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The Same CCAAT Box-Binding Factor Binds to the Promoter of Two Coordinately Regulated Major Histocompatibility Complex Class II Genes

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Using competition mobility shift, methylation interference, and proteolytic clipping DNA binding assays, we demonstrate that the protein binding the major histocompatibility complex Aβ CCAAT box is indistinguishable from the protein previously named NF-Y, which binds the major histocompatibility complex Eα CCAAT box. Although the two CCAAT boxes share the same 10-base core sequence, termed the Y box, their flanking sequences, known to be important for binding, are very different.

The GCCAAT recognition motif, commonly referred to as the CCAAT box, is found in the promoter region of most eucaryotic genes and is important for the correct initiation of transcription (2, 4, 14). The CCAAT box is the recognition site for a family of multisubunit DNA-binding proteins (5, 7, 8, 10, 13–16, 19, 20, 23, 24). While the CCAAT box-binding proteins share a common specificity for the CCAAT core sequence, their binding is also influenced by sequences flanking the CCAAT box (10, 15, 20).

The Y box, one of two highly conserved sequences in the promoter of all major histocompatibility complex class II genes, appears to play an important role in the regulation of coordinate and tissue-specific gene expression (3, 11). The Y box consists of a 10-bp consensus sequence, CTGATTG GCT, which contains a CCAAT box on the opposite strand (10). Although the Y box is highly conserved among class II genes, the flanking sequences are not, thereby allowing for significant variation in CCAAT box-binding factor affinity and specificity (10, 15). Protein-DNA interactions have been observed to occur at or near the Y box within the promoters of several class II genes (6, 8, 10, 17a, 17b, 22, 26, 28). A Y-box-binding protein termed NF-Y has been partially purified by affinity chromatography, using the Eα Y-box region (10, 16). In these studies, it was shown that the flanking regions as well as the core sequence of the Y box were important to the binding of this factor (10). In fact, mutation of flanking residues that differ between Aβ and Eα Y boxes was shown to significantly diminish NF-Y binding (10). More recently, partial purification of the Aβ Y-box-binding protein has been reported previously (6). Since the flanking regions of the Aβ Y box differ significantly from those of the Eα Y box at positions that would be expected to diminish NF-Y binding (Fig. 1), it was important to establish whether the CCAAT box-binding factors interacting with these two coordinately regulated genes are the same or different proteins. Furthermore, since the Aβ Y-box region contains an additional CCAAT sequence not found in the other class II genes (Fig. 1), it was important to determine whether this second CCAAT sequence had any effect on protein binding to the Aβ Y box.

To compare the nuclear factors binding to the Eα or Aβ Y-box region, we used two 36-bp oligonucleotides, which were centered around the Y box of each gene, in a variety of binding assays (Fig. 1). Extracts were prepared from cell lines which were class II expressing (A20/2J) (18), nonexpressing (Ltk$^-$) (30), or inducible with gamma interferon (WEHI-3) (29). All the cell lines were cultured as previously described (17), and cell extracts were prepared by the method of Dignam et al. (9). Although both cytoplasmic (S100) and nuclear fractions behaved identically in mobility shift assays (data not shown), greater binding activity was present in the S100 fraction; therefore, this fraction was utilized in all the experiments described.

Initial comparison of the migration of the protein-DNA complex formed using either the Aβ or Eα oligomers showed identical migration rates in all extracts (data not shown). In order to further characterize the specificity of binding, competition mobility shift assays were performed (27). The Aβ or Eα oligomers were individually end labeled by using [γ-^{32}P]ATP (ICN Pharmaceuticals, Irvine, Calif.) and T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, Md.) and then incubated with S100 cell extract in the presence of nonspecific competitor, poly(dI · dC), or with unlabeled Aβ, Eα, or AB-75 oligomers. AB-75 is a 45-bp oligomer, 5'GGCTACAGAACTTTGCTTTCTGAAGGG GGCACAGCAGGTGTGAGT, from a region of the Aβ promoter that is 75 bp upstream of the start site of translation and contains no Y-box consensus. This oligomer does not specifically bind any proteins from nuclear extracts in the mobility shift assay (data not shown). Both unlabeled Aβ and Eα oligomers competed equally for protein binding to the labeled Aβ oligomer (Fig. 2A) or the labeled Eα oligomer (Fig. 2B). In contrast, the AB-75 control oligomer did not compete for binding to either labeled oligomer (Fig. 2). Densitometric analysis confirmed that both Aβ and Eα oligomers competed equally and at the same molar concentrations (data not shown). Thus, the Eα and Aβ Y-box-binding proteins have identical specificities and affinities for the Eα and Aβ Y-box sequences, as determined by gel mobility shift competition experiments. Extracts from the cell lines Ltk$^-$, WEHI-3, and WEHI-3 stimulated with gamma interferon were also evaluated by competition mobility shift assays, and identical results were obtained (data not shown).

