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Complex Protein Binding within the Mouse Immunoglobulin Heavy-Chain Enhancer

CRAIG L. PETERSON[1] AND KATHRYN L. CALAME[1,2]*

Molecular Biology Institute[1] and Department of Biological Chemistry,[2] University of California at Los Angeles, Los Angeles, California 90024

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We have begun to purify and characterize several proteins which bind to the mouse immunoglobulin heavy-chain enhancer to understand the molecular interactions important for enhancer activity. Three proteins which bind to different sites on the immunoglobulin heavy-chain enhancer have been chromatographically separated and partially purified. One protein binds a site which has not been reported previously and does not bind to other reported protein-binding sites on the immunoglobulin heavy-chain enhancer. Binding-site boundaries for the three partially purified proteins have been precisely mapped by methylation interference, DNase I footprinting, and orthophenanthroline/copper chemical nuclease footprinting. We have also characterized these three proteins with respect to dissociation rate constants.

Transcriptional enhancer elements have been defined as *cis*-acting DNA sequences which stimulate the initiation of transcription from heterologous promoters in an orientation- and distance-independent fashion (2, 3, 6, 16, 19, 24, 37). Enhancers have been identified which are tissue specific, such as the immunoglobulin heavy chain (IgH) enhancer, or inducible, such as the β-interferon enhancer (2, 10, 11, 14, 23). Deletion analyses of several enhancers suggest that enhancers contain multiple independent elements which act together to elicit transcriptional stimulation (17, 38, 40). Though the precise mechanism of enhancer function has not been determined, in vivo and in vitro studies have demonstrated that enhancer function requires interactions between *trans*-acting proteins and enhancer sequences (22, 29, 31–33).

We have previously demonstrated by exonuclease III protection and gel retardation assays that a 1-kilobase *Xba*I fragment containing the mouse immunoglobulin heavy-chain enhancer contains seven binding sites for cellular proteins (26). One of these binding sites, an octamer sequence also found in immunoglobulin and nonimmunoglobulin gene promoters, is recognized by several proteins, one of which is B-cell specific (20, 35). Four binding sites, designated B, C1, C2, and C3, are clustered just 5′ of the octamer; sites A and D are located further 5′ and 3′, respectively, of the central cluster of binding sites (Fig. 1). Our earlier study showed that sites A, D, C1, and C3 were recognized by general enhancer proteins which also bound to the simian virus 40 or immunoglobulin kappa light-chain enhancers while sites B and C2 were bound by a protein(s) which appears to be more specific for the IgH enhancer. We (26) and others (30, 34) have also found that, with the exception of the octamer-binding protein, immunoglobulin heavy-chain enhancer-binding proteins do not have a B-cell-specific distribution.

To understand more precisely the nature of protein-DNA interactions important for IgH enhancer activity, we have begun to purify several enhancer-binding proteins, focussing on those which appear to be more specific for the IgH enhancer. Here we report the identification of an additional protein binding site, E, 5′ to site B and the chromatographic separation, partial purification, and characterization of proteins binding to sites E, B, and C2.

MATERIALS AND METHODS

Fractionation of nuclear extracts. Nuclear extracts were prepared from plasmacytoma M603 grown subcutaneously in C×D2 F1 mice as previously described (26, 36), except the detergent lysis buffer contained 30% sucrose. For the assay in Fig. 1A, lane a, extract was prepared from plasmacytoma cell line MPC11.

Nuclear extracts were prepared and heat treated as previously described (26), with two modifications: (i) nuclear extracts were dialyzed into chromatography buffer C {20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 8.0, 0.2 mM EDTA, 0.2 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.5 mM dithiothreitol [DTT], 100 mM NaCl, 20% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 10 U of aprotinin per ml, 0.5 μg of leupeptin per ml, 0.5 μg of pepstatin A per ml} before heat treatment; (ii) heated extracts were clarified at 100,000 × *g* for 60 min. Heated extracts from approximately 60 g of M603 tumor tissue were adjusted to 350 mM NaCl and then chromatographed successively on a 6-ml DEAE-Sephacel (Pharmacia) column and a 5-ml heparin agarose column as previously described (26).

The heparin agarose 0.6 M NaCl step elution (H0.6) fraction obtained from approximately 60 g of M603 tumor was desalted on a 30-ml Sephadex G-25 (Pharmacia) column into Mono Q buffer (50 mM Tris [pH 8.1], 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 10 U of aprotinin per ml, 0.5 μg of leupeptin per ml, 0.5 μg of pepstatin A per ml, 0.1 mM phenylmethylsulfonyl fluoride) containing 30 mM NaCl, clarified by centrifugation for 15 min at 12,000 × *g*, and chromatographed on a FPLC HR5/5 Mono Q column (Pharmacia) equilibrated in the same buffer. The column was washed with 5 ml of Mono Q buffer containing 30 mM NaCl and then developed with a 25-ml linear 30 to 500 mM NaCl gradient in Mono Q buffer. The salt gradient was followed by a 5-ml step elution with Mono Q buffer containing 1 M NaCl. Fractions were collected into glass tubes which had been treated with dichlorodimethylsilane (silanized). Fractions were assayed by gel retardation, and active fractions were

* Corresponding author.

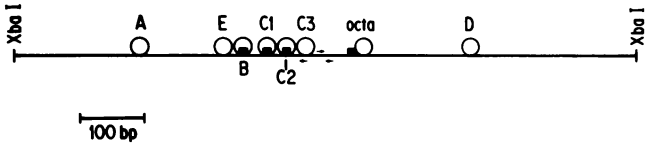

FIG. 1. Schematic representation of protein-binding sites on the IgH enhancer. Circles designate previously identified protein-binding sites (26) and an additional site, E, from this work. Closed boxes represent the four homology blocks identified by in vivo protein-binding studies (7). Arrows designate simian virus 40 enhancer core homologies (17).

pooled. Acetylated bovine serum albumin (13) was added at a 200-μg/ml final concentration to all pools, but only to 0.5 ml of pool [22–28]. The remainder of pool [22–28], 3 ml, was stored overnight at 0°C for subsequent chromatography. Pools to be stored were dialyzed into chromatography buffer C (50 mM Tris [pH 7.5], 0.2 mM EDTA, 0.2 mM EGTA, 100 mM NaCl, 0.5 mM DTT, 50% glycerol, and proteinase inhibitors as in Mono Q buffer) and stored at −20°C.

