

## MUTATION OF RFXAP, A REGULATOR OF MHC CLASS II GENES, IN PRIMARY MHC CLASS II DEFICIENCY

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### ABSTRACT

**Background** Major - histocompatibility - complex (MHC) class II deficiency is an autosomal recessive primary immunodeficiency disease in which MHC class II molecules are absent. It is a genetically heterogeneous disease of gene regulation resulting from defects in several transactivating genes that regulate the expression of MHC class II genes. The mutations responsible for MHC class II deficiency are classified according to complementation group (a group in which the phenotype remains uncorrected in pairwise fusions of cells). There are three known complementation groups (A, B, and C).

**Methods** To elucidate the genetic defect in patients with MHC class II deficiency that was not classified genetically, we performed direct complementation assays with the three genes known to regulate the expression of MHC class II genes, *CIITA*, *RFX5*, and *RFXAP*, and the relevant mutations were identified in each patient.

**Results** Mutations in the *RFXAP* gene were found in three patients from unrelated families, and the resulting defect was classified as belonging to a novel complementation group (D). Transfection with the wild-type *RFXAP* gene restored the expression of MHC class II molecules in the patients' cells.

**Conclusions** Mutations in a novel MHC class II transactivating factor, *RFXAP*, can cause MHC class II deficiency. These mutations abolish the expression of MHC class II genes and lead to the same clinical picture of immunodeficiency as in patients with mutations in the other two MHC class II regulatory genes. (N Engl J Med 1997;337:748-53.)

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**M**AJOR - histocompatibility - complex (MHC) class II deficiency, also referred to as the bare lymphocyte syndrome, is a rare autosomal recessive immunodeficiency disease in which a lack of all MHC class II molecules results in the inability to generate T-cell-dependent cellular and humoral immune responses.<sup>1-3</sup> Affected patients are extremely susceptible to viral, bacterial, and fungal infections, which usually involve the respiratory and gastrointestinal tracts. Symptoms begin in the first year of life.<sup>2,3</sup>

The clinical manifestations of the disease result from the lack of MHC class II molecules, but the primary genetic defect does not involve the MHC

class II genes themselves or their promoters. Instead, the affected genes encode transactivating factors that regulate the expression of MHC class II genes. The demonstration that the disease does not segregate with the MHC class II locus established MHC class II deficiency as a disease of gene regulation.<sup>4,5</sup> Although clinically homogeneous, primary MHC class II deficiency is genetically heterogeneous. This was shown by cell-fusion experiments that identified three distinct complementation groups (i.e., groups in which the phenotype remains uncorrected in pairwise fusions of cells). These groups were named A, B, and C.<sup>6-8</sup> Mutant cells lacking MHC class II molecules have been produced in vitro, and they can also be classified according to complementation groups. One such mutant cell line (6.1.6) belongs to a fourth complementation group (D), which differs from the groups found thus far in MHC class II deficiency.<sup>6-8</sup>

MHC class II molecules present antigens to T lymphocytes and are essential for the activation of T cells. The expression of MHC class II genes is under very tight and complex regulation. Epithelial cells in the thymus, dendritic cells, and B lymphocytes constitutively express MHC class II genes. In many other types of cells, the expression of these genes can be induced by certain stimuli, especially interferon- $\gamma$ .<sup>9</sup> In all these situations, the expression of MHC class II genes is controlled and directed by the MHC class II transactivator *CIITA*.<sup>10,11</sup>

Studies of cell lines derived from patients with MHC class II deficiency and mutant cell lines have identified three key regulatory factors: *CIITA*, *RFX5*, and *RFXAP*.<sup>10-14</sup> *CIITA* is defective in complementation group A. The molecular defect in groups B, C, and D is a deficiency of regulatory factor X (RFX), a multimeric protein complex that binds to promoters of MHC class II genes.<sup>5</sup> One subunit of the RFX complex, *RFX5*, is mutated in complementation group C.<sup>12</sup> The mutant cell line 6.1.6 (complementation group D) has a mutation in *RFXAP*, a 36-kd

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subunit of the RFX complex.<sup>13</sup> The identification of these regulatory genes has allowed us to study several patients with MHC class II deficiency whose genetic defect had not been previously classified or was thought to belong to complementation groups distinct from A, B, and C.<sup>8,15</sup> Our results show that in all the patients with bona fide MHC class II deficiency that we have studied, the mutations responsible belong to one of the four known complementation groups — A, B, C, or D.

