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# NF-HB (BSAP) Is a Repressor of the Murine Immunoglobulin Heavy-Chain 3′α Enhancer at Early Stages of B-Cell Differentiation

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**We have identified a nuclear factor expressed in pro-B-, pre-B-, and B-cell lines that binds to two sites within the murine immunoglobulin heavy-chain (IgH) 3′α enhancer (3′αE). These sites were defined by oligonucleotide competition in an electrophoretic mobility shift assay (EMSA) and methylation interference footprinting. The 3′αE-binding factor is indistinguishable from NF-HB (B-lineage-specific nuclear factor that binds to the IgH gene) and the B-lineage-specific transcription factor BSAP by several criteria, including similar cell type distribution of binding activity, cross-competition of binding sites in EMSA, similar protein size as demonstrated by UV cross-linking, and sequence identity of one of the 3′αE-binding sites with a BSAP-binding site within the promoter of the sea urchin late histone gene H2A-2.2. These observations indicate that 3′αE is one of the mammalian targets for NF-HB (BSAP). Transient-transfection assays with chloramphenicol acetyltransferase gene constructs containing 3′αE and mutant 3′αE, in which one of the NF-HB binding sites was inactivated by site-specific mutagenesis, showed ca. five- to sixfold-enhanced activity of mutated 3′αE over parental 3′αE in B-cell lines (NF-HB$^+$), while no significant difference was observed in plasmacytoma cells (NF-HB$^-$). We conclude from these observations that NF-HB (BSAP) acts as a repressor of the mouse IgH 3′αE.**

The first tissue-specific eukaryotic enhancer was defined in the immunoglobulin heavy-chain (IgH) locus, located upstream of the μ switch region, i.e., Eμ (5, 17). Several *cis*-acting elements within Eμ are important for its activity, only some of which are lymphoid cell specific. The latter include the highly conserved octamer motif (ATTTGCAT), also present in all Ig promoters (14, 46), and μB (32, 37).

Although the importance of Eμ in B-lymphoid cell-specific Ig expression has been well established, various observations have shown that in a number of myeloma cell lines, IgH gene transcription can continue despite its absence (2, 12, 25, 43). These and other observations (20) indicated that the IgH gene might contain additional regulatory elements. Indeed, a second B-cell-specific enhancer located ~16 kb downstream of the $C_\alpha$ gene in the mouse has been identified (11, 33, 39). The role of this second enhancer, 3′αE, in normal Ig gene expression remains to be elucidated.

Insight into the action of 3′αE has come from various observations. Dariavach et al. (11) showed that upon transfection of reporter constructs containing 3′αE into B-cell and plasma cell lines, 3′αE was active only in the plasma cells. Additionally, our examination of the methylation status of the 3′αE and the sequences flanking it has shown that at early stages of B-cell differentiation, represented by pre-B- and B-cell lines, the region 3′ of the $C_\alpha$ gene (3′α region) was hypermethylated; in contrast, in plasmacytoma cell lines, and independent of the specific isotype of Ig produced, the 3′α region was undermethylated (16). Moreover, we have observed the acquisition of a DNase I-hypersensitive site near 3′αE in plasmacytoma cells (16). Together, these observations suggested that 3′αE might play a role late in normal B-cell development at the transition between B and plasmacytoma cells, when B cells are activated by antigen and T-cell help. This is in contrast to Eμ, which is active at all stages of B-cell differentiation (15).

Comparison of the sequence of 3′αE with that of Eμ revealed the presence of a number of common motifs. By analogy to Eμ, *trans*-acting factors binding to these sequences were candidates for 3′αE activation. Recent studies by Grant et al. (19) of rat 3′αE have shown the importance of several of these elements, namely, μB, octa, μE1, and μE3 (13) and also μA. None, however, appears to be differentially expressed during B-cell development. In order to understand the function of 3′αE during B-cell differentiation, we have been interested in identifying *trans*-acting factors that might be important for its function in a developmentally regulated manner.

Our laboratory has identified an early B-lineage-specific nuclear protein, NF-HB (B-lineage-specific nuclear factor that binds to the IgH gene), which binds to various sequences within and 3′ of the IgH gene locus (31). By several criteria, NF-HB has been shown to be indistinguishable from a B-cell-specific transcription factor, BSAP (7), which was originally identified as a mammalian homolog of a sea urchin tissue-specific transcription factor, TSAP (6). Recently, Adams et al. have cloned the BSAP gene and found the protein to be encoded by the *Pax-5* gene, a member of a multigene family encoding paired-domain proteins in vertebrates (1). Originally identified in *Drosophila* cells (9), evolutionarily conserved *Pax* proteins (one of which is BSAP) are strongly implicated as important regulators for early development (4, 44). *Pax-5* (BSAP) has been shown to be expressed not only in B lymphocytes but also in the developing central nervous system and in adult testis (1).

Here we show that NF-HB (BSAP) protein binds to murine 3′αE at two sites and functions as a negative regulator in B-cell lines; in terminally differentiated plasmacy-

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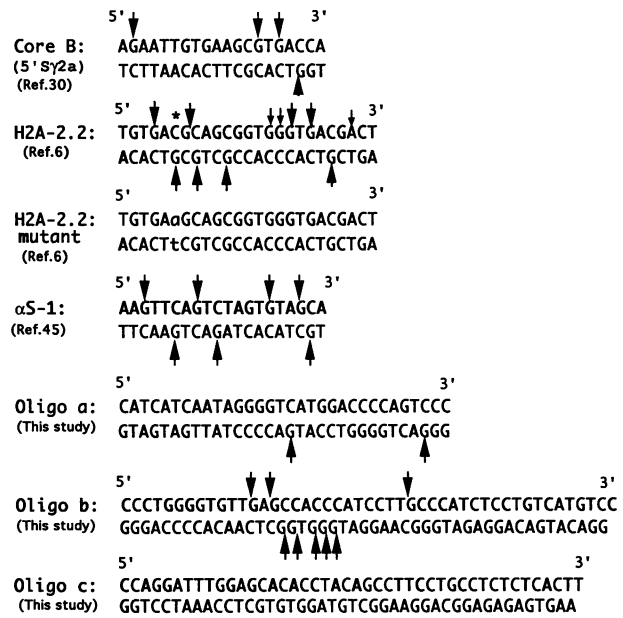


FIG. 1. Oligonucleotides used in EMSAs for direct binding or competition. All oligonucleotides except oligonucleotide c represent NF-HB binding sites. Arrows indicate contact point G residues, as identified by methylation interference footprinting. The H2A-2.2 mutant contains a single-base-pair alteration of the "invariant C" (asterisk), the single invariant nucleotide among four BSAP (TSAP) binding sites in promoters of the sea urchin late histone genes H2A-2 and H2B-2 (6).

toma cells, when NF-HB (BSAP) is no longer present, 3′αE is fully active.

## MATERIALS AND METHODS

**Cell lines.** Murine cell lines representing various stages of B-cell development included BascG2/C7 (pro-B); 3-1 and 18-81 (pre-B); W231, BCL1, A20, and M12.4.1 (B-cell lymphomas); MPC11, P3X, J558L, and S194 (plasmacytomas); EL-4 and BW5147 (T lymphomas); and MEL (murine erythroleukemia). All pro-B-, pre-B-, and B-cell lines and S194 were grown in RPMI 1640 (Whittaker Bioproducts Inc., Walkersville, Md.) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, Kans.), 50 μM β-mercaptoethanol, 1% penicillin-streptomycin, and 2 mM L-glutamine. Other plasmacytomas and non-B-cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All cell lines were grown in suspension culture at 37°C in an atmosphere of 7 to 8% $CO_2$.

