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Evidence for a trans-acting factor that regulates the transcription of class II major histocompatibility complex genes: Genetic and functional analysis

(*HLA-DRα* gene/cell fusion/complementation analysis/DNA transfection/DNA-binding proteins)

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ABSTRACT **The study of specific trans-acting transcription factors in prokaryotes and lower eukaryotes has been greatly facilitated by genetic analysis of mutant strains deficient in such factors. We have developed such a system to study mammalian trans-acting factors that regulate the transcription of class II major histocompatibility complex genes, using the mutant cell lines RM2 and RM3. These cells, derived from the human B-cell line Raji, specifically fail to transcribe their class II major histocompatibility complex genes. Here we show that a transfected *HLA-DRα* class II major histocompatibility complex gene, like the endogenous *HLA-DRα* genes, is efficiently transcribed in Raji cells but not in RM2 or RM3 cells, demonstrating that the mutant cells are deficient in a specific trans-acting factor required for transcription of these genes. HLA-DR expression in RM2 and RM3 cells is rescued by fusion to another B-cell line but not by fusion to each other. Thus, the defects in the two cell lines are recessive and noncomplementing and define a locus whose wild-type product we designate TF-X1. We show that TF-X1 influences the activity of a 24-base-pair B-cell-specific cis-acting transcription element in the HLA-DRα promoter. However, in three different biochemical assays, we detect no difference between wild-type and mutant cells in the DNA-binding proteins that interact with these DNA sequences. Thus, the defective version of TF-X1 may be a DNA-binding protein that binds to the HLA-DRα promoter but fails to activate transcription. Alternatively, TF-X1 may not be a DNA-binding protein at all.**

Control of transcription in prokaryotes and lower eukaryotes is largely mediated by sequence-specific DNA-binding proteins that interact with target sequences within or adjacent to structural genes and by proteins that interact directly with RNA polymerases (1, 2). Many of these trans-acting factors were first identified genetically by the isolation of mutant strains whose expression of specific genes was altered due to deficient regulatory proteins. The combination of such genetic methods with biochemical analyses of the identified proteins has enabled elegant molecular dissection of transcriptional control networks in these organisms (3, 4). In mammals, genetic analysis of trans-acting transcription factors has been frustrated by the diploidy and long generation times of cultured cells and by the difficulty of devising an efficient assay for mutations in specific trans-acting transcription factors. Because accurately regulated mammalian *in vitro* transcription has been elusive in most systems, a genetic approach may be extremely useful for functional characterization of trans-acting factors and for identification of factors that are not DNA-binding proteins.

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The class II genes of the major histocompatibility complex (MHC) constitute one of the best-characterized nonhormonally regulated mammalian systems for the characterization of mutations in specific trans-acting factors that may regulate transcription (5–9). The class II MHC regulon, comprising at least 13 coregulated genes whose protein products (designated HLA-DP, -DQ, and -DR) function as restriction elements in presentation of antigens to T cells and in T-cell–B-cell interactions, is expressed constitutively only in B cells and thymic epithelial cells (9). We have previously described two B-cell lines, RM2 and RM3, produced independently by mutagenesis of the B-cell line Raji with ethane methylsulfonate followed by selection for class II negative cells. Expression of mRNA and protein encoding the α and β chains of HLA-DR, HLA-DQ, and HLA-DP is very low in RM2 cells and undetectable in RM3 cells, even though the α- and β-chain genes encoding these class II proteins are grossly intact in RM2 and RM3 cells. *In vitro* nuclear run-on transcription experiments and measurement of mRNA stability demonstrated conclusively that the failure of class II expression in these cells is due to decreased transcription (5). The defect in RM2 and RM3 cells is specific for class II MHC gene expression: other B-cell markers, such as Igκ mRNA and cell-surface CD19 and CD21, are expressed at normal levels in the mutant cells as are class I MHC mRNA and protein (ref. 5; unpublished data). Here we further characterize RM2 and RM3 cells and the function of the trans-acting factor deficient in these cells. We designate the wild-type version of this factor TF-X1, for *t*rans-acting *f*actor controlling *X* box transcriptional activity.

