

An Isotype-specific *trans*-acting Factor Is Defective in a Mutant B Cell Line that Expresses HLA-DQ, but not -DR or -DP

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Summary

The B lymphoblastoid cell line clone 13 (a subclone of the mutant cell line P3JHR-1) has been found to express high levels of HLA-DQ; by contrast, HLA-DR and -DP antigens are not expressed and cannot be induced by interferon γ . Northern blot analysis using gene-specific probes indicated that the lack of surface expression of the DR and DP antigens is due to a marked decrease in the levels of steady-state RNA for both the α and β chains. Southern blots demonstrated that none of the transcriptionally repressed genes are grossly deleted. Preparation of interspecific transient heterokaryons between clone 13 and the class II antigen-positive murine B cell lymphoma, A20, resulted in reactivation of the DRA gene and surface expression of both the DR and DP molecules. The efficiency of the DRA promoter relative to the DQB promoter is markedly and specifically diminished in clone 13 (P3JHR-1) as compared with the parental cell line, Jijoye, as assayed both by transient expression of appropriate chloramphenicol acetyltransferase gene (CAT) constructs and by *in vitro* transcription analysis. These data clearly demonstrate the existence of an isotype-specific *trans*-acting factor, and provide direct evidence that the highly homologous class II genes have distinct regulatory mechanisms.

Immune responsiveness to foreign antigen is controlled by a set of heterodimeric integral glycoproteins encoded within the class II region of the MHC (1). These class II molecules participate in the immune response at two levels: in the selection of the T lymphocyte repertoire in the thymus, and in the presentation of foreign antigens to T lymphocytes in the periphery (2). The molecular basis of immune recognition at both levels is suggested by the hypothetical three-dimensional structure of the class II molecule, inferred from the structure of the class I molecule, HLA-A2 (3, 4). A foreign antigen binding cleft is predicted to be formed between the COOH-terminal α helices of the first $\alpha 1$ and $\beta 1$ domains of the α and β polypeptides that form the molecule, with the floor of the cleft formed by the NH₂-terminal β strands. Immune recognition, then, involves the discrimination by T lymphocytes of class II molecules that have bound foreign antigen from those molecules that harbor self peptides.

In view of the central role the class II molecules play in the immune response, it is not surprising that dysregulation of their tissue-specific and developmental patterns of expression results in severe impairment of the immune system and the homeostasis of the organism. Normally, class II molecules are expressed only on cells of the immune system, although they are inducible on many cells by certain lymphokines

such as IFN- γ or IL-4. The complete loss of class II gene expression in man results in a severe combined immunodeficiency syndrome termed bare lymphocyte syndrome (5), and the aberrant expression of class II molecules on normally class II-negative cells is hypothesized to trigger or exacerbate certain types of autoimmune disease (6). The elucidation of class II gene regulation, then, is not only of interest from the standpoint of eukaryotic transcription, but is significant in view of the consequences that defective class II gene regulation has on the functioning of the immune system.

Rapid progress in our understanding of class II gene regulation has occurred in the past few years. Comparison of the 5' flanking regions of all of the class II genes analyzed to date reveal several conserved motifs, most notably, the X and Y boxes of the proximal promoter (-180 to +10) (7, 8). Deletion and mutational analysis have demonstrated that these motifs are indispensable for class II gene expression, and some of the genes encoding sequence-specific DNA binding proteins that bind to these motifs in gel retardation and footprinting assays have been cloned (9). In spite of this progress, several basic questions regarding class II gene regulation remain unsolved. For example, are the seven functional class II genes (Fig. 1 C) that are encoded over a region 1 megabase in size regulated by the same or distinct mechanisms? The

similarities of the conserved motifs, and the usual coordinate expression of the three class II isotypes in man (both tissue specifically and developmentally), are clues that there is likely overlap in the transcription factors that regulate the various genes. Further evidence comes from several mutant cell lines, where defects in *trans*-acting factors affect the expression of all of the class II genes (10, 11). On the other hand, gel retardation analysis suggests that different factors bind the conserved motifs in the various genes, and differential inducibility of the class II isotypes on HeLa cells, melanoma lines, and dermal fibroblasts by IFN- γ has been observed (12–14). In addition, some cell lines derived from patients with bare lymphocyte syndrome, certain leukemic cells, and a subset of normal cells of the myelomonocytic lineage exhibit differential expression of class II isotypes; i.e., they express HLA-DR and -DP, but not -DQ (which is normally expressed at a low level relative to DR) (15).

In this report, a B lymphoblastoid cell line (B-LCL),¹ clone 13 (a subclone of the mutant B cell line, P3JHR-1) that exhibits the reciprocal differential expression of the class II isotypes is described; i.e., it expresses HLA-DQ, but not -DR or -DP and is the first such line reported. Interspecific heterokaryons and transient transfection of reporter plasmids demonstrate that the defect in clone 13 affects an isotype-specific *trans*-acting factor, clearly illustrating that the regulatory machinery for the genes are distinct. The existence of the wild-type parental line, Jijoye, provides a new isogenic cell pair with a novel mutant phenotype for the dissection of class II gene regulation.

Materials and Methods

Cell Lines. Raji, RJ 2.2.5, A20, Jijoye, and clone 13 cells were all maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 20 mM Hepes, 5×10^3 U/100 ml penicillin and streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate. The cells were split 1:5 every 3 d. HeLa cells were maintained in DMEM media. A20 is a class II-positive murine hybridoma. RJ 2.2.5 is derived from the Burkitt's lymphoma cell line Raji (DR3, DR10) after irradiation and immunoselection with class II-specific mAbs and complement. Raji cells express all three class II isotypes, while RJ 2.2.5 cells do not express any class II antigen. Jijoye cells (also originally from a Burkitt's lymphoma patient) were originally isolated by Pulvertaft and designated then as P₃. Jijoye cells also express all three class II isotypes. Hinuma et al. (16) then isolated subclones of the line in semisolid media and generated a line designated P₃J-HR-1, which is a high producer of viral capsid antigen. Clone 13 cells were derived by further subcloning of the P₃J HR-1 cell line with the aim of producing isogenic cell lines with varying efficiencies of conversion from the latent to lytic cycles of the virus (17). The HLA phenotype of the clone 13 cell line is the focus of this report.

