Identification of the transcription factors NF-YA and NF-YB as factors A and B that bound to the promoter of the major histocompatibility complex class II gene I-Aβ

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The Y box is a conserved sequence in the promoter of major histocompatibility complex (MHC) class II genes, which contains a CCAAT sequence (CCAAT box). Previously, we partially purified the DNA-binding protein that recognizes the Y box of the I-Aβ gene and showed that it consisted of two components (factors A and B) both of which were necessary for optimal DNA binding. The genes for the heteromeric protein NF-Y (NF-YA and NF-YB), which binds to the I-Eα Y box have been cloned. We subsequently isolated the genes for NF-YA and NF-YB using oligonucleotides designed from the published sequences. NF-YA and NF-YB were tested for binding to the I-Aβ and I-Eα Y boxes. While neither NF-YA or NF-YB alone bound to the Y box, when the components were mixed the complex bound to the I-Aβ Y box with high affinity. Moreover, NF-YA and NF-YB could be complemented for binding to DNA by factor B or factor A, respectively. These results suggest that the active binding protein is NF-YA in factor A extracts and NF-YB in factor B extracts. Finally, antibodies against NF-YA and NF-YB were shown to induce a supershift when nuclear extracts were added to the double-stranded oligodeoxynucleotide covering the Y box of the I-Aβ gene. Antisense expression constructs of both NF-YA and NF-YB were made and their effect on expression from the I-Aβ promoter was tested. Either antisense construction, when transfected into cells, lowered the expression of a reporter gene linked to the I-Aβ promoter. This study provides direct evidence of the identification of NF-YA and NF-YB as the previously described factors A and B. Moreover, these results strongly implicate NF-Y in the expression of the MHC class II gene I-Aβ.

INTRODUCTION

The major histocompatibility complex (MHC) class II genes are expressed in a cell-type-specific manner and in some cells their expression is modulated by cytokines (reviewed in [1–3]). This complex pattern of transcription depends on the presence of at least three *cis*-acting elements located immediately upstream of each class II gene: the W, X and Y boxes. The expression of MHC class II genes has been regarded as co-ordinately regulated with respect to their tissue distribution and inducibility [4,5]. However, there is also evidence for gene-specific regulatory mechanisms among the class II gene family. The rates of transcription of the four murine class II genes are not equal [6] and in some class II-deficient patients, some isotypes are expressed but not others [7–9]. This suggests that common and specific isotype *trans*-acting factors regulate the expression of MHC class II genes.

The Y box was initially defined as a conserved sequence element that contains the sequence CCAAT (CCAAT box) [1–3]. Disruption of this sequence by deletion or mutation results in a reduced level of expression from constructs when transfected into tissue-culture cells [10–19] or tested in transgenic mice [20]. This conclusion has recently been confirmed by transcription assays *in vitro* [21,22]. Evidence that the Y box is a binding site for a nuclear factor has been obtained both *in vitro* [23–31] and *in vivo* [32]. We had previously shown that a nuclear factor bound to the I-Aβ Y box [19] and that this factor was made up of two components, both of which were required for binding to the DNA [28]. The I-Aβ Y box factor was also similar to NF-Y, a nuclear factor that recognized the Y box of the mouse MHC class II I-Eα gene [24]. Recently, NF-Y has been shown to consist of two components and the genes for these components have been isolated [33]. We attempted to determine whether the components of the factor NF-Y (NF-YA and NF-YB) were the same as the constituent elements of a complex that bound to the I-Aβ Y box.

To address these issues we isolated the genes for the two components of NF-Y (NF-YA and NF-YB). The products of these genes were found to bind to the I-Aβ Y box in a manner similar to the partially purified factors A and B [28]. Specific antibodies against the NF-Y components were shown to recognize the proteins that bind to the Y box of the I-Aβ gene. A significant drop in expression from the I-Aβ promoter was observed when antisense constructs for either NF-YA or NF-YB were tested in a co-transfection assay using a reporter gene linked to the I-Aβ promoter. Thus, by these criteria NF-Y is related to the partially purified factors A and B and is an important factor for the expression of I-Aβ.

MATERIALS AND METHODS

Cells and nuclear extract preparation

In these studies we used the mouse B-lymphocyte cell line A20-2J, which was maintained in Dulbecco's modified Eagle's medium containing 5% (v/v) fetal-calf serum. A20-2J cells express cell-surface I-Aβ as previously shown [34].

