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Immunology

Defective HLA class II expression in a regulatory mutant is partially complemented by activated *ras* oncogenes

(histocompatibility complex genes/retroviral vectors/trans-acting factors)

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ABSTRACT **The human B-cell line RJ2.2.5, derived by mutagenesis from a Burkitt lymphoma cell line and selected for loss of HLA class II antigen expression, was infected with recombinant retroviruses containing either the Harvey murine sarcoma virus oncogene v-Ha-*ras* or the human neuroblastoma homolog *NRAS*. Both activated *ras* genes partially complemented the regulatory defect in RJ2.2.5 and specifically increased the expression of the *DR* and *DQ* subsets of *HLA* class II genes. Blot-hybridization analysis and RNase mapping indicated that HLA-DQ α-chain mRNA in the infected cell lines was increased to a level at least 50% that of the parent B-cell line, Raji. The levels of HLA-DR and -DQ β-chain RNA also were increased but to a lesser extent. In contrast, we detected no effect of *ras* on the quantities of other class II, class I, or invariant-chain mRNAs. Fluorescence-activated cell sorter analysis with antibodies recognizing HLA-DR, -DQ, and class I antigens supported these observations. Enhancement of HLA class II gene expression by *ras* genes may have important implications for regulation of the immune system in response to transformation.**

Major histocompatibility complex (MHC) class II antigens are highly polymorphic cell surface glycoproteins that are critical elements in regulating cellular interactions in the immune system (1, 2). Developmental expression of class II antigens is normally limited to B lymphocytes, macrophages, activated T cells, and some hematopoietic precursors (3, 4). The primary function of class II antigens on these cells is to act as restricting elements for the presentation of foreign antigen to helper T cells (5).

HLA class II antigens are heterodimers composed of α and β chains of 34 and 29 kDa. The genes encoding these polypeptides are members of a large multigene family comprising at least 14 individual genes in a single linkage group on chromosome 6 (2). Biochemical and serological studies have provided evidence for expression of at least three distinct class II heterodimers, HLA-DR, HLA-DQ, and HLA-DP (hereafter called DR, DQ, and DP antigens), on the surface of a single cell. Several other class II genes are transcribed and may encode additional polypeptides, although their products have not been defined biochemically.

Transcription of individual HLA class II genes is regulated coordinately in most cases, suggesting that a common mechanism governs their expression (6). This hypothesis is supported by the existence of a recessive genetic defect not linked to HLA, called "bare-lymphocyte syndrome," that leads to a complete lack of expression of all class II genes (7–9). Two groups have also derived class II-negative mutants *in vitro* from class II-positive B-cell lines (10–12). HLA class II RNA is absent or greatly reduced in these mutants, although the cells have no apparent rearrangement or loss of the appropriate structural genes (13). Such variant cell lines may contain regulatory defects analogous to the naturally occurring mutation(s) in the bare-lymphocyte syndrome.

One HLA class II-negative variant, RJ2.2.5, was isolated by γ irradiation of the Burkitt lymphoma cell line Raji and selection with anti-class II antibodies and complement (12). Subsequently, it was shown that *HLA-DR*, *HLA-DQ*, and *HLA-DP* (hereafter called *DR*, *DQ*, and *DP* genes) expression in RJ2.2.5 could be restored by fusion with either mouse spleen cells or the class II-positive mouse lymphoma, M12.4.1 (14, 15). These results implied that a factor or factors present in the fusion partners could act in trans, across species barriers, to reactivate class II gene expression in the mutant cell line. Segregation analysis of the RJ2.2.5-spleen cell hybrids led to the conclusion that the complementing mouse gene was unlinked to the MHC (16). Recently, it was shown that stable transfection of mouse genomic DNA into RJ2.2.5 could also induce reexpression of the human class II genes (17). However, the mechanism by which class II gene expression was restored in each of these cases has not yet been determined.

