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Proteins Binding to Site C2 (μE3) in the Immunoglobulin Heavy-Chain Enhancer Exist in Multiple Oligomeric Forms

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We describe the purification to near homogeneity of proteins binding to site C2 (μE3) in the immunoglobulin heavy-chain enhancer. Proteins binding to this site produce four protein-DNA complexes which are distinguished by their mobility in gel retardation assays and their elution properties in an anion exchange column. DNA affinity-purified preparations of three chromatographically separated pools, containing different subsets of the four complexes, each contained three polypeptides of 42.5, 44, and 45 kilodaltons (kDa). UV crosslinking of protein to enhancer DNA demonstrated that site C2-binding activities in the three different pools bound DNA through proteins of similar sizes (about 45 kDa), even though the protein-DNA complexes formed by these binding activities were quite distinct. Gel exclusion chromatography and equilibrium binding analyses indicated that the distinct protein-DNA complexes were due to different oligomeric forms of the individual subunits and that a larger multimeric form bound with high affinity to the heavy-chain enhancer site C2, while a smaller species had a much lower affinity for heavy-chain enhancer sequences. Purified protein has been used to map high-affinity binding sites for site C2-binding proteins within an immunoglobulin heavy-chain promoter and at site KE3 in the kappa light-chain enhancer.

The transcriptional regulation of murine immunoglobulin heavy-chain (IgH) genes requires two B-cell-specific regulatory elements: a promoter located upstream of all IgH variable region (V_H) gene segments and a transcriptional enhancer located between the joining (J_H) and the mu constant region (C_μ) gene segments (K. Calame and S. Eaton, Adv. Immunol., in press).

The IgH enhancer is a prototypic enhancer that functions in *cis* in an orientation- and distance-independent manner (3, 10, 19, 20). Although it is able to activate heterologous promoters, it has been shown to activate V_H promoters preferentially (9). In order to understand the molecular mechanism by which this enhancer acts in a B-cell-specific manner to preferentially activate V_H promoters, we have been identifying and characterizing the nuclear proteins which bind to the IgH enhancer.

A combination of in vitro protein-binding studies and in vivo functional studies from several laboratories have identified a minimum of six different proteins which bind to the enhancer and are necessary for its activity (18, 35; Calame and Eaton, in press). One protein, which binds to the conserved octamer sequence (17, 28, 29, 34), displays a B-cell-specific tissue distribution. A family of octamer-binding proteins also binds to IgH and light-chain promoters and to promoters and enhancers in other nonimmunoglobulin genes. These have been purified in several laboratories (8, 29, 36; J. H. Hanke, N. F. Landolfi, P. W. Tucker, and J. D. Capra, Proc. Natl. Acad. Sci. USA, in press). A second IgH enhancer protein, μEBP-E, has recently been purified to homogeneity (24). It is a 45-kilodalton (kDa) protein which also binds to V_H promoters and to a site near the KE1 motif (31) within the kappa light-chain enhancer.

In this paper we describe the purification and characterization of proteins binding to a third site in the IgH enhancer, site C2. This in vitro protein-binding site is identical to site μE3 identified by in vivo footprinting studies (7). Site-directed mutagenesis of site C2 results in an enhancer which is only 40% as efficient as the wild-type IgH enhancer in transient transfection experiments, emphasizing the importance of this enhancer factor (35). Previously, we identified several site C2-binding proteins which could be separated by fast protein liquid chromatography (FPLC)–anion-exchange chromatography, suggesting that protein-binding to this site might be rather complex (23). We now report the purification to near homogeneity of polypeptides which associate to form multimeric proteins that bind to site C2. Proteins which bind to site C2 as apparent dimers and tetramers are present in nuclear extracts from plasmacytoma cells. Significantly, we have shown by quantitative Scatchard analyses that the larger form has a 5- to 14-fold higher affinity for site C2 than the smaller form, suggesting that regulation of site C2 enhancer-binding activity could be modulated by formation of different multimeric species. Chemical nuclease footprinting experiments have demonstrated that purified site C2-binding proteins also bind to sequences within a V_H promoter and to the KE3 motif (31) of the kappa light-chain enhancer.

MATERIALS AND METHODS

Purification of μEBP-C2. Nuclear extracts were prepared from the plasmacytoma cell line M603, which was grown as subcutaneous tumors in BALB/c × D2 mice, essentially as described previously (23), except that the protease inhibitors tosyl-lysine chloromethyl ketone (TLCK) and tosyl-phenylalanine chloromethyl ketone (TPCK) were added to the extraction buffers at a final concentration of 50 μg/ml. Nuclear extracts were chromatographed on DEAE-Sephacel, heparin-Sepharose, and FPLC Mono Q as previously described (23), but with several modifications. The crude nuclear extracts (60 ml) were not dialyzed or heat treated,

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but were loaded directly onto a 10-ml DEAE-Sephacel column equilibrated in TEN buffer (50 mM Tris [pH 7.5], 0.2 mM EDTA, 15% glycerol, 0.5 mM dithiothreitol [DTT], 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 2 μg of pepstatin A per ml, 5 μg of TLCK per ml, and 5 μg of TPCK per ml) containing 350 mM NaCl. The DEAE flowthrough was dialyzed into TEN buffer containing 100 mM NaCl (TEN100) and 5 mM $MgCl_2$ (TEN100+Mg), clarified, and loaded onto a 30-ml heparin-Sepharose column equilibrated in the same buffer. The column was washed with 3 volumes of TEN100+Mg, and protein was eluted with 3 volumes of TEN600+Mg (600 mM NaCl). This H0.6 fraction (35 ml) was dialyzed into Mono Q buffer (TEN buffer at pH 8.1) containing 100 mM NaCl. The dialyzed H0.6 fraction was loaded onto a Pharmacia 10/10 Mono Q column, washed with 25 ml of Mono Q buffer containing 100 mM NaCl, and then developed with a 160-ml linear 100 to 400 mM NaCl gradient.

A site C2 oligonucleotide affinity column was constructed by the methods of Kadonaga and Tjian (13). The double-stranded oligonucleotide contained the sequence 5′-CCT TGCCACATGACCTGCTT-3′, 3′-ACGGTGTACTGGACG AAGGA-5′. The annealed oligonucleotides were ligated to an average length of 125 base pairs (bp), and approximately 20 to 30 μg of ligated double-stranded oligonucleotide was coupled per ml of Sepharose.

Mono Q pools a (15 ml), b (10 ml), and c (20 ml) were dialyzed against two changes of 4 liters of TEN80 buffer containing 0.01% Nonidet P-40 (NP-40). The dialyzed pools were clarified, poly(dI-dC:dI-dC) was added to 40 μg/ml final concentration, and the three pools were each loaded onto 2.5-ml site C2 oligonucleotide affinity columns. The columns were washed successively with 5 column volumes of TEN80, TEN200, and TEN1000 plus NP-40. Fractions were collected into dichlorodimethlysilane-treated plastic Eppendorf tubes and assayed by the standard gel retardation assay. Fractions from each column containing their distinct site C2-binding activities were individually pooled, dialyzed against two changes of TEN80 plus 0.01% NP-40, and repassed over the affinity columns under identical conditions as the first pass (same concentration of nonspecific DNA). Site C2-binding proteins were stored at 0°C for several weeks with negligible losses in binding activity, although pool a appeared to be less stable than other pools.

For the experiment shown in Fig. 4, affinity-purified site C2-binding protein (two affinity passes) from pool C was rechromatographed on a 1-ml Pharmacia FPLC HR 5/5 Mono Q column. Pool c (5 ml) was dialyzed into Mono Q buffer containing 100 mM NaCl and 0.01% NP-40 and loaded onto the Mono Q column, and activity was eluted with a 30-ml linear 100 to 300 mM NaCl gradient in the same buffer. Fractions (0.5 ml) were collected as described for affinity purification.

