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Two distinct genetic loci regulating class II gene expression are defective in human mutant and patient cell lines

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Heterokaryons were prepared and analyzed shortly after cell fusion using two mutant class-II-negative human B cell lines (RJ 2.2.5 and 6.1.6) and a cell line (TF) from a patient with a class-II-negative Bare Lymphocyte Syndrome. The resulting transient heterokaryons were analyzed by using an anti-HLA-DR monoclonal antibody to assess the cell surface expression of HLA-DR (the major subtype of class II antigens) by immunofluorescence microscopy and by using uniformly ^{32}P-labeled SP6 RNA probes in Northern blots and RNase protection assays to assess mRNA synthesis. We find that class II gene expression in a B cell line from a Bare Lymphocyte Syndrome patient (TF) is rescued by a B cell line which expresses class II antigens indicating that this disease, at least in part, is caused by a defect(s) in a genetic locus encoding a factor(s) necessary for class II gene expression. Secondly, reciprocal genetic complementation was demonstrated in the heterokaryons 6.1.6 × RJ 2.2.5 and TF × RJ 2.2.5 (but not in TF × 6.1.6) by detection of cell surface DR by immunofluorescence microscopy and by a novel class II mRNA typing technique which allows characterization of distinct class II alleles. Thus, the two mutants generated *in vitro* have defects at two different genetic loci encoding specific regulatory factors necessary for human class II gene expression. One of these mutant cell lines, but not the other, complements the defect in the patient cell line, TF.

Key words: Bare Lymphocyte Syndrome/mutant B cells/transient heterokaryons/genetic complementation/HLA class II RNA typing

Introduction

The fundamental function of the immune system is to discriminate between 'self' and 'non-self' and to destroy 'non-self'. In man, the key self–non-self recognition system is encoded by a chromosomal segment called the major histocompatibility complex (MHC) (Benacerraf, 1981; Dausset, 1981; Snell, 1981), which contains genes coding for two classes of extensively polymorphic cell surface antigens, each of which is developmentally regulated in a distinct manner. Class I antigens are expressed on virtually all nucleated cells in the adult and function primarily as restriction elements in antiviral immunity. In contrast, class II antigens have a restricted tissue distribution and are normally expressed on peripheral B cells and thymic epithelium. Class II expression can be induced on human T cells by mitogens or antigens (Ko *et al.*, 1979) and on macrophages, fibroblasts and endothelial cells by γ-interferon (Pober *et al.*, 1983). These cell surface glycoproteins are two-chain heterodimers (α, 34 kd and β, 29 kd) which play a key role in the immune response by presenting foreign antigens to T cells (reviewed by Kaufman *et al.*, 1984; Korman *et al.*, 1985). Induction of class II antigens leads to the ability of a cell to become an antigen-presenting cell in a syngeneic situation or to act as target for rejection in an allogeneic situation (as in organ transplantation). Furthermore, complete loss of class II gene expression or inappropriate class II gene expression occurs in several human diseases [Bare Lymphocyte Syndrome in the first case (Griscelli *et al.*, 1984; Hadam *et al.*, 1984; Touraine *et al.*, 1984; Zegers *et al.*, 1984), autoimmune disease in the second (Hanafusa *et al.*, 1983)].

Although the class II gene family has been intensively studied for its immunological interest, the molecular mechanisms of class II gene expression have only been studied in recent years. Studies of the *cis*-acting elements which regulate expression of class II genes demonstrate an unusual complexity of regulatory DNA sequences; much less is known about *trans*-acting factors which regulate class II gene expression and how this regulation results in class II gene expression (Gillies *et al.*, 1984; Boss and Strominger, 1986; Miwa and Strominger, 1987; Dorn *et al.*, 1987a,b; Miwa *et al.*, 1987; Sherman *et al.*, 1987; Widera *et al.*, 1987).

Two separate mutants of human B lymphoblastoid lines, 6.1.6 and RJ 2.2.5, were generated *in vitro* by immunoselection against parental DR antigens (Gladstone and Pious, 1978; Accolla, 1983). These mutant lines do not express any of the three subtypes of class II antigens (DR, DQ and DP) encoded by genes spanning ~1000 kb in the class II region of the human MHC on chromosome 6. More recently two additional examples of similar mutations were reported (Calman and Peterlin, 1987). Southern blots have shown that all of the α and β chain genes encoding these antigens are intact in these two mutants, but Northern blots revealed that they have little or no class II mRNA. Somatic cell hybridization of each of these mutant cell lines with normal or unrelated human B lymphocytes or normal mouse B cells resulted in stable hybrid cell lines which express the class II genes of the mutant cells. Therefore, a *trans*-acting factor provided by the fusion partner permits re-expression of the class II genes in the mutant cells. The mutants were therefore suggested to have a defect in a genetic locus encoding regulatory factor(s) required for class II gene expression (Gladstone and Pious, 1980; Accolla *et al.*, 1985). Activation

of specific alleles of class II genes in T cells has also been observed in some stable B × T cell hybrids, indicating that class II genes in T cells can be activated by the same factors active in B cells (Salter *et al.*, 1985). Finally, B cells from patients with a class-II-negative Bare Lymphocyte Syndrome have a phenotype similar to that of the two mutant cell lines (Griscelli *et al.*, 1984; Hadam *et al.*, 1984; Touraine *et al.*, 1984; Zegers *et al.*, 1984). In these patients, the absence of expression of class II antigens on lymphocytes and macrophages explains the immunological unresponsiveness and the clinical course often ending in death from infections. Limited family studies have indicated that the genetic defect does not segregate with the MHC, implicating a defect in a gene encoding a *trans*-acting factor(s) which controls the expression of class II genes (dePreval *et al.*, 1985). There have been no reports using somatic cell fusion techniques to demonstrate directly that B cells from patients with the Bare Lymphocyte Syndrome have a defect which might be rescued by a *trans*-acting factor from normal B cells.