Nuclear extracts were prepared as described previously [25]. After washing in phosphate buffer, pelleted cells were frozen in solid CO_2 and ethanol and stored at -70 °C until use. Pellets containing about 10^9 cells were thawed and resuspended at 4 °C

Abbreviations used: CAT, chloramphenicol acetyltransferase; MHC, major histocompatibility complex; DTT, dithiothreitol; SV40, simian virus 40.
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in two packed cell volumes of solution I [0.3 M sucrose, 10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF]. Cells were lysed in a Dounce homogenizer using about 30 strokes of the tight pestle. The homogenate was centrifuged at 1600 ***g*** for 10 min to pellet crude nuclei. The nuclei were resuspended in 2 vol of solution II (10 mM Hepes, pH 7.9, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, 0.5 mM PMSF). The crude nuclei were extracted at 4 °C for 30 min with continuous stirring, followed by centrifugation at 100000 ***g*** for 1 h. The supernatant was transferred to cellulose dialysis tubing (Spectrum Medical Industries, Los Angeles, CA, U.S.A.; molecular mass cutoff 1000 Da) for dialysis in solution III (20 mM Hepes, pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol, 0.5 mM PMSF) overnight. The extracts were cleared by centrifugation at 10000 ***g*** for 10 min. The supernatant (protein concentration of 0.5 to 2 mg/ml) was frozen in aliquots and stored at −70 °C.

Plasmid constructions

The chloramphenicol acetyltransferase (CAT) reporter constructions were made using the KS$^+$-SV2 CAT vector [35] in which the simian virus 40 (SV40) enhancer was removed by digestion with *Sph*I and *Pst*I. This new vector, which retained the SV40 promoter, was called KS1. The constructions used in these studies, KSI-WXY (−146 to −26), KSI-WX (−146 to −69), and KSI-WX mut Y (−146 to −32), contained DNA sequence from the mouse MHC class II gene promoter I-Aβ [25]. The constructions were generated using specific oligodeoxynucleotides and PCR to generate the appropriate fragment from the I-Aβ promoter for insertion into the vector [36]. The DNA fragments were ligated directionally into *Xba*I plus *Sma*I-digested KS1 vector. All the constructions were confirmed by DNA sequencing. The DNA fragments used for the gel-electrophoresis DNA-binding studies were generated by synthesizing a double-stranded oligodeoxynucleotide containing the Y box [25] and cloning this into the *Eco*RV site of the KS$^+$ vector (Stratagene, La Jolla, CA, U.S.A.). The clones NF-YA, NF-YA.1 and NF-YB were isolated by PCR using oligodeoxynucleotides prepared from the sequences of NF-YA and NF-YB [33] and RNA prepared from the plasmacytoma MOPC 315. The PCR products were cloned into the KS$^+$ vector and sequenced. The genomic clone containing a portion of the NF-YA gene was isolated by PCR using oligodeoxynucleotides flanking the six-amino-acid segment that is absent from clone NF-YA but present in clone NF-YA.1, and genomic DNA from the plasmacytoma MPC II. Sense and antisense constructions of NF-YA and NF-YB were prepared by inserting the NF-YA (*Bam*HI plus *Cla*I) or NF-YB (*Sal*I plus *Eco*RI) genes in the appropriate orientation into the expression vector pJ6 [37]. The pJ6 vector contains the rat β-actin promoter. Oligodeoxynucleotides were synthesized on a model 380A Applied Biosystems DNA synthesizer (Foster City, CA, U.S.A.).

Transfection assays

A20-2J cells were transfected using the DEAE-dextran method [38]. Aliquots of DNA (2 μg) from the CAT construct and plasmid pCH110 (3 μg), a β-galactosidase expression plasmid (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ, U.S.A.) used to measure the efficiency of transfection, were added to each 10-cm-diam. plate of cells. In the co-transfection experiments with sense or antisense construction of NF-YA or the antisense construction of NF-YB, 2 μg of the indicated plasmid was used. CAT assays were performed as described in [38]. Briefly, cells were isolated 48 h after the DNA was added and then lysed by three freeze–thaw cycles using solid CO_2/ethanol and 37 °C. The extracts (10–60 μl standardized using β-galactosidase activity) were incubated with [^{14}C]chloramphenicol and acetyl-CoA for 60 min at 37 °C, followed by extraction with ethyl acetate. The ethyl acetate was removed by vacuum evaporation and the sample resuspended in 20 μl of ethyl acetate for TLC. The quantification of acetylation was performed using the AMBIS Radioanalytical Imaging System (AMBIS, San Diego, CA, U.S.A.).