Gel retardation assays and OP/copper footprinting. Gel retardation probes were 5′-end labeled by T4 polynucleotide kinase and gel purified as described previously (23). IgH enhancer probes (200-bp *Dra*I-*Dde*I, 190-bp *Pst*I-*Hin*fI, and 165-bp *Hin*fI-*Dde*I) were derived from the 1.0-kb *Xba*I fragment (7), the V1 promoter probe (106-bp *Rsa*I-*Bam*HI) was from a 600-bp BamHI fragment (6), and the Cκ enhancer probe (125-bp *Sau*3A-*Dde*I) was derived from the rearranged MOPC41 kappa gene (30). A site C2 oligonucleotide-containing probe was developed to simplify the quantitation of site C2-binding activities. Annealed site C2 oligonucleotides containing the sequence shown above were end-filled with the Klenow fragment of *Escherichia coli* polymerase and cloned into the *Hin*cII site of pUC8 to generate the plasmid pUC-C2. Single-insertion clones were isolated, and one used to create the 50-bp *Hin*dIII-*Eco*RI probe end labeled at the *Eco*RI site.

Gel retardation assays were performed in 15-μl volumes as previously described (23), except that the reaction mixes contained final concentrations of 20 mM Tris (pH 7.5), 10% glycerol, and 80 mM NaCl, and affinity-purified fractions were assayed with 100 μg of acetylated bovine serum albumin per ml. Low levels of $MgCl_2$ have been observed to greatly inhibit binding activity. In addition, samples were loaded while the gels were running. Quantitation of site C2-binding activity from pool c was done with 1.6 fmol of the 50-bp site C2 probe and poly(dI-dC:dI-dC) concentrations of 5 μg for the crude nuclear extract, DEAE flowthrough, and H0.6 fractions, 1 μg for the Mono Q fractions, and 200 ng for first- and second-pass oligonucleotide affinity pools. Two different amounts of protein in the linear range for binding were used for each protein fraction. Gel retardation complexes 3 and 4 were excised from the dried gels after autoradiography, and the radioactivity was quantitated by liquid scintillation.

Treatment of gel retardation gels with the orthophenanthroline-copper chemical nuclease, DNA isolation, and gel electrophoresis were performed essentially as described previously (15, 23), except that NA-45 nitrocellulose membranes were used in place of DE81 cellulose papers.

UV crosslinking. pUC-C2 plasmid was labeled internally with [α-^{32}P]dATP and [α-^{32}P]dCTP by priming with random oligonucleotides and the Klenow fragment of *E. coli* DNA polymerase. In addition to the labeled deoxynucleotides, reaction mixes contained 0.5 mM dGTP and 0.5 mM deoxybromouridine (dBUdr). Labeled DNA was desalted on Sephadex G-25 and digested with *Pst*I and *Bam*HI, and the 40-bp fragment was isolated from polyacrylamide gels and purified as for gel retardation probes. Approximately 1 ng of this labeled probe was then used in a binding reaction (40 μl) with partially purified or affinity-purified protein. Poly(dI-dC:dI-dC) (200 ng) was included in reactions with affinity-purified protein (1 μg for Mono Q pools). After 30 min at 23°C, the reaction mixture was cooled to 0°C and then spotted as a droplet onto a Saran Wrap-covered transilluminator UV source and exposed to UV light for 2 min at 0°C. After irradiation, samples were either mixed with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer (250 mM Tris [pH 6.8], 20% glycerol, 20% β-mercaptoethanol, 4% SDS) or processed for DNase I digestion. Reaction mixes which were treated with DNase I were first supplemented with 5 mM CaCl and 5 mM $MgCl_2$ and then 125 μg of DNase I per ml was added. The reaction mixes were incubated at 37°C for 20 min and then an equal volume of 2× SDS sample buffer was added. Samples were boiled for 5 min and electrophoresed on SDS-polyacrylamide gels. The gels were fixed in 10% methanol–10% acetic acid, dried, and exposed to film (Kodak XAR-5). High-molecular-weight ^{14}C-labeled protein standards were obtained from Bethesda Research Laboratories (BRL).

Glycerol gradient sedimentation and FPLC-Superose 12 chromatography. Linear glycerol gradients (4.8 ml, 10 to 30% glycerol in a buffer containing 50 mM Tris [pH 7.5], 0.2 mM EDTA, 500 mM NaCl, 0.5 mM DTT, 0.01% NP-40, and proteinase inhibitors as in TEN buffers) were prepared and centrifuged in a Beckman SW50.1 rotor for 26 h at 40,000 rpm at 0°C; 100 μl of affinity-purified site C2-binding protein from pool c was mixed with 100 μl of glycerol gradient buffer without glycerol and 10 U of *E. coli* polymerase I. A marker gradient was sedimented in parallel which contained 50 μg of

ovalbumin, 50 μg of cytochrome *c*, 50 μg of bovine serum albumin, and 10 U of *E. coli* polymerase I. Markers were adjusted to the same volume and glycerol concentration as the samples. From 31 to 33 150-μl fractions were collected from the top of the tubes, and binding activity was measured by the gel retardation assay. Markers were assayed by SDS-polyacrylamide gel electrophoresis (PAGE) and silver staining, or in the case of *E. coli* DNA polymerase, enzyme activity was assayed by a standard nick translation assay with nicked calf thymus DNA as the substrate.

A Superose 12-prepacked column (Pharmacia FPLC system) was equilibrated in TEN700 buffer with 0.005% NP-40 but without DTT and calibrated with blue dextran, β-amylase, yeast alcohol dehydrogenase, bovine serum albumin, ovalbumin, and cytochrome *c* at concentrations of 1 mg/ml in final volumes of 100 μl at a flow rate of 0.5 ml/min. Prior to application onto the column, 5 ml of affinity-purified site C2-binding proteins (2 to 3 μg) was concentrated to 150 μl by (i) dialyzing either pool a or pool c into TEN100 buffer plus 0.01% NP-40, (ii) loading this onto a 0.1-ml Q-Sepharose column equilibrated in the same buffer, and (iii) eluting protein in 150 μl of TEN700 plus 0.005% NP-40 without DTT. This step concentrated site C2-binding activities approximately 25-fold, with an 80% yield. Concentrated protein (120 μl) was injected onto the calibrated column, and 150-μl fractions were collected; 2 μl was assayed for binding activity in a standard gel retardation assay.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (16). High-molecular-weight protein standards were obtained from BRL, and silver staining was done by the technique of Ansorge (1). To obtain the most accurate molecular weight determinations for site C2-binding proteins, affinity-purified material was electrophoresed on multiple gels of different acrylamide concentrations (12, 16, 18, 14 to 18% linear gradient, and 12 to 18% linear gradient). The apparent sizes of the 42.5- to 45-kDa site C2-binding proteins varied slightly with acrylamide concentration, so the apparent sizes determined from protein standards from each of these gels were averaged to obtain our best estimates for the three proteins.

Equilibrium binding studies. Saturation binding curves were obtained by using the standard gel retardation assay with the 50-bp *Hin*dIII-*Eco*RI probe. The amount of protein sample was kept constant, while probe DNA was varied from 2×10^{-11} to 1×10^{-9} M. No nonspecific DNA was included in the binding reactions. Standard gels were electrophoresed in parallel which contained 14 different dilutions of probe DNA to generate a standard curve for converting integration values to probe molarity for every exposure. Binding data were quantitated by densitometry with a Pharmacia-LKB ultrascan laser densitometer (model 2222). Standard curves were generated by linear regression with the 1987 Data Analysis program copyrighted by John Lighton. Scatchard analyses of saturation binding data were plotted (2), and a best-fit line was obtained by linear regression with the Data Analysis program. In this graph, the slope is equal to $-1/k_d$ and the *x*-intercept is equal to the total number of binding sites for DNA. Four experiments were quantitated for each pool to produce the best estimate for binding at site C2, and the margin of error was determined from the standard deviation of these results.

