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Repression of Major Histocompatibility Complex I-Aβ Gene Expression by dbpA and dbpB (mYB-1) Proteins

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The induction of major histocompatibility complex class II gene expression is mediated by three DNA elements in the promoters of these genes (W, X, and Y boxes). The Y box contains an inverted CCAAT box sequence, and the binding activity to the CAAT box is mediated by factor NF-Y, which is composed of subunits NF-YA and NF-YB. We have found that transfection of either dbpA or dbpB (mYB-1) or both inhibits I-Aβ gene expression. Although the genes for some members of the Y-box family of binding proteins have been isolated by screening an expression library using the Y-box sequence, under our conditions no binding of dbpA or dbpB to the Y box of the I-Aβ or I-Eα promoter was detected. This suggested that repression of I-Aβ gene expression by dbpA and dbpB was not due to competition for binding to the Y-box sequence. The results suggest two other mechanisms by which dbpA and dbpB can inhibit transcription from the I-Aβ promoter. When dbpA was added, the binding of NF-YA to DNA increased, which could be explained by interaction between these two proteins whose purpose is to increase the binding affinity of NF-YA for DNA. However, this complex was unable to stimulate transcription from the I-Aβ promoter. Thus, dbpA competed for the interaction between NF-YA and NF-YB by binding to NF-YA. When dbpB factor was added together with NF-YA and NF-YB, the binding of the NF-YA–NF-YB complex was reduced. This suggested that dbpB may compete with NF-YB for interaction with NF-YA. These results provide an example of how dbpA and dbpB may regulate transcription of promoters that utilize NF-Y as a transcription factor.

Major histocompatibility complex (MHC) class II proteins have a key role in the immune response (44). They participate in the generation of the T-cell repertoire in the thymus and are required for antigen presentation to T lymphocytes. Class II proteins are normally expressed in a limited number of cell types, which include B, thymic epithelial, dendritic, and glial cells and, also, activated macrophages (21). The aberrant expression of class II proteins has been implicated in immune dysfunction. The absence of class II expression in humans (27) or in experimental models (13, 51) leads to severe combined immunodeficiency. The abnormal expression of class II molecules may be linked to the development of autoimmune diseases (5, 22). Upstream of all MHC class II genes there are at least three *cis*-acting elements that are essential for the transcriptional regulation of these genes (4, 24, 46). The elements have been referred to as W, X, and Y, and nuclear factors have been shown to bind each element. The W sequence is also known as H, S, or Z.

The Y element contains an inverted CCAAT motif, and the protein that binds to this site is factor NF-Y (16, 17). NF-Y is composed of two subunits of approximately 32 kDa (NF-YA) and 42 kDa (NF-YB) (8). The two subunits have been cloned by protein purification and micro sequencing by using the Eα promoter (30). The protein sequence reveals stretches with 70% sequence homology to regions of the *Saccharomyces cerevisiae* transcription factors HAP2 and HAP3, heterodimers that control cytochrome gene transcription in yeast cells. NF-YA and NF-YB are also the binding factors for the Y box of the I-Aβ gene (10).

The protein YB-1 was identified by expression cloning (λgt11), using an oligonucleotide covering the Y box of the DRA gene promoter (15). Its binding characteristics have not been defined as clearly as those of NF-Y, although analysis with a few mutant templates suggests it also recognizes the CCAAT motif. The protein and cDNA sequences of YB-1 and NF-YA or NF-YB are completely different. A family of Y-box factor proteins that have structural and functional homology to YB-1 have been identified (52). Y-box proteins from *Escherichia coli* and from vertebrates recognize specific DNA sequences that regulate the transcriptional activity of prokaryotic and eukaryotic promoters. Besides the CCAAT element, these proteins also bind to several unrelated sequences and to single-stranded DNA and mRNA (52).

Sakura and colleagues also used expression cloning to isolate two DNA-binding proteins: dbpA and dbpB (43). These were found to interact with the enhancer of the epidermal growth factor receptor gene, which contains an inverted CCA AT box (33). YB-1 and dbpB are identical. It has recently been reported that YB-1 represses the gamma interferon (IFN-γ)-induced expression of MHC class II genes (46).

In the present report, we examine the ability of dbp proteins to regulate the expression of the MHC class II gene I-Aβ. Our results indicate that dbp proteins are able to repress I-Aβ expression by two different mechanisms that block the formation of the NF-YA–NF-YB complex, which is necessary for the transcription of the I-Aβ gene. First, dbpA can replace NF-YB, leading to a nonfunctional NF-YA–dbpA complex, and second, dbpB (mYB-1) can interact with NF-YB through a protein-protein interaction that inhibits the formation of the NF-YA–NF-YB complex.

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MATERIALS AND METHODS

Cells. The cell line A20-2J (mouse B lymphocyte) was used. Mouse bone marrow-derived macrophages were produced in vitro, using L-cell-conditioned medium as previously described (6). After 6 days in culture, bone marrow-derived macrophages were washed and incubated without L-cell-conditioned medium for 4 days. For IFN-γ stimulation studies, 300 international reference units of murine IFN-γ (a gift from Genentech, Inc., South San Francisco, Calif.) per ml was added to the medium for 24 h. Under these conditions, IFN-γ induces the expression of mRNA for I-Aβ and I-A surface expression (7).

