

## Evidence for Multiple Major Histocompatibility Class II X-Box Binding Proteins

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**The X box is a loosely conserved DNA sequence that is located upstream of all major histocompatibility class II genes and is one of the *cis*-acting regulatory elements. Despite the similarity between all X-box sequences, each promoter-proximal X box in the mouse appears to bind a separate nuclear factor.**

The major histocompatibility (MHC) class II genes are expressed in a cell-type-specific manner (9), and their expression is regulated by the lymphokines gamma interferon (11, 27) and interleukin-4 (18, 19). A number of *cis*-acting regulatory elements have been identified both upstream and within the introns of several class II genes (1, 2, 4, 8, 12, 23, 25, 26). One of these elements is the X box, which consists of about 13 base pairs of loosely conserved sequence located at -80 to -100 base pairs from the transcription start site (16, 22). The presence of the X-box sequence is necessary for the efficient and accurate transcription of the MHC class II genes (8). An additional X-box sequence, referred to as the promoter-distal X box (X'), has been identified further upstream in three mouse MHC class II genes, I-A $\beta$ , I-E $\beta$ , and I-E $\alpha$  (6, 7, 24). By using gel electrophoresis DNA-binding and DNA-footprinting assays, several investigators have shown that nuclear proteins footprint to the various X-box sequences (13, 17). In the case of the I-A $\alpha$  X box, a cDNA clone coding for a protein that binds to the I-A $\alpha$  X box has been identified (14). A cDNA clone has also been isolated for the X-box binding factor that recognizes the X box of the human lymphocyte antigen DR $\alpha$  gene (20). This X-box binding protein is thought to be defective in patients with combined immunodeficiency, a disease in which there is no MHC class II expression (15, 21).

**DMS footprint of the I-A $\beta$  X-box binding protein.** The I-A $\beta$  promoter-proximal X box was shown to bind a factor present in nuclear extracts, prepared from the B-cell lymphoma A20-2J, by a direct DMS footprinting assay (Fig. 1). A 320-base-pair end-labeled fragment, 12.33.2 (6), containing the I-A $\beta$ <sup>b</sup> promoter-proximal X box was incubated with nuclear extracts and poly(dI · dC) for 15 min at room temperature, after which dimethyl sulfate was added and the incubation was extended for 1 min before 2-mercaptoethanol was added to stop the reaction. The samples were treated with piperidine and run on a 6% polyacrylamide-urea gel as described previously (6). The methylation protection assay revealed protection of one guanine residue on the coding strand, while modification and cleavage at a second guanine toward the 5' side of the X box were enhanced. On the noncoding strand, two guanines were protected from methylation; one was located toward the center of the X-box sequence, and the other was located just 3' of the X-box sequence.

In the I-A $\beta$ <sup>b</sup> haplotype, the sequence recognized by this nuclear factor was characterized by the direct repeat 5'-

GACAGA-3'. The single guanine in both repeats, located on the noncoding strand, was protected, suggesting that both repeats may be involved in binding. Although a survey of other X-box sequences revealed several with one copy of the sequence 5'-GACAGA-3', none of those examined contained the direct repeat. Interestingly, the X-box sequence of the HLA-DR $\alpha$  gene contained five of the six bases (5'-ACAGA-3'), yet the protein that footprints to this X-box sequence gives a different footprint pattern than the one seen in this study (20). This suggests that although the sequence 5'-GACAGA-3' may be important for the binding of a nuclear factor to the I-A $\beta$  X box, it may not be an essential element for the binding of nuclear factors to other X-box sequences.