The affinity of the tetramer and dimer forms of site C2-binding proteins for nonspecific DNA was determined by the method used by Prywes and Roeder (25). Binding reaction mixes contained 1.0×10^{-10} M probe, approximately 3×10^{-10} M protein (determined by Scatchard analysis), and six different concentrations of poly(dI-dC:dI-dC). The molar quantity of nonspecific DNA was calculated by assuming that each base pair represented the start of a new nonspecific binding site. Data were quantitated as above, and the concentration of nonspecific DNA which produced 50% inhibition of binding was determined.

RESULTS

Different forms of site C2-binding activity interact identically with enhancer sequences. As a source of site C2-binding proteins, we used nuclear extracts from the mouse plasmacytoma cell line M603. Since these cells are grown as solid tumors in mice, large amounts of cells can be processed for the purification of low-abundance proteins. Previously we described the partial purification of three protein fractions, μEBP-C2a, μEBP-C2b, and μEBP-C2c, which bound to site C2, by using a protocol which included heating at 68°C and chromatography on DEAE-Sephacel, heparin-Sepharose, and FPLC Mono Q (23). We showed that these chromatographically distinct site C2-binding fractions were eluted from the Mono Q column between 120 and 200 mM NaCl and gave rise to DNA-protein complexes of differing mobility in the gel retardation assay. Two changes have now been introduced into this purification scheme: (i) the heat step is omitted so that possible proteolysis during this step can be avoided, and (ii) a shallower salt gradient is used for elution from the Mono Q column so that all three site C2-binding activities can be resolved.

Figures 1B and C show a representative gel retardation assay of a portion of the Mono Q gradient elution of site C2-binding proteins with a 200-bp *Dra*I-*Dde*I IgH enhancer probe (Fig. 1A). As shown previously, a complex pattern of gel retardation complexes was formed by proteins in the gradient fractions; four different chromatographically distinct site C2 gel retardation complexes were routinely observed (Fig. 1B and C). Competitions with unlabeled site C2 oligonucleotide showed that all of the predominant retardation complexes observed in this section of the NaCl gradient were due to proteins binding at site C2 (data not shown; complexes denoted by bracket in Fig. 1B). The minor protein-DNA complexes in fractions 21 to 29 were due to the protein μEBP-E, which binds to site E in the IgH enhancer (Fig. 1B, arrow labeled E) (24). For the further purification of these multiple site C2-binding species, we created three protein pools as shown in Fig. 1C. Pool a contains primarily complex 1, pool b contains complexes 2 and 3, and pool c contains complexes 3 and 4.

Previous chemical nuclease footprinting studies indicated that identical coding strand sequences at site C2 were protected from nuclease by proteins in complexes 1 and 3 plus 4 (23). Since complex 2 was not analyzed and complexes 3 and 4 were not analyzed individually, we have now extended our footprinting experiments to examine the interaction of proteins with site C2 from all four gel retardation complexes on the noncoding strand. These experiments are shown in Fig. 2. Uncomplexed DNA is labeled f (free), and DNA isolated from the gel retardation complexes is labeled b (bound). The results clearly show that proteins in complex 1, complex 2, complex 3, and complex 4 all resulted from protein binding to identical sequences at site C2 in the enhancer. Interestingly, all of the complexes also induced OP/Cu hypersensitivity 7 and 14 bp from the 3′ boundary of site C2 (arrows). This hypersensitivity was not dependent on DNA sequence, since it was observed in vector sequences

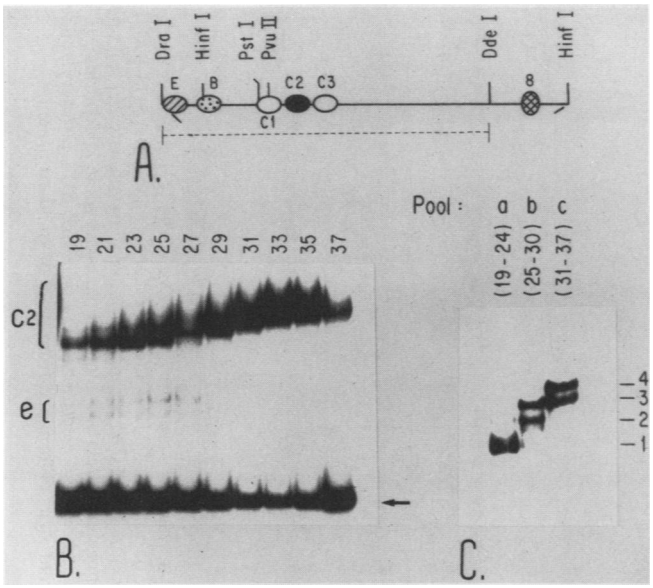

FIG. 1. FPLC Mono Q separates multiple site C2-binding proteins. (A) Schematic representation of protein-binding sites on the IgH enhancer. Circles designate previously identified protein-binding sites (24). Binding sites B, C1, and C2 represent in vitro protein binding to the homology blocks μE1, μE2, and μE3, respectively, identified by in vivo protein-binding studies (7). The dotted line indicates the *Dra*I-*Dde*I fragment used as a probe in binding assay. (B) Gel retardation assays of Mono Q gradient fractions. All assays used the 200-bp *Dra*I-*Dde*I IgH enhancer probe, 3 μl of the indicated gradient fractions, and 1 μg of poly(dI-dC:dI-dC). Brackets labeled C2 and E indicate protein-DNA complexes due to binding at site C2 and site E, respectively. Unlabeled arrow indicates DNA probe uncomplexed with protein. (C) Gel retardation assay of Mono Q pools. Numbers in parentheses indicate gradient fractions which were pooled, and numbers designate specific site C2 gel retardation complexes. Reactions were identical to those in panel A, except that twice the time of electrophoresis was used. Protein-DNA complexes due to protein binding at site E are not shown.

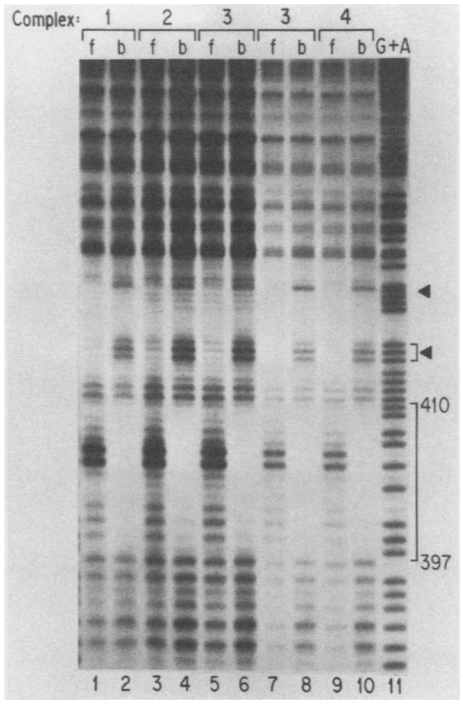

FIG. 2. OP/Cu chemical nuclease footprinting of site C2 protein-DNA complexes. Lanes labeled f represent chemical nuclease-cleaved DNA isolated from the free region of the gel retardation gel, and lanes labeled b represent DNA isolated from the indicated protein-DNA complex. Mono Q pools as shown in Fig. 1 containing binding activity which produced complex 1 were obtained from pool a, complex 2 was from pool b, complex 3 (lane 6) was from pool b, complex 3 (lane 8) was from pool c, and complex 4 was from pool c. Solid triangles indicate bases which were hypersensitive to the OP/Cu nuclease in DNA isolated from each of the protein-DNA complexes. Brackets indicate sequences protected from nuclease cleavage, and numbering of sequences is from Ephrussi et al. (7). Lane labeled G+A indicates Maxam and Gilbert purine chemical sequencing ladder.

on the 256-bp C2-pUC probe (Fig. 2, even-numbered lanes) as well as within IgH enhancer sequences on the 165-bp *Hin*fI-*Dde*I enhancer probe (see Fig. 8, lane 2). Hypersensitivity to OP/Cu suggests that protein binding at site C2 causes distortion of the DNA helix 7 to 14 bp downstream from the binding site.

