pear to be involved in the modulation of the Ca^{2+} channel by protein kinase A. Along with our finding that stimulation of protein kinase C did not alter I_{Ca}, these results provide further evidence that cardiac K^+ and Ca^{2+} channels are regulated differently (*22*).

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24. We thank T. Begenisich and J. Arena for their critical reading of the manuscript. Financial support was provided by National Science Foundation grant PCM-8116822.

3 May 1988; accepted 2 August 1988

Distinct Cloned Class II MHC DNA Binding Proteins Recognize the X Box Transcription Element

HSIOU-CHI LIOU, MARK R. BOOTHBY, LAURIE H. GLIMCHER

The class II (Ia) major histocompatibility complex (MHC) antigens are a family of integral membrane proteins whose expression is limited to certain cell types. A pair of consensus sequences, X and Y, is found upstream of all class II genes, and deletion of each of these sequences eliminates expression of transfected genes. Furthermore, the absence of a specific X box binding protein in patients with severe combined immunodeficiency disease whose cells lack class II suggests an important role for these proteins in class II regulation. Here, the cloning of two λgt11 complementary DNAs encoding DNA binding proteins (murine X box binding proteins λmXBP and λmXBP-2) is reported. Both phage-encoded fusion proteins bind specifically to the X box of the Aα, but not to Eα or Eβ class II genes. These two independent isolates do not cross-hybridize. The λmXBP complementary DNA hybridizes to two RNA species, 6.2 and 3.0 kilobases in mouse, that are expressed in both Ia positive and Ia negative cells. By means of DNA blot analysis with the λmXBP complementary DNA insert and probes generated from each end of this complementary DNA insert, λmXBP was found to arise from a multigene family. These data illustrate the high degree of complexity in the transcriptional control of this coordinately regulated gene family.

THE MURINE MAJOR HISTOCOMPATIBILITY complex (MHC) locus encodes four coexpressed class II genes: Aα, Aβ, Eα, and Eβ whose expression is coordinately regulated in response to a number of stimuli. Constitutive class II expression in B cells is under the control of at least one dominant locus located on mouse chromosome 16 that encodes a cross-species transacting factor (*1*). Control regions for eukaryotic genes are subject to trans-regulation by sequence-specific DNA binding proteins (*2*). Several DNA binding activities specific for sequences upstream of class II genes, including the X and Y boxes, have been identified (*3–7*). DNA binding proteins have been identified that recognize the consensus X box transcription element of human (*5, 6*) and murine (*3, 7*) class II genes; the proteins that bind class II Aα X boxes differ from those that bind Eα and Eβ X boxes (*7*). Patients with severe combined immunodeficiency disease, whose cells do not express class II genes, lack an X box binding protein as indicated by gel retardation assays (*8*). We have now identified cDNA clones that direct the synthesis of X(Aα) binding proteins in order to better characterize the proteins that bind these transcription elements.

A method developed by Singh *et al.* (*9*) enabled these investigators to molecularly clone a DNA binding protein whose recognition properties overlap those of transcriptional factors H2TF1 and NF-κB. We, therefore, screened a λgt11 mouse spleen cDNA expression library with ^{32}P-labeled X(Aα)44, a 44 bp oligonucleotide containing the Aα X box as well as the interspace sequence separating the X and Y boxes (Fig. 1B). From 750,000 plaques examined, two clones remained positive after three rounds of screening and were negative with a control probe BRE(Aα)39. The frequency of the message is therefore at least 1/400,000, which is consistent with the predicted frequency for rare messages encoding other transcription factors [1/100,000 in (*9*)].

