Two DNA-Binding Proteins Discriminate between the Promoters of Different Members of the Major Histocompatibility Complex Class II Multigene Family

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The regulation of major histocompatibility complex (MHC) class II gene expression is a key feature of the control of normal and abnormal immune responses. In humans, class II α- and β-chain genes are organized in a multigene family with three distinct subregions, HLA-DR, -DQ, and -DP. The regulation of these genes is generally coordinated, and their promoters contain highly conserved motifs, in particular the X and Y boxes. We have identified five distinct proteins that bind to specific DNA sequences within the first 145 base pairs of the HLA-DRA promoter, a segment known to be functionally essential for class II gene regulation. Among these, RF-X is of special interest, since mutants affected in the regulation of MHC class II gene expression have a specific defect in RF-X binding. Unexpectedly, RF-X displays a characteristic gradient of binding affinities for the X boxes of three α-chain genes (DRA > DPA > DQA). The same observation was made with recombinant RF-X. We also describe a novel factor, NF-S, which binds to the spacer region between the X and Y boxes of class II promoters. NF-S exhibited a reverse gradient of affinity compared with RF-X (DQA > DPA > DRA). As expected, RF-X bound well to the mouse IEx2 promoter, while NF-S bound well to Iαα. The drastic differences in the binding of RF-X and NF-S to different MHC class II promoters contrasts with the coordinate regulation of HLA-DR, -DQ, and -DP genes.

Major histocompatibility complex (MHC) class II genes, or immune response genes, encode highly polymorphic transmembrane glycoproteins that are directly responsible for the recognition of antigens by the receptors of T lymphocytes. The immune response is controlled on one hand by the extensive structural diversity of MHC molecules, which is responsible for allelic differences in the efficiency of T-cell stimulation by a given antigen and for the existence of high- and low-responder phenotypes (4, 5). In addition, normal and abnormal immune responses are controlled by the quantitative regulation of MHC class II gene expression. Since expression of class II molecules at the cell surface confers the ability to stimulate T cells, the tissue specificity and level of MHC class II expression control T-cell activation (6, 21).

Because of this biological relevance, there is great interest in the mechanisms responsible for the regulation, developmental control, and tissue specificity of MHC class II gene expression. Finally, since MHC class II genes form a multigene family, the mechanisms responsible for the global regulation observed for the entire family of HLA-DR, -DQ, and -DP α- and β-chain genes (8) are of interest. Rare cases of dissociated expression of HLA-DR versus -DQ have also been described elsewhere (13).

In transfection experiments, a DNA sequence containing less than 160 base pairs upstream of MHC class II genes is sufficient to confer both B-cell-specific expression (23, 30) and inducibility by gamma interferon (3, 28, 30). Within this region, three highly conserved sequences are observed in human and mouse class II genes: the X and Y boxes (29) and a heptamer motif, also referred to as the Z or W box (26). Binding to this promoter region of the DRA gene, an octamer-binding protein and two factors binding to the X and Y boxes have been described elsewhere (25, 28). The study of regulatory mutants known to be affected in a trans-acting factor controlling class II gene expression (11, 20) has revealed a specific defect in the binding of RF-X, a factor which normally binds to the class II X box (25). It is likely, therefore, that impaired binding of RF-X is responsible for the class II-negative phenotype of these regulatory mutants. We have recently cloned an RF-X cDNA (24), and a direct test of this proposition has become possible.

The objectives of this study were first to compare the binding of RF-X with the promoters of α-chain genes from the DR, DQ, and DP subregions and then to search for the existence of other DNA-binding proteins specific for this functionally relevant promoter region. Unexpectedly, we observed that RF-X binds very differently to the X boxes of individual class II genes, showing an affinity that is strong for DRA, intermediate for DPA, and very weak for DQA. We have also identified a novel factor, NF-S, which binds to the spacer region immediately 3' of the X box. Interestingly, NF-S exhibits a reverse gradient of binding affinity, binding strongly to DQA, less well to DPA, and only very weakly to DRA. Thus, the α-chain promoters of the three different MHC class II subregions exhibit distinct patterns of binding affinity for the two protein factors RF-X and NF-S.

