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Novel Mutations Within the RFX-B Gene and Partial Rescue of MHC and Related Genes Through Exogenous Class II Transacti
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MHC class II deficiency or bare lymphocyte syndrome is a severe combined immunodeficiency caused by defects in MHC-specific regulatory factors. Fibroblasts derived from two recently identified bare lymphocyte syndrome patients, EBA and FZA, were found to contain novel mutations in the RFX-B gene. RFX-B encodes a component of the RFX transcription factor that functions in the assembly of multiple transcription factors on MHC class II promoters. Unlike RFX5- and RFXAP-deficient cells, transfection of exogenous class II transactivator (CIITA) into these RFX-B-deficient fibroblasts resulted in the induction of HLA-DR and HLA-DP and, to a lesser extent, HLA-DQ. Similarly, CIITA-mediated induction of MHC class I, \( \beta_2 \)-microglobulin, and invariant chain genes was also found in these RFX-B-deficient fibroblasts. Expression of wild-type RFX-B completely reverted the noted deficiencies in these cells. Transfection of CIITA into Ramia cells, a B cell line that does not produce a stable RFX-B mRNA, resulted in induction of an MHC class II reporter, suggesting that CIITA overexpression may partially override the RFX-B defect. The Journal of Immunology, 2000, 164: 3666–3674.

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Abbreviations used in this paper: BLS, bare lymphocyte syndrome; CREB, cAMP response element binding protein; \( \beta_2 \)-m, \( \beta_2 \)-microglobulin; CIITA, class II transactivator; Ii, invariant chain; CAT, chloramphenicol acetyltransferase.

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MHC class II genes. CIITA augments IFN-γ induction of MHC class I and β2m genes (8–10).

Four distinct genetic complementation groups have been described in BLS (A–D) (33), and the genes affected in these complementation groups have been identified. BLS patients of complementation group A are defective in CIITA (27, 34). The genes affected in groups B, C, and D all encode subunits of the heterotrimeric phosphoprotein RFX (35). BLS groups B, C, and D are defective in RFX-B (also referred to as RFXANK) (36, 37), RFX5 (38, 39), and RFXAP (40, 41), respectively. RFX-B is the smallest (33 kDa) of the three subunits that constitute the RFX complex (36, 37). Despite evidence demonstrating that RFX-B interacts with DNA (42), the protein has no known DNA binding domain (36, 37). RFX-B contains three ankyrin repeats that may be important in the formation of the RFX complex and the assembly of the multiprotein complex involving RFX, X2BP, and NF-Y on X-Y DNA. Alternatively, these ankyrin repeats may mediate interactions of the multiprotein/DNA complex with the MHC CIITA.

Here we describe the molecular characterization of BLS patients that were found to belong to BLS complementation group B. The defects in RFX-B resulted in absence of MHC class II expression and reduced levels of MHC class I expression. Expression of exogenous CIITA, in the absence of wild-type RFX-B, resulted in the induction of appreciable amounts of HLA-DR and also of HLA- DP in RFX-B-deficient fibroblasts. Exogenous CIITA alone was able to transactivate the MHC class I and the β2m promoter in RFX-B-deficient fibroblasts as well. These results reveal that certain mutations in RFX-B can function in CIITA-mediated transactivation of MHC class II, class I, and β2m promoters and that CIITA overexpression may override the deficiency of BLS group B patients.