Cell Fusion. 10^7 cells of each type were combined in a 50-ml centrifuge tube and washed three times with serum-free media. The cells were then disaggregated by passage through an 18-gauge needle (three times), washed one more time in serum-free media, and pelleted by gentle centrifugation. The cell pellet was resuspended by tapping and warmed to 37°C. 1 ml of PEG 1500 (50% [wt/vol],

Boehringer Mannheim Biochemicals, Indianapolis, IN) was added dropwise over a 1-min period. 50 ml of serum-free media was then added slowly over a 3-min period. After allowing the cell suspension to sit at room temperature for 5 min, the fused cells were collected by centrifugation at 250 g for 3 min. 50 ml of prewarmed complete media was then added to the cell pellet (resuspending by gentle tapping) and the cells were incubated at 37°C for 48 h before they were analyzed.

mAbs and Fluorescence Microscopy. The following mAbs were used for immunofluorescence microscopy: LB3.1 (anti-DR), B7/21 (anti-DP), and Genox 3.53 (anti-DQ). The three mAbs used for FACS[®] analysis (Becton Dickinson & Co., Mountain View, CA) of cell surface class II expression (LB3.1, Genox 3.53 and Leu-10 [anti-DQ], and B7/21) were all of the IgG1 subclass. Fused cells were washed gently with PBS and incubated with monospecific antibody for 30 min on ice. The cells were then washed twice with PBS and incubated with FITC-conjugated goat anti-mouse antibody. After washing twice with PBS, the cells were immediately analyzed under a fluorescence microscope at the appropriate wavelength.

Preparation of Total Cellular RNA and High Molecular Weight DNA and Southern and Northern Blot Analysis. High molecular weight DNA was isolated by Sarcosyl disruption of the cell membranes followed by three phenol, two phenol/chloroform, an one chloroform extraction. Purified DNA was dialyzed with four changes against 10 mM Tris (pH 8.0) and 1 mM EDTA. Total cellular RNA was isolated after disruption of the cells with 6 M guanidium isothiocyanate, 5 mM NaCitrate (pH 7.0), 0.1 M β -mercaptoethanol, and 0.5% Sarcosyl. RNA was purified by centrifugation through a 1.2-ml cushion of 5.7 M CsCl at 35,000 rpm for 12 h at room temperature. The pelleted RNA was extracted several times with 4:1 chloroform/butanol and ethanol precipitated and stored at -70°C before use. 10 μ g of high molecular weight DNA was digested overnight with fivefold excess restriction endonuclease, phenol-extracted, and ethanol precipitated before separation on 1% agarose gels in 1 \times TAE buffer. After visualization on an ultraviolet transilluminator the gels were denatured and neutralized and transferred to GeneScreen Plus hybridization membranes according to the manufacturers suggested protocols (DuPont Co., Wilmington, DE). Total cellular RNA was separated on 1% formaldehyde agarose gels and transferred to GeneScreen Plus membranes as recommended. Both Northern and Southern blots were prehybridized, hybridized, and washed as recommended.

Plasmids and Nucleic Acid Probes. For Northern blot analysis, short DNA fragments corresponding to divergent regions of the mRNA were used for specific detection of transcripts. These fragments were derived from SP72 subclones constructed in this laboratory (18). The precise fragments that were random hexamer labeled to generate specific probes were DPA (Bgl 2-Pst), DPB (Sst-Hae III), DQA (Pst-Pst), DQB (PvuII-PvuII), DRA (Sst-EcoRI), and DRB (HindIII-Rsa). Class I heavy chain transcripts were detected using the Pst fragment of pHLA2. For CAT assay, the plasmids used were DQB160CAT (19) and DRA1028CAT, DRA300CAT, and DRA141CAT (20). For Southern blot analysis, full-length cDNAs were labeled by the random hexamer method. DRA and DRB cDNAs were kindly donated by D. Monos, Harvard University (Monos et al., manuscript in preparation) and the DPA and DPB cDNAs were kindly provided by D. Kappes (Harvard University). The cDNAs were excised from vector sequences and purified by binding to glass beads before labeling.

Immunoisolation of HLA Antigens. Class II antigens were isolated using the modified solid-phase immunoisolation technique. Briefly, mAbs were immobilized to the surface of 96 U-bottomed

¹ Abbreviation used in this paper: B-LCL, B lymphoblastoid cell line.

plastic microplates (Falcon Labware, Oxnard, CA) via goat anti-mouse antibody. After washing, 1% Triton X-100 lysate of surface iodinated cells (cells were labeled by lactoperoxidase-catalyzed method) containing 1 mM PMSF was applied, incubated at 4°C overnight, and washed with lysis buffer and PBS. HLA antigens bound to the immobilized mAbs were eluted with reducing sample buffer and boiled before SDS-PAGE in the discontinuous buffer system.

Transient Transfection of Plasmid DNA and CAT Assay. 10⁷ recipient cells were washed extensively with serum-free media. 20 µg of cesium-chloride-purified, supercoiled plasmid DNA was added to the cells in a 1-ml volume of serum-free RPMI 1640 containing either 20 µl of lipofectin or 200 µg of DEAE Dextran (Bethesda Research Laboratories, Bethesda, MD, and Pharmacia Fine Chemicals, Piscataway, NJ, respectively). 5 µg of plasmid pXGH5, a mammalian expression vector encoding human growth hormone, was cotransfected with reporter constructs to control for variability in transfection efficiency. In the case of lipofection, cells were incubated for 3 h at 37°C before addition of 50 ml complete medium. For DEAE Dextran transfection, the cells were incubated for 1 h at 37°C before addition of medium. 48 h after transfection, the cells were washed with serum-free media and pelleted. The cells were resuspended in 300 µl of 0.25 M Tris and freeze-thawed three times. After centrifugation in a microfuge to remove debris, 150 µl of the cell extract was incubated with 20 µl 10 mM acetyl coenzyme A (Pharmacia Fine Chemicals) and 2 µl of ¹⁴C-chloramphenicol (New England Nuclear, Boston, MA) (49 mCi/mmole, 0.1 mCi/ml) for 4 h at 37°C. The chloramphenicol was then extracted with 1 ml of ethyl acetate, speed vacuum dried, and spotted onto thin-layer chromatography plates. After the solvent front was allowed to travel three-quarters of the length of the plate, the plate was removed from the chromatography tank, allowed to dry, and subjected to autoradiography.