MATERIALS AND METHODS

Cells and Plasmids. Raji, RM2, and RM3 cells were grown as described (5). DBB is an Epstein–Barr virus (EBV)-transformed B-cell line from an HLA-DR7 homozygote. Plasmids RSV-CAT, pSV2-CAT, and pTE2ΔSal/Nru (herein designated tk-CAT) contain the chloramphenicol acetyltransferase (CAT) gene transcribed from the Rous sarcoma virus, simian virus 40 (SV40) early region, and herpes simplex virus thymidine kinase (TK) promoter, respectively (10, 11). p(−136/+31)CAT contains HLA-DRα promoter sequences from positions −136 to +31 linked to the CAT gene. p(−116/−92)tk-CAT contains HLA-DRα promoter sequences from positions −116 to −92 linked to the TK promoter and CAT gene. pDM3 and pDM10 were constructed by insertion of a 6.0-kilobase (kb) *Xba* I fragment containing the *HLA-DRα* gene into the *Hin*dIII site of

Abbreviations: MHC, major histocompatibility complex; EBV, Epstein–Barr virus; CAT, chloramphenicol acetyltransferase; TK, herpes simplex virus thymidine kinase; SV40, simian virus 40; mAb, monoclonal antibody.

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pHEBo (12), followed by *Hin*dIII digestion (to remove exons 2–4 and associated introns) and religation to form pDM3 and pDM10, which differ only in the orientation of the *HLA-DRα* minigene relative to the other plasmid sequences.

Stable Transfection and mRNA Analysis. Plasmid DNA (10 μg) was introduced into 2×10^7 cells by electroporation without carrier DNA as described (13). Three days later, hygromycin B was added to 400 μg/ml. Cells were maintained in selective medium for several weeks. From surviving cells, poly(A)$^+$ RNA was isolated and analyzed by transfer blot (Northern blot) hybridization as described (14). Hybridization probes were a cloned HLA-DRα cDNA and a 1.9-kb *Eco*RI–*Eco*RV fragment of pHEBo containing the hygromycin-resistance (*hph*) gene.

Complementation Analysis. For complementation analysis, 2.0-cm^2 wells were coated with *Mytilus edulis* adhesive protein (Cell-Tak; Biopolymers, Farmington, CT) as recommended by the manufacturer; 1.5×10^6 cells of each fusion partner were added in 0.5–1.0 ml of RPMI 1640 medium. After 1 hr at 37°C, cells were fused essentially as described, except that phytohemagglutinin was omitted (15). Twenty-four hours later, cells were stained by indirect immunofluorescence as described (5), using monoclonal antibody (mAb) 20-1E7.4 supernatant (from Susan Radka, Genetic Systems, Seattle, WA), which recognizes all known HLA-DR types except DR1, DR4, DR7, and DRw9, or 5 μg of mAb MOPC 195 per ml, which recognizes no known antigen; this was followed by staining with 1 mg of goat anti-mouse IgG, F(ab')$_2$ per ml conjugated with fluorescein or rhodamine. Stained cells were examined and photographed using a Leitz fluorescence microscope.

Transient Transfection and CAT Assay. Plasmid DNA (10 μg) was introduced into 2×10^7 cells by electroporation without carrier DNA as described (13). Seventy-two hours later, CAT enzymatic activity was measured in cell lysates as described (11).

Preparation of Nuclear Protein Extracts. Nuclear protein fraction 2 was prepared from Raji, RM2, and RM3 cells as described (16).

DNase I Protection ("Footprint") Assay. DNase I footprinting was performed essentially as described (17). Each reaction mixture contained 25,000 cpm (≈5 fmol) of uniquely 5′ or 3′ ^{32}P-end-labeled DNA (a 0.3-kb *Xba* I–*Sac* I fragment containing the HLA-DRα promoter) and 40 μg of nuclear protein extract.

Gel Mobility-Shift Assay. Gel mobility-shift electrophoresis, using ^{32}P-labeled oligonucleotide X3 [a 25-base-pair (bp) double-stranded fragment containing HLA-DRα promoter sequences from positions −109 to −93 flanked by *Bam*HI and *Bgl* II restriction sites] and nuclear protein extracts, was performed essentially as described (18). Control samples contained either 50 μg of predigested proteinase K per ml or 20 ng (1.3 pmol) of unlabeled competitor DNA, which was either X3 itself or oligonucleotide Z5 (a 32-bp fragment containing sequences from positions −130 to −109 of the HLA-DRα promoter and flanked by the same restriction sites as X3).