## METHODS

### Patients and Cell Lines

The ABI fibroblast cell line was derived from a Turkish patient (Patient 1).<sup>15</sup> The ZM fibroblast and B-cell lines were derived from a Moroccan patient (Patient 2) (Fondanèche MC, et al.: unpublished data). The DA cell line was derived from an Algerian patient (Patient 3).<sup>16,17</sup> Cells were cultured, transfected, selected with hygromycin, and analyzed by flow cytometry (ABI and DA cells) or immunofluorescence (ZM cells) as described previously.<sup>10-13</sup> Transfected ABI and HeLa cells were stimulated with recombinant interferon- $\gamma$  (GIBCO) for 72 hours. Transfected ZM fibroblasts were treated with interferon- $\gamma$  for 48 hours.

### Preparatory Measures and Assays

The plasmids used to transfect the ABI and ZM fibroblast-cell lines were pREP4 (Invitrogen) and pREP4-RFXAP. The RFXAP complementary DNA (cDNA)<sup>13</sup> was cloned between the *Bam*HI and *Hind*III site of pREP4. For DA cells, the plasmids used for transfection were pCD<sup>10</sup> and pCD-RFXAP.<sup>13</sup> The <sup>32</sup>P-labeled riboprobe used to detect RFX5 messenger RNA (mRNA) was transcribed with T7 RNA polymerase from a Bluescript plasmid (Stratagene) containing a 372-bp fragment of RFX5 (nucleotides 739 to 1110). The <sup>32</sup>P-labeled riboprobes used to detect mRNA of CIITA, HLA-DRA, and guanylate-binding protein have been described previously.<sup>10,18,19</sup>

Preparation of whole-cell extracts,<sup>20</sup> procedures for electrophoretic mobility shift assays,<sup>12,21</sup> binding conditions for RFX, nuclear factor Y (NF-Y),<sup>22</sup> and the double-stranded oligonucleotides (WX2 and Y) used as probes<sup>23,24</sup> have been described previously. Binding reactions were carried out with 10  $\mu$ g of whole-cell extract. For ABI cells, decreasing amounts of nonspecific competitor DNA were used.

### RNase Protection Experiments

Cytoplasmic RNA was extracted from 5 million cells as described previously.<sup>25</sup> To generate the probes, the plasmids were linearized and transcribed in the presence of [<sup>32</sup>P]uridine triphosphate with T7 RNA polymerase in the case of RFX5, CIITA, and guanylate-binding protein; T3 RNA polymerase in the case of TATA-binding protein; or SP6 RNA polymerase in the case of HLA-DRA. The specific activity of the HLA-DRA probe was 30 times less than that of the other probes. For each sample, 30  $\mu$ g of cytoplasmic RNA was analyzed as described previously.<sup>11</sup>

### Amplification and Sequencing

Full-length RFXAP cDNA clones were isolated by a reverse-transcription-polymerase-chain-reaction (PCR) assay and analyzed for mutations as described previously.<sup>13</sup> The *RFXAP* gene was also examined for mutations by direct sequencing of PCR-amplified fragments derived from genomic DNA. The following primers were used: RFXAPC5 (5'ATGGAGGCGCAGGGGTAG3'), which is situated at the translation-initiation codon (nucleotides 116 to 133 of RFXAP cDNA), and RFX5APDA2 (5'TGCAGGTCTTGCTCATGCTG3'), which is situated between nucleotides 521 and 540 of RFXAP cDNA. PCR was performed with the Expand

high-fidelity PCR system (Boehringer Mannheim). Sequencing was performed directly with the Applied Biosystems PRISM dye terminator cycle-sequencing kit and an Applied Biosystems DNA sequencer.