For further fractionation of site C2 binding activity, pool [22–28] was desalted on a 30-ml Sephadex G-25 column into mono-S buffer (50 mM $NaPO_4$, pH 6.8, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol, and proteinase inhibitors as in Mono Q buffer) containing 50 mM NaCl, clarified as described above, and chromatographed on an FPLC HR5/5 Mono S column (Pharmacia) equilibrated in the same buffer. The column was washed with 5 ml of Mono S buffer containing 50 mM NaCl and then developed with a 25-ml linear gradient containing 50 to 500 mM NaCl in Mono S buffer. The gradient elution was followed by a 5-ml step elution with Mono S buffer containing 1 M NaCl. Fractions were collected into silanized plastic microcentrifuge tubes. Fractions were assayed by gel retardation, fractions 30 to 35 were pooled, and 200 μg of acetylated bovine serum albumin per ml was added. Mono S pool [30–35] was frozen in small samples in liquid N_2 and stored at −80°C. All protein determinations were by the method of Bradford, with bovine serum albumin as the standard (4).

All chromatography steps were performed at 0 to 2°C, except for the FPLC Mono Q fractionation step; cold buffers were circulated over the column, which was set up at room temperature. The FPLC system was placed at 4°C for Mono S chromatography.

Gel retardation assays. A 200-base pair (bp) *Dra*I-*Dde*I restriction fragment 5′-end labeled at the *Dde*I site was used for most gel retardations, except for a coding strand-labeled probe which was this same fragment but 3′-end labeled with the Klenow fragment of *Escherichia coli* polymerase I and [α-^{32}P]dATP and [α-^{32}P]dCTP (see reference 8 and Fig. 8 for position on IgH enhancer). The gel retardation assays were performed as previously described with the following modifications (26). Binding reactions were performed in 12 μl, containing binding buffer (50 mM Tris [pH 7.5], 5 mM $MgCl_2$, 0.5 mM DTT), 0.5 to 1.0 ng of 5′-end-labeled probe DNA, variable amounts of poly(dIC:dIC) competitor DNA (Pharmacia), 50 to 70 mM NaCl, and protein extract. Binding reactions with unfractionated nuclear extracts routinely contained 3 to 5 μg of poly(dIC:dIC) competitor, heated and H0.6 fractions contained 1 to 2 μg, and Mono Q and Mono S fractions contained 200 ng. Binding reactions were incubated at 24°C for 30 min prior to loading onto 6% acrylamide gels in 1× TBE (89 mM Tris, 89 mM borate, 2 mM EDTA). Gels were electrophoresed at 20-mA constant current at 24°C.

Quantitative estimates of binding activity were obtained with the gel retardation assay. For each purification step, a three-point titration of binding activity was compared with the previous step. One unit of binding activity is defined as the amount of gel retardation activity in 10 μg of the crude nuclear extract.

Methylation interference and DNase I footprinting assays. Methylation interference assays were performed as described previously (12), except that DE81 paper (Whatman) was used for the electroelution.

DNase I footprinting assays were performed in 40-μl volumes, and reaction mixtures contained gel retardation binding buffer, 0.5 to 1 ng of probe DNA, 200 ng of poly(dIC:dIC) competitor DNA, 50 to 70 mM NaCl, and protein extract. Reactions lacking extract contained chromatography buffer C instead of extract. Binding reactions were incubated as described for gel retardation assays, and then 2 to 200 ng of DNase I (Worthington Diagnostics), diluted fresh from a 1-mg/ml stock into 50% glycerol–2 mM $MgCl_2$, was added and incubation was continued for 1 min. The amount of DNase I had to be titrated for each Mono Q pool to produce equivalent digestions. The DNase I digestions were stopped with 5 volumes of urea-sodium dodecyl sulfate stop (175 mM NaCl, 5 mM Tris [pH 7.5], 10 mM EDTA, 3.5 mM urea, 1% sodium dodecyl sulfate), phenol extracted, and ethanol precipitated. Samples were then loaded onto 8% acrylamide–8 M urea gels. The noncoding strand-labeled probe was a 360-bp *Rsa*I-*Dde*I restriction fragment 5′-end labeled at the *Dde*I site. The coding strand probe was this same fragment but 3′-end labeled at the *Dde*I site with Klenow fragment and [α-^{32}P]dATP and [α-^{32}P]dCTP (see Fig. 8 and reference 8 for position of this fragment on the enhancer sequence).

Dissociation rate constant analysis. Dissociation rate constants were determined by a modified version of the technique of Fried and Crothers (9). Standard binding reactions were incubated until equilibrium, 30 min at 24°C. For 24°C determinations, an 80-fold molar excess of unlabeled 1.0-kilobase *Xba*I-*Xba*I restriction fragment (2 to 3 μl) was added, and samples representing six to eight time points were loaded onto standard 6% TBE gels at 24°C while the gels were running. For assays at 0°C, following the incubation at 24°C, the binding reactions were cooled to 0°C before addition of precooled competitor DNA. These gels were loaded and electrophoresed at 2°C. These assays contained 6 to 10 time points. In all assays the zero time point received 2 to 3 μl of TE-1 (10 mM Tris [pH 7.5], 0.1 mM EDTA) instead of competitor. Autoradiographs were quantitated by densitometry, and data were plotted according to the first-order rate equation of Riggs et al. (27).

OP/copper footprinting. A preparative-scale gel shift reaction [5-fold in extract, poly(dIC:dIC), and volume, but 10-fold in probe] was electrophoresed on a standard gel retardation gel. The whole gel was then treated with the 1,10-phenanthroline (OP)/copper reagent which cleaves DNA within the gel, using a procedure developed by M. Kuwabara and D. Sigman (submitted for publication). The DNA bands were isolated by electroelution onto DE81 paper as described for methylation interference, and isolated DNA was electrophoresed on 10% acrylamide–8 M urea gels. Purine sequencing reactions were performed by the method of Maxam and Gilbert (21).

