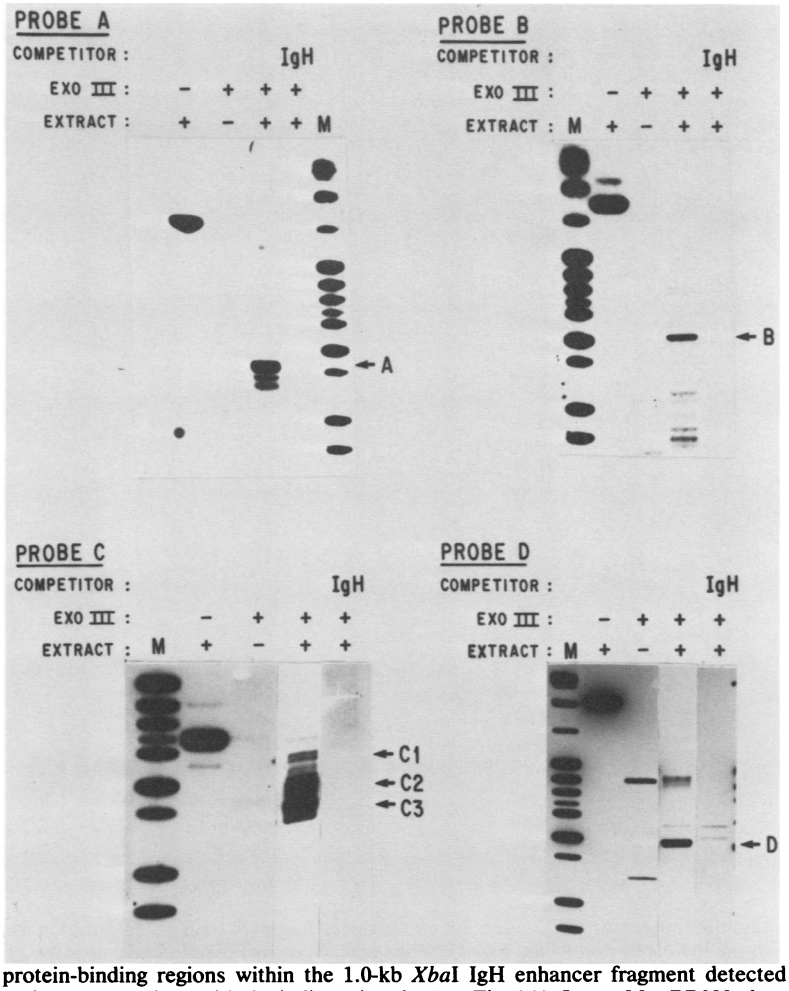

FIG. 2. Identification of protein-binding regions within the 1.0-kb *Xba*I IgH enhancer fragment detected by exoIII protection. Each lettered panel represents a set of exoIII reactions with the indicated probe (see Fig. 1A). Lanes M, pBR322 plasmid DNA digested with *Hpa*II and end labeled as markers. For each panel, the first lane contained probe incubated with extract but not exoIII digested. The second lane contained probe and no extract but was digested with exoIII. The third and fourth lanes contained probe incubated with 15 μg of DE.25 extract and digested with exoIII; lane 3 contained 3 μg of pBR322-*Hpa*II DNA and lane 4 (labeled IgH) contained 2 μg of pBR322-*Hpa*II and 1 μg of the *Xba* 1.0-kb IgH enhancer fragment (see the text for details). Arrows indicate the major extract-dependent DNA fragments protected from exoIII digestion. Several extract-independent bands were seen with probe D, in addition to the specific band, and one of these seemed to disappear with addition of IgH enhancer DNA. This extract-independent stop was unique in this property and was inconsistent between experiments; for this reason it was not examined further.

first control reaction (shown in lane 2 for each set) lacked protein extract and usually showed only the small-limit digestion products of the probe which run off the gel (for example, panels A through C), although some probes did show extract-independent protected fragments larger than limit digestion products (for example, probe D). A second control reaction was also run (shown in lane 4 for each set). This control involved prior incubation of the extract with an excess of unlabeled IgH enhancer DNA before addition of the labeled probe, allowing the unlabeled fragment to compete for sequence-specific binding proteins. The reaction mixtures in lane 3 lacked sequence-specific competitor but were preincubated with extra pBR322 DNA so that each reaction mixture was preincubated with equivalent weights of nonspecific (lane 3) or nonspecific plus sequence-specific (lane 4) competitor DNA. Protected fragments which were