Thus, we have shown that the four different protein-DNA complexes, contained in three different chromatographic pools, are formed by proteins binding to the same DNA sequence, site C2, and result in the same alterations of DNA helix structure downstream of the binding site. It seems likely that similar or identical protein-DNA interactions are involved in each complex and that the observed differences in gel shift mobilities and chromatographic properties result from differences in protein charge or mass which do not affect the nature of the DNA bases which are contacted by protein.

Purification of proteins which bind to IgH enhancer site C2. We used separation on the FPLC Mono Q column and added a DNA-binding site affinity column for the final purification of site C2-binding proteins. A site C2 oligonucleotide affinity column was prepared according to the procedure of Kadonaga and Tjian (13). Pools a, b, and c from the Mono Q column (Fig. 1) were dialyzed, mixed with a large excess of poly(dI-dC:dI-dC), and then applied to three individual 2.5-ml oligonucleotide affinity columns. The columns were washed extensively with 80 and 200 mM NaCl buffers, and proteins with high affinity for the matrix were eluted with buffer containing 1 M NaCl. Fractions were assayed by gel retardation; a representative first pass on the affinity matrix for Mono Q pools a, b, and c is shown in Fig. 3A.

These results indicate that gel retardation complexes 1 to 4 are all able to bind to the site C2 affinity matrix and that each pool retains its characteristic pattern of gel retardation complexes, even at high levels of binding activity. That is, purification of pool a, which contains primarily complex 1, always gave the same proportion of complex 1 and never produced more of complexes 2 to 4; the same was true for the proportion of complexes 2 to 4 in pools b and c. Fractions which contained binding activity from the first pass over the affinity column were combined for each pool and rechromatographed on the affinity columns to remove low levels of contaminating proteins.

Samples from each of the Mono Q pools and affinity column fractions were precipitated with trichloroacetic acid, electrophoresed on SDS-polyacrylamide gels, and silver stained. A representative set of results are shown in Fig. 3B. It is apparent from the Mono Q load and flowthrough lanes that the majority of the proteins present in each of the Mono Q pools were unable to bind to the site C2 affinity matrix

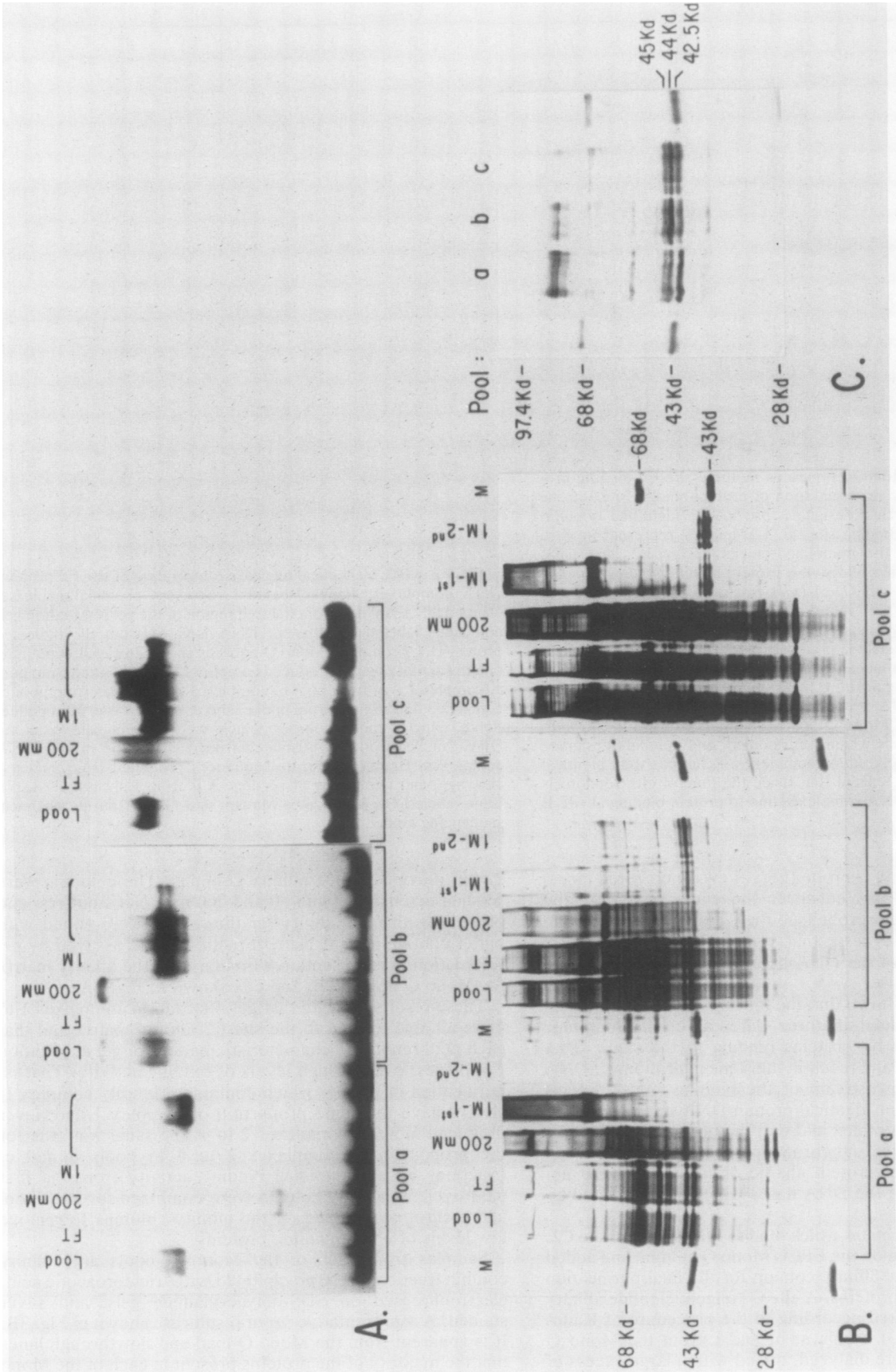

FIG. 3. Affinity purification of proteins which produce multiple site C2 protein-DNA complexes. (A) Gel retardation assays. Reactions used the 200-bp *Dra*1-*Dde*1 IgH enhancer probe, 1 μl of the indicated fractions from each pool, and 1 μg of poly(dI-dC:dI-dC) for load and flowthrough (ft) lanes, and 50 ng of poly(dI-dC:dI-dC) for all other reactions. (B) SDS-PAGE of affinity purification step. A 100-μl amount of each fraction was TCA precipitated for each pool with a final concentration of 15% TCA, 0.2 mg of sodium deoxycholate per ml, and 100 μg of bovine insulin per ml. Samples were electrophoresed on 12% SDS-PAGE and silver stained. Lanes labeled M represent protein size markers (sizes shown in kilodaltons). (C) SDS-PAGE of affinity second-pass pools: 1.2 ml of pool a, 0.4 ml of pool b, and 0.3 ml of pool c were TCA precipitated as above and electrophoresed on a 16% SDS–polyacrylamide gel. These amounts of protein contain approximately equal quantities of site C2-binding activity. Numbers indicate apparent sizes determined from plots of standard proteins.

