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RFXAP, a novel subunit of the RFX DNA binding complex is mutated in MHC class II deficiency

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Major Histocompatibility Complex class II (MHC-II) deficiency is a disease of gene regulation that provides a unique opportunity for the genetic dissection of the molecular mechanisms controlling transcription of MHC-II genes. Cell lines from MHC-II deficiency patients have been assigned to three complementation groups (A, B and C) believed to reflect the existence of distinct essential MHC-II regulatory genes. Groups B and C, as well as an *in vitro* generated regulatory mutant representing a fourth group (D), are characterized by a specific defect in the binding activity of RFX, a multimeric DNA binding complex that is essential for activation of MHC-II promoters. RFX5, a subunit of RFX, was recently shown to be mutated in group C. We have now isolated a novel gene, *RFXAP* (RFX Associated Protein), that encodes a second subunit of the RFX complex. *RFXAP* is mutated in the 6.1.6 cell line (group D), as well as in an MHC-II deficiency patient (DA). This establishes that group D is indeed a fourth MHC-II deficiency complementation group. Complementation of the 6.1.6 and DA cell lines by transfection with *RFXAP* fully restores expression of all endogenous MHC-II genes *in vivo*, demonstrating that *RFXAP* is a novel essential MHC-II regulatory gene.

Keywords: Bare Lymphocyte Syndrome/gene expression/human disease gene/MHC class II deficiency/RFX proteins

Introduction

Major Histocompatibility Complex Class II (MHC-II) molecules are heterodimeric transmembrane glycoproteins consisting of α and β chains. In man there are three MHC-II isotypes, HLA-DR, -DP and -DQ. MHC-II molecules play a key role in the immune system. They present exogenous antigenic peptides to the receptor of CD4+ T helper lymphocytes, thereby triggering the antigen specific T cell activation events required for the initiation and sustenance of immune responses (Benacerraf, 1981; Janeway *et al.*, 1984). Considering this pivotal function, it is not surprising that correctly regulated expression of MHC-II molecules represents a crucial parameter in the control of the immune response. This is exemplified by the findings that ectopic or aberrantly high levels of MHC-II expression is associated with autoimmune diseases (Bottazzo *et al.*, 1986), while a lack of MHC-II expression results in a severe immunodeficiency syndrome called MHC-II deficiency, also referred to as the Bare Lymphocyte Syndrome (BLS) (reviewed in Griscelli *et al.*, 1993; Reith *et al.*, 1995; Mach *et al.*, 1996; Reith *et al.*, 1997).

The α and β chain genes encoding the HLA-DR, -DP and -DQ MHC-II molecules are normally expressed in a coordinate and tightly regulated cell-type specific and inducible fashion (reviewed in Glimcher and Kara, 1992; Mach *et al.*, 1996). Constitutive expression is largely restricted to a limited number of cell types including professional antigen presenting cells such as B cells, cells of the macrophage/monocyte lineage and dendritic cells, and certain other specialized cell types such as epithelial cells of the thymus. The majority of other cell types are MHC-II negative but can be induced to express MHC-II genes in response to a variety of stimuli, of which the most potent and well studied is interferon-γ. This pattern of constitutive and inducible expression is controlled at the level of transcription by a highly conserved promoter proximal enhancer consisting of four *cis*-acting DNA sequences referred to as the S, X, X2 and Y boxes (reviewed in Benoist and Mathis, 1990; Glimcher and Kara, 1992; Mach *et al.*, 1996).

The identification of *trans*-acting factors controlling MHC-II gene transcription via the promoter proximal enhancer has been greatly facilitated by a genetic approach, namely the analysis of cell lines that are characterized by regulatory defects abolishing transcription of MHC-II genes (reviewed in Reith *et al.*, 1995; Mach *et al.*, 1996). The majority of these MHC-II regulatory mutants are cell lines derived from patients suffering from MHC-II deficiency, a rare autosomal recessive disease characterized by the lack of constitutive and inducible MHC-II expression in all cell types and tissues (reviewed in Griscelli *et al.*, 1993; Reith *et al.*, 1996). The genetic lesions responsible for this lack of expression lie not in the MHC-II genes themselves, but in *trans*-acting regulatory genes required for their transcription (de Preval *et al.*, 1985; Reith *et al.*, 1995; Mach *et al.*, 1996). Cell lines from MHC-II deficiency patients have been classified into at least three different complementation groups (groups A, B and C) by means of somatic cell fusion experiments (Table I) (Hume and Lee, 1989; Benichou and Strominger, 1991; Seidl *et al.*, 1992; Lisowska-Grospierre *et al.*, 1994). These complementation groups are believed to reflect the existence of three different essential MHC-II regulatory genes. In addition to MHC-II deficiency cell lines, several regulatory mutants have also been generated experiment-

Table I. Genetic, biochemical and molecular heterogeneity in MHC class II deficiency

Complementation group	Prototypical patient cell line	Prototypical *in vitro* mutant	Binding of RFX[a]	Promoter occupation[b]	Affected gene
A	BCH BLS-2	RJ2.2.5	+	occupied	CIITA[c]
B	BLS-1 Ra Na	none	–	bare	?
C	SJO Ro TF	none	–	bare	RFX5[d]
D	DA[e]	6.1.6	–	bare	RFXAP

[a]Reith *et al.* (1988); Stimac *et al.* (1991); Herrero-Sanchez *et al.* (1992); Hasegawa *et al.* (1993).
[b]Kara and Glimcher (1991, 1993).
[c]Steimle *et al.* (1993).
[d]Steimle *et al.* (1995).
[e]Patient shown to be mutated in RFXAP and allocated to group D in this paper.

ally by mutagenesis of established B cell lines (Gladstone and Pious, 1978; Accolla, 1983; Calman and Peterlin, 1987). One of these mutant cell lines, 6.1.6 (Gladstone and Pious, 1978), was reported to be the sole representative of a fourth group (Table I, group D) believed to correspond to a fourth regulatory gene (Benichou and Strominger, 1991; Seidl *et al.*, 1992). No patients belonging to group D have been described so far.

The molecular defects responsible for complementation group A reside in the gene encoding CIITA (Steimle *et al.*, 1993). CIITA is a non-DNA binding transactivator that functions as a molecular switch controlling both cell-type specific and inducible MHC-II gene transcription (Steimle *et al.*, 1993, 1994; Chang *et al.*, 1994, 1996; Chin *et al.*, 1994; Silacci *et al.*, 1994). In contrast, the defects in complementation groups B, C and D all lead to a deficiency in RFX, a nuclear protein complex that binds to the X box of MHC-II promoters (Reith *et al.*, 1988; Stimac *et al.*, 1991; Herrero Sanchez *et al.*, 1992; Hasegawa *et al.*, 1993; Durand *et al.*, 1994). Affinity purification has shown that RFX is a heteromeric complex consisting of at least two different subunits of 75 kDa and 36 kDa (Durand *et al.*, 1994). The lack of RFX binding activity in complementation group C has recently been shown to result from mutations in the gene encoding the 75 kDa subunit of RFX (Steimle *et al.*, 1995). This gene was called *RFX5* because it is the fifth member of a growing family of DNA binding proteins sharing a novel and highly characteristic DNA binding domain called the RFX motif (Steimle *et al.*, 1995; Emery *et al.*, 1996a,b).