In the present studies, the question of whether normal B cells containing factors needed for class II gene expression could rescue class-II-negative patient B cell lines has been examined. Moreover, the question of whether the two *trans*-acting factor-defective mutant B cell lines generated *in vitro* and a B cell line derived from a patient with Bare Lymphocyte Syndrome have defects in the same genetic locus, has been studied by carrying out fusion of these cells with each other. Technically, isolating stable lines after cell fusion has two major problems. First, many generations of cell growth are required before the hybrids can be isolated and analyzed and chromosomes in the hybrids are selectively lost during passage in culture. Second, the stable hybrids are synkaryons in which the nuclear components of one parental cell type are physically mixed with those of the other, leaving open several possibilities for correction of genetic defects. To overcome these problems, the transient heterokaryon assay described by Blau *et al.* (1983) and further successfully applied to the study of human globin (Baron and Maniatis, 1986) and β-interferon gene expression (Enoch *et al.*, 1986) was used in this study. The advantage of this approach is that in heterokaryons the nuclei of two parental cell types remain separate and distinct and they retain a full complement of both parental sets of chromosomes. Thus, gene activation, if it occurs, must occur in *trans*.

Results and discussion

De novo synthesis of DRα in patient B cells (TF) after fusion with the human mutant B cell line 9.22.3

The B cell line, TF, was derived from a Dutch patient by transformation of peripheral blood lymphocytes with Epstein–Barr virus (EBV). TF, the first child of healthy consanguineous parents, was diagnosed as having a class-II-negative Bare Lymphocyte Syndrome (and was nursed in a semi-sterile environment). His B cells and the cell line derived from them failed to express all class II antigens (Table I). Fluorescence activated cell sorter (FACS) profiles using a panel of different anti-DR, -DQ and -DP monoclonal antibodies (mAbs) revealed that DR, DQ and DP class II antigens were all absent on TF cell surfaces. Furthermore, Northern blots using DR, DQ and DP cDNA probes revealed that no class II mRNA could be detected in TF cells. The family study, i.e. two healthy parents with two diseased children, suggests that the disease is autosomal recessive (Zegers *et al.*, 1984; Rijkers *et al.*, 1987). Interestingly, early in life, class II antigens were detectable on mitogen-activated T lymphocytes of the patient, suggesting that class II genes themselves are not deleted in TF cells. The global inability to express all the three subsets of class II genes may result from genetic defect(s) which arrest class II gene expression, as has been demonstrated for the mutant cell lines RJ 2.2.5 and 6.1.6. To test the possibility that the defect might be rescued by providing a missing regulatory factor(s), the cell line TF was fused to another mutant B cell line, 9.22.3, to prepare transient heterokaryons. 9.22.3 cells, derived from the human B cell line T5-1, have a unique phenotype (Table I). All of the DR and DQ genes were deleted in one MHC haplotype while only the DRα gene was deleted in the second MHC haplotype.

Thus, DQ and DP are normally expressed on the surface of 9.22.3 cells (although at characteristically lower levels normally than DR); DR is, however, not expressed, because the DRα genes were physically deleted in both chromosomes. Thus 9.22.3 cells should contain all the regulatory factors necessary for class II gene expression. Therefore, DR antigen expressed on the surface or DRα mRNA synthesized in the heterokaryon TF × 9.22.3 should result from *trans*-activation of the intact DRα gene in TF cells. This expression would be mediated by diffusible factors from

Table I. Properties of B cell lines used

		Origin	Gene expression[a]		
			DR	DQ	DP
Cell line	DR type				
T5-1	DR1, DR3	EBV-transformed B cell	+	+	+
6.1.6	(DR1, DR3)[b]	Derived from T5-1	–	–	–
9.22.3	(DR1, DR3)	Derived from T5-1	–	+	+
Raji	DR3, DR10	EBV-transformed B cell (Burkitt's lymphoma)	+	+	+
RJ 2.2.5	(DR3, DR10)	Derived from Raji	–	–	–
TF	DR2?, DR5?	Bare Lymphocyte Syndrome	–	–	–
Fused cells	Expected DR type				
6.1.6 × RJ 2.2.5	DR1, DR3, DR10				
TF × RJ 2.2.5	DR2, DR5?, DR3, DR10				

[a] + and – refer to transcriptional and translational expression.
[b] Parentheses refer to the DR type of the parental cell line. The genes are intact but not expressed in 6.1.6 and RJ 2.2.5. In 9.22.3 one DRβ chain and both α chain genes are deleted.

9.22.3 cells transported to the TF nuclei through the cytoplasm, since the nuclei of the two cell types remain separate and distinct in the heterokaryons.

Transient heterokaryons, prepared by fusing TF cells and 9.22.3 cells with PEG1500, were analyzed shortly after cell fusion. The efficiency of fusion was ~1–10%. Cell surface expression of HLA-DR was detected 48 h after cell fusion employing immunofluorescence microscopy with the anti-DR-specific mAb L243 and goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated $F(ab)_2$ fragment (Figure 1). To confirm this observation, total cellular RNA was isolated 24 h after fusion and Northern blot analysis was carried out by using uniformly ^{32}P-labeled SP5 DRα RNA probes. DRα mRNA synthesized *de novo* was found in the

