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Immunology

Reactivation by a *trans*-acting factor of human major histocompatibility complex Ia gene expression in interspecies hybrids between an Ia-negative human B-cell variant and an Ia-positive mouse B-cell lymphoma

(gene regulation)

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ABSTRACT **Raji, a human B-lymphoma cell line, expresses high levels of class II (Ia) antigens. The expression of Ia antigens is totally abolished at the level of specific mRNA accumulation in RJ 2.2.5, a variant cell line derived from Raji after mutagenesis and immunoselection. We report here that the human Ia antigen expression can be restored in interspecies somatic cell hybrids between RJ 2.2.5 cells and BALB/c-derived M12.4.1 (I-A$^+$, I-E$^+$) B-lymphoma cells. Two hybrid clones were studied in detail. In both clones Ia molecules of the DR and NG2 type were easily detected by cell surface immunofluorescence and specific immunoprecipitation. In contrast, the DQ1 molecules were not detected with the above technique. DNA hybridization experiments using specific probes indicated that α-chain *DR* and β-chain *DQ* genes were present in the hybrids. However, RNA hybridization experiments revealed that β-chain DQ mRNA was present in the hybrids at very low amount compared to α-chain DR-specific mRNA. These results indicate that at least several genes of the class II gene cluster are coordinately regulated by *trans*-acting factor(s) that operate across species barriers. The basis of the mechanisms controlling the expression of class II antigens in these human–mouse hybrids might be related to the extinction (lack of expression) or activation of tissue-specific traits that take place when genomes of cells of dissimilar developmental potentials are brought together.**

Human Ia antigens are a heterogeneous family of polymorphic major histocompatibility complex (MHC)-encoded molecules made of two distinct subunits, an α chain of 33,000–36,000 daltons and a β chain of 24,000–28,000 daltons (reviewed in ref. 1). The apparent involvement of Ia antigens in a variety of immune functions (2) as well as in the susceptibility to certain diseases (3) has encouraged investigations to analyze the structural heterogeneity of Ia antigens at the protein and at the gene level. It is now well documented that the human Ia system consists of a heterogeneous family of molecules (4–10) encoded by distinct structural genes (11–13) that are all clustered in the short arm of chromosome 6 (14). At least four Ia heterodimers, each one composed of distinct α and β subunits, can be coexpressed on the cell surface of a single B cell (15). In contrast to MHC class I gene products (the HLA-A, -B, -C) that are expressed on the surface membrane of virtually all cells, Ia (or MHC class II) molecules are expressed only on a limited number of cells, primarily of lymphoid or myeloid origin. However, in certain pathological conditions, other cells, including tumor cells of nonlymphoid or nonmyeloid origin, can express "de novo" class II gene products (reviewed in ref. 16). The timing of class II gene expression appears developmentally regulated; no information on the factors intervening into these developmental processes is as yet available. In other systems it is presently believed that regulatory genes are required either for the shut-off of developmentally active genes (extinguisher genes) (17, 18) or for the activation of their expression (activator genes) (19, 20). The effect of these regulatory genes appears to be independent of species barriers, as assessed in somatic cell hybrids, and may relate to the different stages of differentiation of the parent cells of the hybrids (reviewed in ref. 21).

We have recently isolated from the human B-lymphoma cell Raji a variant—namely, RJ 2.2.5—that after mutagenesis and immunoselection had lost the expression of Ia antigens (22). Although the cell line used for immunoselection was heterozygous for some Ia antigens and although the immunoselection was performed by using monoclonal antibodies specific for distinct families of the human Ia molecular pool, the variant had lost the expression of the entire assessable repertoire of class II antigens. Gladstone and Pious (23) generated variants from a DR1,3 heterozygous B-cell line by immunoselection with an alloantiserum directed against only the DR1 specificity. Some of these variants were lacking both DR1 and DR3 specificities and, interestingly, could reexpress both DR markers after somatic cell hybridization with a partner cell with different DR phenotype (24). Further studies on the DR1,3-negative variant could not elucidate the precise step(s) during biosynthesis of DR antigens affected in the variant (25). Studies on our RJ 2.2.5 cells based on genomic DNA analysis by means of hybridization with specific cDNA probes demonstrated, within the limit of sensitivity of the method employed, that the class II gene structures were not altered in the variant as compared to the parental cell line; in contrast, mRNA measurements showed that the variant did not produce (or failed to accumulate) class II-specific mRNAs (26). Therefore, we concluded that a defect in the accumulation of the mRNAs of most, if not all, class II genes residing on both copies of chromosome 6 was responsible for the coordinate loss of expression of Ia molecules.