We have now isolated a novel gene that encodes the 36 kDa subunit of RFX. This subunit was called RFX Associated Protein (RFXAP) because it is a subunit of the RFX complex and interacts with RFX5, yet it does not contain the characteristic RFX DNA binding motif. In transfection experiments, the RFXAP cDNA specifically restores expression of all MHC-II genes to normal levels in the 6.1.6 cell line (group D). Each allele of the *RFXAP* gene in 6.1.6 contains a frameshift mutation. In addition we demonstrate that the RFXAP cDNA also fully complements a cell line from an MHC-II deficiency patient, DA (Touraine *et al.*, 1978; Touraine and Bétuel, 1981). Patient DA has a homozygous frameshift mutation within the *RFXAP* gene. These results thus provide unequivocal evidence for the existence of a fourth MHC-II deficiency complementation group (group D) and demonstrate that it is due to mutations of *RFXAP*, a novel regulatory gene encoding the 36 kDa subunit of the RFX complex.

Results

Isolation of RFXAP cDNA clones

To isolate cDNAs encoding the 36 kDa subunit of RFX, the RFX complex was purified by affinity chromatography and four tryptic peptide sequences (Figure 1, P1–P4) were obtained from the 36 kDa polypeptide present in the purified fraction (see Materials and methods). A homology search identified an EST (Figure 1, HS790163) from the IMAGE consortium (Lennon *et al.*, 1996). HS790163 contains a complete polyadenylated 3′ untranslated region preceded by a short truncated open reading frame containing the last five amino acids of P3 followed by the entire sequence of P4 (Figure 1). A probe generated using the EST sequence was then used to isolate cDNA clones from a human B cell cDNA library (see Materials and methods). Four full-length cDNA clones encoding the 36 kDa subunit were isolated (Figure 1, clones 1, 2, 8 and 10). At the 5′ extremity these clones end within a 51 nucleotide region. All four clones were polyadenylated, but at three different positions in the 3′ flanking region.

The cDNAs have a 272 amino acid open reading frame containing all four peptide sequences (P1 to P4) near the C-terminal end. The initiation codon conforms perfectly to the Kozak consensus (A at position –3, G at position +4) (Kozak, 1989) and is preceded by an in-frame TAG stop codon, indicating that the coding region is complete. Although the predicted molecular weight of the encoded protein is only 30 kDa, *in vitro* translation experiments have confirmed that the apparent molecular weight in SDS–PAGE is indeed 36 kDa (data not shown).

The cDNA clones encode a novel protein exhibiting no significant sequence homology to any other known proteins. In particular, it does not belong to the RFX family of DNA binding proteins (Steimle *et al.*, 1995; Emery *et al.*, 1996a,b); it does not contain the characteristic RFX DNA binding domain, and in fact exhibits no homology to RFX5 or any of the other known RFX proteins. It is nevertheless part of the RFX complex and was therefore called RFXAP. The only features of interest in the RFXAP sequence are three regions that are rich in acidic amino

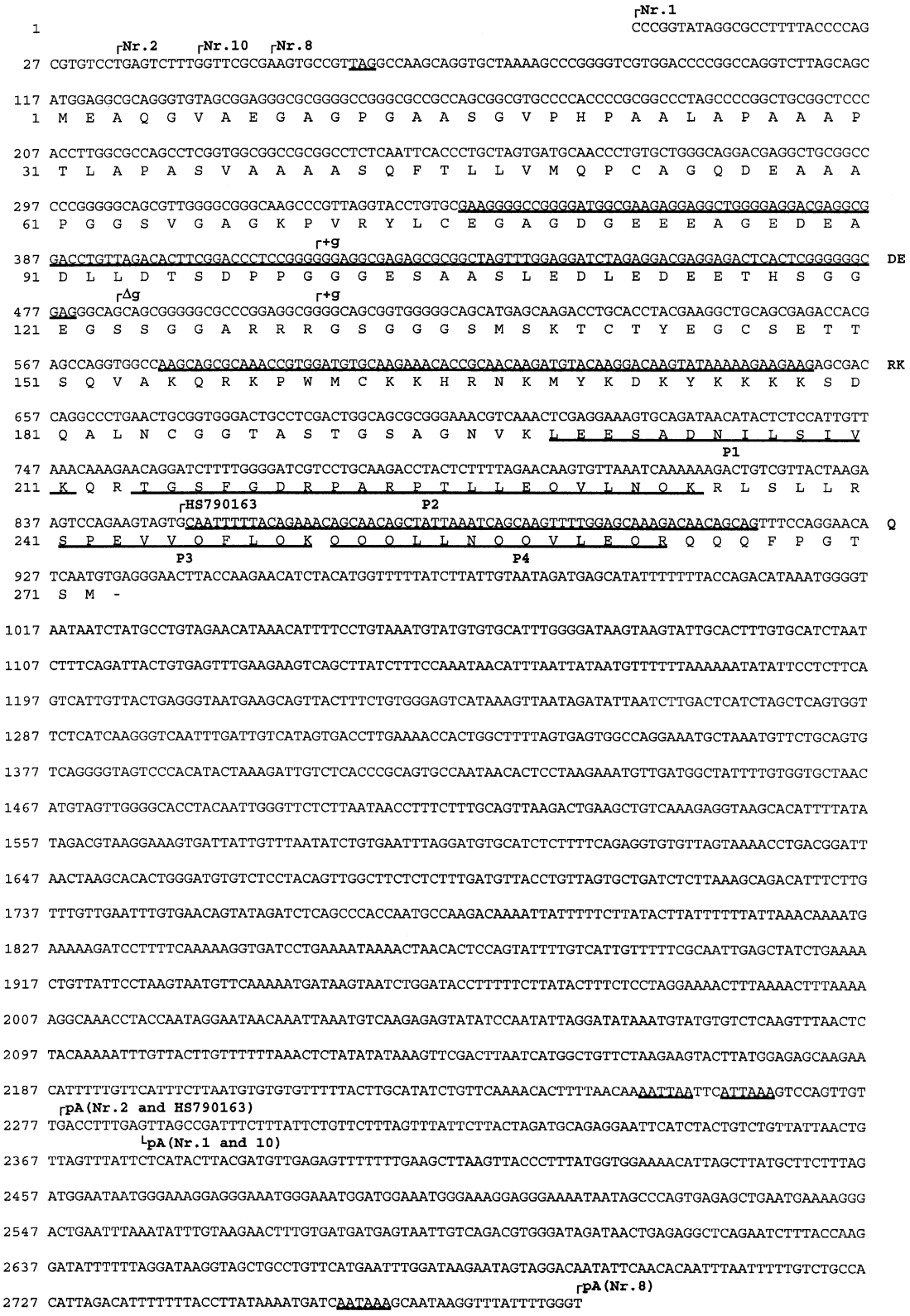

Fig. 1. Nucleotide and predicted amino acid sequences of RFXAP. The in-frame TAG stop codon preceding the translation initiation codon and the TGA stop codon terminating the open reading frame of RFXAP are underlined. Regions rich in acidic amino acids (DE), basic amino acids (RK) and glutamine (Q) are indicated. Tryptic peptide sequences (P1-P4) derived from the 36 kDa polypeptide present in the purified RFX fraction are underlined. The 5′ and 3′ ends of the EST (HS790163) and the four full-length RFXAP cDNA clones (Nrs 1, 2, 8 and 10) are indicated above the nucleotide sequence. All 3′ extremities are polyadenylated (pA). Potential polyadenylation signals preceding the three different 3′ ends are underlined. Positions of mutations in 6.1.6 (+g at nucleotides 418 and 508) and patient DA (Δg at nucleotide 484) are indicated (see Figures 4 and 6).

