

Octamer Transcription Factors 1 and 2 Each Bind to Two Different Functional Elements in the Immunoglobulin Heavy-Chain Promoter

LORENZ POELLINGER AND ROBERT G. ROEDER*

*Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, 1230 York Avenue,
New York, New York 10021*

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Immunoglobulin heavy-chain genes contain two conserved sequence elements 5' to the site of transcription initiation: the octamer ATGCAAAT and the heptamer CTCATGA. Both of these elements are required for normal cell-specific promoter function. The present study demonstrates that both the ubiquitous and lymphoid-cell-specific octamer transcription factors (OTF-1 and OTF-2, respectively) interact specifically with each of the two conserved sequence elements, forming either homo- or heterodimeric complexes. This was surprising, since the heptamer and octamer sequence motifs bear no obvious similarity to each other. Binding of either factor to the octamer element occurred independently. However, OTF interaction with the heptamer sequence appeared to require the presence of an intact octamer motif and occurred with a spacing of either 2 or 14 base pairs between the two elements, suggesting coordinate binding resulting from protein-protein interactions. The degeneracy in sequences recognized by the OTFs may be important in widening the range over which gene expression can be modulated and in establishing cell type specificity.

The immunoglobulin heavy- and light-chain genes are transcribed in a developmental-stage- and B-lymphocyte-specific fashion. The octanucleotide sequence 5'-ATG CAAAT-3' and its precise inverse are well conserved in all sequenced immunoglobulin heavy (V_H)- and light (V_L)-chain promoters, respectively (8, 23), and have been implicated in tissue-specific promoter function in vivo and in vitro by deletion analysis (1, 2, 8, 10, 19, 20, 24). Octamer motifs also function in the heavy-chain enhancer (11) as well as in non-tissue-specific promoters such as those of the histone H2B (14) and small nuclear RNA genes (for a review, see reference 3). In addition, the octamer motif is required for adenovirus DNA replication (22). In vitro, the octamer element is recognized by both a ubiquitous and a B-cell-specific factor (for reviews, see references 9 and 28). A ubiquitous factor of about 90 kilodaltons (kDa) and a B-cell-specific factor of about 60 kDa have been purified to apparent homogeneity and shown to stimulate RNA synthesis from octamer-containing promoters in vitro and have been designated OTF-1 and OTF-2, respectively (9, 27).

Recently, it has been reported that the octamer motif is able, by itself, to confer lymphoid-cell-specific activity to a heterologous promoter when linked immediately upstream of the TATA box (6, 32) or when acting at a distance (11). However, it is not yet known whether the lymphoid-cell-specific activity of this element is governed by the lymphoid-cell-specific factor OTF-2 alone or whether this factor acts in concert with the ubiquitous OTF-1 or additional (as yet unidentified) factors (or both) to achieve cell type specificity. Interestingly, immunoglobulin V_H promoters contain, in addition to the octamer element, a second, strongly conserved sequence motif (5'-CTCATGA-3') referred to as the heptamer element (7, 28). This sequence motif is located at variable distances (2 to 22 base pairs [bp]) upstream of the octamer element and was shown to be required for the full lymphoid-cell-specific activity of the immunoglobulin V_H promoter (1, 7). In gel mobility shift assays with crude nuclear extracts from a B-lymphoid cell line, DNA frag-

ments containing both the heptamer and octamer elements generated four distinct octamer-specific protein-DNA complexes (15, 16), in contrast to the two dominating complexes (reflecting bound OTF-1 and OTF-2, respectively) observed with probes containing only the octamer motif (11, 27, 29). Moreover, DNase I or methidiumpropyl-EDTA footprint analyses revealed an extended area of protection, including both sequence elements, in at least one of the four heptamer- and octamer-generated protein-DNA complexes (16, 17, 26).

In this report, we show that heptamer- and octamer-dependent DNA-binding activities copurify. This suggests that a single protein (presumably one or both of the OTFs) has binding specificity for each of the two separated DNA sequences, rather than the heptamer sequence being recognized by a distinct factor that acts coordinately with the OTFs. This suggestion was confirmed by the finding that purified OTFs, either alone or in combination, bind to the heptamer and octamer sequences and form homodimeric or heterodimeric complexes. However, binding to the heptamer sequence required the presence of an intact octamer motif and occurred with either 2 or 14 bp between the two elements. Thus, both the immunoglobulin octamer and heptamer elements constitute the site of coordinate protein-DNA interaction by the OTFs.

MATERIALS AND METHODS

Cell culture and extract preparation. HeLa cells were grown in Spinner flasks in Joklik medium supplement with 5% bovine calf serum. Namalwa (human Burkitt's lymphoma) cells were grown in Spinner flasks in RPMI 1640 medium supplemented with nonessential amino acids, glutamine, and 10% fetal bovine serum. Nuclear extracts were prepared essentially as described by Dignam et al. (5).

Oligonucleotides and DNA affinity column. To prepare an octamer- and heptamer-containing probe for DNA-binding studies, two 38-bp oligonucleotides were synthesized. Oligonucleotide 1 (5'-GGATCCACCCTGTCTCATGAATATGCAAATCAGGTGAG-3'), when annealed to oligonucleotide 2 (5'-TGGGACAGAGTACTTATACGTTTAGTCCACTCCCTAGG-3'), forms 32 bp of the V_H BC11 promoter (13,

* Corresponding author.

15) extending from positions -73 to -42 from the initiation site. Mutant oligonucleotides containing clustered point mutations in either the heptamer or octamer motifs or both (see Fig. 2A) were also synthesized. In addition, a double-stranded oligonucleotide corresponding to positions -86 to -45 of the murine V_H promoter (7) was prepared. This oligonucleotide contained a 14-bp spacing between the heptamer and octamer elements (see Fig. 2A). An oligonucleotide-Sepharose column containing the octamer motif of the V_L T1 gene (27) was used for DNA affinity chromatography.

Chromatographic procedures. Namalwa cell nuclear extract was fractionated essentially as described for purification of OTF-2 (27), except that care was taken not to separate the ubiquitous and lymphoid-cell-specific heptamer- and octamer-binding activities. Specific DNA-binding activity was monitored by gel mobility shift assays by using an end-labeled oligonucleotide containing the wild-type heptamer and octamer motifs of the BC11 V_H promoter (see Fig. 2A). Namalwa nuclear extract (20 ml [corresponding to approximately 10¹⁰ nuclei and 160 mg of protein]) was adjusted to 0.42 M NaCl and passed over a 10-ml DEAE-cellulose column (DE 52; Whatman, Inc.) equilibrated with 20 mM Tris hydrochloride (pH 7.9)-25% glycerol-0.42 M NaCl-1.5 mM MgCl₂-0.2 mM EDTA. The flowthrough material was collected, diluted with BC buffer (20 mM Tris hydrochloride [pH 7.9]-0.2 mM EDTA-20% [vol/vol] glycerol-0.5 mM phenylmethylsulfonyl fluoride-1 mM 2-hydroxyethylmercaptan) and loaded at 50 mM KCl onto a 10-ml heparin-agarose column. The column was developed with eight column volumes of a 50 to 500 mM KCl gradient in BC buffer. Active fractions (100 to 300 mM KCl) were pooled, diluted to 50 mM KCl with BC buffer containing 0.03% Nonidet P-40 (NP-40), and loaded onto a 3-ml single-stranded DNA-agarose column (Bethesda Research Laboratories, Inc.) equilibrated with BC buffer-50 mM KCl-0.03% NP-40. Heptamer- and octamer-binding activity was eluted with 10 column volumes of a 50 to 1,000 mM KCl gradient in the same buffer. Peak fractions (150 to 275 mM KCl) were pooled, diluted with BC buffer-0.03% NP-40 to a final concentration of 100 mM KCl, and loaded onto a 0.75-ml specific oligonucleotide column (containing a κ -light-chain octamer motif [27]) equilibrated in BC buffer-100 mM KCl-0.03% NP-40. The column was washed with 10 column volumes of equilibration buffer and developed with 10 column volumes of a 100 to 1,000 mM KCl gradient in BC buffer. Eluted material was frozen in liquid nitrogen and stored at -70°C.