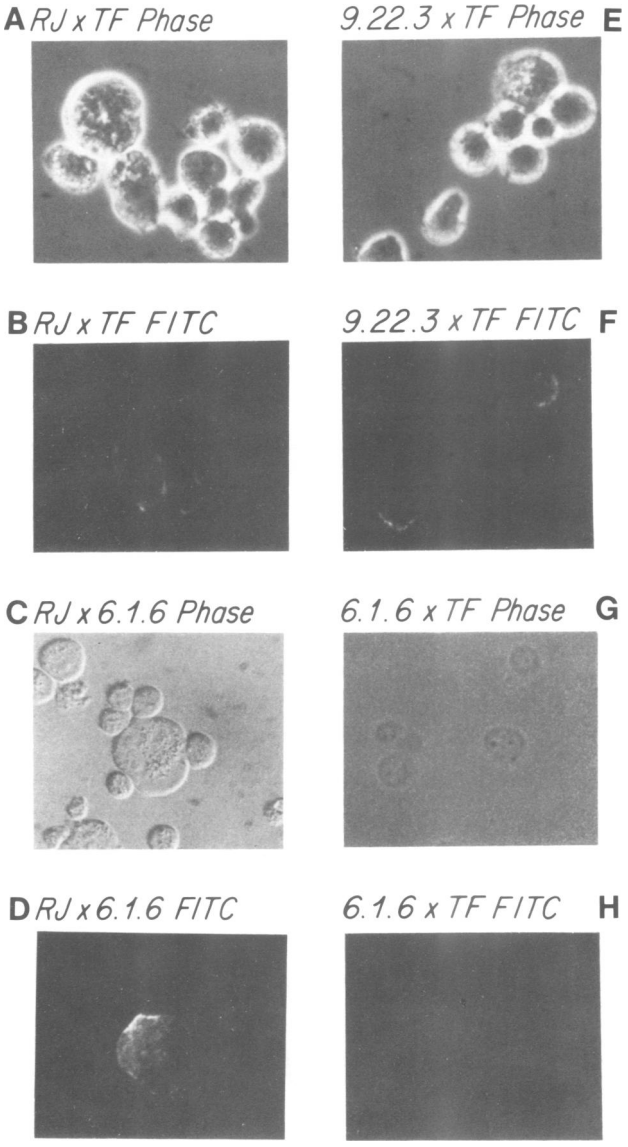


Fig. 1. Expression of HLA class II DR antigens on transient heterokaryons. Fused cells were assayed, after PEG treatment for 48 h, for surface DR antigen expression by immunofluorescence using anti-DR Mab L243 as described in Materials and methods. Phase-contrast views of the fusions of RJ 2.2.5 and TF cells (**A**), RJ 2.2.5 and 6.1.6 cells (**C**), 9.22.3 and TF cells (**E**) and 6.1.6 and TF cells (**G**). FITC UV fluorescence views of surface DR expression on the fused RJ 2.2.5 and TF cells (**B**), RJ 2.2.5 and 6.1.6 cells (**D**), 9.22.3 and TF cells (**F**) and 6.1.6 and TF cells (**H**).