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      *****  Y BOX
A-beta AGCTTCAGCTCCAATGCTGATTGGTTCCTCACTTGG
E-alpha AGTCTGAAACATTTTTCTGATTGGTTAAAAGTTGAG
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FIG. 1. Sequence of the coding strand of the 36-bp Aβ and Eα oligomers corresponding to the $A\beta^d$ (21) and $E\alpha^k$ (10) regions containing the Y box. The Y-box sequence is underlined. The upstream CCAAT element on the coding strand of the Aβ oligomer is indicated by asterisks.

In order to compare the binding protein's contact points with the Aβ and Eα Y box, methylation interference assays were performed (1). Single-stranded oligomers were end labeled, annealed, methylated with dimethyl sulfate (Aldrich, Milwaukee, Wis.) and gel purified. Mobility shift assays were performed, and the DNA from the bound and free bands was electroeluted, phenol extracted, chloroform extracted, and ethanol precipitated. The DNA was then cleaved via piperidine treatment and separated on a 12% acrylamide 8 M urea sequencing gel, dried, and autoradiographed. Figure 3 shows the methylation patterns for both the coding and noncoding strands of the Aβ and Eα oligomers. Both the Eα and Aβ oligomers appear identical with respect to the methylation interference patterns. This includes an enhancement of binding in the same relative position outside of the Y box for both oligomers despite the differences in the flanking sequences. This demonstrates that the factor binding to the two oligomers contacts the DNA at the same points within the Y box and possibly its flanking regions.

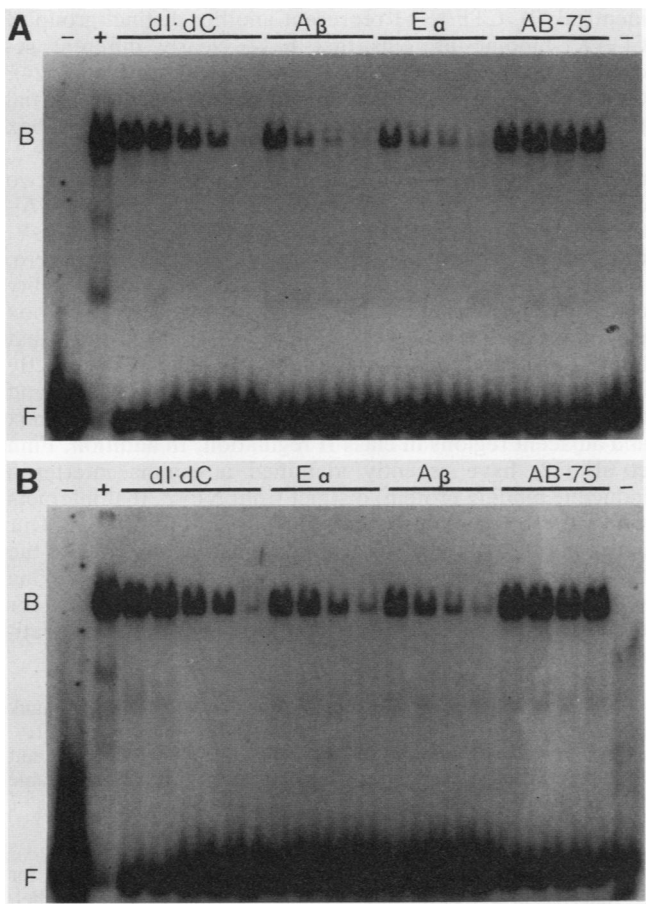

FIG. 2. Cross-competition assays for protein binding to the Aβ or Eα oligomers. (A) The labeled A-β oligomer was incubated with A20 S-100 extract, and protein binding was inhibited with poly(dI · dC) (50, 100, 500, 1,000, or 5,000 ng) or unlabeled Aβ, Eα, or AB-75 oligomers at 2, 5, 10 or 20× molar excess (from left to right). Only 0.065 ng of the labeled probe was used for each lane. Lane − received no extract, and lane + received extract without competitor DNA. For the Aβ, Eα, and AB-75 lanes, 100 ng of poly(dI · dC) was used in all lanes. (B) Exactly as described above for panel A, except the labeled Eα oligomer was used as a probe. B, Bound complex; F, free complex.