FIG. 1. (A and B) Restriction maps of the 1.0-kb *Xba*I fragment containing the entire IgH enhancer. Lines labeled A, B, C, D, and 8 below each restriction map represent DNA restriction fragments used as labeled probes in exoIII protection assays (A) and gel retardation assays (B). Asterisks designate the position of the 5′ label for exoIII probes. ExoIII probes designed to detect binding at the conserved octamer region are not shown, but included a 303-bp *Pst*I-*Eco*RI fragment (5′ label at *Pst*I site), a 190-bp *Pst*I-*Hin*fI fragment (5′ label at *Pst*I site), a 170-bp *Dde*-*Eco*RI fragment (5′ label at *Eco*RI site), and a 185-bp *Mbo*II-*Taq*I fragment (5′ label at the *Mbo*II site). Solid circles on probe fragments indicate where specific protein binding has been determined (see Fig. 2, 3, and 4). Abbreviations: X, *Xba*I; R, *Rsa*I; H, *Hin*fI; Ps, *Pst*I; Dd, *Dde*I; E, *Eco*RI; Dr, *Dra*I; T, *Taq*I. (C) Identification of protein-binding site boundaries on the sequence of the IgH enhancer. The sequence is taken from Ephrussi et al. (9), and position 1 corresponds to the 5′ *Xba*I site shown in Fig. 1. Open circles above the sequence designate guanine residues where tissue-specific DMS protection was detected in vivo, and solid circles represent DMS enhancement (9). Lettered brackets designate the orientation and position (±2 bp) of in vitro extract-dependent exoIII stops shown in Fig. 2. The conserved octanucleotide sequence is underlined and labeled 8.