Gel-electrophoresis DNA-binding assay

For gel-retardation assays, the DNA fragment containing the Y box sequence of the I-Aβ gene (320 bp), used previously to characterize the Y box DNA-binding protein [25,28], was used. We also used a 33 bp double-stranded oligodeoxynucleotide covering the Y boxes of the I-Aβ and the I-Eα genes. For these assays, DNA (10000 c.p.m. of ^{32}P-labelled, approx. 0.1 ng) and nuclear extract or proteins prepared by *in vitro* transcription/translation using a rabbit reticulocyte lysate system (Promega, Madison, WI, U.S.A.) were mixed to a total volume of 20 μl in a buffer containing 12 mM Hepes (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 0.12 mM EDTA, 0.3 mM PMSF, 0.3 mM DTT and 12% glycerol. A sample (2 μg) of poly(dI-dC) (Pharmacia LKB Biotechnology) 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 = 89 mM Tris, 89 mM boric acid, 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 exposed to X-ray film (XAR-5 from Kodak). The quantification of the radioactivity was performed using the AMBIS Radioanalytical Imaging System (AMBIS).

In some experiments an antiserum against NF-YB or the monoclonal antibody YA1a (anti-NF-YA) was added to nuclear extracts and gel retardation was determined using an oligonucleotide covering the Y box of the I-Aβ gene. The antibodies were kindly provided by Drs. D. Mathis, C. Benoist and R. Mantovani (INSERM U184, Strasbourg, France) [22]. In some controls, we used PU.1 transcribed/translated as previously reported [39].

RESULTS

The presence of the Y box sequence was examined for its influence on the expression of the mouse MHC class II gene I-Aβ. A 124 bp fragment from the promoter of this gene was inserted into the CAT vector, KS1, which contains the SV40 promoter and gave us a better signal in the CAT assays. 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 [17–19]. This 124 bp fragment contained, in addition to the Y box, two additional boxes, W and X, both of which have been shown to be important for the expression of several other MHC class II genes [1–3]. When this CAT construct was transfected into A20-2J B-lymphocytes a CAT activity of 18% was obtained (Figure 1). This was in contrast to a CAT activity of 1.0% when the KS1 vector alone was transfected into the same cells. A substantial drop in CAT activity was observed in those constructions in which the Y box was either deleted (KS1-WX) or mutated (KS1-mutant). When the Y box sequence was deleted CAT activity decreased to 9%. Previously we had shown that a mutation in the Y box, which changed two guanines to thymidines, disrupted binding of a nuclear factor to the Y box [25]. When this same mutation was incorporated into

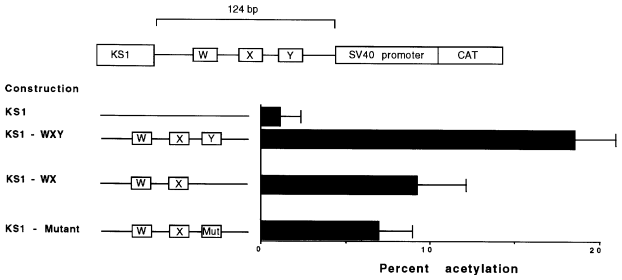

Figure 1 The Y box is an important *cis*-acting element for expression from the I-Aβ promoter

A CAT reporter plasmid (KSI) was used for the insertion of various fragments of the I-Aβ promoter. The constructions were, KSI (vector); KSI-WXY (−146 to −26); KSI-WX (−146 to −69); and KSI-WX mut (−146 to −32 with a mutation in the Y box). The constructions were transfected into A20-2J B-lymphocyte cells and after 48 h CAT activity was assayed by TLC. CAT activity was quantified using an AMBIS Radioanalytical Imaging System. Each determination was made three times and the values represent the means of three independent experiments. In each case, the S.D. was below 8% of the mean values.

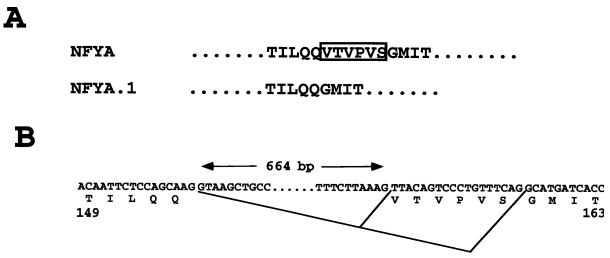

Figure 2 (A) The protein NF-YA, which has six additional amino acids not present in NF-YA.1, and (B) the sequence of genomic DNA which contains the splice donor site and acceptor sites for the NF-YA and NF-YA.1 genes

The predicted amino acid sequence is shown. Numbering of amino acids follows that of NF-YA. A 664 bp intron exists between the splice donor site and splice acceptor site of NF-YA. The splice acceptor site for NF-YA.1 is 18 nucleotides downstream.