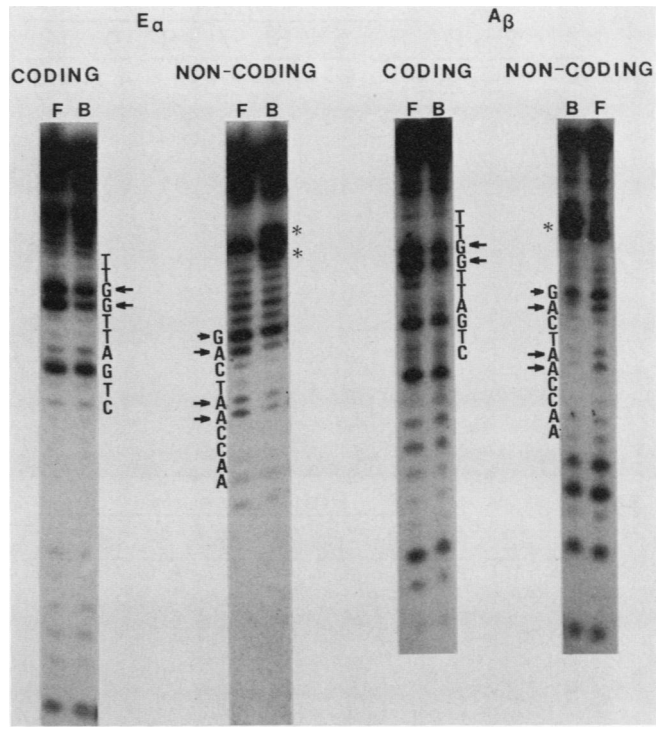

FIG. 3. Comparison of the protein contact points on the Eα and Aβ Y boxes. Methylation interference assays of the Eα oligomer or the Aβ oligomer were conducted. Binding reactions were performed with methylated oligomers that were 5′ end labeled with ^{32}P on the coding or noncoding strands. Preparative mobility shift assays separated the bound complex (B) from the free (F) oligomers, and these bands were excised from the gel, electroeluted, cleaved, and run on a 12% acrylamide sequencing gel. The sequence shown corresponds to the Y box. The arrows indicate bases on which methylation interfered with the formation of protein DNA complexes, and the asterisks indicate bases on which methylation enhanced protein binding.

In order to directly compare the protein structures of the two binding proteins, we utilized a proteolytic clipping mobility shift assay (25). Cell extracts were incubated with the labeled oligomers and then treated with trypsin, chymotrypsin, or proteinase K in increasing concentrations. Upon electrophoresis, this method generates a pattern of degradation products unique for a given DNA-binding protein (25). For all three enzymes, both binding proteins showed nearly identical cleavage products, and stepwise cleavage occurred at the same enzyme concentration (Fig. 4). With the highest enzyme concentrations, the Aβ complex migrated slightly faster than the Eα complex. However, this is the result of the influence of the oligomers and not the bound proteins. The unbound Aβ oligomer consistently migrated faster than the