MATERIALS AND METHODS

Cell culture and nuclear extracts. The Epstein-Barr virus-transformed human normal B-cell lines Mann and HHK and the class II-negative B-cell lines Robert, Nacera, and Ramia from combined immunodeficiency (CID) patients were grown as previously described (25). Nuclear extracts were prepared by the method of Dignam et al. (12), with the exception that nuclear proteins were extracted at 1 M NaCl. Large amounts of human class II-expressing lymphocytes were obtained by leukopheresis of blood from a patient with chronic lymphocytic leukemia (CLL). In this case, nuclear

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Oligonucleotides. The xDR, xDP, and xDQ oligonucleotides were synthesized on a Gene Assembler (Pharmacia) by using the phosphoramidite method. Complementary strands were combined, boiled for 10 min in 10 mM Tris hydrochloride (pH 7.5)—1 mM EDTA—5 mM MgCl2, and allowed to anneal by cooling to room temperature over a period of 3 to 4 h. The double-stranded oligonucleotides contained a 5' overhang and were end labeled either by using the DNA polymerase I Klenow fragment and either [α-32P]dATP or [α-32P]dTTP or by using [γ-32P]ATP and T4 polynucleotide kinase. All probes were gel purified before use.

Oligonucleotides xEα and xAα were derived from murine class II promoters and corresponded to the X(Eα)33 and X(Aα)44 oligonucleotides described by Liou et al. (19).

Gel retardation assays. The standard 20-μl binding reaction mixture consisted of 12% glycerol, 12 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.9), 60 mM KCl, 5 mM MgCl2, 0.12 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 0.3 mM dithiothreitol, 1 μg of poly(dI-dC)·poly(dI-dC), 0.5 μg of sonicated denatured Escherichia coli DNA, 20,000 cpm of 32P-labeled DNA (0.1 to 0.5 ng), and the amount of nuclear extract indicated in the figure legends.

32P-end-labeled probes were added to the reaction mixture after 5 min of preincubation at 0°C of the nonspecific competitor and the nuclear extract. Binding was allowed to proceed for 30 min on ice. Samples were then analyzed on 4% acrylamide and 0.25× TBE, as previously described (25). For competition experiments, the preincubation was carried out in the presence of 1 to 100 ng of the competitor DNA.

Methylation interference assays. The double-stranded oligonucleotides, 32P-labeled at one of their 5' ends, were partially methylated with dimethyl sulfate for 2 min, as described by Maxam and Gilbert (22).

Binding reactions and gel electrophoresis were set up as described in the preceding section with the exception that the reactions were scaled up by a factor of eight and contained 2 × 106 cpm of the appropriate methylated probe. Following complex separation, the DNA was transferred electrophoretically overnight onto Whatman DE81 paper in 0.25× TBE at 18 V, 50 mA (Trans-Blot system; Bio-Rad Laboratories). Radioactive bands were localized by autoradiography of the wet DE81 paper, cut out, and eluted in 450 μl of 20 mM Tris hydrochloride (pH 8)—2 mM EDTA—1.5 M NaCl with 10 μg of RNAse for 2 h at 37°C. Piperidine cleavage and sequencing gel analysis were performed according to published protocols (22).

RESULTS

Two distinct proteins bind to DPA, DQA, and DRA promoters with different affinities. In order to assess the binding of nuclear factors to the X box of different α-chain gene promoters, we used a series of oligonucleotides of identical size centered on the X box of the DRA, DQA, and DPA genes. All three oligonucleotides contained the entire spacer region between the X and Y boxes and had the X consensus sequence at exactly the same position (Fig. 1). These three oligonucleotides therefore allowed direct comparisons of the binding affinities of DRA, DQA, and DPA promoter sequences. The binding patterns of the xDR, xDP, and xDQ oligonucleotides obtained by gel retardation assays with nuclear extracts from B lymphocytes are shown in Fig. 2.

