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mXBP/CRE-BP2 and c-Jun Form a Complex Which Binds to the Cyclic AMP, but Not to the 12-*O*-Tetradecanoylphorbol-13-Acetate, Response Element

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Proto-oncogene products c-Fos and c-Jun form a complex which binds with high affinity to the 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response DNA element and which stimulates transcription of phorbol ester- inducible genes. We have previously identified, by screening a λgt11 expression library, murine protein mXBP, which binds to a sequence which overlaps the 3′ end of the murine class II major histocompatibility complex Aα gene X box, a conserved transcription element found upstream of all class II genes. Here, we demonstrate that the target sequence for mXBP is a consensus cyclic AMP response element (CRE). mXBP is a member of the leucine zipper family of DNA-binding proteins and has significant homology to oncoproteins c-Fos and c-Jun. The inferred amino acid sequence of mXBP shows near identity to human CRE-BP1, except it does not contain an internal proline-rich domain. Immunoprecipitation and glutaraldehyde cross-linking studies show that mXBP/CRE-BP2 can form a complex with c-Jun. Complex formation is dependent on intact leucine zipper domains in both proteins. mXBP–c-Jun complexes can coexist with c-Fos–c-Jun complexes and can bind with high affinity to CRE, but not to TPA response DNA element, sequences. These results suggest that changes in the expression of mXBP/CRE-BP2, c-Fos, and c-Jun, which alter the ratio of mXBP–c-Jun to c-Fos–c-Jun complexes, would affect the relative expression of cyclic AMP and phorbol ester-responsive genes. This provides support for a combinatorial model of gene regulation, whereby protein-protein interactions which alter the DNA binding specificity of protein complexes can expand the flexibility of cellular transcriptional responses.

Gene expression is regulated through sequence-specific binding of proteins to *cis*-acting DNA elements (for a review, see references 33, 46, and 50). Palindromic DNA sequence TGACTCA (12-*O*-tetradecanoylphorbol-13-acetate [TPA] response DNA element [TRE] or AP-1 site) mediates transcriptional activation in response to the phorbol ester-activated, protein kinase C-dependent, signal transduction pathway (2, 15). Closely related sequence TGACGTCA (cyclic AMP [cAMP] response element) (CRE) mediates transcriptional activation in response to the distinct cAMP-activated, protein kinase A-dependent signaling pathway (2, 15, 25). These two major signal transduction systems regulate multiple cellular responses, including proliferation, and may act in concert with or counteract each other in a variety of cells (55, 59).

Protein CREB, which binds to CREs as a monomer or homodimer, is phosphorylated by protein kinase A after treatment of cells with cAMP and stimulates transcription of cAMP-responsive genes (20, 26, 52, 73). Several proteins, including c-Jun/AP-1, Jun B, and Jun D, bind to TREs as homodimers and stimulate transcription (4, 6, 24, 40, 53, 61, 68). Affinity of binding and transcriptional activation are dramatically increased when c-Jun heterodimerizes with c-Fos or Fra-1, proteins which do not homodimerize and which do not specifically bind to DNA in the absence of c-Jun (7, 10, 11, 23, 53, 64). Dimerization of these proteins is mediated by a conserved leucine zipper structure (19, 34, 38, 56, 66, 70). Recent "domain swap" experiments in which the leucine zippers of c-Fos, c-Jun, and yeast transcription factor GCN4 were interchanged indicate that the leucine zipper determines dimerization specificity and that dimerization is a prerequisite for binding (35, 54, 67). The inability of c-Fos and Fra-1, c-Fos and c-myc, GCN4 and c-Jun, GCN4 and c-Fos, c-Fos and CREB, and CREB and GCN4 to form heterodimers provides further support for the specificity of interactions mediated by leucine zipper domains (56, 70). This specificity most likely resides in the residues which lie between the leucines (35, 54, 67).

In vitro dimerization of leucine zipper proteins correlates with the ability to complex in vivo, and the formation of c-Fos–c-Jun complexes in vivo has been correlated with transactivation of genes with an active TRE (7, 64). Phorbol esters and mitogens stimulate transcription of both c-Fos and c-Jun and phosphorylation of c-Fos and thus may lead to the formation of an active c-Fos–c-Jun complex (3, 37, 62, 72). However, the mechanism by which c-Fos and c-Jun mediate transcription is not well understood. These proteins can repress as well as activate transcription by binding to the same TRE sequence in different genes (2, 18). In addition, the c-Fos–c-Jun complex can bind to CREs, depending on flanking sequences (53, 58; see below), but the CRE appears incapable of mediating transcriptional activation by phorbol esters (15, 25). The TRE, however, can mediate transcriptional responses to both phorbol esters and, to a lesser extent, cAMP (15, 25), although not in all cell types (32). This appears puzzling, since CREB does not bind to TREs. Electrophoretic mobility shift assays with JEG-3 cell nuclear extracts have detected a common factor(s), which binds to

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both TREs and CREs, and another factor(s), which binds only to the CRE (25). An additional level of complexity is introduced by the ability of c-Fos and c-Jun to regulate their own promoters (1, 72) and by the ability of CREB to regulate the c-Fos promoter (I. Verma, unpublished data). Thus, the mechanism by which closely related TRE and CRE sequences may serve to couple transcriptional responses to phorbol esters and cAMP most likely involves complex regulatory networks.

In this report, we show that mXBP/CRE-BP2, a protein with a leucine zipper domain, which was recently isolated by screening a spleen cell λgt11 expression library with a murine class II major histocompatibility complex probe (42), binds specifically to the CRE. The interaction of mXBP with c-Jun redirects c-Jun to bind to CREs preferentially over TREs as part of an mXBP–c-Jun complex. This protein-protein interaction may represent one mechanism which links cellular transcriptional responses to phorbol esters and cAMP.

MATERIALS AND METHODS

Plasmids and sequencing. Standard recombinant technology was used (45). A 963-base-pair (bp) *Eco*RI fragment from clone λmXBP (42) was subcloned into pBluescript-KS (Stratagene Cloning Systems) to generate pBS-MX. Both strands of the insert were sequenced by the Sanger dideoxynucleotide protocol modified for use with Sequenase, as recommended by the manufacturer (U.S. Biochemical). Plasmid pATH11-mXBP was constructed by subcloning the 963-bp *Eco*RI fragment into pATH11 (16). c-jun cDNA (37) and c-fos cDNA (49) were subcloned into pGEM4 (Promega Biotec) to generate plasmids T7jun and T7fos.

Cell culture and nuclear extracts. 69.9.15 pre-B-cell lymphoma cells used for preparation of nuclear extracts were harvested at logarithmic growth phase from suspension cultures in RPMI 1640 medium containing 8% fetal bovine serum. Nuclear extracts were prepared essentially as described by Dignam et al. (17). Final protein concentrations were typically 2 to 3 mg/ml.

Production of β-galactosidase–λmXBP fusion protein (λmXBP). A lysogen of phage λmXBP (42) in *Escherichia coli* Y1089r$^-$ was formed, isolated, induced to overproduce β-galactosidase–mXBP fusion protein (λmXBP) with isopropyl-β-D-thiogalactopyranoside (IPTG), and lysed as described previously (31, 42). Final protein concentrations were typically 8 to 10 mg/ml.

DNase I footprinting and methylation interference. A 220-bp Aα promoter fragment, Hf220 (5), 5′ end labeled on the sense strand, was used for DNase I footprinting with 69.9.15 pre-B-cell nuclear extract and λmXBP extract as previously described (5). For methylation interference experiments, the same DNA fragment 5′ end labeled on the coding strand was partially methylated at guanine residues in a 2-min reaction, as detailed by Maxam and Gilbert (47), except the reaction was quenched with 1.5 M sodium acetate (pH 7.0), 1 M β-mercaptoethanol, and 100 μg of poly(dI · dC) (Pharmacia) per ml. Methylated DNA was precipitated and suspended in 10 mM Tris (pH 7.6) and 1 mM EDTA. Binding and gel electrophoresis were carried out as described below, except the conditions were scaled up three- to fivefold. After exposure of the wet gel at 4°C for 4 h, the complex and free bands (B and F, respectively; see Fig. 3) were excised and DNA was eluted and ethanol precipitated. The pellet was rinsed with 70% ethanol, dried, and redissolved in 100 μl of 1 M piperidine. Base cleavage reactions were carried out for 40 min at 90°C, followed by removal of piperidine by lyophilization. After two additional rounds of lyophilization from water, equal counts of products were analyzed on 8% polyacrylamide gels in the presence of 8 M urea and were subsequently dried and autoradiographed.