Materials and Methods

Cell lines

BLS patient EBA was 8 mo of age at the time he was referred to the Leiden University Medical Center for alellic bone marrow transplantation (43). Patient FZA was 14 years of age at the time of cell sampling. FACS analysis revealed that <1% of his lymphocytes stained weakly positive for HLA-DR. Patient EBA was characterized by ophthalmopathy as DR7/DRI1, and patient FZA was characterized by ophthalmopathy as DR4/CDR10. Primary fibroblasts from a skin biopsy of the two patients were first transformed with an SV40 ori plasmid and subsequently stably cotransfected with pCMVBEVNA and pRSVneo (44). SV40-transformed fibroblasts derived from BLS group C patients OSE (DR17/DR4) and SSI (DR7/DR10) and BLS group D patient ABI (DR16/DR7) were described previously (44–47). SV40-transformed JHV (DR17/DR11) and ABL fibroblasts (DR1/DR15) were derived from BLS group A and B patients, respectively (48). The BLS group B patient-derived B cell line Ramia was described previously (48). Fibroblasts and B lymphoblastoid cell lines were grown in Iscove’s modified DMEM (Life Technologies, Paisley, U.K.) supplemented with 10% (v/v) FCS (Life Technologies) penicillin (100 IU/ml), and streptomycin (100 µg/ml).

Transient heterokaryon analysis

The generation and analysis of transient fibroblast homo- and heterokaryons were essentially as described before (44). Upon treatment of fibroblasts with polyethylene glycol 4000, cells were cultured in the absence or presence of 500 U/ml of IFN-γ for 48 h. RNA was isolated from the cells and subjected to RT-PCR using HLA-DRB haplotype-specific oligonucleotides (located in exon 2) as 5′ primers and a generic HLA-DRB oligonucleotide (located in exon 3) as the 3′ primer. The RT-PCR products were size-fractionated on an 1% agarose gel, transferred to Hybond N+ membranes (Amerham, Little Chalfont, U.K.), and hybridized with biotin-labeled HLA-DRB haplotype-specific probes. The generic 3′ primer as well as the 5′ primers and biotin-labeled hybridization oligonucleotides and the critical washing temperatures were described before (44–46). To assure that the quality and the amount of the various cDNAs were similar, GAPDH-specific RT-PCR and hybridization was performed as described previously (44).

RNA and DNA hybridization analysis

For Figs. 1 and 2, total cellular RNA was prepared using RNAzolB (Cima/ Biotech, Laboratories, Houston, TX) following the manufacturer’s instructions. Twenty micrograms of total RNA was separated on an 1.2% agarose gel containing 2.2 M formaldehyde, transferred to a Hybond N membrane (Amerham), and hybridized using probes that were labeled with [32P]dCTP by random priming (DuPont-NEB, Brussels, Belgium). Transfer and hybridization were performed according to the instructions of the membranes. The human cDNA probes for MHC class I, MHC class II, invariant chain (Ii), β2m, and GAPDH were described before (43–46). For Fig. 7, RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA). Total RNA (10 µg) was used for Northern blot analysis. Random primed RFX-B cDNA (Rediprime II; Amersham) spanning exons 3–10 was used as a probe, and hybridization was conducted in UltraHyb (Ambion, Austin, TX). The blots were reprobed for GAPDH to verify equal loading of RNA. Southern blot analysis of RT-PCR products was conducted as described (49) using an end labeled primer as probe. Primers corresponding to exons 4 and 5 were 5′-CCAC CACTCTCACCACAACGG and 5′-CTGTCCACATCCACGCTGCGAC CACAG, respectively.

Plasmids and DNA transfections

The following plasmids were used in this study: pREP4-CIITA (9, 31), pSVK-FLAG-CIITA (50), pRFX-B (37), pRFX-BS1 (37), and pRFX5 (37) (pRFX5 contains an insertion of the FVX-DR DNA into pCMV3.1). The base vectors for the above plasmids, pREP4 (Invitrogen, Carlsbad, CA), pcDNA3.1 (Invitrogen), and pSVK (Pharmacia, Uppsala, Sweden) were used as controls. The promoter reporter constructs pGL3-B250, pGL3-β2m, pGL3-II, pDRCAT, and pDRCATwt have been described previously (8, 9, 31, 46). Stable cell lines were generated in fibroblasts by the calcium phosphate precipitation method (51) or electroporation (31) with the indicated plasmid and were selected in either 50 µg/ml G418 (Life Technologies) or 50 µg/ml hygromycin (Boehringer Mannheim, Mannheim, Germany) for an average of 2 wk. Resistant colonies were pooled and used for analysis of MHC surface expression.