We, with others, previously demonstrated that the introduction of activated v-*ras* genes via the Harvey or Kirsten murine sarcoma viruses (Ha-MSV or Ki-MSV) into normal human melanocytes induced *de novo* the expression of DR, DQ, and DP antigens (18). Further, it appeared that in melanocytes, the mechanism of induction of HLA class II gene expression by *ras* was not dependent on the production of γ interferon in response to infection with the viruses. To extend our observations on the effect of *ras* genes on the expression of class II genes, we have used selectable retroviral vectors to introduce activated v-Ha-*ras* and human *NRAS* genes into the mutant HLA class II-negative cell line RJ2.2.5. In this report we show that RJ2.2.5 cells containing activated *ras* genes display increased surface expression of DR and DQ class II antigens as well as increased levels of mRNA for their respective α and β chain genes.

METHODS

Cell Lines and Viruses. The human Burkitt lymphoma cell line Raji and a mutant induced by γ irradiation and selected for lack of HLA class II gene expression, RJ2.2.5, have been described (12, 13). The homozygous lymphoblastoid B-cell line LBF was obtained from N. Flomenberg of Memorial Sloan–Kettering Cancer Center. All human cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Whittacker M.A. Biolabs, Walkersville, MD), 2 mM glutamine, and 100 μg of penicillin and of streptomycin per ml (all from GIBCO).

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Abbreviations: MHC, major histocompatibility complex; *neo*, neomycin-resistance gene; FACS, fluorescence-activated cell sorter; Ii chain, invariant chain; v-Ha-*ras*, *ras* oncogene of Harvey murine sarcoma virus.

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Supernatants of recombinant ecotropic retroviruses containing v-Ha-*ras* (originally from K. B. Marcu, State University of New York, Stony Brook) and *NRAS* were obtained from E. Fleissner of Memorial Sloan–Kettering Cancer Center. The viruses were constructed by using the G418-selectable retrovirus vector pZIPneo (19) and either the *Sac* I–*Pst* I fragment of the v-Ha-*ras* gene (20, 21) or a genomic clone of the human *NRAS* gene (22, 23). Tissue culture supernatants were used to infect the helper virus-free amphotropic retroviral packaging cell line ψ-AM (24). Cell lines producing control virus were made by transfecting the G418-resistant retrovirus vector pDOL (25) into the ψ-AM cell line by the $Ca_3(PO_4)_2$ technique (26). Individual ψ-AM clones were titered for virus production of NIH 3T3 fibroblasts. High-titer clones produced $\approx 10^4$ G418-resistant colony-forming units per ml.

RJ2.2.5 was infected with the recombinant retroviruses by incubation of 5×10^6 cells in 2 ml of viral supernatant containing Polybrene (10 μg/ml; Aldrich) for 90 min at 37°C. The cells were then pelleted by centrifugation and resuspended in 10 ml of complete medium; 48 hr after infection, the cells were replated at a density of 10^6 cells per ml in complete medium supplemented with 500 μg of G418 per ml. Cell lines were established from all cultures 2–3 weeks after beginning selection.

Analysis of RNA and DNA. Cytoplasmic DNA and high molecular weight DNA were isolated by a modification of the method of Favaloro *et al.* (27). DNA was extracted from the nuclei by standard procedures (28). For Southern analysis, high molecular weight DNAs (10 μg) were digested with restriction endonucleases according to the manufacturer's conditions and were fractionated by electrophoresis in 0.75% agarose gels. After electrophoresis the DNA was depurinated in 0.25 M HCl and transferred to Zetabind filters (AMF-CUNO, Meriden, CT) in 0.4 M NaOH (29). Total cytoplasmic RNA (4 μg) was electrophoresed in 1% agarose/formaldehyde gels as described (28) and transferred to Zetabind filters in 3.0 M NaCl/0.30 M sodium citrate.