In vitro transcription and translation and gel electrophoresis. Plasmids carrying various cDNAs were digested with appropriate restriction enzymes, and the linearized DNA template was transcribed in vitro with an mRNA capping kit, as recommended by the manufacturer (Stratagene Cloning Systems). The mRNA was suspended in 25 μl of distilled water, of which 1 to 10% was translated in vitro with a micrococcal nuclease-treated rabbit reticulocyte lysate (Promega Biotec). Each 50-μl reaction mixture, containing 35 μl of lysate, 5 μl of translation-grade [^{35}S]methionine (Dupont, NEN Research Products), 1 μl of 0.1 M dithiothreitol, and 1 μl of a 1 mM amino acid mixture minus methionine (Promega Biotec) was incubated with the appropriate mRNA at 30°C for 60 min. Equivalent amounts of individual mRNAs were used for separate translations or cotranslations. In vitro-translated proteins were resolved on sodium dodecyl sulfate (SDS)–8% polyacrylamide gels (36) along with ^{14}C-labeled molecular mass markers (Amersham Corp.). The gels were fixed with 30% methanol and 10% acetic acid, treated with En^{3}Hance (Dupont, NEN Research Products), dried, and autoradiographed.

Generation of antibodies. *E. coli* cells harboring the plasmid PATH11-mXBP, encoding for a fusion protein, TrpE-mXBP, were grown in conditions of tryptophan starvation as previously described (27). An induced band with an expected apparent molecular mass of 82 kilodaltons (kDa) was detected by SDS-polyacrylamide gel electrophoresis (as described above), except the gel was stained with Coomassie brilliant blue for 20 min. This band was isolated from the gel (approximately 1 mg of fusion protein), and a rabbit antiserum to this protein was prepared by the Berkeley Antibody Company. An antiserum collected after the second bleeding was used in experiments. Anti-c-Fos monoclonal antibody 14Cl has been previously described (14), and 4883 is an affinity-purified rabbit antiserum directed against a murine c-Jun peptide corresponding to residues 6 through 24 (W. W. Lamph, unpublished data).

Preparation of DNA fragments and DNA binding assays. Oligonucleotides were synthesized on a Biosearch synthesizer by standard phosphoramidite techniques and deprotected for 16 h in saturated ammonium hydroxide at 55°C. Complementary strands were annealed in 0.5 M NaCl at 65°C, and double-stranded oligonucleotides were purified on 12% polyacrylamide gels. One hundred-nanogram samples were end labeled with T4 polynucleotide kinase (New England BioLabs, Inc.) and [γ-^{32}P]ATP (Dupont, NEN Research Products), phenol-chloroform extracted, ethanol precipitated, and suspended in distilled water, and trichloroacetic acid-precipitable radioactivity was measured. A total of 10,000 to 20,000 cpm of ^{32}P-labeled oligonucleotide, representing approximately 0.05 to 0.2 ng of DNA, was used in each binding reaction. Binding was carried out in 10 μl of a solution containing 14 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.9), 20% glycerol (vol/vol), 75 mM KCl, 50 mM NaCl, 10 mM Tris (pH 7.9), and 1 mM EDTA with 4 μg of poly(dI · dC) (Pharmacia). A total of 1 μl of bacterial or nuclear extract or unlabeled translation products was incubated with ^{32}P-labeled oligonucleotide for 20 min at room temperature. Alternately, equal volumes of different translation products were mixed and incubated for 30 min at 37°C, and 1 μl of the

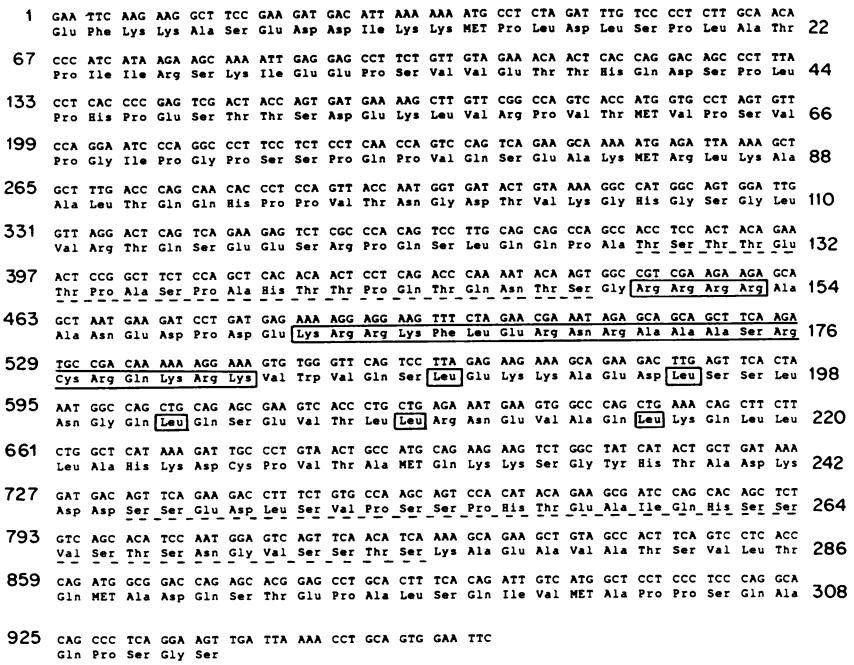

FIG. 1. cDNA sequence and inferred amino acid sequence of mXBP. Numbers on the left refer to the first nucleotide listed on each line, while numbers on the right refer to the last amino acid appearing on each line. Basic regions and the leucines of the leucine zipper are boxed. Serine- and threonine-rich regions are underlined with a broken line. A potential protein kinase A phosphorylation site (Lys Lys Ala Ser) is located at residues 3 through 6. The GenBank accession number for mXBP is M31629.

mixture was used. Unmixed translation products were also incubated, in parallel, for 30 min at 37°C. Samples were resolved on 4% polyacrylamide gels in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA) at 11 V/cm at room temperature for 1.5 h. For greater resolution, samples were loaded onto 7 to 8% polyacrylamide gels with 4% stacker and run in Tris-glycine buffer as described by Laemmli (36), except that SDS was omitted. Electrophoresis was carried out at 4°C at 11 V/cm for 12 to 15 h. Gels were dried and autoradiographed.

Immunoprecipitation. ^{35}S-labeled in vitro-translated proteins in 300 μl of RIPA buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA, 10 mM KCl) were incubated for 15 min at 4°C with 40 μl of a 50% protein A-Sepharose slurry (Pharmacia). After the Sepharose beads were removed, 2 μl of antiserum was incubated with the mixture at 4°C for 90 min. After addition of 60 μl of 50% protein A-Sepharose slurry and incubation for 60 min at 4°C, the Sepharose bands were washed four times with RIPA buffer, twice with RIPA buffer containing 1 M NaCl, and once in RIPA buffer. The Sepharose beads were boiled for 5 min in 50 μl of sample buffer (62.5 mM Tris [pH 6.8], 10% glycerol [vol/vol], 2% SDS, 5% 2-mercaptoethanol), and the sample was resolved on SDS–8% polyacrylamide gels (36).

Protein cross-linking. Mixed or unmixed in vitro translation products were incubated at 37°C for 30 min, and then the volume of each sample was brought up to 5 μl with rabbit reticulocyte lysate to which no exogenous mRNA had been added. Each 5-μl sample was added to 15 μl of 0.013% electron microscopy-grade glutaraldehyde (Sigma Chemical Co.) in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2PO_4 \cdot 7\ H_2O$, 1.4 mM KH_2PO_4) and was incubated for 2.5 h at room temperature. Samples were resolved on SDS–7% polyacrylamide gels as described above.

RESULTS

mXBP/CRE-BP2 is a member of the leucine zipper family of DNA-binding proteins. A 963-bp insert originally isolated from a spleen cell λgt11 expression library (42) was subcloned into the *Eco*RI site of pBluescript-KS, and the nucleotide sequence of both strands was determined by the dideoxynucleotide chain termination method. The nucleotide sequence and inferred amino acid sequence of the only long open reading frame are shown in Fig. 1. The inferred amino acid sequence of this partial cDNA clone shows >99% identity to human CRE-BP1 (43), except it does not contain the amino-terminal 94 amino acids or internal residues 150 through 247, which are rich in proline. The structural relationship between mXBP/CRE-BP2 and CRE-BP1 is shown in Fig. 2B. Northern (RNA blot) analysis reveals mXBP mRNA species of 6.2 and 3.0 kilobases (42), and preliminary characterization of independently isolated mXBP cDNA clones reveals different blocks of coding regions at the 5′ ends, suggesting alternative splicing may generate a family of mXBP/CRE-BP proteins. This is reminiscent of CTF/NF-1, where alternative splicing generates several related peptides differing in a proline-rich activator domain (48). For the sake of simplicity, we will refer to the clone shown in Fig. 1 as mXBP for the remainder of this manuscript.

mXBP has a heptad repeat of five leucines between amino acid residues 187 and 226. Chou-Fasman analysis (8) predicts α helix as the preferred secondary structure of this region. Eight out of ten amino acid residues on one face of this putative α helix are hydrophobic. Amino terminal to this region is a highly basic region where 15 out of 31 residues are lysine or arginine. Flanking these regions are two stretches, where 11 out of 21 (residues 128 to 148) or 15 out of 31 (residues 245 to 275) amino acids are serine or threonine.