Our RJ 2.2.5 variant may be interpreted either as a cell line having lost a functional activator gene or as a cell line reexpressing a previously silent extinguisher locus; these two possibilities can be eventually discriminated by somatic cell hybridization experiments. We report here the generation of interspecies human–mouse somatic cell hybrids obtained by fusion of human RJ 2.2.5 Ia-negative variant cells (22) with

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Abbreviations: MHC, major histocompatibility complex; FACS, fluorescence-activated cell sorter; HAT, hypoxanthine/aminopterin/thymidine; mAb, monoclonal antibody.

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mouse M12.4.1 Ia-positive B-lymphoma cells (27) and their subsequent characterization by fluorescence-activated cell sorter (FACS), DNA, RNA, and protein analyses.

MATERIALS AND METHODS

Cell Lines and Somatic Cell Fusion. The cell lines used in this study were the human B-cell line Raji (class II phenotype: DR3,w6; DQ1; NG2); its Ia-negative derived variant RJ 2.2.5 (22); and the mouse BALB/c-derived B-lymphoma line M12.4.1, a hypoxanthine/guanine phosphoribosyltransferase-lacking cell line that dies in selective medium containing hypoxanthine/aminopterin/thymidine (HAT) (27). Cells were propagated in RPMI 1640 medium containing 10% fetal calf serum, glutamine, and antibiotics. Hybrids between RJ 2.2.5 and M12.4.1 cells were obtained by fusion in PEG (Merck, M_r 1000) as described (28). Because human cells are much more sensitive than rodent cells to ouabain (29), selection of hybrids was performed in HAT medium containing 1 μM ouabain.

Screening and Analysis of Hybrids. Cells growing in selective HAT/ouabain medium were analyzed by indirect immunofluorescence with the FACS (FACS II; B. D. Electronics, Mountain-View, CA) as described (22). The analysis was performed by using the following monoclonal antibodies: D1-12 (specific for DR, see ref. 15); D4-22 (NG2 and DR, see ref. 15); BT3/4 (DQ1, see ref. 5); 25.9.17 (mouse I-$A^{b,d,p,q}$, see ref. 30); B9.12.1 (HLA-A, -B, -C, see ref. 31); and S13.11 (H2-K^d; S. Tonkonogy, personal communication). Hybrids of interest were sorted by FACS and cloned in limiting dilution conditions. Cells were externally labeled by lactoperoxidase-catalyzed iodination (32), immunoprecipitated at 4°C with various monoclonal antibodies (mAbs) covalently bound to Sepharose 4B beads, and eluted in 100 μl of $NaDodSO_4$ sample buffer (33) for 2 min at 100°C. The eluted material was then analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) as described (15). High molecular weight DNA was extracted according to published procedures (34). Aliquots of 10 μg of DNA were digested with *Hin*dIII restriction enzyme (Boehringer, Federal Republic of Germany) and separated by horizontal gel electrophoresis in Tris acetate buffer (34). The DNA was transferred to nitrocellulose membranes (GeneScreen Plus, New England Nuclear) and hybridized to labeled DNA probes under stringent conditions according to the specifications of the manufacturers. cDNA encoding the DR α chain (35) and a DQ β chain (36) were used. DNA labeling with [α-^{32}P]dCTP and [α-^{32}P]dATP by nick-translation was performed according to Rigby *et al.* (37). Cytoplasmic RNA was prepared as described (38) and hybridized in 50% formamide.