Protein lysates made from the first of the two positive clones (λmXBP) were tested for DNA binding activity in a gel retardation assay. A λcOVA (chicken ovalbumin λgt11 cDNA clone) protein lysate and a B cell nuclear extract that contained X box binding activity were included as negative and positive controls, respectively. A DNA binding activity was detected specifically in the lysate of the λmXBP lysogen (Fig. 1A). This activity was sequence-specific since competition for retarded band was provided by unlabeled X(Aα)44 but not by Y(Aα)37, an oligonucleotide containing the Y box plus eight bases of the interspace-element (Fig. 1A, lanes 3 to 6). Furthermore, no retarded bands unique to λmXBP were detected when labeled Y(Aα)37 and BRE(Aα)39 oligonucleotides were used as probes (Fig. 1A, lanes 9 to 18). The faint bands present in all lanes with both the Y(Aα)37 and BRE(Aα)39 probes probably represent nonspecific binding. These complexes were eliminated with amounts of poly(dI-dC) that did not affect the formation of the X(Aα) complexes. The activity was induced by isopropyl-β-D-thiogalactopyranoside (IPTG), indicating that it was linked to the lac Z fusion gene (Fig. 1A, lanes 3, 4, 19, and 20). Neither the nonspecific λcOVA (with or without IPTG induction) nor Y1089 host lysates showed any corresponding retarded bands (Fig. 1A, lanes 2, 7, and 8). To obtain direct evidence that the β-galactosidase fusion protein en-

H.-C. Liou, Department of Cancer Biology, Harvard School of Public Health, Boston, Massachusetts, 02115. M. R. Boothby and L. H. Glimcher, Department of Cancer Biology, Harvard School of Public Health, and Department of Medicine, Harvard Medical School, Boston, MA 02115.

coded by λmXBP was responsible for the sequence-specific DNA binding activity, we added antibody to β-galactosidase to the DNA binding reaction; DNA binding activity was retained and the mobility of the complex decreased in a dose-dependent manner (Fig. 1A, lanes 19 to 23). In contrast, control antibody to cOVA did not affect the mobility of the retarded bands (lanes 24 to 26). These results provide evidence that the novel binding activity observed is derived from an mXBP–β-galactosidase fusion protein. Thus, λmXBP encodes a fusion protein of lac Z with a DNA binding domain that specifically recognizes the X box of the Aα gene.

Differences among the major protein species that bind to the X box of the class II Aα, Eα, and Eβ genes can be detected by gel mobility and cross-competition (*7*), although one species appears to be communal. To determine which of the X box binding proteins λmXBP represents, we performed gel retardation assays with the X(Eα)33 and X(Eβ)32 probes (Fig. 1B). The λmXBP fusion protein did not specifically bind X(Eβ) or X(Eα) probes (Fig. 2), whereas in the same experiment (Fig. 1A) it did bind X(Aα). The faint bands observed are nonspecific: they also appeared in λcOVA lanes and in the absence of IPTG. These results demonstrate directly that a cloned X box binding domain discriminates between the Aα sequence and the related Eα and Eβ sequences. The second murine spleen cDNA isolated from the λgt11 library, λmXBP-2, also encodes an X(Aα) DNA binding domain which binds only to the X(Aα) and not to the X(Eβ) or X(Eα) (*10*). Deoxyribonuclease I (DNase I) footprint and methylation interference analyses of these two clones and native B cell nuclear extract have very similar patterns (*10*). Specifically, residues spanning the X box region and the interspace element were protected from DNase I cleavage relative to the unretarded DNA. By methylation interference analysis, contact sites to two G residues in the X box and one G residue in the interspace element were observed from all three samples (native nuclear extract, clones mXBP, and mXBP-2). Surprisingly, the inserts of these two clones did not cross-hybridize. Thus, at least two distinct X(Aα) DNA binding domains are encoded by messenger RNAs expressed in mouse spleen cells. That this is a minimum estimate is suggested by our finding that a human λgt11 cDNA clone that binds to the murine Aα X box also does not cross-hybridize with λmXBP or λmXBP-2, but detects murine RNA species of different sizes (*11*).

The expression of the mouse gene mXBP was analyzed by RNA blot hybridization (Fig. 3A). Two transcripts (approximately 6.2 and 3.0 kb) were observed with polyadenylated [poly(A)$^+$] RNA from both Ia positive (spleen cells, P388D1 and R8205.C2) and Ia negative (HT-2, P815)