**Gel electrophoresis DNA-binding assay of X-box binding proteins.** To determine if the protein that recognizes the promoter-proximal X box of the I-A $\beta$  gene could recognize the promoter-distal I-A $\beta$  X-box sequence, we prepared 33-base-pair double-stranded oligonucleotides that included each X-box sequence and some flanking sequence (Fig. 2A). The double-stranded oligonucleotides were end labeled, and a gel electrophoresis DNA-binding assay was performed by using nuclear extracts prepared from the B-cell lymphoma A20-2J (6). Incubation of either the I-A $\beta$  X- or I-A $\beta$  X'-containing oligonucleotides with nuclear extract generated a DNA-protein complex that migrated more slowly than did the free DNA. Competition experiments indicated that the interaction between DNA and nuclear extract was specific; DNA sequences of identical composition effectively competed with the labeled oligonucleotides (Fig. 2B), but other DNA sequences (e.g., pBR322 or oligonucleotides with irrelevant DNA sequences) did not compete with the labeled oligonucleotides (data not shown). We found, however, that the I-A $\beta$  X-box-containing oligonucleotide, at 100 times molar excess, did not compete for the binding of the factor that recognized the oligonucleotide containing the I-A $\beta$  X' sequence, nor did the I-A $\beta$  X'-containing oligonucleotide compete for the binding of a factor that recognized the I-A $\beta$  X-box-containing oligonucleotide. On the basis of this result, we extended the analysis to include the promoter-proximal X boxes of the I-A $\alpha$ , I-E $\alpha$ , and I-E $\beta$  genes. In each case, the X-box-containing oligonucleotides bound nuclear factors that were competed for by the same X-box-containing oligonucleotide but were not competed for by any of the other X-box containing oligonucleotides (Fig. 2B). These results suggest that there may be multiple X-box DNA-binding factors, each of which specifically recognizes one of the X-box-containing oligonucleotides used in this study.

**Molecular weight determination of X-box binding factors.**

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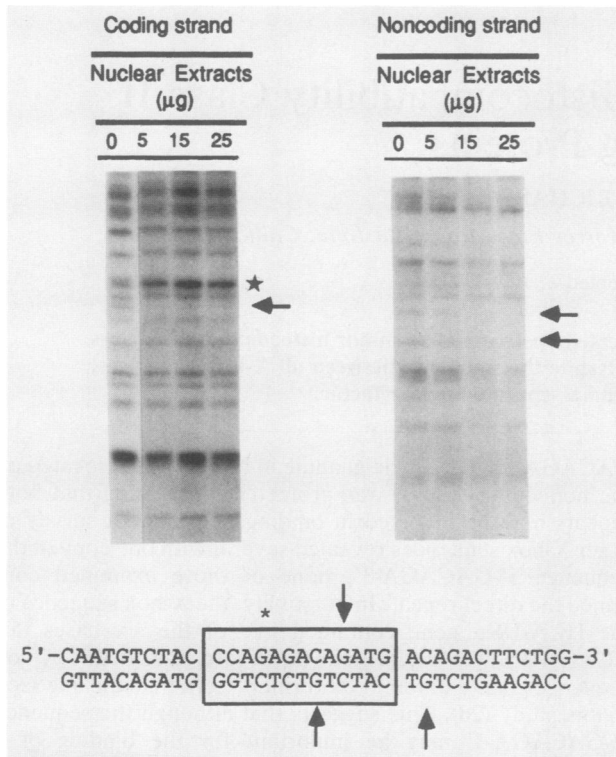


FIG. 1. DMS protection assay of the promoter-proximal X box of the I-A $\beta$  gene. A direct DMS protection assay was performed by using end-labeled DNA (12.33.2) on the coding and noncoding strands of DNA. Lane 0, Guanine cleavage pattern without added nuclear extract. The arrows indicate those guanine residues protected from DMS modification. The star indicates a guanine residue where enhanced cleavage occurs. At the bottom is a summary of the results. The X-box sequence is boxed.