**Plasmids and oligonucleotides.** The murine 3′αE had been cloned in the *Kpn*I and *Bam*HI sites of vector pBS (Stratagene, San Diego, Calif.), and the two fragments I and II were also cloned in pBS (33).

Complementary oligonucleotides were synthesized chemically with a model 380B DNA synthesizer and annealed. The oligonucleotide concentration was quantitated spectrophotometrically under conditions in which a concentration of 20 μg/ml yields an optical density at 260 nm of 1.0. The octamer (ATT TGC AT) and μE5 (CAG CTG CAG GTG) oligonucleotides contain three and two copies, respectively, of the motif. Figure 1 shows the sequences of the oligonucleotide-binding sites for NF-HB (BSAP) used in this study.

**EMSA.** For the electrophoretic mobility shift assay (EMSA), nuclear extract was prepared by the mini-extract method of Schreiber et al. (41) with slight modification. Typically, cells were grown to a density of $1 \times 10^6$ to $2 \times 10^6$/ml, and approximately $10^7$ cells were taken to prepare the mini-extract and frozen immediately without dialysis. The protein concentrations of all the extracts were determined by the Bradford assay with bovine serum albumin as the standard (Bio-Rad, Richmond, Calif.).

DNA probes used for EMSA were labeled with α-$^{32}$P-deoxyribonucleotides (NEN, Boston, Mass.) to a specific activity of $\sim 5 \times 10^7$ cpm/μg by the Klenow fill-in reaction and then gel purified.

EMSA was performed by the method of Singh et al. (42) with minor modifications. Approximately 8 to 10 μg of crude nuclear proteins was incubated with 5 μg of poly(dI-dC). Poly(dI-dC) (Pharmacia) was present in the binding buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9], 100 mM NaCl, 10% glycerol, 0.5 mM $MgCl_2$, and 1 mM dithiothreitol) in a reaction volume of 15 μl. After incubation for 15 min at room temperature, 30,000 cpm (~1 to 5 ng) of end-labeled DNA probe was added and incubated for an additional 20 min at room temperature. Finally, protein-DNA complexes were separated on a 4.5% polyacrylamide gel in 1× TBE buffer (89 mM Tris base, 89 mM boric acid, 0.2 mM EDTA [pH 8.0]) at 20 mA.

For competition analysis, unless otherwise indicated, a 100-fold molar excess of unlabeled competitor DNA or annealed double-stranded oligonucleotide was added to the preincubation mixture before the radioactive probe was added.

**Methylation interference footprinting analysis.** The methylation interference footprinting was carried out by the procedure of Jamieson and Sen (24) with minor modifications. The one-end-labeled DNA was partially methylated for 2 min with dimethyl sulfide, and the partially methylated DNA was used as a probe in the binding reaction for the EMSA. The DNA in the protein-DNA complex and unbound DNA were eluted on a DEAE membrane (DE81), purified, and cleaved at the G residues with 10% piperidine by the method of Maxam and Gilbert (35). Samples were analyzed by 7.5% polyacrylamide denaturing sequencing gel electrophoresis.

**UV cross-linking experiment.** Core B oligonucleotide of 5′$S_{\gamma 2a}$-176 (GTTACCTATC TTGTAAGAAC CAGGGAT CAG AATTGTGAAG CGTGACCAT) and oligo b (CCCT GGGGTG TTGAGCCACC CATCCTTGCC CATCTCCTG TCATGTCC) of 3′αE, both containing the NF-HB binding sites, were annealed to primers before the complementary strand was synthesized in the presence of 5′-bromo-dUTP and [α-$^{32}$P]dCTP (47). Uniformly labeled DNA was incubated with W231 nuclear extracts in four separate EMSA binding reactions with and without competition. Following electrophoretic separation in a 7.5% acrylamide gel, the protein-DNA complexes were cross-linked in situ in the gel by exposure to UV light for 10 min in a UV Stratalinker 1800 (Stratagene), and then the gel was autoradiographed overnight. On the following day, the retarded complexes or the region corresponding to it for the reactions with self-competitions were excised and analyzed by sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis (SDS–8% PAGE) (28), followed by autoradiography. Prestained markers (Sigma) were run in the same gel for determining the molecular weight of the cross-linked protein. The core B oligonucleotide used in this experiment was longer than that generally used in EMSA (Fig. 1).