Promoter activity assays

For luciferase containing reporter plasmids, in each of four wells of a six-well plate, 1.5 × 106 fibroblast cells were transfected by the calcium phosphate method, with a DNA mix containing 1 µg firefly luciferase pGL3 reporter plasmid (pGL3-DRA, pGL3-B250, pGL3-β2m, or pGL3-II), 1 µg Renilla luciferase pRL-TK control plasmid (Promega, Madison, WI), and 1 µg of pREP4 or 0.5 µg of pREP4-CIITA. Cells were harvested 3 days after transfection. Luciferase activity was determined using the dual-luciferase reporter assay system (Promega) and a luminescenceometer (Tropix, Bedford, MA).

For chloromphenicol acetyltransferase (CAT)-based reporter constructions, cells were cotransfected with 10 µg pRFX-B, 10 µg pSVK-FLAG-CIITA; 10 µg pDRCATwt, a reporter that contains the SXY box of class II promoter (52); and 0.5 µg of pGL3-β2m promoter vector (Promega), which encodes the luciferase gene driven by a constitutive promoter (SV40). Control transfections were conducted similarly with the pDRCAT reporter that does not contain the SXY box of class II promoter (52). Cell lysates were prepared 72 h posttransfection, and 5% of the lysate was analyzed for expression of luciferase product using the Luciferase Assay System (Promega). The remaining sample was analyzed for CAT protein using an ELISA (Boehringer Mannheim) according to the manufacturer’s instructions. The data were normalized to the expression of luciferase.

Flow cytometric analysis

To measure MHC class II and class I surface expression, cells were stained with mAbs against the HLA-DR backbones (B8.11.2) (53), -DQ backbone (SPV-L3) (54), -DP backbone (B7/21) (55), or MHC class I (WT6/32) and FITC-conjugated goat anti-mouse IgG (Becton Dickinson, Mountain View, CA). Cells stained with the corresponding conjugated or unconjugated Ig isotype were used as controls. In some experiments, the Abs described in Nagarajan et al. (37) were used. Approximately 5000 cells were analyzed on a FACScan flow cytometer (Becton Dickinson) in each assay.

RT-PCR

PolyA RNA was made using PolyA Tract mRNA isolation system (Promega). Reverse transcriptase reactions were conducted using Superscript II (Life Technologies), and PCR was conducted using native Pfui polymerase
(Stratagene, La Jolla, CA). The RFX-B cDNA from exons 2–10 was amplified using primers with restriction site overhangs; 5′-CTAGTCTA GACAGATCCTGGAGGTGC GG and 5′-CCGAGATCCCCGGAGCG GCTCTCCTC. Thirty cycles of 30 s at 95°C, 30 s at 55°C, 1 min at 72°C, and a final extension at 72°C for 7 min, were used to amplify the RFX-B cDNA for all RT-PCR samples. GAPDH was amplified from the same reverse transcription reaction as a control, using the primers 5′-CCATGGGGAAAAAGTTAGGATCG and 5′-GAGGGATGGGTG TCCGGTGTGTAC.

RFX-B cDNAs obtained by RT-PCR from patient RNAs were cloned into the pCRBlunt vector (Invitrogen) for sequencing and also cloned into the mammalian expression vector pcDNA3.1’ at XbaI/EcoRI sites.