Methylation Interference Assay. Methylation interference analysis was carried out essentially as described (19), using a 45-bp *Hin*dIII–*Bam*HI restriction fragment containing HLA-DRα promoter sequences from positions −115 to −81, end-labeled with ^{32}P at either its 5′ or 3′ end.

RESULTS

Deficient Expression of a Stably Transfected *HLA-DRα* Minigene in RM2 and RM3 Cells. To investigate whether RM2 and RM3 cells are deficient in a trans-acting factor required for transcription of class II MHC genes, we constructed plasmids pDM3 and pDM10 (Fig. 1*A*). Both of these plasmids

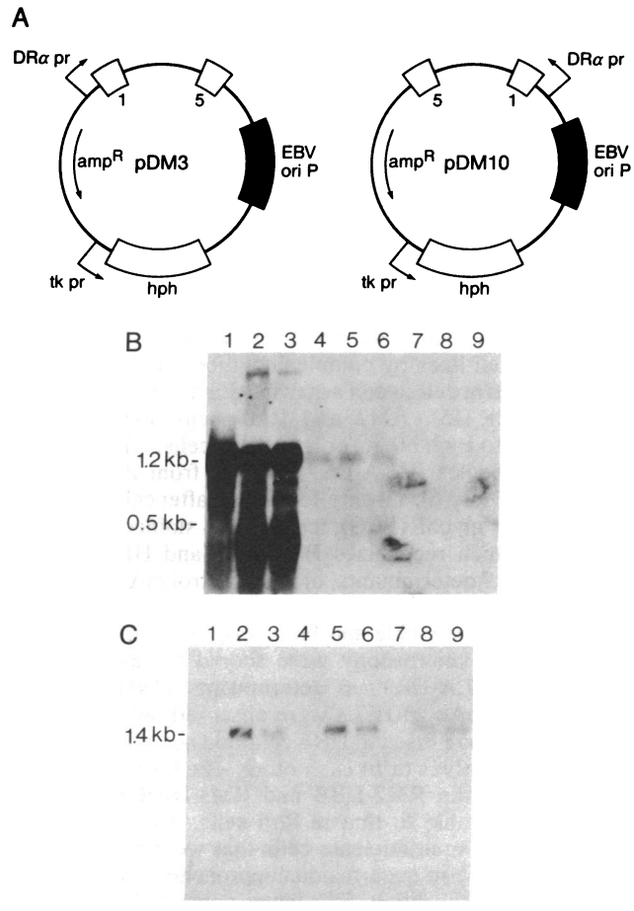

FIG. 1. Stable transfection assay. (*A*) Structure of plasmids pDM3 and pDM10. The *HLA-DRα* minigene, hygromycin-resistance (*hph*) gene, ampicillin-resistance gene (amp^R), and EBV origin of replication (oriP) are indicated. pr, Promoter. (*B* and *C*) Northern blot hybridization with HLA-DRα cDNA probe and *hph* probe, respectively. Lanes 1, Raji; lanes 2, Raji transfected with pDM3; lanes 3, Raji transfected with pDM10; lanes 4, RM2; lanes 5, RM2 transfected with pDM3; lanes 6, RM2 transfected with pDM10; lanes 7, RM3; lanes 8, RM3 transfected with pDM3; lanes 9, RM3 transfected with pDM10.

contain an *HLA-DRα* minigene, consisting of 266 bp of 5′ flanking sequences, all of exon 1, part of introns 1 and 4, all of exon 5, and 0.3 kb of 3′ flanking sequences. The minigene gives rise to an mRNA of 0.5 kb, which can be distinguished from the 1.2-kb transcript of the endogenous *HLA-DRα* gene. pDM3 and pDM10 also contain the EBV origin of replication and the hygromycin-resistance (*hph*) gene under the control of the TK promoter. The two plasmids differ only in the orientation of the *HLA-DRα* minigene. The EBV origin of replication permits the plasmid to exist stably as an episome in transfected Raji, RM2, and RM3 cells (12).