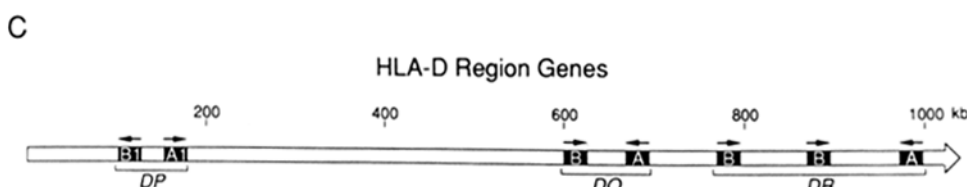
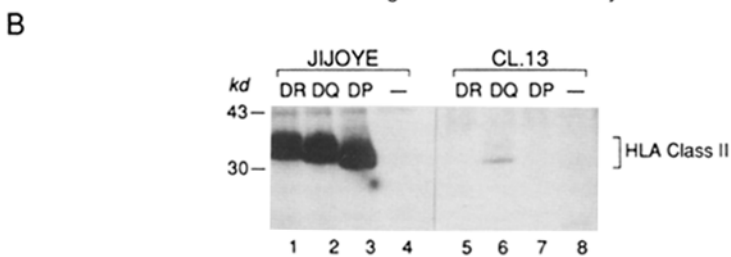
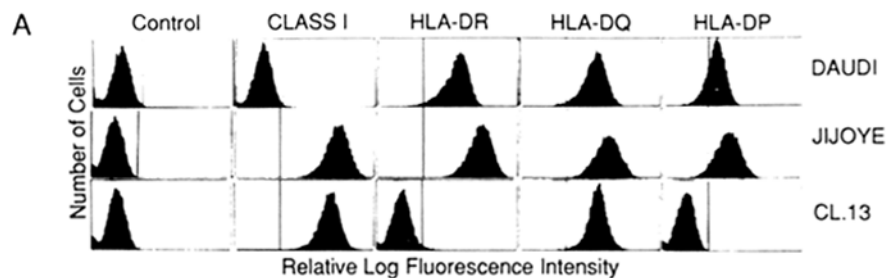
Preparation of Nuclear Extracts, Gel Retardation, and In Vitro Transcription Analysis. 500 ml of log phase cells was pelleted at 2,000 rpm for 10 min. The cell pellet was washed twice in ice-cold PBS and resuspended in 25 ml nuclear isolation buffer I (10 mM Tris-HCl, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, and 1 mM DTT). 0.3 ml of ice-cold 10% NP-40 was added dropwise (while vortexing at lowest setting) and incubated on ice for 20 min. The cell lysate was layered onto 12 ml of ice-cold nuclear isolation buffer I containing 1.7 M sucrose and centrifuged at 13,000 g for 15 minutes in an SW27 rotor (Beckman Instruments, Inc., Palo Alto, CA). Purified nuclei were then resuspended in 3 ml of ice-cold 20 mM Hepes, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT, and homogenized with 10 strokes of a dounce homogenizer on ice. The suspension was then rocked for 30 min at 4°C and centrifuged for 30 min at 25,000 g in an SS34 rotor. The supernatant was then dialyzed against 150 ml of transcription buffer (-rNTPs): 12 mM Hepes, pH 7.9, 12% glycerol, 0.3 mM DTT, 0.12 mM EDTA, and 60 mM KCl for 5 h. MgCl₂ was omitted from these preparations to be used for gel-retardation assay, as MgCl₂ has been found to inhibit gel-retardation. For the gel-retardation assay, complementary oligonucleotides (described in Fig. 6 A) were annealed by boiling for 2 min in 0.5 M NaCl, 20 mM Tris-Cl, pH 7.5, and 1 mM EDTA, followed by incubation overnight at 65°C. The oligonucleotides were then ethanol precipitated and gel purified. The double-stranded oligonucleotides were then labeled by polynucleotide kinase and separated from unincorporated ³²P by spun-column chromatography on Biogel P-2. DNA binding was allowed to proceed for 20 min at room temperature in a 10-µl volume containing 5 µg nuclear extract and 5,000 cpm radiolabeled oligonucleotide in the

presence of 4 µg poly(dL) dC (in a buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, and 5% glycerol). The protein-DNA complexes were then resolved from free DNA by separation on a 5% polyacrylamide gel. The products were then visualized by autoradiography of the fixed, dried gel. For *in vitro* transcription analysis, 25 µl of nuclear extract was added to 1 µl template DNA (SmaI-cleaved pSmaI fragment of the adenovirus major late promoter and DdeI-cleaved DRA1028CAT or DQB2500 CAT plasmids) and incubated at room temperature for 30 min to allow preinitiation complex formation. 10 µl of 10× rNTP solution was added to preinitiation complexes: 6 mM ATP, CTP, UTP, and 2 µl of 250 µM α-[³²P]-GTP. Transcription buffer was then added to a final volume of 50 µl. Transcription was then allowed to proceed for 2 h at 30°C. Nucleic acids were purified by sequential phenol, phenol/chloroform, and chloroform/isoamyl alcohol extractions, and precipitated by addition of 50 µl 10.5 M ammonium acetate, pH 5.2, 1% SDS, 1 µg tRNA, and 250 µl ice-cold ethanol. After precipitation and washing with 70% ethanol, the RNA was heated to 68°C for 5 min and loaded onto a 4% acrylamide gel containing 7 M urea. The gel was then fixed in 10% acetic acid, 10% methanol, dried for 1 h at 90°C, and autoradiographed.

Results

HLA Phenotype of Clone 13 and its Related Cell Lines. During studies of the inducibility of class II genes, clone 13 cells (a subclone of the mutant EBV-transformed cell line, P3JHR-1, whose parent was the Burkitt's lymphoma cell line Jijoye) were found to express very high levels of surface HLA-DQ and class I antigens but not HLA-DR or HLA-DP (Fig. 1 A). P3J-HR-1, which had been derived without mutagenesis by plating Jijoye in semisolid media and screening for a high producer of EBV viral capsid antigen, had the same phenotype. By contrast, the original Burkitt's lymphoma isolate, Jijoye, expressed all three class II molecules. Analysis of several other B-LCLs by FACS analysis indicates that the level of cell surface expression of HLA-DQ on clone 13 was comparable with that on other B-LCLs examined. Both Leu-10 and Genox antibodies were used to detect HLA-DQ by FACS analysis, and both indicated that high levels of HLA-DQ are expressed on the surface of clone 13 cells. Expression of HLA-DQ on the surface of clone 13 was demonstrated unequivocally by immunoaffinity isolation of the molecule from surface-iodinated clone 13 cells with the anti-HLA-DQ antibody BT3/4 (Fig. 1 B). Using a panel of mAbs that recognize different DQ alleles, the molecule expressed on clone 13 cells was shown to be DQ1 (data not shown).

Marked Reduction in the Transcription of the α and β Chain Genes of HLA-DR and -DP in Clone 13 in the Absence of Gross Deletion of the Structural Genes. The level at which expression of HLA-DR and -DP antigens is blocked in clone 13 cells was investigated by Northern blot analysis using gene-specific probes derived from the divergent 3' untranslated regions of the various genes. The DRA and DRB genes, as well as DPA and DPB, were transcribed at high levels in Jijoye and Raji cells, while transcripts for all four genes were undetectable in clone 13 and RJ2.2.5 cells (a class II-negative mutant of Raji) (Fig. 2). In contrast, the DQA and DQB genes were transcribed at high levels in Jijoye clone 13 and Raji.



cosmid cloning of the different loci. The transcriptional orientation of the genes is indicated by arrows. The remaining 2.5 megabases of the MHC containing the class I, III, TNF, and newly identified genes are located in the direction of the large arrow.