**Genomic DNA PCR and analysis of homozygosity**

Genomic DNA from the cell lines was made as described in Ausubel et al. (56). PCR of the RFX-B gene consisted of 30 cycles of 10 s at 92°C, 30 s at 60°C, and 2 min at 68°C. The last cycle was extended for 7 min. DNA fragments encoding exons 4–8 were amplified using the primers, 5′-CCACCCACCCTCCAAACGG and 5′-CCCATTTACGTGCTCC CGCG-3′. To analyze the mutations in FZA and family members, exon 6 was amplified using primers corresponding to introns 7 and 8: 5′-GAACT GCCTGGAGATGGCAGATG and 5′-GCAGGACGGGACTACCTGC CTC, respectively. The PCR products were purified and sequenced. To detect homozygosity in EBA, PCR was conducted using Taq polymerase and primer sets specific for the mutated or wild-type sequence in exon 5. A wild-type primer forward located in exon 4, 5′-CCACCCACCCTCCAC CAAACCAC, was used with either the wild type 5′-GCTCTTCCAGGCTG GTCCAGCTC or mutated 5′-GCTCTTCCAGGCTGCTCCAGCTA reverse primers.

**Results**

**Lack of MHC class II and reduced class I expression in BLS patients**

Patients FZA and EBA presented themselves with an apparent loss of MHC class II surface expression on lymphocytes, although in the case of FZA <1% of the lymphocytes stained weakly for HLA-DR. Immortalized fibroblast cell lines were established from skin biopsies as previously described (44). To examine the expression characteristics of MHC class II (HLA-DRA), MHC class I, li, and β2m genes in fibroblasts derived from BLS patients FZA and EBA, RNA was isolated from untreated and IFN-γ-treated cells and subjected to Northern blot analysis (Fig. 1). Compared with a normal control fibroblast cell line WSI, no HLA-DRA mRNA was detected upon induction of FZA and EBA fibroblasts with IFN-γ. Furthermore, li expression both in FZA and EBA could not be detected upon treatment with IFN-γ. The amount of constitutively expressed MHC class I and β2m transcripts were greatly reduced. The expression characteristics of MHC and accessory genes in FZA and EBA is similar to our previous observations in other BLS patients that contain defects in the RFX5 and RFXAP subunits of RFX (3, 8, 44–46).

**FZA and EBA belong to BLS complementation group B**

Somatic cell fusion experiments were performed to determine the complementation group assignment of FZA and EBA. FZA fibroblasts were fused with fibroblasts derived from BLS patients JHV, ABL, SSI, and ABI, which were representative for complementation groups A, B, C, and D, respectively. The transient heterokaryons were analyzed for restoration of MHC class II expression by RT-PCR and Southern blotting for HLA-DRB. As shown in Fig. 2, A and B, both FZA and EBA were complemented by JHV, SSI, and ABI, but not by ABL and EBA. These results demonstrate that both FZA and EBA belong to complementation group B and suggest that they contain defects in the RFX-B subunit of the RFX complex (36, 37).

**CIITA-mediated transactivation of MHC genes in the absence of wild-type RFX-B**

IFN-γ induces MHC class II genes through the induction and expression of the transactivator CIITA. Expression of exogenous CIITA via plasmid transfection results in the induction of MHC class II expression of all three isotypes in many cell types (47, 57). To determine whether overexpression of CIITA could result in MHC class II expression, EBA fibroblasts were stably transfected with pREP4-CIITA. Following hygromycin selection, these cells were found to express HLA-DR and HLA-DP, and to a lesser extent HLA-DQ (Fig. 3). Expression of all three MHC class II isotypes in the absence of functional RFX has not been observed in fibroblast cells with defects in RFX5 or RFXAP (46, 47). CIITA-transfected RFX5 or RFXAP-deficient cells were found to express HLA-DR (results not shown; see Refs. 46 and 47). In addition, these CIITA-transfected EBA cells also displayed a moderate increase in MHC class I cell-surface expression, which was also not noted in CIITA-transfected RFX5 and RFXAP-deficient fibroblasts.