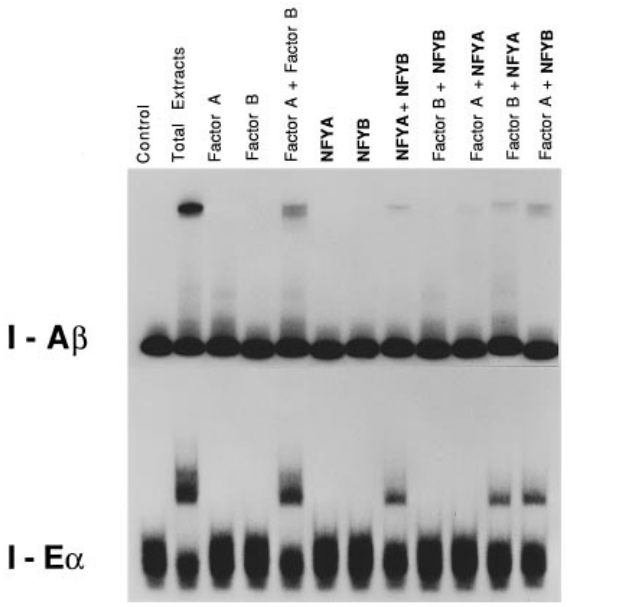

Figure 3 Recombinant NF-YA and NF-YB complement factors A and B in a gel-electrophoresis DNA-binding assay

The probe was a 320 bp fragment of the I-Aβ promoter that contained the Y box or a double-stranded oligodeoxynucleotide containing the Y box of the I-Eα gene. Total nuclear extracts as well as factor A and factor B were prepared from the B-cell line A20-2J. The factors NF-YA and NF-YB were prepared by *in vitro* transcription/translation. Retarded complexes were detected by autoradiography.

the CAT vector containing the W and X boxes (KS1-mutant), CAT activity was decreased to 7%. These results indicated that the region of the I-Aβ promoter encompassing the W, X and Y boxes could stimulate the expression of the CAT gene and that the Y box was an important element for expression from this promoter.

We cloned the genes for the two components of NF-Y (NF-YA and NF-YB) from the mouse plasmacytoma MOPC 315. The sequence of the gene for NF-YB was identical to that published previously [33]. For NF-YA we obtained two groups of clones from the same library, one of which had a DNA sequence that was identical to that previously published [33]. The other group of clones contained a deletion of 18 nucleotides corresponding to six amino acids from position 154 to 159. The gene containing this deletion was named NF-YA.1 (Figure 2A).

Based on the available sequence information it was likely that the six-amino-acid deletion in NF-YA.1 resulted from an alternative splicing event. To test this possibility oligonucleotide primers flanking the region coding for the six-amino-acid deletion were designed and mixed with genomic DNA and isolated from the plasmacytoma MPC11. PCR was then performed. A fragment of DNA of about 700 bp was generated, cloned into the KS$^+$ vector and sequenced. The sequence revealed a 664 bp intron between the codons for amino acids 153 and 154 (Figure 2B). Furthermore, the sequence revealed that in the NF-YA.1 clone in which the six amino acids were deleted the same splice donor site was used but a new splice acceptor site was used 18 nucleotides downstream from the splice acceptor site used for NF-YA. The results indicated that the six amino acids found to be deleted in NF-YA.1 could be accounted for by an alternative splicing event. In addition, the six amino acids were not encoded in a separate exon but were included in the sequence of NF-YA and deleted in the sequence of NF-YA.1 by the use of an alternative splice acceptor site.

We had previously shown that the nuclear factor that bound to the I-Aβ Y box was composed of two components, called factors A and B, which could be separated by FPLC using a Mono Q column [28]. While neither factor A nor factor B alone bound efficiently to DNA containing the Y box sequence of the I-Aβ gene, when the factors were mixed the complex bound a DNA fragment containing the Y box with high affinity (Figure 3). We wanted to know if the products of the cloned genes NF-YA and NF-YB could bind to the Y box of the I-Aβ gene and if they could complement the binding of factors A or B to the DNA. To answer these questions the genes for NF-YA and NF-YB were expressed *in vitro* using T7 polymerase to generate RNA and rabbit reticulocyte lysate for the preparation of protein. Neither of the proteins, when added individually to labelled Y box-containing DNA fragment, bound well to DNA (Figure 3). When NF-YA was mixed with NF-YB, however, the complex bound to the Y box-containing DNA with high affinity. We then tested the ability of NF-YA or NF-YB to complement factors A and B, which we had previously characterized. To prevent any confusion we have used the nomenclature consistent with NF-YA and NF-YB. When factor B was mixed with NF-YB or factor A was mixed with NF-YA and added to the probe no