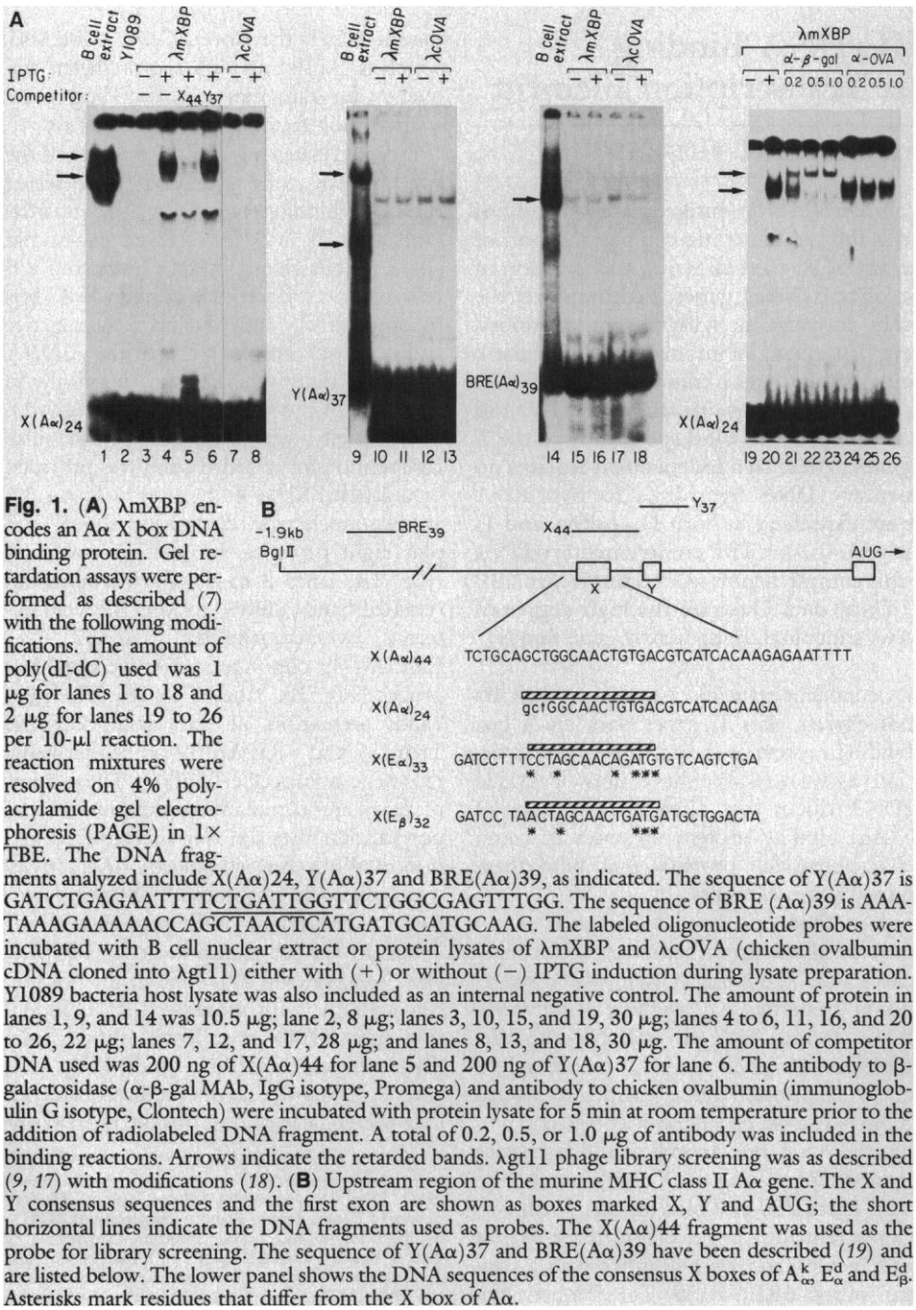

Fig. 1. (**A**) λmXBP encodes an Aα X box DNA binding protein. Gel retardation assays were performed as described (*7*) with the following modifications. The amount of poly(dI-dC) used was 1 μg for lanes 1 to 18 and 2 μg for lanes 19 to 26 per 10-μl reaction. The reaction mixtures were resolved on 4% polyacrylamide gel electrophoresis (PAGE) in 1× TBE. The DNA fragments analyzed include X(Aα)24, Y(Aα)37 and BRE(Aα)39, as indicated. The sequence of Y(Aα)37 is GATCTGAGAATTTTCTGATTGGTTCTGGCGAGTTTGG. The sequence of BRE (Aα)39 is AAATAAAGAAAAACCAGCTAACTCATGATGCATGCAAG. The labeled oligonucleotide probes were incubated with B cell nuclear extract or protein lysates of λmXBP and λcOVA (chicken ovalbumin cDNA cloned into λgt11) either with (+) or without (−) IPTG induction during lysate preparation. Y1089 bacteria host lysate was also included as an internal negative control. The amount of protein in lanes 1, 9, and 14 was 10.5 μg; lane 2, 8 μg; lanes 3, 10, 15, and 19, 30 μg; lanes 4 to 6, 11, 16, and 20 to 26, 22 μg; lanes 7, 12, and 17, 28 μg; and lanes 8, 13, and 18, 30 μg. The amount of competitor DNA used was 200 ng of X(Aα)44 for lane 5 and 200 ng of Y(Aα)37 for lane 6. The antibody to β-galactosidase (α-β-gal MAb, IgG isotype, Promega) and antibody to chicken ovalbumin (immunoglobulin G isotype, Clontech) were incubated with protein lysate for 5 min at room temperature prior to the addition of radiolabeled DNA fragment. A total of 0.2, 0.5, or 1.0 μg of antibody was included in the binding reactions. Arrows indicate the retarded bands. λgt11 phage library screening was as described (*9*, *17*) with modifications (*18*). (**B**) Upstream region of the murine MHC class II Aα gene. The X and Y consensus sequences and the first exon are shown as boxes marked X, Y and AUG; the short horizontal lines indicate the DNA fragments used as probes. The X(Aα)44 fragment was used as the probe for library screening. The sequence of Y(Aα)37 and BRE(Aα)39 have been described (*19*) and are listed below. The lower panel shows the DNA sequences of the consensus X boxes of A^k_α, E^d_α and E^d_β. Asterisks mark residues that differ from the X box of Aα.

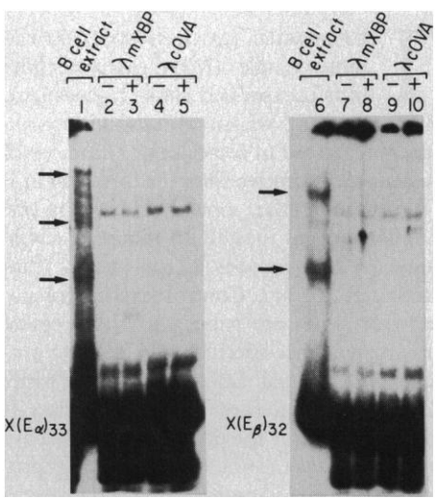

Fig. 2. mXBP does not bind I-E X boxes. Gel retardation assays were performed exactly as in lanes 1 to 18, Fig. 1B, except that either X(Eα)33 or X(Eβ)32 were used as labeled probes. The probe in lanes 1 to 5 was X(Eα)33, and in lanes 6 to 10 was X(Eβ)32.