RESULTS

A sensitive gel retardation assay has been used throughout purification for rapid analysis of DNA-binding activity. We

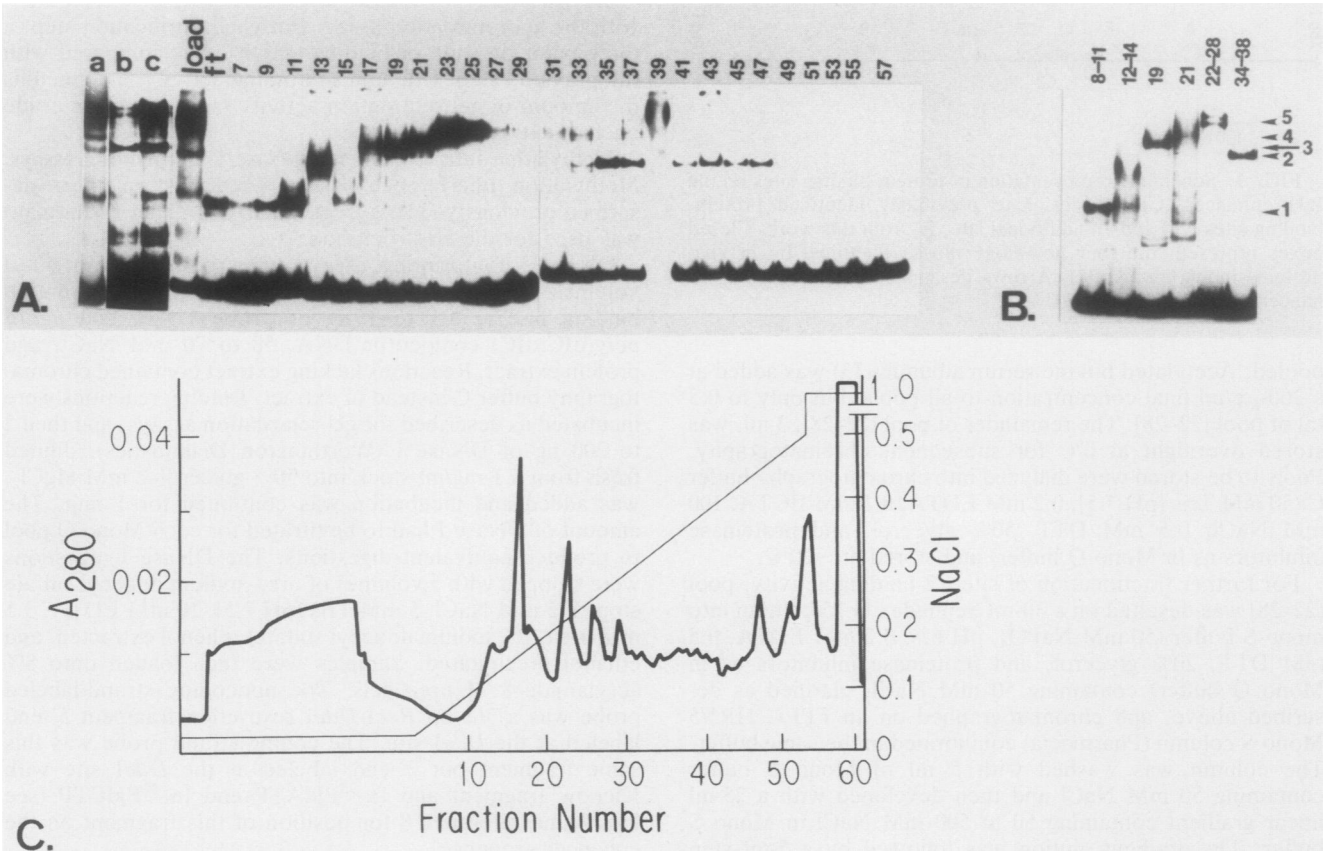

FIG. 2. Separation of IgH enhancer-binding proteins by FPLC Mono Q chromatography. (A) Gel retardation assays. All gel retardation assays used the 200-bp *Dra*I-*Dde*I probe. Binding reactions included 2 μl of plasmacytoma MPC11 nuclear extract and 4 μg of poly(dIC:dIC) (lane a), 1 μl of M603 nuclear extract and 4 μg of poly(dIC:dIC) (lane b), 3 μl of heated M603 extract and 2 μg of poly(dIC:dIC) (lane c), 1 μl of H0.6 fraction and 1 μg of poly(dIC:dIC) (load), 2 μl of the Mono Q flowthrough pool and 200 ng of poly(dIC:dIC) (lane ft), or 2 μl of the indicated Mono Q gradient fractions and 200 ng of poly(dIC:dIC). (B) Gel retardation activity of Mono Q pools. Numbered lanes represent the fractions which were pooled. Reactions contained 2 μl of the indicated pools and 200 ng of poly(dIC:dIC). Arrows designate primary gel retardation complexes. (C) Mono Q gradient elution profile. The conductivity plot was taken directly from the chart recorder; actual conductivity lags four fractions from the recorded value (an observed value for fraction 20 is the actual value for fraction 16).

have limited our purification at this point to proteins which bind to a 200-bp *Dra*I-*Dde*I subfragment of the enhancer, using this fragment as a probe for our gel retardation assays. This fragment contains four previously identified binding sites: B, C1, C2, and C3 (Fig. 1). Competition studies using a variety of assays (26, 30, 34, 39) suggest that several different proteins bind to the sites contained on this probe.

Protein degradation is a potentially serious problem during purification of DNA-binding proteins because partially degraded proteins may retain binding activity. Although this problem cannot be definitely ruled out until antibodies to the factors are available or the factor genes are cloned, we find no evidence that our extract preparations are seriously degraded. We initially investigated two different methods for isolation of nuclei prior to extract preparation: hypotonic lysis (7) and Nonidet P-40–sucrose lysis (36). We also compared binding activity of crude nuclear extracts prepared from solid plasmacytoma tumors with that of extracts prepared from plasmacytoma or fibroblast cells grown in culture. A complex pattern of protein binding was observed with the *Dra*I-*Dde*I probe in a gel retardation assay with these extracts; however, there was no distinguishable difference among the patterns observed in these extracts (data not shown). Thus, we conclude that neither the method of preparing nuclei nor the tissue source of the nuclei has a significant effect on the number or intactness of the proteins which bind to our probe. Since extracts from Nonidet P-40–sucrose lysis nuclei consistently appeared more active and since we desired a convenient source of B cells, we chose to begin the purification with extracts from Nonidet P-40–sucrose-lysed M603 tumor cells.

FPLC Mono Q chromatography separates multiple IgH enhancer-binding proteins. As previously described, proteins binding to sites B, C1, C2, and C3 on the *Dra*I-*Dde*I fragment of the IgH enhancer are relatively heat stable; therefore, an initial purification step involved heating nuclear extracts at 67°C for 10 min (26). This results in no loss in binding activity (Fig. 2A, lanes b and c). This heated extract was chromatographed on DEAE-Sephacel to remove residual nucleic acid, and the eluate from the DEAE-Sephacel column was then chromatographed on heparin agarose as previously described (26). These purification steps retain all gel-retarded complexes observed in the original crude nuclear extracts from M603 tumors (lane b) or from crude extracts made from nuclei prepared from different cell types or by different lysis procedures (data not shown).