Nuclear extract preparation. Nuclear extracts were prepared as described previously (11). In brief, cells were lysed in a homogenizer (Dounce). The crude nuclei were extracted at 4°C with a buffer containing 0.4 M NaCl for 30 min with continuous stirring; this step was followed by centrifugation at 105 × *g* for 1 h. The supernatant was dialyzed, and extracts were cleared by centrifugation at 104 × *g* for 10 min. The supernatant (protein concentration of 1 to 5 mg/ml) was frozen in aliquots and stored at −70°C.

Transfection assays. A20-2J (mouse B-lymphocyte) cells were transfected in suspension in 1 ml, using the DEAE-dextran method as previously described (9). Fifteen micrograms of the experimental DNA, 3 μg of the plasmid pCH110, a β-galactosidase expression vector used to measure transfection efficiency, and 30 μg of DEAE-dextran were added to each plate. In the cotransfection experiments with dbpA and dbpB or retinoic acid receptor, 2 μg of the indicated plasmid was added. Chloramphenicol acetyltransferase (CAT) assays were performed, using a standard protocol (25). Briefly, cells were isolated 48 h after the addition of DNA and subjected to three freeze-thaw cycles in dry ice-ethanol and a water bath at 37°C. The extracts (10 to 60 μl; standardized by β-galactosidase activity) were incubated with [^{14}C]chloramphenicol and acetyl coenzyme A for 60 min at 37°C and then for 7 min at 60°C; this was followed by extraction with ethyl acetate. The samples were dried and resuspended in 20 μl of ethyl acetate for thin-layer chromatography. Acetylation was quantified with a radioanalytic imaging system (AMBIS, Inc., San Diego, Calif.). Each experiment was performed at least three times, and the mean of the experiments is shown in each figure.

Plasmid constructions. The CAT reporter constructions were made using the KS+−SV2CAT vector (48) from which the simian virus 40 (SV40) enhancer region but not the SV40 promoter (*Sph*I to *Pst*I) had been removed. The constructions KS1-WXY (−124 to −26), KS1-Y mutant, KS1-X mutant, and KS1-W mutant (mutations in the Y, X, and W boxes as indicated in Fig. 1) containing all or part of the I-Aβ promoter, were generated using specific oligonucleotides or by PCR using the plasmid 12.33.2, which contains the I-Aβ promoter (11). The DNA fragments were ligated directionally into *Xba*I-*Sma*I-digested KS1 vector. DNA fragments used for the gel electrophoresis DNA binding assay were generated by cloning. Double-stranded oligonucleotides were synthesized on a DNA synthesizer (model 380 A; Applied Biosystems Inc., Foster City, Calif.) and cloned into the *Bam*HI site of the vector pGEM1. The sequence of the 33-bp oligonucleotide covering the Y box of the Eα gene was the following: 5′-GATC AACATTTTCTGATTGGTTAAAAGTTGAG-3′. The plasmid containing the gene for the retinoic acid receptor (pECE RARβ) was obtained from M. Pfahl (La Jolla Cancer Research Foundation) (26), and the plasmids containing the dbpA and dbpB genes (pECE dbpA and pECEdbpB) were kindly provided by M. Fukuda (La Jolla Cancer Research Foundation). The plasmids containing NF-YA and NF-YB were obtained by PCR, using oligonucleotides prepared from the sequence of the plasmid vectors containing NF-YA and NF-YB, as described previously (10). Finally, PU.1 and the PU.1 binding site with the CAT construction were described previously (32). The pECE vector contains the SV40 promoter and enhancer (17). Transcription and translation of NF-YA, NF-YB, dbpA, and dbpB were performed in vitro as described by the manufacturer (Promega Biotec). RNA was transcribed with T3 RNA polymerase. The RNA template (2 μg) was added to a rabbit reticulocyte lysate (Promega Biotec) to generate [^{35}S]methionine-labelled protein in vitro. Protein production was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Gel electrophoresis DNA binding assays were performed directly with the product translated in vitro. The relative amount of protein for NF-Y or dbp was measured, taking into account the amount of radiolabelled methionine incorporated during the translation in vitro, quantified by using an imaging system (AMBIS, Inc., San Diego, Calif.), and then diluted to equal concentrations. In some cases the gels were dried and placed in contact with two sheets of Kodak XAR film to distinguish the ^{35}S label from the ^{32}P label.