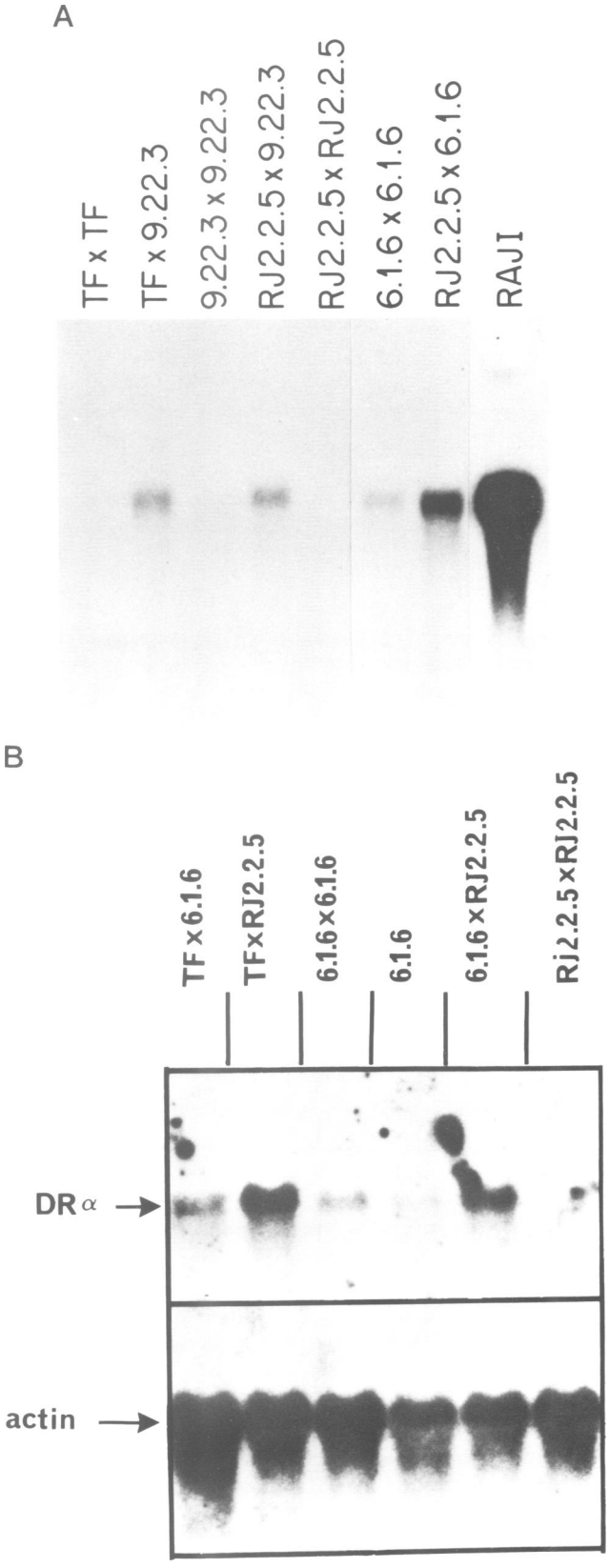
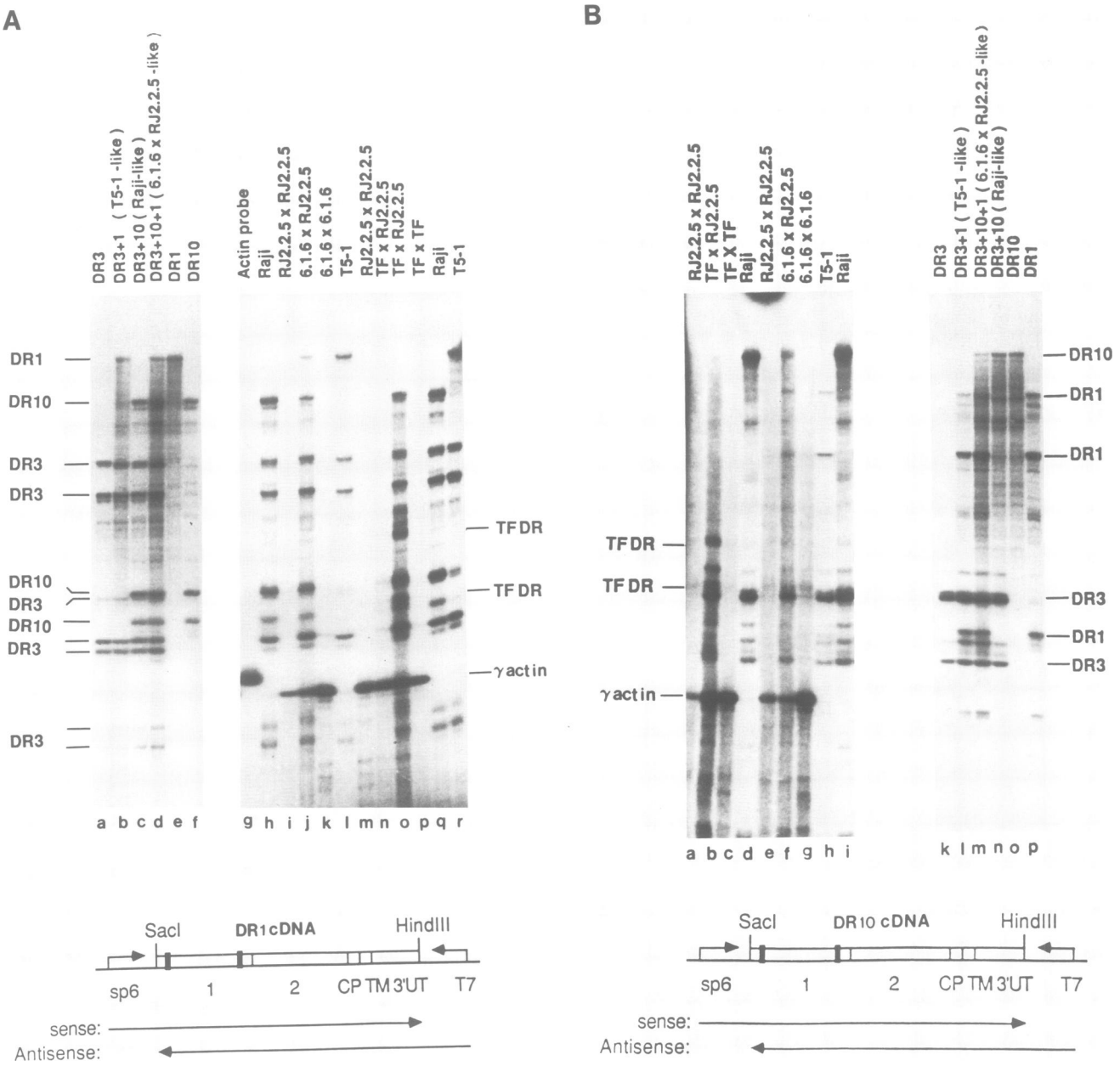


Fig. 2. (**A** and **B**) Detection of DRα RNA synthesis *de novo* by Northern blot analysis. Total cellular RNA was isolated from Raji cells and different populations of fused cells as indicated in the figure 24 h after PEG-mediated cell fusion. Thirty micrograms of each RNA was electrophoresed on 1% formaldehyde agarose gel, transferred to GeneScreen membrane (NEN Research Products), and hybridized with uniformly ^{32}P-labeled DRα cDNA probe, and reprobed after washing with γ-actin DNA probe.



heterokaryons (Figure 2). Furthermore, neither switching culture medium from 9.22.3 cells to TF cells nor mixing 9.22.3 cells with TF cells without PEG treatment induced DRα mRNA synthesis (data not shown). Moreover, the rescue of TF cells' HLA class II DR expression is not an artefact of PEG treatment, because transcription of DRα genes was not observed when 9.22.3 cells and TF cells were fused to themselves to form homokaryons. Thus, one or more diffusible regulatory factors from 9.22.3 cells are capable of activating the HLA class II DRα genes in TF cells.

Activation of class II genes in HLA class-II-negative variants after fusing them to each other

In order to ask whether the genetic defects in the mutants 6.1.6 and RJ 2.2.5 and in the patient cell line TF were at the same locus, different transient heterokaryons, 6.1.6 × RJ 2.2.5, TF × RJ 2.2.5 and TF × 6.1.6, were prepared and analyzed either at the protein level by observing surface expression of HLA class DR antigens by immunofluorescence microscopy using anti-DR-specific mAb and FITC-conjugated goat anti-mouse $F(ab)_2$ fragments, or at the RNA transcription level by probing Northern blots with uniformly ^{32}P-labeled DRα DNA probes. The immunofluorescent labeling pattern revealed that *de novo* DR antigens were expressed on the surface of resulting heterokaryons from 6.1.6 × RJ 2.2.5 and TF × RJ 2.2.5, but not on 6.1.6 × TF (Figure 1). Northern blots using a ^{32}P-labeled DRα probe (and re-probed with γ actin cDNA as an internal control) further demonstrated DRα mRNA synthesized *de novo* in 6.1.6 × RJ 2.2.5 and TF × RJ 2.2.5, but not in 6.1.6 × TF (Figure 2). It should be noted that the mutation in the 6.1.6 cell line is either 'leaky' or this cell line reverts at some frequency leading to the presence of a small amount of DRα message (Figure 2 and Levine *et al.*, 1985). Control fusions, 6.1.6 × 6.1.6, RJ 2.2.5 × RJ 2.2.5 or physically mixing RJ 2.2.5 and 6.1.6, or RJ 2.2.5 and TF (not shown), did not result in *de novo* expression of DRα mRNA. To confirm further the results obtained by immunofluorescence microscopy and Northern