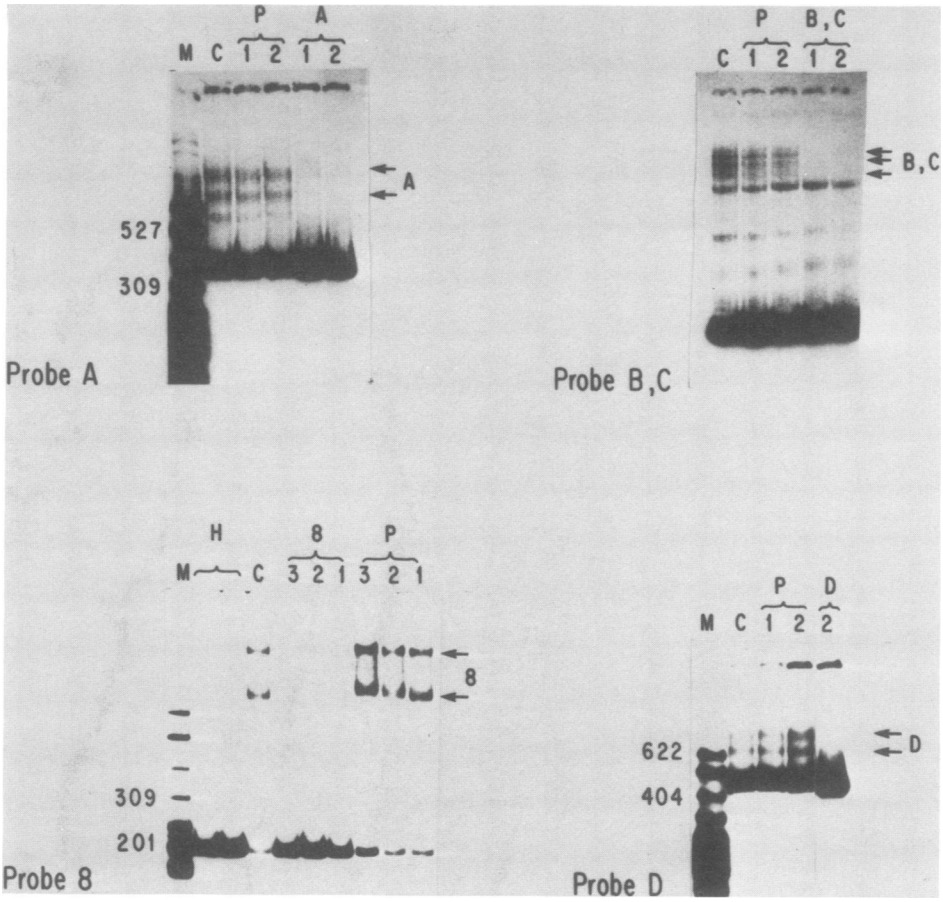

FIG. 3. Identification of protein-binding regions within the 1.0-kb enhancer fragment by a gel retardation assay. Each panel represents a set of gel retardation assays with the indicated probe (see Fig. 1B). Lanes C, Standard reaction mixtures for each probe. Lanes 1, 2, and 3, Reaction mixtures which were preincubated with unlabeled restriction fragments containing site A (350-bp *Xba*I-*Hin*fI fragment), site B/C (220-bp *Hin*fI fragment), site octamer (lanes 8) (220-bp *Hin*fI fragment), and site D (430-bp *Hin*fI-*Xba*I fragment). The competitor fragment (lanes P) represents pBR322-*Hpa*II DNA. The molar ratios of competitor fragments were 100:1 (lanes 1) or 200:1 (lanes 2) for probes A, B/C, and D, or 50:1 (lane 1), 100:1 (lane 2) and 200:1 (lane 3) for probe 8. Arrows indicate the major gel-retarded bands that were specifically competed with. Probes A, B/C, and D, 1.5 μg of HO.6 extract and 3 μg of poly(dI-dC) · poly(dI-dC); octamer probe, 4 μg of crude lymphoid nuclear extract and 4 μg of poly(dI-dC) · poly(dI-dC), except for the lanes labeled H, which received 1.5 μg of HO.6 and 2 or 3 μg of poly(dI-dC) · poly(dI-dC).

found to be both extract dependent and susceptible to competition with IgH enhancer were considered to result from the binding of sequence-specific proteins to IgH enhancer DNA.

The probes used for exoIII protection experiments (Fig. 1A) are capable of detecting protein-binding sites throughout the 1.0-kb enhancer fragment. Each panel in Fig. 2 illustrates a typical set of exoIII binding assays for the end-labeled probes shown in Fig. 1. These assays used a nuclear extract from plasmacytoma HPCM2 (13) which had been partially purified by ion-exchange chromatography on DEAE-Sephacel. Similar results were seen with crude nuclear extracts (see Fig. 5). Control lanes lacking extract or preincubated with IgH competitor are shown for each probe. Protected DNA fragments that were extract dependent and competed for by excess IgH enhancer DNA are indicated by the arrows adjacent to each panel. All the extract-dependent, sequence-specific fragments we observed were resistant to at least 8,000 U of exoIII per ml but were susceptible to treatment of the extract with trypsin (data not shown). Probe fragments which did not show extract-dependent, sequence-specific binding included the 165-bp *Dde*-**Eco*RI, the 185-bp **Mbo*II-*Taq*I, the 160-bp **Xba*-*Rsa*I, and the 230-bp *Mbo*II-**Xba*I fragments (the asterisk indicates the 5′-end-labeled site) (data not shown).

Four separate regions of the IgH enhancer were shown by this assay to bind protein. Probe A showed an IgH enhancer-specific fragment of 155 bp; probe B showed a 162-bp fragment; probe C showed a group of protected fragments ranging from 175 to 140 bp; and probe D showed a 162-bp protected fragment. The positions of these four binding-site regions within the 1.0-kb *Xba* fragment are indicated in Fig. 1. Based on competition data presented below, we believe that a minimum of three binding sites occur within the complex C region. Therefore, these four probes identified six spedific binding sites within the IgH enhancer.

However, some expected exoIII-protected fragments were not found. Probe B contains sequence corresponding to the A, B, and C binding sites. As seen in Fig. 2, smaller protected fragments corresponding to the C binding region were routinely seen with probe B, but a larger protected fragment corresponding to the A site was only apparent after