## RESULTS

### Defect in the Binding of RFX to MHC Class II Promoters in Patient 1

A fibroblast cell line (ABI) from Patient 1, with an unidentified genetic defect,<sup>15</sup> was studied for the expression of mRNA for the MHC class II gene *HLA-DRA* and the transactivators CIITA and RFX5. Figure 1A shows that the level of RFX5 mRNA was normal in the ABI cell line, and that interferon- $\gamma$  induced similar degrees of expression of CIITA mRNA in ABI and control cells. By contrast, interferon- $\gamma$  induced the expression of HLA-DRA mRNA in control cells but not in ABI cells. Thus, in Patient 1 the molecular defect does not affect RFX5 mRNA or the inducibility of CIITA mRNA by interferon- $\gamma$ .

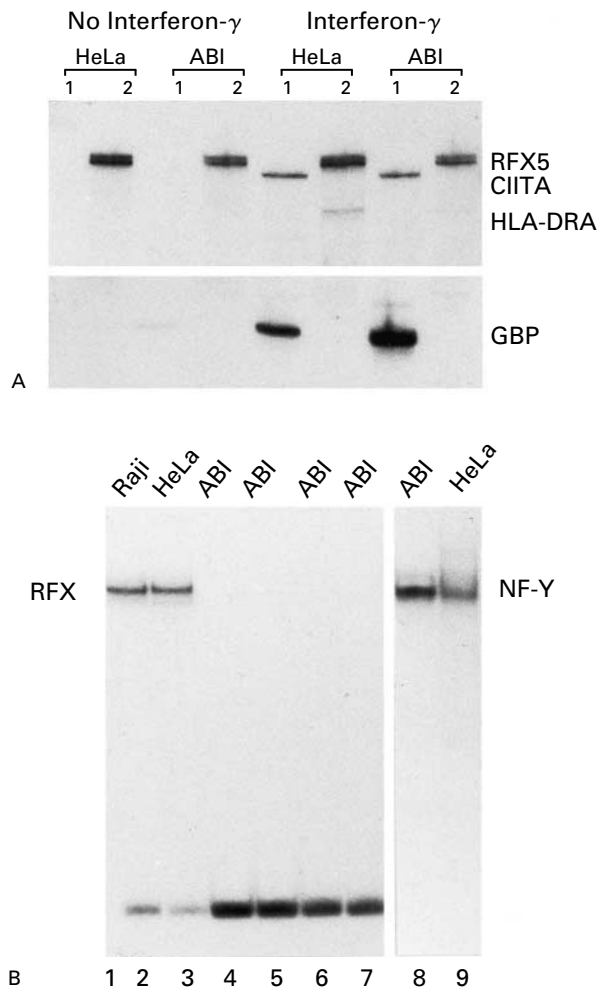
Patients with MHC class II deficiency of complementation groups B and C (as well as the mutant cell line 6.1.6, classified in group D) all have a defect that impairs the binding of RFX to the X box of MHC class II promoters.<sup>5</sup> The X box is a *cis*-acting DNA sequence characteristic of MHC class II promoters. We therefore used electrophoretic mobility shift assays to test cell extracts, which normally contain the RFX protein, for binding to an oligonucleotide containing the DNA sequence of the X box (Fig. 1B). Extracts from control HeLa and Raji cells bound RFX normally, whereas no binding to the X-box oligonucleotide was detected with extracts from ABI cells. In contrast, the NF-Y was detected in the extracts of both ABI and control cells. Patient 1 thus had the same specific defect in RFX binding that has been reported in complementation groups B, C, and D.