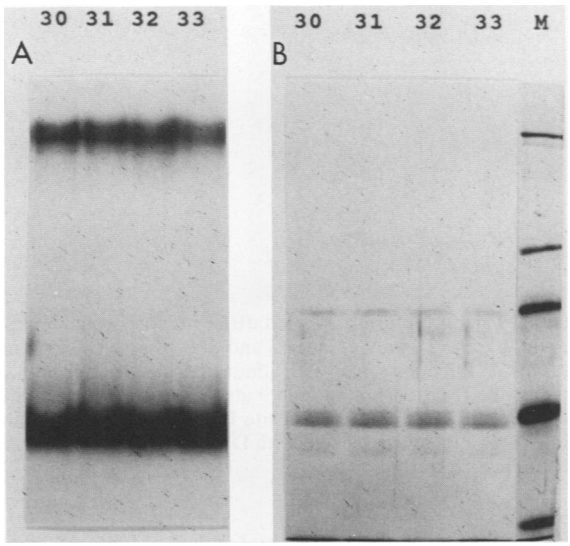

FIG. 4. Copurification of three polypeptides with site C2-binding activity. (A) Gel retardation assays of purified site C2-binding activity (complexes 3 and 4) after rechromatography on Mono Q resin. Reactions used the 200-bp *Dra*I-*Dde*I probe and 1.5 μl of the indicated gradient fractions (0.5 ml). No nonspecific DNA was included in the reactions. (B) SDS-PAGE of gradient fractions: 400 μl of the indicated fractions were TCA precipitated as described above and electrophoresed on an 8% SDS–polyacrylamide gel, and the gel was silver stained. Lane M, size markers (28, 43, 68, and 97.4 kDa).

under the conditions employed. Each affinity-purified pool contained three predominant polypeptides of 42.5, 44, and 45 kDa, although additional variable bands were present. Specifically, a protein of 80 kDa was present at high levels in this preparation of pool a. However, the amount of this protein was variable between preparations, and its abundance never correlated with the level of site C2-binding activity. Pool a always contained larger amounts of this 80-kDa protein but the least site C2 binding activity; the opposite was true for pool c. In addition, Fig. 4 shows that when the second affinity column fraction of pool c was rechromatographed on the FPLC Mono Q column, only the 42.5-, 44-, and 45-kDa proteins coeluted with site C2-binding activity; the 80-kDa protein did not elute within the peak of site C2-binding activity.

When equal amounts of binding activity from each affinity-purified pool were loaded on adjacent lanes and separated by SDS-PAGE, only the 42.5- to 45-kDa polypeptides were present at levels consistent with the amount of site C2-binding activity (Fig. 3C). In addition, the molecular weights of these polypeptides in different pools appeared to be identical. The proportion of the three major polypeptides varied in the different pools—pool a contained predominantly the 42.5- and 44-kDa polypeptides, pool b contained predominantly the 44-kDa polypeptide, and pool c contained all three polypeptides in approximately equal amounts. These data suggest that site C2-binding proteins may be oligomeric proteins comprising three polypeptides. The overall purification of site C2-binding activity from pool c was estimated to be 26,600-fold from the crude nuclear extract (Table 1).

Another explanation for these data might be that the three polypeptides are proteolytic degradation products of a single site C2-binding protein. However, an analysis of the amino acid composition of these three polypeptides indicates that these polypeptides are not proteolytic degradation or covalent modification products of a single protein, but are distinct gene products (J. Tomich, unpublished results).

It is formally possible that only one of these three proteins actually represents the site C2-binding activities and that the other proteins are not involved. However, we note that (i) these three polypeptides consistently copurified with site C2-binding activity in multiple preparations and (ii) these three polypeptides also copurified with site C2-binding activity when the Mono Q pools were chromatographed on a Mono S cation-exchange column prior to affinity purification (data not shown).

UV crosslinking of protein to site C2 DNA. To determine directly the size of the polypeptide(s) responsible for binding to site C2, we performed UV crosslinking experiments with a 50-bp probe containing site C2 (11). Protein fractions from either the Mono Q or affinity column pools were incubated with internally ^{32}P-labeled dBUdr-substituted probe DNA in the presence of excess nonspecific DNA. Reaction mixes were exposed to high-intensity UV light, DNA not crosslinked to protein was removed by DNase I digestion, and the reaction products were electrophoresed on SDS-PAGE. The results are shown in Fig. 5.

Polypeptides of the same sizes were crosslinked to the site C2 probe when either Mono Q pools or affinity-purified fractions were used (Fig. 5). In all cases a predominant polypeptide of approximately 44 to 48 kDa was crosslinked to the site C2 probe. Assuming that 5 to 10 bp of DNA is inaccessible to DNase I (11), the size of the crosslinked polypeptide was approximately 42 to 46 kDa. When equivalent amounts of protein from a Mono Q fraction which did not contain site C2-binding activity were used, no protein was crosslinked to the probe (lane 7). The 42- to 46-kDa crosslinked protein(s) was the result of sequence-specific interactions, since crosslinking of this species was greatly diminished by the addition of double-stranded oligonucleotide containing site C2 (lane 9), but not a double-stranded oligonucleotide containing the IgH enhancer site B (lane 10).

TABLE 1. Purification of site C2-binding proteins (pool c)

Protein fraction	Total protein (mg)	Total activity (U)[a]	Sp/act (U/μg of protein)	Total purification (fold)	Total yield (%)
Nuclear extract	480	85,714	0.179		100
DEAE-Sephacel	440	78,571	0.179	1	92
Heparin-Sepharose	129	33,000	0.256	1.43	39
FPLC Mono Q	7.7	37,714	4.9	27.4	44
Site C2 affinity					
1st pass	0.021	33,333	1,587	8,866	39
2nd pass	0.004	20,000	4,760	26,592	23

[a] One unit is defined as the amount of protein fraction required to bind 0.4 fmol of probe into gel retardation complexes 3 and 4 under standard assay conditions.

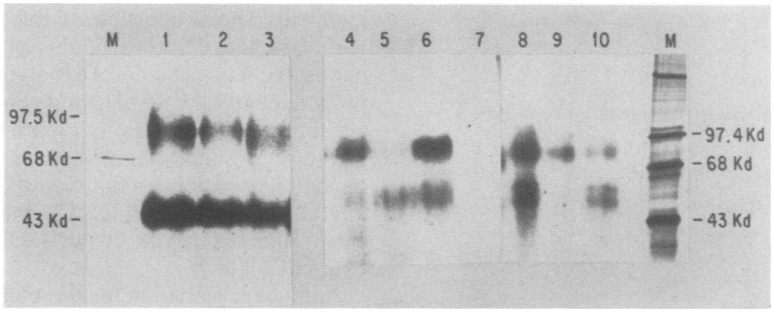

FIG. 5. UV crosslinking of site C2-binding proteins. Reaction mixes contained 1 ng of uniformly labeled dBUdr-incorporated 50-bp site C2 probe and 4 μl (2 ng) of protein from the first-pass affinity pool a (lane 1), pool b (lane 2), pool c (lanes 3 and 8 to 10), or 3 μl (600 ng) of Mono Q pool a (lane 6), pool b (lane 5), or pool c (lane 4) or Mono Q fraction 60 (lane 7). Reaction mixes with affinity-purified protein contained 1 μg of poly(dI-dC:dI-dC), and other reaction mixes contained 4 μg. In addition, the reaction mix in lane 9 also received 80 ng of site C2 double-stranded monomer oligonucleotide, and the reaction mix in lane 10 received the same amount of a site B double-stranded monomer oligonucleotide. Binding reaction mixes were irradiated with UV light for 2 min. Lanes 1 to 3 were treated with DNAse I prior to SDS-PAGE, but lanes 4 to 10 were not digested. Lanes M, ^{14}C-labeled protein standards obtained from BRL.

Identical competition results were obtained for pool a as well (data not shown).

In addition to the species at 42 to 46 kDa, a protein species of 75 to 85 kDa was also crosslinked. This protein(s) was more evident in the Mono Q pools and was partially removed by the affinity purification step (compare lanes 4 to 6 and 1 to 3). In addition, crosslinking of this protein in different preparations was variable, and the inability of the site C2 oligonucleotide to compete for crosslinking (lane 9) suggests that this band is due to contamination with a non-sequence-specific DNA-binding protein.

Since DNA crosslinked to the protein makes it impossible to achieve high resolution, we cannot determine which of the three affinity-purified polypeptides (Fig. 3C) bound to DNA; however, the results clearly establish that at least one of them does bind to site C2.