RESULTS

Analysis of Hybrid Cell Surface Phenotype by Flow Cytometry. Human RJ 2.2.5 and mouse M12.4.1 cells were fused by PEG-mediated cell fusion as described in *Materials and Methods*. Cells from two hybrid cultures, M/R 51 and M/R 23, derived from two independent fusions and positively reacting with the D1-12 mAb, were sorted by using the FACS, cloned under limiting dilution conditions and analyzed. Fig. 1 shows that both hybrids expressed significant amount of HLA-DR determinants (as defined by D1-12 mAb) as compared to Ia-negative RJ 2.2.5 cells. However, although virtually all M/R 51.1 cells were positive for D1-12-defined epitopes, only a fraction of M/R 23.2 cells expressed these epitopes (70% in the experiment shown). This pattern was not modified after a second cloning and four additional sortings of the two hybrid clones over a 6-month period. M/R 23.2

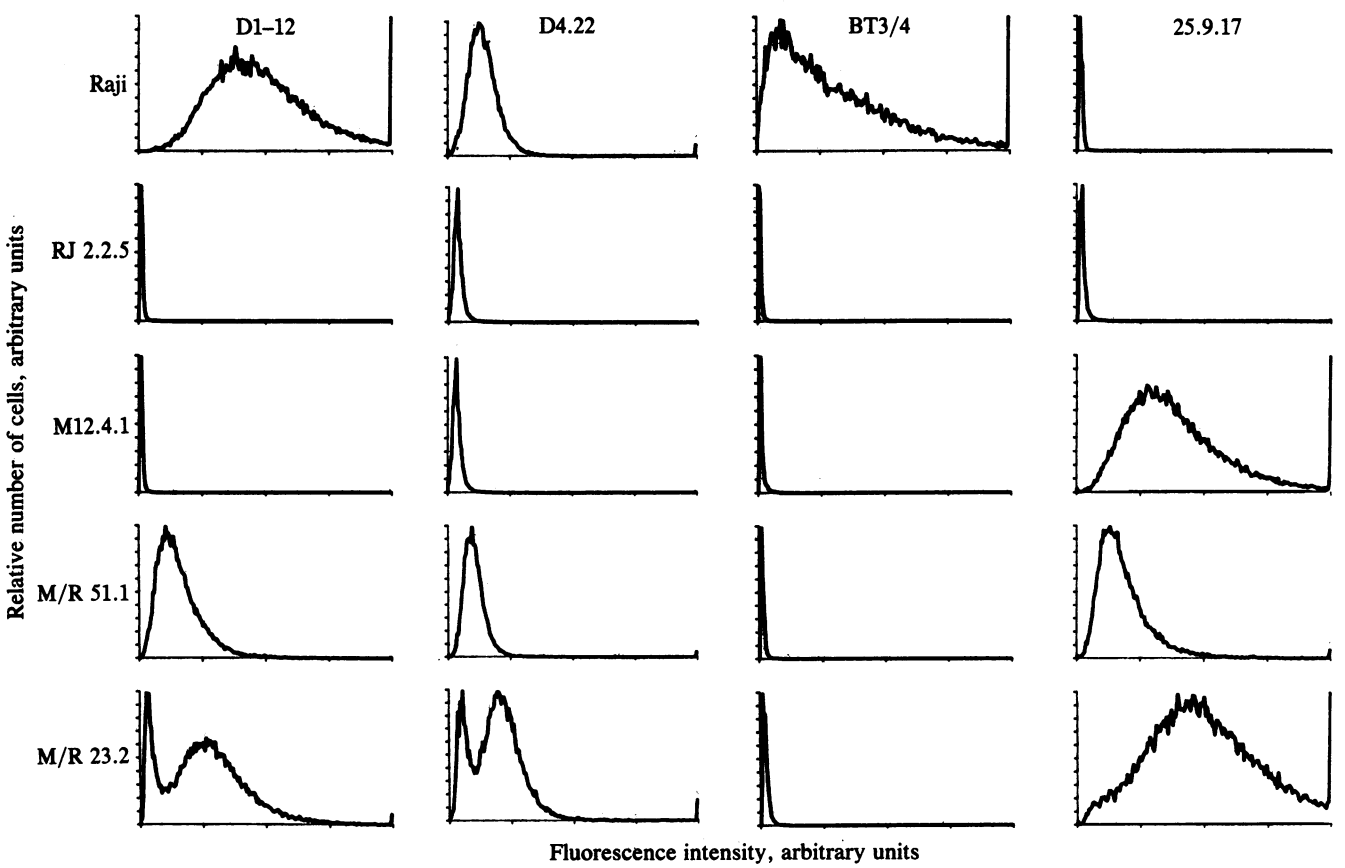

FIG. 1. Flow microfluorimetric analysis of human × mouse cell hybrids and their parental cells after staining with anti-class II mAbs. Cell lines are listed from top to bottom and mAbs are listed from left to right.