**Site-directed mutagenesis by PCR and cloning of mutant**

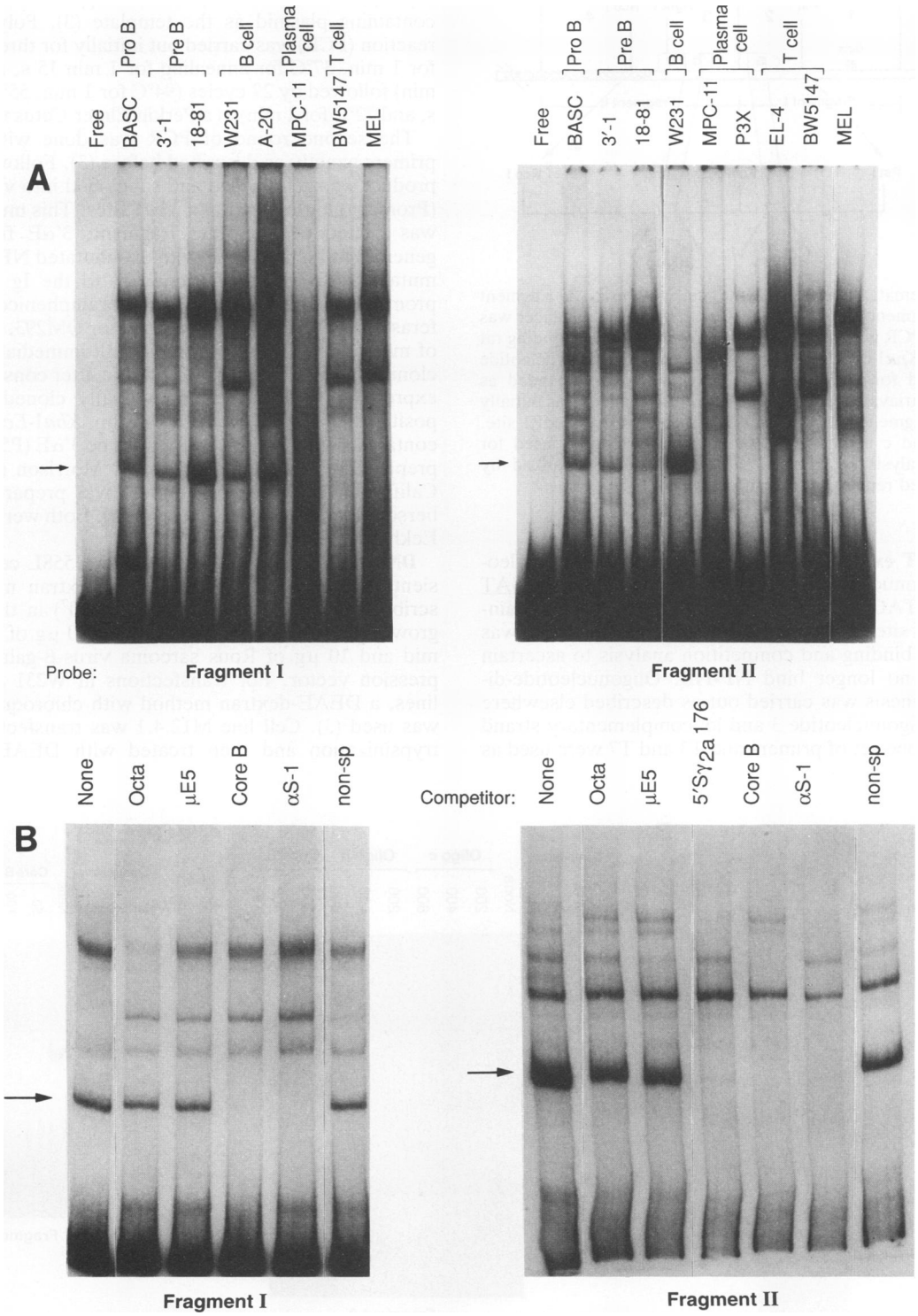


FIG. 2. (A) 3′αE binds to an early B-cell-specific nuclear factor. EMSA was carried out with end-labeled fragments I and II. Crude nuclear extracts (8 to 10 μg) from a variety of B-cell lines representing different developmental stages were used in each binding reaction in the presence of 5 μg of poly(dI-dC). Free α-$^{32}$P-labeled DNA was separated from bound complexes by electrophoresis in TBE buffer on a nondenaturing 4.5% polyacrylamide gel. The arrow indicates a single pro-B-, pre-B-, and B-cell-specific DNA-protein complex. (B) NF-HB interacts with 3′αE. Unlabeled DNA fragments were used as competitors in the binding reaction with W231 nuclear extract. Nonspecific (non-sp) oligonucleotide (TAC TGG GTT ACC CGG GGC ACC TG) is the binding site for a yeast ribosomal protein. Competitors were added at a 100-fold molar excess to each binding reaction mix prior to addition of the radiolabeled DNA. The arrow indicates the B-cell-restricted complex.

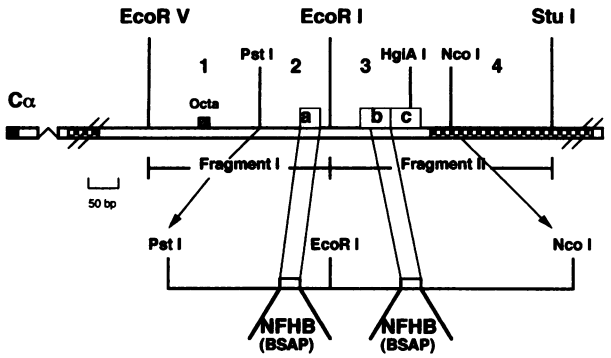


FIG. 3. Schematic diagram of the murine 3′αE. A fragment containing a segment (*Eco*RV-*Stu*I) of the murine 3′α enhancer was cloned (33) by PCR with primers derived from the corresponding rat elements (39) (*Kpn*I and *Bam*HI sites are synthetic oligonucleotide extensions used for the PCR). The map shown is expanded as described by Dariavach et al. (11). The 600-bp enhancer was initially divided into fragments I and II, based on the internal *Eco*RI site. Boxes a, b, and c represent synthetic oligonucleotides used for competition analysis by EMSA. Note that 3′αE is flanked by ~340-bp inverted repeats (checked boxes).

**3′αE into CAT expression vector.** The mutant oligonucleotide 3 of oligonucleotide b (CCCTGGGGTA TTATCGAT AA TATCCTTACC CATCTCCTGT CATGTCCT) containing a new *Cla*I site (underlined) because of the mutation, was used for both binding and competition analysis to ascertain that it could no longer bind NF-HB. Oligonucleotide-directed mutagenesis was carried out as described elsewhere (3). Mutant oligonucleotide 3 and its complementary strand were used as one set of primers and T3 and T7 were used as the other set of primers from the pBS vector side in two separate reaction tubes, with the pBS 3′αE fragment II-containing plasmid as the template (3). Polymerase chain reaction (PCR) was carried out initially for three cycles (94°C for 1 min, 37°C for annealing for 1 min 15 s, and 72°C for 2 min) followed by 27 cycles (94°C for 1 min, 55°C for 1 min 15 s, and 72°C for 1 min) in a Perkin Elmer-Cetus thermal cycler.

The second round of PCR was done with T3 and T7 primers exactly as described before (3). Following PCR, the product was gel purified and subcloned into vector pGem7z (Promega) in the *Kpn*I and *Xba*I sites. This mutant fragment was ligated to the other fragment, 3′αE fragment I, to generate full-length 3′αE with one mutated NF-HB site. The mutant 3′αE was then cloned 5′ to the Ig light-chain $\lambda_1$ promoter in the enhancerless chloramphenicol acetyltransferase (CAT) gene expression vector QM293. The sequence of mutant 3′αE was confirmed both immediately after subcloning of the PCR product and also after construction of the expression vector. Also individually cloned at the same position in QM293 were a 678-bp *Xba*I-*Eco*RI fragment containing Eμ (QM351) and wild-type 3′αE (P5). QM351 was prepared by M. Anderson and S. Morrison (University of California, Los Angeles), and P5 was prepared by R. Lieberson (Hunter College, New York). Both were gifts from L. Eckhardt (Hunter College).

**DNA transfections and CAT assay.** J558L cells were transiently transfected by the DEAE-dextran method as described previously (33). Cells ($2 \times 10^7$) in the logarithmic growth phase were transfected with 10 μg of reporter plasmid and 10 μg of Rous sarcoma virus-β-galactosidase expression vector. For transfections in W231 and S194 cell lines, a DEAE-dextran method with chloroquine treatment was used (3). Cell line M12.4.1 was transfected after brief trypsinization and then treated with DEAE-dextran (8).

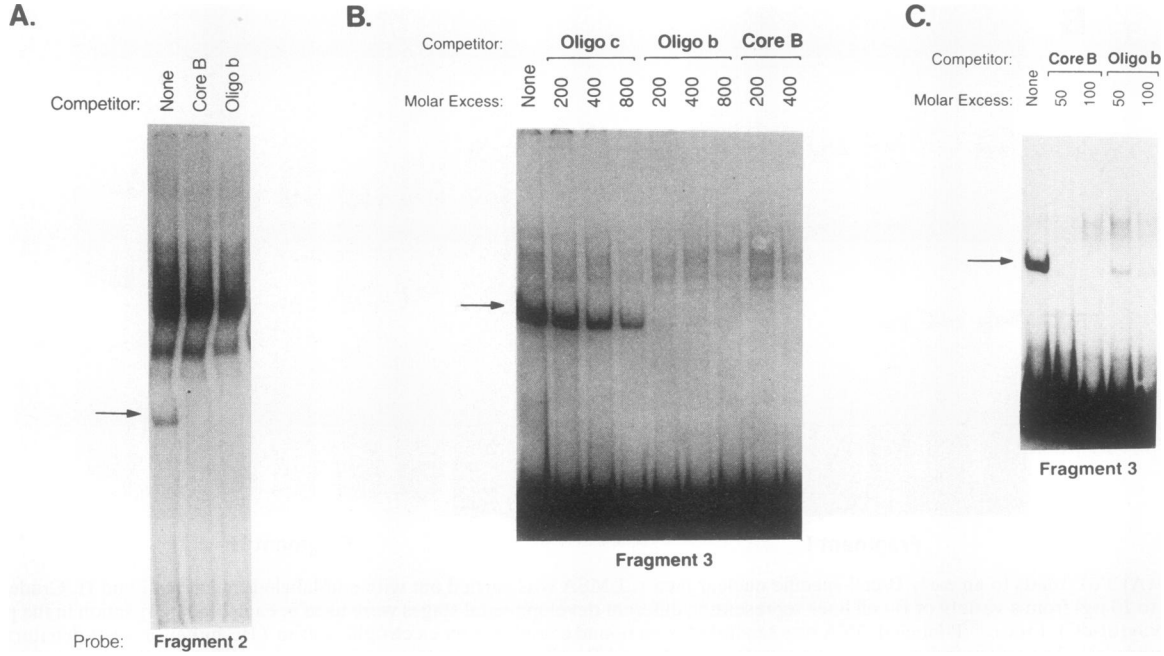


FIG. 4. NF-HB binding sites reside in two adjacent fragments. Fragments I and II were subdivided into four fragments, 1 through 4 (see Fig. 3), and each fragment was used for binding reactions with W231 nuclear extracts, followed by competition. (A) Competition of fragment 2 with unlabeled core B and oligonucleotide b sequences at a 100-fold molar excess. (B) Competition of fragment 3 with oligonucleotides b and c (see Fig. 1 and 3) and core B. (C) Competition analysis of fragment 3 with oligonucleotide b and core B at lower concentrations. The arrows indicate an NF-HB binding complex.