To further characterize the X-box binding proteins, we determined their apparent molecular weights. Total nuclear proteins from A20-2J cells were fractionated on a 12% sodium dodecyl sulfate-polyacrylamide gel, and the gel was cut into 2.5-mm slices (50 fractions), after which the protein was extracted from each slice and renatured as described previously (5, 10). A DNA-binding assay was performed with a portion of protein eluted from each gel slice by using each of the labeled oligonucleotides. The portion of the gel corresponding to free and bound DNA was cut from the gel, the radioactivity was determined by liquid scintillation counting, and the percent binding was determined. At least one DNA-binding activity was recovered from the gel that specifically recognized each of the oligonucleotides tested (Fig. 3). The factor recognizing the I-A $\alpha$  X box was located in several fractions with the peak of activity corresponding to an apparent molecular mass of 37 kilodaltons (kDa). For the I-A $\beta$  X box, one peak of activity was found with an apparent molecular mass of 43 kDa. In the case of the I-E $\alpha$  X box, two peaks of binding activity were detected, with apparent molecular masses of 43 and 64 kDa. For the I-E $\beta$  X box, one peak of binding activity with an apparent molecular mass of 59 kDa was observed. The binding activity that recognized the I-A $\beta$  X' box had an apparent molecular mass of 43 kDa.

Possibly the most interesting explanation for these results is that a number of different X-box binding proteins exists in the mouse, and each recognizes a different X-box sequence. This idea is further supported by recently published results

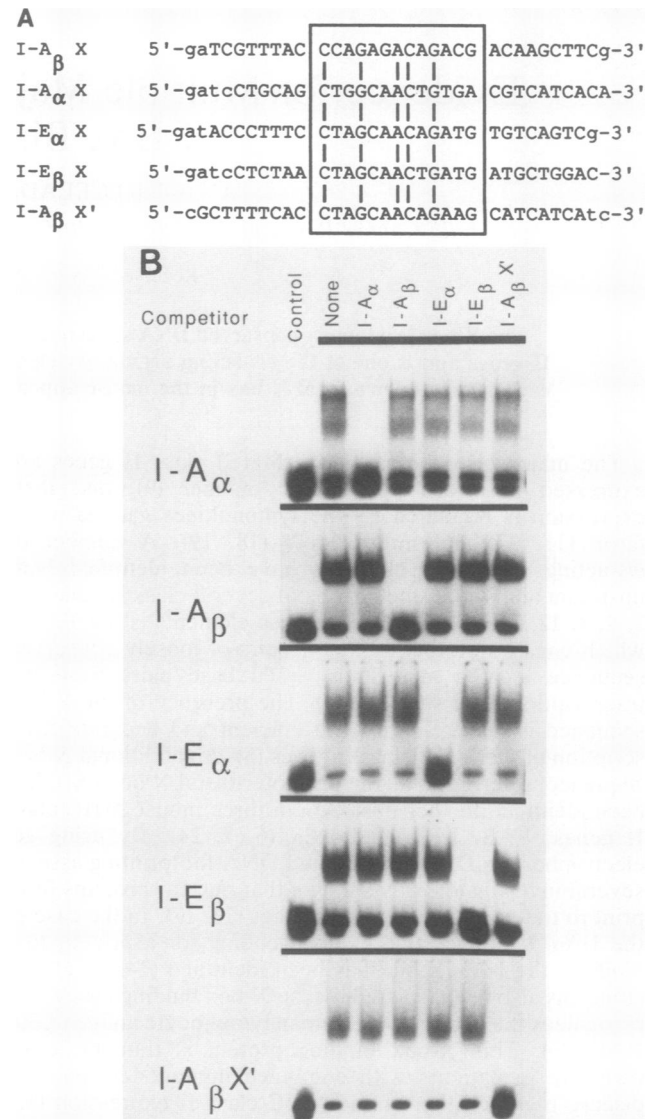


FIG. 2. (A) DNA sequence of one strand of the double-stranded oligonucleotides used in the study. The X-box sequence for each oligonucleotide is outlined. Those bases that are conserved between all X boxes are connected by vertical lines. The lowercase letters refer to bases that are not in the sequence flanking the X boxes but have been included in the sequence of the oligonucleotide. The orientation of each X box with respect to the gene is as shown except for I-A $\beta$  X', which is reversed. (B) Gel electrophoresis DNA-binding assay using the X-box oligonucleotides from the different mouse class II genes. Nuclear extracts were prepared from the B-cell lymphoma A20-2J and used in a binding assay as described elsewhere (6). The double-stranded oligonucleotides listed in panel A were used as competitors or were radiolabeled and used as probes. The competitors are listed at the top; the probes are listed on the left. Reactions contained 0.5  $\mu$ g of nuclear extract, 2  $\mu$ g of poly(dI · dC), and either no competitor or competitor at 100 times molar excess. The control was the labeled oligonucleotide in the absence of nuclear extract.