### Effect of Transfection with the *RFXAP* Gene on the Expression of MHC Class II Molecules in the ABI Cell Line

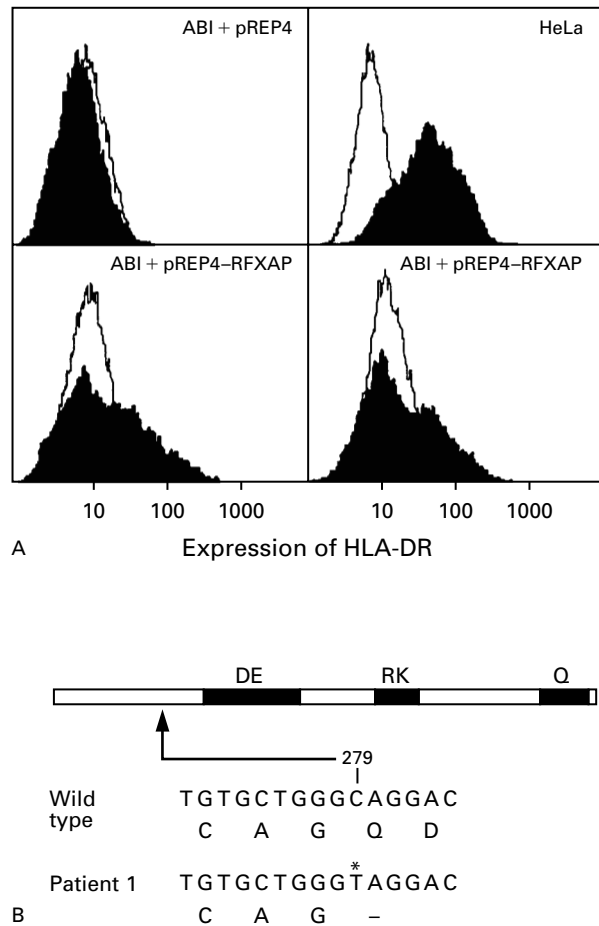
In view of the defect in RFX binding in ABI cells, we investigated whether transfection with cDNA encoding the two subunits of RFX (RFX5 and RFXAP) would allow interferon- $\gamma$  to induce the expression of MHC class II genes in ABI cells. The cDNA encoding *RFX5*, the gene affected in complementation group C, had no effect (data not shown), whereas transfection with the cDNA of RFXAP enabled interferon- $\gamma$  to induce the expression of the *HLA-DR* gene in ABI cells (Fig. 2A). The wild-type *RFXAP* gene can thus correct the defect in the expression of MHC class II in cells from Patient 1.

### The *RFXAP* Gene in Patient 1

To characterize the *RFXAP* gene in Patient 1, the entire coding region of RFXAP mRNA from ABI