Glycerol gradient sedimentation. Since site C2-binding activities are clearly complex in that they have different chromatographic properties, give rise to different gel shift complexes, and comprise different polypeptides, we wished to determine the native molecular weights of site C2-binding proteins under different conditions. Glycerol gradient sedimentation under dilute conditions and in high-salt buffer was used to achieve conditions in which noncovalently associated polypeptide complexes would be expected to dissociate. Marker proteins were either cosedimented with site C2-binding proteins (pool c) or sedimented in a parallel gradient. Site C2-binding activity was monitored by gel retardation with a 190-bp *Pst*I-*Hin*fI IgH enhancer probe. The results (Fig. 6A) show that gel shift complexes 3 and 4 were formed from gradient fractions which cosedimented with ovalbumin at 3.5 S. In similar analyses, proteins producing complexes 1 and 2 comigrated with proteins producing complexes 3 and 4. Assuming a globular protein of average partial specific volume, a value of 3.5 S would correspond to a protein of 42 to 45 kDa.

These data are in good agreement with the sizes of polypeptides seen as major species purified by affinity chromatography and show that only polypeptides in the range from 42 to 45 kDa are required for binding to site C2. The gradient results also confirm the UV crosslinking experiments showing that one or more polypeptides of 42 to 46 kDa bind to site C2. Furthermore, they demonstrate that possible associations between the different polypeptides, if they exist, are of a noncovalent nature, since the complexes can be dissociated on these high-salt gradients.

Determination of the native molecular weights of site C2-binding proteins. It seemed likely that native site C2-binding proteins could be oligomers of one or more of the three 42- to 45-kDa polypeptides and that these oligomers were dissociated during the glycerol gradient sedimentation. Reassociation of multimers might then occur during the binding assay. In fact, we observed that glycerol gradient sedimentation under low-salt conditions resulted in a very broad peak of site C2-binding activity centered at approximately 4.6 S (80 kDa), consistent with multimer formation (data not shown). It is likely that the low concentration of site C2-binding activity (estimated at approximately 10^{-9} M) favors partial dissociation during glycerol gradient sedimentation even in low salt concentrations. To determine a nondissociated native molecular weight for the site C2-binding proteins, we first concentrated site C2-binding pools 25- to 40-fold by ion-exchange chromatography and then carried out rapid size separations on an FPLC-Superose 12 gel exclusion column. Fractions were assayed by gel retardation, and the results are shown in Fig. 6B for affinity-purified pools a and c.

After a 30-fold concentration, pool c (gel shift complexes 3 and 4) eluted as a single, discrete peak of binding activity from the Superose 12 column with an apparent size of 170 kDa. In conjunction with the molecular mass determinations of individual polypeptides made on SDS gels and glycerol gradients, this size indicates that site C2-binding protein in complexes 3 and 4 is most likely a tetramer. It is possible that the protein in complexes 3 and 4 is a trimer of each of the three polypeptides. However, since a tetramer structure is more consistent with the predicted monomer molecular weight, in the following discussion we refer to the larger complex as a tetramer. In contrast, protein in pool a (primarily complex 1) eluted from the column with an apparent size of 100 kDa, consistent with a dimer of 42.5- to 45-kDa subunits. These multimers were stable at this protein concentration and were not the result of nonspecific interactions, since the column was equilibrated in buffer containing 700 mM NaCl. In addition, these larger sizes are not due to an abnormal shape of the polypeptide subunits, since chromatography on a Sephacryl-300 gel exclusion column under dilute conditions indicated sizes similar to those found in sedimentation analyses (data not shown).

Since the sizes of the three polypeptide subunits which potentially form these complexes were so similar, these data do not allow us to determine which polypeptides are in-

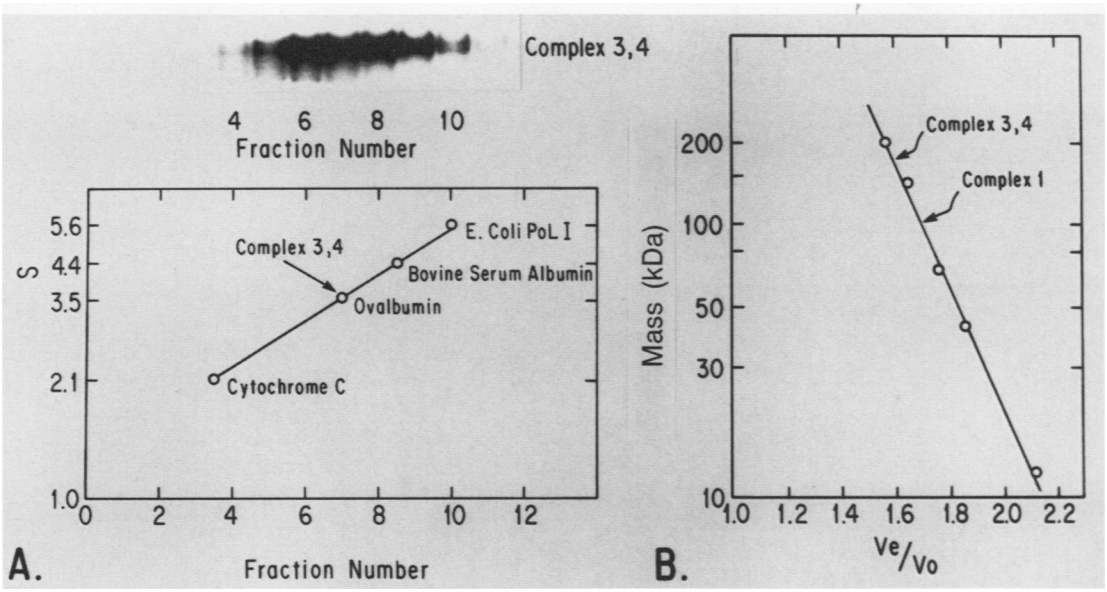

FIG. 6. Glycerol gradient sedimentation and gel exclusion chromatography. (A) Glycerol gradient sedimentation. Affinity-purified site C2-binding protein from pool c was sedimented in a 10 to 30% linear glycerol gradient. Gradient fractions (3 μl) were assayed in the standard gel retardation assay with a 190-bp *Pst*I-*Hin*fI IgH enhancer probe (upper portion of panel A). Only the bound portion of the gel retardation experiment is shown. Bottom portion of panel A depicts the sedimentation positions of marker proteins. Pol I, Polymerase I. (B) Gel exclusion chromatography. An FPLC-Superose 12 HR 10/30 column was calibrated with proteins of known molecular weight as described in the text. The void volume (V_0) was determined from the elution position of blue dextran, and the elution volume (V_e) for each protein was determined from the peak of absorbance at 280 nm. Site C2-binding activities (complexes 1, 3, and 4) were concentrated 25- to 40-fold by ion-exchange chromatography prior to injection onto the column. Fractions were assayed by the standard gel retardation assay as described in the text. Marker proteins were cytochrome *c*, ovalbumin, bovine serum albumin, yeast alcohol dehydrogenase, and β-amylase.

volved in the individual oligomeric complexes. However, the data clearly show that all the site C2-binding activities we have detected result from oligomers of 42- to 44-kDa polypeptide subunits. The activity responsible for gel shift complex 1 is most likely a dimer, and complexes 3 and 4 are tetramers. We have not determined the native size of protein giving complex 2.