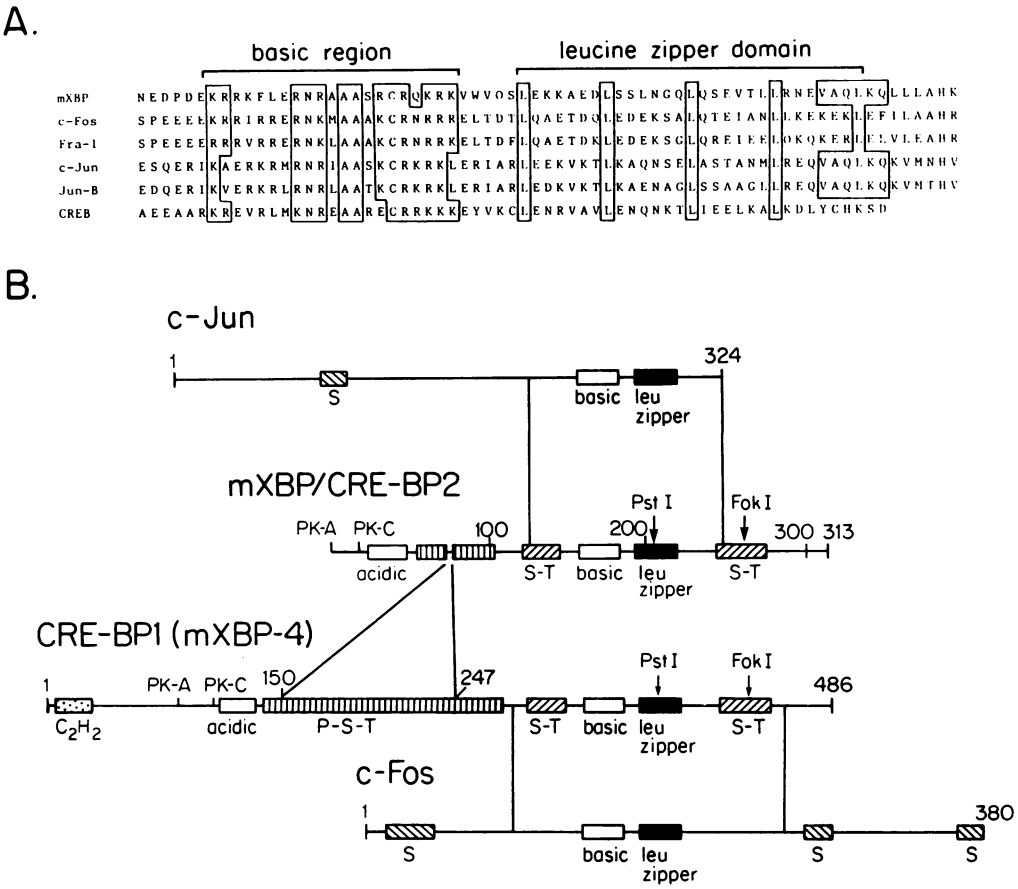

FIG. 2. mXBP shares homology with the leucine zipper family of DNA-binding proteins. (A) Alignment of amino acid homology between mXBP, c-Fos (49), Fra-1 (10), c-Jun (37), Jun B (61), and CREB (26), starting at amino acids 156, 133, 103, 251, 263, and 264, respectively. The highly conserved, mainly basic residues, the leucines of the leucine zipper, and a six-amino-acid stretch of identity among mXBP, c-Jun, and Jun B are boxed. (B) Diagram of structures of c-Jun, mXBP/CRE-BP2, CRE-BP1, and c-Fos aligned by regions of homology. The structure shown for CRE-BP1 is based on the sequence reported by Maekawa et al. (43) which corresponds to a clone, mXBP4, also isolated in our laboratory. mXBP/CRE-BP2 does not contain an internal domain corresponding to residues 150 through 247 of CRE-BP1. This domain is part of a sequence (labeled P-S-T) which is rich in proline (19%) as well as serine and threonine (21%). Leucine zipper, basic, acidic, serine-and-threonine-rich (S-T) and serine (S)-rich regions are indicated. PK-A and PK-C are potential sites for phosphorylation by protein kinase A and protein kinase C, respectively (43). C_2H_2 is a zinc finger containing two cysteines and two histidines. Vertical lines delineate regions of homology of mXBP with c-Jun and c-Fos, as discussed in the text.

Similar serine-and-threonine-rich motifs have been described for transcription factors Spl (13) and Oct 1 (69) and are schematized in Fig. 2B. mXBP also contains a potential protein kinase A phosphorylation site (Lys Lys Ala Ser; residues 3 through 6) and an acidic domain, where 7 out of 25 amino acids are acidic (residues 29 through 54).

The homology of mXBP with c-Fos, Fra-1, c-Jun, Jun B, and CREB in the leucine repeat and basic regions is shown in Fig. 2A. There is a striking conservation of basic amino acids and leucine residues in all six proteins. Pairwise comparison in these domains shows that c-Fos and Fra-1 are 80% identical, as are c-Jun and Jun B. mXBP shows 33% identity with c-Fos, 30% identity with c-Jun, and 30% identity with CREB. This limited homology of mXBP with c-Fos and c-Jun extends outside this domain (Fig. 2B). The mXBP amino acid sequence from residues 110 to 258 shares 24% identity and 69% homology with c-Fos, if conservative amino acid substitutions are discounted. The mXBP amino acid sequence from residues 122 to 237 shares 26% identity and 78% homology with c-Jun, if conservative amino acid substitutions are discounted.

DNA binding specificity of β-galactosidase-mXBP fusion protein (λmXBP) and proteins in pre-B-cell nuclear extracts. Protein lysates from a λgt11 cDNA clone coding for mXBP (λmXBP) have specific binding activity for oligonucleotides containing Aα X box and interspace sequences (42; see Fig. 4A). DNase I footprint and methylation interference analysis of λmXBP and native pre-B-cell nuclear extract showed similar patterns (Fig. 3). The DNase I footprints for λmXBP and pre-B-cell extracts were in the same region of X box and interspace, and methylation of a G in the X box inhibited binding by λmXBP and pre-B-cell extracts. Methylation at other Gs in the X box and interspace interfered with binding by λmXBP but not with binding by pre-B-cell extracts. This may reflect the ability of several proteins or protein complexes in B-cell extracts to bind to this site (42; see below). The region protected from DNase I cleavage and G residues where methylation interfered with λmXBP binding spanned a sequence overlapping the X box and interspace and was centered around an 8-bp palindrome TGACGTCA, which is identical to the CRE (Fig. 4A). We synthesized a set of mutant oligomers (Fig. 4A) and used gel retardation assays to extend the analysis of binding specificity and comparison of λmXBP and nuclear extracts.

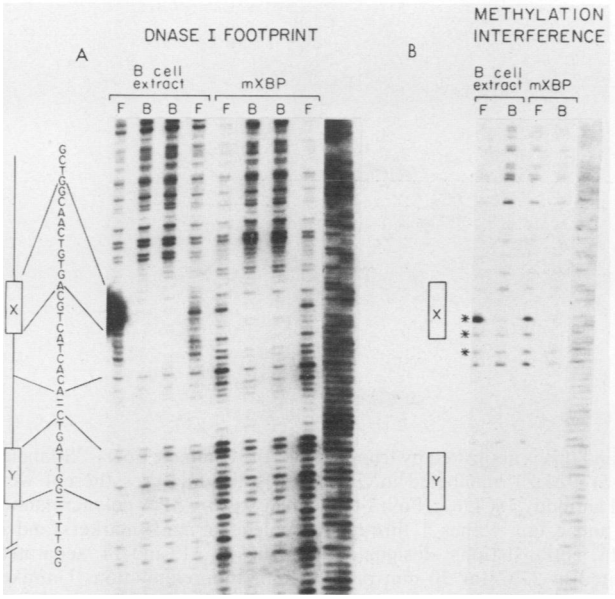

FIG. 3. DNase I footprinting and methylation interference analysis of pre-B-cell nuclear extracts and λmXBP. DNase I footprinting (A) and methylation interference (B) analyses were performed with a ^{32}P-labeled 220-bp DNA fragment which spans the Aα promoter, as described in Materials and Methods. DNase I treatment was for 3 min at 37°C, with a final DNase I concentration of 1 μg/ml. Cleavage products of unbound (free) DNA (F) and complexed DNA (B), with either pre-B-cell 69.9.15 nuclear extracts or λmXBP protein, are shown. Maxam and Gilbert (47) sequencing ladders G+A and A>C are shown to the right of each panel. The diagram outlines the protected region in bound DNA. G residues where methylation interferes with protein binding are starred.