To separate these enhancer-binding proteins, the heparin

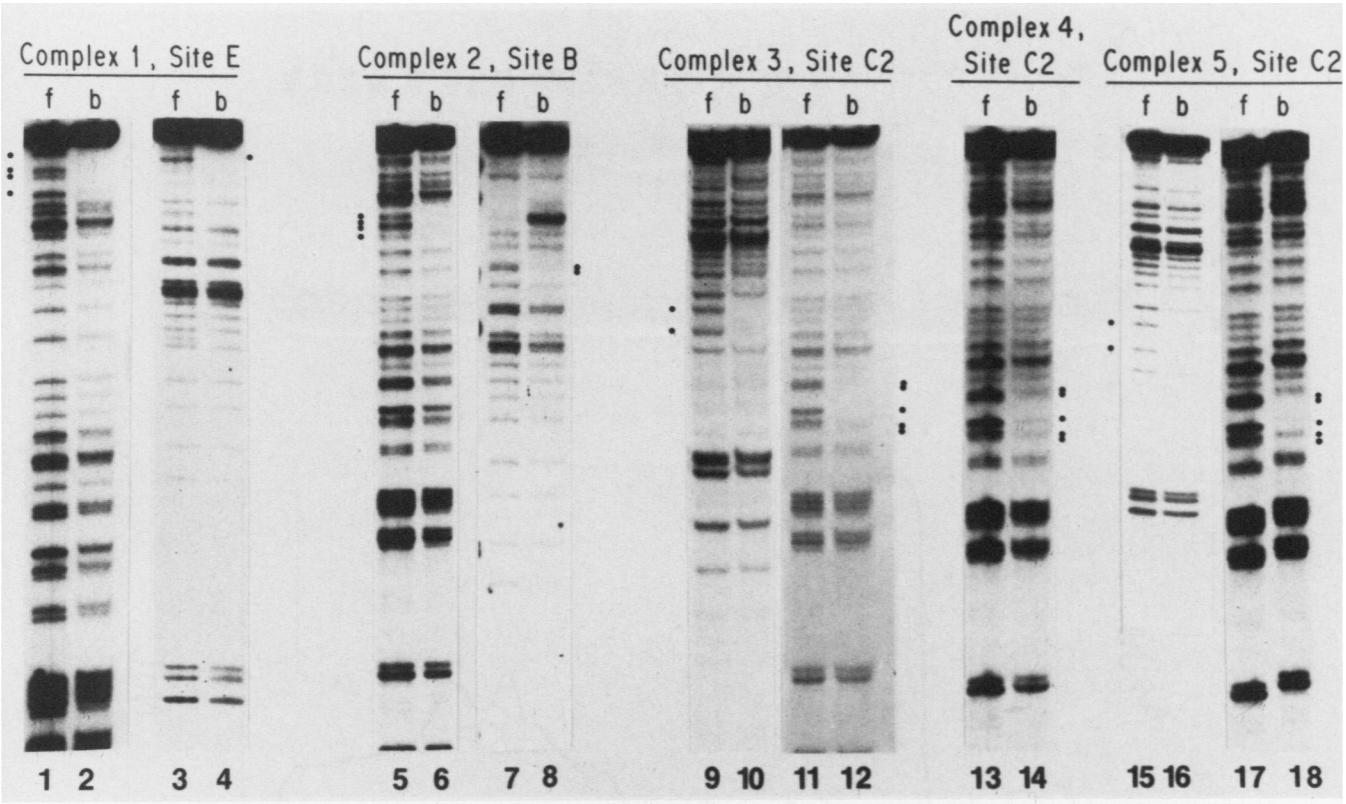

FIG. 3. Methylation interference analysis of gel retardation complexes from Mono Q gradient elution. Lanes labeled f represent DNA isolated from the free region of the gel retardation gel, while lanes labeled b represent DNA isolated from the indicated gel retardation complex. Dots designate guanine residues absent from the bound lanes and presumably inhibit protein binding when methylated. Assays shown in lanes 1, 2, 5, 6, 11 to 14, 17, and 18 utilized the 200-bp *Dra*I-*Dde*I probe labeled on the coding strand, while assays in lanes 3, 4, 7 to 10, 15, and 16 used this fragment labeled on the noncoding strand. Complex 1 activity was obtained from Mono Q pool [8–11]; complex 2, from pool [34–38]; complex 3, from pool [19]; complex 4, from pool [21]; and complex 5, from pool [22–28]. The intense band in lane 8 which is not present in lane 7 represents partially reannealed probe DNA.

agarose fraction was loaded onto an FPLC Mono Q anion-exchange column. Binding activities were eluted with a linear NaCl gradient, and fractions were assayed by gel retardation (Fig. 2A). This high-resolution chromatography step resulted in the separation of proteins which produce five different gel retardation complexes, providing direct evidence that numerous chromatographically distinct proteins bind to this portion of the IgH enhancer. Similar gel retardation profiles for the Mono Q gradient elution were observed in six other preparations. Peak fractions of proteins generating electrophoretically distinct gel retarded complexes were pooled (Fig. 2B): protein responsible for the lower complex, complex 1, is found in pools flowthrough (ft), [8–11], and [12–14]; protein responsible for complex 2, in pool [34–38]; protein for complex 3, in pool [19]; protein for complex 4, in pool [21]; and protein for complex 5, in pool [22–28].

Methylation interference analysis localizes three protein-binding sites. To map the binding sites for the different gel retardation complexes, we used the methylation interference assay as described by Gilman et al. (12) (Fig. 3). This assay allows the identification of guanine residues that are in close contact with bound protein because methylation at the N7 position of these guanines interferes with protein binding. Pairs of lanes showing cleavage at guanine residues in free and bound DNA isolated from each complex are shown in Fig. 3. Guanine residues which interfered with protein binding are indicated by dots.

Methylation interference analysis of complex 1 localized a new protein-binding site which we have designated site E; there is one guanine in close contact with the protein on the noncoding strand at position 333 and four guanines on the coding strand at positions 325, 330, 332, and 337 (numbering system of Ephrussi et al. [8] in which the 5′ *Xba*I site is position 1) (Fig. 3, lanes 1 to 4). This binding site was not identified by earlier exonuclease III experiments probably due to limitations discussed previously (26). Protein binding at site E does not represent a tissue-specific binding activity because it can be detected in nuclear extracts from mouse fibroblasts and HeLa cells (data not shown). Competition experiments have shown that the simian virus 40 enhancer is unable to compete for binding to site E under conditions in which the heavy-chain enhancer does compete for binding (data not shown).

Complex 2 represents protein binding to the previously identified site B. This site is the same as site μE1 reported by Weinberger et al. (39). The methylation interference pattern showed that four guanines at positions 372, 375, 376, and 379 are in close contact with the protein on the coding strand and two guanines at positions 377 and 378 are in close contact with the protein on the noncoding strand (Fig. 3, lanes 5 to 8).