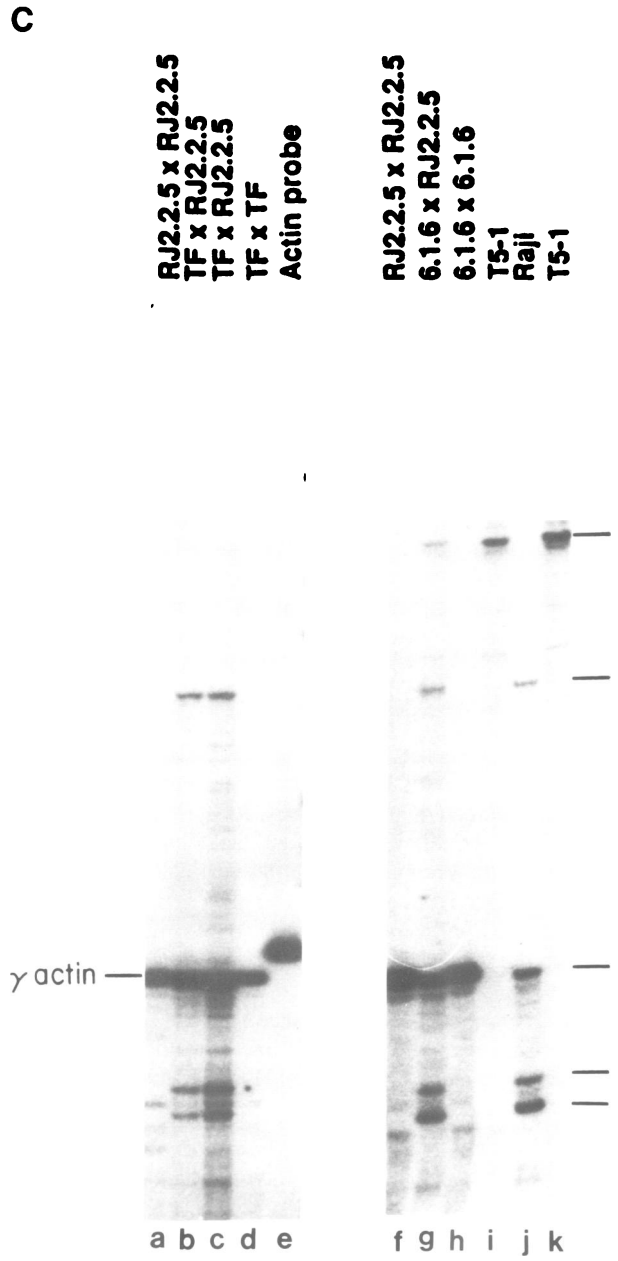


Fig. 3. HLA class II RNA typing by RNase protection assay. (**A**) Typing with uniformly ^{32}P-labeled DR1β RNA probe (see Materials and methods). Total cellular RNA was isolated 24 h after PEG-mediated cell fusion from the heterokaryons derived from fused RJ 2.2.5 cells (**lanes i** and **m**), fused 6.1.6 cells (**lane k**) and fused TF cells (**lane p**), and from the heterokaryons derived from 6.1.6 fused with RJ 2.2.5 cells (**lane j**) and TF fused with RJ 2.2.5 cells (**lanes n** and **o**). The cell fusion efficiency was different in lanes n and o; the sample in lane n had a low fusion efficiency and was used as a control. Total cellular RNA isolated from Raji (**lanes h** and **q**) and T5-1 (**lanes i** and **r**) cells served as positive controls. Sense RNA synthesized *in vitro* by SP6 polymerase (**lanes a–f**) were used to assemble the expected DR phenotypes (see Materials and methods) and for the assignment of the bands to different DRβ RNAs. Thirty micrograms each of the homokaryon and heterokaryon RNAs, 1 μg Raji RNA or T5-1 RNA and ~10 pg each of sense RNA synthesized *in vitro* which assembled different DR phenotypes (with RNA concentration brought up to 30 μg by adding yeast tRNA) were hybridized with uniformly ^{32}P-labeled DR1β RNA probe and excess low specific activity ^{32}P-labeled γ-actin RNA probe (**lane g**) at 43°C for 12 h. The RNA hybrids were digested with RNase A (42 mg/ml) at 30°C for 1 h. The digested RNA products were analyzed on 6% polyacrylamide–urea sequencing gels. (**B**) Typing with uniformly ^{32}P-labeled DR10β RNA probe. Total cellular RNA isolated from the homokaryons (**lanes a, c, e** and **h**), heterokaryons (**lanes b** and **f**) and from Raji cells (**lanes d** and **i**) and T5-1 cells (**lane h**) as described in (A), were hybridized with uniformly ^{32}P-labeled DR10β RNA probes and excess low ^{32}P specific activity γ-actin RNA probe, digested with RNase A as described in (A). Different DRβ sense RNAs synthesized *in vitro* were also used to mimic DR phenotypes. (**C**) Typing with uniformly ^{32}P-labeled DQ2β RNA probe. Total cellular RNAs used in (A) and (B) were also used to hybridize with a 560-base DQ2β RNA probe and excess low specific activity γ-actin RNA probe (**lane e**) and digested as described in (A). **Lanes a, d, f and h** are different homokaryon RNAs indicated in the figure. **Lanes b, c** and **g** are heterokaryon RNAs (the samples in lanes b and c had slight differences in fusion efficiency). **Lanes i, j** and **k** are T5-1 and Raji RNA used as positive controls.