Gel electrophoresis DNA-binding assay. DNA probes were end labeled with the Klenow fragment of DNA polymerase I and purified by electrophoresis on 6% polyacrylamide gels. Binding reactions were carried out in 20- μ l volumes for 30 min at 30°C. The reaction mixtures contained 4 fmol of end-labeled probe, various amounts of partially purified or purified protein preparations, and 1.5 μ g of poly(dI-dC) double-stranded heteropolymer (Pharmacia, Inc.) in 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.9)-60 mM KCl-2 mM dithiothreitol-4% Ficoll. Typically, protein-DNA complexes were resolved on 4% (30:0.8 cross-linked) low-ionic-strength native polyacrylamide gels as previously described (27). Usually, the polyacrylamide gels contained 0.03% (vol/vol) NP-40.

Sucrose gradient centrifugation. Linear 5 to 20% (wt/vol) sucrose gradients were prepared in BC buffer containing 0.03% NP-40 and 10 instead of 20% (vol/vol) glycerol. Samples were applied in a total volume of 100 μ l containing approximately 100 fmol of either purified OTF-1 or OTF-2

and 100 μ g of bovine serum albumin. The gradients were centrifuged at 320,000 \times g to a cumulative centrifugal effect of 1.8 \times 10¹² rad²/s in a Beckman L8-70 ultracentrifuge. Bovine serum albumin (4.4 S) and rabbit aldolase (7.8 S) were used as external sedimentation standards.

UV cross-linking. Purified OTF-1 was photoaffinity labeled essentially as described previously (4, 11, 11a, 12, 33). A bromodeoxyuridine (BrdU)-substituted heptamer element was prepared by annealing oligonucleotide 1 with the complementary primer 5'-CTCACCTGATTTGCATAT-3'. To prepare a mutant probe, the upper strand of oligonucleotide H⁻O⁺, which contained clustered point mutations in the heptamer element (see Fig. 2A), was annealed to the same primer. BrdU was incorporated by filling in with the large fragment of *Escherichia coli* DNA polymerase I and α -³²P-labeled dATP, dCTP, dGTP, and BrdUTP. OTF-1 (20 fmol) was incubated for 30 min at 30°C with 10 fmol of BrdU-substituted specific or mutant probes in a mixture containing 20 mM HEPES, 4% Ficoll, 2 mM EDTA, 0.2 mM dithiothreitol, 4 mM spermidine, 100 μ g of bovine serum albumin per ml, and 0.03% NP-40 in a total volume of 10 μ l. The samples were placed 5 cm from a UV lamp and irradiated at 1,600 μ W/cm² at 260 nm and 4°C for 20 min. After the addition of 1 μ l of a solution of 120 mM CaCl₂-24 mM MgCl₂ and 1 μ l of a mixture of 2 U of DNase I and 0.05 U of micrococcal nuclease per ml of 5 mM Tris hydrochloride (pH 7.5)-50 mM NaCl-35% (vol/vol) glycerol-50 ng of bovine serum albumin per ml-10 mM CaCl₂, the samples were digested for 20 min at 30°C. The reaction was stopped by the addition of 1 μ l of 0.5 M EDTA and 3 μ l of sodium dodecyl sulfate sample buffer. The mixture was then boiled for 3 min, electrophoresed on an 8% polyacrylamide-sodium dodecyl sulfate gel, and analyzed by autoradiography.

RESULTS

Copurification of ubiquitous and lymphoid-cell-specific heptamer- and octamer-dependent DNA-binding activities. To characterize and purify factors that interact with the conserved heptamer and octamer sequence motifs of immunoglobulin V_H promoters, Namalwa and HeLa cell nuclear extracts were prepared and fractionated by passage through DEAE-cellulose columns at a high salt concentration. The unbound fractions were diluted and subsequently applied to heparin-agarose columns at 50 mM KCl. The columns were washed at the same ionic strength and eluted with linear KCl gradients. DNA-binding activities were monitored by a gel mobility shift assay which used a 44-bp oligonucleotide containing the heptamer and octamer sequence motifs of the murine BC11 V_H promoter region (see Fig. 2A) as the specific DNA probe. With heparin-agarose-fractionated Namalwa nuclear extracts, four distinct protein-DNA complexes were observed and numbered according to their R_f values (Fig. 1A). These complexes appeared to be similar to the four migration-retarded species previously observed with crude B-lymphocyte nuclear extracts (15, 16). (Although additional, faster-migrating complexes were also observed, they varied in intensity in different experiments and could be attributed to nonspecific DNA-binding activities in the various column fractions.) The factors forming the four specific complexes C1 to C4 coeluted from the heparin-agarose column in the 100 to 240 mM KCl region of the gradient (Fig. 1A, lanes 7 to 12). In contrast, an analysis of HeLa nuclear extracts fractionated in an identical manner revealed only two major retarded complexes that corresponded in mobility to complexes C1 and C3 of Namalwa