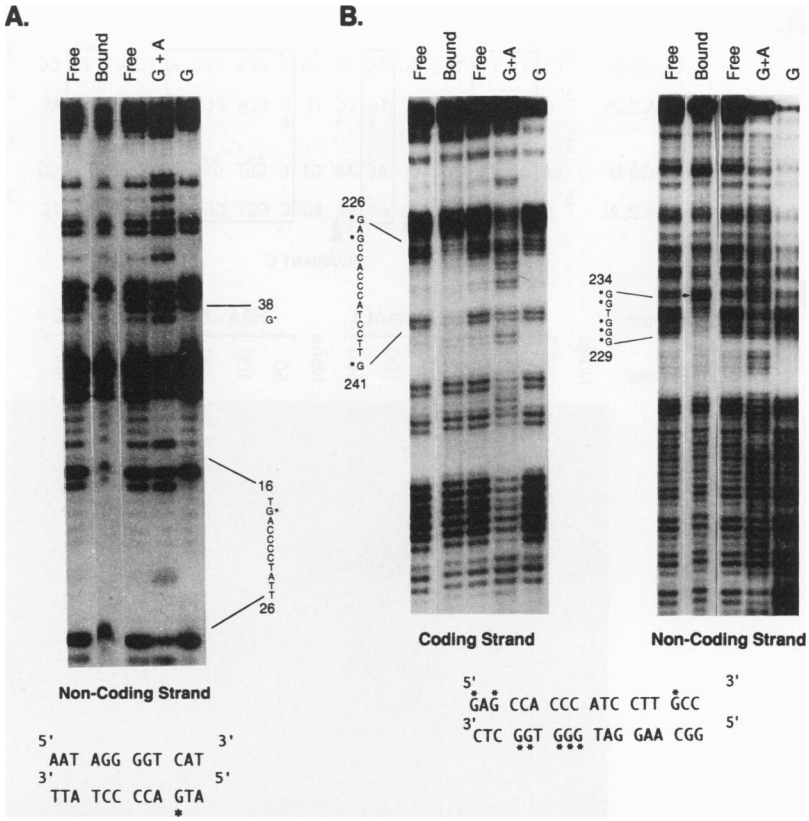


FIG. 5. Methylation interference footprint analysis of B-cell-specific complexes. (A) One-end-labeled fragment 2 (*Pst*I-*Eco*RI restriction fragment) was used for footprinting analysis with W231 nuclear extract. (B) End-labeled strands of fragment 3 (*Eco*RI-*Hgi*AI restriction fragment) were used for footprinting. Methylated G residues that interfered with binding are indicated by asterisks. A G residue with enhanced binding due to methylation is indicated by the arrow in the second lane for the noncoding strand.

After 42 to 44 h of incubation at 37°C, the cells were harvested, resuspended in 100 μl of 0.25 M Tris-HCl, pH 7.5, and lysed by the freeze-thaw method (3). A 30-μl amount of the resulting extract was used for the β-galactosidase assay with chlorophenol red β-D-galactopyranoside as the substrate (23).

The remainder of the extracts were heated at 65°C for 10 min and then centrifuged at 12,000 × *g* for 10 min in order to inactivate an endogenous acetylase activity before the supernatant was used in the CAT assay. The protein concentrations of the cellular extracts were determined by the Bio-Rad protein assay. CAT activity was measured for equal amounts of β-galactosidase units by the two-phase fluor method, as modified by Han et al. (21), and also by the solvent extraction method (3). At least three to five separate experiments were carried out for each cell type, and one of the sets was assayed by thin-layer chromatographic separation (3).

## RESULTS

**Identification of NF-HB (BSAP) binding sites in 3′αE.** 3′αE was divided into two fragments by digestion with *Eco*RI. These fragments were analyzed by EMSAs with nuclear extracts prepared from murine cell lines representing different stages of B-cell development. B-cell-specific binding to both fragments I and II was present (Fig. 2A, arrow) in pro-B- (BascG2/C7), pre-B- (3-1 and 18-81), and B-cell (W231 and A20) lines and absent from plasmacytoma cells (MPC11 and P3X), T-cell lines (EL-4 and BW5147), and nonlymphoid cells (MEL).

Our laboratory has previously identified a nuclear binding protein, NF-HB (BSAP), with the same cell type distribution. To determine whether the early-B-lineage-specific 3′αE binding protein we had observed was related to NF-HB, we carried out competition analysis by EMSA with unlabeled NF-HB binding sites. As shown in Fig. 2B, NF-HB binding sites from 5′$S_{\gamma 2a}$ (5′$S_{\gamma 2a}$-176 and core B) and 5′ of Sα (αS-1) (45) competed with the B-cell-specific complex observed with both 3′αE fragments I and II. Other sequences known to have B-cell-specific binding activity, including the octamer and μE5, failed to compete with this complex. These observations indicated that either NF-HB or NF-HB-like binding activity was likely involved in binding to two sites within 3′αE. Interestingly, a precise octamer is present in fragment I. Competition with unlabeled octamer identifies a shifted complex that appears to be Oct-1, since the complex is formed in both B-cell and non-B-cell extracts (Fig. 2).

NF-HB sites were localized by using unique *Pst*I and *Hgi*AI sites in 3′αE fragments I and II, respectively, to generate four subfragments, 1, 2, 3, and 4 (Fig. 3). B-cell-specific NF-HB binding was restricted to fragments 2 and 3 (Fig. 4A and B) and, within fragment 3, to oligonucleotide b, one of two 50-bp oligonucleotides tested (Fig. 1 and 4B). B-cell-specific octamer-binding activity (Oct-2) was detectable with fragment 1 (data not shown).