Figure 1. (A) Cell surface expression of the HLA molecules on Jijoye and clone 13. (A) FACS analysis of cell surface expression of class I (W6/32), HLA-DR (LB 3.1), HLA-DQ (Genox 3.53), and HLA-DP (B7/21) on Daudi, Jijoye, and clone 13 cells. The same results were obtained with the anti-DQ mAb Leu-10. All antibodies were of the IgG subclass. (B) Immunoprecipitation of HLA antigens from surface iodinated Jijoye (lanes 1–4) and clone 13 (lanes 5–8) cells using mAbs L243 (anti-DR; lanes 1 and 5), BT3/4 (anti-DQ; lanes 2 and 6), B7/21 (anti-DP, lanes 3 and 7), and MEM-18 as a negative control (anti-CD14; lanes 4 and 8). Isolated antigens were separated by SDS-PAGE and subjected to autoradiography. Position of HLA antigens is indicated. The difference in intensity of bands in lanes 1–4 as compared with lanes 5–8 is due to different extent of labeling of the cells in the two experiments and to different times of exposure of the autoradiograms. (C) Organization of the HLA-D (class II) region genes in the human MHC. The linkage map was generated by pulsed-field gel electrophoresis and

Class I genes were also transcribed in all of the cells. To determine whether the decreased transcription of the α and β chain genes of HLA-DR and -DP in clone 13 cells resulted from gross deletion of any of the genes. Southern blot analysis was next performed using full-length cDNAs as probes. None of the genes examined was grossly deleted in any of the cells (Fig. 3). Although some of these genes exhibit restriction fragment length polymorphism, the isogenic cell pairs, Jijoye-clone 13 and Raji-RJ2.2.5, had identical RFLP patterns.

Preparation of Interspecific Transient Heterokaryons between Clone 13 and the Class II-Positive Murine Hybridoma A20 Activates de novo Expression of HLA-DR and -DP Antigens. Transient fusion of cells from different species has been used to demonstrate the existence of *trans*-acting factors in several systems (21). Transient interspecific clone 13 \times A20 heterokaryons were prepared by fusion with polyethylene glycol (PEG) (see Materials and Methods). 48 h after cell fusion, cell surface expression of HLA-DR and -DP antigens was assessed by immunofluorescence microscopy using the mAbs LB3.1 and B7/21, respectively, followed by FITC-conjugated IgG as the fluorochrome. Transient homokaryons, clone 13 \times clone 13 and A20 \times A20, prepared in parallel served as controls. Neither the clone 13 nor A20 transient homokaryons expressed HLA-DR or -DP antigen on their cell surface (and moreover, the mAbs LB3.1 and B7/21 do not crossreact with the murine class II molecules I-A and I-E expressed on the surface of A20) (Fig. 4). The interspecific transient heterokaryon be-

tween clone 13 and A20, however, expressed both HLA-DR and -DP antigen de novo. To show unequivocally that transcription of a previously silent human class II gene was activated by fusion with A20, total cellular RNA was isolated from both homokaryons and the interspecific heterokaryon 48 h after fusion and analyzed by Northern blotting. Transcripts from the DRA gene were undetectable in total cellular RNA from clone 13, from A20, and from the A20 and clone 13 homokaryons. DRA transcripts were detected, however, in RNA isolated from the clone 13 \times A20 heterokaryons (Fig. 5).

Transient Transfection of CAT Constructs and In Vitro Transcription Analysis Provide Additional Evidence of an Isotype-specific Trans-activator Defect in Clone 13. To examine further the defect in HLA-DR expression in clone-13 cells, CAT constructs bearing progressive 5' deletions (-1028, -300, and -141 bp of upstream sequence) of the DRA promoter or 160 bp of upstream sequence from the DQB1 gene were transfected into clone 13 and Jijoye cells (Fig. 6A). All of the CAT constructs were transcribed efficiently in Jijoye cells. Transfection of the DQB160CAT plasmid into clone 13 cells resulted in the expression of comparable levels of CAT activity with that observed when transfected into Jijoye cells. On the other hand, transfection of any of the DRACAT plasmids into clone 13 cells resulted in only 5% of the CAT activity observed in Jijoye cells. In vitro transcription analysis using two different nuclear extract preparations from Jijoye and clone 13 indicated that DRACAT transcription could not be supported

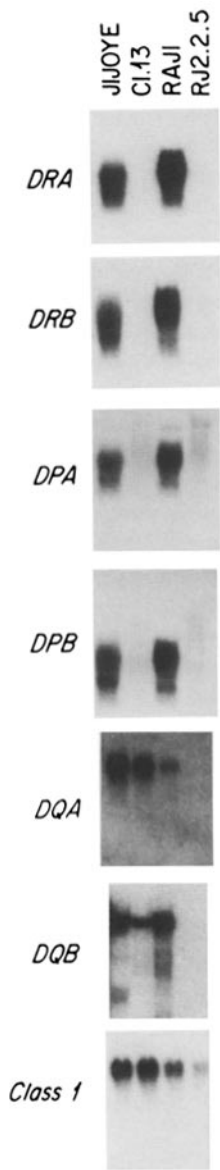


Figure 2. Transcription of the various HLA genes in Jijoye, clone 13, Raji, and RJ2.2.5 cells. 10 μ g of total RNA isolated from each cell line was separated on formaldehyde gels, transferred to GeneScreen Plus hybridization membranes, and probed with random-hexamer-primed, radiolabeled probes specific for the indicated transcripts. The Northern blots were then washed at high stringency and the resulting autoradiograms are presented.