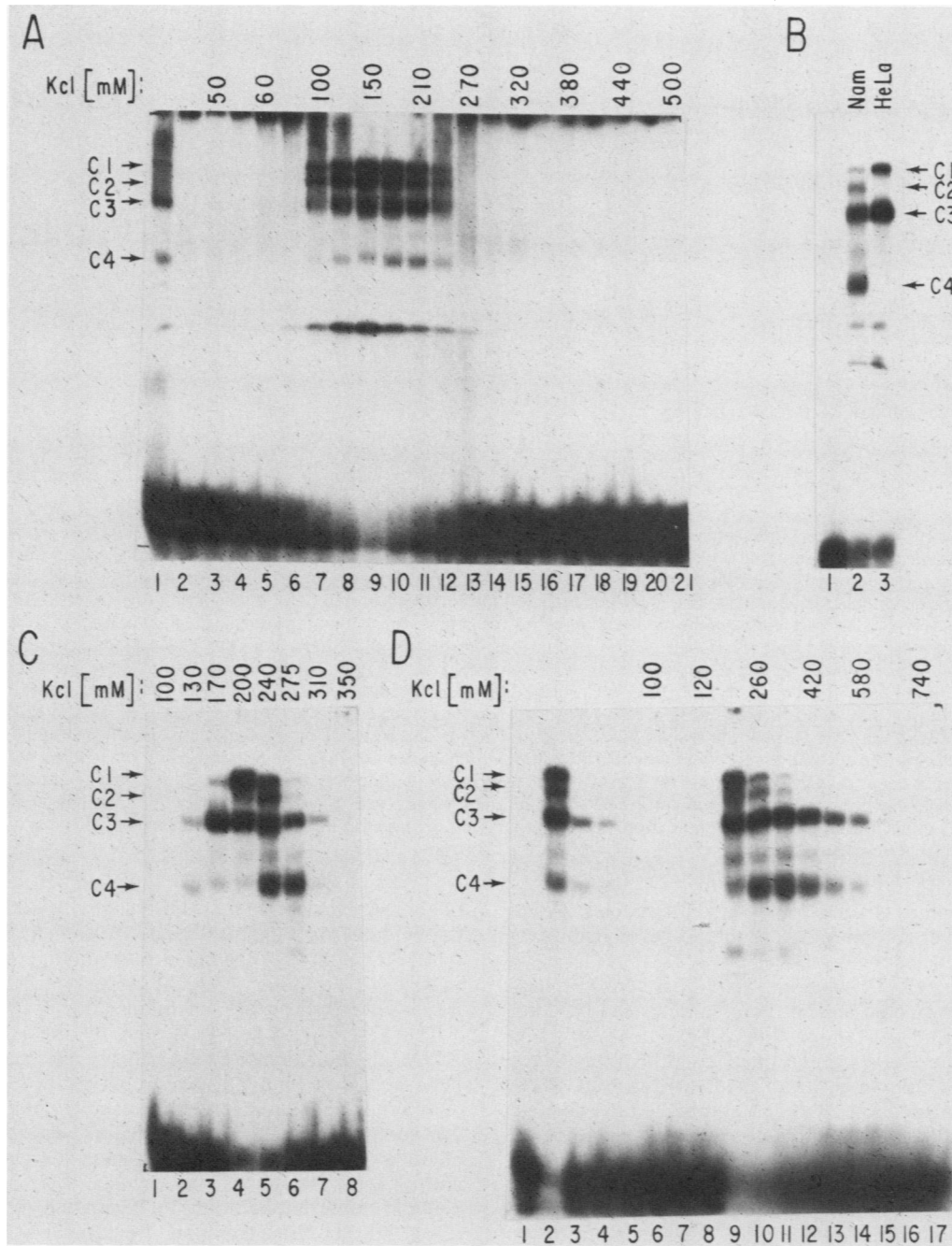


FIG. 1. Copurification of heptamer- and octamer-dependent DNA-binding activities. Extracts from approximately 10^{10} Namalwa or HeLa cell nuclei were passed over DEAE-cellulose at high ionic strength before further fractionation. (A) Heparin-agarose chromatography of Namalwa nuclear extract. DNA-binding reactions were carried out with an end-labeled wild-type BC11 promoter probe spanning both the heptamer and octamer elements (see Fig. 2A). The probe was incubated with 2- μ l aliquots of the column fractions and analyzed by gel mobility shift. The salt concentrations of the fractions are indicated above the lanes. C1 to C4 indicate specific protein-DNA complexes, whereas unbound probe runs at the bottom of the autoradiogram. Lanes: 1, input material; 2 to 4, wash fractions; 5 to 21, gradient elution fractions. (B) Comparison of the DNA-binding activities of heparin-agarose-fractionated nuclear extracts from Namalwa and HeLa cells. Namalwa and HeLa nuclear extracts were fractionated by heparin-agarose chromatography as described above. Aliquots (2 μ l) of the peak DNA-binding fractions (eluting at 180 mM KCl) were assayed for DNA-binding activity as for panel A. The incubations were performed with no added protein (lane 1), Namalwa nuclear extract peak fraction (lane 2), or HeLa nuclear extract peak fraction (lane 3). (C) Single-stranded DNA-agarose chromatography of heptamer- and octamer-binding activities. Peak fractions from the heparin-agarose column of panel A were analyzed by single-stranded DNA-agarose chromatography and assayed for DNA-binding activity as for panel A. (D) DNA affinity chromatography. Peak fractions from the single-stranded DNA-agarose column in panel C were chromatographed on an oligonucleotide affinity column containing the octamer motif of the T1 V_L promoter. The DNA-binding assay was carried out as described for panel A with no added protein (lane 1), input material (lane 2), flow-through fraction (lane 3), wash fractions (lanes 4 to 6), or salt gradient elution fractions (lanes 7 to 17).