Gel electrophoresis DNA binding assay. The DNA fragment containing the Y-box sequence (320 bp) was used to characterize the Y-box DNA-binding protein (11). In some cases, a plasmid containing a 33-bp synthesized oligonucleotide (5′-gaTCCAATGCTGATTGGTTCCTCACTTGGGACg-3′) covering the Y box was digested with *Hin*dIII, labelled by filling in the ends with α-^{32}P-labelled deoxynucleoside triphosphates and Klenow polymerase, and digested with *Eco*RI. A 70-bp fragment containing the desired protein binding site was then isolated from a 6% polyacrylamide gel (9). For the gel electrophoresis DNA binding assays, DNA (10,000 cpm of ^{32}P labelled; approximately 0.1 ng) and nuclear extract or proteins, prepared by transcription-translation using a rabbit reticulocyte lysate system in vitro (Promega, Madison, Wis.), were mixed in a total volume of 20 μl in a buffer containing 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9), 60 mM KCl, 0.12 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 0.3 mM dithiothreitol, and 12% glycerol. Two micrograms of poly(dI-dC) (Pharmacia) was also added to each reaction mix. Samples were incubated at 20°C for 30 min and then applied to a 6% polyacrylamide gel (acrylamide-bisacrylamide, 30:1)–0.25× TBE (1× TBE is 89 mM Tris, 89 mM boric acid, and 2 mM EDTA) containing 5% glycerol, and electrophoresis was carried out at 4°C and 250 V for 3 h. After electrophoresis, the gels were dried and placed in contact with X-ray film (XAR-5 from Kodak). Radioactivity was quantified with a radioanalytic imaging system (AMBIS, Inc.).

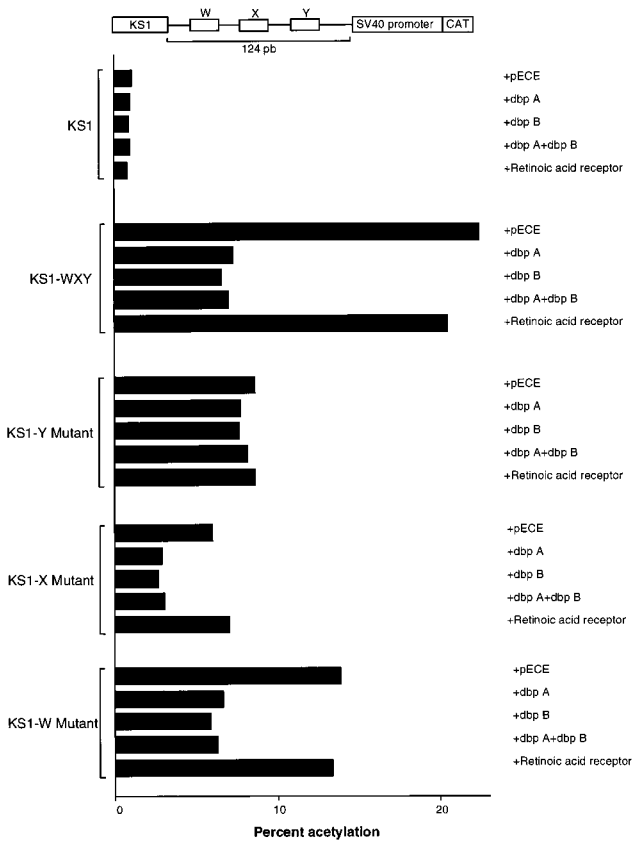


FIG. 1. Repression of I-Aβ promoter expression by dbp proteins. A reporter plasmid (15 μg) (top) containing a fragment of the I-Aβ promoter (WXY −124 to −26) or the same fragment with a mutation in the Y box (CTGATTGG to CTGATTTT), X box (CCCAGAGACAGATG to AGTCGTCTGACTC), or W box (GAGCCTT to TCTAACG) was transfected into A20-2J cells, together with the β-galactosidase expression plasmid and 2 μg of the pECE vector, the dbpA, the dbpB, the dbpA-dbpB, or the retinoic acid receptor expression vectors as indicated. The amount of cell extract used for CAT assays was normalized according to the level of β-galactosidase expression. The CAT enzymatic activity was quantitated with an imaging system (AMBIS, Inc.). The results are the mean averages of four experiments; the standard deviation in all cases is less than 15% of the corresponding value. The KS1 vector contains the SV40 promoter but not the I-Aβ promoter.

RESULTS

To examine the possible role of dbp proteins in I-A expression, we determined the transcription steady state from the I-Aβ promoter. Using the I-Aβ promoter linked to the CAT gene, we observed low CAT activity when this construction was transfected into the B-cell line A20-2J. Because we were interested in the cell type-specific enhancing activity of the I-Aβ promoter, we linked a 124-bp fragment of the I-Aβ promoter containing the W, X, and Y boxes to the SV40 promoter (Fig. 1), which then gave us a better signal in the CAT assay. This type of construction has been used by others to obtain more efficient expression of other MHC class II genes, including the I-Eα and I-Aα genes (19, 20, 50). Each CAT construction was

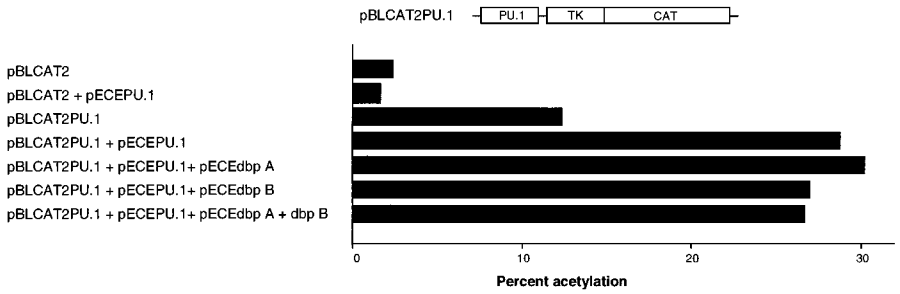


FIG. 2. Transactivation by the PU.1 DNA-binding protein is not repressed by dbpA and dbpB proteins. A reporter plasmid (15 μg) (top) containing the PU.1 binding site linked to the thymidine kinase (TK) promoter was transfected into A20-2J cells, with the β-galactosidase expression plasmid and 2 μg of the pECE vector or the PU.1, dbpA, or dbpB expression vector as indicated. The results are the means of three experiments; the standard deviation is less than 12% of the corresponding value.