cells selected on the basis of a D1-12-negative surface phenotype retained such a negative phenotype, thus indicating the occurrence of a unidirectional reversion from the positive to the negative expression of HLA-DR determinants (data not shown). The reactivity of the two hybrid clones with mAb detecting human Ia molecules distinct from DR was analyzed. Fig. 1 shows that the reactivity pattern obtained with the D4-22 mAb closely paralleled the one observed with the D1-12 mAb. All M/R 51.1 cells expressed D4-22-specific epitopes, although to a lower level than Raji cells; in contrast, two distinct populations of cells were observed in the M/R 23.2 hybrid clone at all purification stages, the first one expressing undetectable amounts of D4-22-defined epitopes and the second one expressing relatively large amounts of these epitopes. Fig. 1 also shows the FACS profiles obtained after staining the various cell populations with an anti-DQ1 reagent, the BT3/4 mAb. Both M/R 51.1 and M/R 23.2 hybrid clones were negative for cell surface expression of BT3/4-defined epitopes. Control experiments with the 25.9.17 mAb, specific for I-A^b antigens and crossreactive with I-A^d antigens (30), demonstrated that M/R 51.1 and M/R 23.2 displayed I-A-specific epitopes. Reactivity with the 25.9.17 mAb was conserved during further purification of the two hybrid clones. Moreover, both hybrids expressed HLA class I antigens, as detected by the B9.12.1 mAb (31), and mouse H-2K^d antigens, as detected by the S13.11 mAb (see Fig. 2). It is noteworthy that M/R 23.2 cells showed a biphasic distribution after staining with B9.12.1 mAb similar to that described for the D1-12 mAb. M/R 23.2 cells that had been selected on the basis of a D1-12-positive or -negative phenotype were found to be B9.12.1-positive and B9.12.1-negative, respectively (data not shown).

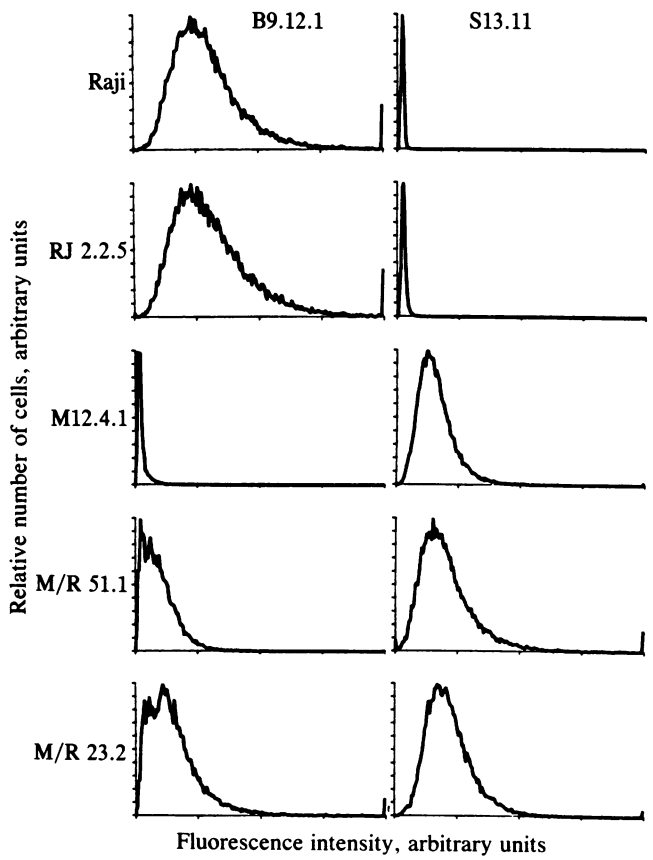

FIG. 2. Flow microfluorimetric analysis of human–mouse cell hybrids and their parental cells after staining with anti-class I mAbs. Cell lines, listed from top to bottom, were tested with either the B9.12.1 mAb (left histograms) or the S13.11 mAb (right histograms).