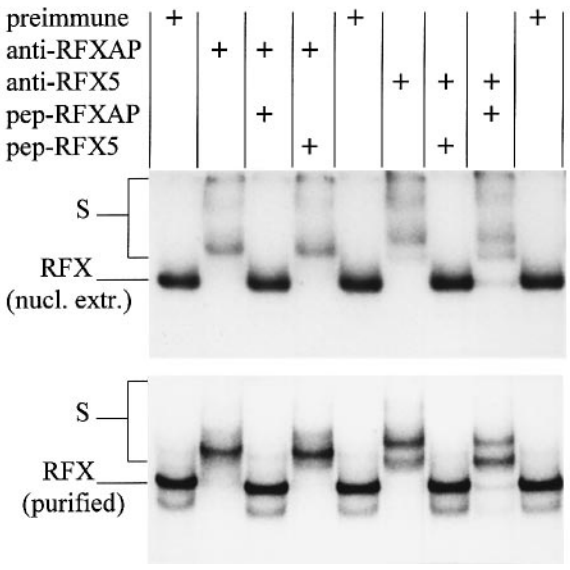

Fig. 2. RFXAP is part of the RFX complex. EMSA was performed with a double stranded probe containing the X box binding site of the DRA gene, and either a crude nuclear extract (top) or affinity purified RFX (bottom). Binding reactions were supplemented (+) as indicated above with preimmune serum, anti-RFXAP antiserum or anti-RFX5 antiserum. Specificity of the anti-RFXAP and anti-RFX5 antisera was confirmed by preincubation (+) of the antisera with the peptides (pep-RFXAP and pep-RFX5) against which they were raised. Positions of RFX–DNA complexes (RFX) and RFX–DNA complexes supershifted (S) by the antisera are indicated. Only the regions of the gels containing protein–DNA complexes are shown.

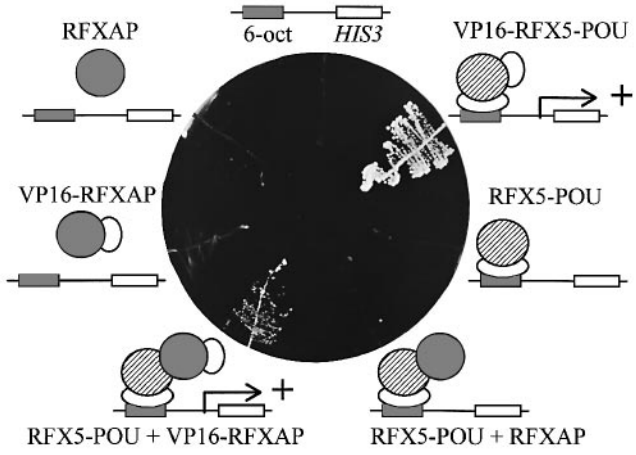

Fig. 3. RFXAP interacts with RFX5. A yeast strain (6OCT-HIS3) carrying a *HIS3* gene controlled by six octamer motifs was transformed with expression vectors directing synthesis of the various different versions of RFX5 (crosshatched) and/or RFXAP (solid) proteins indicated schematically at the side. These proteins include wild type RFXAP, RFXAP fused to the activation domain of VP16 (VP16-RFXAP), RFX5 fused to the POU DNA binding domain of Oct-1 (RFX5-POU) and RFX5 fused to both the POU DNA binding domain and the VP16 activation domain (VP16-RFX5-POU). Activation of the *HIS3* gene was assayed by analyzing growth on a plate containing AT. Growing colonies are observed only when the yeast contains the positive control VP16-RFX5-POU (upper right segment) or both VP16-RFXAP and RFX5-POU (lower left segment). In the other segments only a background representing the remains of the yeast that were streaked on the plate are visible.

acids (39%), glutamine (52%) and basic amino acids (54%) respectively (Figure 1). The acidic and glutamine-rich stretches are reminiscent of transcriptional activation domains. The basic region contains a sequence (KKHRN-KMYKDKYKKKKS) conforming to the consensus motif for bipartite nuclear localization signals (underlined), namely two basic amino acids followed by a 10 amino acid spacer and a second cluster of basic amino acids (Dingwall and Laskey, 1991).

RFXAP is part of the RFX complex

To confirm that RFXAP is indeed a subunit of the RFX complex, a polyclonal rabbit antiserum was generated against a peptide containing the last 26 amino acids of RFXAP. This antiserum was tested for its reactivity with the RFX complex in electrophoretic mobility shift assays (EMSA) using an X box DNA probe and either affinity purified RFX or a crude B cell nuclear extract (Figure 2). The antiserum supershifts RFX efficiently. Specificity of the supershift is demonstrated by the fact that it can be competed for by addition of the RFXAP peptide against which the antiserum was generated, but not by an unrelated peptide from RFX5. That the complex recognized by the RFXAP antibody is indeed RFX was confirmed by the fact that the same complex is supershifted specifically by a peptide antiserum directed against the RFX5 subunit of RFX.

RFXAP interacts with RFX5

To determine whether RFXAP interacts directly with RFX5, we first performed co-immunoprecipitation experiments with *in vitro* synthesized proteins. These experiments failed to reveal a stable protein–protein interaction between these two subunits of RFX (data not shown). We therefore used a sensitive two hybrid system in yeast to demonstrate that RFXAP interacts with RFX5. A yeast strain (6OCT-HIS3) carrying a *HIS3* allele controlled by six octamer motifs was used (Strubin *et al.*, 1995). 6OCT-HIS3 cannot grow in the presence of aminotriazole (AT), a competitive inhibitor of HIS3, unless transcription of the *HIS3* gene is activated (Figure 3). Activation of *HIS3* and growth in the presence of AT can for example be conferred by expression of the VP16-RFX5-POU fusion protein, which consists of RFX5 fused to the transcription activation domain of VP16 and the POU DNA binding domain of Oct-1 (Figure 3). The ability of 6OCT-HIS3 to grow on plates containing AT was evaluated after transformation with expression vectors directing the synthesis of various versions of RFX5 and RFXAP. Growth is observed when 6OCT-HIS3 is co-transformed with expression vectors for the two fusion proteins RFX5-POU (POU DNA binding domain of Oct-1 fused to the C-terminus of RFX5) and VP16-RFXAP (activation domain of VP16 fused to the N-terminus of RFXAP). On the other hand, no growth is observed when the yeast contain either RFX5-POU alone or VP16-RFXAP alone (Figure 3). These results demonstrate that RFXAP interacts *in vivo* with the RFX5-POU fusion protein. Additional controls have confirmed that RFXAP interacts with the RFX5 moiety of RFX5-POU, rather than with the POU DNA binding domain (data not shown).