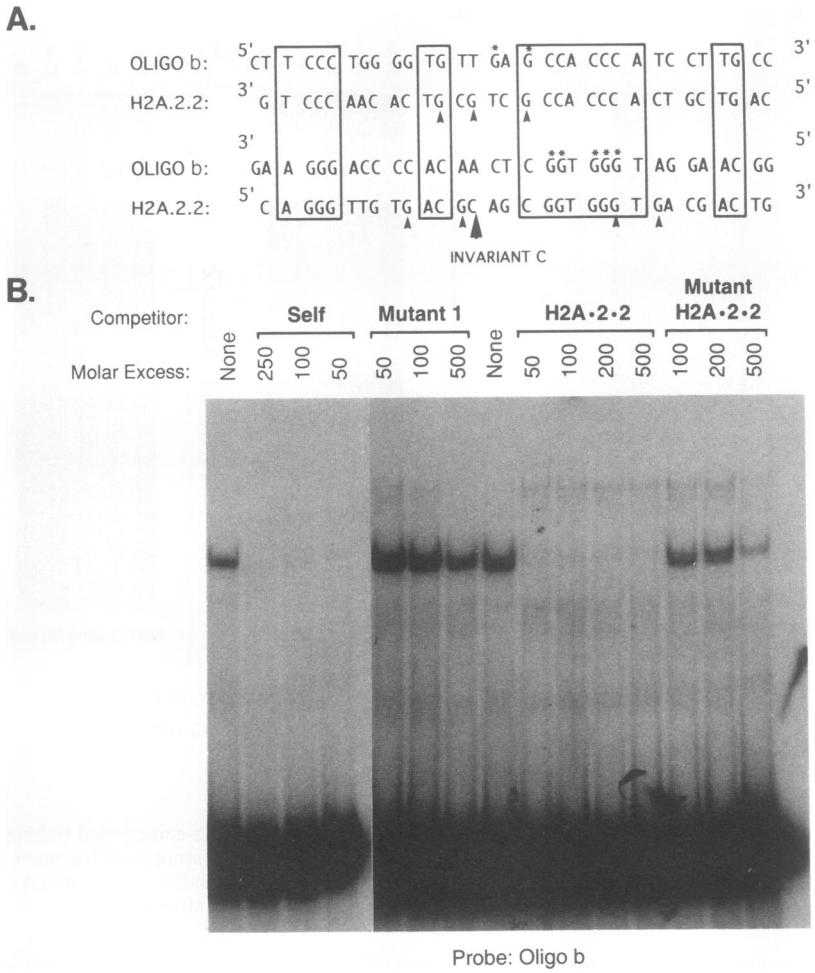


FIG. 6. Structural and functional similarities between the binding sites for NF-HB in fragment 3 (3′αE) and BSAP in H2A-2.2. (A) Methylation interference footprinted regions were aligned for maximum homology. Boxed regions denote identical sequences. Asterisks and arrows indicate the contact residues for NF-HB and BSAP, respectively. Note that the two sequences are in inverted orientations. "Invariant C" refers to the single invariant nucleotide among four BSAP (TSAP) binding sites in promoters of sea urchin late histone genes H2A-2 and H2B-2 (6, 7) and Fig. 1. (B) Competition by EMSA with W231 nuclear extracts and oligonucleotide b. The sequence of mutant 1 of oligonucleotide b is shown in Fig. 8A. Mutant H2A-2.2 contains a single-base-pair alteration at the invariant C (Fig. 1).

To determine the binding sites of NF-HB in 3′αE more precisely, we carried out methylation interference footprinting analysis of fragments 2 and 3 with nuclear extracts from the B-cell line W231. For fragment 2, only two G residues on the noncoding strand showed marked interference due to methylation (Fig. 5A). These G residues were separated by a stretch of G residues that do not show any preference for protein binding. Moreover, as the footprinted region resides at the 5′-most end of fragment 2, footprinting on the coding strand could not be carried out because of technical difficulties. To confirm the location of the binding site in fragment 2, an oligonucleotide of the protected region and its complementary strand were synthesized (oligonucleotide a, Fig. 1) and used as unlabeled competitors along with oligonucleotide b (fragment 3) for binding to oligonucleotide b; both oligonucleotides a and b were found to compete at a 100-fold molar excess (data not shown). Footprint analysis of fragment 3 showed that within a segment contained in oligonucleotide b, methylation at three G residues in the coding strand and five G residues in the noncoding strand (as marked by asterisks, Fig. 5B) markedly interfered with binding and hence represent contact points with the DNA-binding site on NF-HB.

Like other NF-HB (BSAP) binding sites, including those originally identified in the upstream regions of the sea urchin late histone genes H2A-2 and H2B-2 (6), the two NF-HB binding sites in 3′αE, represented by oligonucleotides a and b, bear no sequence identity to each other (see Fig. 1 for comparison of NF-HB [BSAP] binding sites used in this study). Thus, it was particularly striking that comparison of the footprinting-protected region for fragment 3 with a BSAP binding site in the sea urchin late histone gene promoter H2A-2.2 (6, 7) showed an eight of eight base identity together with additional flanking region sequence homology (Fig. 6A, boxed regions). Competition analysis by EMSA with oligonucleotide b as the probe confirmed (as shown in Fig. 6B) that H2A-2.2 was an effective competitor, while a mutant of H2A-2.2, containing a single base change of C to A, competed only at higher concentrations. Interestingly, both core B (from 5′ of $S_{\gamma 2a}$) and the H2A-2.2 binding site have greater affinity for NF-HB (BSAP) than do either of the 3′αE binding sites (29a). For example, as shown in Fig. 4C,

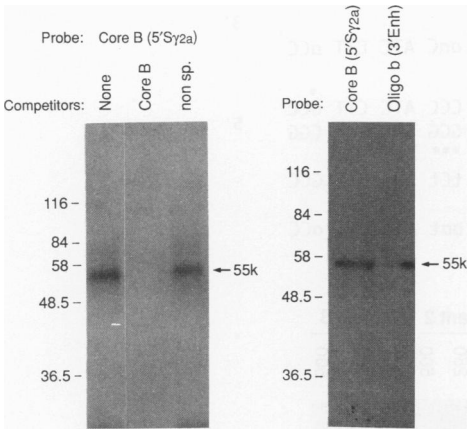


FIG. 7. NF-HB and BSAP bind to a similar-sized protein. Uniformly labeled and 5′-bromo-dUTP-substituted DNA probes (core B and oligonucleotide b) were used for EMSA with the W231 nuclear extract. Cross-linking of the DNA to protein was done by UV irradiation of the gel in situ after electrophoretic separation of protein-DNA complexes (see Materials and Methods). Retarded complexes were excised and analyzed by SDS–8% PAGE. In some reactions, unlabeled competitors were used at a 100-fold molar excess. Sizes are shown in kilodaltons.

core B but not oligonucleotide b could compete for NF-HB binding at a concentration as low as a 50-fold molar excess. Other competition studies have indicated that oligonucleotide a binds about half as well as oligonucleotide b to core B and H2A-2.2 (29a).

To further document that the 3′αE-specific binding protein was NF-HB (BSAP), we used UV cross-linking to determine the molecular weights of the proteins involved in the complexes formed by both oligonucleotide b (3′αE fragment 3) and core B (5′$S_{\gamma 2a}$). As shown in Fig. 7, in both core B and oligonucleotide b complexes, we detected polypeptides of 55 kDa. In previously reported experiments, BSAP was estimated to be 50 kDa by the same method (7). We conclude from these data that NF-HB (BSAP) binds to two sites within 3′αE. In some experiments, a faint band of ~80 kDa that could be competed with by NF-HB binding sites was also detected (see left panel of Fig. 7).

Recent studies of the rat 3′αE have identified a number of elements important for its transcriptional activity (19). Among these is a sequence, μA, that is located in the same vicinity as one of the NF-HB binding sites in fragment 3. Thus, μA was a candidate for a rat NF-HB binding site. However, EMSA competition analysis has shown that the rat μA sequence did not compete for NF-HB binding in murine 3′αE (Fig. 8C), indicating that it might either be rat specific or represent a very low affinity binding site.

**NF-HB has a role in downregulation of 3′αE activity in vivo.** To understand the role of NF-HB in 3′αE function, we mutated the NF-HB binding site of fragment 3 (oligonucleotide b) and determined its effect on transcriptional enhancement in a CAT assay. Based on the footprinted region, three mutant oligonucleotides were synthesized (Fig. 8A). As shown in Fig. 8B, mutants 1 and 2, having fewer mutations, still contained residual binding activity in the EMSA, while mutant 3, containing mutations in all the contact residues, failed to compete. We substituted the NF-HB binding site in 3′αE fragment 3 with the mutant oligonucleotide 3 (Fig. 8B) by PCR and prepared an intact mutant 3′αE. Mutant 3′αE, parental 3′αE, or Eμ was then cloned 5′ to the promoter in an enhancerless expression vector, QM293. QM293 contains the CAT gene as a reporter under the control of the Ig $\lambda_1$ light-chain promoter.