Each of the plasmids was transfected into Raji, RM2, and RM3 cells by electroporation, and stable transfectants were isolated by selection in hygromycin-containing medium. RNA from transfected cells was purified and analyzed by Northern blot hybridization using a radiolabeled HLA-DRα cDNA probe. Raji cells transfected with either pDM3 or pDM10 expressed high levels of mRNA from the endogenous *HLA-DRα* genes and the introduced *HLA-DRα* minigene (Fig. 1*B*), whereas the levels of both transcripts were lower by a factor of ≈14 in RM2 transfectants and undetectable in RM3 transfectants, as determined by scanning densitometry. When an identical Northern blot was hybridized to the radiolabeled *hph* probe, equivalent levels of *hph* mRNA were

seen in Raji, RM2, and RM3 cells transfected with pDM10 (Fig. 1C). For pDM3, the level of *hph* mRNA was slightly higher in Raji than in the mutants; this may be attributable to a distance-dependent effect of cis-acting HLA-DRα upstream promoter elements (which function in Raji but not in the mutant cells) upon the TK promoter, which is closer to the *HLA-DRα* minigene in pDM3 than in pDM10. These results demonstrate that the selective absence of class II MHC gene expression in these mutant cells is due to a deficient trans-acting factor and not to a cis-acting defect within the endogenous class II genes.

Complementation Analysis of RM2 and RM3 Cells. We next examined the genetic characteristics of the deficient loci in the mutant cell lines by complementation analysis. Using a 24-hr short-term cell fusion assay in which both donor nuclei remain intact (15), RM2 and RM3 cells were fused to themselves, to each other, or to DBB B cells, which express class II molecules (HLA-DR7) distinct from those of Raji cells (HLA-DR3, -6). Twenty-four hours after cell fusion with polyethylene glycol (PEG), cells were stained with mAb 20-1E7.4, which recognizes HLA-DR3 and HLA-DR6 but not HLA-DR7 determinants, or with control mAb MOPC 195 and examined by immunofluorescence microscopy.

Cells that were viable and binucleate or multinucleate by phase-contrast microscopy were scored for expression of cell-surface HLA-DR3, -6 determinants (Table 1). Cells staining with mAb 20-1E7.4 were observed only when RM2 or RM3 cells were fused to DBB cells and not when they were fused to themselves or to each other. The level of HLA-DR expression in the RM2-DBB and RM3-DBB hybrids was almost comparable to that in Raji cells. The frequency of binucleate and multinucleate cells that were heterokaryons staining positive in such fusions approached the theoretical value of 50% (the other 50% being noncomplementing homokaryons). No cells expressing HLA-DR3, -6 determinants were seen when PEG was omitted. These results demonstrate that the defects in RM2 and RM3 cells are recessive and map to the same complementation group.

Localization of Regulatory DNA Sequences That Interact with TF-X1. A transient expression assay was used to determine whether TF-X1 exerts its function by way of the HLA-DRα promoter. Expression of CAT activity from the HLA-DRα promoter [plasmid p(−136/+31)CAT] was reduced by a factor of 5 in RM2 and a factor of 10 in RM3 when compared to Raji cells (Table 2). In contrast, three different control promoters (TK, Rous sarcoma virus, and SV40 early promoters) gave equivalent CAT activities in the three cell lines.

We have previously described a 24-bp upstream sequence within the HLA-DRα promoter (positions −116 to −92) that is responsible for high levels of HLA-DRα promoter activity in normal B cells (20). This B-cell-specific cis-acting regulatory element consists of the X box (positions −108 to −95), which is conserved among all class II MHC genes, and the upstream pyrimidine tract (positions −116 to −109). In Raji cells, the CAT activity of p(−116/−92)tk-CAT, which contains this element linked to the TK promoter and CAT gene, was 40 times higher than the activity of the TK promoter alone (Table 2). In striking contrast, the relative CAT activity of this plasmid in RM2 and RM3 cells was lower by a factor of 5–10 than in Raji cells and was comparable to its activity in class II negative non-B cells (20). Experiments with other combinations of HLA-DRα promoter sequences gave equivalent results: efficient expression was observed only in wild-type Raji cells and only when both the X box and pyrimidine tract were included (data not shown).

Table 1. Complementation analysis of RM2 and RM3 cells

Fusion partner 1	Fusion partner 2	PEG	% cells DR3, -6$^+$
RM2	DBB	+	44
RM2	DBB	−	0
RM3	DBB	+	40
RM3	DBB	−	0
RM2	RM3	+	0
RM2	RM2	+	0
RM3	RM3	+	0
DBB	DBB	+	0

Twenty-four hours after fusion with PEG-1000, viable cells containing two or more nuclei were scored for HLA-DR3, -6 expression. Such cells comprised 0.1–0.5% of the total cell population when PEG was included and <0.01% when PEG was omitted. Data for each fusion represent results for at least 50 binucleate/multinucleate cells compiled from at least three independent experiments.