RFXAP contains an acidic region reminiscent of transcription activation domains such as that of VP16, which are known to function in both yeast and mammalian cells. To determine whether the acidic region of RFXAP could function as an activation domain in yeast, 6OCT-HIS3

was co-transformed with expression vectors for RFX5-POU and wild type RFXAP. In contrast to the result obtained with VP16-tagged RFXAP, no growth is observed with wild type RFXAP, indicating that the acidic region of RFXAP does not function as an activation domain in yeast (Figure 3).

RFXAP restores MHC-II expression in the 6.1.6 cell line (complementation group D)

RFX binding activity is defective in MHC-II deficiency complementation groups, B, C and D (Reith *et al.*, 1988; Stimac *et al.*, 1991; Herrero Sanchez *et al.*, 1992; Hasegawa *et al.*, 1993; Durand *et al.*, 1994). In complementation group C, the lack of RFX binding activity is due to mutations of its RFX5 subunit (Steimle *et al.*, 1995). It therefore seemed likely that mutations of RFXAP could account for the lack of RFX binding activity in one of the two remaining complementation groups (B or D). An RFXAP expression vector (pCD-RFXAP) was therefore transfected into BLS-1, a cell line from complementation group B, and into the 6.1.6 cell line representing complementation group D. As negative controls, pCD-RFXAP was also transfected into cell lines from complementation groups A (RJ2.2.5) and C (SJO), which carry mutations in CIITA and RFX5 respectively (Steimle *et al.*, 1993, 1995; Mach *et al.*, 1996). Expression of MHC-II is restored in the 6.1.6 cell line (group D), while no reactivation of MHC-II expression is observed in the cell lines from the other three complementation groups (Figure 4A). Complementation of 6.1.6 with pCD-RFXAP restores expression of all three MHC-II isotypes (HLA-DR, -DP and -DQ) to levels similar to those observed in the control B cell line Raji (Figure 4B), indicating that all MHC-II α and β chain genes are reactivated coordinately. As expected from this reactivation of MHC-II expression, binding activity of the RFX complex to the X box of MHC-II promoters is also restored in the complemented cells (data not shown). In contrast to the drastic effect of RFXAP on MHC-II, no effect on cell surface expression of MHC class I (MHC-I) genes is observed (Figure 4B).

RFXAP is mutated in 6.1.6

The specific complementation obtained in 6.1.6 suggested that RFXAP was likely to be mutated in this cell line. The entire coding region of RFXAP was therefore amplified from 6.1.6 by RT–PCR, subcloned and sequenced. Analysis of eight independent subclones revealed the presence of a frameshift mutation in each *RFXAP* allele (Figure 5). One allele contains an additional G inserted into a run of six G residues (nucleotides 413–418). The second allele contains an additional G inserted into a run of four G residues (nucleotides 505–508). The resulting frameshifts lead to the use of premature out-of-frame stop codons situated at nucleotide 433 in the first allele and nucleotide 638 in the second. The nature of the two mutations identified is consistent with the fact that the ICR-191 mutagen was used to generate the 6.1.6 cell line (Gladstone and Pious, 1978). ICR-191 is a frameshift mutagen that leads preferentially to the introduction of an additional G:C base pair in runs of G:C base pairs (Miller, 1983).

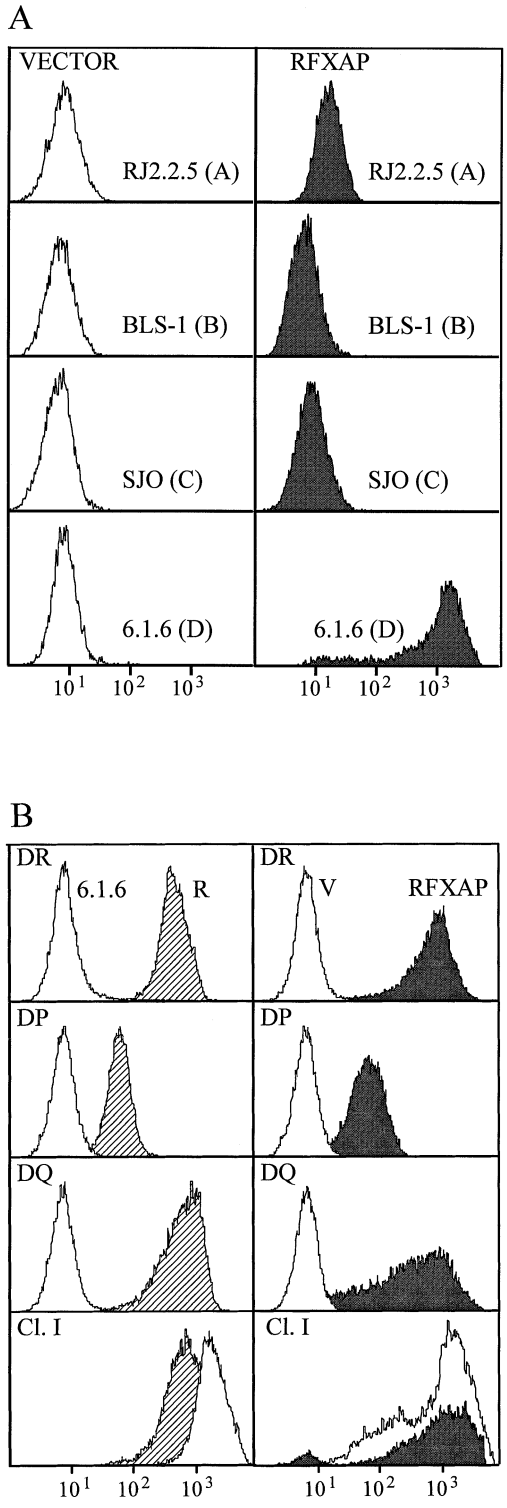

Fig. 4. Complementation of the 6.1.6 cell line by RFXAP. (**A**) RFXAP complements only the 6.1.6 cell line representing complementation group D. Cell lines from complementation groups A (RJ2.2.5), B (BLS-1), C (SJO) and D (6.1.6) were transfected with empty pCD expression vector (open profiles) or pCD-RFXAP (solid profiles). Transfected cells were stained for HLA-DR expression and analyzed by FACScan. (**B**) pCD-RFXAP restores expression of all three MHC-II isotypes in 6.1.6 cells. 6.1.6 cells (open profiles in left panels), control Raji cells (R, crosshatched profiles), 6.1.6 cells transfected with empty pCD vector (V, open profiles in right panels) and 6.1.6 cells transfected with pCD-RFXAP (RFXAP, solid profiles) were analyzed by FACScan for expression of all three MHC-II isotypes (DR, DP and DQ) and MHC-I molecules (Cl. I).