**RFX-B regulates constitutive and CIITA-mediated transactivation of MHC and accessory genes**

To further investigate the role of RFX-B in constitutive and CIITA-mediated transactivation of MHC class II, MHC class I, and accessory genes, transient transfection experiments were performed using FZA and EBA fibroblasts with either HLA-DRA promoter-driven CAT reporter constructions or HLA-B7, β2m, and li promoter-driven luciferase reporter constructs. For comparison to FZA and EBA, another BLS group B cell line, Ramia, was also analyzed. Ramia cells are EBV-transformed B lymphocytes and like FZA and EBA are negative for class II expression (48). Transient cotransfection of Ramia cells with the HLA-DRA reporter or a control reporter, an RFX-B expression plasmid or a plasmid expressing a splice variant of RFX-B, RFXBΔ5, showed that RFX-B but not the control vectors could complement the defect in Ramia cells (Fig. 4A). RFX-BΔ5 expression showed a slight
Because Ramia cells are B cells, exogenous CIITA expression was not required. FZA and EBA fibroblasts were similarly cotransfected; however, in these experiments a CIITA expression vector was required for expression. Similar to the flow cytometry experiments in Fig. 3, transfection of the CIITA expression vector alone results in a moderate level of expression (Fig. 4A). As with Ramia cells, transfection of RFX-B rescues the class II defect. Moreover, transfection of the RFX5 subunit in FZA fibroblasts did not result in rescue, demonstrating the specificity of the complementation.

Complementation of FZA and EBA fibroblasts with RFX-B alone has a significant impact on the constitutive levels of expression of MHC class I and B2m promoters. This is in contrast to the HLA-DRA and Ii promoter, where RFX-B had no effect on the constitutive level of expression. In combination with CIITA, all of the above mentioned promoters displayed a substantial increase in the level of expression in these RFX-B-deficient fibroblasts. Interestingly, both the MHC class I and B2m promoters were transactivated by CIITA in the absence of wild-type RFX-B. This is in contrast to RFX5-and RFXAP-deficient cells that lacked CIITA-mediated transactivation in the absence of these wild-type proteins. Together, these results suggest that RFX-B plays a critical role in the constitutive and CIITA-mediated transactivation of MHC class I and B2m genes.

Expression of RFX-B leads to surface expression of MHC class II in both Ramia B cells and FZA fibroblasts

Stable cell lines expressing RFX-B were created to determine whether RFX-B could fully complement the defect of the endogenous MHC class II genes in both Ramia and FZA cells. Flow cytometric analysis of vector-transfected or RFX-B-transfected cell lines showed that RFX-B could complement all three MHC class II isotypes (Fig. 5). FZA cells coexpressing both CIITA and RFX-B showed enhanced expression of MHC class II compared with CIITA-expressing cells. Thus, mutations in the RFX-B gene are likely responsible for the lack of class II expression in these cells.

Ramia, FZA, and EBA have different RFX-B mutations

The above results could suggest that the mutant RFX-B alleles produce proteins that could provide some function in the presence of excess CIITA. Alternatively, excess CIITA may be sufficient to drive class II expression in the absence of functional RFX-B protein. To begin to distinguish between these hypotheses, the nature of the defect and the genotypes of these cell lines were determined.
Previous analysis of RFX-B-deficient cells identified two mutant alleles, both of which affected the splicing of exon 6. One allele contained a 26-bp deletion in a region encompassing the splice acceptor site of exon 6 (36, 37). The second allele identified was a 56-bp deletion of the DNA encompassing the splice donor site 5’ to exon 6 (36). Both of these mutations cause a frame shift and a premature stop codon. To determine the mutations in each of these cell lines, RFX-B cDNA from each of the cell lines was generated by RT-PCR; however, as discussed below, full-length RT-PCR products were obtained from FZA only. Shorter transcripts were obtained from EBA and Ramia. The RT-PCR products were sequenced after cloning into PCR blunt vector. Both FZA and EBA DNA sequences each contained a single base pair substitution (Fig. 6A). FZA contained a T→C transition in exon 8, resulting in a Leu195→Pro substitution. This mutation alters a conserved leucine residue in the third ankyrin repeat of the RFX-B protein. Sequence analysis of EBA’s RT-PCR product showed that it corresponded to the sequence of RFX-BD5 as observed in wild-type Raji cells. This suggested that EBA could either contain a splice acceptor/donor mutation flanking exon 5 or a point mutation in exon 5 that could result in an unstable full-length transcript. To delineate the defect in EBA, genomic DNA was isolated from EBA cells and exons 4–8 of RFX-B gene were amplified by PCR. A transversion, which changed GAG (Glu103)→TAG (amb) was found in exon 5, thereby encoding a truncated protein. The mutation in Ramia was found to be the same 26-bp deletion described above (Fig. 6A). A smaller RT-PCR product, lacking both exons 5 and 6, was also found in Ramia cells.