blots, RNase protection was carried out using ^{32}P-labeled DRα and DPα RNA probes. These results further demonstrated that class II genes were activated in fusing RJ 2.2.5 × 6.1.6 and TF × RJ 2.2.5 (not shown and see below). Repeated fusions of TF and 6.1.6 did not result in any *de novo* DRα mRNA synthesis. The genetic complementation between 6.1.6 and RJ 2.2.5 and between TF and RJ 2.2.5 resulting in *de novo* synthesis of HLA class II molecules strongly suggests that at least two specific factors are involved in regulation of class II gene expression in B cells. TF cells may share the same genetic defect as 6.1.6 cells and both clearly had a different genetic defect(s) for class II gene expression from RJ 2.2.5 cells.

Reciprocal genetic complementation in heterokaryons 6.1.6 × RJ 2.2.5 and TF × RJ 2.2.5 demonstrated by a class II RNA typing technique

To prove further that at least two regulatory factors are needed for HLA class II gene expression and that the complementaion in the fusions 6.1.6 × RJ 2.2.5 and TF × RJ 2.2.5 was reciprocal, typing of class II gene products was required to demonstrate that the class II genes of both fusion partners had been turned on in the transient heterokaryons. HLA class II molecules are highly polymorphic. The polymorphism of DRβ, DQα, DQβ and DPβ molecules has been intensively studied, earlier from protein studies but most extensively from cDNA sequences (reviewed by Kappes and Strominger, 1988). DPα has a very limited polymorphism and DRα is essentially non-polymorphic. The polymorphism of DRβ predominantly occurs in the first domain (β1) in three clusters located at amino acids 9–13, 25–38 and 67–74. In addition, small nucleotide mismatches occur at unpredictable positions along the genes. The classic method of establishing reciprocal complementation in the fusions would be to use allele-specific mAbs to type the expressed surface molecules. However, reagents for only a few DR alleles are available. In order to accurately type expressed class II products in transient heterokaryons 6.1.6 × RJ 2.2.5 and TF × RJ 2.2.5, mRNA typing has been developed as an alternative. This technique takes

advantage of the polymorphism of class II genes and generates specific digestion patterns from each allelic mRNA by using one allele-specific ^{32}P-labeled DRβ RNA probe hybridized with total cellular RNA, followed by RNase A or RNase T digestion (but not the combination of the two enzymes as in a typical RNase protection assay; RNase A and T1 together often result in a very complicated digestion pattern as a result of the mismatches between the allele-specific DRβ probe and other allelic DRβ mRNAs). Unlike DNA restriction enzyme digestion, the RNA bands from some digested alleles may not be additive because the RNase digestion rates are different from one mismatch to the other, depending on the size of the mismatch and the nucleotide composition of each mismatch (D.Melton, personal communication). This technique is able both to type already known class II products and to detect unknown expressed class II products (e.g. the DR alleles in TF cells). T5-1, the parent of 6.1.6 cells is DR1,3 and DQw1. Raji, the parent of RJ 2.2.5 cells is DR3,10 and DQw? (Table I). Therefore, the expected DR phenotype in the transient heterokaryon 6.1.6 × RJ 2.2.5 was DR1,3,10 and in the transient heterokaryon RJ 2.2.5 × TF it was DR3,10,X,Y (unknowns).

Three DRβ RNA probes (DR1β, DR3β and DR10β) were used to type expressed DRβ mRNA in the transient heterokaryons. Long probes containing most of the DRβ cDNA fragments were employed in order to generate distinguishable digestion products of DRβ mRNA. First a T7-polymerase-synthesized anti-sense DR1β RNA probe was used to examine the mRNA in the heterokaryons 6.1.6 × RJ 2.2.5 and its two parents, T5-1 and Raji (Figure 3A). Comparing the Raji and T5-1 digestion patterns, the common bands should represent the DR3 phenotype while the difference bands should represent DR1 and DR10. DR1β mRNA should be fully protected while DR3β and DR10β mRNA should not. Accordingly, a fully protected DR1β band was seen with T5-1 RNA (lanes l and r). Digested products common to Raji (lanes h and q) and T5-1 (lanes l and r) could be assigned as DR3, and digested products found uniquely in Raji (lanes h and q) could be assigned as DR10. The digestion products of RNA from the 6.1.6 × RJ 2.2.5 heterokaryons (lane j) included all of the DR3 and DR10 bands, as well as the fully protected DR1 band. No protected RNA bands were seen in the controls: RJ 2.2.5 × RJ 2.2.5 homokaryons (lanes i and n), 6.1.6 × 6.1.6 homokaryons (lane k). When the same technique was applied to type RNA from TF × RJ 2.2.5 heterokaryons, several new bands were seen in the digestion in addition to the expected DR10 and DR3 bands (lanes m−q). These new bands should represent the unknown DR alleles of TF cells. An identical conclusion was drawn when a T7-polymerase-generated DR10β probe was employed in the analysis (Figure 3B).

To validate this assay further, a reconstitution experiment *in vitro* mimicking the same experiment was carried out. Unlabeled DR1β-, DR3β- and DR10β-sense RNAs were synthesized *in vitro* from the SP6 promoter in the construction used, hybridized with T7-polymerase-synthesized anti-sense DR1β [^{32}P]RNA probe and then digested with RNase A under the same conditions used for the heterokaryons. The resulting digestion products (Figure 3A and B, lanes a−f) were nearly identical to those seen in the heterokaryon typing experiments (lanes g−r). They also provided clear confirmation of the assignment of bands to DR1β, DR3β and DR10β.