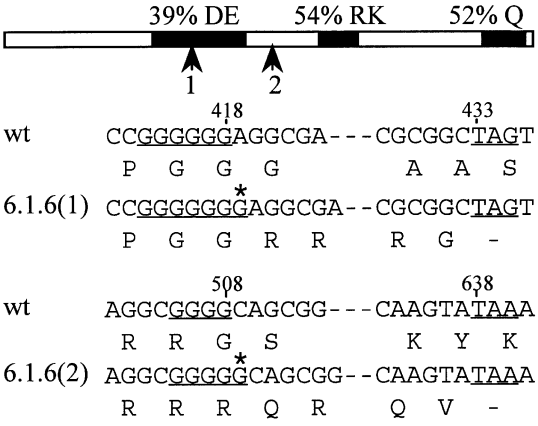

Fig. 5. RFXAP is mutated in the 6.1.6 cell line. A schematic map of RFXAP is shown at the top. The positions of regions rich in acidic amino acids (39% DE), basic amino acids (54% RK), glutamine (52% Q) and the two mutations identified in 6.1.6 (arrowheads) are indicated. Sequences of the relevant regions of wild type RFXAP (wt) and the two alleles of 6.1.6 (1 and 2) are shown. Each allele contains a G insertion (star) in a run of G nucleotides (underlined). The resulting frameshifts lead to the use of premature out-of-frame stop codons (underlined) further downstream. Nucleotide positions of the additional G and the out-of-frame stop codons are indicated above the sequences.

RFXAP complements a cell line from an MHC-II deficiency patient

No MHC-II deficiency patients have yet been assigned to the same complementation group as 6.1.6 (group D). However, cell lines from several patients have not yet been classified in the known complementation groups (A, B and C). To determine whether MHC-II deficiency patients might belong to group D, we transfected pCD-RFXAP into a number of such unclassified MHC-II deficiency cell lines. One of these cell lines, DA, is an EBV transformed B cell line established from one of the first MHC-II deficiency patients described (Touraine *et al.*, 1978; Touraine and Bétuel, 1981). Patient DA presented a clinical phenotype and immunological characteristics typical of MHC-II deficiency, although a lack of expression of MHC-II genes was not reported at the time (Touraine *et al.*, 1978; Touraine and Bétuel, 1981). The DA cell line clearly has the same MHC-II negative phenotype as EBV transformed B cell lines from other MHC-II deficiency patients, namely a complete lack of expression of HLA-DR, -DP and -DQ (Figure 6A). Transfection of DA with pCD-RFXAP restores a normal MHC-II positive phenotype (Figure 6B). Cell surface expression of the three MHC-II isotypes is restored to wild type levels, indicating that the α and β chain genes for HLA-DR, -DP and -DQ are all reactivated coordinately. In contrast to the severe deficiency in MHC-II, the level of cell surface MHC-I expression is normal in the DA cell line (Figure 6A). As observed in the case of 6.1.6, complementation by pCD-RFXAP has no effect on the level of MHC-I expression (Figure 6B).

RFXAP is mutated in patient DA

The specific complementation obtained in the DA cell line suggested that *RFXAP* was likely to be mutated in this patient. The entire coding region of *RFXAP* was therefore amplified from DA by RT–PCR, subcloned and sequenced. Six independent subclones were analyzed and

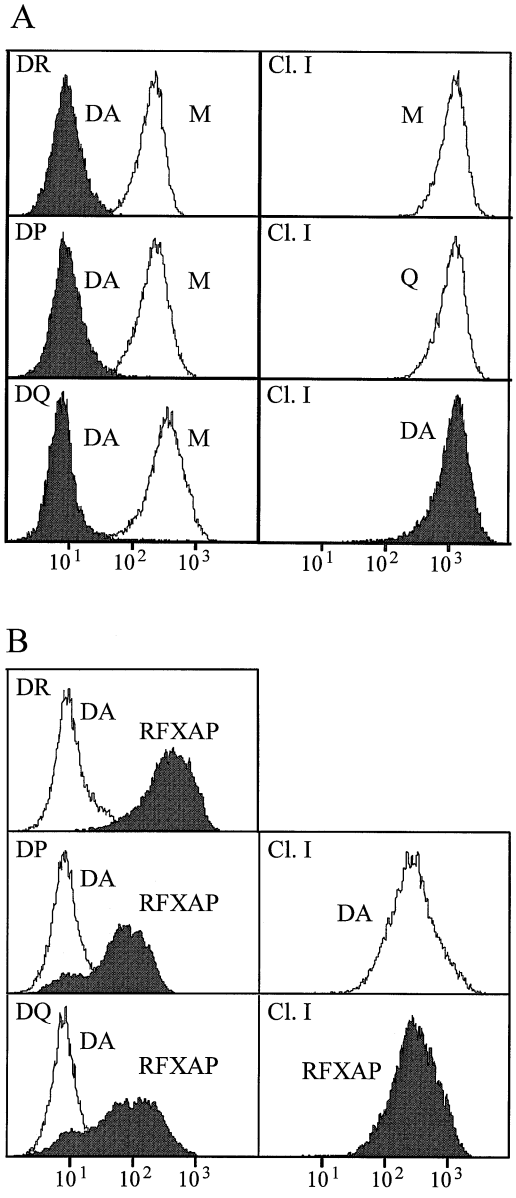

Fig. 6. RFXAP complements a cell line from an MHC-II deficiency patient. (**A**) The EBV transformed B cell line from patient DA is MHC-II negative, but expresses normal levels of MHC-I molecules. FACScan analysis of the expression of all three MHC-II isotypes (DR, DP and DQ, left panels) and MHC-I molecules (Cl. I, right panels) on the DA cell line (DA, solid profiles) and the control MHC-II positive EBV transformed B cell lines Mann and QBL (M, Q, open profiles). (**B**) RFXAP restores expression of MHC-II in DA, but does not affect expression of MHC-I molecules. FACScan analysis of the expression of all three MHC-II isotypes (DR, DP and DQ, left panels) and MHC-I molecules (Cl. I, right panels) in DA (open profiles) and DA transfected with the pCD-RFXAP (solid profiles).

all were found to contain a deletion of a G residue at nucleotide 484 (Figure 7A). The resulting frameshift leads to the use of a premature out of frame stop codon at nucleotide 525, and thus to the synthesis of a severely truncated and inactive protein of only 136 amino acids.