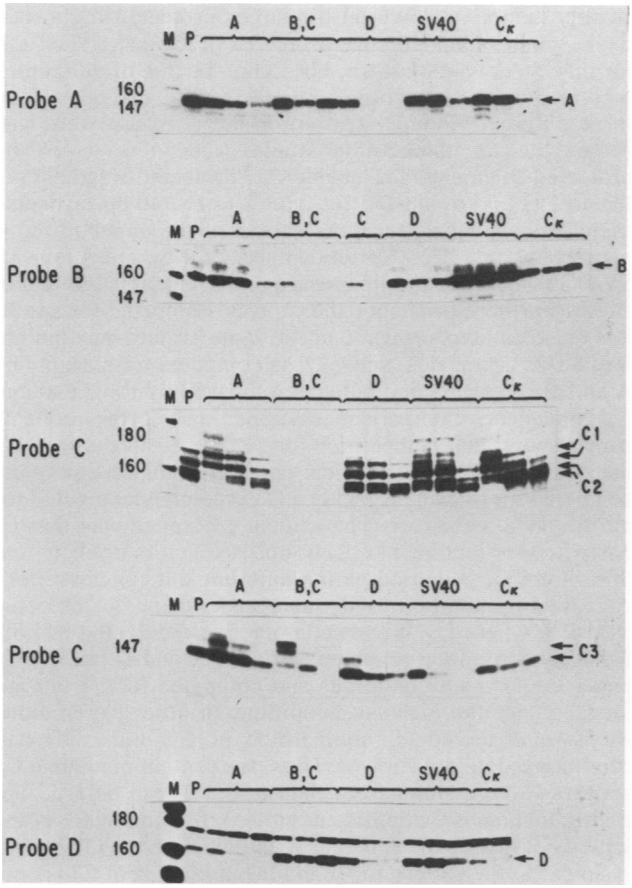

FIG. 4. Competition experiments. Each panel shows a set of parallel exoIII reactions that were preincubated with various unlabeled restriction fragments that contain the indicated binding site(s) (for details, see text). Arrows indicate the protein-binding sites that were identified in Fig. 2. Lanes P, Competitions with pBR322-*Hpa*II DNA present at an additional molar excess of 2,000-fold for probes A and B, 2,500-fold for probe D, and 1,000-fold for probe C. Sets of three reactions for other competitors represent a molar excess of competitor to probe of 70-fold, 140-fold, and 330-fold, from left to right. Sets of two reactions represents a molar excess of 70-fold and 140-fold. Part of the sample in lane 5 of panel 2 (probe B) was lost; this does not represent competition. Unlabeled competitor fragments included (see Fig. 1 for IgH enhancer subfragments) site A (350-bp *Xba*I-*Hin*fI fragment), sites B/C (220-bp *Hin*fI fragment), site C (190-bp *Pst*I-*Hin*fI fragment), site D (430-bp *Xba*I-*Hin*fI fragment), SV40 (440-bp *Pvu*II fragment [18]), and C_κ (1,000-bp *Hin*dIII-*Hpa*I fragment [35]). In addition to the indicated competitor fragments, reaction mixtures in panel 1 (probe A) contained 3 μg of HO.6 extract and 2 μg of pBR322-*Hpa*II DNA; reaction mixtures in panel 2 (probe B) contained 0.7 μg of HO.6 extract and 1 μg of pBR322-*Hpa*II; reaction mixtures in panel 3 (probe C) contained 3.7 μg of HO.6 and 1 μg of pBR322-*Hpa*II; reaction mixtures in panel 4 (probe C) contained 1 μg of QS.3 and 1 μg of pBR322-*Hpa*II; and reaction mixtures in panel 5 (probe D) contained 1.5 μg of HO.6 and 2 μg of pBR322-*Hpa*II. Reactions in panel 3 were performed at 90 mM NaCl, and panel 4 reaction mixtures were at 110 mM NaCl to allow better resolution of sites C1, C2, and C3.

long exposure and was very minor compared with the B and C sites (data not shown). In addition, we labeled probes A through D on the alternate strand to search for the opposite boundaries of the six identified binding sites, but no specific binding was detected. Finally, the heavy-chain enhancer contains an octanucleotide sequence which is also present in the promoter regions of immunoglobulin light- and heavy-chain genes and has been suggested to have a role in transcriptional regulation (10, 33). We used several probes in this region (Fig. 1) but failed to detect an exoIII-protected fragment corresponding to protein binding at the octamer, although protein binding at this site has been reported by Singh et al. (45). Therefore, we decided to use a second assay for protein binding to verify the sites suggested by the exoIII protection assay and to search for protein binding at the octamer region.

Gel retardation assays detect protein binding at the octamer site. Gel retardation assays are based on the fact that DNA fragments which have proteins bound to them have an altered electrophoretic mobility in polyacrylamide gels. Our assay conditions were similar to those of Singh et al. (45). Poly(dI-dC) · poly(dI-dC) was added in excess to all reaction mixtures to compete for nonspecific DNA-binding proteins. In addition, we compared the ability of pBR322 DNA and unlabeled enhancer DNA to compete with the labeled enhancer fragment for bound proteins. This control is similar to the competitor control used for the exoIII protection assays and demonstrates the sequence specificity of the bound protein detected in the assay.

Figure 3 shows the results of gel retardation assays with the labeled fragments indicated in Fig. 1. The A, B/C, octamer, and D probes all showed sequence-specific protein binding, as evidenced by retarded bands which were competed for by IgH enhancer DNA but not by plasmid DNA (sequence-specific retarded bands are indicated by arrows for each probe). An *Xba*I-*Rsa*I probe from the 5′ end of the enhancer region and an *Mbo*II-*Xba*I probe from the 3′ end of the enhancer showed no binding in the gel retardation assay (data not shown). The extracts used in the exoIII protection experiments (Fig. 4) and for gel retardation with probes A, B/C, and D (Fig. 3) had been heated at 67°C for 10 min and retained full binding activity. However, when heated extracts were used with the octamer probe, binding to the octamer was destroyed (Fig. 3, lane H), suggesting that protein binding at this site is different from that of the protein(s) binding to sites A through D.