At least two discrete retarded bands, termed B1 and B2, were detected by using labeled xDR as a probe. Two complexes of identical electrophoretic mobility were also observed by using either xDP or xDQ oligonucleotides, although with xDQ, the slowest-migrating complex (B1) was only detectable after prolonged exposure (data not shown).

The intensity of the upper band (B1), which corresponded to RF-X (see below), was stronger with the xDR probe than with xDP and was barely detectable with xDQ. In contrast, complex B2 was strong with xDQ and xDP but very faint with xDR. Identical patterns in the formation of both complexes were obtained with nuclear extracts from two Epstein-Barr virus-transformed B-lymphocyte lines (QBL and HHK) and from B cells from CLL patients.

FIG. 1. Map of MHC class II promoter region. Boxes W, X, and Y represent conserved upstream sequences found in all class II promoters. The region encoding mRNA is represented by a hatched box, and the transcriptional start is indicated by an arrow. Relative positions and sequences of the x oligonucleotides derived from DRA (9), DPA (14), and DQA (2) promoters are shown below.

FIG. 2. B-cell nuclear factors that bind to xDR, xDP, and xDQ probes. Shown are the results of the gel retardation assays performed by incubating 32P-end-labeled xDR, xDP, and xDQ oligonucleotides with 8 μg of B-cell Mann nuclear extract. B1 and B2 indicate the positions of bound complexes.
To further characterize these bands, we carried out cross-competition experiments. Binding was performed with either labeled xDR, xDP, or xDQ probes, and in each case, the abilities of all three unlabeled oligonucleotides to compete for the formation of the specific complexes were tested (Fig. 3). Competition with a DNA fragment of similar size but of unrelated sequence was used as a control. Comparison of band intensities at the same molar excess of competitor revealed that the formation of complex B1 was inhibited most efficiently by xDR, slightly less by xDP, and only very weakly by xDQ. The same pattern was observed whether xDR (Fig. 3A) or xDP (Fig. 3B) was used as the labeled oligonucleotide. Competition by xDQ, although weak, was significant when compared with that obtained by the non-specific competitor DNA (Fig. 3A), which confirms that RF-X can interact with the X box of the DQA promoter.

In contrast, these same three oligonucleotides competed in reverse order for the formation of complex B2: xDQ was a more efficient competitor than xDP, and xDR had practically no effect. The same result was obtained whether xDR, xDP, or xDQ was used as the labeled oligonucleotide (Fig. 3). The formation of complex B2 on the xDR probe was efficiently inhibited by both xDP and xDQ templates, but not by an irrelevant oligonucleotide, showing that the weak interaction with xDR sequences is indeed specific.

Using a screening procedure based on direct expression, we recently cloned an RF-X cDNA and expressed recombinant RF-X in E. coli (24). This recombinant protein exhibits the same characteristic gradient of binding affinities for xDR, xDP, and xDQ, as observed for RF-X in B-cell extracts.

The close correlation between the relative affinities measured in competition assays and the relative binding efficiencies revealed by gel retardation experiments suggests that the xDR, xDP, and xDQ oligonucleotides all bind the same two proteins but with very different affinities.

Analysis of DNA-protein contact points. To identify the sequences and precise contact points that are involved in the formation of complexes B1 and B2, we performed methylation interference assays. The methylated residues that interfere with the binding of a specific protein were underrepresented in DNA from the complexes (B1 and B2) relative to the unbound probe (F). The results obtained with the xDP oligonucleotide as a probe are shown in Fig. 4.

The methylation interference profile reveals that when methylated, five G residues within the X box interfered with the formation of B1 (Fig. 4A). These contact points within the class II X box correspond to those previously reported for the binding of the regulatory factor RF-X on the X box of the DRA promoter (25). Analysis of complex B2 allowed us to assign the binding site of the second protein to a sequence situated immediately 3' of the X box, in the spacer region between the X and Y boxes (Fig. 4B). It was thus named...
NF-S. Methylation of one G residue on the coding strand and two residues on the noncoding strand immediately downstream of the X box interfered with the formation of this complex on xDP. Methylation of the same G residues on the xDQ oligonucleotide also prevented B2 formation (Fig. 4B).