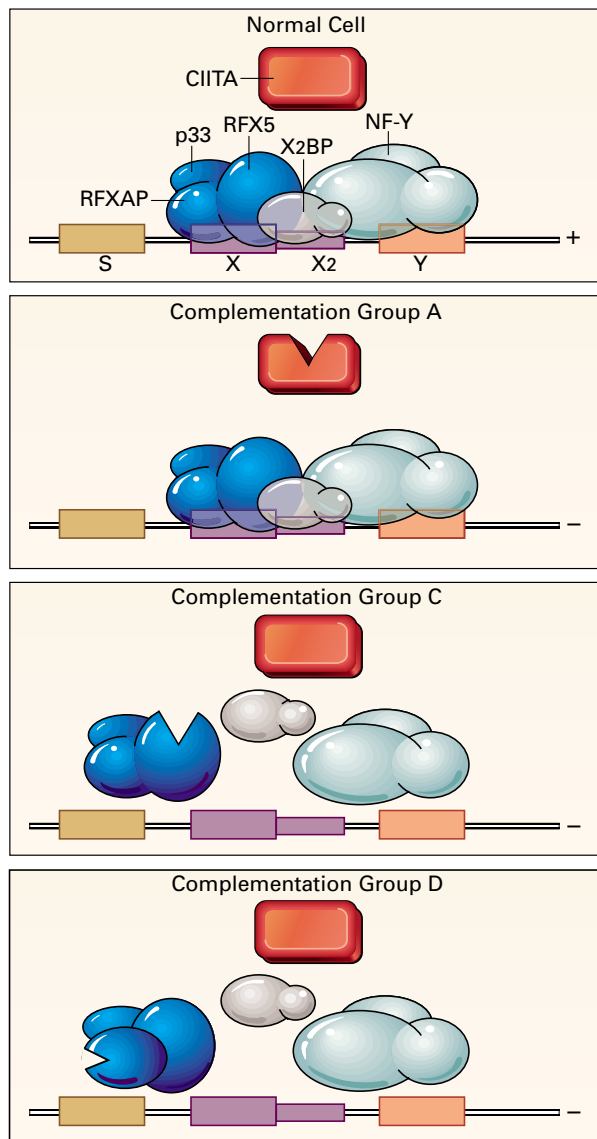


**Figure 1.** Analysis of the Expression of CIITA and RFX in Patient 1. The induction of CIITA is normal in Patient 1, as shown in Panel A. RNase protection experiments were performed with total RNA extracted from the HeLa cell line, in which the induction of CIITA is normal, and the ABI cell line from Patient 1. Each lane 1 shows probes for CIITA and guanylate-binding protein (GBP) mRNA. The GBP probe was used as a positive control for induction by interferon- $\gamma$ . Each lane 2 shows probes for RFX5 and HLA-DRA mRNA. The exposure time for GBP differed from that for the other probes (24 hours vs. 48 hours). In Panel B, there is no binding of the RFX complex in cells from Patient 1 (ABI). In lanes 1 through 7, extracts from ABI cells; a normal B-cell line (Raji), which is positive for MHC class II molecules; and the HeLa cell line were analyzed by an electrophoretic mobility shift assay with an X-box oligonucleotide (WX2) as a probe. Binding reactions with the ABI extract were done with decreasing amounts of nonspecific competitor DNA (concentrations in lanes 4, 5, 6, and 7, respectively: 1.0, 0.8, 0.7, and 0.5  $\mu$ g of poly[dIdC].poly[dIdC] per reaction, and 0.5, 0.4, 0.35, and 0.25  $\mu$ g of single-stranded *Escherichia coli* DNA per reaction). In lanes 8 and 9, binding of nuclear factor Y (NF-Y) to a Y-box oligonucleotide was analyzed in the two extracts.



**Figure 2.** Complementations of HeLa Cells and Cells from Patient 1 (ABI) by RFXAP. In Panel A, ABI cells from Patient 1 were transfected with pREP4, an expression vector, and the expression of MHC class II molecules was induced with 5000 U of interferon- $\gamma$  per milliliter (upper left-hand corner), or ABI cells were transfected with pREP4-RFXAP and expression was induced with either 1000 U of interferon- $\gamma$  per milliliter (lower left-hand corner) or 5000 U of interferon- $\gamma$  per milliliter (lower right-hand corner). Expression of MHC class II molecules by control HeLa cells was induced with 1000 U of interferon- $\gamma$  per milliliter (upper right-hand corner). Cells were stained for HLA-DR and analyzed by flow cytometry (FACSscan). The open profiles are those of uninduced cells and the solid profiles those of cells treated with interferon- $\gamma$ . Panel B shows the RFXAP protein. The position of regions rich in acidic amino acids (39 percent aspartic acid and glutamic acid; DE), basic amino acids (54 percent arginine and lysine; RK), and glutamine (52 percent; Q) are indicated. Both cDNA clones and PCR-amplified genomic DNA from Patient 1 contained a point mutation at nucleotide 279 (asterisk) that leads to a premature stop codon (TAG).





**Figure 4.** The MHC Class II Promoter and the Transcription Factors Affected in Complementation Groups A, C, and D.

*CIITA* is mutated in complementation group A.<sup>10</sup> Mutations in *CIITA* do not affect binding of transcription factors to the promoter *in vivo*. *CIITA* presumably controls transcription by contacting promoter-bound transcription factors, but its mechanism of action is unknown. RFX5 and RFXAP are two subunits of the RFX complex and are mutated in complementation groups C<sup>12</sup> and D,<sup>13</sup> respectively. A third subunit of the RFX complex is shown. X2BP and nuclear factor Y (NF-Y) are proteins that bind to the X2 and Y boxes and interact with the RFX protein complex.<sup>21,24</sup> The S box is a less well characterized binding site. A deficiency of any of the components of the RFX complex does not allow transcription of class II MHC genes. Plus and minus signs indicate transcriptional activity.

**TABLE 1.** GENETIC DEFECTS IN PATIENTS WITH MHC CLASS II DEFICIENCY, ACCORDING TO COMPLEMENTATION GROUP.

VARIABLE	COMPLEMENTATION GROUP*			
	A	B	C	D
Prototypical cell line	BLS-2	BLS-1	SJO	ABI
No. of unrelated families identified†	5	15	3	6‡
Prototypical <i>in vitro</i> mutant	RJ 2.2.5	None	None	6.1.6
Affected gene	<i>CIITA</i>	?	<i>REFX5</i>	<i>REFXAP</i>
Chromosomal location	16p	?	1q	13q

\*The nomenclature for the complementation groups follows that used by Mach et al.,<sup>5</sup> Benichou and Strominger,<sup>6</sup> and Lisowska-Grospierre et al.<sup>8</sup>

†The numbers are based on somatic-cell-fusion experiments or the identification of a mutation, or both.