Multiple retarded bands, which were observed with each probe shown in Fig. 4, could result either from protein-protein interactions involving a protein bound at one site or from binding of protein to multiple sites within the probe fragment. The gel retardation data alone do not distinguish between these possibilities. The multiple bands observed for the A and D probes, which showed one site of exoIII protection, could result from either multiple binding sites or protein-protein interactions. Singh et al. also noted multiple retarded bands with octamer probes, although they only mapped one binding site (45), suggesting protein-protein interactions. The complex banding pattern obtained with the B/C probe is consistent with multiple binding sites mapped in that region by exoIII protection assays (Fig. 2 and 4).

Evidence of binding to the A site probe and the octamer probe was of particular interest, since exoIII protection assays had detected binding at A with only one of two probes and had failed to detect binding to any octamer probe. The gel retardation results confirm those of Singh et al. (45) for the octamer binding site and are consistent with our exoIII protection results for the A, B, C, and D binding sites. Although we have not mapped the activity binding within the octamer probe further, we assume that it binds to the octamer region as previously shown by Singh et al. (45).

Multiple heavy-chain enhancer-binding proteins. ExoIII competition experiments were designed to compare the

TABLE 1. Summary of exoIII competition binding results[a]

Binding site	Competitor fragment binding					
	A	BC	C	D	SV40	C_κ
A	+	−	ND[b]	+	+	+
D	+	−	ND	+	+	+
C1	+/−	+	ND	+/−	+	+
B	−	+	+	−	−	−
C2	−	+	ND	−	−	−
C3	+/−	+	ND	+/−	+	−

[a] Data from Fig. 4. See legend to Fig. 4.
[b] ND, Not done.

ability of a particular protein to bind to two different DNA fragments. Increasing molar amounts of unlabeled competitor restriction fragments were tested for each labeled probe. Three kinds of information can be derived from these studies. First, by using subfragments of the IgH enhancer, we can estimate how many different proteins bind at sites A through D. Second, we can determine whether any of these proteins have the ability to bind to other enhancer sequences. Finally, the complex C region may be resolved into discrete binding sites by demonstration of differential competition at various sites. Since these assays were carried out with partially purified extracts, we have not attempted to interpret the results quantitatively in terms of relative binding affinities.

The results of the competition experiments are shown in Fig. 4 and summarized in Table 1. For each probe, we compared the ability of different unlabeled DNA fragments to compete for binding at three different molar ratios of competitor. A control lane in which a high molar ratio of pBR322 DNA was used as competitor is also included for each probe for comparison with the enhancer-containing competitor fragments. The unlabeled competitor fragments tested included (refer to Fig. 1 for IgH enhancer subfragments) site A (350-bp *Xba-Hinf* fragment), sites B/C (220-bp *Hinf* fragment), site C only (190-bp *Pst-Hinf* fragment), site D (430-bp *Hinf-Xba* fragment), the SV40 early region containing the enhancer sequences (440-bp *Pvu*II fragment [18]), and the immunoglobulin kappa light-chain enhancer (1,000-bp *Hpa*I-*Hin*dIII fragment [35]). A *Pvu*II fragment from the vector PA10CAT2 (24) containing the SV40 early region but lacking the SV40 enhancer region did not compete in these experiments (data not shown).

As shown in the probe A panel, site D, the SV40 enhancer and the kappa enhancer competed for binding at site A; sites B/C did not compete. Similarly, protein binding at site D (probe D panel) was competed for by site A, the SV40 enhancer, and the C_κ enhancer but not by sites B/C. In contrast, only the B/C and C-only sites were able to compete for protein binding at site B (probe B panel); fragments containing site A, D, the SV40 enhancer, and the C_κ enhancer did not compete. (Lower signal in the third lane of probe B, fragment A, competition is due to underloading of the gel; no competition for the B site was observed with the A fragment in other experiments.) These results suggest that the same protein binds at sites A and D but that a different protein binds at site B. The protein which bound to sites A and D appears to be a general enhancer protein since it also bound to SV40 and C_κ enhancers. The B site protein recognized only the IgH enhancer sequence but not the SV40 or C_κ enhancer.