DNA Fragments and Probes. The probes used for DNA and RNA blot-hybridization analyses were as follows: DP α-chain cDNA, gift of H. Erlich (Cetus, Emeryville, CA); DR α-chain cDNA, pDRH2 (30); DQ β-chain cDNA, gift of E. O. Long (31); invariant chain (Ii chain) cDNA, p33.1 (32); Tn*5*-neomycin resistance gene (*neo*), the *Hin*dIII–*Eco*RI fragment of pZIPneoSV(X)1 (21); v-Ha-*ras* (22); and class I HLA-B cDNA (33). All fragments were labeled either by nick-translation (34) or by random priming with hexanucleotide fragments (35). Hybridization of the Zetabind filters was conducted as recommended by AMF-CUNO. Final washes were in 0.015 M NaCl/0.0015 M sodium citrate/1% $NaDodSO_4$ at 60°C. The DQ α-, DQ β-, DP β-, and DR β-chain-specific oligonucleotides are complementary to 5′ regions of the coding sequences that are not homologous to other class II loci (36). The oligonucleotide probes were labeled with [^{32}P]ATP to an activity of 1×10^9 cpm/μg with phage T4 polynucleotide kinase (Boehringer Mannheim; 3′ exonuclease free) and hybridized as described (36).

Fluorescence Analysis. The antibodies L243, Leu-10, and control mouse IgG were purchased from Becton Dickinson and used for staining as recommended by the manufacturer. The antibody W6/32 was obtained as tissue culture supernatant from R. W. Knowles (Sloan–Kettering Institute). The cells were analyzed on an EPICS V fluorescence-activated cell sorter (FACS).

RESULTS

Introduction of *ras* Genes into RJ2.2.5. Recombinant retroviruses containing the G418-resistance gene either alone or in combination with v-Ha-*ras* or *NRAS* were used to infect the HLA class II-deficient human B-cell mutant RJ2.2.5. The G418-resistant cell lines that were established from the cultures will be referred to as RJ-Hras and RJ-Nras, and the control virus-infected cell line as RJ-DOL. Each of the lines has been maintained in culture in the absence of selection for at least 6 months with no loss of ability to grow in G418-containing medium and with no change in phenotype. Genomic DNA was analyzed by Southern blotting to verify the presence of intact proviruses and to determine if the cell lines arose from multiple infected clones. The enzyme *Xba* I cuts once in the long terminal repeats (LTR) of all three proviruses, generating viral genome fragments of ≈4, 5, and 5.5 kilobases, respectively, for RJ-DOL, RJ-Hras, and RJ-Nras. Hybridization with the G418-resistance gene (Tn*5*-*neo*) indicated that all three cell lines contained intact provirus (Fig. 1). By comparison to the intensity of hybridization of a single-copy gene (*NRAS*), we estimated that individual cells contained only one copy of the virus (data not shown). Because only one *Eco*RI site is present internally in each provirus, a polyclonal-infected population would contain multiple distinct junction fragments between viral and cellular DNA, generating a smear on a Southern blot rather than distinct bands when hybridizing with Tn*5*-*neo*. Fig. 1 shows that both RJ-Hras and RJ-DOL appeared to be derived from many independent infections, while RJ-Nras consisted of two predominant clones. Presumably, this resulted from either a low efficiency of infection or poor survival after the selection. We have not detected any phenotypic differences between the RJ-Nras and -Hras RJ2.2.5 cell lines, although one is biclonal and the other polyclonal in character. This observation suggests that there was relatively little effect of the integration site on the level of virus expression in our system.

Expression of the recombinant viral genes and endogenous *HRAS* genes in RJ-DOL, RJ-Hras, RJ-Nras, and the normal class II-positive lymphoblastoid B-cell line LBF was as-

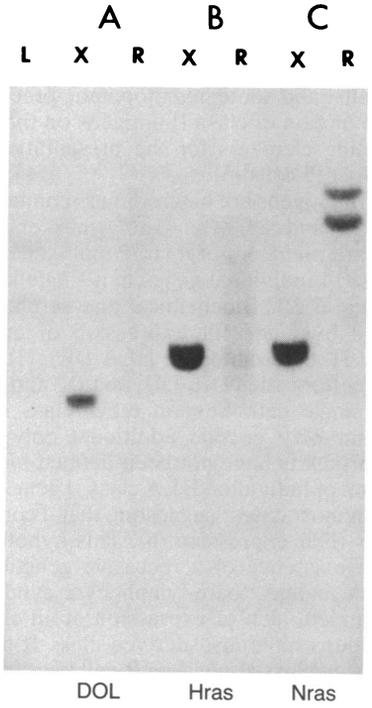

FIG. 1. Southern blot analysis of proviral integration sites. High molecular weight DNAs from RJ-DOL (*A*), RJ-Hras (*B*), and RJ-Nras (*C*) cell lines were digested with either *Xba* I (lanes X) or *Eco*RI (lanes R) and hybridized with the Tn*5*-*neo* gene (19). Molecular weight markers were phage λ DNA digested with *Hin*dIII and labeled with [^{32}P]dCTP (lane L).