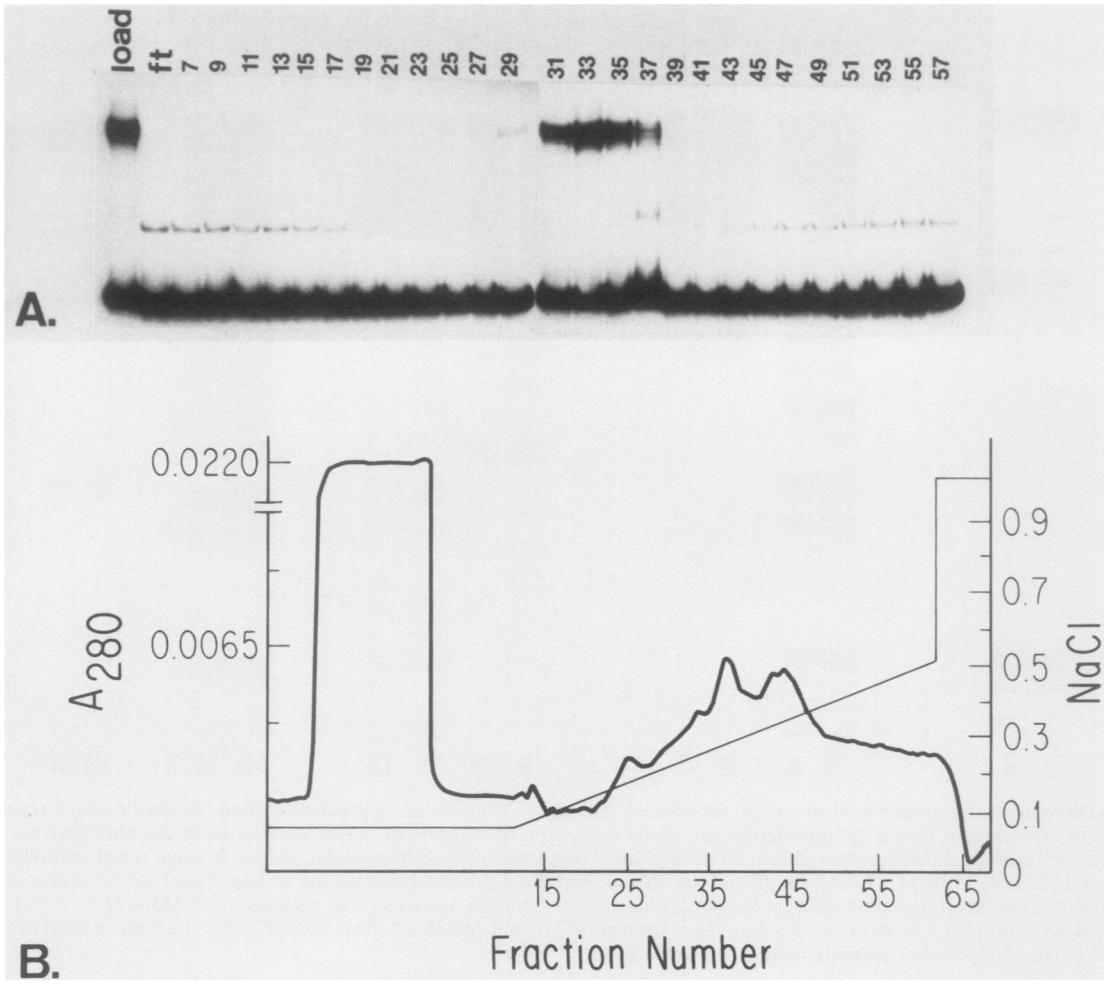

FIG. 4. FPLC Mono S chromatography of the site C2 binding protein producing complex 5 binding activity. (A) Gel retardation assays. Reactions included 2 μl of Mono Q pool [22–28] (load), 2 μl of the Mono S flowthrough pool (lane ft), or 2 μl of the indicated Mono S gradient fractions. All assays contained 200 ng of poly(dI:dC). (B) Mono-S gradient elution profile. The conductivity plot was obtained as in the legend to Fig. 2 and needs the same correction to obtain the actual conductivity measurement.

Complexes 3, 4, and 5 all localized protein binding to the previously identified site C2. The methylation interference pattern was similar to protein binding at site μE3 reported by Sen and Baltimore (34); two guanines are in close contact with the protein at positions 401 and 408 on the noncoding strand, and five guanines at positions 398, 399, 404, 406, and 407 are in close contact on the coding strand (Fig. 3, lanes 9 to 18). Noncoding strand contacts for complex 4 were identical to complexes 3 and 5 (data not shown).

In summary, methylation interference analysis has allowed us to identify gel retardation complexes which represent protein binding at previously identified sites B and C2 of the IgH enhancer, as well as a new site, site E, which was not previously detected (results summarized in Fig. 7). Gel retardation complexes which represent binding at previously identified sites C1 and C3 have not been identified in the Mono Q eluate and have not been studied further at present.

FPLC Mono S cation-exchange chromatography allows further purification of site C2 binding activity. We further purified the protein which produces complex 5 by chromatography on FPLC Mono S cation exchange (Fig. 4). Pool [22–28], which contains complex 5 binding activity, was loaded onto the Mono S column, and a linear NaCl gradient was used to elute site C2 binding activity. Complex 5 chromatographed as a homogeneous peak of binding activity, with no generation of complexes 3 and 4 (Fig. 4). The small amount of site E binding protein (complex 1) in Mono Q pool [22–28] eluted from the Mono S column at a higher NaCl concentration than the site C2 binding protein (Fig. 4, lane 37). The slow-migrating band in fractions ft to 17 and 45 to 57 is a probe artifact and does not represent sequence-specific protein binding.

Mono S chromatography of complex 5 binding activity resulted in an additional 15.4-fold purification of this site C2 binding protein with a yield of 83%. When complex 5 binding activity is quantitated throughout its purification, the overall purification of this site C2 binding protein is approximately 4,310-fold from the nuclear extract, with an apparent yield of 40% (Table 1). After Mono Q chromatography, purifications of site E and B binding proteins are similar to that shown for complex 5 (site C2) binding activity.