To study the complex C region, we used two different NaCl concentrations because we had noted previously that slightly higher salt favored the larger protected fragments, while smaller fragments were favored in low salt. The basis for this effect is not known, but it may be due to differential binding affinities of proteins bound in this region. At least three different binding sites within the C region were suggested by the competition studies. The 175- to 167-bp protected fragments are labeled C1, protected fragments of 161 to 159 bp are labeled C2, and 146- to 140-bp protected fragments are labeled C3. As shown in the upper probe C panel, binding at C2 was not competed for by site A, site D, SV40 enhancer, or C_κ enhancer, a pattern identical to that of site B. Furthermore, since the C probe competed for site B, it is reasonable to suggest that the same protein may bind to B and C2. In contrast, site C1 was competed for partially by A and D fragments and competed for well by the SV40 and C_κ enhancers. Comparison of upper and lower probe C panels shows that competition for binding at sites C1 and C3 had a similar but not identical pattern: both were partially competed for by sites A and D and completely competed for by the SV40 enhancer. The critical difference was that C_κ competed for binding at C1 but not at C3. It is unlikely that this difference is caused by the different salt concentrations or extract preparation used, since sites where C_κ competed and sites where C_κ did not compete were detected in both conditions—i.e., C_κ competed for sites A and D but not C3 under the low-salt conditions and competed for C1 but not for C2 under the high-salt condition. In other experiments we have observed C_κ competition at C1 under low-salt conditions (data not shown). Thus, the protein binding at C3 appears to be distinct from that at C1. These data do not clearly distinguish whether the protein binding at C1 is the same as or distinct from that at A and D, since the C1 pattern is similar to the pattern for A and D but binding at C1 is only partially competed for by sites A or D.

The octamer-binding protein was distinguished from the proteins binding at A through D by its heat lability (Fig. 3). Singh et al. (45) showed that the octamer-binding protein binds to both the kappa promoter and the heavy-chain enhancer. The sequence also occurs in heavy-chain promoters, SV40 enhancer, and regulatory sites of other genes (45). Thus, this protein appears to be a general promoter-enhancer factor.

In summary, the competition experiments suggest that a minimum of three different proteins bind in the A, B, C, and D regions. This number could be an underestimate since there may be differences between the proteins which were not detected by binding competition. The octamer-binding protein is distinct from proteins binding to A through D, as demonstrated by its heat lability, making a minimum total of four proteins which bind in the IgH enhancer. Three of the four bound to other enhancers or promoters and appear to be general factors.

IgH enhancer-binding proteins found in extracts from nonlymphoid cells. Three kinds of in vivo studies—DNase I hypersensitivity studies, functional competition studies, and dimethyl sulfate (DMS) protection studies—have suggested that one or more B-cell-specific factors are required for IgH enhancer activity (9, 28, 29). However, it has already been shown that the octamer-binding protein is not B cell specific (45). We wished to determine the tissue distribution of the proteins binding to sites A through D. When crude nuclear extracts were prepared from B cell, fibroblast, T cell, and macrophage lines and tested for binding activity, there were no apparent differences in the binding of proteins to the *Xba* 1.0-kb enhancer fragment (Fig. 5). Each labeled panel corresponds to the probe used in the set of reactions.