cotransfected together with the β-galactosidase expression plasmid pCH110 into the B-cell line A20-2J. All CAT values were than normalized to the level of β-galactosidase expression to correct for any differences in transfection efficiency. The KS1 vector exhibited a level of CAT activity of <1%, while KS1 containing the W, X, and Y boxes exhibited a CAT activity level of 22.5% (Fig. 1). When a mutation of the Y, X, or W box was generated in the context of all three boxes, CAT activity levels fell to 8.6, 6.3, and 14.0%, respectively, suggesting that these elements play an important role in the expression of the I-Aβ gene. The Y-box mutation was designed to disrupt the binding of NF-Y to the Y box, as measured by a gel electrophoresis DNA binding assay (11).

To determine the effect of dbp proteins on I-Aβ expression, we cotransfected expression plasmids of either dbpA or dbpB or both into A20-2J cells together with the CAT constructions. In all cases, there was a decrease in the level of CAT activity, from 22.5% to 7.3% with dbpA, to 6.5% with dbpB, and to 7% with both dbpA and dbpB. Cotransfection of a construction able to express the retinoic acid receptor together with any of the I-Aβ constructions had no effect on CAT activity. When the Y box was mutated, in cells cotransfected with dbpA, dbpB, or both, the level of CAT expression activity was not lowered further: 8.6% versus 7.7% (dbpA), 7.5% (dbpB), and 7.5% (dbpA plus dbpB). However, when the X and Y boxes were mutated, in cells cotransfected with dbpA, dbpB, or both, the level of CAT expression activity was lower. Thus, dbpA and dbpB were able to repress CAT activity only in those constructions containing the Y-box sequence.

We attempted to determine whether the suppressive effect of dbpA and dbpB was specific for the I-Aβ promoter and not due to a general down regulation of transcription. To do so, the effect of plasmids containing dbpA and dbpB was tested on a vector containing the PU.1 binding site. PU.1 is a B-lymphocyte and macrophage tissue-specific transcription factor involved in the regulation of different genes (32). When the vector pBLCAT2, which contains the CAT gene linked to the thymidine kinase promoter, was transfected into B lymphocytes, CAT activity was at background levels (2.3%) (Fig. 2). Cotransfection of an expression construct containing the PU.1 gene (pECE PU.1) did not alter the basic levels of CAT expression. When a PU.1 binding site was inserted upstream of the thymidine kinase promoter, the level of CAT activity rose (12.3%). When, however, the CAT vector containing the PU.1 binding site was cotranfected with the PU.1 expression plasmid, the level of CAT activity increased to about 30%. Cotransfections of either dbpA or dbpB or both did not alter the levels of CAT expression in this assay (Fig. 2).

We also tested the effect of dbp proteins on I-A expression induced by IFN-γ. Bone marrow-derived macrophages were transfected with the KS1-WXY CAT vector and incubated with 300 international reference units of murine IFN-γ per ml for 24 h. Under these conditions, IFN-γ induces the expression of mRNA for I-Aβ and I-A surface expression (7). The KS1 containing the W, X, and Y boxes exhibited a CAT activity level of 2.3%, and it exhibited a CAT activity level of 22.5% in the presence of IFN-γ (Fig. 3). The transfection of dbpA, dbpB, or both had an inhibitory effect on IFN-γ induction of CAT activity. The repressive activity of dbp proteins on the CAT expression of the KS1-WXY construction is probably mediated through the Y box. Mutation of the Y box reduces the IFN-γ-induced CAT activity level from 22.5 to 7.1%. The cotransfection of dbpA or dbpB or both did not reduce the CAT activity when the KS1-Y mutant was used (6.8, 7.0, and 6.3%, respectively). Thus, the dbp proteins were able to repress CAT activity only in constructions containing the Y-box sequence.