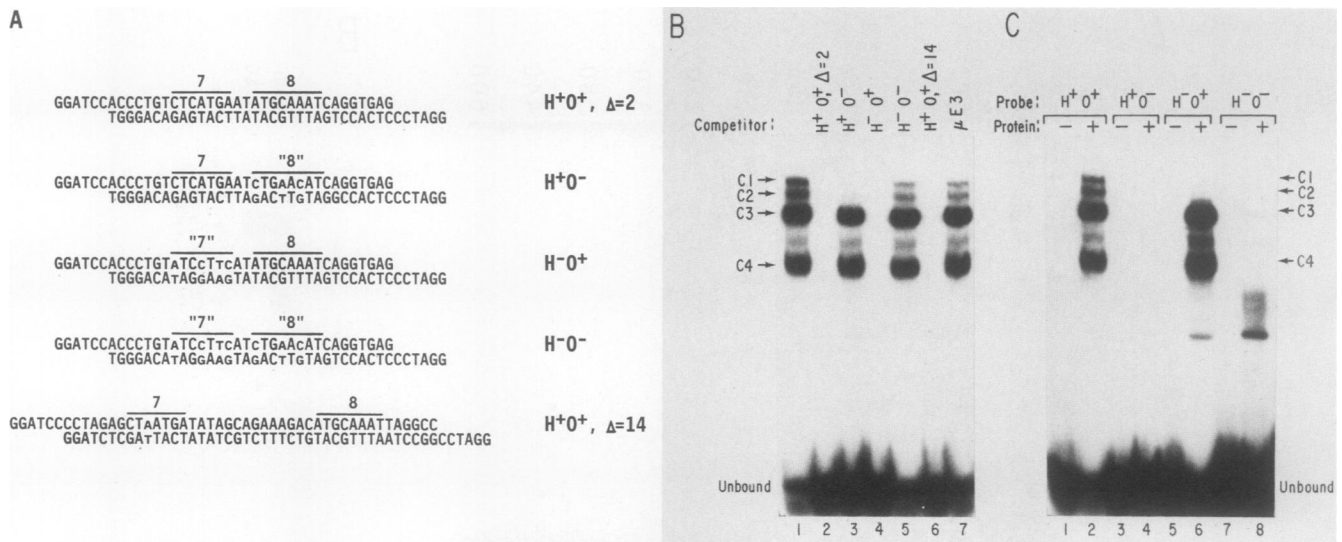


FIG. 2. Specificity of heptamer- and octamer-binding factors in heparin-agarose-fractionated Namalwa nuclear extract. (A) Nucleotide sequences of wild-type and point-mutated oligonucleotides used in this study. H^+O^+ ($\Delta = 2$) is the wild-type probe spanning the octamer and heptamer elements of the murine BC11 promoter in which the two sequence elements occur at a 2-bp distance from each other. The octamer (O) and heptamer (H) elements are indicated. The H^+O^- oligonucleotide carries clustered point mutations in the octamer element of the BC11 promoter, oligonucleotide H^-O^+ carries a point-mutated heptamer element of the same promoter, and oligonucleotide H^-O^- contains both mutated octamer and heptamer elements. Small capital letters indicate point-mutated nucleotides. The oligonucleotide H^+O^+ ($\Delta = 14$) contains the octamer and heptamer of the V1 promoter with a 14-bp spacing between the two elements. It may be noted that the V1 promoter contains a transversion in the third base of the heptamer (indicated by a small capital letter). (B) Competition experiments. The end-labeled H^+O^+ ($\Delta = 2$) probe was incubated with 2 μ l of heparin-agarose-fractionated Namalwa nuclear extract (Fig. 1A) and subjected to gel mobility shift analysis (lane 1). Lanes 2 to 7 show the results of identical analyses in the presence of a 500-fold molar excess of unlabeled competitor DNA as indicated above the autoradiogram. (C) Direct binding studies. The indicated wild-type or point-mutated BC11 promoter probes were incubated with 2 μ l of heparin-agarose-fractionated Namalwa nuclear extract (+ lanes) or with buffer alone (- lanes) and analyzed by the gel migration shift assay. The intermediary band migrating between C3 and C4 has been observed previously with Namalwa cell nuclear extract (11) and represents either a distinct OTF-1 degradation product or an additional (as yet unidentified) octamer-specific factor. The appearance of this band is not heptamer dependent.

cells (Fig. 1B; compare lanes 2 and 3). Thus, species C2 and C4 were tentatively identified as lymphoid-cell-specific protein-DNA complexes.

The Namalwa cell DNA-binding activity eluted from heparin-agarose was pooled and further characterized by chromatography on single-stranded DNA-agarose. A slight separation of the four factors generating C1 to C4 was achieved by elution of the column by a salt gradient (Fig. 1C). Thus, the factor responsible for C4 eluted at a higher salt concentration (around 250 mM KCl) than those responsible for the slower-migrating species C1 to C3 (elution centered around 200 mM KCl). During further purification, the factors responsible for all four complexes were efficiently retained on a DNA affinity column containing the octamer motif of the immunoglobulin T1 V_L promoter (27). Upon application of a salt gradient, all of the factors involved in formation of the four complexes coeluted over a range of 200 to 250 mM KCl (Fig. 1D), indicating similar affinities for the affinity resin.

Specificities of heptamer- and octamer-binding factors. The specificities of the four copurifying factors responsible for formation of C1 to C4 were tested by performing gel mobility shift reactions in the presence of the different competitor oligonucleotides shown in Fig. 2A. Oligonucleotide H^+O^+ ($\Delta = 2$) contained the wild-type sequence from positions -73 to -42 of the murine BC11 promoter (13, 15), with the heptamer sequence element 2 bp upstream of the octamer; H^+O^- contained three octamer element point mutations which are known to be detrimental to octamer function in vivo and OTF binding in vitro (11); H^-O^+ contained four transversions in the heptamer element; and H^-O^- was a

double mutant combining the clustered point mutations of the heptamer and octamer sequence elements. Oligonucleotide H^+O^+ ($\Delta = 14$) spanned positions -86 to -45 of the murine V1 promoter, with the heptamer and octamer elements separated by 14 nucleotides (7).

The four complexes generated by the wild-type BC11 H^+O^+ oligonucleotide probe with a peak fraction from the heparin-agarose column were abolished completely by any competitor fragment containing an intact octamer motif (Fig. 2B; compare lanes 1, 2, 4, and 6). In contrast, the oligonucleotide containing point mutations in the octamer element and an intact heptamer sequence (H^+O^-) competed, at a 500-fold molar excess, only for formation of complexes C1 and C2 and not for formation of complexes C3 and C4 (Fig. 2B; compare lanes 1 and 3). However, competition for formation of C1 and C2 was not observed at a 100-fold molar excess of the unlabeled H^+O^- oligonucleotide, whereas a 100-fold molar excess of any octamer-containing oligonucleotide effectively competed for generation of all of the complexes (data not shown). Neither the double mutant (H^-O^-) nor an oligonucleotide ($\mu E3$) spanning a distinct factor interaction site within the immunoglobulin heavy-chain enhancer (31) competed for binding (Fig. 2B; compare lanes 1, 5, and 7). In direct-binding experiments, mutations in the octamer element eliminated formation of any of the retarded complexes (Fig. 2C; compare lanes 2, 4, and 8), whereas mutations in the heptamer element eliminated only C1 and C2 but not C3 and C4 (Fig. 2C; compare lanes 2 and 6). In fact, the electrophoretic mobilities of complexes C3 and C4 were identical, respectively, to those of the OTF-1 and