In another experiment an SP6-polymerase-synthesized 560-bp DQ2β anti-sense RNA in the SP65 vector was used as a probe. This probe contained part of the intracytoplasmic sequence and the entire 3′ untranslated region (UT) of the DQ2β gene. The 3′ UT of the DQ2β gene consists of ~350 nt to the 3′ end. This sequence is extremely homologous to the 3′ ends of other DQβ alleles, but it does have short nucleotide mismatches compared with other DQβ gene 3′ UT sequences. This probe was used to detect DQ messages in the heterokaryons 6.1.6 × RJ 2.2.5 and TF × RJ 2.2.5 and in the parental cells Raji and T5-1. A protected 350-base DQβ message fragment was found in T5-1 (lanes i and k). Instead of this band, short protected DQβ fragments were found in Raji (lane j). As expected, two different lengths of protected DQβ messages found in T5-1 (lanes i and k) and Raji (lane j) were also found in the heterokaryon 6.1.6 × RJ 2.2.5 (lane g). Thus the activation of DQ genes in the heterokaryon 6.1.6 × RJ 2.2.5 was reciprocal, confirming the conclusion reached from analysis of the RNA products of the DR genes. However, the RNA digestion pattern in the heterokaryon TF × RJ 2.2.5 did not reveal any distinct TF DQβ message fragment. Presumably, there were no significant nucleotide mismatches in the 3′ UT of the DQβ transcripts between RJ 2.2.5 and TF cells.

These experiments thus demonstrate that the two class-II-negative human mutant cell lines, RJ 2.2.5 and T5-1, have mutations in two different genes and that their genetic defects are reciprocally complemented following cell fusion. Moreover, a cell line from a patient with a class-II-negative Bare Lymphocyte Syndrome, TF, can also be complemented by a factor present in human B cells, and thus also contains a mutation in a gene encoding a *trans*-acting factor regulating class II gene expression. This mutation in TF cells is reciprocally complemented by the cell line containing one of the two mutations generated *in vitro* (RJ 2.2.5) but not by the other (6.1.6).

Several laboratories have been involved in studies of DNA-binding proteins which bind to the promoter region of class II genes, in particular to the conserved elements X and Y (for example, Dorn *et al.*, 1987a). In previous studies we have in particular investigated the binding of nuclear proteins from the two mutant cell lines RJ 2.2.5 and 6.1.6 to these elements and compared them to the two parental lines (Miwa *et al.*, 1987). No differences were detected. This failure could result from several causes, among which are: (i) the proteins which are defective in the two mutant cell lines are not DNA-binding proteins but regulate transcription of class II genes in another way, (ii) the two mutant cell lines do have mutations in DNA-binding proteins which render them non-functional but do not affect their DNA-binding domains, (iii) the resolution of the methods employed was not sufficient to detect the differences. Further investigation will be required to clarify the various possibilities.

Materials and methods

Cell lines and cell fusion

The 9.22.3 human B cell line, generated by mutagenesis *in vitro* and immunoselection from the T5-1 cell line, had all DR and DQ genes deleted in one MHC haplotype, while only the DRα gene was deleted in the second

MHC haplotype (Pious *et al.*, 1985). Its surface phenotype was DR^-, DQ^+, DP^+. TF cells are B cells derived from a Dutch patient TF who had been diagnosed as having a Bare Lymphocyte Syndrome (Zegers *et al.*, 1984). The 6.1.6 cell line was derived from the human B cell line T5-1 by mutagenesis *in vitro* followed by immunoselection for loss of DR1 antigen expression (Gladstone and Pious, 1978). The RJ 2.2.5 line was derived from the human B cell line Raji by a similar technique with immunoselection *in vitro* for loss of DR3 antigen expression. Both mutants, 6.1.6 and RJ 2.2.5, failed to express the two DR haplotypes as well as DQ and DP while Ii, the class-II-associated invariant chain, had decreased expression in both. Cell fusions had demonstrated that both were lacking a *trans*-acting factor regulating class II gene expression (Gladstone and Pious, 1980; Accolla *et al.*, 1985). All cell lines were maintained in RPMI 1640 medium plus 10% fetal calf serum (FCS).

Since all cell lines used in this study were grown in suspension medium, the transient heterokaryons were essentially prepared by the 'suspension cell fusion' procedure (Shay, 1982; Lane *et al.*, 1986). Briefly, $\sim 1 \times 10^7$ mutant B cells were combined with 1×10^7 of another mutant B cell line in a 50-ml centrifuge tube, then disaggregated by gentle passage through an 18-gauge syringe needle four or five times and finally centrifuged and washed twice with serum-free medium. The cell pellet was dispersed by gentle tapping after completely removing the medium. Prior to fusion, the cells were warmed in a 37°C water bath for 1 min. One milliliter of PEG1500 (50% w/v, Boehringer Mannheim Biochemicals) was applied to the cell suspension over a 60-s period at 37°C while gently shaking the tube. The fusion process was halted by slowly adding 50 ml of serum-free medium which took 3 min. The cell suspension was then allowed to stand for 8 min at room temperature. After gentle centrifugation at 250 *g* for 2–4 min, the cells were resuspended in 50 ml of RPMI/20% FCS, followed by incubation at 37°C for 24 h (or as indicated for a particular experiment).

Immunofluorescence microscopy and FACS profile

Cells were washed with phosphate-buffered saline 48 h after cell fusion and incubated with anti-DR-specific mAb L243 (Lampson and Levy, 1980) in RPMI medium containing 2% FCS and 0.02% sodium azide for 40 min, followed by washing the cells twice with the medium (2% FCS and 0.02% sodium azide). The washed cells were further stained with FITC- or Texas-red-labeled sheep anti-mouse $F(ab')_2$ (Cappel, Organon Teknika Corp.). Cell surface expression of DR antigens in the fused cell population was evaluated by fluorescence microscopy at the appropriate UV wavelength.