Before carrying out the functional assays with these constructs, we performed an EMSA with both the parental and mutated 3′αE (with the *Pst*I-*Nco*I restriction fragment from both of them) to assess the effect of mutation on the binding activity for NF-HB. As shown in Fig. 8C, mutation of one of the two binding sites of NF-HB, i.e., fragment 3 (oligonucleotide b), revealed lack of binding to the other nonmutated site, namely, fragment 2 (oligonucleotide c).

Vectors were transiently transfected into B-cell lymphomas or into plasmacytomas. The activity of mutated and wild-type 3′αE was compared with that of Eμ. We predicted that in plasmacytoma cell lines (J558L and S194), in which NF-HB was absent, mutation of the NF-HB binding site in 3′αE should have little effect, while in B-cell lines (W231 and M12.4.1), in which NF-HB is present, mutated 3′αE should display a difference in activity. In agreement with these predictions, we observed that in S194 plasmacytoma cells [IgG2b(κ); NF-$HB^-$], the activities of all three enhancers, Eμ, 3′αE, and mutated 3′αE, were comparable (~80-fold induction). In J558L, the mutated 3′αE was ~60% as active as parental 3′αE, and 3′αE was only 15% as active as Eμ. In both the W321 and M12.4.1 B-cell lines (NF-$HB^+$), we observed that mutated 3′αE exhibited a significant increase in enhancer activity over wild-type 3′αE (~five- to sixfold (Fig. 9 and 10). These results indicate that NF-HB acted as a repressor of 3′αE activity.

The activity of mutated 3′αE differed markedly in the two B-cell lines tested. In W231, mutated 3′αE was comparable in activity to Eμ, while in M12.4.1, mutated 3′αE was about five times more active than Eμ. These differences in activity largely reflected differences in the basal activity of 3′αE in the two cell lines. In W231, wild-type 3′αE was inactive, consistent with previous reports, while in M12.4.1, an $HGPRT^-$ variant of M12 (18), 3′αE activity was comparable to that of Eμ (~26-fold induction). In the B-cell lines we had examined previously, i.e., W231 and BCL1, the 3′α region was hypermethylated, while in plasmacytoma cell lines (in which 3′αE is active), this region was undermethylated (16). We found that in M12.4.1, the 3′α region was less methylated than in either W231 or BCL1 (16). Such a pattern of methylation is more typical of plasmacytoma cells (data not shown), and thus M12.4.1 may represent a more mature B-cell stage.

## DISCUSSION

The studies reported here document active repression of 3′αE at early stages of B-cell differentiation through interaction with a B-lineage-specific DNA-binding protein. The 3′αE binding protein is indistinguishable from NF-HB (BSAP), a B-cell-specific transcription factor, in several aspects. First, binding activity is limited to early stages of B-cell differentiation. Second, both NF-HB and BSAP binding sites cross-competed in an EMSA with 3′αE binding sites (Fig. 2B and 6B); in fact, compared with the sites within 3′αE, both NF-HB and BSAP binding sites were of higher affinity (Fig. 4C). A mutant BSAP binding site was also mutant with respect to 3′αE binding (Fig. 6B). In addition, UV cross-linking experiments have shown that a similar-sized protein of ~55 kDa binds to 3′αE, core B of 5′$S_{\gamma 2a}$ (Fig. 7), and the promoter of the sea urchin late histone gene H2A-2.2 (BSAP). Lastly, one of the binding sites within 3′αE, as determined by methylation interference footprint-

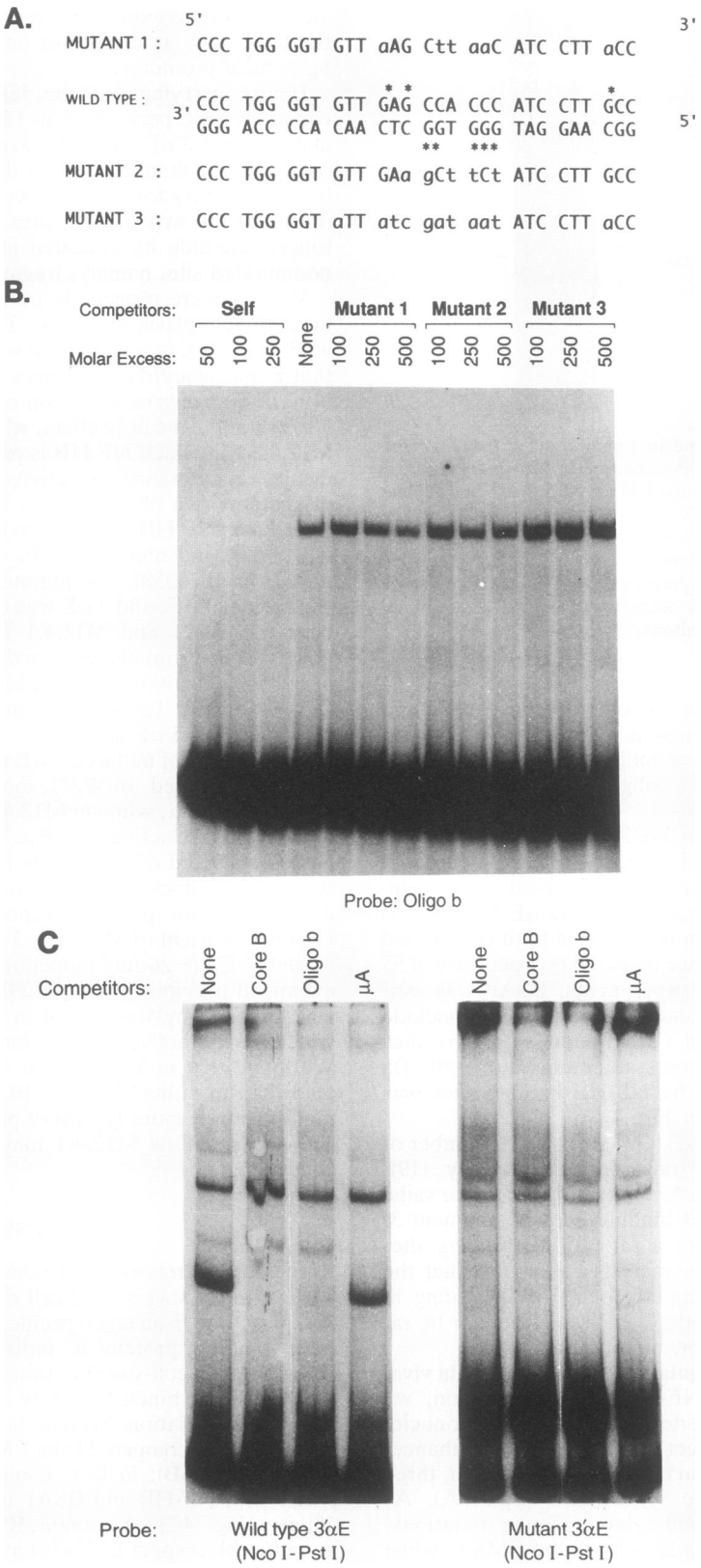


FIG. 8. Mutational analysis of the footprinted region in oligonucleotide b. (A) Three mutants were synthesized with a gradual increase in the number of mutations in the footprinted region; lowercase letters denote mutated residues. (B) Competition analysis by EMSA was done with unlabeled mutant oligonucleotides with oligonucleotide b as the probe. (C) The *Pst*I-*Nco*I restriction fragments from both the wild-type (containing both NF-HB binding sites) and the mutated (containing one mutated NF-HB binding site) 3'αE were each end labeled and used in an EMSA with specific competitors.