which suggest that different proteins bind to the X-box sequences of I-A $\alpha$ , I-E $\alpha$ , and I-E $\beta$  (3). An alternative explanation for these results is that the oligonucleotides bind different proteins but the X box contributes very little to the binding specificity of the DNA-binding proteins. Because of the information available on the interaction of X-box binding

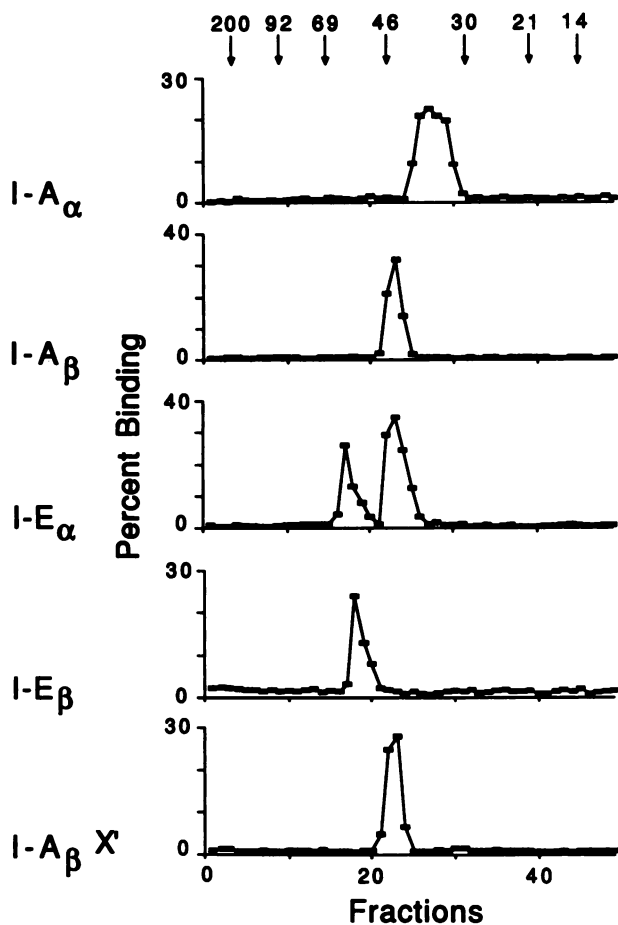


FIG. 3. Determination of the molecular weights of X-box binding activities recognizing the different X-box-containing oligonucleotides. Total nuclear extract prepared from A20-2J cells was fractionated on a 12% sodium dodecyl sulfate-polyacrylamide gel. The gel was cut into slices 2.5 mm wide, and the proteins were eluted and renatured as described previously (5, 10). A gel electrophoresis DNA-binding assay was performed by using each of the labeled oligonucleotides listed in Fig. 2A and a portion of protein eluted from each gel fraction. The percent binding was determined by cutting the areas of the gel corresponding to the positions of bound and free DNA and counting the radioactivity in the gel pieces. Molecular mass markers are indicated at the top (in kilodaltons).

proteins with X-box sequences, we do not believe this to be the case. The X-box binding protein that interacts with the I-A $\alpha$  X box footprints to an area covering the X box and sequences located to the 3' side (3). The footprint of the I-A $\beta$  X-box binding protein reported here clearly shows that the protein footprints to the X-box sequence. Finally, an oligonucleotide containing the I-E $\alpha$  X-box sequence is recognized by a nuclear factor in the B-cell lymphoma M12; however, when the X-box sequence within this oligonucleotide is changed, there is no binding of the oligonucleotide by A factor in the nuclear extract from M12 cells (8). The problem of these alternatives will be resolved only when the individual X-box binding proteins are further characterized. It will be interesting to compare the different X-box binding proteins and to determine what role they play in MHC class II gene expression. The results presented here strongly suggest that the MHC class II X-box sequences and the proteins that recognize these sequences may be more complex than originally thought.

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