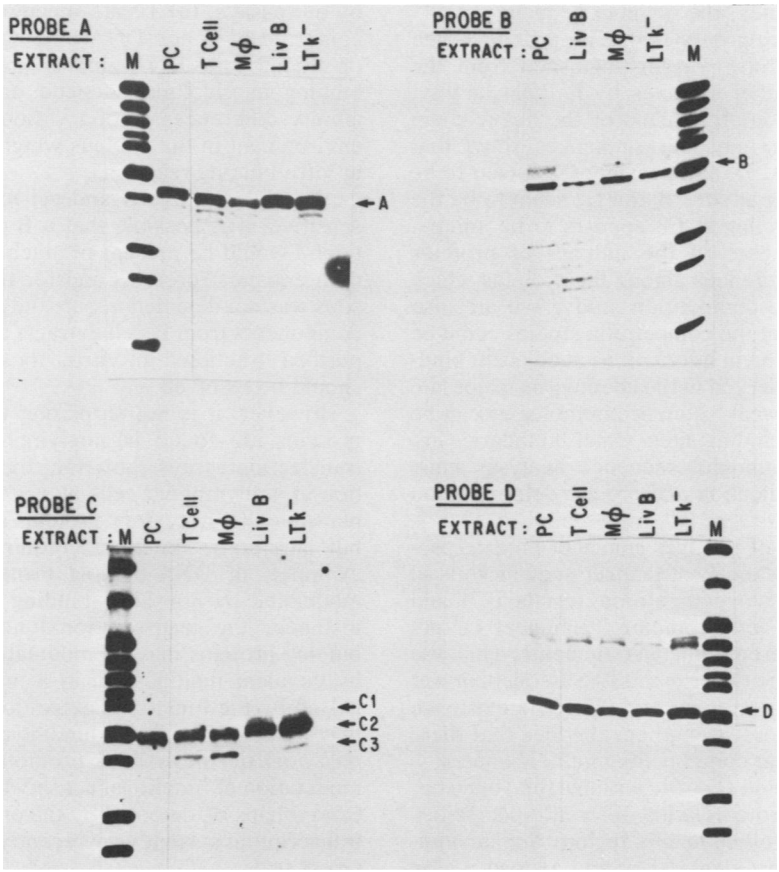

FIG. 5. Tissue distribution of IgH enhancer-binding proteins. Each panel shows reactions for each probe shown in Fig. 1A. These reactions used 50 μg (2×10^6 to 3×10^6 cell equivalents) of crude nuclear extracts and 10 μg of pBR322-*Hpa*II. Binding to sites C1 and C3 was apparent at longer exposures, but it is more difficult to see all three binding sites at one protein concentration with crude extracts than with more purified extracts. Cell lines: PC, plasmacytoma P3X-AG.63; T cell, EL4; Mφ, Pu5; Liv B, fibroblast; Ltk−, fibroblast.

DISCUSSION

Comparison of two assays for DNA-binding proteins. We used two assays to detect cellular proteins in crude extracts which bind to the IgH enhancer in vitro. Each assay has strengths and weaknesses which are worth noting. ExoIII protection depends on competition between exoIII and another protein; the nature of this interaction is not well understood. Differences in relative affinity for DNA between exoIII and the binding protein may explain why we detected protein binding at site A when it was located near the center of probe A but not when it was located at the end of probe B. (Presumably, however, protein binding at site C1 competes more effectively with exoIII because binding at site C1 was detected when it was at the end of probe C.) Similarly, low affinity relative to exoIII might explain why the octamer-binding protein could not be detected with exoIII. Our inability to detect binding on both DNA strands by the exoIII assay suggests that another limitation of the assay may be that exoIII digestion is not inhibited when a protein does not contact a sufficient number of bases along a given strand. It is possible that the proteins that bind to sites A through D may bind to only one strand, as has been observed for the transcription factors SP1 and TFIIIA (14, 38), although it is more likely that weaker binding occurs to the alternate strand, which the exoIII assay did not detect. Footprinting studies are under way to define the binding regions on both strands more precisely.

Our results indicate that exoIII protection assays detect only a subset of all DNA-protein interactions. This limitation does not, however, invalidate the binding sites which are detected. Verification of binding at sites A through D by gel retardation and the demonstration of sequence-specific competition establish the validity of the sites detected by exoIII. In addition, exoIII protection has the advantage of showing the approximate location of bound proteins, and at least in some cases, binding at more than one site on a single probe can be distinguished. In contrast, the gel retardation assay appears to detect a larger subset (possibly all) of binding sites. It has the disadvantages that binding sites cannot be located within the probe and that multiple binding sites that occur on a single probe cannot be resolved. Our approach of using both assays circumvents the problems of each, although we still cannot be certain that we have detected all the protein-binding sites within the heavy-chain enhancer. These two assays will allow further purification and biochemical characterization of the binding proteins which we have identified.

At least four proteins with different specificities bind to the IgH enhancer. A combination of exoIII and gel retardation assays allowed us to identify seven different binding sites for cellular proteins within the 1.0-kb heavy-chain enhancer

sequence. One of these sites, the octamer sequence at 545 bp, has been identified previously (45). The protein which bound to the octamer probe was distinguished from the proteins binding to the other six sites by its heat lability. Competition studies with subfragments of the heavy-chain enhancer and with other enhancer sequences show that proteins binding at sites A, D, and possibly C1 appear to be the same; proteins binding at sites B and C2 seem to be the same; and the protein binding at C3 appears to be unique. This is a minimum estimate of the number of proteins because there may be differences among the proteins which were not detected by the competition study. We are also aware that interpretation of the competition studies could be complicated by protein-protein interactions at adjacent binding sites similar to that observed in the adenovirus major late promoter (40). Although weak sequence homologies can be found between common binding sites, exact boundaries are not established, and meaningful sequence analyses must await further protein purification and precise determination of binding domains.