DNase I footprinting delineates the binding site boundaries for sites E and C2. DNase I footprinting of Mono Q pools was used to further identify the binding-site boundaries for

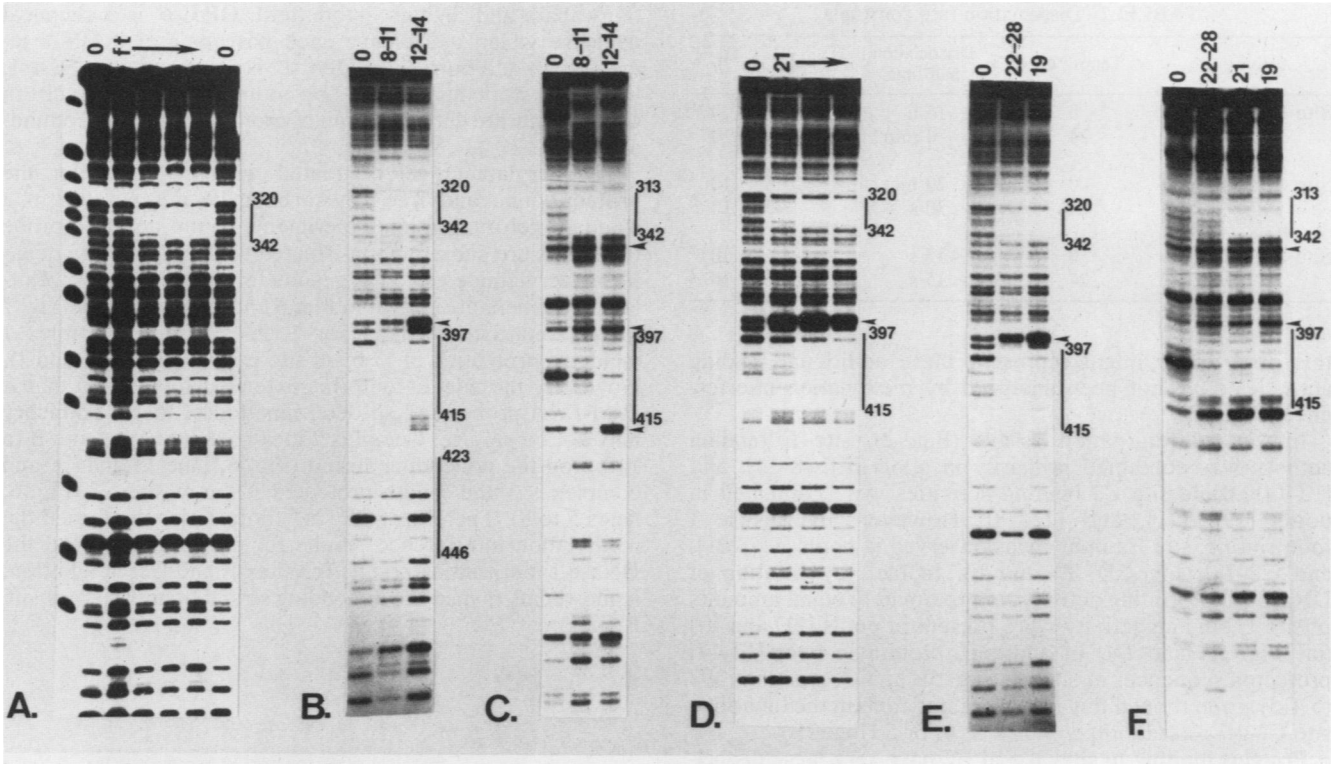

FIG. 5. DNase I footprinting analysis of Mono Q pools. Assays shown in panels A, B, D, and E utilized a noncoding strand-labeled probe, while assays in panels C and F utilized a coding strand-labeled probe. Lanes labeled 0 contained buffer instead of extract in the binding reactions. (A) Titration of binding activity in the Mono Q flowthrough pool. Binding reactions included 2 (lane 2), 4 (lane 3), 8 (lane 4), or 16 (lane 5) μl of Mono Q pool [ft]. The lane labeled M represents pBR322 plasmid DNA digested with *Hpa*II and end labeled as markers. Binding reactions shown in panels B, C, E, and F included 12 μl of each of the indicated pools. Binding reactions in panel D included 3 (lane 2), 6 (lane 3), or 12 (lane 4) μl of Mono Q pool [21]. Sequences protected from DNase I cleavage are indicated by vertical bars, and the endpoints of the protected region were determined from G+A sequencing ladders.

binding sites E, B, and C2 and to determine whether additional binding activities were present in these pools which did not produce stable gel retardation complexes. Figure 5 shows the results of such experiments, using Mono Q pools containing site E binding activity and the three different site C2 binding activities. Pools containing site B binding activity (pool [34–38], Fig. 2) did not show DNase I footprinting even when 20-fold more extract than necessary to obtain the gel retardation complex shown in Fig. 2B was used (data not shown).

The boundaries of the E and C2 binding sites on both DNA strands were well defined by this analysis. Site E binding protein in the Mono Q pool [ft] produced strong protection from DNase I at positions 320 to 342 on the noncoding strand at site E (Fig. 5A). The site E binding protein in Mono Q pools [8–11] and [12–14] also protected these same bases at site E from DNase I (Fig. 5B). On the coding strand, site E binding protein in pools [8–11] and [12–14] protected nucleotides 313 to 342 from DNase I at site E and showed a hypersensitive site at position 344 (Fig. 5C). These results are consistent with the presence of gel retardation complex 1 in these pools (Fig. 2B).

Site C2 binding proteins in pools [19] (complex 3), [21] (complex 4), and [22–28] (complex 5) each protected nucleotides 397 to 415 on the noncoding strand (Fig. 5D and E) and on the coding strand (Fig. 5F) of the C2 binding site. DNase I-hypersensitive sites were evident at position 395 on both strands and at position 416 on the coding strand. All three pools of site C2 binding produced identical footprints, in agreement with the identical methylation interference patterns for gel retardation complexes 3, 4, and 5. Site C2 binding proteins never completely protected site C2 from DNase I cleavage even at large excesses of gel retardation activity. These data are included in the summary binding site map in Fig. 7.

Interestingly, although the gel retardation pattern of pool [8–11] is rather simple (Fig. 2B), a complex binding pattern is seen with DNase I footprinting (Fig. 5B and C). Protein(s) in pool [8–11] partially protected sequences in the C2 region (397 to 415), the C3 region (423 to 446), and other simian virus 40 enhancer core homologies 3′ to site C3 (Fig. 5B and C; data not shown). The footprint from this pool on the C2 region did not show the characteristic hypersensitive sites on the noncoding strand at position 395 and on the coding strand at position 416. Minor gel retardation complexes in

TABLE 1. Purification of site C2 binding activity

Purification step	Sp act (U/μg of protein)[a]	Total protein (mg)	Total yield (%)	Total purification (fold)
Nuclear extract	0.1	162.0	100	1
Heat, 10 min at 67°C	0.9	18.0	100	9
DEAE-Sephacel	0.9	16.4	91	9
Heparin agarose	1.8	8.15	80	18
FPLC Mono Q	27.9	0.315	48	279
FPLC Mono S	431.0	0.0085	40	4,310

[a] See materials and methods for unit definition.