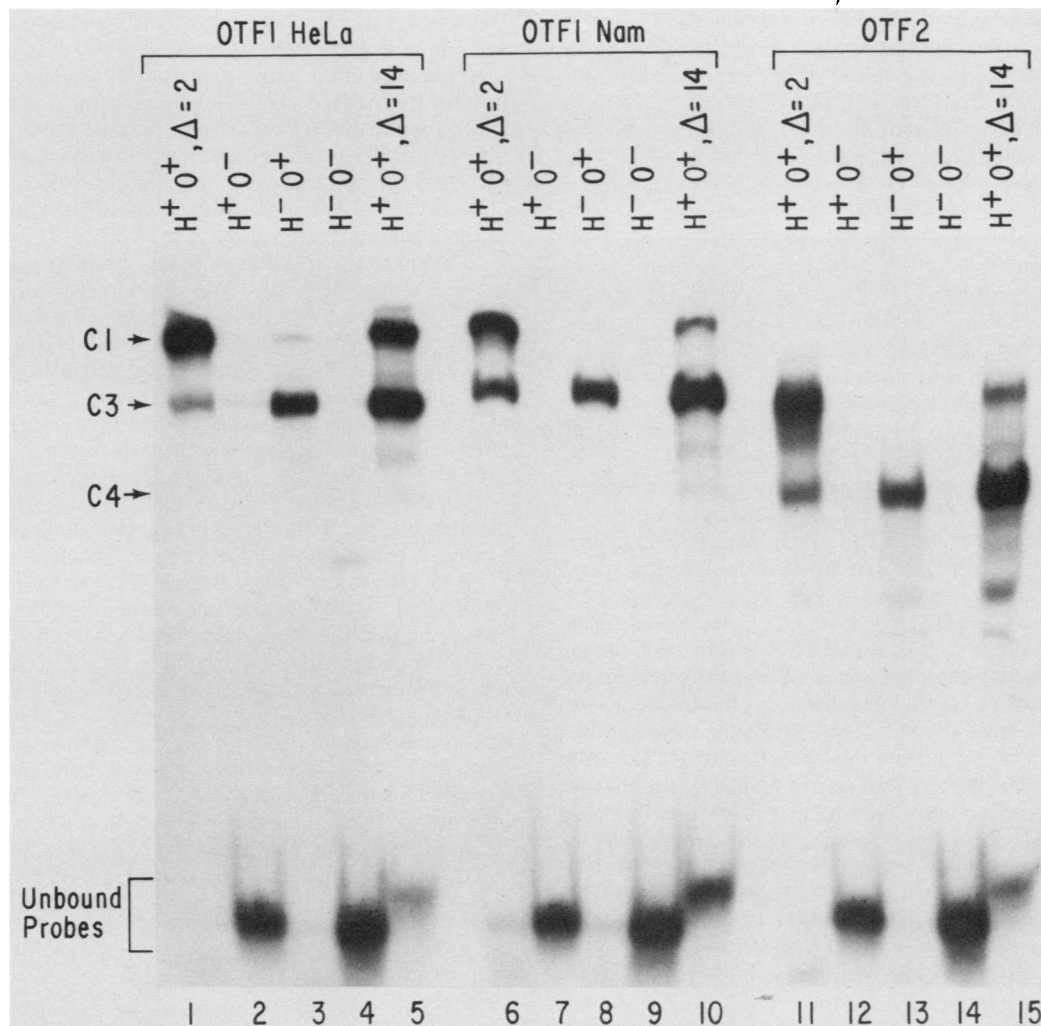


FIG. 3. DNA-binding studies with affinity-purified OTF proteins. Affinity-purified OTF-1 from HeLa cells (lanes 1 to 5) or from Namalwa cells (lanes 6 to 10) or affinity-purified OTF-2 (lanes 11 to 15) was incubated with five different end-labeled wild-type or point-mutated V_H promoter probes as indicated. Protein-DNA complexes thus generated were analyzed by the gel mobility shift assay.

OTF-2 complexes formed with an identically sized immunoglobulin heavy-chain enhancer fragment containing only the octamer element (data not shown). Thus, while C3 and C4 formation depended only upon an octamer element, it appears that the formation of C1 and C2 was both heptamer- and octamer-dependent and that the heptamer sequence had an intrinsic, albeit low, ability to compete for a factor(s) involved in C1 and C2 formation.

Binding of affinity-purified OTF-1 and OTF-2 to the heptamer sequence. Given the fact that the heptamer- and octamer-binding activities copurified and that the octamer element alone was sufficient to compete for formation of all the specific complexes, we next addressed the possibility that OTF-1 and OTF-2 could each recognize both the heptamer and octamer elements. To this end, the purified ubiquitous factor (OTF-1) from both HeLa cells (9) and B lymphocytes (C. Scheidereit, unpublished data) and the purified lymphoid-cell-specific OTF-2 (27) were used in direct-binding experiments with wild-type and mutant oligonucleotides as the specific probes. Each of these purified factors generated a more slowly migrating, heptamer-dependent complex in addition to the bona fide octamer-dependent complexes characteristic of each factor (C3 for OTF-1 and

C4 for OTF-2) (Fig. 3, lanes 1, 6, and 11). The slower- and faster-migrating complexes generated by either OTF-1 or OTF-2 were detected with both a 2-bp and a 14-bp spacing between the heptamer and octamer sequence elements, respectively (Fig. 3; compare lanes 1, 6, and 11 with lanes 5, 10, and 15, respectively). This analysis also revealed that the slower-migrating heptamer-dependent complex formed with OTF-2 comigrated with the faster-migrating of the two complexes (presumably monomeric C3) generated with OTF-1 (Fig. 3; compare lanes 3, 8, and 11). Thus, only three distinguishable retarded species, corresponding to the above-characterized complexes C1, C3, and C4, were detected with the purified OTFs, as opposed to the four complexes observed in cruder fractions (see above).

Mixtures of purified OTF-1 and OTF-2 generate heteromeric complexes. To investigate the mechanism(s) by which OTF-1 and OTF-2 interact with both the heptamer and octamer sequence motifs, we tested whether they could form native dimer structures in the absence of DNA or whether the OTFs could be associated with an additional (unidentified) factor which might be important in facilitating OTF interactions with the heptamer element. Sucrose gradient centrifugation was used to determine the sedimentation

coefficients of the individual, affinity-purified OTFs and to score possible large protein-protein complexes. The distribution of the proteins was assayed by gel mobility shift analysis of the recovered fractions. Purified OTF-1, a 90-kDa protein under denaturing conditions (9), sedimented as a 4S-to-4.5S entity when assayed with either an H^+O^+ or an H^-O^+ probe (Fig. 4A). OTF-2, which is a 60-kDa protein under denaturing conditions (27), sedimented as a slightly slower-migrating species centered in the 4S region of the gradient, as assessed by DNA-binding experiments using either the wild-type BC11 H^+O^+ (Fig. 4B) or the mutant H^-O^+ (data not shown) oligonucleotides as specific probes. Given the recently demonstrated identity between OTF-1 and the adenovirus replication factor NF-III (21) and the Stokes radius of 6.1 nm for NF-III (22), the native mass for OTF-1 was calculated to be approximately 100 kDa. Thus, the sedimentation behavior of both OTF-1 and OTF-2 appeared to be consistent with that of asymmetric monomers. Importantly, for both OTF-1 and OTF-2, the activities which generated the heptamer-dependent complexes (C1 for OTF-1 and C3 for OTF-2) were coincident with the fraction which generated the heptamer-independent octamer complexes (C3 for OTF-1 and C4 for OTF-2) (Fig. 4A, lanes 3 and 4, and Fig. 4B, lanes 4 to 6). This suggests that formation of the heptamer-dependent complexes does not require preformed dimeric structures of OTFs but rather that it can occur with the monomeric forms of the OTFs. To further substantiate this contention, we mixed the peak fraction of OTF-1 from the sucrose gradient with the corresponding fraction of OTF-2 in an approximately 1:1 molar ratio prior to incubation with the H^+O^+ DNA probe. By this procedure, we detected an additional gel-retarded species, C2 (Fig. 4B, lane 22), that was not observed with the individual, purified OTFs (Fig. 3 and Fig. 4B, lanes 5 and 21). We conclude that complex C2 most likely represents a heterodimer of OTF-1 and OTF-2; this would explain why this complex appears as a B-lymphocyte-specific species in cruder fractions (Fig. 1B).