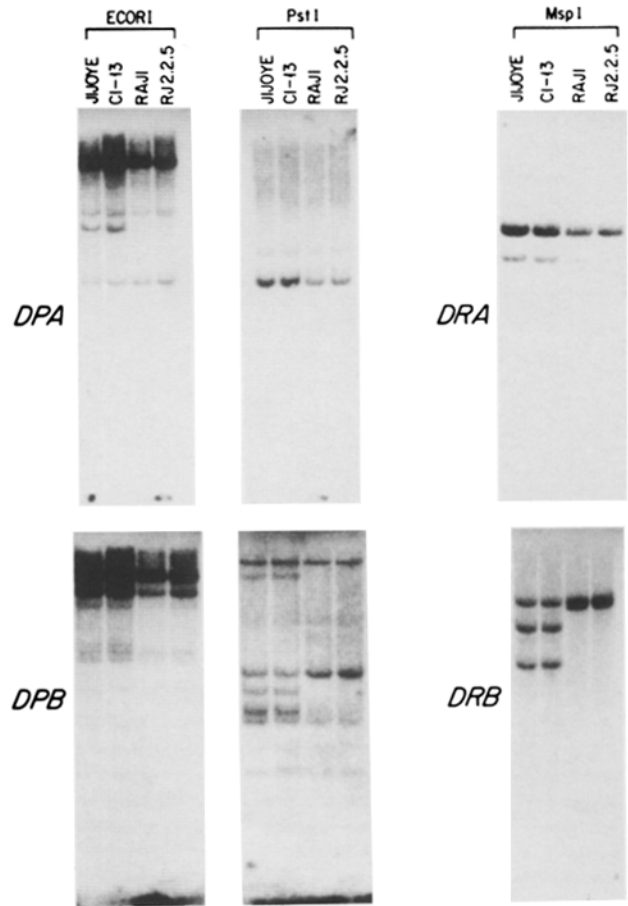


Figure 3. Genomic Southern blots of high molecular weight DNA isolated from the cell lines Jijoye, clone 13, Raji, and RJ2.2.5. 20 μ g of genomic DNA isolated from the various cell lines was cleaved by overnight digestion with the indicated restriction endonucleases. The cleaved DNA was then separated on 1% agarose gels, transferred to GeneScreen Plus hybridization membranes, and probed with random hexamer-primed, radiolabeled cDNAs of the indicated genes. After high stringency washing, the filters were exposed to X-ray film, and the autoradiograms are presented.

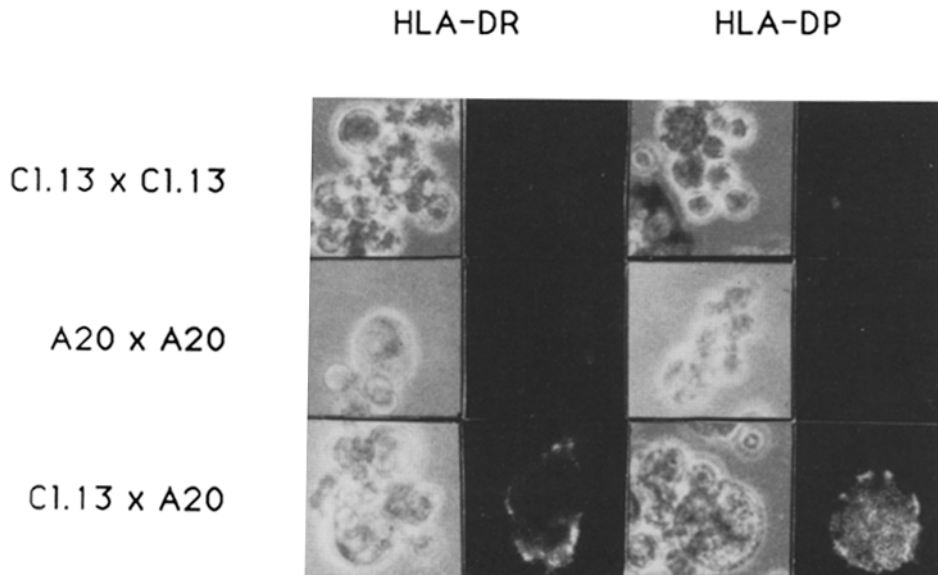


Figure 4. Interspecific transient heterokaryons between clone 13 and A20 cells express HLA-DR and -DP molecules de novo on their cell surface. 48 h after fusion with PEG 1500, clone 13 and A20 homokaryons and clone 13 \times A20 heterokaryons were assayed for expression of HLA-DR and -DP antigens using LB3.1 and B7/21 (respectively) mAbs as primary antibody. FITC-conjugated goat anti-mouse immunoglobulin was used as secondary antibody. After gently washing, the homokaryon and heterokaryon preparations were analyzed under an immunofluorescence microscope and both phase/contrast and immunofluorescent photographs were taken. Phase/contrast and immunofluorescence photographs of transiently fused cells from the clone 13 and A20 homokaryon fusions and the clone 13 \times A20 heterokaryon fusion are presented.

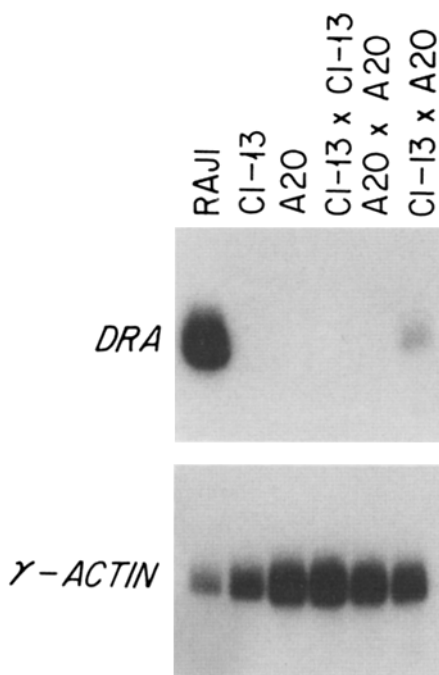


Figure 5. The DRA gene is activated de novo in the clone 13 × A20 heterokaryon. 10 μg of total RNA isolated from the indicated cell lines and from the indicated fusions (48 h after fusion) was fractionated on a formaldehyde gel. After transfer to a nylon membrane, the Northern blot was first probed with the radiolabeled DRA-specific fragment. After high stringency washing, the Northern blot was exposed to X-ray film. After exposure for 3 d, the same filter was stripped of the DRA probe by boiling in distilled water, and reprobed with the γ-actin-specific probe. After high stringency washing, the blot was exposed to X-ray film for 4 h. The autoradiograms are presented. The fusion efficiency of ~10% accounts for the relatively low level of DRA mRNA in the heterokaryon.