Experiments were performed to determine whether the mutations were homozygous within these patients. The mutation in FZA generates a novel BsiWI restriction site. DNA from FZA and cell lines from the parents of FZA, JZA, and MZA was isolated and amplified across exon 8 by PCR. The resulting DNA was subjected to BsiWI digestion and analyzed by gel electrophoresis (Fig. 6B). Wild-type DNA (Raji) and EBA DNA were analyzed in parallel. The results show that each of FZA’s parents carries one wild-type and one mutant allele and that FZA is homozygous for the point mutation. To determine whether EBA was homozygous for the nonsense codon, wild-type and mutation-specific PCR primers were generated and used to amplify genomic DNA by PCR (Fig. 6C). The results showed that EBA DNA was amplified by the mutation-specific primer only. Control reactions included wild-type DNA from Raji cells and DNA from FZA, which is wild type at this position. Thus, both FZA and EBA are homozygous for
Ramia was also found to be homozygous by Southern blot analysis (data not shown), as described in Nagarajan et al. (37).

RNA analysis of the group B cell lines shows distinct expression patterns. The 26-bp deletion mutation that is also found in Ramia cells was shown previously to result in destabilization and loss of the RFX-B mRNA. To determine whether the same were true for the FZA and EBA mutations, RNA analyses by Northern blotting and RT-PCR were performed (Fig. 7). Northern blot analysis of Ramia, FZA, and EBA showed two patterns. Ramia and EBA were found to express undetectable and very low levels of RFX-B mRNA by Northern blot, respectively, whereas, FZA expressed wild-type RFX-B mRNA levels (Fig. 7A). RT-PCR of exons 2–10 was conducted to determine whether RNA could be detected in EBA cells (Fig. 7B). RT-PCR of RFX-B from the wild-type Raji cells shows both the full-length RFX-B band (upper) and the minor splice variant, RFX-BΔ5 (lower band). FZA produces an identical pattern to the wild type. Surprisingly, EBA displays only the lower band. To demonstrate that this lower band was in fact the splice variant, a Southern blot was performed on this gel using probes specific for exon 5, which is missing in the splice variant, and exon 4, which is present in the splice variant. The result showed that EBA expresses the splice variant, but not the full-length mRNA that would contain the nonsense codon. As described above, this result was verified by sequencing the RT-PCR product. Thus, by expressing the splice variant, EBA cells can bypass the nonsense codon generated by the mutation. However, this transcript is not stable in cells and is expressed at very low levels, suggesting that if any protein is synthesized it would be low in abundance.