As previously shown (42), native extract generated one major and two minor retarded bands and λmXBP extract generated one major retarded band when radiolabeled wild-type Aα-27 oligomer was used as the probe (Fig. 4B). When point mutant Aα-M5 was used as the probe, there was a parallel decrease in intensity of retarded bands with both λmXBP and native extract (Fig. 4B). Quantification of radioactivity in corresponding gel slices showed an 85% decrease in the retarded fraction for the major band of native extract and a 78% decrease for λmXBP (data not shown). The upper two bands with native extract and the λmXBP retarded band were not detectable when oligomer Aα-M1, containing three point mutations, was used as the probe (Fig. 4B).

The mutant oligomers were assayed for their ability to compete with binding to radiolabeled wild-type Aα-27 probe. Figure 4C shows the specific competition of the retarded bands of both native extract and λmXBP by excess unlabeled Aα-27 oligomer. Aα-M2 oligomer, containing a G→A transition outside the CRE, competed as well as did the wild type, but oligomer Aα-M3 or Aα-M4, with G→A changes in nucleotides within the CRE, competed less well (Fig. 4D, E, and F). Oligomer Aα-M5, containing the mutations present in Aα-M2 and Aα-M3, competed with a similar pattern as Aα-M3 (Fig. 4G), confirming that the G nucleotide, which is mutated in Aα-M2, is not critical for binding. Mutant Aα-M1, which contains point mutations in both half-sites of the CRE palindrome, competed extremely poorly (Fig. 4H).

These experiments show that nucleotides within the CRE palindrome are critical for binding of λmXBP and proteins in pre-B-cell nuclear extracts. The DNA binding specificity of

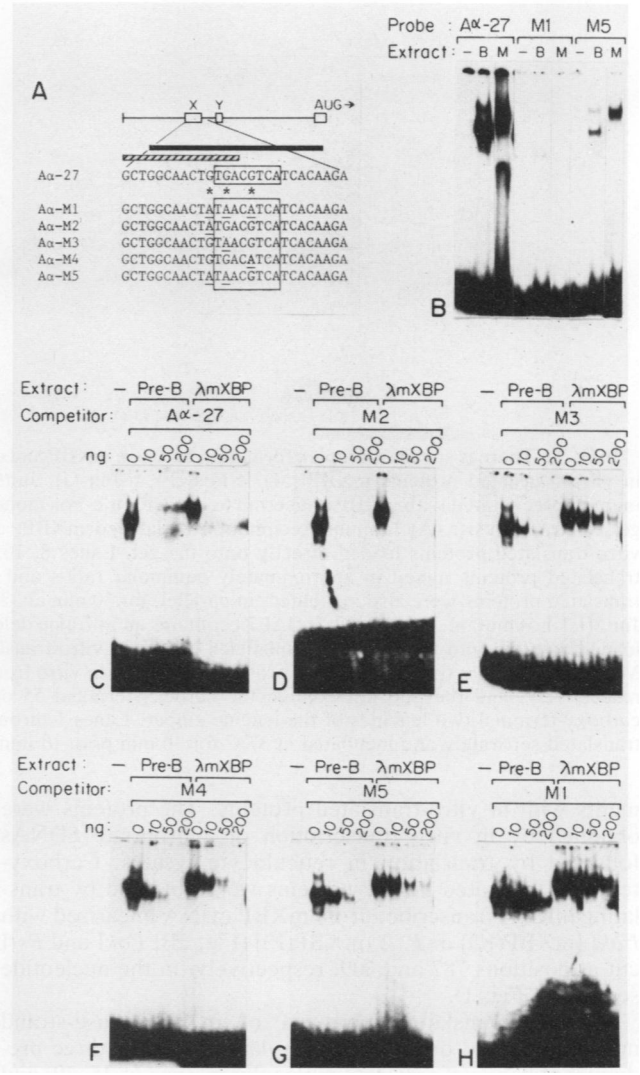

FIG. 4. DNA binding specificity of pre-B-cell nuclear extracts and λmXBP. Pre-B-cell lymphoma cell line 69.9.15 nuclear extracts (B) and λmXBP bacterial extracts (M) were assayed for binding to 20,000 cpm (approximately 0.2 ng) of ^{32}P-labeled DNA by gel retardation analysis. (A) Oligonucleotide Aα-27 from the Aα promoter region and mutants thereof which were used as ^{32}P-labeled probes or unlabeled competitors in gel retardation assays. The striped bar covers the X box. The solid bar covers the region protected from DNase I digestion. G residues where methylation interferes with nuclear extract or λmXBP binding are starred. Mutated bases are underlined. (B) DNA binding of extracts with Aα-27 and mutant Aα-M1 (M1) and Aα-M5 (M5) probes. Symbol: –, no extract. (C through H) Competition of binding to Aα-27 by mutated oligonucleotides. A total of 10, 50, or 200 ng of unlabeled oligonucleotides Aα-27, Aα-M2, Aα-M3, Aα-M4, Aα-M5, or Aα-M1 was added during incubation of extracts with the ^{32}P-labeled Aα-27 probe. Extract was omitted in the first lane of each gel.

λmXBP paralleled that of native extract for all cases tested. Results demonstrating the ability of λmXBP to bind specifically to CRE elements present upstream of several other genes are presented elsewhere in this issue (34a).

mXBP associates with c-Jun but not with c-Fos. The partial homology of mXBP to c-Jun and c-Fos suggested that it might complex with one of these proteins. To approach this question, we performed immunoprecipitation experi-

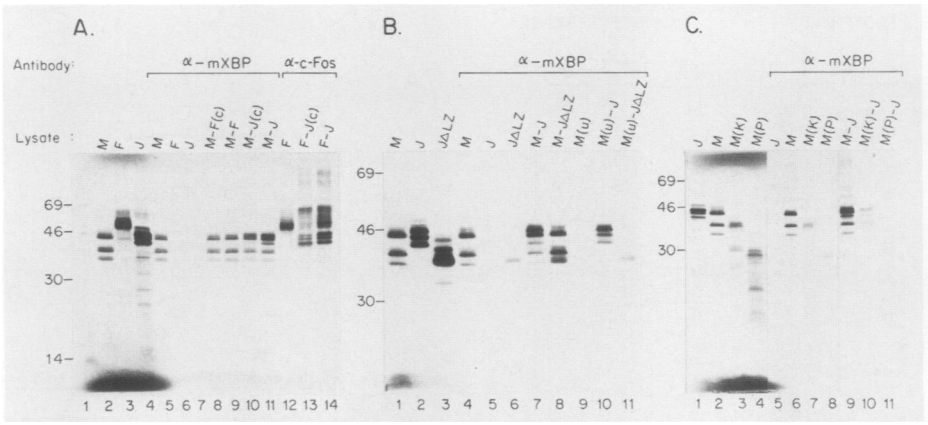

FIG. 5. There is specific complex formation between mXBP and c-Jun, and this is abolished by truncation of the leucine zipper. ^{35}S-labeled in vitro-translated proteins mXBP (M), c-Fos (F), c-Jun (J), JunΔ(L1-L5) (JΔLZ), unlabeled mXBP [M(u)], and mixtures thereof were immunoprecipitated with mXBP antiserum (α-mXBP) or c-Fos monoclonal antibody 14Cl (α-c-Fos; 14) and analyzed by SDS-polyacrylamide gel electrophoresis. (A) Immunoprecipitation of labeled mXBP, c-Fos, and c-Jun. Lanes 1 through 4, molecular mass markers and in vitro-translated proteins loaded directly onto the gel. Lanes 8, 10, and 13, cotranslations, designated (c); lanes 9, 11, and 14, separately translated proteins mixed in approximately equimolar ratios and incubated at 37°C for 30 min prior to immunoprecipitation. Unmixed translated proteins were also incubated, in parallel, for 30 min at 37°C. (B) Immunoprecipitation of labeled or unlabeled mXBP and c-Jun or JunΔ(L1-L5) mutant. Jun Δ(L1-L5) (JΔLZ) contains an in-frame deletion of the leucine zipper. Lanes 1 through 3, in vitro-translated proteins loaded directly onto the gel. M(u), unlabeled mXBP in vitro-translated protein. (C) Immunoprecipitations with truncated mXBP proteins. M(K) and M(P) are truncated mXBP proteins translated in vitro from mRNA transcribed from mXBP cDNAs linearized with *Fok*I and *Pst*I, respectively, and they do not contain the carboxy-terminal 55 or 110 amino acids, respectively (Fig. 2B). M(P) does not contain the carboxy-terminal two leucines of the leucine zipper. Lanes 1 through 4, proteins loaded directly onto the gel; lanes 9 through 11, proteins translated separately and incubated at 37°C for 30 min prior to immunoprecipitation.

ments with in vitro-translated proteins. The proteins were obtained by in vitro transcription of appropriate cDNAs followed by translation in reticulocyte lysates. Carboxy-terminal truncated mXBP proteins were obtained by translating mRNA transcribed from mXBP cDNA linearized with *Fok*I [mXBP(K)] or *Pst*I [mXBP(P)] (Fig. 2B; *Fok*I and *Pst*I cut at positions 787 and 609, respectively, in the nucleotide sequence; Fig. 1).