sessed by RNA blot hybridization. Viral transcripts detected with the Tn*5-neo* probe were present in large amounts in the infected RJ cell lines (Fig. 2). In addition, the viral transcripts detected with the v-Ha-*ras* probe in RJ-Hras appeared to be more abundant than the endogenous RJ-Hras transcripts, although the v-Ha-*ras* probe is of rat cell origin and may not detect the human transcripts efficiently.

Analysis of HLA Class II Transcripts in Cells Infected by Retroviral Constructs. Retrovirus-infected cell lines were tested by blot-hybridization analysis for the presence of mRNA expressed by genes for HLA class II antigens, Ii chain, MHC class I antigens, and β_2-microglobulin. Because of the homology between α- and β-chain genes of different subloci, blots were hybridized either with sublocus-specific oligonucleotides or with cDNA probes and were washed under stringent conditions to eliminate cross-hybridization. Expression of these mRNAs in the control G418-resistant cell line RJ-DOL was indistinguishable from the uninfected RJ2.2.5 (data not shown). RJ-DOL (Fig. 3, lanes b) expressed greatly reduced amounts of DR and DQ α- and β-chain mRNAs when compared with the parent cell line, Raji (lanes a). However, in the RJ cell lines containing activated *ras* genes, there was a substantial increase of each of these mRNAs. DQ α chain was induced to levels at least 50% that of Raji, while the other mRNAs were increased to a lesser extent. For both DR and DQ, the induced mRNAs have the same mobilities as those present in Raji. RNase protection experiments using antisense transcripts of an internal fragment of the *DQ* α-chain gene reinforced our finding that DQ α-chain was induced after introduction of *ras* genes into RJ2.2.5 (data not shown). RJ-Hras and RJ-Nras had comparable abilities to increase *DQ* and *DR* expression, although we have not determined if overexpression of an unactivated *ras* gene is able to have a similar effect (Fig. 3; data not shown).

In contrast to the other HLA class II genes, mRNAs for DP α chain and DO β chain were readily detectable in RJ-DOL, and their expression did not appear as greatly affected by the mutation in RJ2.2.5 (Fig. 4 *A* and *C*). However, DP β-chain mRNA was absent from all of the RJ2.2.5-derived cell lines (Fig. 4*B*). There was no significant change in the levels of DP α-, DP β-, or DO β-chain mRNAs in the *ras* virus-infected cell lines. These observations suggest that class II genes *DP* and *DO* are regulated in a fashion distinct from *DR* and *DQ* and support previous work on expression of the *DO* β-chain gene (31). Similarly, Ii chain expression is generally regulated coordinately with class II genes (2), yet its level was only slightly decreased in RJ-DOL and was not elevated in the RJ-ras cell lines (Fig. 4*D*). To verify that the mutation in RJ2.2.5 and the introduction of *ras* genes did not simply affect general RNA metabolism, duplicate blots were hybridized with HLA class I and β_2-microglobulin genes. As shown in Fig. 4*E*, expression of HLA class I genes was unaltered in RJ-DOL and in the *ras*-containing cell lines. Likewise, β_2-microglobulin gene expression was unchanged (data not shown).