UV cross-linking of affinity-purified OTF-1 to the heptamer sequence element. To identify unambiguously the OTFs as the active heptamer-binding species, purified OTF-1 was specifically cross-linked by UV irradiation to an H^+O^+ probe containing a BrdU-substituted heptamer element. The BrdU-substituted probe was prepared by annealing the upper strand of the BC11 H^+O^+ probe with a primer encompassing the octamer sequence and the 2 bp between the heptamer and octamer elements. In this manner, BrdU could be selectively incorporated over the heptamer. Substitution with BrdU did not change the specific binding activity, as assessed by the electrophoretic mobility shift assay (data not shown). The BrdU-substituted oligonucleotide was incubated with an excess of affinity-purified OTF-1 from HeLa cells, irradiated with UV light, and subjected to nuclease digestion. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the digested material revealed a nuclease-resistant species of approximately 95 kDa (Fig. 5, lanes 2), consistent with the molecular mass of purified HeLa OTF-1 (90 to 100 kDa) (9, 30). Formation of this band was blocked by addition of the unlabeled H^+O^+ oligonucleotide prior to exposure to UV irradiation (Fig. 5, lane 3) but not by the double mutant H^-O^- oligonucleotide (lane 4). A specific band of a size identical to that of the one described above was detected upon UV cross-linking of OTF-1 to a BrdU-substituted octamer element (data not shown). Moreover, it was not possible to cross-link OTF-1 to an oligonu-

cleotide (H^-O^+) containing a BrdU-substituted, point-mutated heptamer element (Fig. 5, lane 6).

Generation of the large, heptamer-dependent complex by an in vitro synthesized OTF-2 fragment. An OTF-2 fragment of approximately 40 kDa was synthesized by in vitro translation of mRNA produced by in vitro transcription of OTF-2 cDNA clone 15 (26a). The specific DNA-binding properties of the expressed fragment were indistinguishable from those of the bona fide, affinity-purified 60-kDa OTF-2 as assessed by competition experiments using the histone H2b octamer motif as a specific probe (26a). To detect binding activity for the heptamer motif, a reticulocyte lysate primed with sense RNA derived from clone 15 was incubated with the BC11 H^+O^+ oligonucleotide. When examined with nondenaturing polyacrylamide gel electrophoresis, the expressed OTF-2 cDNA fragment generated two strong bands with faster mobilities than the bands generated with affinity-purified OTF-2 (Fig. 6A, lane 2). Both the upper and lower complexes were competed for by an unlabeled H^+O^+ oligonucleotide but not by the H^-O^- double mutant (Fig. 6A, lanes 3 and 4, respectively). Moreover, incubation of the expressed OTF-2 fragment with an H^-O^+ mutant oligonucleotide as the DNA probe only generated the lower band, indicating that the upper band represented heptamer-dependent complex formation (Fig. 6A, lane 5). Control experiments performed with lysates primed with clone 15 antisense RNA (Fig. 6A, lane 6) or with no synthetic RNA at all (data not shown) failed to reveal any octamer-specific DNA-binding activity other than a weak band resulting from complex formation with the endogenous OTF-1, which was also detectable in lysates primed with the sense RNA (Fig. 6A, compare lanes 2 and 6).

To determine whether the expressed OTF-2 cDNA fragment could generate heptamer-induced hybrid complexes with purified, wild-type OTFs, mixing experiments were performed. Incubation of the wild-type BC11 H^+O^+ probe with a mixture of in vitro-translated clone 15 mRNA and OTF-2 generated an intermediate species migrating between the dimeric OTF-2 complex (C3) and the expressed homodimer on native 4% polyacrylamide gels with a slightly lower degree of cross-linking (40:1) compared with above (30:08) (Fig. 6B, lanes 3, 4 and 6). The mobility of this intermediate species most likely resulted from binding to the H^+O^+ oligonucleotide of a heteromeric structure consisting of the 40-kDa expressed OTF-2 and the wild-type 60-kDa cellular OTF-2. By analogy, incubation of the H^+O^+ probe with a mixture of expressed, truncated OTF-2 and the 90-kDa purified OTF-1 resulted in formation of a complex of slightly faster mobility than the cellular OTF-1-OTF-2 heterodimer (C2) (Fig. 6B, lanes 3, 4, 5, 7, and 8). Interestingly, a slight separation was indicated on these gels between the heptamer-dependent dimeric form of OTF-2 (migrating in the C3 region; see above) and the monomeric form of OTF-1 (also migrating in the C3 region) (Fig. 6B; compare lanes 4 and 5). This supports our conclusion that in more crude fractions containing both OTFs, C3 harbors both monomeric OTF-1 and dimeric OTF-2. Finally, formation of the hybrid complexes of expressed OTF-2 and cellular OTFs was heptamer dependent, since they were not detected in binding experiments using the mutant H^-O^+ oligonucleotide as the specific probe (Fig. 6B; compare lanes 6, 7, 12, and 13).

DISCUSSION

In our effort to characterize and understand the molecular mechanisms underlying lymphoid-cell-specific gene tran-