Biochemical Studies. Hybrid M/R 51.1 and M/R 23.2 cells were surface labeled with ^{125}I by the lactoperoxidase-catalyzed iodination method. Lysates obtained from these cells after Nonidet P-40 detergent solubilization were precipitated first with D1-12 mAb and then with D4-22 mAb. This procedure was used because D4-22 mAb recognizes an epitope shared by NG2 and DR (NG1) molecules (15). Thus, after elimination of D1-12-reactive DR molecules, the D4-22 mAb reacts only with NG2 molecules.

Both M/R 51.1 and M/R 23.2 immunoprecipitates obtained with D1-12 mAb consisted of typical α–β Ia heterodimers (Fig. 3, lanes c and d, respectively). As expected, D1-12 mAb did not precipitate Ia-like molecules in lysates from M12.4.1 (lane b) or RJ 2.2.5 cells (data not shown). The D1-12-specific Ia heterodimers detected in the two hybrids were similar but not identical to the corresponding molecules found in Raji cells (lane a). In particular, the β chains derived from the hybrid cells migrated in $NaDodSO_4$/PAGE in nonreducing conditions with an apparent molecular mass of 27,500 daltons, as compared to 26,000 daltons for the corresponding subunit derived from Raji cells. After elimination of the D1-12-reactive molecules, additional α–β heterodimers corresponding to NG2 molecules were precipitated by D4-22 mAb in both M/R 51.1 and 23.2 cell lysates (lanes g and h, respectively). These molecules were similar to the corresponding α–β heterodimers present in Raji cells (lane e). No NG2 molecules were found in M12.4.1 (lane f) or RJ 2.2.5 cell extracts (data not shown). It must be noted that the amount of DR and NG2 molecules immunoprecipitated from M/R 23.2 cell lysates was consistently higher than that of the corresponding molecules obtained from M/R 51.1 cell extracts (compare lane d with lane c, and lane h with lane g, respectively). Immunoprecipitation experiments were also performed by using the BT3/4 anti-DQ1 mAb. In agreement with the results obtained by flow microfluorometry, BT3/4 mAb did not precipitate detectable amounts of antigens present in cell lysates derived from M/R 51.1 or M/R 23.2 cells (data not shown).

DNA and RNA Analysis of Hybrid Cells. The ability of the two hybrids to produce human Ia molecules was correlated with the presence of human class II genes as determined by Southern hybridization techniques (39). As shown in Fig. 4, both M/R 23.2 and 51.1 hybrids (lanes c and g, respectively) contained an α-chain *DR* gene indistinguishable, by restric-

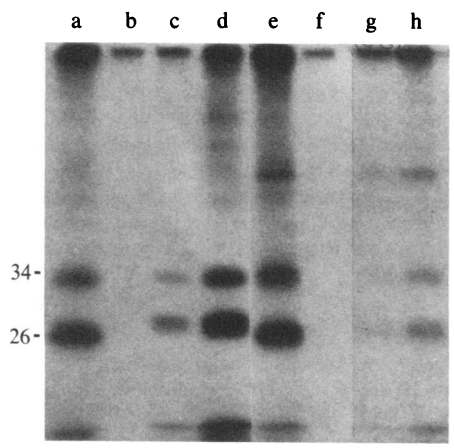

FIG. 3. Autoradiograph of 11% $NaDodSO_4$/polyacrylamide slab gels of unreduced molecules immunoprecipitated with D1-12 mAb (lanes a–d) or D4-22 mAb after depletion of the D1-12-reactive molecules (lanes e–h). Nonidet P-40 extracts of the following ^{125}I-surface-labeled cells were analyzed: Raji cells, lanes a and e; M12.4.1 cells, lanes b and f; hybrid 51.1 cells, lanes c and g; hybrid 23.2 cells, lanes d and h. Molecular masses are indicated in kilodaltons.