In vitro translation products of mXBP sense-strand mRNA migrated on SDS-polyacrylamide gels as three predominant bands of apparent molecular masses of 45, 39, and 36 kDa (Fig. 5A, lane 2). This was larger than the 32.5-, 27-, and 24.5-kDa apparent molecular masses predicted if translation initiated at the first three AUG codons. We believe that these species are mXBP proteins with three distinct amino termini, most likely secondary to initiation of translation in vitro at each of the first three AUG codons, because these species were not seen after translation of antisense RNA and did not change after incubation at 37°C (not shown); they were immunoprecipitated by mXBP antiserum (Fig. 5A, lane 5) but not by preimmune, c-Fos, or c-Jun antisera (not shown); carboxy-terminal truncated proteins migrated with the predicted decrease in apparent molecular mass (Fig. 5C, lanes 3 and 4); and in vitro-translated mXBP specifically binds CREs (see below). Anomalous migration of the mXBP proteins may be secondary to posttranslational modification, similar to the posttranslational modification of c-Fos or GCN4 (3, 28).

c-Fos and c-Jun in vitro translation products migrated as reported on SDS-polyacrylamide gel electrophoresis (Fig. 5A, lanes 3 and 4) (65). Cotranslation of c-Fos with c-Jun or incubation with c-Jun at 37°C for 30 min resulted in a shift in apparent molecular mass of the predominant c-Fos-specific band from 51 to 62 kDa (data not shown; see also Fig. 5A, lanes 13 and 14). mXBP mRNA was cotranslated with c-Fos or c-Jun or was translated separately and then incubated with c-Fos or c-Jun at 37°C for 30 min. Immunoprecipitations with mXBP antiserum were then performed to assay for complex formation. mXBP antiserum efficiently immunoprecipitated mXBP but not c-Jun or c-Fos (Fig. 5A, lanes 5 through 7; Fig. 5B, lanes 4 and 5). This antiserum immunoprecipitated both mXBP and c-Jun from a mixture of these proteins (Fig. 5B, lane 7), indicating the presence of an mXBP–c-Jun complex. Since the bands specific to mXBP and c-Jun partially overlap, we repeated this immunoprecipitation with unlabeled mXBP protein. The immunoprecipitation of labeled c-Jun in the presence of unlabeled mXBP (Fig. 5B, lanes 9 and 10) confirmed that c-Jun is complexed to mXBP. c-Fos did not coprecipitate with mXBP (Fig. 5A, lanes 8 and 9). Monoclonal antibody 14Cl (14) directed against c-Fos immunoprecipitated the c-Fos–c-Jun complex (Fig. 5A, lanes 13 and 14). These results demonstrate the selective formation of mXBP–c-Jun and c-Fos–c-Jun but not mXBP–c-Fos complexes.

The specificity of the mXBP–c-Jun interaction was further investigated with proteins with truncated leucine zipper domains. In vitro translation products of JunΔ(L1-L5), a mutant containing an in-frame deletion of the entire leucine zipper domain of c-Jun (56), were synthesized and shown to migrate on SDS-polyacrylamide gels in accordance with their apparent molecular masses (Fig. 5B, lane 3). A trace amount of JunΔ(L1-L5) was immunoprecipitated with mXBP antiserum (Fig. 5B, lane 6); trace amounts of immunoprecipitated c-Jun were also observed on prolonged exposures (data not shown). There was no significant increase over background immunoprecipitation of JunΔ(L1-L5) when it was incubated with mXBP antiserum in the presence of labeled or unlabeled mXBP (Fig. 5B, lanes 6, 8, and 11). In contrast, there was a dramatic increase in the immunoprecipitation of c-Jun in the presence of mXBP (Fig. 5B, lanes 5, 7, and 10). Immunoprecipitation experiments with truncated mXBP proteins confirmed these findings. Truncated

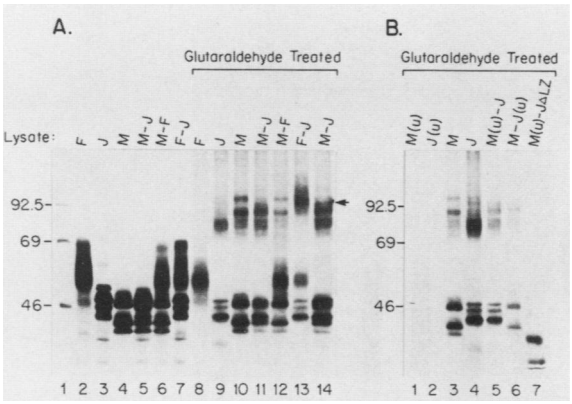

FIG. 6. Glutaraldehyde cross-linking of mXBP and c-Jun proteins. In vitro-translated proteins and mixtures thereof were incubated at 37°C for 30 min, cross-linked with glutaraldehyde, and resolved on SDS–7% polyacrylamide gels. ^{35}S-labeled proteins mXBP (M), c-Fos (F), c-Jun (J), JunΔ(L1-L5) (JΔLZ), or unlabeled proteins mXBP [M(u)] and c-Jun [J(u)] were used. JunΔ(L1-L5) is a mutant containing an in-frame deletion of the leucine zipper. (A) Samples in lanes 2 through 7 were not treated with glutaraldehyde. Sample in lane 14 is identical to sample in lane 11, except that in lane 14, an additional 1.5 μl of reticulocyte lysate to which no exogenous mRNA had been added was included during glutaraldehyde cross-linking. (B) Glutaraldehyde cross-linking of labeled and unlabeled mXBP, c-Jun, and JunΔ(L1-L5).

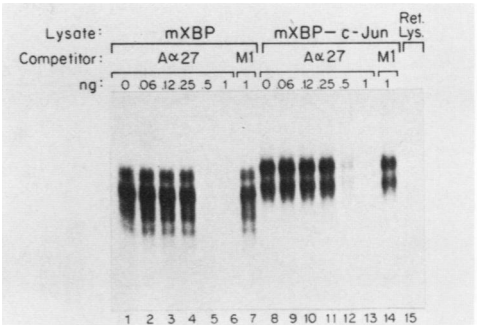

FIG. 7. mXBP and mXBP–c-Jun complex bind specifically to the CRE. In vitro-translated proteins and complexes were assayed for binding to 20,000 cpm (approximately 0.06 ng) of ^{32}P-labeled Aα-27 oligonucleotide by gel retardation analysis. Protein complexes were formed by mixing equal volumes of unlabeled proteins and incubating at 37°C for 30 min. A total of 1 μl of lysate was used in all binding reactions. Various amounts of unlabeled Aα-27 and Aα-M1 oligonucleotides (Fig. 4A) were included in the binding reactions as noted. In lane 15, rabbit reticulocyte lysate with no added mRNA was used.

mXBP in vitro translation products mXBP(K) and mXBP(P) were synthesized with comparable efficiency but with less efficiency than mXBP (Fig. 5C, lanes 2 through 4). mXBP(K) is truncated at a *Fok*I site and contains an intact leucine zipper; mXBP(P) is truncated at a *Pst*I site and does not contain the carboxy-terminal two leucines of the leucine zipper (Fig. 2B). Both truncated proteins were immunoprecipitated efficiently by mXBP antiserum (Fig. 5C, lanes 7 and 8). c-Jun coprecipitated with mXBP(K), but not with mXBP(P), when approximately equal amounts of mXBP(K) or mXBP(P) were used (Fig. 5C, lanes 10 and 11). Thus, alteration of the leucine zipper domain of either c-Jun or mXBP significantly decreased the formation of the mXBP–c-Jun complex. This is similar to the interaction of c-Fos and c-Jun, where intact leucine zipper domains have been shown to be essential for complex formation (19, 34, 56, 66, 70).