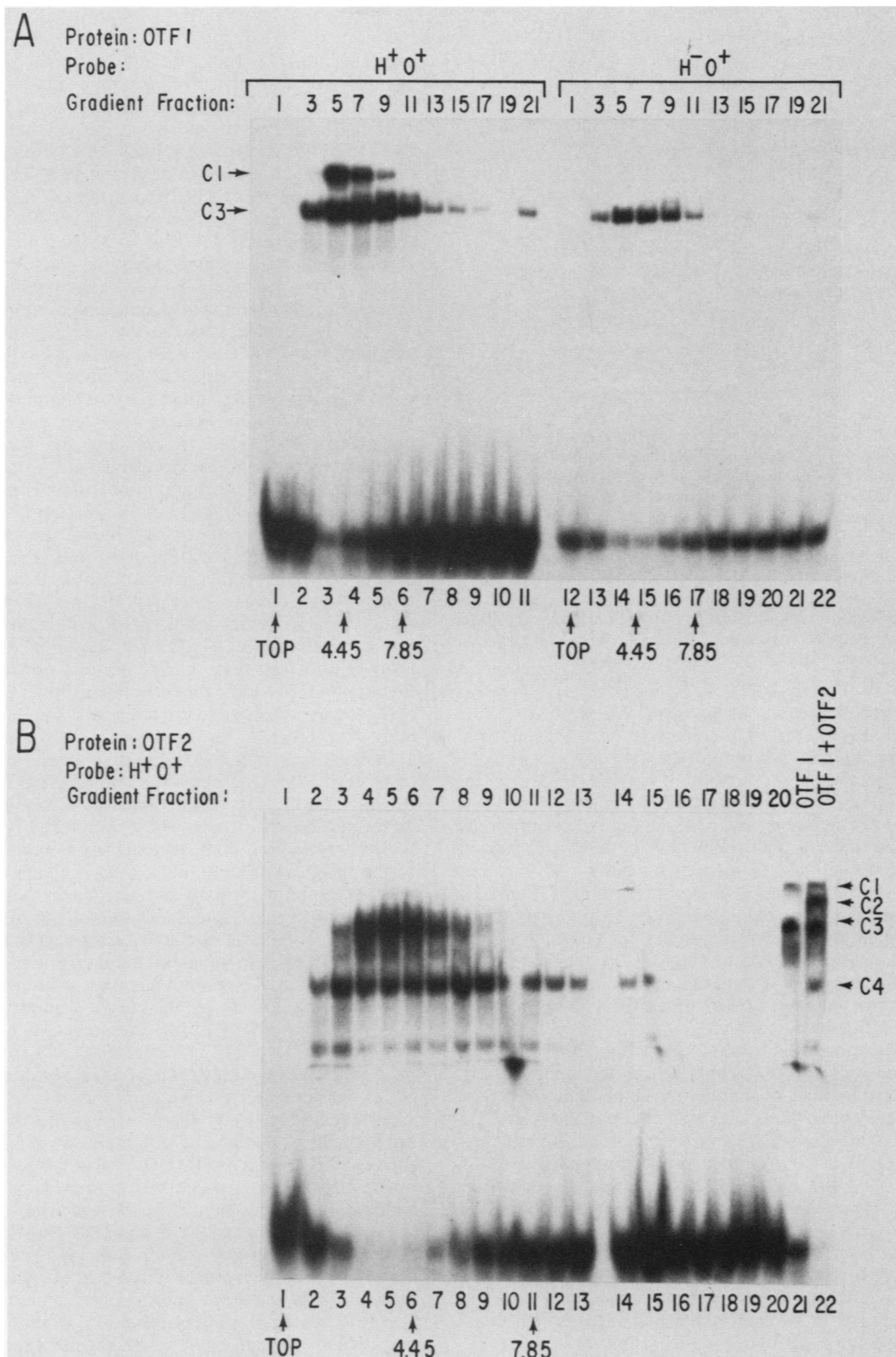


FIG. 4. Sucrose gradient sedimentation of affinity-purified OTFs. OTFs were analyzed by gradient centrifugation as described in Materials and Methods. Fractions (5 μ l) were assayed for DNA-binding activity by the gel mobility shift assay. External sedimentation standards were centrifuged in parallel with the OTFs; the top of the gradient and the sedimentation positions of the standards are indicated. (A) Sedimentation analysis of purified HeLa OTF-1. Odd-numbered gradient fractions were incubated with either the wild-type BC11 H^+O^+ probe (lanes 1 to 11) or the mutant H^-O^+ probe (lanes 12 to 22). (B) Sedimentation analysis of purified OTF-2. Fractions were incubated with the wild-type BC11 probe. Lanes 1 and 20 correspond to the top and the bottom of the gradient, respectively. For reference, fraction 6 of gradient-fractionated OTF-1 (see panel A) was incubated either with probe alone (lane 21) or with probe and fraction 5 of gradient-fractionated OTF-2 (lane 22).

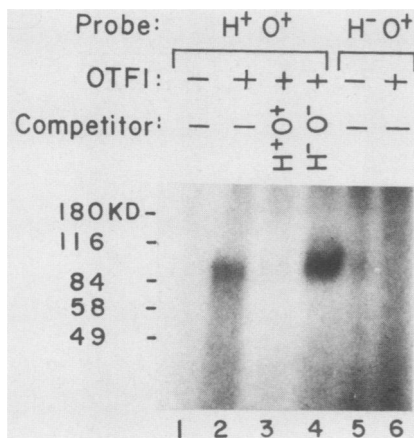


FIG. 5. UV cross-linking of OTF-1 to the heptamer element. Affinity-purified HeLa OTF-1 was incubated with a ³²P-labeled BrdU-substituted heptamer element within the wild-type BC11 H⁺O⁺ probe in the absence of competitor (lane 2) or in the presence of a 200-fold molar excess of the unlabeled H⁺O⁺ (lane 3) or mutant H⁻O⁺ (lane 4) oligonucleotides. OTF-1 was also incubated with a BrdU-substituted mutant heptamer probe (H⁻O⁺; lane 6). In control reactions, the probes were incubated without any protein (lanes 1 and 5). The samples were irradiated and processed as described in Materials and Methods prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Molecular masses (in kilodaltons) are indicated on the left.

scription, we have attempted to identify and purify a factor(s) specific for the strongly conserved heptamer motif of the immunoglobulin V_H promoter. Both this sequence element and the similarly conserved octamer element are important determinants for V_H promoter function (1, 7). It is possible that the heptamer element acts in concert with the octamer to confer cell type specificity on the V_H promoter, as indicated by deletion experiments on the V47 V_H gene (19). This gene contains two octamer motifs, but only deletion of the TATA-proximal octamer and more than half of the immediately adjacent heptamer proved to be detrimental to transcription (19). In support of coordinate function of the heptamer and octamer elements, coordinate DNA binding to both sequence motifs of the BC11 V_H promoter has been described (15, 16).

Here we demonstrated that the ubiquitous OTF-1 and the lymphoid-cell-specific OTF-2 bind to the heptamer motif of V_H promoters. Significant binding to the heptamer required an intact, adjacent octamer and occurred both on the BC11 promoter probe with a 2-bp spacing between the two elements and on the V1 promoter probe containing a 14-bp spacing. In the case of the BC11 promoter, the last four bases of the heptamer, the two intervening nucleotides and the first two nucleotides of the octamer, showed a six-of-eight match with the consensus octamer (17). However, this alignment is not possible in the V1 promoter containing the 14-bp spacing between the two elements. Thus, the heptamer represents an OTF-binding element which bears no obvious sequence similarity to the octamer.

It is unlikely that OTF specificity for the heptamer was conferred by any additional cellular factor associated with the OTFs. The affinity-purified OTF proteins sedimented on sucrose gradients as apparent monomers, and formation of the heptamer-dependent protein-DNA complexes did not require any preformed, larger homomeric or heteromeric protein structures. Moreover, UV cross-linking experiments with the heptamer motif revealed only a species correspond-

ing in size to OTF-1 and not any additional factor or a larger complex of unknown protein composition. Thus, we conclude that the same protein can recognize two apparently different DNA sequences, as has been shown previously for the C/EBP protein from rat liver (18 and references therein) and the yeast HAP1 activator protein (25). The two sequence motifs recognized by C/EBP are the CCAAT homology of several promoters and the enhancer core homology common to many enhancers, whereas HAP1 exhibits specificity for two dissimilar upstream activation sites of two different yeast genes. In contrast, heptamer binding of an OTF seemed to be coordinated with an interaction of the protein with the adjacent octamer sequence such that the octamer site must first be occupied for heptamer binding to occur. In the absence of an adjacent octamer element, the OTFs exhibited only weak affinity for the heptamer sequence, as indicated by the requirement of a very large excess of the heptamer motif alone to compete for formation of the heptamer-dependent species C1 and C2. Consequently, it is possible that protein-protein interactions between the OTFs facilitated and stabilized interaction with the heptamer, resulting in cooperativity in binding. In support of the possibility of a physical contact between the heptamer- and octamer-associated factors, footprinting analysis of a heptamer- and octamer-generated protein-DNA complex revealed a large, extended area of protection covering both sequence elements (16, 17, 26). Possibly relevant to this issue is the fact that specific protein-protein interactions involving OTF-1 have recently been shown to occur. Thus, OTF-1 can associate with a herpes simplex virus *trans*-activating protein, resulting in the formation of a stable complex on an octamer-related sequence element that is conserved among herpes virus immediate early genes (11a).