Table 2. Transient expression assay

Plasmid	Raji	RM2	RM3
tk-CAT	1.0	1.0	1.0
RSV-CAT	37.9 ± 11.1	28.5 ± 5.7	44.4 ± 10.5
pSV2-CAT	14.3 ± 5.3	10.4 ± 4.9	13.8 ± 3.8
p(−136/+31)CAT	5.4 ± 1.9	1.1 ± 0.2	0.5 ± 0.1
p(−116/−92)tk-CAT	40.6 ± 2.0	5.6 ± 1.9	8.6 ± 0.5

Results are expressed as relative CAT activities (with the activity of tk-CAT set equal to 1.0) and represent the arithmetic mean ± SEM of three independent experiments.

These results strongly suggest that TF-X1 influences the transcriptional activity of the pyrimidine tract and X box of the HLA-DRα promoter, either by interacting directly with these DNA sequences or by indirect means (see *Discussion*). The magnitude of the difference in expression between Raji and mutant cells was greater in the stable transfections than in the transient assays, as has been observed in other systems (21–23). These differences do not affect our conclusions and are likely due to differences in copy number and chromatin structure in the two assays and the difficulty of quantifying very low signal levels in the CAT assay.

Proteins That Bind to a B-Cell-Specific Promoter Element Are Not Grossly Altered in RM2 and RM3 Cells. To determine whether the defect in class II expression in RM2 and RM3 cells was due to an absent or grossly abnormal DNA-binding protein that directly interacts with HLA-DRα promoter sequences, we examined such binding by using DNase I footprinting, gel mobility-shift and methylation interference assays. In DNase I footprinting analysis of both strands of the HLA-DRα promoter (Fig. 2A; summarized in Fig. 3) nuclear proteins from Raji, RM2, and RM3 cells protected conserved sequences, including the X box, Y box, and ATTTGCAT octamer, but not the pyrimidine tract, from nuclease digestion. No difference was observed between Raji, RM2, and RM3 protein extracts in this assay, suggesting that none of the DNA-binding proteins detected in this assay was absent in the mutant cells.

In an electrophoretic gel mobility-shift assay using a radiolabeled X box oligonucleotide probe (Fig. 2B), nuclear protein extracts from Raji, RM2, and RM3 cells exhibited identical patterns: two major DNA–protein complexes, and a minor species whose mobility was intermediate between the two major bands, were observed. Formation of these bands was inhibited by proteinase K and by unlabeled X box oligonucleotide but not by an unrelated oligonucleotide. These data agree well with data obtained by others for the DRα (24) and murine I-E_α (25) promoters. An identical banding pattern was observed with an oligonucleotide containing the X box and the pyrimidine tract (not shown). Gel-mobility shift analysis of proteins binding to the Y box and octamer likewise revealed no detectable difference between Raji and mutant proteins (not shown).