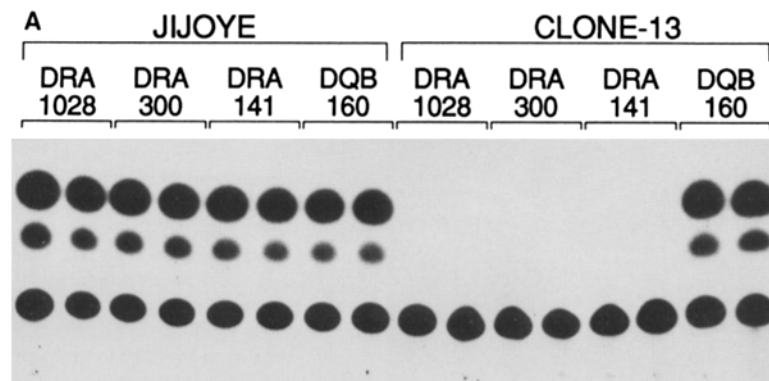
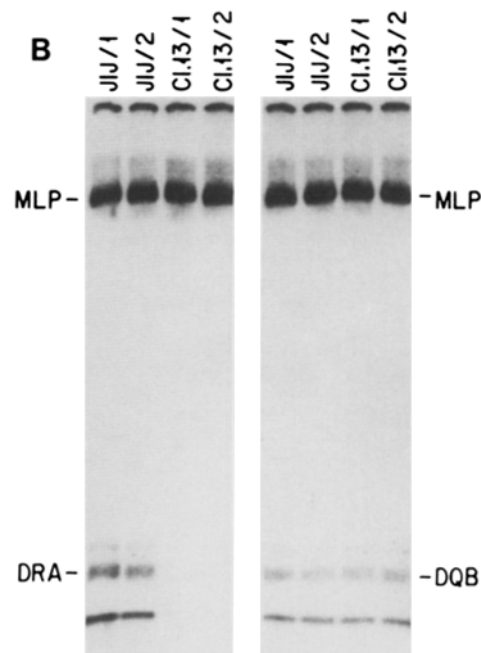


Figure 6. (A) Assay of CAT activity in extracts from Jijoye and clone 13 cells transiently transfected with the DQB160CAT and three DRACAT reporter plasmids. Cell extracts were incubated with C^{14} -chloramphenicol, as described in Materials and Methods, and the ethyl acetate-extracted material was separated on TLC. The autoradiogram of the dried TLC plate is presented. CAT activity was measured by counting the radioactivity of excised portions of the TLC plate corresponding to the acetylated form of chloramphenicol (3-acetate chloramphenicol). (B) In vitro transcription of the DRA1028CAT and DQB2500CAT plasmids in two different nuclear extract preparations from Jijoye and clone 13 cells. MLP indicates the in vitro transcript from the adenovirus major late promoter, and DRA and DQB the in vitro transcripts generated from the linearized CAT plasmids.

in clone 13 nuclear extract (in contrast to Jijoye nuclear extract), while DQBCAT transcription was transcribed with equal efficiency in Jijoye and clone 13 extracts (Fig. 6 B).

Gel Retardation Assays Using Oligonucleotides Spanning the Proximal Promoter of the DRA Gene Reveal No Detectable Differences in Sequence-specific DNA Binding in Nuclear Extracts from Jijoye and Clone 13 Cells. Since only 141 bp of upstream sequence are sufficient for efficient activity of the DRA promoter in B-cells (Fig. 6 A), gel retardation assays using oligonucleotides spanning this region were performed in an attempt to identify whether the putative defective transactivator might bind to this proximal promoter. However, no differences in DNA binding were detectable in nuclear extracts prepared from Jijoye and from clone 13 cells using the three oligonucleotides DRAS, DRAX, and DRAY+OCT, which cover the region from -37 to -143 (Fig. 7).

Treatment with IFN-γ Is Unable to Induce the Expression of HLA-DR or -DP Antigens on Clone 13 Cells. IFN-γ can induce the expression of class II molecules on many cell types that are normally class II negative. To test whether the defect in HLA-DR and -DP expression in cells could be bypassed after treatment with IFN-γ, clone 13 (as well as HeLa cells, which are normally class II negative as a positive control) was incubated in the presence of 400 U/ml human rIFN-γ for 48 h. IFN-γ treatment induced low-level HLA-DR and -DP (but not -DQ) expression on HeLa cells, but did not induce the expression of any class II isotype on clone 13 cells (Fig. 8).



A

[-----S Box oligo-----] [-----X Box oligo-----] [-----Y Box + Oct oligo-----]
 -143
 GTATCTTGTGCTCTGGACCCCTTGGCAAGAACCCTTCGCGTCAATCTCAAATATTATTTTCTGATTGGCCAAAGAGTAATGATTGGCTTTTAAAT
 S BOX X BOX Y BOX OCTAMER
 37

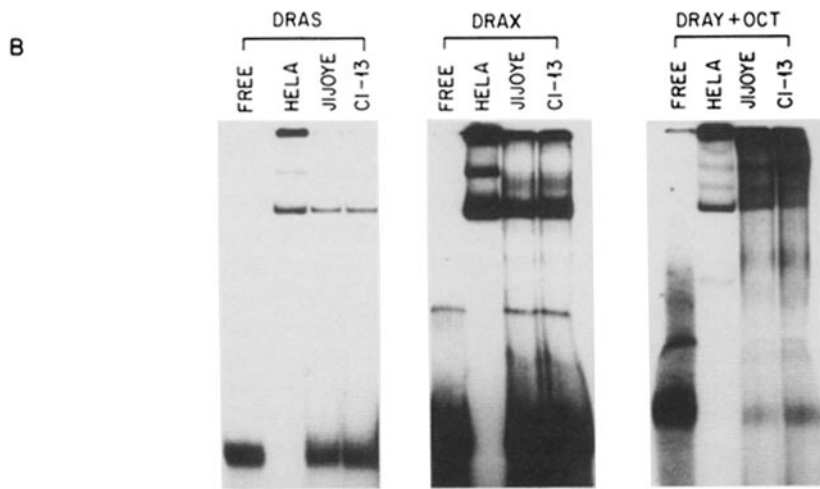


Figure 7. Gel-retardation analysis of nuclear extracts prepared from HeLa, Jijoye, and clone 13 cells using overlapping probes from the DRA proximal promoter. (A) The nucleotide sequence of the DRA proximal promoter with the conserved *cis*-acting motifs W-box, X-box, Y-box, and the octamer consensus sequence are boxed. The precise nucleotides contained in the indicated oligonucleotides are: W-box (5'GTA-TCTTGTGCTCTGGACCCCTTGGCAAGAACCCTTC3'), X-box (5'AG-AACCCTTCCCCTAGCAACAGATGCGTCAATCTCAAATATT3'), and Y-box + octamer (5'TCTCAAAT-ATTTTCTGATTGGCCAAAGAGTAATGATTGCAATTTAAT3'). (B) Autoradiograms of protein-DNA complexes and free radiolabeled oligonucleotide after incubation of the indicated double-stranded oligonucleotide with the indicated nuclear extract.