On the basis of these results and the data obtained from gel retardation assays, we conclude that two distinct DNA-binding factors interact specifically with neighboring sequences in a short region of the DRA, DPA, and DQA promoters. One factor, binding to the X box, corresponds to the previously identified protein RF-X. Its binding is thus not restricted to DRA and also takes place with the X boxes of DP and DQ α-chain gene promoters, although with very different affinities. The second factor, NF-S, binds next to the X box of the different HLA α-chain gene promoters with affinities that are the opposites of those observed for RF-X.

**Binding of RF-X and NF-S is not mutually exclusive.** Since RF-X and NF-S recognize adjacent sequences, we asked whether both proteins could bind simultaneously to the same DNA fragment. Titration experiments were performed with increasing amounts of B-cell nuclear extract. For this, we took advantage of the high activity of RF-X and NF-S found in nuclear extracts from B cells obtained from CLL patients. The oligonucleotide xDP was used as a probe because it has a high affinity for both RF-X and NF-S. With increasing amounts of protein, B1 and B2 complexes increased as the amount of free fragment decreased. When almost no more free fragment was available for binding, formation of complex B2 progressively decreased as two slower-migrating complexes (C1 and C2) were formed (data not shown). Competition experiments with DRA and DQA oligonucleotides (Fig. 5) and methylation interference assays (data not shown) showed that the formation of complex C1 seems to be due to the simultaneous binding of NF-S and RF-X.

**RF-X and NF-S also bind to murine class II α-gene promoter sequences.** Competition experiments were performed to explore the relative affinities of RF-X and NF-S for the promoters of the murine MHC class II α-chain genes (Fig. 6). For competitors, we used oligonucleotides containing the X box and X-Y spacer sequence of murine Iαa and IEd α genes (see Materials and Methods), which are the mouse equivalents of human HLA-DQA and -DRA genes, respectively.

Binding of RF-X was inhibited more efficiently by the xEd oligonucleotide than by the same molar excess of xAα, showing a preferential affinity of RF-X for IEdα over Iαa promoter sequences. Upon competition with an excess of xAα, binding of NF-S (B2) was significantly reduced, whereas an equivalent molar excess of xEd had virtually no effect. The differential binding of RF-X and NF-S to IEdα and IAα sequences, therefore, correlates with the results obtained with the corresponding human DRA and DQA class II genes.
HLA class II regulatory mutants show defective binding of RF-X but normal binding of NF-S. Mutations affecting the regulation of MHC class II gene expression have been described previously. In particular, patients with HLA class II deficiency (CID), a type of primary immunodeficiency (10), are characterized by a defect in a trans-acting regulatory factor controlling HLA class II gene expression (11). Cells from CID patients show a specific defect in the binding of the X-box-binding factor RF-X (25). We compared the binding of RF-X and NF-S in nuclear extracts from normal B cells and cells from a CID patient (Fig. 7). With CID nuclear extracts, there was no detectable binding of RF-X with the xDR, xDP, or xDQ oligonucleotide, confirming the earlier data obtained with DRA X box sequences. On the other hand, NF-S from the CID extract binds normally. We have also observed a normal NF-S binding pattern with extracts from other CID patients, from another type of regulatory mutant, RJ 2.2.5 (1), and from a fibroblastic cell line (data not shown).

DISCUSSION

The control of gene expression involves multiple DNA-binding factors which interact with specific enhancer and promoter target sequences (17). These factors may cooperate functionally, interact with one another, bind to other protein cofactors, and be the subject of functionally relevant biochemical modifications (16, 18). This complexity in DNA-protein interactions is of special interest in the case of a multigene family, such as MHC class II genes, in which one can observe either a global regulation of all class II genes (8) or a specific control of certain members of the multigene family (13). A number of proteins are capable of binding to the promoter of the DRA gene. Homologous enhancer and promoter sequences of α-chain genes of the three class II subregions DR, DQ, and DP have now been compared for their abilities to interact with these specific factors. In the cases of RF-X and of the newly described factor NF-S, very different binding affinities were observed for different members of the class II gene family. Indeed, RF-X and NF-S show a rather surprising reverse gradient of affinities for the promoter sequences of DRA, DPA, and DQA (Fig. 8).