**RFX-B<sup>FZA</sup> can provide a low level of expression**

Because FZA produces normal RFX-B mRNA levels, it is possible that the RFX-B<sup>FZA</sup> protein can stimulate a low level of MHC class II expression. Similarly, the RFX-BΔ5 splice variant, which is found in EBA, may also be able to provide some level of MHC class II expression. To assay these variants, the RFX-B<sup>FZA</sup> mRNA and the splice variant were cloned into the mammalian expression vector pcDNA3.1. Ramia cells, which are deficient for both the wild-type and splice variant, were cotransfected with the above expression vectors and a DRA promoter-dependent reporter construct (pDRCATwt) or its control vector (pDRCAT). The results showed that the splice variant could provide a small increase in expression of the MHC class II promoter-dependent reporter over...
the background associated with this vector (Fig. 8). However, RFX-B^{FZA} overexpression resulted in almost one-third of the wild-type activity. Thus, the ability to transactivate MHC class II expression by the mutant proteins may be partially achieved when the levels of CIITA reach a threshold level, such as that in normal B lymphocytes, or through the overexpression of CIITA from an exogenous promoter.

RFX-B-independent activation of MHC class II expression

As stated above, Ramia cells produce an unstable RFX-B mRNA that is not detectable by Northern analysis. Thus, these cells are most likely devoid of RFX-B protein. However, Ramia cells do express normal B cell levels of CIITA. Thus, this cell line could serve as a model for overexpression of CIITA in the absence of RFX-B. Therefore, to determine whether CIITA could transactivate in the absence of RFX-B, a CIITA expression vector was transfected into Ramia cells (Fig. 9). This resulted in a 4- to 5-fold increase in the activity of the cotransfected DRA reporter. As above, transfection of the RFX-B expression vector yielded a 12-fold increase in expression. Interesting, expression of both RFX-B and CIITA vectors resulted in a 26-fold increase, suggesting that CIITA expression was limiting in these cells. Moreover, mRNA from wild-type RFX-B or the mutant alleles expressed in FZA or EBA were not found to be induced by treatment of the cells with IFN-γ or in cells transfected with CIITA (data not shown). These experiments eliminate the possibility that CIITA or IFN-γ treatment might contribute to class II expression through an increase in RFX-B levels. Thus, these data demonstrate that CIITA overexpression can partially override the RFX-B defect and provide a mechanism by which EBA cells can express class I, class II, and β₂m genes following CIITA transfection but not following IFN-γ treatment.

Discussion

The cloning of the RFX-B/ANK gene (36, 37) completed the identification of the genes responsible for the four major BLS complementation groups. BLS group B, the largest of the complementation groups, was associated initially with two deletion mutations in the RFX-B gene, both of which involved the splicing of exon 6 (36, 37). Disease progression in this group is variable, and thus investigating the genetic basis of patients in this group may shed light on the regions of RFX-B required for expression of MHC and their related genes. The analysis of cells derived from two new patients, FZA and EBA, showed the ability of these cells to induce MHC class I and all MHC class II genes in response to overexpression of CIITA, a property not observed with other BLS complementation groups. A third patient cell line, Ramia, belonging to the same complementation group was also analyzed. Ramia cells showed no class I expression despite normal constitutive levels of CIITA. As discussed below, the relative phenotype caused by the mutations in FZA and EBA are distinct from that of Ramia and may explain the variability of disease seen in BLS group B patients. This may correlate with residual levels of MHC class II expression as noted on lymphocytes of patient FZA and EBV B lymphoblastoid cell lines of EBA (results not shown), which is corroborated by the in vitro expression studies presented in Figs. 4 and 8.

The partial restoration of MHC class II, class I, II, and β₂m expression following overexpression of CIITA in the FZA and EBA fibroblasts suggests that the mutant RFX-B proteins are either partially active or that the CIITA overexpression can compensate in some manner for a mutated RFX-B protein. The data argue for a combination of these two view points. Unlike RFX-B mRNA in EBA and Ramia, RFX-B^{FZA} mRNA is expressed at normal levels and is not rapidly degraded. The fact that one-third of wild-type activity is observed when the RFX-B^{FZA} mRNA in Ramia cells suggests that the mutant protein is produced. The development of an antiserum to RFX-B will allow confirmation of this prediction. Thus, with regard to RFX-B^{FZA}, it is likely that the protein is partially functional. The RFX-B^{FZA} mutation changes Leu₁⁹⁵ → Pro in the third ankyrin repeat of the RFX-B protein. These ankyrin repeats are conserved domains that are involved in protein-protein interactions and have α helical structures. The proline substitution in RFX-B^{FZA} may affect the α helix within the third repeat. We have recently found that the third ankyrin repeat is required for association of RFX-B with the other two RFX subunits, RFX-5 and RFXAP (DeSandro, Nagarajan, and Boss, unpublished data). It is not known at the current time whether FZA mutation specifically affects the interactions between the three subunits, although this is a likely prediction from our current data.