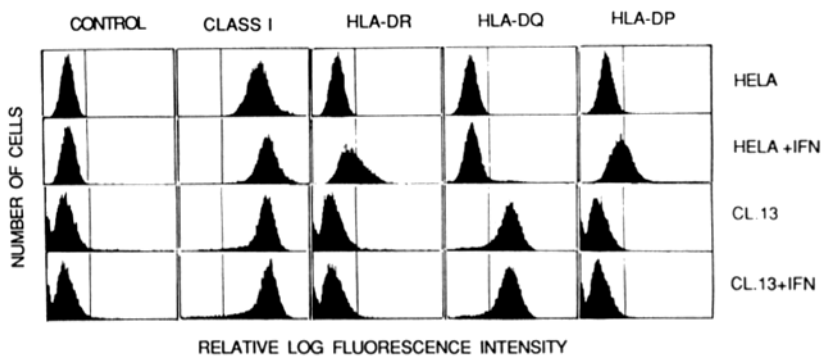


Figure 8. FACS analysis of the cell surface expression of HLA antigens on HeLa and clone 13 cells with and without treatment with 400 U/ml rIFN- γ for 48 h. The same mAbs used in Fig. 1 were used in these experiments.

Discussion

The class II region of the MHC of all vertebrates studied to date contain multiple genes that encode the α and β chain polypeptides that constitute a class II heterodimer. The isolation and sequence analysis of the genes encoding these polypeptides facilitated the identification of conserved *cis*-acting elements in the proximal-promoters of these genes. Three elements, called W, X, and Y boxes, are found in all class II genes. The similarities of the proximal promoters of the class II genes has been interpreted by some to suggest that the members of the class II multigene family are coordinately regulated (22). Further support for this hypothesis has come from the analysis of several mutant cell lines, where defects in the class II transcription machinery have generally affected the expression of all of the class II genes. On the other hand, cell lines that express DR and DP without DQ have been reported. Three reports indicate that the different class II isotypes are activated at different times in cell differentiation (23–25), and the fourth (15) indicates that an extremely low level of DQA transcription occurs in a cell line isolated from

a patient with bare lymphocyte syndrome (BLS-1). Further clues that the class II genes may not be coordinately regulated come from crosscompetition experiments where synthetic oligonucleotides corresponding to the conserved *cis*-acting elements from the different genes are analyzed for their ability to compete with each other in gel retardation assays (12, 13). These studies suggest the existence of multiple DNA-binding proteins with differential affinity for the conserved elements in the various genes.

In this report, a B-LCL, clone 13, is described that expresses high levels of the HLA-DQ1 antigen on its cell surface, in the absence of HLA-DR and -DP expression (Fig. 1). This cell line exhibits a marked reduction in the transcription of the α and β chain genes of HLA-DR and -DP in the absence of gross deletion of their structural genes (Figs. 2 and 3). The parental Burkitt's lymphoma line, Jijoye, from which clone 13 was derived, expresses high levels of all three class II isotypes (Fig. 1 A). Two lines of evidence strongly suggest that the defect in HLA-DR and -DP expression in

clone 13 cells affects an isotype-specific *trans*-activator. First, preparation of interspecific transient heterokaryons between clone 13 and the class II-positive murine hybridoma, A20, activates the de novo cell surface expression of the HLA-DR and -DP antigens and the transcription of the DRA gene (Figs. 4 and 5). Second, transient transfection and in vitro transcription analysis of CAT constructs demonstrates that the DRA promoter is specifically downregulated by the mutation in clone 13 (Fig. 6). The interspecific heterokaryon results demonstrate that a mouse homologue to the mutant factor exists, and that this murine homologue is sufficiently conserved to activate human class II genes.

The expression of high levels of HLA-DQ antigen on clone 13 in the face of marked reduction in the transcription of the genes encoding the HLA-DR and -DP antigens clearly demonstrates that the defective transactivator exhibits isotype specificity. Three other reports suggest that the regulation of HLA-DQ genes might be different from the HLA-DR and -DP genes (15, 18, 23). Symington et al. (23) found that DR and DP antigens are sometimes expressed in the absence of DQ. Moreover, HLA-DR and -DP expression can be induced by IFN- γ on certain cell types without the induction of HLA-DQ (14, 18, 26). These results have been interpreted by some to suggest that *trans*-activators in addition to those required for the expression of HLA-DR and -DP are required for HLA-DQ transcription. Indeed, previous to this report, the only example of transcription of an HLA-DQ gene in the absence of DR or DP transcription was the detection of a very low level of DQA transcription in BLS-1 (15). BLS-1 is quite different from clone 13, however, as the DQB gene is not expressed and transcription of the DQA gene is markedly reduced. Clone 13, then, is the first example of a cell expressing high levels of surface HLA-DQ in the absence of HLA-DR and -DP. The clone 13 phenotype suggests that HLA-DR and -DP genes require a *trans*-activator that is not critical for the transcription of HLA-DQ genes. Taken together, these data demonstrate that the class II genes have different regulatory mechanisms.

Since the region where the mutant *trans*-activator acts maps into the -140 region (Fig. 6A), gel retardation analysis using overlapping oligonucleotide probes spanning this region were performed to try to identify differences in sequence-specific binding in nuclear extracts from Jijoye and clone 13 cells; no differences were detected with any of the probes (Fig. 7). Similar negative results have been obtained by this method using the in vitro class II-negative mutant cell lines RJ2.2.5 and 6.1.6 (27) and four cell lines derived from patients with bare lymphocyte syndrome (28). These negative results suggest that the defect in clone 13 (and many other class II-negative cell lines) could involve *trans*-activator mutations in regions separate from the DNA-binding domain, or affect activators that do not bind directly to DNA. In addition, the inability to rescue the expression of HLA-DR and -DP after treatment with IFN- γ demonstrates that the defective factor is also required for inducible expression of these genes. Complementation strategies involving gene transfer and/or biochemical fractionation of nuclear extracts will be required to identify the defective or absent *trans*-activator in clone 13.

At face value, redundancy occurs in the immune response region of the MHC. Multiple genes of similar structure may have evolved as a safeguard against mutation in these vital genes. Such a view would imply that the class II isotypes have identical and interchangeable function. Indeed, mice lacking the I-E antigen are generally immunocompetent, and the production of xenogeneic class II molecules in transgenic mice (29) results in the generation of T cells restricted to the new class II isotype. An alternative possibility is that there might be divergent functions for different class II isotypes. For example, the HLA-DQ antigen has been proposed to play major role in the suppressor T cell response to streptococcal cell wall antigen (30). The compelling evidence of differential regulation of the class II genes provides additional reason to consider divergence in the biological roles of the class II isotypes.

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References

1. Kappes, D., and J.L. Strominger. 1988. Human class II major histocompatibility complex genes and proteins. *Annu. Rev. Biochem.* 57:991.
2. Benacerraf, B. 1981. Role of MHC gene products in immune regulation. *Science (Wash. DC)*. 212:1229.
3. Bjorkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L.