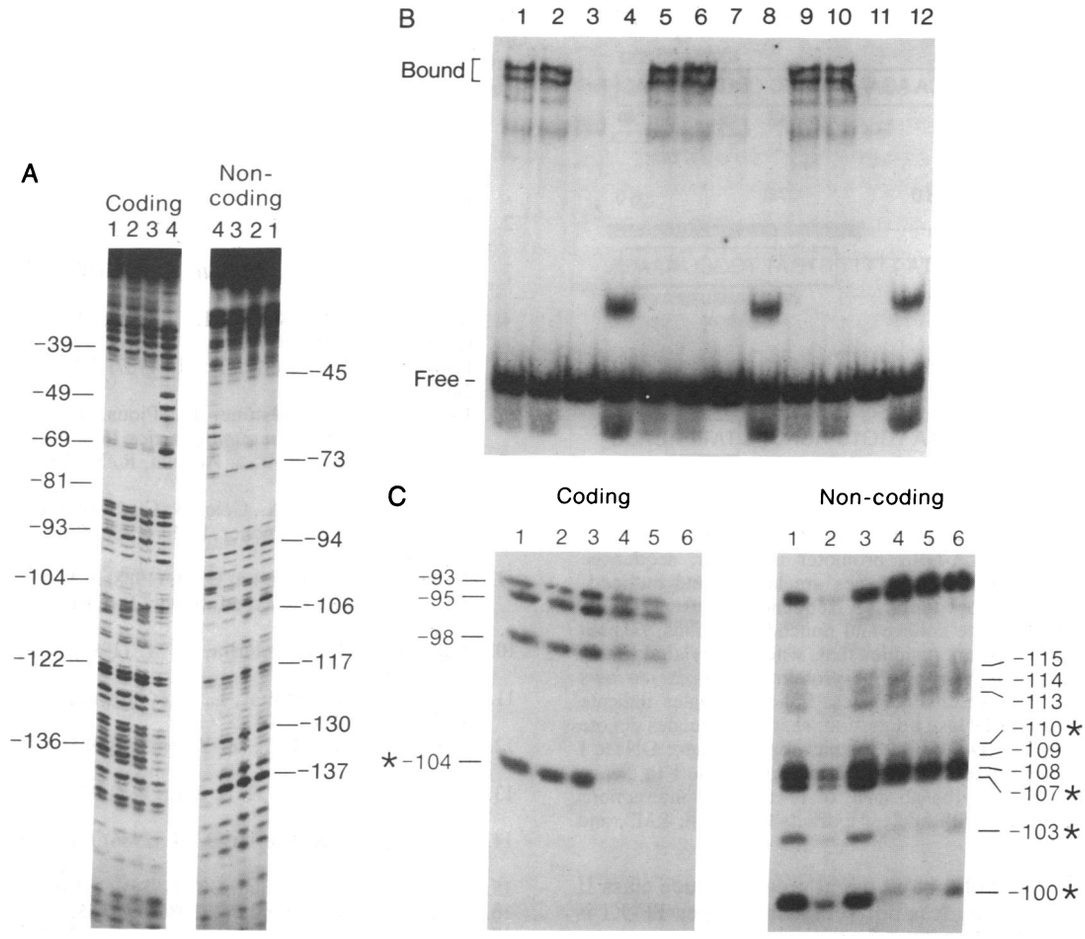

FIG. 2. DNA-binding proteins that interact with the HLA-DRα promoter. (*A*) DNase I footprinting assay. Results for the coding and noncoding strands are shown. Lanes 1, Raji; lanes 2, RM2; lanes 3, RM3; lanes 4, no protein. (*B*) Gel mobility-shift assay of X box oligonucleotide. Lanes 1–4, Raji; lanes 5–8, RM2; lanes 9–12, RM3. Lanes 1, 5, and 9, no competitor; lanes 2, 6, and 10, nonspecific competitor (Z5 oligonucleotide); lanes 3, 7, and 11, specific competitor (X3 oligonucleotide); lanes 4, 8, and 12, proteinase K. (*C*) Methylation interference assay. Bands whose relative intensity is significantly lower in the bound than in the free lane (after correcting for differences in the total radioactivity loaded in each lane) are indicated with asterisks; these represent putative DNA–protein contact points. Lanes 1–3, free DNA; lanes 4–6, bound DNA. Lanes 1 and 4, Raji; lanes 2 and 5, RM2; lanes 3 and 6, RM3.

Finally, we used a methylation interference assay to identify protein–DNA contact points within the X box and pyrimidine tract (Fig. 2*C*; summarized in Fig. 3). For protein extracts from Raji, RM2, or RM3 cells, methylation of guanine residues at position −104 on the coding strand or at position −110, −107, −103, or −100 on the noncoding strand inhibited formation of the DNA–protein complexes detected by gel mobility-shift (the bound fraction in this experiment contained all three bands, since they were not sufficiently well resolved to be eluted separately). Thus, for the subset of DNA–protein contact points detectable by this assay, the interactions between X box DNA sequences and their binding protein(s) were indistinguishable in Raji and mutant cells.