TABLE 2. Dissociation rate constants

Activity	Temp (°C)	Dissociation half-time	K_d (s^{-1})
Site E binding	0	15 h	1.0×10^{-5}
	24	4 min	2.9×10^{-3}
Site C2 binding	0	20 min	5.8×10^{-4}
	24	30 s	2.3×10^{-2}
Site B binding	0	<15 s	$>4.6 \times 10^{-2}$
	24	<15 s	$>4.6 \times 10^{-2}$

this pool which might represent these additional binding activities have not been analyzed by methylation interference (Fig. 2B).

In the gel retardation assays (Fig. 2), site E binding activity was contained primarily in pools [ft], [8–11], and [12–14], while site C2 binding activities were contained in pools [19], [21], and [22–28]. However, by DNase I footprinting, site E binding was observed in pools [19], [21], and [22–28] (Fig. 5D, E, and F). In fact, by titration of DNase I footprinting activity, approximately equal amounts of site E binding activity were present in pools [21] and [ft] (cf. Fig. 5A and D). In addition, protein in pool [12–14] protected sequences at site C2 (Fig. 5B and C, positions 397 to 415), even though this pool does not contain the identified site C2 gel retardation complex 3, 4, or 5 (Fig. 2B).

Proteins binding to sites B and C2 have very large dissociation rate constants. The inability to detect binding at site B by DNase I footprinting with pools that were active by gel retardation, as well as the inability to obtain complete protection from DNase I at site C2, led us to investigate the dissociation rate constants of these two proteins under DNase I footprinting conditions. If these proteins have very large dissociation rate constants under our assay conditions, then complete protection from DNase I cleavage would not be expected. As a control, an analysis of site E binding was also investigated, since this protein completely protects its binding site from DNase I digestion.

To determine dissociation rate constants, we used the gel retardation method described by Fried and Crothers (9). Dissociation rate constants were determined for all three proteins at 24° and 0°C (Table 2). Site E binding protein had dissociation rate constants of 2.9×10^{-3} s^{-1} at 24°C and 10^{-5} s^{-1} at 0°C, similar to those observed with other high-affinity DNA-binding proteins (5, 9). In contrast, site B binding protein had an extremely large dissociation rate constant even at 0°C, $>4.6 \times 10^{-2}$ s^{-1}. Site C2 binding protein had an intermediate dissociation rate constant at 0°C, 5.8×10^{-4} s^{-1}, smaller than that of site B protein but 58-fold larger than that for site E protein. These results are in agreement with the DNase I footprinting results, since rapid dissociation at 24°C of the proteins at sites B and C2 would be expected to decrease their ability to protect sequences from DNase I digestion. The ability to observe B and C2 binding activities by gel retardation suggests that the gel matrix stabilizes these binding interactions.

Delineation of binding-site boundaries by OP/Cu^+ chemical footprinting. Since site B and C2 binding proteins have very large dissociation rate constants, we decided to utilize an additional method for mapping the binding site boundaries that would take advantage of the apparent stability of protein-DNA complexes in the gel. We used a new technique which has been recently developed for application to gel retardation that utilizes the chemical OP and copper ion (Kuwabara and Sigman, submitted). OP/Cu^+ is a chemical nuclease which can cleave each base pair of a DNA sequence. In addition, since OP/Cu^+ is smaller than DNase I, it is not as sterically hindered by bound protein and results in a more accurate determination of protein binding-site boundaries.

The acrylamide gel is treated with OP/Cu^+ after the protein-bound and free DNAs have been separated in a standard gel retardation experiment; bound and free probe fragments are subsequently isolated and separated on denaturing acrylamide gels. The results for an OP/Cu^+ footprinting experiment are shown in Fig. 6 and summarized in Fig. 7 for gel retardation complexes 1, 2, 3, and 5. Complex 1 yielded a protection of 17 bp at site E (Fig. 6, lanes 1 and 2). However, the site E footprint extended to the end of the *Dra*I-*Dde*I probe, and so determination of the 5′ boundary may not be precise. Complex 2 localized binding at site B to 14 bp on the noncoding strand (Fig. 6, lanes 3 and 4), and complexes 3 and 5 both protected 11 bp at site C2 (Fig. 6, lanes 5 to 8). Therefore, OP/Cu^+ footprinting confirmed the methylation interference results for all three sites and the DNase I footprinting results for sites E and C2; in addition, it allowed us to map the noncoding strand boundaries for site B.

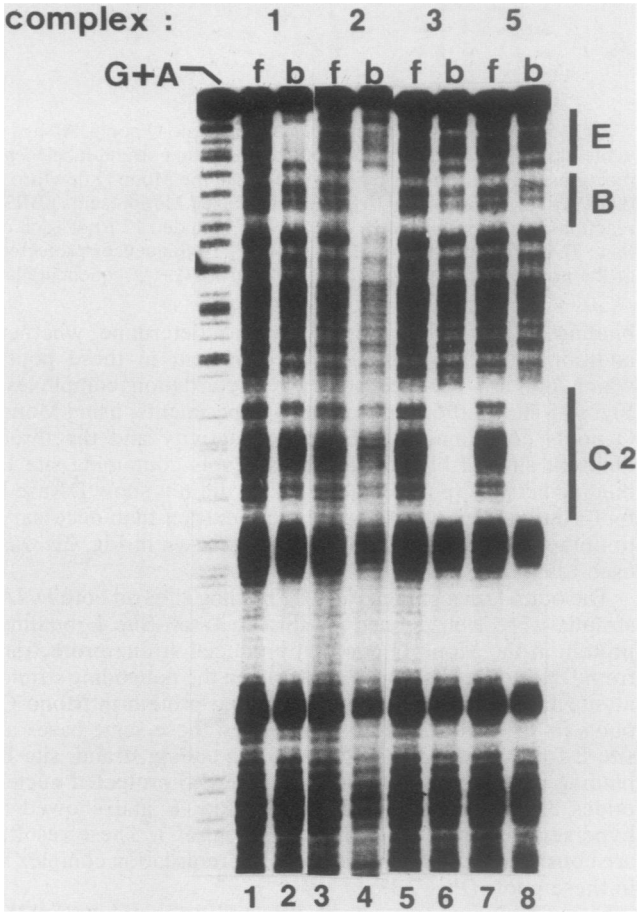

FIG. 6. OP/Cu^+ chemical footprinting analysis of Mono Q pools. Reactions in lanes labeled f contained DNA isolated from the free region of the gel retardation gel, and reactions in lanes labeled b contained DNA isolated from the indicated gel retardation complexes. Binding activities were obtained from the same pools as indicated for Fig. 3. Lane labeled G+A indicates a purine sequencing ladder.