It has been reported that some proteins of the family of Y-box factors are able to bind the Y box of the Eα gene promoter (18, 23). To investigate whether dbp proteins also bind the Y box of the I-Aβ promoter, which could account for the transcriptional repression of the I-Aβ gene, a gel electrophoresis DNA binding assay was performed. When nuclear extracts prepared from the B-cell line A20-2J and a probe covering the Y box were used, a retarded band was observed. Cold probe competed for the binding of nuclear factor and eliminated the appearance of the retarded band in the gel electrophoresis DNA binding assay. A double-stranded DNA fragment with a mutation of two G's within the Y box (CTG ATTGG) did not compete for binding, showing that the binding to the Y box is specific. We had previously shown that the nuclear factor that bound to the I-Aβ Y box was composed of

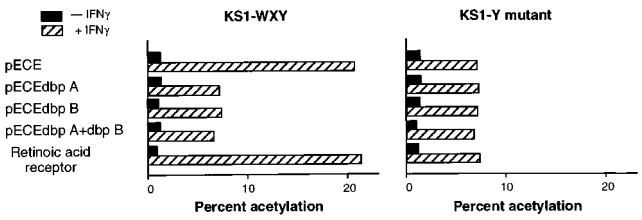


FIG. 3. The dbp proteins repress the IFN-γ induction of I-Aβ expression on macrophages. Bone marrow-derived macrophages were transfected with 15 μg of the KS1-CAT plasmids described in the legend to Fig. 1, together with 2 μg of the pECE vector or the pECE vector containing the sequence for dbpA, dbpB, or the retinoic acid receptor. The results are the means of four experiments; the standard deviation is less than 16% of the corresponding value.

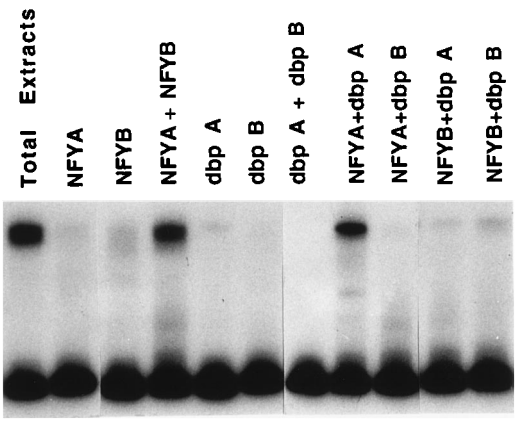
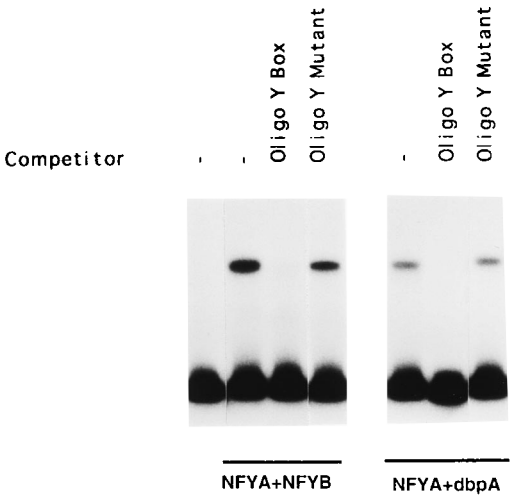


FIG. 4. Gel electrophoresis DNA binding assay of nuclear extracts of in vitro transcribed-translated NF-YA, NF-YB, dbpA, or dbpB proteins. Nuclear extracts were prepared from A20-2J cells. The probe was a 320-bp fragment of the I-Aβ promoter that contained the Y box (11). Retarded complexes were detected by autoradiography. The relative concentration of protein for NF-Y or dbp was measured, taking into account the amount of radiolabelled methionine incorporated during the in vitro translation, quantitated with an imaging system (AMBIS, Inc.), and then diluted to equal concentrations. For each condition, 1 μl of recombinant proteins was added alone or in combination.



Oligo Y Box 5'-gaTCCAATGCTGATTGGTTCCTCACTTGGGACg-3'
Oligo Y Mutant 5'----------TT----------3'

FIG. 5. The NF-YA–NF-YB and NF-YA–dbpA complexes bound to the Y box. A gel electrophoresis DNA binding assay was performed with in vitro transcribed-translated NF-YA, NF-YB, and dbpA proteins and a probe, a 320-bp fragment of the I-Aβ promoter that contained the Y box. We used the indicated Y-box and Y-mutant 33-bp oligonucleotides covering the Y box (CTGATTGG) as competitors (100-fold excess). The mutation of the two G's by two T's is sufficient to inhibit the protein binding to the Y box (11).

two components called factors A and B, which could be separated by fast protein liquid chromatography using a monoQ column (8). These factors correspond to the proteins NF-YA and NF-YB. The genes for NF-YA and NF-YB were expressed in vitro with T7 polymerase to generate RNA and rabbit reticulocyte lysate for the preparation of protein. Neither protein bound well to DNA when added individually to the labelled Y-box-containing DNA fragment (Fig. 4). When NF-YA was mixed with NF-YB, however, the complex bound with high affinity to the Y-box-containing DNA. The relative amount of proteins produced by transcription-translation in vitro was calculated, taking into account the amount of radiolabelled methionine incorporated during the translation in vitro. No retarded band was found when we added dbpA or dbpB, either alone or together, to the assay, even when 10 times more protein was used than in assays on the binding of NF-YA and NF-YB. Because some of the previous work on YB-1 binding proteins was done with the Y box of the Eα promoter (18, 23), we also used a 33-bp oligonucleotide covering this area. A specific retarded band was obtained when NF-YA and NF-YB were used as described previously (10). However, no retarded band was obtained when dbpA or dbpB was used, either alone or together.