The EBA mutation provides an opportunity to examine the role of the RFX-B splice variant. The stop codon caused by the single base pair mutation results in a frame shift and an unstable mRNA for the full-length transcript. However, because the splice variant fortuitously removes the exon encoding the mutation, it is unaffected by the mutation. A low level of the splice variant mRNA can be detected by overexposure of a Northern blot. RT-PCR readily detects this transcript as shown in Fig. 7. Expression of the
splice variant mRNA does not normally rescue RFX function or MHC class II expression. This could be due to instability of the RFX-B RNA or to the protein that it encodes. However, in the presence of excess CIITA, a low but significant level of MHC class II and an increase in MHC class I expression can be detected in EBA cells. Additionally, overexpression of the splice variant in several of the experiments presented showed a low level of MHC class II expression. One interpretation of this result is that the low level of splice variant-generated protein may function in these as- says. In contrast, the mutation in Ramia, which results in a frame shift in exon 6 and unstable mRNA, is completely devoid of RFX activity. The splice variant would also be affected by this mutation as well, as it includes exon 6. Thus, these three mutations may be grouped into three categories with regard to the transcriptional potential of RFX-B: partial activity in FZA, low activity in EBA, and no activity in Ramia.

The finding that IFN-γ treatment of FZA and EBA cells did not induce MHC class I or II expression but that CIITA expression in these cell lines did was intriguing and suggested that CIITA may partially override the RFX-B defect. This hypothesis was tested by transfecting CIITA into Ramia cells, which lack RFX-B. The results showed a 4- to 5-fold increase in expression of the MHC class II reporter gene and demonstrated that CIITA could compensate for the RFX-B defect. We have found in other experiments that CIITA expression from the pcDNA3.1 vector provides at least a 3-fold increase in the level of CIITA mRNA in transfected cells compared with IFN-γ-treated cells (data not shown). Additionally, in stable cell lines, CIITA is constitutively expressed and always present, allowing accumulation of CIITA protein. Thus, the effect is likely to be greater in the EBA and FZA cell lines stably trans- fected with CIITA.

What is CIITA’s role in these experiments? It is possible that CIITA stabilizes weak interactions at the class I or even class II promoters. There is evidence to suggest that CIITA expression could enhance the in vivo footprint in cells that are normally class II negative (58–60). Thus, in the case of the EBA and Ramia RFX-B alleles, CIITA may stabilize the promoter complex in the absence of a functional RFX-B protein. In doing so, CIITA would then be able to activate transcription. The FZA allele is expressed and may provide partial activity to the RFX complex, as indicated by partial rescue of Ramia cells. Because MHC class I genes use other elements for their expression and induction by IFN-γ, which are located upstream to the SXY motifs, it is possible that these elements provide additional stability or aid in the assembly of the SXY-specific factors when CIITA is expressed. Thus, deficiencies in RFX-B may not be sufficient to prevent transcription of MHC class II genes under all conditions. Therefore, it is not surprising that some of the RFX-B-deficient patients have lived into adult- hood. The analysis of the three patient cell lines in this report provide evidence for single base pair substitution within an im- portant regulatory element. Thus, this analysis questions whether there are other alleles for complementation group B. It is possible that such mutations may be plentiful in humans and that there is allowance at this locus and protein structure for expression and a viable immune system.

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