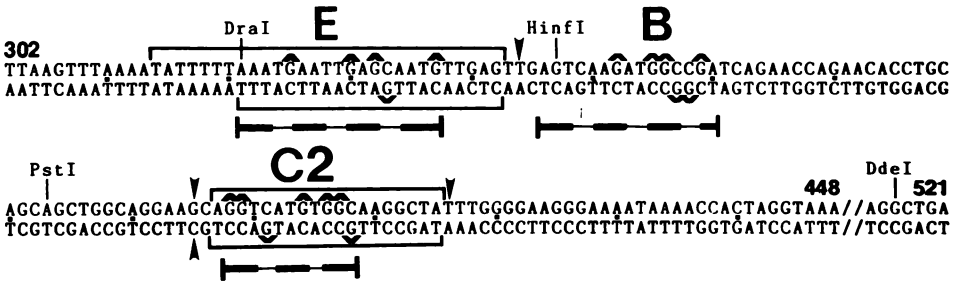

FIG. 7. Summary of protein-binding-site data on the sequence of the IgH enhancer. Inverted v's designate guanine residues identified by methylation interference (Fig. 3). Thin horizontal lines represent binding-site boundaries identified by DNase I footprinting (Fig. 5). Arrows indicate hypersensitive sites for DNase I. Boxed lines designate binding-site boundaries obtained from OP/Cu^+ chemical footprinting (Fig. 6).

DISCUSSION

Identification of a new binding site on the IgH enhancer. Fractionation of IgH enhancer-binding proteins has allowed us to separate and distinguish proteins which bind to previously identified sites B and C2. These proteins differ in their elution from the FPLC Mono Q column and their dissociation rate constants. Thus, it is clear that, even though they could not be distinguished in crude extracts by competition studies (26), they are distinct proteins. Furthermore, an additional binding activity was identified which binds to a new site, site E. This binding site was not detected by previous exonuclease III experiments (26), gel retardation assays (34), in vitro DNase I footprinting (1, 29), or in vivo mapping studies (7), although in most of the previous studies the probes used did not include the E-binding site. Site-directed mutagenesis studies of site E, as well as sites B and C2, are currently under way to assess directly the functional significance of these binding sites. Preliminary data suggest that all three binding sites are required for efficient enhancer function (B. Tsao, personal communication).

An analysis of the DNA sequence of site E revealed a 10-of 13-bp homology to the herpes simplex virus thymidine kinase CAAT box element (15). Only limited homology was observed between site E and the consensus sequence for nuclear factor I (28). Graves et al. have partially purified a protein from rat liver which binds to the herpes simplex virus thymidine kinase CAAT box and appears to be distinct from nuclear factor I (15). Site E binding protein may be related or homologous to this promoter factor. Until such homology can be tested directly, we propose that the site E binding protein be designated μEBP-E, for mu enhancer-binding protein, site E. In addition, the protein binding at site B will be designated μEBP-B, for mu enhancer-binding protein, site B.

Multiple gel retardation complexes correspond to binding at site C2. Gel retardation complexes 3, 4, and 5, which all showed binding to site C2, have different mobilities in the gel retardation assay and the proteins producing these complexes elute from the Mono Q column at different salt concentrations (Fig. 2B). These data are consistent with the possibility that the proteins producing complexes 3, 4, and 5 are three different proteins or are modified forms of a single protein which bind to site C2. Alternatively, it is also possible that complexes 3 and 4 result from proteolytic degradation of the protein producing complex 5. Several observations are inconsistent with the possibility of proteolytic degradation: (i) complexes 3, 4, and 5 are detected in crude nuclear extracts prior to heating (Fig. 2A, lanes a and b); (ii) the ratio of complexes 3 and 4 to the more abundant complex 5 does not appear to change during purification; and (iii) storage of complex 5 pools either on ice for several days or in 50% glycerol at −20°C for several months or further purification on the Mono S column does not result in the appearance of additional amounts of complexes 3 and 4 (Fig. 4; data not shown). However, until gene clones or antibodies to the site C2 binding proteins become available, the possibility of proteolysis cannot be ruled out.

We propose that the site C2 binding proteins be designated μEBP-C2a (complex 5), μEBP-C2b (complex 4), and μEBP-C2c (complex 3) for mu enhancer-binding proteins, site C2. It is possible that μEBP-C2a to c are the same as the NF-μE3 factor identified by Sen and Baltimore (34), but until these proteins are purified and their identity is established, we will retain different names.

Chromatographically separable proteins binding to the same DNA sequence have also been reported for proteins binding to the conserved octamer sequence found in the heavy-chain enhancer and immunoglobulin heavy- and light-chain promoters (20, 35). In the case of the octamer-binding proteins, one protein was found to be B-cell specific. Although proteins binding at site C2 are not tissue specific (26, 34), there may be important differences between them in terms of their transcriptional competence, binding affinities, or ability to interact with other transcription proteins.

Possible protein-protein interactions involving enhancer-binding proteins. Protein-protein interactions involving enhancer-binding proteins could play a key role in enhancer activity since the binding or transcriptional activity of enhancer proteins may be modulated by interactions with each other or with non-DNA-binding proteins. The large dissociation rate constants observed for μEBP-B and μEBP-C2 suggest that protein-protein interactions may be necessary to stabilize binding in vivo. In addition, glycerol gradient sedimentation analyses indicate that μEBP-E and μEBP-C2a to c interact either with each other or with other nuclear proteins in the heparin agarose fraction (C. Peterson, unpublished results). Further purification and characterization of the proteins binding to sites E, B, and C2 will be required to determine directly the nature and importance of the protein-protein interactions suggested by our present data.

In summary, the results presented here show that protein binding to the IgH enhancer involves a complexity of DNA-protein and potential protein-protein interactions. We have mapped the binding site for a new protein, μEBP-E, and demonstrated that proteins binding at sites B and C2 are distinct, thus bringing to a minimum of six the number of proteins binding to the IgH enhancer. We have determined the dissociation rate constants for three IgH enhancer-binding proteins and have discussed preliminary evidence

for protein-protein interactions. In addition, we show data suggesting the existence of several forms of a protein binding at site C2. The purification scheme we have developed, in conjunction with recently described DNA affinity columns (5, 18), should allow complete purification of these proteins. Ultimately, biochemical amounts of pure material corresponding to each IgH enhancer-binding protein will probably be required to fully understand the mechanism of action of this important regulatory element.

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