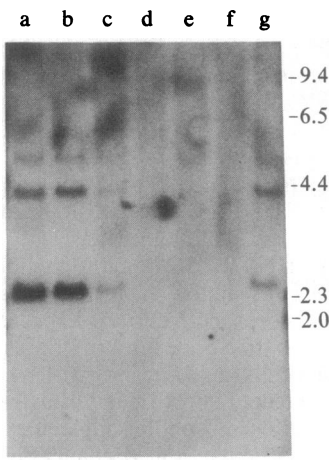

FIG. 4. Hybridization of *Hin*dIII-digested genomic DNA from various cell lines with the DR α-10 plasmid probe. Lanes: a, Raji DNA; b, RJ 2.2.5 DNA; c, hybrid M/R 23.2 DNA; d, Ia-negative hybrid M/R 23.2 DNA; e, mouse kidney DNA; f, M12.4.1 DNA; g, hybrid M/R 51.1 DNA. Molecular size standards are indicated in kilobases.

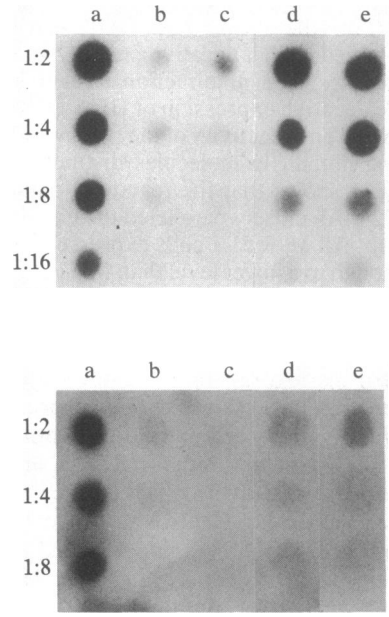

FIG. 5. RNA dot-blot hybridization with DR α-10 (*Upper*) or DC β-1 (*Lower*) probes. RNA was extracted according to White and Bancroft (38) from the following cell lines: Raji, lane a; RJ 2.2.5, lane b; Ia-negative M/R 23.2-derived hybrid, lane c; M/R 23.2 hybrid, lane d; and M/R 51.1 hybrid, lane e. The dot blot in *Upper* was exposed 2 days, whereas the dot blot in *Lower* was exposed 7 days. Numbers on the left represent serial dilutions. Cells (3×10^6) of RJ 2.2.5 and M12.4.1 and cells (1.5×10^6) of the other cell lines were extracted in a volume of 50 μl; 5 μl of each sample was diluted and spotted onto nitrocellulose according to White and Bancroft (38).

tion enzyme pattern, from the α-chain *DR* gene copy carried by Raji (lane a) and RJ 2.2.5 (lane b) cells. This experiment was carried out under stringency conditions such that no crossreaction between the human cDNA probe and the relevant murine Ia genes present in the genomic DNA extracted from mouse kidney cells (lane e) or from the mouse cell line M12.4.1 (lane f) could be detected. Southern hybridization experiments also showed that *DR* β-chain, *DQ* α-chain, and *DQ* β-chain genes were present in the M/R 23.2 and 51.1 hybrids (data not shown). Analogous experiments were performed by using DNA from M/R 23.2 hybrid cells that had been negatively selected for reactivity with D1-12 mAb as described, and no hybridization to the *DR* α-chain probe used was observed (lane d). Similarly, no hybridization was observed with *DR* β-chain, *DQ* α-chain, and *DQ* β-chain probes (data not shown). Hybridization experiments using human-specific and mouse-specific probes indicated that these Ia-negative cells contained both human and mouse DNA in amounts comparable to those present in Ia-positive M/R 23.2 hybrid cells (unpublished experiments).

To assess whether the absence of expression of DQ1 molecules in hybrid cells was related to a lack of transcription of specific mRNAs, measurements of mRNA were then obtained. Fig. 5 shows the results of such an analysis. The two hybrids expressed detectable amounts of α-chain DR mRNA (*Upper*, lanes d and e). In contrast, the RJ 2.2.5 cell variant failed to express detectable amounts of α-chain DR mRNA (lane b), in agreement with our earlier studies (26). As expected, Ia-negative M/R 23.2 hybrid cells did not express α-chain DR mRNA (lane c). When the same cytoplasmic RNA preparations were assayed by hybridization with the β-chain DQ-specific probe (*Lower*), some specific mRNA was detectable in M/R 23.2 Ia-positive (lane d) and M/R 51.1 (lane e) hybrid cells but at a comparatively much lower level than α-chain DR mRNA. In agreement with data shown previously (26), negative results were obtained with the RJ 2.2.5 cells (lane b); the M/R 23.2 Ia-negative hybrid was also negative for β-chain DQ mRNA (lane c).