Glutaraldehyde cross-linking of in vitro translation products and subsequent SDS-polyacrylamide gel electrophoresis were used to determine the apparent molecular mass of the mXBP–c-Jun complex and to investigate the ability of mXBP to form homodimers. Cross-linking of c-Jun, but not c-Fos, proteins generated an additional species of greater apparent molecular mass (Fig. 6A, lanes 8 and 9). This confirms that c-Jun, but not c-Fos, can form homodimers, as previously shown (53, 70). Cross-linking of mXBP proteins generated additional species of apparent molecular masses of 95, 85, and 74 to 82 kDa (Fig. 6A, lane 10). This is approximately twice the apparent molecular mass of mXBP monomers and shows that mXBP can form a homodimer. Cross-linking a mixture of mXBP and c-Fos generated a pattern identical to that seen with mXBP alone (Fig. 6A, lanes 10 and 12). Cross-linking a mixture of mXBP and c-Jun generated an additional pattern of bands in the 87- to 93-kDa and 79- to 84-kDa ranges of apparent molecular mass (Fig. 6A, lanes 11 and 14). This is not merely a summation of c-Jun and mXBP specific patterns as the species of lowest mobility in Fig. 6A, lane 10, was absent, and a species with a new apparent molecular mass can be detected (Fig. 6A, lane 14; marked with arrow). Because of the overlapping patterns of bands, glutaraldehyde cross-linking was performed with mixtures of mXBP and c-Jun in which only one protein was radiolabeled. In both cases, when either only mXBP or only c-Jun was radiolabeled, an identical pattern of additional bands in the 87- to 93-kDa and 79- to 84-kDa ranges of apparent molecular mass was generated (Fig. 6B, lanes 5 and 6). This shows that these additional species, which have apparent molecular masses in the range for heterodimers of mXBP (apparent molecular masses of 45, 39, and 36 kDa) and c-Jun (apparent molecular mass of 41 to 46 kDa), contain both mXBP and c-Jun proteins. These additional species were not generated after glutaraldehyde cross-linking of unlabeled mXBP and radiolabeled mutant JunΔ(L1-L5), which lacks the leucine zipper domain (Fig. 6B, lane 7).

The mXBP–c-Jun complex binds specifically to the CRE site in Aα upstream region. The DNA-binding specificity of mXBP translation products and the mXBP–c-Jun complex were investigated with gel retardation assays. Resolution was increased by increasing polyacrylamide concentration, with a stacker, and a Tris-glycine buffer system, as described in Materials and Methods. Electrophoresis was carried out for approximately 10 times the length of time required to run free probe off the gel. Under these conditions, a retarded band caused by a binding activity present in reticulocyte lysate was run off the gel. Binding of mXBP to radiolabeled probe Aα-27 generated at least five retarded bands (Fig. 7, lanes 1 through 4; possible reasons for the generation of at least five retarded complexes are discussed further below). This binding was specific, as the retarded complexes were competed away by an 8- or 16-fold excess of unlabeled Aα-27 (Fig. 7, lanes 5 and 6) but not by mutant Aα-M1 competitor used in 16-fold excess (Fig. 7, lane 7) or 260-fold excess (data not shown).

When the mXBP–c-Jun complex was formed by incubating separately translated proteins at 37°C for 30 min and the complex was then bound to Aα-27 probe, a new pattern of retarded bands with unique mobilities was generated (Fig. 7, compare lane 8 with lanes 1 and 7). Identical results were obtained when the two mRNAs were cotranslated (data not shown). Incubation or cotranslation of mXBP with c-Fos

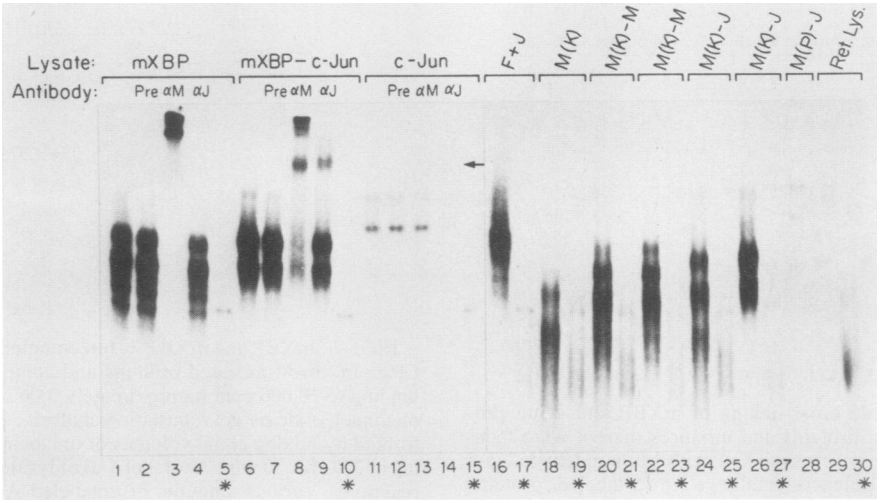

FIG. 8. Reactivity with antisera and mobility shifts with truncated proteins confirm that mXBP–c-Jun complex binds to the CRE. In vitro-translated mXBP (M), c-Fos (F), c-Jun (J), and mixtures thereof were assayed for binding to 20,000 cpm (approximately 0.05 ng) of ^{32}P-labeled DNA. The ^{32}P-labeled probe was Aα-27 oligonucleotide except in starred (*) lanes 5, 10, 15, 17, 19, 21, 23, 25, 27, and 30, where the ^{32}P-labeled Aα-M3 oligonucleotide (Fig. 4A) was used to control for specificity. In lanes 2 through 14, different antisera were included in the binding reactions. A total of 1 μl of preimmune serum (pre), 1 μl of mXBP antiserum (αM), or 2 μl of c-Jun affinity-purified antiserum 4883 (αJ) was preincubated with the binding reaction for 5 min prior to adding ^{32}P-labeled DNA. Preimmune serum and mXBP antiserum were diluted 1:4 in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7\ H_2O$, 1.4 mM KH_2PO_4) prior to being used. In lanes 18 through 28, truncated mXBP proteins M(K) and M(P) and mixtures of these truncated proteins and mXBP or c-Jun were used in the DNA binding reactions. M(K) and M(P) are truncated mXBP proteins which do not contain the carboxy-terminal 51 and 110 amino acids, respectively (see legend to Fig. 5). Lanes 20, 21, 24, and 25, Cotranslated proteins. Otherwise, protein complexes were formed by incubating equal volumes of separately translated unlabeled proteins at 37°C for 30 min. Lanes 29 and 30, Rabbit reticulocyte lysate with no added mRNA.

resulted in a pattern of retarded bands identical to that seen with mXBP alone (data not shown). The new retarded complexes were specific, as they were competed by unlabeled Aα-27 but not by mutant Aα-M1 (Fig. 7, lanes 13 and 14).

Confirmation that the mXBP–c-Jun complex binds to DNA with antisera and truncated proteins. The alteration in gel shift pattern with mXBP–c-Jun compared with that of mXBP alone was consistently reproducible with numerous independent preparations of proteins. However, since the shift in mobility for some bands was not dramatic, we sought to confirm that the mXBP–c-Jun complex was binding to Aα-27.

Specific binding of antibodies to protein-DNA complexes may alter the mobility of a retarded band in gel retardation assays (42). The change in mobility would depend on antibody class, affinity, titer, and ability to cross-link its substrate. Retarded bands with slower mobility were generated when mXBP was preincubated with mXBP antiserum, but not when it was preincubated with c-Jun antiserum, prior to binding to radiolabeled Aα-27 DNA (Fig. 8, lanes 3 and 4). Preincubation of c-Jun with c-Jun antiserum, but not with mXBP antiserum, generated a new retarded complex with slower mobility, which was seen on longer exposure (Fig. 8, lane 14, marked by arrow). When the mXBP–c-Jun complex was preincubated with either mXBP or c-Jun antiserum, retarded bands with slower mobility were generated (Fig. 8, lanes 8 and 9). These results confirm that both mXBP and c-Jun proteins are present when the mXBP–c-Jun complex binds to Aα-27 DNA. The patterns in lanes 8 and 9 (Fig. 8) are not identical, most likely because the c-Jun antiserum has a dramatically lower titer (L. Ivashkiv, unpublished data) and possibly lower affinity for the mXBP–c-Jun complex; the pattern generated with mXBP antiserum varied with different dilutions of antiserum (L. Ivashkiv, unpublished data).

Further evidence for binding of an mXBP–c-Jun complex was obtained with protein mXBP(K), in which the carboxy-terminal 51 amino acids are truncated but in which the leucine zipper remains intact. The pattern of bands generated when the mXBP(K)–c-Jun complex was bound to Aα-27 DNA is clearly different from the pattern generated with mXBP(K) or c-Jun alone (Fig. 8, compare lanes 24 and 26 with lanes 11 and 18). The generation of one predominant and intermediate pattern suggests that mXBP(K)–c-Jun was binding to Aα-27 DNA as a heterodimer with a mobility intermediate between that of mXBP(K) and c-Jun homodimers. When mXBP(P), in which the leucine zipper is truncated, and c-Jun were incubated at 37°C for 30 min and used in binding assays, no new retarded band was generated (Fig. 8, lane 28), though retarded bands comigrating with c-Jun-specific bands (Fig. 8, lane 11) were seen. c-Fos–c-Jun heterodimers also bound specifically to the Aα-27 probe (Fig. 8, lanes 16 and 17).