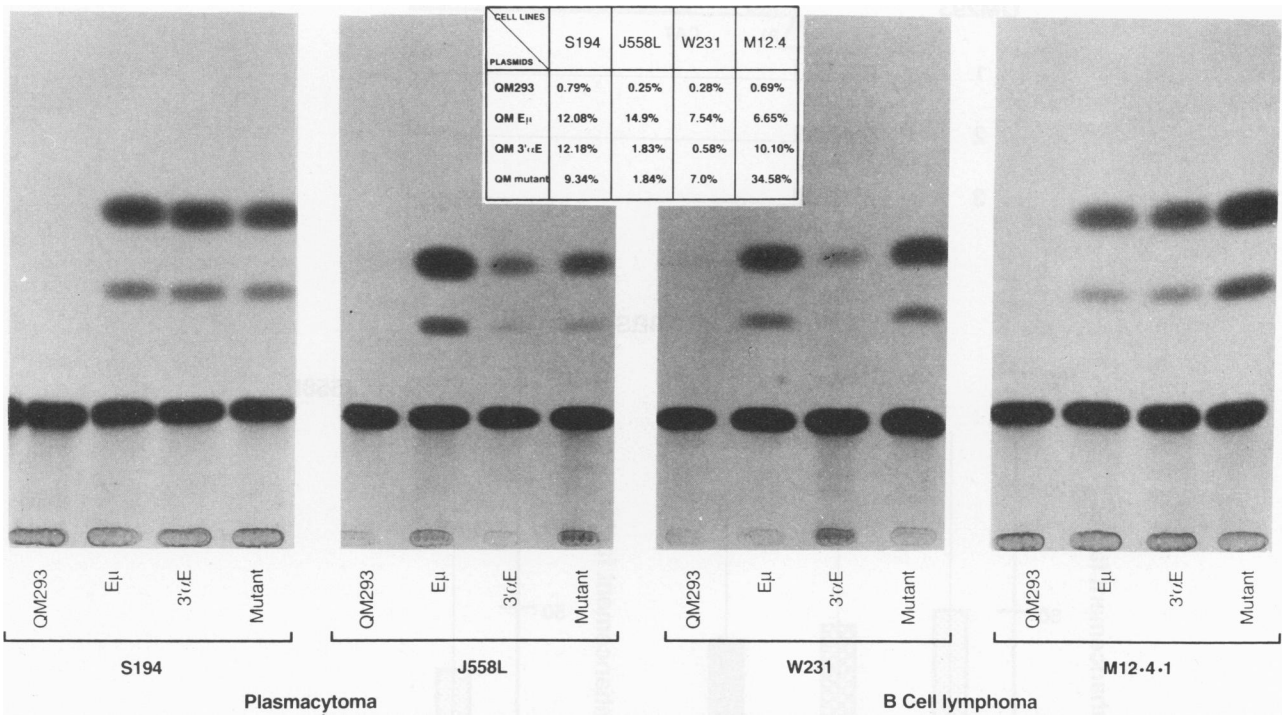


FIG. 9. NF-HB acts as a repressor of 3′αE. Thin-layer chromatographic separation of acetylated chloramphenicol from chloramphenicol. The reporter plasmids and cell lines are indicated below each lane. The inset represents the percent conversion of chloramphenicol to acetylated chloramphenicol, as determined with a Phosphorimager and the Image-quant program (Molecular Dynamics).

ing, bore an eight-for-eight base identity with the BSAP binding site in H2A-2.2. As noted, this observation is in marked contrast to the limited sequence homology identified by comparisons of BSAP binding sites. From these data, we conclude that 3′αE is one of the mammalian targets for BSAP.

Transient-transfection assays of cell lines, as reported previously (11, 33, 39), demonstrated 3′αE activity in plasmacytoma cell lines and inactivity in B-cell lines. The inverse relationship between the presence of NF-HB in pro-B-, pre-B-, and B-cell lines and the activity of 3′αE in plasmacytoma cells prompted our investigation of a possible negative regulatory role of NF-HB (BSAP) in 3′αE function. To test this hypothesis, we mutated one of the binding sites of NF-HB in 3′αE (fragment 3 [oligonucleotide b]) and analyzed the effect of this mutation on 3′αE function in transient-transfection assays with CAT reporter constructs. With the B-cell lines, in which NF-HB was present, transfection of 3′αE containing a crippled NF-HB binding site resulted in considerable augmentation of 3′αE activity (about five- to sixfold) over that with the nonmutated counterpart. Hence, we conclude that NF-HB is a negative regulator of 3′αE activity.

Since putative AP-2 binding sites lie adjacent to each NF-HB binding site, we considered that one mechanism by which NF-HB could repress 3′αE would be by blocking AP-2 binding. This could be accomplished either by direct steric hindrance of AP-2 sites by NF-HB binding or as a result of protein-protein interactions between NF-HB and AP-2 analogous to those described for simian virus 40 T antigen and AP-2 (36). However, using an oligonucleotide for 3′αE that contains one of the putative AP-2 sites (oligonucleotide b), we could not detect AP-2 binding on an EMSA even with the addition of purified AP-2 protein. Thus, while the possibility of competition between NF-HB and AP-2 has not been fully ruled out, it seems likely that repression might be effected by a different mechanism.

One possibility is that NF-HB could repress 3′αE through simultaneous interaction with the two binding sites within 3′αE to form a DNA loop. The formation of a loop might physically preclude the interaction of *trans*-acting factors with other (positive) regulatory binding sites situated within the loop region. Candidates include μB and μA, which have been shown to be important for rat 3′αE function (19). Although a μA binding site has not been demonstrated for the mouse (Fig. 8C), a μB binding site is located in the same position in both rat and mouse. Mutation of a single NF-HB binding site (fragment 3) resulted in loss of binding to the other site, supporting the notion of a physical interaction between the two sites. Physical interaction could be fostered through direct interaction between NF-HB molecules or indirectly through association of NF-HB with other proteins. Our studies have identified the interaction of NF-HB with other ubiquitous proteins to form heteroprotein complexes. Such complexes are present in pre-B- and B-cell lines and are generated within lipopolysaccharide-treated spleen cells (31). It should be noted, however, that our experiments have shown no evidence of cooperativity between NF-HB and 3′αE binding sites. EMSAs of fragment I (306 bp) and fragment II (290 bp), each containing one NF-HB binding site, and the *Nco*I-*Pst*I fragment (260 bp) (containing both NF-HB binding sites) were carried out on gels containing the same percentage of polyacrylamide. For each of these, only one NF-HB shifted complex was identified, and the mobility of this complex was comparable in all three instances. Furthermore, as described in the Results, studies of the mutant *Nco*I-*Pst*I fragment that retains a single unmutated NF-HB (BSAP) binding site in fragment I revealed no