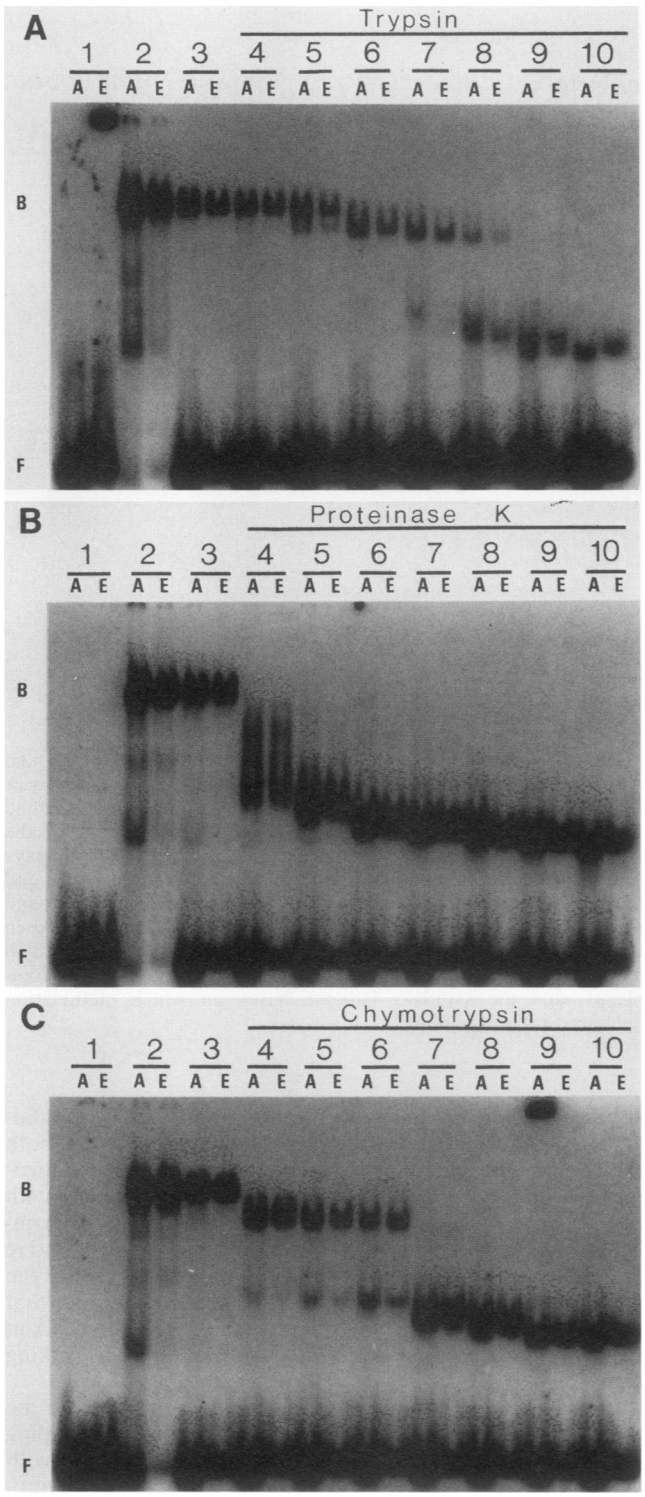

FIG. 4. Proteolytic clipping mobility shift assay of the labeled A-β (A) and E-α (E) oligomers. Binding reactions were performed with the indicated oligomers and then cleaved with increasing concentrations of trypsin (A), chymotrypsin (C) or proteinase K (B). Treatment groups: 1, probe only [without extract or poly(dI · dC) competitor DNA]; 2, probe plus A20/2J extract; 3, probe plus A20/2J extract and 500 ng of poly of poly(dI · dC); 4 to 10, the same as group 3, except 1, 3, 10, 30, 100, 300, or 1,000 ng of enzyme, respectively, were added after protein-DNA complex formation and 10 min prior to loading on the native polyacrylamide gel. B, Bound complex; F, free complex.

unbound Eα oligomer. These results indicate that the protein structure, as defined by the cleavage sites of three different enzymes, is nearly identical for both proteins.

By examining both DNA binding specificity and the structure of the bound protein, we were unable to detect any differences between the CCAAT box-binding factors binding to the Aβ or Eα Y boxes. While we have not provided rigorous biochemical proof of the identity of the two factors, other members of the CCAAT box-binding family that have been described differ sufficiently to be easily detected by our methods. Thus, CBP was identified on the basis of binding to the murine sarcoma virus long terminal repeat, which has the identical 10-base Y-box sequence as that found in the class II genes (14). However, NF-Y and CBP are clearly different factors based on migration in mobility shift assays and show a different pattern of methylation interference. Furthermore, their respective binding sites do not equally cross-compete for one another (10). CP-1 and CP-2 also recognize a number of CCAAT-containing sequences, including those with core sequences identical to the class II Y box, i.e., the H-2K^b promoter (7). CP-1 and CP-2 are clearly distinct proteins, but CP-1 has not yet been distinguished from NF-Y and may be identical (7). CTF/NF-1 represent another distinct group of CCAAT-binding proteins that have clearly different sequence specificities from those previously described (7, 24). Celada and Maki (5) have recently reported the partial purification of a factor based on binding to the Aβ Y-box region. Analogous to the results obtained for CP-1 and CP-2, their results demonstrate that the factor consists of two components, both of which are required for binding. Although this factor has not been positively identified as NF-Y, it seems likely based on the results we have presented here.

One of the central questions in major histocompatibility complex class II gene regulation is, what role does the Y box play in tissue-specific or inducible expression? The fact that NF-Y is a ubiquitous protein makes it difficult to directly implicate it in regulation. However, both transfection and transgenic experiments have clearly implicated the Y box and adjacent regions in class II regulation. In addition, Finn et al. (12) have recently identified a gamma interferon inducible binding protein, distinct from NF-Y, that interacts with the Y box and upstream sequences of the Eβ gene. Our data, that the same CCAAT box-binding factor binds to the CCAAT box of both Aβ and Eα, in spite of different flanking sequences, further supports a model that NF-Y is critical for the proper coordinate regulation of the major histocompatibility complex class II genes.

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