We tested the abilities of dbpA and dbpB (mYB-1) to complement NF-YA or NF-YB. When NF-YA was mixed with dbpB or NF-YB was mixed with dbpA and the proteins were added to the probe, no retarded complex was observed (Fig. 4). The retarded complex DNA–NF-YA–NF-YB was almost at the same position as the retarded complex with dbpA. This is probably due to the large fragment (320 bp) used as a probe. In contrast, when NF-YA was mixed with dbpA and added to the probe, a retarded complex in the gel electrophoresis DNA binding assay was obtained (Fig. 4). Competition experiments showed that the retarded bands are specific to the Y box. The bands were eliminated when cold oligonucleotides with the sequence of the Y box were added but not when oligonucleotides with the mutated Y box were added to the assay (Fig. 5). No retarded complex was observed when NF-YB was mixed with dbpB. These results demonstrated that the complex consisting of NF-YA and NF-YB bound to the Y box of the I-Aβ promoter and that a similar complex could also be formed using NF-YA and dbpA.

In order to characterize the interaction between the NF-YA and dbpA proteins and the DNA Y box, we used a 33-bp synthetic oligonucleotide covering this area. When we incubated large amounts of transcribed-translated NF-YA in vitro together with the Y-box oligonucleotide, a retarded band could be seen (Fig. 6). As previously described, NF-YA alone is able to bind DNA. Furthermore, the dimethyl sulfoxide protection footprint indicated that NF-YA binds the two G's in the Y box (8). When we included the dbp A protein in the gel retardation experiments with the Y-box oligonucleotide, a supershift was observed (Fig. 6). The amount of the supershift is proportional

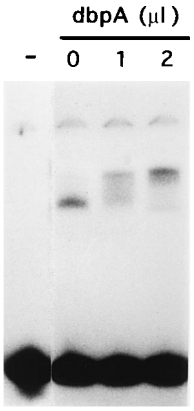


FIG. 6. Demonstration of the interaction between NF-YA and dbpA proteins. A gel electrophoresis DNA binding assay was performed with in vitro transcribed-translated NF-YA and dbpA proteins. The probe was a 70-bp fragment from a vector in which a 33-bp synthetic oligonucleotide containing the Y-box sequence was cloned. In the last three lanes, NF-YA was at a concentration of 4 μl and the concentrations of dbpA were as indicated. −, probe alone.

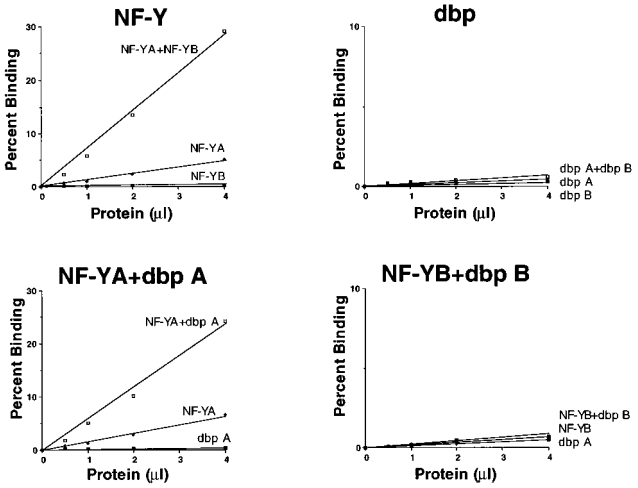


FIG. 7. Titration of recombinant proteins for binding to DNA. A gel electrophoresis DNA binding assay was performed with in vitro transcribed-translated NF-YA, NF-YB, dbpA, and dbpB proteins. There is a binding complementation between NF-YA and NF-YB and also between NF-YA and dbpA, but not for any other combinations. Retarded complexes were quantitated, using an AMBIS radiographic imaging system. NF-YB was used at a constant concentration of 4 μl, while for NF-YA the amounts used in the assay were as indicated (top left panel). dbpA was used at a concentration of 4 μl, while the amounts of NF-YA used in the assay were as indicated (bottom left panel). Percent binding refers to the total amount of DNA added in the assay mixture. The experiment was performed four times; results of a representative experiment are shown.

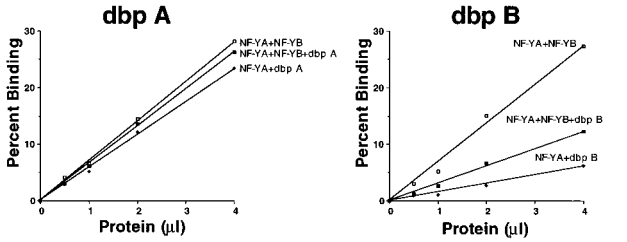


FIG. 8. Competition between NF-YB and dbpA and between NF-YA and dbpB. A gel retardation assay was performed, using a 320-bp fragment of the I-Aβ promoter that contained the Y box and translated-transcribed protein in vitro. NF-YB and dbpA were used at a constant concentration of 4 μl, while for NF-YA, the amounts used were as indicated (left panel). NF-YB and dbpB were used at a constant concentration of 4 μl, while for NF-YA the amounts used were as indicated (right panel). The experiment was performed three times; results of a representative experiment are shown.

to the amount of dbpA protein included in the assay. These results demonstrated that NF-YA interacts with dbpA, increasing the binding capacity of NF-YA.