In addition to DR-specific mAb L243, anti-DQ mAb Leu10 (Becton–Dickinson) and anti-DP mAb B7/21 (Watson *et al.*, 1983) were also used to make class II FACS profiles of TF cells. The immunofluorescence staining was carried out essentially as described above.

Preparation of total cellular RNA and Northern blot analysis

Total cellular RNA was prepared from 50 ml of cell suspensions ($\sim 2 \times 10^7$ cells) by a modification of the procedure of Chirgwin *et al.* (1979) as described by Maniatis *et al.* (1982). Northern blots of the cellular RNAs were prepared following size fractionation on 1% formaldehyde gels. The RNA blots were prehybridized at 42°C for 16 h in 50% formamide, 5 × SSPE, 5% SDS containing 100 μg/ml sheared salmon sperm DNA. The blots were hybridized in the same buffer with uniformly ^{32}P-labeled DRα cDNA probe and re-probed with a γ-actin cDNA probe. The blots were washed once in 6 × SSPE, 0.1% SDS at room temperature for 20 min and three times in 0.1 × SSPE, 1% SDS at 68°C for 2 h.

The uniformly ^{32}P-labeled DRα probe, consisting of a 600-bp *Pst*I fragment from the DRα cDNA coding region (Yang *et al.*, 1987), was generated by random primer extension (Feinberg and Vogelstein, 1984). The γ-actin DNA probe was derived from plasmid pSP6 γ-actin (Enoch *et al.*, 1986) which contains an internal fragment from the human γ-actin gene.

SP6 and T7 probes

The plasmids pSP64, pSP65 and pSP73 have been described previously (Melton *et al.*, 1984; Krieg and Melton, 1987). pSP64–DRα was constructed by ligation of the 600-bp *Pst*I fragment of the HLA–DRα cDNA clone pα-15 (Wake *et al.*, 1982), which includes the first domain and most of the second domain of DRα, into the *Pst*I site of pSP64. The linear DNA template was obtained by cleavage with *Eco*RI. pSP64–DQβ was prepared by subcloning a 500-bp *Pst*I fragment of pDC1.1 (Boss and Strominger, 1984) containing 3′ UT exon of the DQβ gene into pSP64, which should give a 350-bp protected band in the RNA protection assay. The plasmid was linearized by treatment with *Eco*RI. pSP73–DRβ1 was constructed by inserting a 700-bp *Sac*I–*Hin*dIII fragment of the DRβ1 cDNA clone pDR008 from a DR1 homozygous B cell line (Tonnelle *et al.*, 1985) into pSP73 at *Sac*I and *Hin*dIII sites. The linear DNA template of pSP73–DRβ1 was made by cutting the plasmid with *Sac*I in order to use T7 polymerase to synthesize an anti-sense RNA probe or alternatively by treatment with *Hin*dIII to generate sense RNA using SP6 polymerase. pSP73–DRβ10 was prepared by subcloning a 700-bp *Sac*I–*Hin*dIII fragment of the HLA–DRβ10 cDNA clone p-II-β-4 from the DR3,10 B cell line Raji (Gustafsson *et al.*, 1984) into pSP73. The linear DNA template was prepared by digesting pSP73–DRβ10 with *Sac*I or *Hin*dIII to synthesize anti-sense or sense RNA. pSP73–DRβ3 was also constructed by ligating a 700-bp *Sac*I–*Hin*dIII fragment of the DRβ3 cDNA clone p-II-β-3 from Raji into pSP73 and linearized either by *Sac*I or by *Hin*dIII to synthesize anti-sense or sense RNA. pSP6 γ-actin (Enoch *et al.*, 1986) linear template for transcription by SP6 polymerase was prepared by digesting the plasmid with *Hin*fI. RNase mapping of human actin identifies a fragment of 135 nt.

RNA–RNA hybridization and class II DR RNA typing

Uniformly labeled RNA probes were transcribed *in vitro* by either SP6 polymerase or T7 polymerase (Melton *et al.*, 1984; Krieg and Melton, 1987) from linearized DNA templates (see above). SP6 polymerase and T7 polymerase were purchased from Promega-Biotec or BRL, RNase A type III-A from Sigma, RNasin from Promega-Biotec and [α-^{32}P]UTP from Amersham. RNA– RNA hybridization was performed at 42–45°C for 12 h in hybridization buffer [400 mM NaCl, 40 mM Pipes $(Na)_2$, pH 6.4, 10 mM EDTA, 87% formamide]. The temperature change between 42 and 45°C did not significantly alter the digestion pattern (not shown). The RNA digestion with RNase A (42 μg/ml) was carried out at 28–30°C for 1 h in RNase digestion buffer (300 mM NaCl, 10 mM Tris–HCl, pH 7.5, 5 mM EDTA). After further processing of the samples as described (Zinn *et al.*, 1983), the digested RNA products were separated by electrophoresis through 6% denaturing gels.

The same RNAs generated *in vitro* from pSP73–DR1β, pSP73–DR3β and pSP73–DR10β by SP6 polymerase were used in the reconstitution experiment (see text for details). Approximately 10 pg DR3β sense RNA synthesized *in vitro* was either mixed with 10 pg DR1β sense RNA to mimic the T5-1 DR phenotype or mixed with ~ 10 pg DR10β sense RNA to mimic the Raji DR phenotype. To reassemble the DR phenotype of the hybrid synthesized *in vitro*, sense DR1β, DR10β and DR3β RNAs were mixed together. Either DR1β (uniquely found in T5-1 cells) or DR10β (uniquely found in Raji cells) anti-sense RNA synthesized by T7 polymerase was used for RNA–RNA hybridization. The hybridization conditions and further processing of the samples are described above.

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