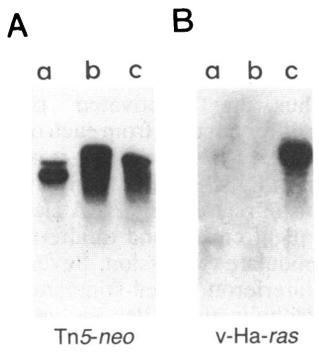

FIG. 2. Expression of viral transcripts in retrovirus-infected RJ2.2.5. (*A*) Four micrograms of cytoplasmic RNA from RJ-DOL (lane a), RJ-Hras (lane b), RJ-Nras (lane c) were electrophoresed on 1% formaldehyde/agarose gels, transferred to Zetabind filters, and hybridized with a Tn*5-neo* probe (20) as described. (*B*) v-Ha-*ras* expression in lymphoblastoid cell line LBF-DOL (lane a), RJ-DOL (lane b), and RJ-Hras (lane c). RNAs were prepared as above and hybridized with a v-Ha-*ras* probe (20).

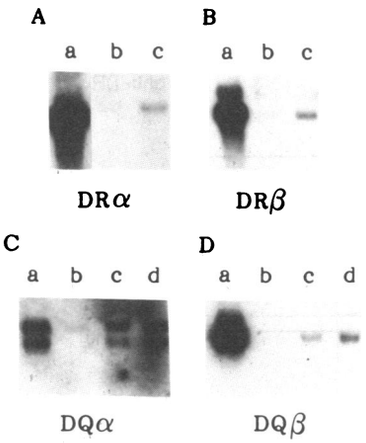

FIG. 3. Expression of DR and DQ RNAs in RJ-DOL and RJ-ras. RNAs from the parent Burkitt lymphoma cell line Raji (lanes a), RJ-DOL (lanes b), RJ-Hras (lanes c), and RJ-Nras (lanes d) were prepared as outlined in Fig. 2. Blots were hybridized with probes for DR α chain (*A*), DR β chain (*B*), DQ α chain (*C*), and DQ β chain (*D*) as specified.

Surface Expression of HLA Class II Antigens. Cell surface expression of class I and class II HLA antigens was quantitated by FACS with anti-HLA (W6/32), anti-DR (L243), and anti-DQ (Leu-10) monoclonal antibodies (Fig. 5). RJ-Hras and RJ-Nras have consistently expressed similar levels of these antigens; thus, for simplicity the fluorescence profiles of RJ-Nras are not shown. As predicted from the RNA hybridization data (Figs. 3 and 4), RJ-DOL expressed no detectable surface DR or DQ but normal levels of class I antigens. As in our earlier experiments, we did not detect any difference in expression of HLA antigens between RJ2.2.5 and RJ-DOL (data not shown), suggesting that the infection and selection process did not alter the phenotype of the

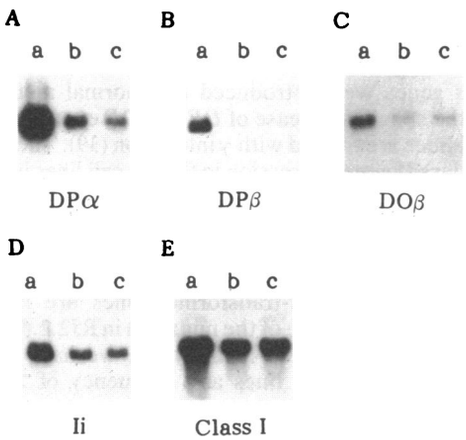

FIG. 4. Analyses of RNAs from RJ-DOL and RJ-ras cells. RNA samples from Raji cells (*A–C*, lanes a) or the normal lymphoblastoid B-cell line LBF (*D–E*, lanes a), RJ-DOL (lanes b), and RJ-Hras (lanes c) were prepared as described in Fig. 2. Blots were hybridized with probes for DP α chain (*A*), DP β chain (*B*), DO β chain (*C*), Ii chain (*D*), and HLA class I (*E*) as described.