To further characterize the interaction between the NF-Y and dbp proteins, we quantified the binding activities of the factors, both separately and together (Fig. 7). For these binding experiments we used a large DNA fragment of 320 bp containing the Y box, because quantitation of the amount of DNA bound to proteins provides more reproducible results than are obtained using the 33-bp oligonucleotide. NF-YA alone bound only small amounts of DNA, while NF-YB, dbp A, or dbp B alone bound little or no DNA. When increasing amounts of NF-YA were added to a constant amount of NF-YB (4 μl), a linear relationship between the amounts of NF-YA and protein-DNA complex was observed. Under these conditions, NF-YB was apparently in excess, since even when large amounts of NF-YA were added to the reaction mixture there was no evidence that the percentage of bound probe reached a plateau (Fig. 7, NF-Y). These results indicate that NF-YA alone binds to DNA but that the affinity between DNA and NF-YA is much greater when NF-YA is associated with NF-YB. This result is in good agreement with a previously published work (8). We observed no significant binding when either dbpA or dbpB or both together were added to DNA (Fig. 7, dbp). In the presence of a constant amount of dbpA there was a linear increase proportional to the amount of NF-YA present in the reaction mixture (Fig. 7, NF-YA + dbpA). No significant binding was found when dbpA was added to increasing amounts of NF-YB. These data suggest that NF-YA has some affinity for the Y box of the I-Aβ gene, and this affinity increases with the addition of dbpA.

We also compared the binding of the complex NF-YA–NF-YB with that of NF-YA–dbpA (Fig. 8). The binding results were quite similar for each complex. When the same comparison was made using NF-YA–NF-YB and NF-YA–dbp B, a large difference in the percentage of complex bound was observed. These data suggest that dbpA can effectively substitute for NF-YB in its interaction with NF-YA and binding to DNA. However, as discussed above, the NF-YA–dbpA complex is not transcriptionally active. We also observed that dbpB cannot substitute for NF-YB in its ability to interact with NF-YA.

DISCUSSION

Several CCAAT binding proteins have been reported to coexist in eukaryotic cells. This is the case for C/EBP, CTF/NFI, NF-Y, CP2, and YB-1. In vitro, the different factors appear to discriminate among different CCAAT-box sequences. Band shift assays and competition studies have shown that among different CCAAT binding proteins, NF-Y displays the highest affinity for the Y box of the MHC class II gene promoter (11, 16). Using different approaches, it has been found that NF-Y is the factor required for the efficient transcription of MHC class II genes. For example, purified NF-Y factor has been shown to be necessary to obtain class II gene transcription in vitro (56). Second, monoclonal antibodies against NF-Y proteins inhibit transcription of the Eα class II gene in vitro (35). Finally, antisense experiments with the NF-Y genes showed that I-Aβ gene expression can be inhibited in vivo (10). All these data strongly suggest that NF-Y proteins play a key role in regulating the expression of MHC class II genes.

The NF-YA and NF-YB proteins are related to the yeast proteins HAP2 and HAP3 (30). These proteins have several features in common. First, there is a large degree of amino acid identity in the DNA binding domains (73% between HAP2 and NF-YA and 66% between HAP3 and NF-YB). Second, in the amino-terminal region of NF-YA there is a glutamine-rich region that is also present in HAP2; these regions could be related to the fusion of DNA, allowing the correct initiation of mRNA (31). Third, both NF-YA and HAP2 have an acidic region near their carboxyl termini. Fourth, the NF-YB and HAP3 proteins have acidic stretches located on both sides of the DNA binding domain.

Recently, it has been reported that YB-1, a protein identified using radiolabelled Y box containing a DNA fragment screen λgt11 expression cDNA library, represses the IFN-γ activation of MHC class II genes (47). This result could partly explain the reverse relationship between the levels of YB-1 and MHC class II gene induction by IFN-γ. One possible mechanism for this repression is based on the affinity of proteins for the same or different DNA sequences (36). In this regard, it has been observed that the NF-Y factor and the F2 factor bind in a mutually exclusive manner to a critical promoter region of

the gene for the IE110k protein of herpes simplex virus (37). Moreover, NF-Y factor can act as a negative regulator, competing with factor 3 for binding to the promoter of the apoA-I gene (39). A *cis*-acting regulatory element that silences expression of the class II I-Aβ gene in a tissue-specific manner, acting on parenchymal cells, has recently been described (1).

The binding specificity of YB-1 was determined using mutations of the Y-box sequence, which abrogate the filter hybridization of a fusion protein that expresses YB-1. However, the binding specificity of YB-1 has not been demonstrated by direct binding assays such as the gel shift or the footprinting methods. The YB-1 protein belongs to a family of Y-box factors, which have structural and functional homologies (52). Among the Y-box factors, there is an 80-amino-acid sequence in the amino-terminal half that is highly conserved. This region corresponds to the DNA binding domain and is known as the cold shock domain because of the high degree of identity with the cold shock proteins of *E. coli* (43). Next to the cold shock domain there is a hydrophilic domain that extends up to the carboxyl termini in which there is an alternation of groups of basic and acidic amino acids that contribute to the protein-protein interactions (43).