‡The number is based on mutation analysis in Patients 1, 2, and 3 and on unpublished cell-fusion results in five families (Fondanèche MC, et al.: unpublished data).

DNA and serves as a general controller of both the constitutive and inducible expression of MHC class II genes.<sup>5,10,11</sup> The expression of *CIITA* is very tightly controlled by several alternative promoters.<sup>14</sup> Mutations in the gene encoding RFX5, a subunit of the ubiquitous RFX complex, cause the defect in complementation group C (Fig. 4).<sup>12,26</sup> Recently, a second subunit of the RFX complex, RFXAP, has been cloned.<sup>13</sup> We found that mutations in the *REFXAP* gene define a novel complementation group — group D (Fig. 4). The genes affected in groups A (*CIITA*) and C (*REFX5*) have been mapped to chromosomes 16 and 1, respectively.<sup>5,26</sup> Using a novel high-resolution mapping method,<sup>27</sup> we have mapped *REFXAP* (group D) to chromosome 13 (Table 1). The defect in complementation group B entails the same lack of binding of the RFX protein to the X box of the promoters of MHC class II genes as in groups C and D. We have recently identified a third protein subunit within RFX, which might be affected in complementation group B.

Mutations in *REFXAP* account for the lack of expression of MHC class II molecules in complementation group D, and all the patients that we studied were homozygous for a mutation in this gene (Fig. 2 and 3). Patients 2 and 3 have the same defect but are from different families, originating from Algeria and Morocco, respectively. An identical mutation, in a homozygous state, in such a rare genetic disease, suggests both consanguinity and common ancestry.

Patient 2 is of special interest because his genetic defect has been shown by cell-fusion experiments to be related to the defect in five other patients from three unrelated families (Fondanèche MC, et al.: unpublished data). Thus, at least eight patients from six

unrelated families have a defect in the *RFXAP* gene and thus belong to complementation group D (Table 1). This complementation group is the largest after group B. It also appears that the four complementation groups account for all currently known types of true MHC class II deficiency (Table 1). A family with an almost asymptomatic form of immunodeficiency and with only selective defects in the expression of certain HLA class II genes has been described,<sup>28,29</sup> but this atypical phenotype probably represents a distinct syndrome.

The clinical manifestations and immunologic abnormalities in MHC class II deficiency are similar in all four complementation groups,<sup>2,3,5</sup> probably because the distinct regulatory factors involved in this disease, although having very different structures and functions, are all essential for the control of the expression of MHC class II genes. Interestingly, the three regulatory genes affected in MHC class II deficiency, *CIITA*, *RFX5*, and *RFXAP*, are not only absolutely essential, with no bypass or alternative pathways, but also highly specific for MHC class II genes. These properties are unusual for transcriptional regulatory factors, which generally control multiple genes and thus have pleiotropic effects.

Symptomatic and prophylactic treatment of infections in patients with MHC class II deficiency does not prevent progressive organ dysfunction, and death is usual before the age of 18 years. Allogeneic bone marrow transplantation is considered the treatment of choice, but the success rate is lower than that for other immunodeficiency syndromes.<sup>30</sup> Now that three of the genes affected in MHC class II deficiency have been identified, gene therapy becomes a possibility in this disease. In the case of complementation group A, it would not be possible to reproduce the complex pattern of physiologic control of *CIITA* expression,<sup>14</sup> and uncontrolled expression of *CIITA* is likely to lead to aberrant expression of MHC class II molecules. In the case of the novel complementation group D, however, the *RFXAP* gene is not regulated, and gene therapy can now be envisaged.

Supported by the Swiss National Science Foundation and the Louis Jeanet Foundation.

We are indebted to M.C. Fondaneche, E. Barras, and M. Zufferey for expert technical assistance.

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