Competition with the octamer motif abolished the heptamer-dependent protein-DNA complexes, consistent with the possibility that both octamer and heptamer interactions of the OTFs involve a single DNA-binding domain. However, we cannot formally exclude the possibility that heptamer binding was lost as a result of the dissociation of a stabilizing octamer-associated factor rather than by direct competition of the octamer sequence for the heptamer-binding domain. To resolve this issue, the delineation of the DNA-binding domain by deletion or mutation analysis (or both) of cloned OTF-1 (A. Pierani et al., manuscript in preparation) and OTF-2 (26a) cDNAs will be of importance in the ultimate identification of OTF residues that contact either the heptamer or the octamer motifs.

Although OTF-1 and OTF-2 represent two different gene products (26a; Pierani et al., manuscript in preparation), they exhibit virtually indistinguishable DNA-binding properties (A. Heguy, C. Scheidereit, and R. G. Roeder, unpublished data). Interestingly, we observed no differences between the abilities of OTF-1 and OTF-2 to form heptamer-dependent protein-DNA complexes. However, our finding that the OTFs recognized sites that display considerable differences in sequence suggests a degeneracy which may serve to create a variation in binding affinities that, in turn, may widen the range over which gene expression can be modulated. Therefore, it is possible that OTFs, by protein-protein interaction, may form homomeric or heteromeric complexes which achieve cooperativity or selectivity (or both) in function. In this context, it will be important to determine whether the B-cell-specific function of V_H promoters is mediated by a heteromeric OTF-1-OTF-2 complex or by homomeric structures and whether additional cellular factors which recognize either DNA or protein are required.

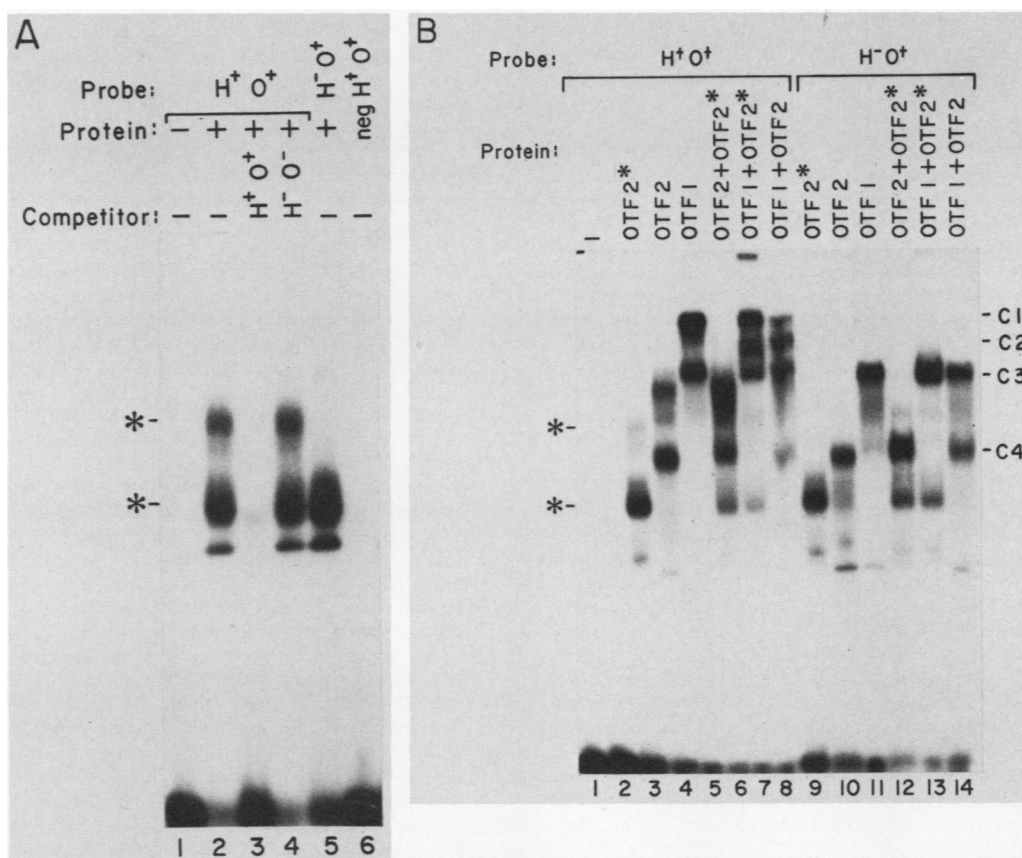


FIG. 6. In vitro expressed OTF-2 cDNA generated a heptamer-dependent slower migrating protein-DNA complex. (A) The wild-type H^+O^+ BC11 probe was incubated with buffer alone (lane 1), with rabbit reticulocyte lysate programmed with in vitro-generated OTF-2 mRNA (lanes 2 to 4), or with lysate programmed with OTF-2 antisense RNA (neg; lane 6). In addition, lysate programmed with OTF-2 mRNA was incubated with the mutant probe H^-O^+ (lane 5). The binding reactions containing in vitro-translated OTF-2 were performed in the absence of competitor (lane 2) or in the presence of a 200-fold molar excess of either specific competitor H^+O^+ (lane 3) or nonspecific competitor H^-O^- (lane 4). All binding reactions were analyzed by native polyacrylamide gel electrophoresis as described above. Specific DNA complexes generated by expressed OTF-2 cDNA are indicated (*). (B) The wild-type BC11 H^+O^+ oligonucleotide (lanes 1 to 8) or the mutant H^-O^+ oligonucleotide (lanes 9 to 14) was incubated in standard binding reactions with in vitro-expressed OTF-2 cDNA (OTF2*) alone (lanes 3 and 9), with mixtures of expressed OTF-2 and purified cellular OTF-2 (lanes 6 and 12), or with mixtures of expressed OTF-2 and purified cellular OTF-1 (lanes 7 and 13). Lane 1 shows a binding reaction performed in the absence of any added protein; lane 2 contains rabbit reticulocyte lysate primed with clone 15 antisense RNA. Lanes 4 and 10 contain purified OTF-2 alone, and lanes 5 and 11 contain purified OTF-1 alone. Purified OTFs were incubated together before addition of specific probe (lanes 8 and 14). All binding reactions were analyzed by native polyacrylamide gel electrophoresis as described above. In a shorter autoradiography exposure, the upper band in lane 6 was resolved into two distinct bands, one comigrating with the C3 complex in lane 4 and one running slightly faster. When the analyses shown in lanes 7 and 8 were repeated under more discriminating electrophoretic conditions, the C2 complex generated by OTF-1 and OTF-2* clearly migrated faster than the C2 complex generated with OTF-1 and OTF-2, whereas the C1 and C3 complexes showed similar mobilities in each case.

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