The ability of a subset of the IgH enhancer-binding proteins to bind to the SV40 and C_κ enhancer sequences is in agreement with the in vivo competition results (27) and provides direct evidence for "common" enhancer factors capable of binding to different enhancer elements. This was suggested previously by the experiments of Sassone-Corsi et al. with an in vitro transcription system (39). The existence of common enhancer factors strengthens the idea that there may be common functional components in all enhancers.

Four sites—B, C1, C2, and C3—fall within a total of 80 bp. Our results show that proteins with different specificities probably bind near each other in this region. Similar mingling of different regulatory elements within a small region has been observed in other systems (21; M. Karin, personal communication) and may provide a means of generating regulatory diversity as well as regulatory specificity. It also suggests that protein-protein interactions may have an important function in regulatory regions such as enhancers and promoters.

Neither assay used in this study relates protein binding to enhancer function. Site-directed mutagenesis studies are under way to establish the functional significance of the seven protein-binding sites. However, previous in vivo competition studies (27) showed that factor binding in the enhancer region is required for enhancer activity. As indicated in Fig. 1C, sites B, C1, C3, octamer, and D are located near or within regions identified as protein-binding sites in vivo by a DMS protection assay (9). The region surrounding site A has not been investigated for DMS sensitivity. Earlier studies by Gillies et al. demonstrated that deletion of a region which includes site D resulted in decreased enhancer activity (15). Similarly, other deletion studies suggest that the 220-bp *Hinf* fragment, which contains sites B, C1, C2, C3, and the octamer, contains sequences responsible for most of the IgH enhancer activity (T. Kadesch, personal communication). Therefore, it is likely that many or all of the protein-binding sites we have identified in vitro are necessary for enhancer function.

Lack of B-cell specificity in the enhancer-binding proteins. The apparent lack of tissue specificity in the occurrence of the enhancer-binding proteins was unexpected, because in vivo studies show tissue-specific DNase I hypersensitivity at the enhancer (29), tissue-specific competition for function (27), and tissue-specific DMS protection (9). We have considered several plausible explanations for our results. (i) A tissue-specific DNA-binding protein may have been missed by our assays. (ii) Tissue-specific enhancer activity may be conferred by a non-DNA-binding protein which modulates or modifies the DNA-binding proteins. (iii) Tissue-specific binding in vivo may depend on subtle concentration or affinity differences among various factors within a microenvironment in the nucleus which is not reproduced by our in vitro binding reaction.

Although the exoIII and gel retardation assays are very sensitive, it is possible that a B-cell-specific enhancer protein(s) would be present in much lower amounts than common enhancer proteins and for this or other technical reasons was not detected in our study. As we continue to purify components from B-cell extracts and are able to mix various purified fractions in vitro, these technical uncertainties should be resolved.

However, it is not surprising that IgH enhancer-binding proteins are found in nonlymphoid cell types. Transient transfection assays show that the IgH enhancer does function in nonlymphoid cells at a very low level (27). It seems plausible that necessary proteins are present in all cell types but lack some functional requirement. There are known examples of DNA-binding proteins that are functionally modulated by non-DNA-binding proteins (16, 20, 25). For instance, the transcriptional activity of IgH enhancer-binding proteins may be modulated in a tissue-specific way by covalent modification in a manner similar to that suggested by the functional activation of heat shock transcription factor, a preexisting binding component of a *Drosophila melanogaster* heat shock promoter (32, 44). Tissue-specific modulation of functional activity that does not affect binding can perhaps be detected by the enhancer-dependent in vitro transcription system very recently described by Scholer and Gruss (42).

Alternatively, if tissue-specific binding of a particular enhancer protein and subsequent enhancer activity are determined kinetically by subtle differences in the concentrations or affinities of different enhancer proteins within a given microenvironment, it may be difficult to duplicate this situation in vitro. Such a situation might be similar to that described by Brown and Schlissel for 5S RNA transcription factors (4). However, purification of the known components and quantitative determination of their abundance and binding affinities in different cells will address this question.

Taken together, our results suggest that for enhancer sequences to activate transcription, complex interactions involving several different proteins are likely to be required. In vitro identification of IgH enhancer-binding proteins provides a starting point for biochemical analysis of these fascinating events.

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ADDENDUM

Recently, Singh and Baltimore (Cell **46**:705–716) have reported binding of proteins to two sites in the IgH enhancer

at positions that appear to correspond to the C2 and octamer sites described in this paper. In addition, Weinberger, Baltimore, and Sharp (Nature (London) 322:846–848) have recently mapped a third binding site on the IgH enhancer which appears to correspond to the site B that we have identified.

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