Equilibrium binding analysis. We wished to determine whether the different oligomeric forms of site C2-binding activity were functionally different. The equilibrium dissociation constants for binding at site C2 of proteins producing the dimeric form (complex 1) and the tetrameric form (complexes 3 and 4) of site C2-binding proteins were determined by the gel retardation assay and Scatchard analysis. A small amount of each site C2-binding protein was titrated with a 50-fold range in concentration of DNA probe to obtain saturation binding curves for each site C2-binding protein (Fig. 7). With this 50-bp probe and a short time of electrophoresis, complexes 3 and 4 comigrated as a single species and were quantitated as such. These saturation data were plotted in the form of a Scatchard plot, where the ratio of protein-bound DNA to free DNA was plotted versus protein-bound DNA for each point in the titration (Fig. 7). The slope of such a line is equivalent to $-1/k_d$, and the *x*-intercept is equal to the molar amount of binding sites for DNA probe present in the reaction. Similar graphs from four total experiments yielded a dissociation constant of 5.9 (±2.9) × 10^{-11} M for the tetrameric species (complexes 3 and 4) and 4.3 (±0.2) × 10^{-10} M for the dimeric form (complex 1). This indicates that the tetrameric form has a 5- to 14-fold-higher affinity for site C2 than the dimeric form.

In addition to investigating the affinities of these two activities for their specific site, we also determined their affinity for nonspecific DNA. These experiments were performed with the gel retardation assay, and data were analyzed as described by Prywer and Roeder (25). In these experiments, the concentration of protein and specific DNA probe is kept constant but the concentration of the nonspecific DNA poly(dI-dC:dI-dC) is varied. The molar concentration which results in 50% inhibition of specific binding is then determined. These experiments illustrated that a 3.3 × 10^5-fold excess of nonspecific DNA decreased binding to 50% when pool a (complex 1) was used, and a 5.9 × 10^5-fold excess was required for pool c (complexes 3 and 4) (data not shown). These concentrations were very similar for both the dimer (pool a) and tetramer (pool c) species, suggesting that their relative affinities for nonspecific DNA are not as significant as the differences observed in their affinities for site C2.

The results from the equilibrium binding studies further emphasize that different site C2 protein-DNA complexes are distinct by functional criteria as well as size and chromatographic properties. Different multimeric forms having different affinities for site C2 might provide opportunities for complex regulatory controls.

Binding sites for site C2-binding proteins are contained within promoters and enhancers. The availability of affinity-purified site C2-binding proteins allowed us to search for other high-affinity binding sites within immunoglobulin regulatory regions. Binding sites for site C2-binding protein were not detected on a kappa promoter probe, kappa enhancer probe containing only the kE1 motif, upstream V1 promoter probes, or on a Vb T-cell receptor promoter probe (C. Peterson and S. McDougall, unpublished results). Previous work of Sen and Baltimore with crude nuclear extracts suggested that a protein which bound to site C2 (μE3) might

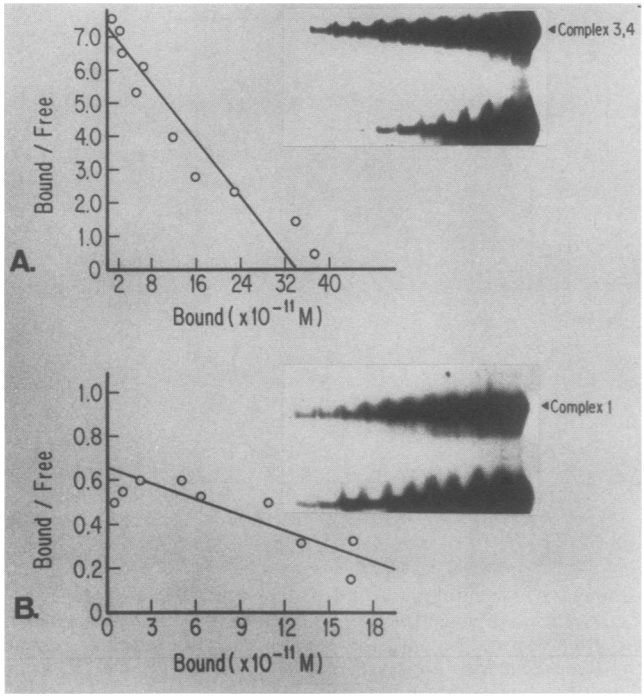

FIG. 7. Equilibrium binding analysis of different site C2-binding proteins. (A) Saturation binding curve and corresponding Scatchard plot for the gel retardation assay and affinity-purified protein from pool c (complexes 3 and 4). Reaction mixes contained 1 μl (approximately 0.35 ng) of protein and the concentration of the 50-bp site C2 DNA probe was varied from 2×10^{-11} M to 1×10^{-9} M. Reactions contained no nonspecific DNA. With this small 50-bp probe and relatively short time of electrophoresis, complexes 3 and 4 comigrated as one species and were quantitated as such. (B) Saturation binding curve for protein from pool a (complex 1). Reaction mixes contained 1.5 μl (approximately 0.35 ng) of protein and the same concentrations of DNA probe as for pool c reactions.

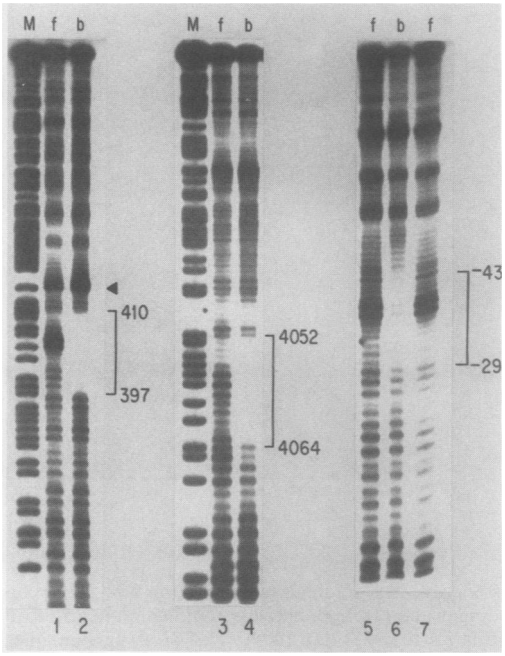

FIG. 8. OP/Cu chemical nuclease footprinting with promoter and enhancer fragments. Lanes 1 and 2 used a 165-bp *Hin*fI-*Dde*I probe from the IgH enhancer, lanes 3 and 4 used a 125-bp *Sau*3A-*Dde*I probe from the kappa light-chain enhancer, and lanes 5, 6, and 7 used a 106-bp *Rsa*I-*Bam*HI probe from the V1 heavy-chain promoter. Lanes labeled f contain DNA isolated from the free region of the gel retardation gel, and lanes labeled b contain DNA isolated from the gel retardation complex. In all reactions, protein was used from the second-pass affinity pool c (complexes 3 and 4). The solid triangle indicates nucleotides hypersensitive to nuclease cleavage, and brackets indicate sequences protected from nuclease. Numbering of sequences for the IgH enhancer is from Ephrussi et al. (7), for the kappa light-chain enhancer is from Parslow and Granner (22), and for the V1 heavy-chain promoter is with respect to the start site for transcription.

also bind to the kappa light-chain enhancer (31). We investigated binding to a kappa enhancer probe by using affinity-purified protein, and a high-affinity binding site was detected (data not shown) (Fig. 8). A kappa enhancer probe produced the same multiple gel retardation complexes as the IgH enhancer probes with the three pools of site C2-binding activity (data not shown). We performed OP/Cu footprinting experiments with purified site C2-binding protein and a kappa enhancer probe, and the results are shown in Fig. 8, lanes 3 and 4. Site C2-binding protein from pool c (complexes 3 and 4) protected sequences from 4064 to 4052 from nuclease cleavage which is centered on the kE3 sequence motif identified by Sen and Baltimore (31). Alteration of this sequence element is known to reduce kappa enhancer function in vivo (18).