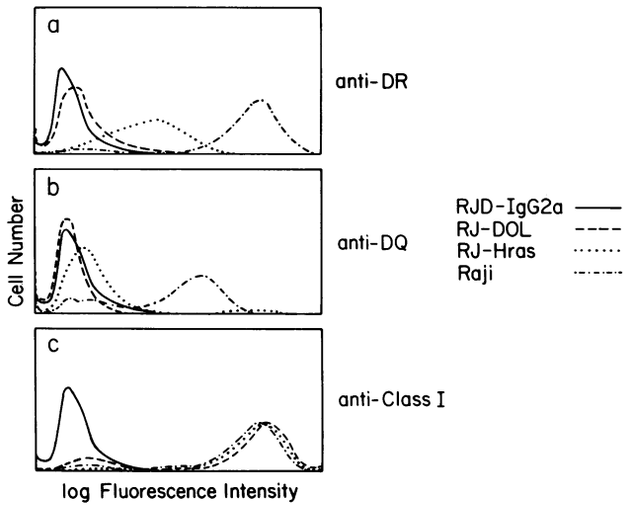

FIG. 5. Surface expression of class II antigens. Fluorescence profiles of DR (*a*), DQ (*b*), and HLA-A,B,C (*c*) expression in RJ-DOL (----), RJ-Hras (····), and Raji (·-·-·). Cells were analyzed by FACS after indirect labeling with either anti-DR L243 (*a*), anti-DQ Leu-10 (*b*), or anti-class I W6/32 (*c*) as described. IgG2a was used as the first antibody in control labelings.

infected cell lines. RJ-Hras and RJ-Nras were essentially 100% positive for DR surface expression, although they stained approximately 1/10th as intensely as Raji (Fig. 5*a*). DQ surface expression was also increased on the *ras* transformants compared to RJ-DOL (Fig. 5*b*).

Adenovirus 12 *E1a* (37) and *myc* (38) oncogenes down-modulate the expression of MHC class I antigens in some cell lineages. Although these oncogenes are very different from *ras* in their structures and apparent functions, it was possible that the activated *ras* products could decrease class I expression through a common pathway. However, there was no apparent alteration in the level of class I expression in RJ-Hras (or RJ-Nras) when compared to RJ-DOL (Figs. 4*E* and 5*c*).

DISCUSSION

We have shown that a high level of expression of activated v-Ha-*ras* or *NRAS* genes in the HLA class II-negative regulatory mutant RJ2.2.5 leads to a specific increase in *DQ* and *DR* expression, as measured by RNA blot hybridization, RNase mapping, and fluorescence. This contrasts with the coordinate induction of the *DR*, *DQ*, and *DP* loci observed when *ras* genes were introduced into normal melanocytes (18) and the specific increase of *DP* and *DR* expression when some cell lines are treated with γ interferon (39). The increase in HLA class II gene expression in the *ras* cell lines is unlikely to be an artefact of the selection procedure because the control cell line, RJ-DOL, like RJ-Hras and RJ-Nras, was established after selection in G418, yet it is indistinguishable in phenotype from the original mutant, RJ2.2.5. It is also improbable that the *ras*-transformed lines are revertants because the reversion rate of the mutation in RJ2.2.5 has been estimated to be $<2 \times 10^{-8}$. Moreover, we have obtained HLA class II-expressing lines at a frequency of $>10^{-4}$, at least in the case of RJ-Hras.

Although both DQ and DR α- and β-chain mRNAs were clearly increased in the *ras* transformants, the relative changes in quantities of DR and DQ RNAs would predict the greatest increase in DQ surface expression. However, the FACS analysis showed only a slight increase in surface DQ expression and a large increase in surface DR. A partial explanation for this finding is the lack of reactivity of the DQ-specific antibody Leu-10 with one of the haplotypes of Raji (*DQw2*) (Fig. 5*b*; refs. 40 and 41). Therefore, this measurement is probably an underestimate of total expression of DQ antigens relative to surface DR expression for the RJ and Raji cell lines. In addition, it is possible that, although the RJ-ras cell lines contain a substantial amount of DQ α-chain mRNA, lower quantities of DQ β-chain limit surface expression of DQ antigens. Another possibility is that the anti-DR antibody L243 detects molecules in addition to authentic DR α–β dimers. Although it appears to recognize a monomorphic determinant present only on DR molecules under normal circumstances (42), L243 may also detect other class II molecules when DR is low as in the RJ-ras lines. Indeed, two-dimensional gel analysis of class II antigens in the RJ-Hras cells indicates that DQ β chains are found in addition to the DR β chains in immunoprecipitates with L243 (data not shown). In contrast to most cells expressing class II antigens, RJ-ras cells express nonequivalent amounts of α- and β-chain mRNAs for all class II subloci. Under these conditions, nonallelic α and β chains may associate to form novel heterodimers, which are also recognized by the L243 monoclonal antibody. For example, the abundant DP α-chain transcripts present in RJ2.2.5 encode α chains that could pair with the induced DR β chains. In normal B cells, however, such complexes may be very rare. Potentially similar "trans" pairing between subloci has been demonstrated in mouse cells transfected with individual α- and β-chain genes from either *I-A* or *I-E* (43). Definitive characterization of the α–β complexes recognized on the RJ-ras cell lines will require further serological and biochemical analysis.