DISCUSSION

We previously described the isolation of class II MHC-negative B-cell lines derived from Raji cells and showed that their class II genes were grossly intact but not transcribed (5). In this study, we have shown that these cells are deficient in a trans-acting factor whose function in augmenting DRα transcription is mediated by way of a B-cell specific cis-acting regulatory element in the pyrimidine tract and X box of the HLA-DRα promoter. We have also shown in cell fusion experiments that the mutations in RM2 and RM3 are noncomplementing and recessive, since class II expression can be rescued by fusion to a wild-type B-cell line. The wild-type product of this locus, which interacts (directly or indirectly) with a B-cell-specific cis-acting transcriptional regulatory sequence in the X box and pyrimidine tract, is designated TF-X1.

TF-X1 may not be a DNA-binding protein, as suggested by the identical *in vitro* interactions of proteins from wild-type and mutant cells with HLA-DRα promoter sequences in three different biochemical assays. For example, TF-X1 could be a protein kinase that modifies the transcriptional activation function of a DNA-binding protein without affecting its binding. TF-X1 could be a secondary binding protein that recognizes specific preformed DNA–protein complexes at the HLA-DRα promoter, as has recently been shown for the protooncogene product FOS (26). Alternatively, the mutated variants of TF-X1 in RM2 and RM3 cells may be DNA-binding proteins that bind normally but fail to activate transcription. Finally, we cannot exclude the possibility that TF-X1 is a DNA-binding protein that escaped detection in all of our assays. In an extensive survey of the *HLA-DRα* gene and its flanking sequences, the pyrimidine tract-X box sequences in the promoter were the most active and B-cell-specific in a transient transfection assay (20, 27). Although this upstream element showed marked B-cell-specific activity, it also had some basal activity as a positive cis-acting element in non-B cells (20). Thus, it may interact with B-cell-specific and ubiquitous trans-acting factors, and mu-

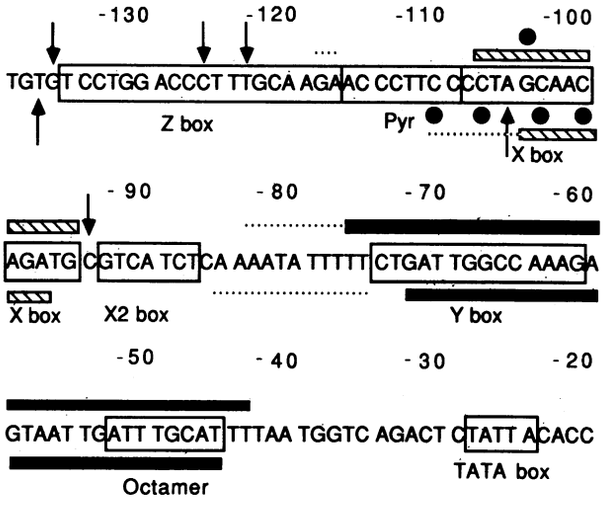

FIG. 3. Summary of DNase I footprint and methylation interference assays. The HLA-DRα promoter is shown; sequences conserved among class II MHC genes are labeled and outlined. Symbols above and below the DNA sequence represent DNA–protein interactions on the coding and noncoding strands, respectively. Solid circles indicate residues that, when methylated, interfere with formation of X box DNA–protein complexes. Arrows represent DNase I-hypersensitive sites. Solid rectangles indicate regions protected from DNase I digestion; shaded rectangles denote incomplete protection. Dotted lines depict regions where DNase I protection cannot be assessed due to failure of DNase I to cleave these sequences even in the absence of protein. All interactions depicted were identical with nuclear extracts from Raji, RM2, and RM3 cells.

tation of either class of factors might greatly reduce class II transcription in B cells. It is not yet clear whether TF-X1 is a B-cell-specific or ubiquitous factor.

Because assays for DNA-binding proteins have not revealed the identity of TF-X1, its characterization may require the cloning of its gene. Transfection of an expression cDNA library constructed from wild-type B-cell mRNA into a population of mutant cells, followed by flow cytometric sorting of cells reexpressing class II MHC determinants, should allow for isolation of the wild-type TF-X1 gene and characterization of its product.

Finally, RM2 and RM3 cells constitute a plausible *in vitro* model of the class II bare lymphocyte syndrome, in which patients' class II MHC genes are grossly intact but are not expressed in B cells due to a defective trans-acting factor (28). Fusion of RM2 or RM3 cells to patients' cells should help to determine whether TF-X1 is deficient in the B cells of some or all of these patients. If so, cloning of the TF-X1 gene may provide a means toward understanding and eventually treating this disease.

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