The case of RF-X is of particular interest because of the genetic defect in RF-X binding in several MHC class II-negative CID lines. In these regulatory mutants, RF-X seems impaired in its binding to the X box and is therefore possibly the trans-acting factor responsible for the global
The unusual X sequences can and DQA, respective of RF-X synthesized in DQA a property provided class observed the binds extracts promoters distance that situated or is mention expression investigation. AMP-responsive-element-related of two form RF-X was described here, or palindrome to RF-X antisense to RF-X class proteins, in all examination of NF-S, shows kinase C and the kinase C motif as a cis-acting element is under investigation. Since the target sequences of RF-X and NF-S are very close and may even partially overlap, one might expect the two factors to interact upon binding, either cooperatively to form a more stable complex or by competing for their adjacent binding sites. On the basis of our in vitro data, this does not seem to be the case. At high concentrations of nuclear extracts, both RF-X and NF-S appear to attach simultaneously to the same DNA fragment, suggesting that their binding is not strictly mutually exclusive. In the absence of RF-X binding (in CID extracts), NF-S retains its DQ >> DP >> DR binding pattern, and in the absence of NF-S, recombinant RF-X alone also retains its gradient of affinities, indicating that their binding characteristics are not interdependent. In addition, several lines of evidence suggest that, under our experimental conditions, interaction between the two proteins is not cooperative. Indeed, simultaneous binding of RF-X and NF-S (complex C1) is only observed when free fragment becomes limiting. Moreover, in competition experiments, the stability of complex C1 (RF-X plus NF-S) is not greater than that of B1 (RF-X alone) or B2 (NF-S alone).

The promoters of the IEα and IAα, the mouse homologs of HLADRα and -DQA, respectively, have conserved the characteristic differences in binding affinities for the two factors, since RF-X binds to IEα better than to IAα and NF-S binds to IAα better than to IEα. These rather unique binding properties confirm the relatedness of the different human and mouse class II genes, as established from the structures of the coding regions. This conservation will also permit the identification of mouse factors homologous to RF-X and NF-S.

Murine DNA-binding proteins recently described and cloned bind to IAα and not to IEα (19). The genes encoding these proteins exhibit a pattern by Southern analysis clearly distinct from that observed with an RF-X cDNA probe (data not shown). Consequently, these newly described genes and factors do not correspond to RF-X, the regulatory factor affected in CID mutants. They may, however, represent mouse homologs of NF-S, because both murine proteins and NF-S have contact points in the spacer region (19) and bind preferentially to IAα rather than to IEα.

In most biological situations, genes of the DR, DQ, and DP subregions of the MHC are regulated in a coordinate fashion. This is true for constitutive expression in B lymphocytes as well as for the expression induced by gamma interferon in a variety of cell types. In this respect, the reverse gradients of affinity of RF-X and NF-S for DRA, DPA, and DQA are particularly puzzling. There is no direct correlation between the levels of DR, DQ, and DP mRNA expression (10) and the different affinities of RF-X and NF-S detected in vitro for these three promoters. However, these differences in binding affinity do not necessarily reflect the state of promoter occupancy in vivo, where it can be influenced by interactions with other factors or by local features of chromatin structure.

An important conclusion from these data is that, in studying the regulation of a multigene family, features observed with one member of a multigene family cannot be extrapolated automatically to the others. The study of different genes in the family can lead to the identification of distinct factors, even when these genes are coregulated. Indeed, the factor RF-X and the RF-X defect in the regulatory mutants would not have been detected with DQA promoter sequences, and NF-S would not have been identified with DRA sequences alone. Perhaps additional factors will be discovered in the study of other MHC class II loci, including β-chain genes, in which the X-Y spacer differs from that of the α-chain genes.
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