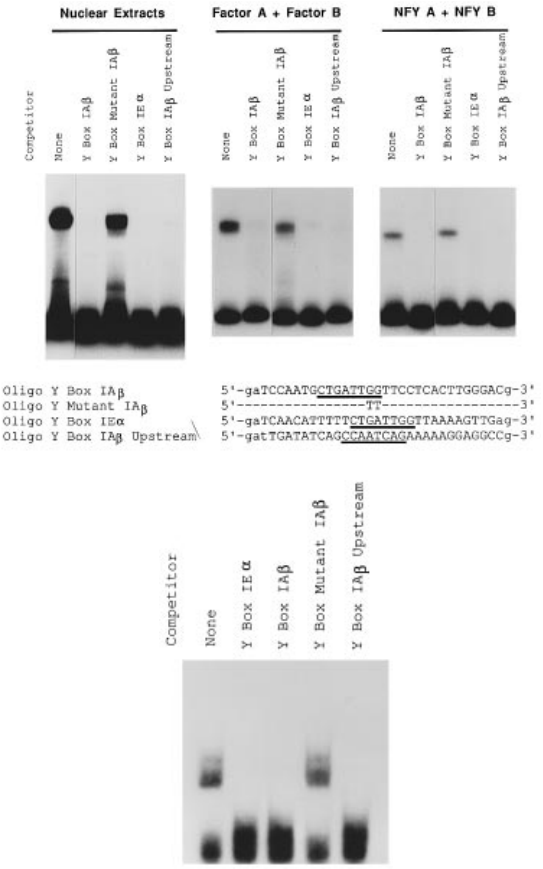

Figure 4 Competition experiments

Mobility shift assays were carried out using a 320 bp fragment of the I-Aβ promoter that contained the Y box (upper panels) or a double-stranded oligodeoxynucleotide containing the Y box of the I-Eα gene (lower panel) and nuclear extracts (1 μg) or recombinant proteins (1 μl). At the bottom of the upper panels, sequences of one strand of the double-stranded oligodeoxynucleotides used for competition are shown. The dashes indicate those bases that are identical between the Y box I-Aβ and Y mutant I-Aβ. The underlined bases show the Y box sequence. The oligodeoxynucleotides for Y box I-Aβ, Y box I-Eα and Y box I-Aβ upstream were used at a 20-fold excess and Y mutant I-Aβ at 200-fold excess.

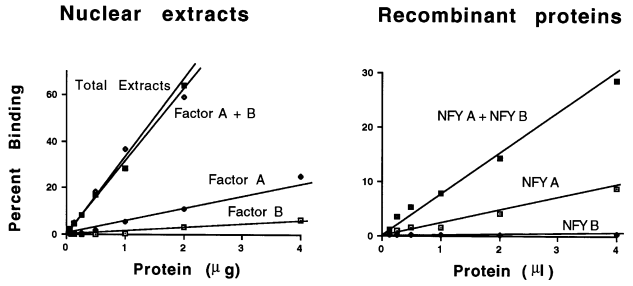

Figure 5 Binding titration of nuclear extracts or recombinant proteins

Left-hand panel: nuclear extracts were used. For factor A and factor B alone the DNA-binding capacity was tested using increasing amounts. The binding of factor A + factor B was tested using an excess of factor B (2 μg) and the indicated amounts of factor A. Right-hand panel: recombinant proteins were used. For NF-YA and NF-YB alone the DNA-binding capacity was tested using increasing amounts. The binding of NF-YA + NF-YB was tested using an excess of NF-YB (2 μl) and the indicated amounts of NF-YA. Binding was measured in a gel-electrophoresis DNA-binding assay using as a probe the I-Aβ Y box.

retarded complex was observed (Figure 3). In contrast, when factor B was mixed with NF-YA or factor A was mixed with NF-YB and added to the probe both mixtures gave retarded complexes in the gel-electrophoresis DNA-binding assay (Figure 3). The intensity of the retarded band produced by the recombinant proteins was less than that produced by the nuclear extracts. This may be due to the limited amount of active recombinant protein produced by the *in vitro* transcription/translation method. The binding of these proteins to DNA produced doublets in the gels. These doublets may represent protein or DNA degradation products or the DNA binding to different forms of NF-YA and NF-YA.1.