When ^{35}S-labeled mXBP was used in gel retardation assays with Aα-27 probe, six retarded complexes were resolved (data not shown). Since the mXBP translation product contains three different molecular mass species, the presence of six bands under nondenaturing conditions is consistent with dimers of the various molecular mass forms binding to Aα-27 DNA. This was explored further with truncated protein mXBP(K) (Fig. 8, lane 18). When mXBP(K), either cotranslated or translated separately and incubated at 37°C for 30 min with mXBP, was bound to Aα-27, additional retarded complexes (Fig. 8, lanes 20 and 22), which migrated at an intermediate position between mXBP complexes (Fig. 8, lane 1) and mXBP(K) complexes (Fig. 8, lane 18), were resolved. Since migration of protein-

TABLE 1. Binding of protein complexes to CRE and TRE elements[a]

Gene	Sequence	Binding of:		
		M	M-J	F-J
Aα-CRE	GCTGGCAACTGTGACGTCATCACAAGA	+	+	+
Aα-M3	GCTGGCAACTGTAACGTCATCACAAGA	Tr	Tr	Tr
Aα-M4	GCTGGCAACTGTGACATCATCACAAGA	Tr	Tr	Tr
E4-CRE	GACTTTAACCGTTACGTCATTTTTTAGT	+	+	−
MT-TRE	GAGCCGCAAGTGAC-TCAGCGCGGGGCG	−	−	+
FS-TRE	AAAAACATGAC-TCAGAGGAAAACATAC	−	−	+
Aα-TRE	GCTGGCAACTGTGAC-TCATCACAAGA	−	−	+
FS-CRE	AAAAACATGACGTCAGAGGAAAACATAC	+	+	+

[a] Summary of binding experiments with approximately 0.1 ng of each ^{32}P-end-labeled probe. The central CRE and TRE sequences are outlined. Base differences from consensus sequences are underlined. Aα-CRE and mutants are identical to Aα-27 and mutants shown in Fig. 4A. E4-CRE is a nonconsensus CRE site found in the adenovirus E4 gene (39). MT-TRE corresponds to sequences in the human metallothionein IIA gene (2). FS-TRE corresponds to FSE2 sequences in the aP2 gene (18). Aα-TRE and FS-CRE are derived from the naturally occurring Aα-CRE and FS-TRE sequences, respectively, by changing the central CRE or TRE elements but retaining the flanking sequences. M, mXBP; J, c-Jun; F, c-Fos; Tr, greatly diminished retarded complex seen on long exposures.

DNA complexes during electrophoresis depends in part on the molecular mass of the DNA-binding protein(s) (29), generation of one predominant intermediate pattern is consistent with mXBP binding as a homodimer, although the complexity of the pattern makes other stoichiometries of association or coexistence of monomers and dimers difficult to exclude.

mXBP controls the DNA binding specificity of the mXBP–c-Jun complex. To explore the DNA binding specificity of the mXBP–c-Jun complex, a series of 26- to 28-bp oligomers corresponding to the CRE or TRE elements and flanking sequences of several genes were synthesized (Table 1) and used in gel retardation assays. To further control for effects of flanking sequences, the Aα-27 oligomer was resynthesized, except the CRE was replaced by a TRE sequence (oligomer Aα-TRE; Table 1); also, a CRE was substituted for a TRE to create oligomer FS-CRE (Table 1). All oligomers were end labeled and assayed by gel retardation for binding to mXBP, mXBP–c-Jun, and c-Fos–c-Jun complexes. The results are summarized in Table 1. In all cases tested, the binding of the mXBP–c-Jun complex was identical to the binding of mXBP, but not to the binding of the c-Fos–c-Jun complex. The mXBP-c-Jun complex did not bind to any of the three TRE elements tested, even when possible nonpermissive effects of flanking sequences were eliminated by using radiolabeled Aα-TRE probe. Binding to TRE elements was not detected even on long exposures which revealed diminished (trace) binding to mutant Aα-M3 or Aα-M4 probes. These results were confirmed by competition experiments which showed that mXBP–c-Jun had a greater than 10-fold-higher affinity for Aα-CRE than for any of the TREs tested (Table 1). c-Fos–c-Jun bound to TRE elements, as expected, and to select CRE sequences, but it did not bind to the nonconsensus CRE of the adenovirus E4 gene.

mXBP–c-Jun and c-Fos–c-Jun complexes can coexist and retain their DNA binding specificities. The mXBP, c-Fos, and c-Jun translation products were mixed in approximately equimolar ratios and were incubated at 37°C for 30 min. Serial immunoprecipitation of this mixture with anti-Fos monoclonal antibody 14Cl, followed by mXBP antiserum, identified separate c-Fos–c-Jun and mXBP–c-Jun complexes (Fig. 9A, lanes 5 and 6). This demonstrated that both complexes have formed and coexist. Oligomers MT-TRE, E4-CRE, and Aα-CRE (identical to Aα-27; Fig. 4A) were used as probes to assay binding of the various complexes. The mXBP–c-Jun complex bound to E4-CRE and Aα-CRE but not to MT-TRE (Fig. 9B, lanes 4 through 6). c-Fos–c-Jun bound to MT-TRE and Aα-CRE but not to E4-CRE (Fig. 9B, lanes 7 through 9). The mixture of both complexes bound to all three probes (Fig. 9B, lanes 10 through 12. The pattern of binding to Aα-CRE (Fig. 9B, lane 12) appears to be a summation of binding patterns to MT-TRE and E4-CRE (Fig. 9B, lanes 10 and 11) and suggests that both mXBP–c-Jun and c-Fos–c-Jun complexes are binding to this probe. The relative ability of these complexes to compete for the same binding site has not yet been analyzed, though the affinity of mXBP–c-Jun for Aα-27 is higher than the affinity of c-Fos–c-Jun for Aα-27 (L. Ivashkiv, unpublished data).

DISCUSSION

We have characterized a DNA-binding protein of the leucine zipper class and have shown that it recognizes a palindromic sequence element TGACGTCA, known as the CRE, in the Aα gene promoter region. λmXBP binds specifically to CRE elements in several genes known to be

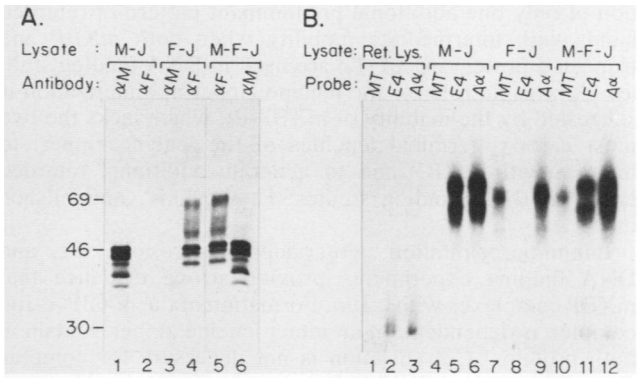

FIG. 9. DNA binding specificity of a lysate containing both mXBP–c-Jun and c-Fos–c-Jun complexes. (A) Formation and coexistence of both complexes. ^{35}S-labeled in vitro-translated proteins mXBP (M), c-Fos (F), and c-Jun (J) were mixed in roughly equimolar ratios, incubated at 37°C for 30 min, immunoprecipitated with mXBP antiserum (αM) or c-Fos monoclonal antibody (αF) 14Cl (14), and analyzed by SDS-polyacrylamide gel electrophoresis. Lanes 5 and 6, Serial immunoprecipitation of one lysate. (B) Specific DNA binding activity. Unlabeled in vitro-translated proteins, incubated as described for panel A, were assayed for binding to 10,000 cpm (approximately 0.2 ng) of ^{32}P-labeled DNA by gel retardation analysis. ^{32}P-labeled oligonucleotides MT-TRE (MT), E4-CRE (E4), and Aα-CRE (Aα) are shown in Table 1. Lanes 1 through 3, Rabbit reticulocyte lysate with no added mRNA.

cAMP inducible (H.-C. Liou, unpublished data) and to the nonconsensus CRE in the adenovirus E4 gene. In immunoblotting experiments, mXBP antiserum reacts with two out of eight species in nuclear extracts which bind radiolabeled Aα-27 probe on the corresponding DNA affinity blot (H.-C. Liou, unpublished data). Thus, mXBP corresponds to one out of several CRE-binding proteins in B-cell nuclear extracts and is distinct from CREB (20, 26). mXBP is nearly identical to CRE-BP1 (43), except it does not contain an internal proline-rich domain. Preliminary characterization of additional mXBP cDNA clones suggests that alternative splicing (9) may generate several forms differing in a region rich in proline residues, similar to what has been found with CTF/NF-1 (63).

The DNA binding specificity of mXBP is similar to that of purified activating transcription factor (ATF), which contains at least four proteins (22, 41). A small amount of AP-1/c-Jun copurifies with ATF on an ATF-specific DNA affinity column, and AP-1/c-Jun and ATF are immunologically related (22). As shown in this study, mXBP forms a complex with AP-1/c-Jun and mXBP antiserum immunoprecipitates, albeit very inefficiently, c-Jun in vitro-translated proteins. Thus, the mXBP protein we have characterized is one member of a family of related CRE-binding proteins, some of which may be the murine counterparts of proteins present in ATF.