The Y-box factors have been identified in rats, mice, cows, frogs, humans, and *E. coli*, and they act as positive or negative regulatory factors. In addition to the inverted CCAAT element (Y box), these proteins are able to bind to several unrelated sequences and also to single-stranded DNA and mRNA (52). Recently, cDNAs encoding Y-box proteins have been isolated after expression screening using a variety of DNA sequences that have little similarity to the Y box, including a basic protein, CT-rich DNA, interferon response elements, and class III gene promoter elements (12, 28, 33, 38, 54). The binding of Y-box proteins to this variety of sequences is puzzling, since previous experiments have indicated a specific requirement for the Y box (15, 23, 29, 45).

In our experiments, we show that both dbpA and dbpB proteins are able to inhibit the expression of the class II I-Aβ gene. However, no binding of these proteins to the Y box of the I-Aβ or the I-Eα promoters was found. This last I-Eα Y box was used in previous studies as a probe for different members of the Y-box protein family (18, 23). In addition, we were unable to shown a binding competition for the Y box between NF-Y and the dbp proteins. Moreover, no significant binding to DNA was found with dbp proteins, either alone or in combination.

Surprisingly, the modest binding of the NF-YA protein to DNA increases drastically in the presence of the dbpA protein, suggesting the formation of a heterocomplex between NF-YA and dbpA that increases the binding affinity of the complex for DNA. In fact, NF-Y and the dbp proteins have been separated, on the basis of their charge, into two components, one anionic (NF-YA and dbpB) and the other cationic (NF-YB and dbpA), using an ionic chromatography system (8, 18). This interaction between these components can be carried out between the carboxy termini of the dbpA protein, a region with acidic and basic modules, and the acidic region of NF-YA. Although dbpA and dbpB have a similar region with acidic and basic modules, the acidic stretches of dbpA induce the formation of a predicted helix (50), while the acidic stretches of dbpB induce the formation of a secondary structure that is rich in turns due to the amino acid sequences and also to the presence of glutamine between each of the basic and acidic modules. The different structures of these areas could explain the interaction between the dbpA and NF-YA and the dbpB and NF-YB proteins.

The formation of the complex NF-YA–dbpA may explain the repression of I-Aβ gene expression when cells were transfected with a gene coding for the dbpA protein. It is well documented that synergism between different transcriptional activators has a major role in transcriptional activation (29). Regulation of MHC class II gene expression requires three separate elements, the boxes W, X, and Y, with stereospecific and distance constraints (49), and also cooperative binding between the different factors that bind to these boxes (40, 41, 53). In this context, the heterodimer between the NF-YA and dbpA proteins could have an inhibitory effect on transcription by disruption of the interactions between the proteins that bind to the W, X, and Y boxes of the MHC class II genes. It is also possible that active repressors could promote local chromatin changes, resulting in repression of transcription. There are several examples showing that protein heterodimers that bind to DNA can function as negative inhibitors of gene transcription. In this regard, the proteins Myc and Mad heterodimerize with Max, and the complex recognizes the same DNA binding site. However, while Myc activates, Mad represses transcription (2). The homeobox-containing genes are positively and negatively regulated through DNA-protein and protein-protein interactions (55).

Some transcriptional activators are down regulated by inhibitory proteins with which they form protein complexes with altered or reduced DNA-binding activity. In our experiments, we found that the presence of dbpB protein reduces the binding to DNA of the NF-YA and NF-YB proteins. One possible explanation is that dbpB in excess is able to interact with NF-YB and, therefore, the complex NF-YA–NF-YB cannot be made. This mechanism may explain the inhibitory effect on I-Aβ expression when the dbpB gene is transfected into cells. There are several examples of a similar repressive mechanism. Among the basic ZIP family of transcription factors, inhibition of transcriptional activity can occur because of dimerization of an activator with repressor subunits that lack a functional basic domain required for DNA binding. Second, c-Jun (a transcriptional activator) is down regulated by JunB (another member of the Jun family). Although the reason for this down regulation was unknown for some time, it now appears to be due to poor DNA-binding activity of c-Jun–JunB heterodimers (14). The dimerization region of the transcriptional activator, CCAAT enhancer binding protein (C/EBP), is highly related to that of the C/EBP-homologous protein (CHOP), allowing heterodimerization of the two proteins. However, CHOP contains two proline substitutions in the region that forms the basic DNA binding domain of the basic ZIP factors. The resulting disruption of the DNA binding domain of CHOP prevents the CHOP-C/EBP heterodimer from binding DNA (42). Third, helix-loop-helix transcription factors are negatively regulated by the Id protein (3). In this case, repression results from recruitment of the helix-loop-helix proteins into Id-containing heterodimers that do not bind DNA. Finally, another example is the inhibition of the protein binding to the X box of the I-Aβ gene by the glucocorticoid receptor protein (9).

The results described here provide a good example of how transcription factors can interact. It is noteworthy that these interactions provide an important opportunity for cross talk between different signal transduction pathways and allow for alteration in gene expression.

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