Because NF-YA and NF-YB were originally recognized as the factors that bound to the Y box of the I-Eα gene, we tested the binding capacity of factor A and factor B to the Y box of the I-Eα gene. As shown in Figure 3 we found similar results using either the Y box of the I-Aβ gene or the I-Eα gene, suggesting that factor A and factor B, as well as NF-YA and NF-YB, recognize the same sequences.

In order to demonstrate the specificity of the binding reactions, competition experiments were carried out using labelled DNA fragments that contained the Y box of the I-Aβ gene (Figure 4, upper panels) or the Y box of the I-Eα gene (Figure 4, lower panel). The binding reaction using total extracts, factor A and factor B or NF-YA and NF-YB, was competed in each case with a 20-fold molar excess of double-stranded oligodeoxynucleotides containing the Y box proximal of I-Aβ gene (-45 to -74), the Y box I-Eα gene or the Y box distal of I-Aβ gene (-1605 to -1630), but not with a 100-fold molar excess of the mutated Y box of I-Aβ that contains two TTs instead of the two GGs that are common to the core sequence of all the Y boxes.

Although factor A and factor B, and NF-YA and NF-YB, bound specifically to the Y box of both the I-Aβ and the I-Eα genes, we wanted to test the relative binding affinities of these factors. Using factor A or NF-YA in excess, we found that the capacity to bind the I-Aβ Y box was dose-dependent on the amount added of factor B (Figure 5, left-hand panel) or NF-YB (Figure 5, right-hand panel). Therefore, the limiting factor in the reaction is the amount of factor B or NF-YB. Under these conditions, we found that the slope of the curves for the binding of factor A and factor B compared with the binding of NF-YA and NF-YB were very similar. This demonstrates that the binding of factor A plus factor B to the Y box is similar if not identical to the binding of NF-YA plus NF-YB to the Y box. These results suggest that the binding affinities of factor A and NF-YA are similar. Similar results were found when the Y box of the I-Eα gene was substituted for the Y box of the I-Aβ gene (results not shown).

Next we tested whether specific antibodies against NF-YA and NF-YB were able to recognize the proteins that bind to the Y box of the I-Aβ gene. The addition of antibodies against either NF-YA or NF-YB to nuclear extracts in a gel-retardation assay generated the appearance of a new complex of higher molecular mass (Figure 6). This supershifted complex probably included a mixture of the antibodies, the proteins NF-YA and NF-YB that bind to DNA and the labelled DNA. No supershifted band was observed when an isotype-matched irrelevant monoclonal antibody was used (Figure 6). The same oligonucleotide that contains the Y box of the I-Aβ gene also contains the PU.1 box [39]. After adding antibodies against either NF-YA or NF-YB no supershifted band was found. These results demonstrate the specificity of the monoclonal antibodies and the identity between factors A and B with NF-YA and NF-YB respectively.

In an additional set of experiments we attempted to determine

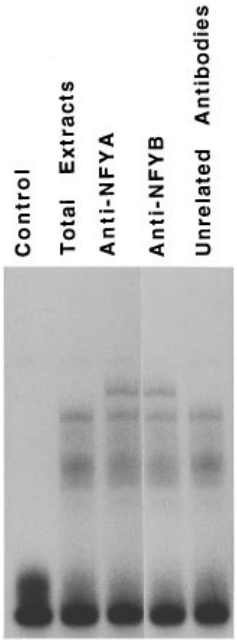

Figure 6 Antibodies against NF-YA and NF-YB produce a supershift with proteins that bind to the Y box of the I-Aβ gene

In the gel-electrophoresis DNA-binding assay the probe was a 33 bp double-stranded oligonucleotide containing the Y box of the I-Aβ gene. Nuclear extracts were prepared from A20-2J B-lymphocytes and 1 μl was mixed with 0.5 μl of each antibody before loading on to the gel. The unrelated antibody was against BSA.