All cDNA clones isolated from DA contain the same deletion, suggesting that the mutation is homozygous. To confirm this, the region of *RFXAP* containing the mutation was amplified by PCR from genomic DNA and RNA. Amplification products derived from DA and a wild type

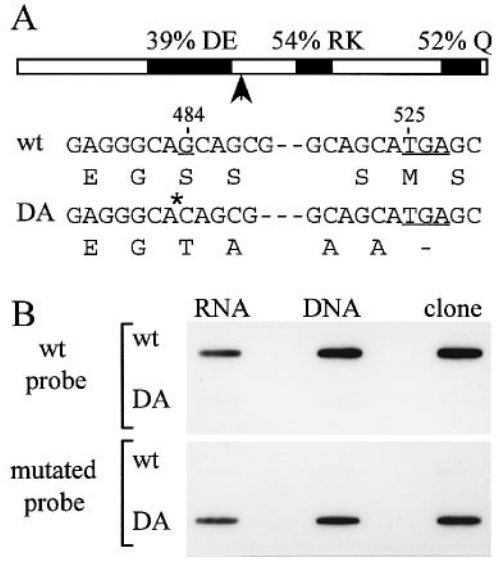

Fig. 7. RFXAP is mutated in patient DA. (**A**) A schematic map of RFXAP is shown at the top. The positions of regions rich in acidic amino acids (39% DE), basic amino acids (54% RK), glutamine (52% Q) and the mutation identified in DA (arrowhead) are indicated. Sequences of the relevant regions of wild type RFXAP (wt) and the RFXAP cDNAs isolated from DA are shown. The sequence from DA contains a deletion of a G residue (star) at nucleotide 484. The resulting frameshifts lead to the use of a premature out-of-frame stop codon (underlined) further downstream at nucleotide 525. (**B**) Only the mutated sequence is present in DA. The region containing the mutation was amplified by PCR from genomic DNA (DNA), total RNA (RNA) and RFXAP cDNA clones (clone) isolated from DA and a wild type cell line (wt). The PCR products were spotted on a filter and hybridized with oligonucleotide probes containing the wild type or mutated sequence.

cell were then either sequenced directly (data not shown) or spotted on a filter and hybridized with oligonucleotides corresponding to the wild type and mutated sequence (Figure 7B). Both direct sequencing of the PCR products and the oligotyping experiment show that only the mutated sequence is present in the DA cell line, indicating that this patient is indeed homozygous for the mutation. This is consistent with the fact that the parents of DA are consanguineous (first cousins) (Touraine *et al.*, 1978; Touraine and Bétuel, 1981). Unfortunately, the parents of DA are not available and it has thus not been possible to confirm that the two parents both carry the same mutation of the *RFXAP* gene. The oligonucleotide containing the mutated sequence hybridizes to the PCR products derived from both genomic DNA and RNA. This indicates that the deletion is actually present in the genomic gene itself, and is thus not due to a splicing defect as was observed in the case of several patients affected in *RFX5* (group C) and *CIITA* (group A).

Discussion

MHC-II deficiency is a genetic disease of gene regulation. It is due to defects in regulatory factors that are essential for both constitutive and IFN-γ inducible expression of MHC-II genes (Reith *et al.*, 1995, 1997; Mach *et al.*, 1996). Together with a number of *in vitro* generated regulatory mutants, MHC-II deficiency patients have been classified into at least four different complementation groups (A, B, C and D) believed to correspond to at least four distinct regulatory genes (Hume and Lee, 1989; Benichou and Strominger, 1991; Seidl *et al.*, 1992; Lisowska-Grospierre *et al.*, 1994). The disease thus provides a genetic approach to identify genes encoding several of the *trans*-acting regulatory factors involved and therefore represents an ideal model system for the dissection of the molecular mechanisms controlling transcriptional activation of MHC-II genes. The relevant regulatory genes can be identified on the basis of a powerful functional criterion, namely the ability to complement the genetic defect and restore expression of the endogenous MHC-II genes. Elucidation of the molecular defects in MHC-II regulatory mutants has now permitted the unequivocal identification of three essential activators of MHC-II gene transcription. Regulation of MHC-II gene expression is thus among the mammalian regulatory systems in which genetic dissection of the transcriptional control mechanism is most detailed.

A complementation cloning procedure was recently used to isolate *CIITA* and *RFX5*, the genes affected in complementation groups A and C respectively (Steimle *et al.*, 1993, 1995). RFX5 is a subunit of RFX, the MHC-II X box binding complex that is deficient in complementation groups B, C and D (Steimle *et al.*, 1995). Here we describe the isolation by a different approach of *RFXAP*, a third essential MHC-II regulatory gene. *RFXAP* encodes a novel 36 kDa protein that is a second subunit of the RFX complex and interacts with the RFX5 subunit. Mutations in *RFXAP* are responsible for the lack of RFX binding activity and MHC-II expression in 6.1.6, the *in vitro* generated cell line assigned to complementation group D. Until now, the existence of group D had been inferred exclusively from somatic cell fusion experiments with the 6.1.6 cell line, which was found to be the only representative of this group (Benichou and Strominger, 1991; Seidl *et al.*, 1992). No patients had been identified in group D. Moreover, the results of cell fusion experiments with the 6.1.6 cell line were difficult to interpret with certainty because it exhibits a leaky phenotype characterized by the presence of a small fraction of MHC-II positive cells (Levine and Pious, 1984; Benichou and Strominger, 1991; Seidl *et al.*, 1992; Lisowska-Grospierre *et al.*, 1994). Consequently, some doubt remained as to whether or not 6.1.6 represented a distinct MHC-II deficiency complementation group. This issue has now been clarified by the isolation of *RFXAP* and the demonstration that it is mutated in the 6.1.6 cell line. Moreover, the isolation of *RFXAP* has allowed us to assign a cell line from an MHC-II deficiency patient (DA) to the same group as 6.1.6. Our results thus provide definitive evidence for the existence of MHC-II deficiency complementation group D, and demonstrate that mutations of the *RFXAP* gene give rise to the same immunodeficiency disease as mutations in *CIITA* and *RFX5*.

RFXAP is a novel protein exhibiting no homology to other known proteins. Although RFXAP interacts with RFX5 in the RFX complex, it does not contain the characteristic RFX DNA binding domain and shares no homology with the RFX5 subunit or with any other proteins belonging to the RFX family of DNA binding proteins (Steimle *et al.*, 1995; Emery *et al.*, 1996a,b). This is rather surprising considering the fact that complexes containing other members of the RFX family (RFX1,

RFX2 and RFX3) are either homodimers or heterodimers in which both subunits belong to the RFX family (Emery *et al.*, 1996a).

Although we could demonstrate an interaction between RFX5 and RFXAP in a yeast two hybrid system (Figure 3), this interaction must be quite weak. It was not observed in co-immunoprecipitation experiments using *in vitro* synthesized proteins and antibodies directed against RFX5 and RFXAP (data not shown). Yet these antibodies are very efficient in recognizing these two subunits of the native RFX complex (Figure 2). Moreover, co-synthesis of RFX5 and RFXAP *in vitro* does not generate a complex capable of binding specifically to the X box (data not shown). At least two explanations could account for these observations. The RFX complex detected in nuclear extracts could contain one or more additional subunits required for stability and/or DNA binding activity. Alternatively, stable association between RFX5 and RFXAP and/or DNA binding activity of the complex could be dependent on a modifying activity, such as a kinase. The latter explanation seems unlikely because preliminary results indicate that overexpression of RFX5 and RFXAP using a vaccinia virus expression system in cells that have endogenous RFX, and should thus contain the putative modifying activity, does not result in increased levels of the RFX complex (data not shown). The existence of other subunits thus appears to be a more likely possibility, and our affinity purified RFX fraction indeed contains a number of unidentified polypeptides in addition to RFX5 and RFXAP (Durand *et al.*, 1994). In this context, it may be significant that three different MHC-II deficiency complementation groups (B, C and D) are characterized by a deficiency in the binding activity of the RFX complex. Mutations in the RFX5 and RFXAP subunits of RFX account for the existence of two of these groups (C and D respectively), but the gene affected in the third group (B) has not yet been isolated. It is tempting to speculate that mutations in an additional subunit of the RFX complex are responsible for the lack of RFX binding activity in group B.