In addition to the site within the kappa light-chain enhancer, a binding site for site C2-binding protein was identified within the V1 heavy-chain promoter. Titrations of purified protein demonstrated that this binding site was equal in affinity to site C2 within the IgH enhancer and that a site C2 oligonucleotide efficiently competed for binding to this site (data not shown). We performed OP/Cu footprinting experiments with this promoter probe, and the results are shown in Fig. 8, lanes 5 to 7. Site C2-binding protein from pool c protected sequences from −43 to −29 with respect to the start site for transcription. A binding site for octamer transcription factor occurs directly 5′ of this site, and the predicted TATA box element for the V1 promoter is directly 3′. Although the function of this site has not yet been tested directly, it is intriguing to speculate that this binding site may be required for efficient transcription and that site C2-binding proteins may interact with an octamer or TATA box transcription factor(s). The results from these chemical nuclease footprinting studies are summarized in Table 2.

DISCUSSION

Protein-binding site C2 within the mouse IgH enhancer is known to be required for optimal enhancer activity in vivo (18, 35). We have shown that protein binding to site C2 is complex, since at least four distinct protein-DNA complexes

TABLE 2. Binding sites for site C2-binding protein[a]

Site	Sequence
IgHE	(397)-**AGGTCATGTGGCAAGGC**-(413)
KE	(4052)-**GTCCCATGTGGTTACAA**-(4068)
V_H V1	(−27)-**TTTTCATGTGATGAGGG**-(−43)

[a] The sequences protected from OP/Cu cleavage by site C2-binding protein are printed in boldface type and aligned for maximal homology. Numbering indicates distance from the transcriptional start site for heavy-chain promoter V1. Numbering for the IgH enhancer is from Ephrussi et al. (7), and numbering for the kappa enhancer follows Parslow and Granner (22).

can be detected by gel shift analyses. Three polypeptides of similar size which appear to produce these four protein-DNA complexes have been purified to near homogeneity. SDS-PAGE, UV crosslinking, and glycerol gradient sedimentation established that one or more of these polypeptides contain a binding site for the site C2 sequence. These polypeptides are able to form oligomers of different sizes, which we show have different affinities for binding at site C2. Purified site C2 proteins also bind to sequences within a heavy-chain promoter and within the kappa light-chain enhancer.

Subunit composition of oligomeric proteins binding to site C2. Three polypeptides of 42.5 to 45 kDa consistently copurified with site C2-binding activity. Unfortunately, the similar sizes of the 42.5- to 45-kDa polypeptides makes it difficult to assess the exact subunit composition of the different site C2 multimeric complexes. One or more of these polypeptides associate to form the low-affinity dimeric binding species in pool a (complex 1) and associate to form the high-affinity tetrameric forms present in pool b (complex 3) and in pool c (complexes 3 and 4). We have not yet determined directly the multimeric structure of proteins which produce complex 2. These oligomeric structures are consistent with the mobility of the protein-DNA complexes in the gel retardation assay, since complex 1 migrates faster than complexes 3 and 4, and all three complexes migrate more slowly than the complex generated by the 45-kDa enhancer-binding protein μEBP-E, which appears to function as a monomer (Fig. 1B) (24).

A heterologous subunit composition for different oligomers is suggested by several results. (i) All three polypeptide subunits consistently copurified with site C2-binding activity in multiple preparations. (ii) Each pool contained substantial amounts of each of the three polypeptides, although different complexes were formed in each pool. Copurification of the three polypeptides strongly suggests that they are all required for binding, but confirmation will require the ability to test each polypeptide individually for binding by using specific antisera or purified components. In pool a, where complex 1 was a dimer, the presence of three polypeptides suggests that two different dimers may be present or that low levels of complex 2 in pool a are responsible for the third polypeptide in the purified pool. Pool c contains two tetramer species, complexes 3 and 4, which appear to be identical in native size but are not of identical mobility in the gel retardation assay.

It is possible that the multiple oligomeric species of site C2-binding activity represent intermediates in the assembly of one of the tetrameric forms. However, several results are inconsistent with this possibility: (i) a 20- to 40-fold concentration of protein which produces complex 1 (dimer) did not result in an increase in the amount of tetrameric form, (ii) the tetramer species which produce complexes 3 and 4 reformed after dissociation during glycerol gradient sedimentation without forming an increased amount of dimeric species, and (iii) each pool always gave rise to the same complexes, even after additional treatment, i.e., dimeric forms were not generated when pool c (complexes 3 and 4) was diluted or rechromatographed on a Mono Q column. It is puzzling that each pool of binding activity apparently contains the same three polypeptides, yet different multimers always form from different pools. It may be that one or more apparently similar polypeptides in different pools differ by covalent modifications which do not alter their mobility in SDS-PAGE.

Multimeric forms are not novel for DNA-binding proteins, but there is little precedent for such oligomers containing heterologous subunits. Recently evidence has been presented which suggests that several protein activities which bind to mammalian CAAT sequence elements may be composed of at least two distinct protein subunits (4). These protein subunits appear to be functionally related to the HAP2 and HAP3 proteins in *Saccharomyces cerevisiae* which also form a heterogeneous complex (5).

Multiple protein-DNA complexes which represent binding to identical sequences are also not unique to proteins which bind to site C2. Several cases are known in which multiple gel shift complexes are seen from binding to a single site, although none are caused by the exact mechanism we describe for site C2-binding proteins. For example, distinct octamer-binding proteins which have different mobilities on SDS-PAGE give rise to several protein-octamer sequence complexes (29; Hanke et al., in press). Distinct *Drosophila* heat shock transcription factor (HSTF) proteins also result in multiple protein-DNA complexes (33). Different HSTF proteins make identical contacts with heat shock-responsive DNA elements (32, 33) but have different transcriptional competence (21). These different protein-DNA complexes are due to extensive phosphorylation of the HSTF protein in heat-shocked cells (33).

Interaction of site C2-binding proteins with DNA. UV crosslinking of protein to DNA has shown that at least one of the 42.5- to 45-kDa polypeptides interacts directly with site C2 DNA sequences. There is no evidence, however, for a single subunit alone binding to DNA, which would generate a faster-migrating gel retardation complex. This suggests that at least two polypeptide subunits must interact prior to DNA binding, similar to *lac* repressor protein (26). It seems likely that the same subunit in each complex binds DNA, since all site C2 multimeric complexes interact with identical DNA sequences and even induce identical hypersensitivity to chemical nuclease.

Quantitative Scatchard analyses show that tetramers have a significantly higher affinity for site C2 than dimers. Different DNA affinity is likely to affect the transcriptional competence of these proteins as well, in a manner similar to that observed for HSTF in HeLa cells (14). Furthermore, our results show that formation of particular site C2-binding multimers is not determined solely by concentration of subunits and suggest that it may be determined by subunit modification or other mechanisms. This leads to the intriguing possibility that fine tuning of IgH enhancer activity could be achieved by the proportion of different site C2-binding multimers present in B cells.

Binding of site C2-binding proteins to V_H promoter and kappa enhancer sequences. Purified proteins which bind to site C2 within the IgH enhancer also bind with high affinity to the KE3 motif within the kappa light-chain enhancer and also to a site within the V1 heavy-chain promoter. The KE3 sequence motif is very similar to the sequence of site C2, as described previously by Sen and Baltimore (31) (Table 2), and it is required for optimal kappa enhancer function in vivo (18). The functionality of the V1 promoter-binding site has not yet been directly determined, and such studies are now in progress.

Binding to multiple immunoglobulin promoter and enhancer elements has also been detected for the IgH enhancer factor μEBP-E, which binds near the KE1 sequence motif within the kappa light-chain enhancer as well as to sites within heavy-chain promoters (24). In addition, octamer-binding protein also binds to the IgH promoter and enhancer. The multiplicity of binding sites for different en-

hancer factors within homologous enhancer and promoter elements suggest that such elements may interact by protein-protein contacts, as suggested by the "looping" model for enhancer function (27). The demonstration that site C2-binding proteins bind as dimers or tetramers would be consistent with such a model, since two dimers bound to different sites might interact; alternatively, a tetramer may contain two binding sites for a site C2 sequence. If one tetramer is able to bind two binding sites simultaneously, than perhaps such a stable loop structure could occur between enhancer and promoter elements.

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