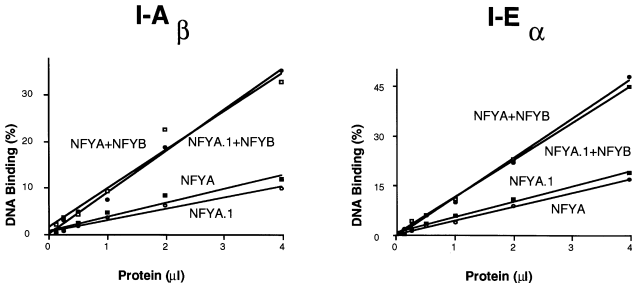

Figure 7 Similar binding activity of NF-YA and NF-YA.1

Binding was measured in a gel-electrophoresis DNA-binding assay using as a probe the I-Aβ or the I-Eα Y boxes.

whether the DNA-binding capacity of NF-YA and NF-YA.1 were similar. In a gel-retardation assay, we observed that NF-YA and NF-YA.1 were equivalent in their binding characteristics to the Y boxes of either the I-Aβ or I-Eα genes and, therefore, the six-amino-acid deletion found in NF-YA.1 appeared not to have a major influence on the binding of NF-Y to the Y box sequence (Figure 7). We cannot rule out the possibility that other functional activities such as protein–protein interactions or transcriptional activation may be affected by the six-amino-acid deletion.

The importance of the Y box for expression from the I-Aβ promoter has been documented by deletion or mutation of the Y box sequence (Figure 1). In order to evaluate the importance of the NF-Y protein for the expression of the I-Aβ gene we prepared antisense constructions of both NF-YA and NF-YB and tested their effect on the expression of a CAT reporter gene

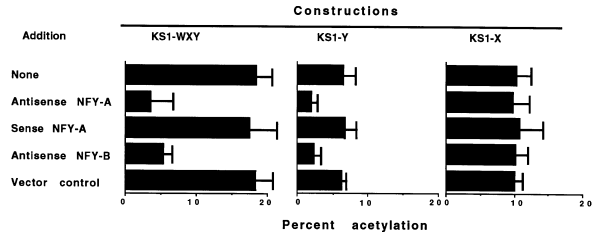

Figure 8 Antisense constructions of NF-YA or NF-YB reduce expression from the I-Aβ promoter

Various I-Aβ promoter segments were inserted into the CAT reporter plasmid KSI. The constructions were; KSI-WXY (−146 to −26), KSI-Y (−75 to −32) and KSI-X (−106 to −78). Sense and antisense orientations of the genes for NF-YA and NF-YB were inserted into the expression vector pJ6. The constructions were transfected into A20-2J cells and after 48 h CAT activity was assayed by TLC. CAT activity was quantified using an AMBIS Radioanalytical Imaging System. Each determination was performed three times and the values represent the means. In each case, the S.D. was below 10% of the mean values.

in various constructions. The NF-YA and NF-YB cDNAs were inserted into the vector pJ6 such that transcription from the rat β-actin promoter gave an antisense RNA of the respective gene. These constructions were transfected into A20-2J cells and antisense RNA was shown to be made using an RNA protection assay (results not shown). When the construction KS1-WXY was transfected into A20-2J cells a CAT activity of 19% was observed (Figure 8). When a plasmid containing the NF-YA gene inserted into the vector pJ6 in the sense orientation was co-transfected into A20-2J cells with the plasmid KS1-WXY, CAT activity remained at about 19%. A similar level of CAT activity was observed when the vector pJ6 was co-transfected with KS1-WXY. When the antisense NF-YA or NF-YB constructs were co-transfected with KS1-WXY, however, a drop in CAT activity was observed to a level of 4% for antisense NF-YA and 5% for antisense NF-YB (Figure 8).

A similar set of experiments was performed using a CAT construction in which the Y box sequence alone was inserted into the KS1 vector. We observed that the plasmid KS1-Y, when transfected into A20-2J cells, gave a CAT activity of 7% (Figure 8). The CAT activity of the KS1 vector was about 0.5% in this experiment (results not shown). When either NF-YA in the sense orientation or the vector pJ6 was co-transfected with KS1-Y, CAT activity was 7% in both cases. The co-transfection with antisense NF-YA resulted in CAT activity of only 2% while with antisense NF-YB it was 3%. These results further support the idea that NF-Y is an important factor for the expression of CAT in those constructions containing an NF-Y DNA-binding site.

As a control the X box sequence from the I-Aβ gene was also inserted into the KS1 vector. Plasmid KS1-X gave a CAT activity of 10% when transfected into A20-2J cells. Similar levels of CAT activity were observed when this plasmid was co-transfected with the vector pJ6, antisense or sense NF-YA constructs, or the antisense NF-YB construct. These results showed that the effects of antisense NF-YA and NF-YB were only seen in those constructions containing the NF-Y DNA-binding site, demonstrating the specificity of the antisense constructions.