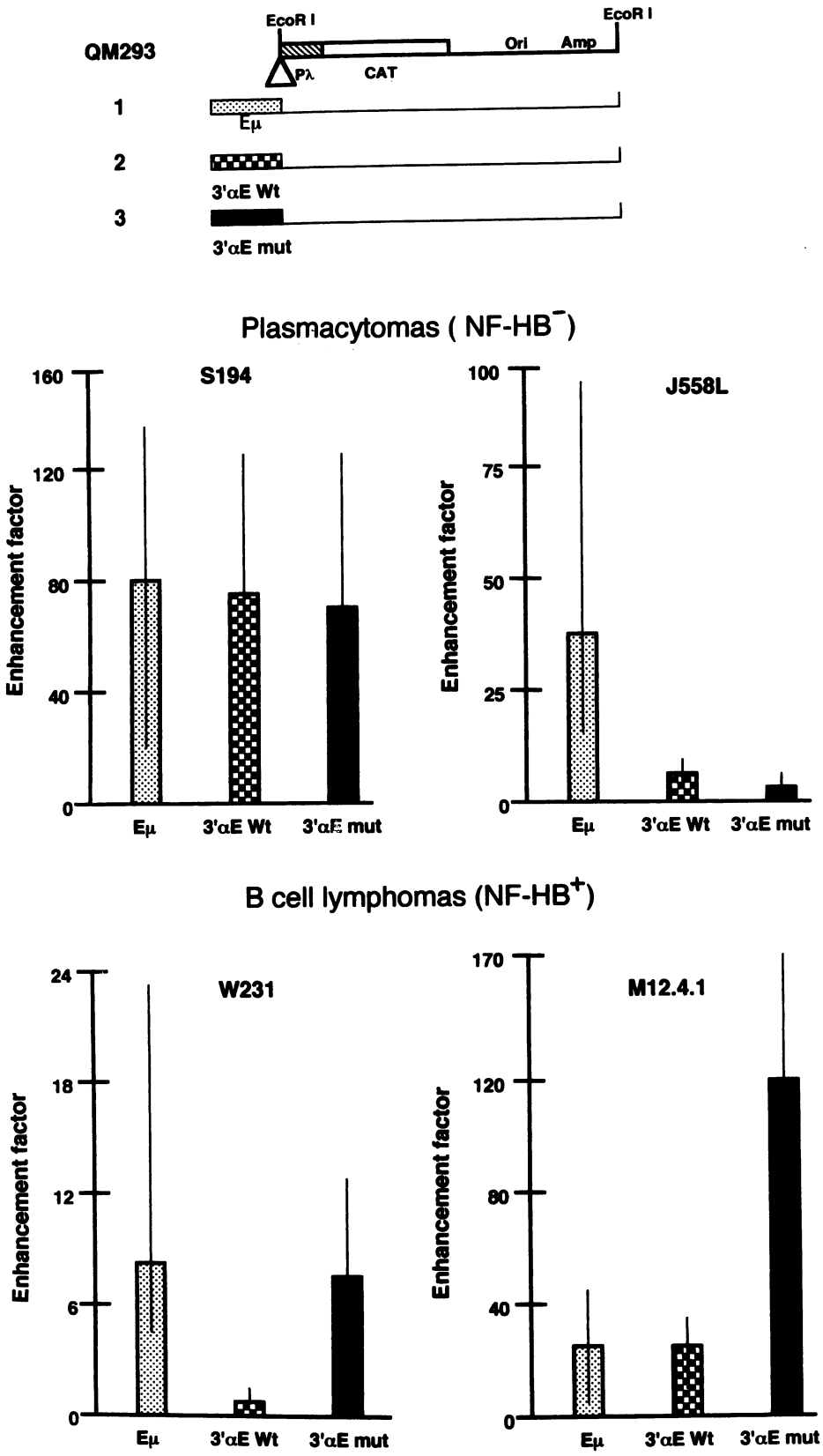


FIG. 10. Enhancement of CAT activity with different reporter plasmids, as diagrammed (the triangle indicates the site of fragment insertion). The enhancement factor was determined by comparing CAT assay values for each of the constructs with that for the enhancerless CAT vector (QM293). Values are averages of three to five independent experiments with at least two different DNA preparations. In all constructions, the fragments to be tested were inserted 5′ to the Ig $\lambda_1$ promoter. The Rous sarcoma virus-β-galactosidase expression vector (10 μg) was cotransfected with 10 μg of the reporter plasmid in each transfection as an index of transfection efficiency. Equal β-galactosidase activity units within each set were used for the CAT assay.

binding. This observation suggests that only the binding site in fragment II is engaged in the wild-type *NcoI-PstI* fragment and raises interesting questions about what physiological conditions might favor the binding sites in fragments I and II to be occupied jointly.

Our previous studies have shown that the degree of methylation of sequences flanking 3′αE is inversely correlated with the stage of B-cell differentiation in which 3′αE is active (16). B (and pre-B) lines showed hypermethylation of the 3′α region compared with that of nonlymphoid tissues, while plasmacytoma cells showed extensive undermethylation (16). However, unlike three other B-cell lines (A20, W231, and parental M12), in which 3′αE was inactive, parental 3′αE in M12.4.1 was as active as Eμ, and the 3′α region was undermethylated. This observation implies that negative regulation of 3′αE can be exerted independently through either methylation or NF-HB production alone. The fact that two mechanisms of repression act on the 3′α region underscores the apparent necessity for strict control of this enhancer-containing region at early stages of B-cell development. The observation that the activity of mutated 3′αE in M12.4.1 is higher than in any other cell line tested suggests that 3′αE may achieve maximal activity during a late B-cell stage, potentially in response to activation by antigen and T cells.

The net balance of multiple (positive and negative) variables, other than the presence of NF-HB, must also influence 3′αE activity, since basal activity, compared with that of Eμ, differs even among plasmacytoma cell lines, in which NF-HB is absent and the 3′α region is undermethylated (see Fig. 2A, fragment II, for a difference in EMSA patterns between MPC11 and P3X). In three plasma cell lines (MPC11, HOPC1 [11], and S194 [this study]), 3′αE was as potent as Eμ, while in the fourth, J558L, 3′αE activity was no more than 25% that of Eμ (33; this report). To what degree the apparent lower activity in J558L may also reflect the particular vector used is not known. The expression vector used for J558L (and S194, W231, and M12.4.1) contains the Ig $\lambda_1$ light-chain gene promoter as the minimal promoter, and J558L is the only λ light-chain producer among the cell lines tested.

In addition to binding sites within 3′αE, NF-HB (BSAP) interacts with multiple sites within the IgH gene cluster, several of which are located in the vicinity of the start sites of germ line transcripts. We have suggested that 3′αE might physically interact with sequences within the $C_H$ cluster through interaction of NF-HB binding sites (31). Our experiments have shown that NF-HB is required for induction by lipopolysaccharide and interleukin-4 of ε germ line transcripts in M12.4.1 (30). Thus, in contrast to its role as a repressor of 3′αE activity, NF-HB is a positive regulator in its interaction with an ε-associated binding site. Interestingly, NF-HB binding sites in 3′αE are of lower affinity than other sites, including those flanking 3′αE(3′α88) and within the IgH cluster (core B-γ2a associated) (31) (see Fig. 4C). In cells in which NF-HB production is diminishing, NF-HB is likely to dissociate from 3′αE while continuing to bind to higher-affinity sites, where it might function in a different regulatory manner.

Binding sites for NF-HB (BSAP) have been identified within other nonimmunoglobulin genes, the expression of which is limited to early stages of B-cell differentiation. These include $V_{preB}$ and $\lambda_5$ (38), CD19 (26), and *blk* (29a). To what degree the various binding sites function as positive or negative regulators is not yet known. There are precedents for a single protein that can act in such a dual fashion in both prokaryotic and eukaryotic cells. These include C1 repressor (40) and CAP (34) of *Escherichia coli* and Rap1 and ABF1 of yeast ribosomal genes (29). Significantly, NF-E1, a human protein, acts as a repressor of the enhancer that lies 3′ of the murine $C_\kappa$ gene (in pre-B cells) (27), while the identical protein, YY-1, mediates E1A activation of the adenovirus P5 promoter (10), and the mouse equivalent, NF-δ, activates ribosomal protein gene promoters (22).

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## ADDENDUM IN PROOF

A third, weaker B-cell-specific enhancer has recently been reported (P. Matthias and D. Baltimore, Mol. Cell. Biol. **13**:1547–1553, 1993). This enhancer interacts with octamer-binding proteins.

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