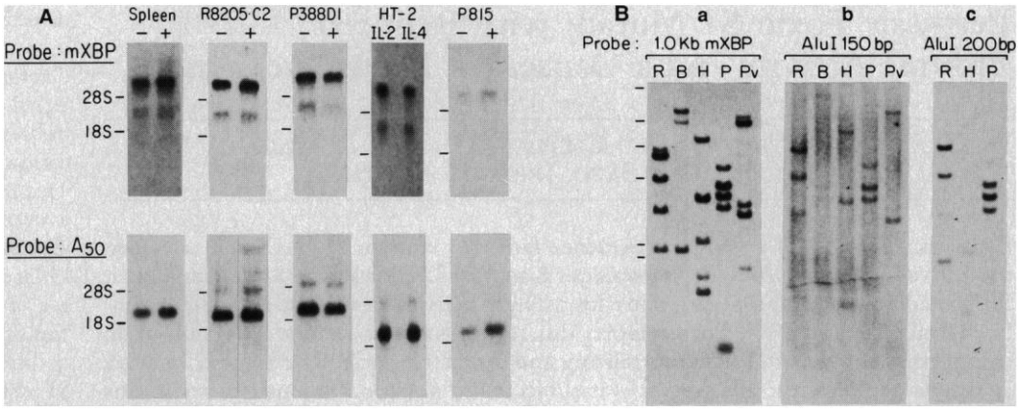

Fig. 3. (**A**) RNA blot analysis with λmXBP cDNA. Cells analyzed include: normal mouse splenocytes; R8205.C2, a pre–B cell line; P388D1, a macrophage-like cell line; HT-2, an IL-2/IL-4–dependent T cell clone; P815, a mastocytoma cell line. Cells were cultured either in the presence (+) or absence (−) of recombinant IL-4 as reported previously (*20*). Total cellular RNA was extracted by the guanidine isothiocyanate method and poly(A)$^+$ RNA was isolated with oligo (dT) columns. Total cellular RNA (10 to 15 μg), or poly(A)$^+$ RNA (1 to 2 μg) was subjected to electrophoresis on 1.3% agarose-formaldehyde gels and transferred to Nytran filters. The RNA filters were probed with nick-translated, ^{32}P-labeled λmXBP cDNA insert. Hybridization was at 37°C in 50% formamide with 10% dextran sulfate for 48 hours; filters were then washed with 2× SSC containing 0.1% SDS at 60° to 65°C. The same set of filters was also probed with the A50 gene, a cDNA clone containing sequences for a constitutively expressed "housekeeping" mRNA of unknown identity (*21*). The position of 28*S* and 18*S* ribosomal RNA is indicated. (**B**) DNA blot hybridization analysis with λmXBP cDNA. Genomic DNA was isolated from a murine B lymphoma M12.A2 and digested with a panel of restriction enzymes (R, Eco RI; B, Bam H1; H, Hind III; P, Pst I and Pv, Pvu II) and transferred to a nitrocellulose filter. The filter was probed with nick-translated λmXBP cDNA insert or short 5′ or 3′ probes derived by Alu I digestion of the λmXBP insert. Hybridization was carried out with 10% dextran sulphate in 50% formamide overnight at 37°C, and the filters washed twice with 2× SSC at 60°C. The positions of λ Hind III size standards (23, 9.4, 6.7, 4.3, 2.2, and 2.0 kb) are indicated. Similar results were obtained with DNA prepared from mouse spleen.

cells. This is consistent with previous observations that the X box binding proteins are constitutively expressed in all cell types (*7*). Induction of Ia by interleukin-4 treatment did not affect mXBP RNA levels, complementing our previous observation that Aα X box DNA binding activity in unfractionated extracts is not changed by lymphokine treatment (*7*). Two RNA species were detected in all the murine cells examined: the larger, 6.2-kb species generated a more intense band than the 3.0-kb species. While we do not yet know that both transcripts are expressed as proteins, these transcripts could be derived from two independent genes with partial homology at the DNA binding domain or may represent alternative splicing of a single species of RNA. To determine whether λmXBP is a member of a multigene family, we performed DNA blot hybridization (Fig. 3B, a). Restriction digests with a panel of enzymes generated multiple prominent bands that hybridized to the labeled λmXBP 1.0-kb insert. A similarly complex hybridization pattern was observed on probing restriction digests of the human and hamster genomes (*11*). Since it was possible that the 1.0-kb cDNA could hybridize to multiple fragments of a single gene with a complex intron-exon organization, short (150 and 200 bp) probes generated from each end of the λmXBP insert were hybridized to the same filter (Fig. 3B, b and c). With several enzymes, at least three bands hybridized with both probes. Thus, the λmXBP cDNA is represented in at least two genes.

The multiplicity of X box binding proteins described above is reminiscent of several other nuclear factors, including proteins that bind the CCAAT motif (*12, 13*), the Kruppel family of zinc finger proteins (*14*), and the *Drosophila* homeo box proteins (*15*). It is striking, though, that distinct proteins bind to the upstream consensus elements of a family of genes like the class II genes, which are coordinately regulated in response to inducing stimuli. Thus, coordinate control may only be achieved by the interplay of multiple distinct regulating proteins at key transcriptional elements (*16*). The further characterization of murine and human X box binding clones should help elucidate the control of this coordinately regulated gene family in normal individuals and in patients with severe combined immunodeficiency disease.

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22. Supported by grants from NIH and a Leukemia Scholar Award (to L.H.G.). We thank N. Nabavi for help with the RNA blot analysis, H. Singh for advice on λgt11 library screening, and L. Blood for manuscript preparation.

7 June 1988; accepted 12 August 1988