DA, the patient shown here to have a homozygous mutation in the *RFXAP* gene, was initially described as exhibiting a reduction in the level of MHC-I expression on PBL, while expression of MHC-II was not analyzed at the time (Touraine *et al.*, 1978; Touraine and Bétuel, 1981). A relatively modest (2- to 3-fold) reduction in MHC-I expression on PBL and other cell types is observed in certain MHC-II deficiency patients and is not restricted to any particular complementation group (Touraine *et al.*, 1992; Griscelli *et al.*, 1993; Reith *et al.*, 1997). We have not observed a reduction in MHC-I on EBV transformed B cells from patient DA (Figure 6A) or patients affected in the *RFX5* (Steimle *et al.*, 1995) or *CIITA* genes (unpublished results). Moreover, complementation of these MHC-II deficiency cell lines with *RFXAP* (Figure 6B), *RFX5* (Steimle *et al.*, 1995) or *CIITA* (unpublished results) restores MHC-II expression to normal levels but does not affect the level of MHC-I. It thus seems unlikely that the reduced MHC-I expression observed in certain patients is a direct consequence of mutations in these MHC-II regulatory factors. It seems more likely that it is an indirect repercussion of the lack of MHC-II expression. It could for instance be due to the general absence of T cell activation or to the repeated infections afflicting the patients.

The previous identification of *CIITA* and *RFX5* has contributed greatly to our understanding of the molecular mechanisms controlling expression of MHC-II genes. However, a number of key questions remain unanswered. Isolation of *RFXAP*, a third essential MHC-II regulatory gene, will certainly improve and complete our comprehension of the system. Two key aspects in particular, the mode of action of *CIITA* and the role of the RFX complex, should benefit from the identification of *RFXAP*.

CIITA is a differentially expressed gene that functions as a molecular switch controlling cell-type specific and inducible MHC-II expression (Steimle *et al.*, 1993, 1994; Chang *et al.*, 1994, 1996; Chin *et al.*, 1994; Silacci *et al.*, 1994). Although the biological function of *CIITA* is now well established, its mode of action remains elusive. Since CIITA is not a DNA binding protein, we have postulated that it functions as a co-activator that is recruited to MHC-II promoters via protein–protein interactions with DNA binding proteins such as RFX, X2BP and NF-Y (Steimle *et al.*, 1993; Reith *et al.*, 1995; Riley *et al.*, 1995, Mach *et al.*, 1996). The fact that CIITA contains an acidic region that can function as a transcription activation domain is consistent with this model (Riley *et al.*, 1995; Zhou and Glimcher, 1995). However, protein–protein interactions between CIITA and proteins bound to the promoter remain to be demonstrated. Since the RFX complex is a potential target for CIITA, isolation of its different subunits (RFX5 and RFXAP), may help elucidate the protein–protein interactions that recruit CIITA to MHC-II promoters.

In contrast to the cell-type specific and inducible expression of CIITA, the RFX complex is detected ubiquitously and both RFXAP and RFX5 mRNAs are expressed in all cell types and tissues that have been analyzed (unpublished data). The RFX complex plays a key role in promoting promoter occupation *in vivo* because it binds cooperatively with other transcription factors (X2BP and NF-Y) recognizing target sites (the X2 and Y boxes) situated adjacent to the X box (Reith *et al.*, 1994a,b; Moreno *et al.*, 1995). The importance of these cooperative interactions is illustrated by the fact that cells containing mutations in RFX5 (group C), RFXAP (group D) and potentially a third subunit (group B) exhibit an unoccupied MHC-II promoter phenotype *in vivo* (Table I) (Kara and Glimcher, 1991; Kara and Glimcher, 1993). The isolation of RFXAP and RFX5 paves the way for structure–function studies that should further our understanding of the cooperative protein–protein interactions that are required for occupation of MHC-II promoters *in vivo*.

Materials and methods

Cell lines and culture

The *in vitro* generated B cell lines 6.1.6 (Gladstone and Pious, 1978) and RJ2.2.5 (Accolla, 1983), B cell lines from patients DA (Touraine *et al.*, 1978; Touraine and Bétuel, 1981), SJO (Casper *et al.*, 1990) and BLS-1 (Hume *et al.*, 1989), and the control B cell lines Raji, Mann and QBL, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin and glutamine. Cells were incubated at 37°C in 5% CO_2.

Purification and sequencing of the RFXAP polypeptide

The procedure for affinity purification of RFX has been described in detail elsewhere (Durand *et al.*, 1994). The purified fraction was estimated

to be enriched ~1500-fold and contained ~10 μg of the 36 kDa RFXAP polypeptide. Purity of the RFX fraction was assayed by EMSA and SDS–PAGE as described previously (Durand *et al.*, 1994). Tryptic peptide sequences (Figure 1, P1–P4) were obtained from the RFXAP polypeptide as described (Rosenfeld *et al.*, 1992).

Isolation and sequence analysis of RFXAP cDNA clones

One of the tryptic peptide sequences (Figure 1, P4) was identified in an EST (clone ym24f10.r1, accession number HS790163) from the IMAGE consortium (Lennon *et al.*, 1996). A PCR fragment amplified with primers based on the sequence of this EST was then used to screen a B cell cDNA library prepared as described (Strubin *et al.*, 1995). To construct this library, cDNA prepared from the B cell line Namalwa was inserted into a yeast expression vector using *Sfi*I adapters (Strubin *et al.*, 1995). Four full-length RFXAP cDNA inserts (Figure 1, numbers 1, 2, 8 and 10) were transferred into a modified Bluescript plasmid (Stratagene) containing a *Sfi*I cassette (Steimle *et al.*, 1993) inserted into the *Eco*RV site of the polylinker. All four cDNA clones were sequenced on both strands at their extremities. One clone (number 8) was sequenced completely on both strands. Sequencing was done with a T7 DNA polymerase kit (Pharmacia). The nucleotide and amino acid sequences of RFXAP were tested for homology to sequences in the EMBL, GenBank, SwissProt and dbEST databases. Sequence analysis was performed with PC/gene (Intelligenetics), the BLAST server (Altschul *et al.*, 1990) and the PROSITE dictionary (Bairoch, 1992).