DISCUSSION

Our previous work on the properties of the human Ia-negative RJ 2.2.5 variant B-cell line suggested that the expression of MHC class II genes might be under the control of a *trans*-acting regulatory element (22, 26). The results obtained in this study demonstrate that expression of at least some human class II genes was restored in RJ 2.2.5–M12.4.1 cell hybrids, as indicated by the presence of mRNA specific for the α-chain DR subunit as well as expression of human α–β Ia heterodimeric structures on the cell surface. As assessed by mAbs, epitopes of two distinct (DR and NG2) molecular subsets of human Ia antigens could be identified in either one of the two hybrid cell lines described in this study. Biochemical analysis showed that the class II molecules expressed in the hybrids were similar to the corresponding molecules expressed on Raji cells. However, a slight difference was observed in the electrophoretic mobility of the β chain of DR-related molecules expressed in the hybrids as compared to the same subunit as Raji cells. Studies are necessary to determine whether this difference is due to distinct posttranslational modifications of human subunits in the mouse background or to other events such as formation of hybrid human–mouse molecules.

α–β DQ1 heterodimers were not detected at the cell surface and measurements of specific β-chain DQ mRNA indicated that β-chain *DQ* genes were transcribed in M/R 51.1 and M/R 23.2 cell hybrids much less efficiently than α-chain *DR* genes. Thus, lack of detectability of DQ1 molecules on the cell surface might either be due to a very low amount of expressed α–β heterodimers (under the threshold of the detection systems used in the present study) or to an absence of α-chain DQ-specific mRNA. In M/R 23.2 cells selected on the basis of a DR-negative cell surface phenotype it was possible to demonstrate the absence of α-chain and β-chain *DR* and α-chain and β-chain *DQ* genes together with loss of human class I antigens, thus suggesting that the loss of the human chromosome 6 was the cause of the instability for the expression of human MHC antigens in the M/R 23.2 original hybrid.

These results demonstrate that the human–mouse cell hybrids described in this study express human Ia antigen subunits encoded by genes donated by the Ia-negative RJ

2.2.5 variant. Thus, the lack of expression of Ia molecules in the variant cannot be explained by structural defects of the class II genes themselves but rather reflects an alteration of a *trans*-acting locus.

Whatever the mechanism might be, it must operate by regulating the level of synthesis or accumulation of class II specific mRNA. Either a negative or a positive loop could be involved in this regulation. Lack of class II gene expression in the RJ 2.2.5 variant may be due to the loss of a factor required to maintain a high concentration of specific mRNA either at the level of synthesis or, less likely, at the level of RNA stability. Conversely, the increased efficiency of a mechanism that normally counteracts overproduction of mRNA (a super-repressor) or the reactivation of a developmentally silent extinguisher gene might lead to the Ia-negative phenotype of RJ 2.2.5. Though reactivation of human class II genes in hybrid cells would imply a complementation by a mouse factor in the first case, segregation of a human chromosome carrying the locus for a repressing factor might be implicated if the second mechanism is correct. Recently, Benham *et al.* (40) have found that hybrid cells that contain an X/6 translocation chromosome as the sole human genetic component in a mouse cell background express class I but not class II gene products, even though the human donor cells were Ia-positive. The authors concluded that expression of human class II genes became extinct. However, the hypothesis of an activator gene lying on another chromosome and necessary for the promotion of class II gene expression cannot be excluded. In the study reported here, segregation of a locus encoding a *trans*-acting repressor factor would have been supported by the finding of Ia-negative hybrid clones giving rise to an Ia-positive progeny; such clones, however, were not found among the original fusion products.

Thus, we presently consider it more likely that reactivation of class II genes is brought about by an activator factor. The findings presented in this study allow us to consider the human Ia system as a model with which to study the regulation of expression of eukaryotic genes. The biological relevance of the regulatory mechanisms discussed here may relate to the mechanisms by which MHC class II antigens contribute to the correct homeostasis of the immune system.

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