The lesion in RJ2.2.5 can be complemented by somatic cell hybridization with HLA class II-positive mouse cells or by transfection of high molecular weight mouse DNA (14–17). Although these approaches, as well as the introduction of an activated *ras* gene, can at least partially restore HLA class II gene expression in RJ2.2.5, it is not clear that they complement the mutation by directly replacing the defective factor. It may be possible to induce class II expression in RJ2.2.5 by either bypassing a defective step in the normal regulatory pathway or activating an independent mechanism. For example, a partial transcriptional defect might be complemented by an increase in class II mRNA stability. Several phenotypic differences between the hybrids, transfectants, and *ras* transformants suggest that they may regain a class II-positive phenotype by distinct mechanisms. DR α-chain expression was nearly quantitatively restored in the hybrids and transfectants, albeit after selection for high expression by FACS (15, 17). In contrast, the amount of DR α-chain mRNA (in the unsorted RJ-Hras cell lines) remained considerably less than in Raji cells. We found that DQ α-chain mRNA was greatly increased in the *ras*-containing cell lines, whereas DR and DQ β-chains were not fully restored to the levels found in Raji cells. Thus, the "reactivated" phenotypes of the various cell lines differ not only from each other but also from the parent of RJ2.2.5, Raji. Therefore, it is unlikely that the original mutation in RJ2.2.5 directly involves a *ras* gene.

Different means of reactivating HLA class II gene expression in RJ2.2.5 might correspond to alternate physiological pathways that modulate expression. Several soluble mediators including γ interferon, B-cell-stimulatory factor 1 (BSF-1), anti-immunoglobulin antibodies, and phorbol esters have been shown to augment class II expression *in vitro* (44–47). Although the biochemical details of the effector pathways for these ligands have not been elucidated, they appear to regulate class II expression in independent and sometimes conflicting ways (48, 49). In the RJ-ras cell lines, the product of activated-*ras* may be acting as an element in the normal signal-transduction pathway for one or more of these factors. This pathway is clearly restricted to certain cell types, as *ras* genes do not induce class II expression in the mouse or

human fibroblasts, mouse or human melanomas, or mouse mammary carcinomas that we have analyzed (ref. 18; unpublished observations). Acute lymphoblastic leukemia T-cell lines, which have been shown to contain activated *NRAS* genes, also do not express class II antigens (22). The effect of *ras* appears to be distinct from the ability to respond to γ interferon and BSF-1, since class II mRNAs are not inducible in RJ2.2.5 with either factor (data not shown). Recent studies have suggested that ras proteins may be involved in regulation of phosphatidylinositol turnover (50, 51). We also have found that treatment of RJ2.2.5 with phorbol myristic acid (PMA), a diacylglycerol analog and activator of protein kinase C, can partially restore class II expression (data not shown). A comparable induction of glucose transporter mRNA in fibroblasts transformed with *ras* or treated with phorbol esters also supports the hypothesis that phorbol esters and *ras* may induce some of their effects through alterations in protein kinase C activity (52).

Quantitative levels of HLA class II antigens at the cell surface may control interactions between antigen-presenting cells and antigen-specific T cells (53). Thus, induction of HLA class II gene expression by *ras* in RJ2.2.5 may have important implications for the immune response to neoplastically transformed cells. Enhanced class II expression in response to oncogene activation may increase the susceptibility of cells to recognition by the immune system. Alternatively, inappropriate or altered expression of class II antigens on transformed cells may enable them to evade recognition by T helper or cytotoxic cells.

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