The symmetry of its binding site, the presence of a leucine zipper, which has been suggested to form a dimerizing surface (38), and the fact that leucine zipper proteins c-Jun (23, 53), GCN4 (29), and CREB (73) can form homodimers, suggested that mXBP monomers may be able to associate into homodimers. The fact that in vitro-translated mXBP protein migrates as three species of similar apparent molecular mass on SDS-polyacrylamide gels complicates analysis of dimerization. Glutaraldehyde cross-linking experiments show that mXBP can form a homodimer in the absence of DNA. The presence of six DNA-binding species under nondenaturing conditions suggests that mXBP can bind to DNA as a homodimer, and this is supported by the generation of only one additional predominant pattern of retarded bands with intermediate mobility when both mXBP and truncated protein mXBP(K) are used in DNA binding studies. A possible role for the leucine zipper in dimerization is suggested by the inability of mXBP(P), which lacks the two most carboxy-terminal leucines of the leucine zipper, to interact with mXBP and to generate additional retarded bands in DNA binding studies (L. Ivashkiv, unpublished data).

Immunoprecipitation, glutaraldehyde cross-linking, and DNA binding experiments provide strong evidence that mXBP complexes with c-Jun. Formation of the mXBP–c-Jun complex is dependent on an intact leucine zipper domain in both proteins. Cotranslation is not necessary for complex formation, as the mXBP–c-Jun complex forms readily when separately translated mXBP and c-Jun are incubated together at 37°C. The most likely mechanisms for complex formation are association of mXBP and c-Jun homodimers into a heterotetramer or interchange of subunits and formation of mXBP–c-Jun heterodimers, although other possibilities exist. Leucine zipper truncations could disrupt tetramer formation by abolishing formation of its component homodimers or could disrupt heterodimer formation if mXBP and c-Jun interact through their leucine zippers. The approximately equimolar coimmunoprecipitation of mXBP and c-Jun is consistent with either tetramer or heterodimer formation. Cross-linking the mXBP–c-Jun protein complex with glutaraldehyde generates products with an apparent molecular mass close to that predicted for mXBP–c-Jun heterodimers. The fact that mXBP–c-Jun–DNA or mXBP(K)–c-Jun–DNA complexes have a mobility on gel retardation analysis which is intermediate between that of mXBP-DNA or mXBP(K)–DNA and c-Jun–DNA complexes suggests that mXBP–c-Jun and mXBP(K)–c-Jun bind to DNA as heterodimers. Thus, the mXBP–c-Jun complex is likely to be a heterodimer, and the leucine zipper is implicated as the structure which mediates or facilitates dimerization. This is analogous to the role of the leucine zipper in c-Fos–c-Jun heterodimerization (19, 23, 34, 53, 56, 58, 64, 66). Fra-1 is also thought to complex with c-Jun through its leucine zipper domain (11). c-Fos and Fra-1 differ from mXBP, as they do not form homodimers and have no intrinsic specific DNA binding activity (11, 23, 70).

Experiments with domain swaps with c-Fos, c-Jun, and GCN4 have shown that the leucine zipper domain of each protein determines its ability to homodimerize or heterodimerize with a particular partner. The specificity of leucine zipper-mediated dimerization is supported by the inability of c-Fos and Fra-1, c-Fos and c-Myc, GCN4 and c-Jun, GCN4 and c-Fos, c-Fos and CREB, and GCN4 and CREB to form complexes (56, 70). To date, all in vitro data on binding of these proteins has accurately reflected their ability to complex and function in vivo (7, 57, 64–66). Therefore, we believe that the mXBP–c-Jun interaction in vitro is likely to reflect an interaction which can occur in cells. When mXBP, c-Fos, and c-Jun are incubated together at 37°C, both mXBP–c-Jun and c-Fos–c-Jun complexes form, coexist in solution, and retain their respective DNA binding specificities. This implies that the relative affinities of c-Fos and mXBP for c-Jun are similar and further supports the notion that the mXBP–c-Jun interaction is physiologically relevant. Preliminary immunoprecipitations from serum-stimulated NIH 3T3 cells with mXBP antiserum have identified a major 63-kDa species, which corresponds to the predicted molecular mass of the peptide encoded by the mXBP cDNA clone most similar to CRE-BP1. There was a faint associated 45-kDa band, an apparent molecular mass consistent with that of c-Jun, which was not present when proteins were denatured prior to immunoprecipitation (L. Ivashkiv, unpublished data). Since serum stimulation induces c-Fos, most of the intracellular pool of c-Jun may be associated with c-Fos under these conditions. Experiments done under conditions where c-Fos is present at very low levels may lead to an increase in this mXBP-associated protein and may allow its identification.

c-Fos and c-Jun are expressed at a low level in most cell types; however, a variety of extracellular stimuli can induce their expression or posttranslational modification (3, 37, 44, 71, 72). Under certain conditions, such as the immediate-early response to serum stimulation, coordinate induction of both genes occurs (61). Fra-1 is closely related to c-Fos and Jun B and Jun D are closely related to c-Jun (10, 24, 60, 61). Fra-1 and Jun B also appear to be coordinately regulated with c-Jun in the immediate-early response, although the kinetics of induction after serum stimulation are different. Jun B and Jun D have a broad tissue distribution but appear to be abundant in the brain. These proteins are thought to interact to form a series of protein complexes which precisely control temporal and possibly tissue-specific expression of genes containing active TRE *cis* elements in response to a variety of extracellular stimuli (11). mXBP mRNA species of 6.2 and 3.0 kilobases are expressed at a low level in all cell lines and tissues tested and are abundant in the

brain (42; L. Ivashkiv, unpublished data). mXBP RNA is present under conditions of serum starvation, when c-Fos RNA is almost undetectable (L. Ivashkiv, unpublished data). The mXBP–c-Jun interaction is more likely to occur under these conditions. However, by analogy with CREB and c-Fos, posttranslational modification of mXBP may be critical in regulating its ability to interact with other proteins or activate transcription. The mXBP clone most similar to CRE-BP1 contains a consensus phosphorylation site for protein kinase A and is likely to be phosphorylated when cells are treated with cAMP. Anomalous migration on SDS-polyacrylamide gels suggests that mXBP may be more extensively modified, possibly by phosphorylation in the serine-and-threonine-rich regions. This would be analogous to phosphorylation of c-Fos in a serine-rich region (3).

mXBP binds to the CRE sequence in the class II major histocompatibility complex Aα promoter either alone, most likely as a homodimer, or when complexed with c-Jun. Aα constructs with mutated CRE sequences, which do not bind any proteins or protein complexes in pre-B-cell nuclear extracts and do not bind in vitro-translated mXBP or mXBP–c-Jun, have decreased enhancer function in transient transfection assays in pre-B cells (L. Ivashkiv, unpublished data). This implies that protein binding at this site is important for regulation of expression of the Aα gene. However, the Aα gene is not known to be cAMP inducible, and the transcriptional effects of binding by mXBP, mXBP–c-Jun, and other CRE-binding factors may be modified by proteins bound at nearby sites, particularly the Y box, which is an inverted CCAAT motif (42). It will be interesting to test if mXBP binding to the Aα gene CRE may contribute to silencing this gene in non-B cells and if binding of mXBP–c-Jun to this site may make this gene responsive to stimuli which induce c-Jun, such as phorbol esters, which have been shown to induce class II gene expression and play a role in B-cell activation (21, 30, 51).

Our data show that the interaction of mXBP with transcription factor and proto-oncogene product c-Jun redirects c-Jun to bind to CREs preferentially over TREs as part of a mXBP–c-Jun complex. This is distinct from the activity of c-Fos, which increases the affinity of c-Jun for TREs, as part of a c-Fos–c-Jun complex (23, 34, 53, 64). Additionally, c-Jun can bind to the adenovirus E4 gene nonconsensus CRE when paired with mXBP but not when paired with c-Fos. Changes in the relative expression and posttranslational modification of these three proteins will determine the relative prevalence of mXBP–c-Jun and c-Fos–c-Jun complexes, as well as mXBP and c-Jun homodimers. The resulting variations in binding of protein complexes of different composition (and possibly different abilities to activate or repress transcription) to CREs and TREs would determine the relative expression of sets of genes which are cAMP or phorbol ester responsive.

Complex interactions between cAMP- and phorbol ester-activated signal transduction pathways have been well documented (55, 59). Coupled transcriptional responses may be mediated by a single factor, such as AP-2, which binds to a sequence distinct from a CRE or TRE (32). Alternatively, distinct factors can bind to closely spaced but different sequences in a single enhancer (12). We propose that protein-protein interactions among related factors such as mXBP, c-Fos, and c-Jun provide an additional mechanism for coupling transcriptional responses to cAMP and phorbol esters. This mechanism allows a limited set of proteins to interact to form complexes of various DNA binding specificities, affinities, and abilities to activate transcription, thus expanding the flexibility of cellular transcriptional responses.

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