Transfections and flow cytometric analysis

The RFXAP cDNA insert (clone number 1) was transferred into the *Sfi*I site of the pCD expression vector (Steimle *et al.*, 1993) to generate the plasmid pCD-RFXAP. The empty pCD expression vector or pCD-RFXAP were transfected by electroporation into RJ2.2.5, SJO, BLS-1, 6.1.6 and DA cells, and transfected cells were selected with hygromycin as described (Steimle *et al.*, 1993). Transfected RJ2.2.5, SJO, BLS-1 and 6.1.6 cells were maintained under hygromycin selection for at least 10 days prior to FACScan analysis (Figure 4). The transfection/selection procedure was less efficient for DA cells; only 30–40% of the DA cells were complemented following transfection and selection with hygromycin for at least 2 weeks (data not shown). After selection with hygromycin, these DA transfectants were therefore sorted for DR expression before FACScan analysis was performed (Figure 6). Sorting for HLA-DR expression was performed using an HLA-DR specific antibody and magnetic beads (Dynal) as described previously (Steimle *et al.*, 1993). The following monoclonal antibodies were used: the HLA-DR specific antibody 2.06 (Charron and McDevitt, 1979), the HLA-DQ specific antibody Tü22 (Ziegler *et al.*, 1986), the HLA-DP specific antibody B7/21 (Watson *et al.*, 1983), and the HLA class I specific antibody W6.32 (Serotec, Oxford, UK). Staining was done as described (Steimle *et al.*, 1993). Viable cells (10 000) were analyzed using a FACScan flow cytometer (Beckton Dickinson). Dead cells were excluded from the analysis by staining with propidium iodide and by their forward and sideways light-scattering properties.

PCR amplification of RFXAP cDNAs from 6.1.6 and DA

The entire coding region of RFXAP was amplified by RT–PCR from total RNA prepared from 6.1.6 and DA cells. Five micrograms of total RNA were reverse transcribed into cDNA in 50 μl reactions using oligo(dT) primers and Superscript II (GIBCO-BRL) as specified by the manufacturer. One to 2 μl of cDNA were then amplified by PCR using the following primers. 5′UT (5′-CAGTAGAATTCGGCCAAGCA-GGTGCTAAAAG-3′) which is situated in the 5′ untranslated region of RFXAP mRNA (nucleotides 62–81) and contains a 5′ extension with an *Eco*RI site (underlined). 3′UT (5′-CAGAGGATCCATGTAGATGTT-CTTGGTAAG-3′) which is complementary to the 3′ untranslated region of RFXAP mRNA (nucleotides 962–941) and contains a 5′ extension with a *Bam*HI site (underlined). PCR was performed with the Expand^{TM} High Fidelity PCR System (Boehringer Mannheim) under the conditions specified by the manufacturer, except that efficient amplification required the addition of 5% DMSO. A touchdown PCR cycle was used: 30 cycles consisting of a 45 s denaturation step at 95°C, a 30 s annealing step at 55–45°C for the first 10 cycles (dropping by 1°C at each cycle) and then at 45°C for the last 20 cycles, and a 45 s extension step at 72°C. PCR products were digested with *Eco*RI + *Bam*HI, subcloned between the *Eco*RI and *Bam*HI sites of a Bluescript plasmid (Stratagene) and sequenced on both strands. For 6.1.6, eight independent subclones were sequenced; three represented allele number 1 and five represented allele number 2 (see Figure 5). For DA, all six independent subclones analyzed contained the same mutation (see Figure 7).

Oligotyping of the mutation in DA

The region containing the mutation identified in DA was amplified by PCR from genomic DNA and total RNA extracted from the DA cell line and the control B cell line Raji. As controls, the same region was amplified from the wild type and mutated RFXAP cDNA clones. Reverse transcription of RNA and PCR amplification were performed as described above. Primers used were as follows. DA1 (5′-GGATGAATTCTAGA-GGACGAGGAGACTCAC-3′) which is situated upstream (nucleotides 447–467) of the mutation and contains a 5′ extension with an *Eco*RI site (underlined). DA2 (5′-GGACTAAGCTTGCAGGTCTTGCT-CATGCTG-3′) which is complementary to a region situated downstream (nucleotides 540–521) of the mutation and contains a 5′ extension with a *Hin*dIII site (underlined). The PCR products were blotted onto nylon membranes (Boehringer Mannheim) using a 'slot blotter' (Schleicher and Schuell), crosslinked to the membrane with the UV-Stratalinker 2400 (Stratagene), and hybridized with ^{32}P-labeled oligonucleotides corresponding to the wild type (5-GAGGGCAGCAGCGGG-3′) and mutated (5-GAGGGCA*CAGCGGG-3′) sequences. Membranes were prehybridized at 54°C for 30 min in tetramethylchloride (TMAC) hybridization buffer (50 mM Tris–HCl pH 8.0, 3 M TMAC, 2 mM EDTA, 5× Denhardt's, 0.1% SDS, 100 μg/ml sonicated denatured herring sperm DNA), hybridized at 54°C for 2 h in the same buffer containing 0.5×10^6 c.p.m. of probe, and then washed in TMAC washing buffer (50 mM Tris–HCl pH 8.0, 3 M TMAC, 2 mM EDTA, 0.1% SDS) at progressively increasing temperature. The final wash was at 52°C.

Antibodies and EMSA supershift experiments

A polyclonal rabbit antiserum directed against a C-terminal peptide of RFX5 (pep-RFX5; LQSSLSQEHKDP, amino acids 600–611) was generated by standard procedures using the KLH-coupled peptide. A polyclonal rabbit antiserum directed against a C-terminal peptide of RFXAP (pep-RFXAP; FLQKQQQLLNQQVLEQRQQQFPGTSM, amino acids 247–272) was prepared by Research Genetics Inc. (Huntsville, USA) using the KLH-coupled peptide. EMSA with crude nuclear extracts and affinity purified RFX were done as described (Durand *et al.*, 1994; Steimle *et al.*, 1995). For supershift experiments, antisera were added to final dilutions of 1/200 after the binding reactions were completed, and samples were then incubated for a further 30 min on ice prior to gel electrophoresis. For competitions with pep-RFX5 and pep-RFXAP, 10 μl of the antiserum were pre-incubated with 1 μg of peptide.

Yeast two hybrid system

Yeast expression vectors encoding the following proteins were constructed by standard methods. pNRFX5POU encodes RFX5-POU, which consists of RFX5 fused at its N-terminus to the nuclear localization signal (NLS) of SV40 large T antigen and at its C-terminus to the POU DNA binding domain of Oct-1 (amino acids 243–461). pNVRFX5POU encodes VP16-RFX5-POU, which is identical to RFX5-POU except that the acidic activation domain (amino acids 413–490) of VP16 (Triezenberg *et al.*, 1988) is inserted between the NLS and RFX5. pNRFXAP encodes RFXAP fused at its N-terminus to the NLS. pNVRFXAP encodes VP16-RFXAP, which is identical to RFXAP except that the VP16 activation domain is inserted between the NLS and RFXAP. The coding regions for all fusion proteins were placed under the control of the 5′ and 3′ flanking sequences of TBP (Cormack *et al.*, 1991). All RFX5 expression vectors were constructed in pRS316 (Sikorski and Hieter, 1989) which contains the *URA3* gene as selectable marker. All RFXAP expression vectors were constructed in pRSADE, a derivative of pRS316 in which *URA3* is replaced with *ADE2* (obtained from E.Gonzalez-Couto and M.Strubin, Geneva, Switzerland). The expression vectors were introduced into 6OCT-HIS3, a yeast strain carrying a *HIS3* allele under the control of six octamer motifs (Strubin *et al.*, 1995). Activation of the *HIS3* gene was assayed by growth on minimal plates containing 5–20 mM aminotriazole (AT).

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