- Strominger, and D.C. Wiley. 1987. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature (Lond.)* 329:506.
4. Brown, J.H., T. Jardetzky, M.A. Saper, B. Samraoui, P.J. Bjorkman, and D.C. Wiley. 1988. A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules. *Nature (Lond.)* 332:845.
 5. Griscelli, C., A. Fischer, A. Durandy, B. Lisowska-Grospierre, C. Bremard, N. Cerf-Bensussan, F. le Deist, A. Marcadet, and C. de Preval. 1986. In *Primary Immunodeficiency Diseases*. M.M. Eibl, and F.S. Rosen, editors. Excerpta Medica, Amsterdam. 41-46.
 6. Pujol-Borrell, R., I. Todd, M. Doshi, G.F. Bottazzo, R. Sutton, D. Gray, G.R. Adolf, and M. Feldmann. 1987. HLA class II induction in human islet cells by interferon-gamma plus tumor necrosis factor or lymphotoxin. *Nature (Lond.)* 326:304.
 7. Saito, H., R.A. Maki, L.K. Clayton, and S. Tonegawa. 1983. Complete primary structures of the E β chain and its gene of the mouse major histocompatibility complex. *Proc. Natl. Acad. Sci. USA*. 80:5520.
 8. Okada, K., J. Boss, H. Prentice, T. Spies, R. Mengler, and J.L. Strominger. 1985a. Gene organization of DC and DX subregions of the human major histocompatibility complex. *Proc. Natl. Acad. Sci. USA*. 82:3410.
 9. Benoist, C., and D. Mathis. 1989. Regulation of MHC class II gene expression: X, Y and other letters of the alphabet. In *Tissue Specific Gene Expression*. Chapter 5. VCH Verlagsgesellschaft, Weinheim, FRG. 5.1-5.10.
 10. Accolla, R.S. 1983. Human B cell variants immunoselected against a single Ia antigen subset have lost expression of several Ia antigen subsets. *J. Exp. Med.* 157:1053.
 11. de Preval, C., B. Lisowska-Grospierre, M. Loche, C. Griscelli, and B. Mach. 1985. A trans-acting class II regulatory gene unlinked to the MHC controls the expression of HLA class II genes. *Nature (Lond.)* 318:291.
 12. Boothby, M., H.C. Liou, and L.H. Glimcher. 1989. Differences in DNA sequence specificity among MHC class II X box binding proteins. *J. Immunol.* 142:1005.
 13. Celada, A., and R.A. Maki. 1989. DNA binding of the mouse class II major histocompatibility CCAAT factor depends on two components. *Mol. Cell Biol.* 9:3097.
 14. Rijkers, G.T., J.J. Roord, F. Koning, W. Kuis, and B.J.M. Zegers. 1987. Phenotypical and functional analysis of B lymphocytes of two siblings with combined immunodeficiency and defective expression of major histocompatibility complex (MHC) class II antigens on mononuclear cells. *J. Clin. Immunol.* 7:98.
 15. Hume, C.R., L.A. Shookster, N. Collins, R. O'Reilly, and J.S. Lee. 1989. Bare lymphocyte syndrome: altered HLA class II expression in B cell lines derived from two patients. *Hum. Immunol.* 25:1.
 16. Hinuma, Y., M. Konn, J. Yamaguchi, D.J. Wudarski, J.R. Blakeslee, and J.T. Grace, Jr. 1967. Immunofluorescence and Herpes-type virus particles in the P3HR-1 Burkitt lymphoma cell line. *J. Virol.* 1:1045.
 17. Heston, L., M. Rabson, N. Brown, and G. Miller. 1982. New Epstein-Barr virus variants from cellular subclones of P3J-HR1 Burkitt lymphoma. *Nature (Lond.)* 295:160.
 18. Blanck, G., M. Lok, K. Kok, E. Downie, J.H. Korn, and J.L. Strominger. 1990. IFN-gamma induction of HLA class II mRNAs in dermal fibroblasts studied by RNase production analysis. *Hum. Immunol.* 29:150.
 19. Sakurai, M., and J.L. Strominger. 1989. Studies of expression of the DQ β promoter and its 5' deletion derivatives in normal and mutant human B cell lines. *Tissue. Antigens.* 34:64-77.
 20. Basta, P.V., P.A. Sherman, and J.P.Y. Ting. 1987. Identification of an interferon-response region 5' of the human histocompatibility leukocyte antigen DR chain gene which is active in human glioblastoma multiforme lines. *J. Immunol.* 138:1275.
 21. Yang, Z., R.S. Accolla, D. Pious, B.J.M. Zegers, and J.L. Strominger. 1988. Two distinct genetic loci regulating class II gene expression are defective in human mutant and patient cell lines. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1965.
 22. Sullivan, K.E., A.F. Calman, M. Nakanishi, S.Y. Tsang, Y. Wang, and B.M. Peterlin. 1988. A model for the transcriptional regulation of MHC class II genes. *Immunol. Today.* 8:289.
 23. Symington, F.W., F. Levine, M. Braun, S.L. Brown, H.A. Erlich, and B. Torck-Storb. 1985. Differential Ia antigen expression by autologous human erythroid and B lymphoblast cell lines. *J. Immunol.* 135:1026.
 24. Marcadet, A., D. Cohen, J. Dausset, A. Fischer, A. Durandy, and C. Griscelli. 1985. Genotyping with DNA probes in combined immunodeficiency syndrome with defective expression of HLA. *N. Engl. J. Med.* 312:1287.
 25. de Preval, C., M.R. Hadam, and B. Mach. 1988. Regulation of genes for HLA class II antigens in cell lines from patients with severe combined immunodeficiency. *N. Engl. J. Med.* 318:1295.
 26. Anichini, A., C. Castelli, G. Sozzi, G. Fossati, and G. Parmani. 1988. Differential susceptibility to recombinant interferon- γ induced HLA-DQ antigen modulation among clones from a human metastatic melanoma. *J. Immunol.* 140:183.
 27. Miwa, K., and J.L. Strominger. 1987. The HLA-DQ β gene upstream region contains an immunoglobulin-like octamer motif that binds cell-type specific nuclear factors. *Nucleic Acids Res.* 15:8057.
 28. Hume, C.R., and J.S. Lee. 1989. Congenital immunodeficiencies associated with absence of HLA class II antigens on lymphocytes result from distinct mutations in trans-acting factors. *Hum. Immunol.* 26:288.
 29. Flavell, R.A. 1988. Sixth HLA/H2 Cloning Workshop, Airlie, Virginia, Abstract 51.
 30. Sasazuki, T. 1989. Immunological Recognition. *Cold Spring Harbor Symp. Quant. Biol.* LIV:69.