DISCUSSION

Several arguments suggest that there is a strong similarity between factors A and B and NF-YA and NF-YB. First, the two factors isolated from nuclear extracts that bound to the I-Aβ Y box have

sizes (34 and 43–45 kDa) [28] similar to the factors that bound to the I-Eα Y box (32 and 40–43 kDa) [33]. Secondly, factors A and B, as well as NF-YA and NF-YB, bound to both the I-Aβ and I-Eα Y boxes (Figure 3). Thirdly, factor A is able to complement NF-YB and factor B complements NF-YA in their ability to bind I-Aβ and I-Eα Y boxes (Figure 3). Fourthly, the binding affinities of factor A and NF-YA, and factor B and NF-YB are very similar (Figure 5). Although the ability of factors A and B to replace NF-YA and NF-YB suggests functional similarity it does not demonstrate sequence identity. However, the finding that antibodies against NF-YA and NF-YB proteins induce a supershift of the nuclear extracts that bound to the I-Aβ Y box, identifies factors A and B as NF-YA and NF-YB respectively.

Recently, using an oligonucleotide covering the Y box of the DRα promoter, the gene for YB1 was cloned [40]. However, under our conditions no binding of the mouse homologue of the YB1 protein was found to bind the Y box of the I-Aβ or I-Eα genes [41].

The Y (CCAAT) box, located within 40 bp upstream of the transcription start site of all MHC class II genes is an important *cis*-acting element for the expression of these genes [10,19]. Deletion or mutation of the Y box sequence reduced or eliminated the expression of a reporter gene when linked to the mouse I-Aα [17], I-Aβ (this paper), I-Eα [19,20], I-Eβ [18] or the human DRα [11,13] genes. When the expression of an I-Eα gene containing a deletion of the Y box was examined in transgenic mice a reduction in expression of the transgene was observed [20]. Recently, the Y box has also been shown to be an essential element for the *in vitro* transcription from the Eα [19,23] and HLA-DRA promoter [21].

While the importance of the Y box for the expression of the MHC class II genes has been firmly established, the involvement of the Y box-binding protein for expression of these genes has not. Using the DRA promoter and an *in vitro* transcription assay it has been demonstrated that a Y box-binding activity is important for transcription from this promoter [21]. Although it is likely that this Y box-binding activity is NF-Y, this has not been demonstrated. In a recent report, a direct approach to show the importance of NF-Y for transcription has been developed using antibodies against NF-YA or NF-YB. Using an *in vitro* system, it has been shown that antibodies against these factors inhibit transcription from the promoter of Eα gene [23]. In this report we have used antisense constructions of both NF-YA and NF-YB to demonstrate that the factor NF-Y is important for transcription from the I-Aβ promoter. These results further support the idea that both the Y box-binding site and the DNA-binding factor NF-Y are important for MHC class II gene expression.

The cloning of the gene for NF-YA revealed that there are at least two forms of NF-YA. The two forms identified here, called NF-YA and NF-YA.1, were shown to differ by six amino acids. This difference could be accounted for by alternative splicing of the primary transcript. Both forms were found to be expressed in the plasmacytoma MOPC 315 and therefore we conclude that at least in these cells both forms can co-exist. Recently, several other isoforms of the mouse NF-YA gene have been identified [42]. Using S1 nuclease analysis from different cell lines or fresh mouse tissues these authors found that the distribution of the two forms does not correlate with MHC class II expression status, but rather breaks roughly along lymphoid/non-lymphoid lines. Thus, it is likely that the alternative splicing of NF-YA seen in the mouse plasmacytoma MOPC 315 is not unique to these cells but appears to occur in other cell types and in other species. The presence of the forms, NF-YA and NF-YA.1, in A20-2J B-lymphocytes could account for the small difference in molecular mass (43 and 45 kDa) in the two forms of factor A partially purified from nuclear extracts from this cell type [28]. Similar results were found using polyclonal antibodies in Western blot analysis [22].

The function of the six amino acids deleted in some forms of NF-YA, but not others, is not known. We did not observe any difference in the binding of either form to DNA. The six-amino-acid segment is not located within a region that has a high degree of amino acid sequence identity with the yeast Hap2 protein, and may code for the DNA-binding domain [22]. Its proximity to a glutamine-rich region which has been found in a number of transcription factors and is thought to be involved in the transactivation function of these proteins suggests that these six amino acids may be important for transactivation [43]. The six amino acids may also be important for specific protein–protein interactions.

Alternative splicing of primary transcripts has been observed for a number of other transcription factors [44–51]. Although the function of the various isoforms remains elusive, for many of these factors there is evidence suggesting that the different isoforms differ in their transcriptional activation properties. Alternative splicing clearly adds a new dimension to the possible mechanisms by which DNA-binding proteins regulate transcription. It will be interesting to know how the splicing events in NF-YA affect its function as a transcriptional activator.

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