Reversion of a transcriptionally defective MHC class II-negative human B-cell mutant

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ABSTRACT

RJ2.2.5, a mutant derived from the human B-lymphoma cell, Raji, is unable to express the MHC class II genes because of a recessive transcriptional defect attributed to the lack of an activator function. We report the isolation of a RJ2.2.5 revertant, namely AR, in which the expression of the mRNAs encoded by these genes is restored. Comparison of the binding of nuclear extracts or of partially purified nuclear preparations from the wild-type, the mutant and the revertant cells to a conserved MHC class II promoter element, the X-box, showed no alteration in the mobility of the complexes thus formed. However, in extracts from RJ2.2.5, and other MHC class II negative cell lines, such as HeLa, the amount of complex observed was significantly higher than in wild-type Raji cells. Furthermore, the binding activity exhibited by the AR revertant was lower than that of the RJ2.2.5 and higher than that of Raji. The use of specific monoclonal antibodies indicated that in all cases c-Jun and c-Fos or antigenically related proteins were required for binding. An inverse correlation between the level of DNA-protein complex formed and the level of MHC class II gene mRNA expressed in the three cell lines was apparent, suggesting that overexpression of a DNA binding factor forming complexes with class II promoter elements may cause repression of class II transcription. A model which reconciles the previously ascertained recessivity of the phenotype of the mutation carried by RJ2.2.5 with the findings reported here is discussed.

INTRODUCTION

The major histocompatibility (MHC) class II antigens are surface heterodimers required for presentation of antigenic peptides to the receptor of T-helper cells; quantitative variation in the expression of these antigens play a central role in immune response (1). The regulatory mechanisms which control the activity of the MHC class II genes at the transcriptional level has therefore received much attention (2,3). In particular, an important contribution to the study of this system was provided by the characterization of mutant cell lines isolated from wild-type B-cell lines (4-6) or from patients carrying the bare lymphocyte syndrome (BLS) (7). In all cases reported, the lesions consisted in a defect resulting in lack of transcription of the mRNA encoded by these genes (8—10). Genetic studies have established that the mutations so far characterized belong to probably four different complementation groups and that some of them are recessive (8—15). Molecular alterations correlating with the phenotype of these mutants have been sought by several laboratories. A defect in RF-X, a regulatory factor binding to the MHC class II promoter-specific X-box, has been reported in EBV-immortalized cells derived from a BLS patient (16,17), while a defect in a X-box binding protein or in a factor required for its binding, may underlie the phenotype of the in vitro selected mutant 6.1.6 (10). In other cases, however, no biochemical difference between the parental cell and the mutant could be pointed out, suggesting that alterations causing lack of transcription do not necessarily involve a defect in the DNA recognition of a transcriptional factor. We have now studied a revertant of RJ2.2.5, a MHC class II negative mutant which is prototypic of one of the four complementation groups so far identified (15). Genetic analysis demonstrated that the lesion of RJ2.2.5 is recessive and correlates with an autosomic locus, namely AIR-1 (8,13). A corresponding gene, air-1, was localized on chromosome 16 in the mouse (14). The defect carried by RJ2.2.5 does not correspond to the absence of any specific binding activity, although CAT-assays indicated that the mutant lacks both promoter and enhancer activity (18—20). Furthermore, in vivo footprinting analysis has shown that MHC class II promoter occupancy is the same in class II-positive Raji and in class II-negative RJ2.2.5 cells (21).

In this paper, we show that there exists an inverse correlation between the amount of mRNA produced by isogenic wild-type, mutant and revertant cells and the amount of a nuclear DNA binding factor containing Jun and Fos. On this basis, we would like to propose that the lack of transcriptional activity in RJ2.2.5 is caused by the accumulation of a DNA binding factor...
recognizing cis-acting regulatory elements of the MHC class II promoter and that the underlying defect consists in the lack of a determinant which prevents such an accumulation.

**MATERIALS AND METHODS**

**Cell lines and antibody staining.** The B-lymphoma cell Raji and its isogenic derivatives RJ2.2.5 (5) and TXII (22) have been described previously; the AR derivative is described in this paper. All cell lines were grown in RPMI1640 medium supplemented with 5% fetal calf serum at 37°C in 5% CO₂. Cell were stained with anti-class I and anti-class II antibodies as previously reported (8); the fluorescence intensity of stained cells was determined cytofluorimetrically (8).

**Preparation of nuclei and gel retardation assays.** Nuclei were prepared according to a procedure which gives transcriptionally active, intact nuclei (23). Briefly, 3 x 10⁷ cells grown in RPMI 1640 supplemented with 5% FCS were harvested, washed twice in 1 x PBS and resuspended in 2 ml of buffer E (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM HEPES pH 7.5, 14 mM β-mercaptoethanol, 0.3 M sucrose, 0.5 mM EGTA and 2mM EDTA); 2 ml of buffer B containing 0.1% NP40 were added (final NP40 concentration: 0.05%) and the mixture was gently pipetted for 1 min on ice. The suspension was then layered onto 5 ml of buffer C (equivalent to buffer E, but containing 30% sucrose) and centrifuged at 2500 rpm at 4°C for 30 min. Nuclear extracts were then obtained by incubating the nuclei in 0.4 ml of buffer E (0.4 M NaCl, 0.5 mM Mg Cl₂, 0.2 mM EDTA, 20 mM HEPES, pH 7.9, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 5ng/µl pepstatin) at 4°C for 30 min. The lysed nuclei were then centrifuged for 30 min at 4°C and 14,000 rpm. The supernatant was subdivided in small aliquots and stored frozen (−80°C). The total amount of protein extracted by this procedure was equivalent to that observed when the procedure of Dignam et al (24) was applied (4–12 µg/10⁶ cells), but the amount of AP-1 activity measured by band-shift experiments was 3–5 fold higher than in the standard procedure. Band-shift experiments were performed in the presence of 600–900 ng of sonicated E.coli DNA as previously reported; 2–3 µg of nuclear protein and 0.03–0.06 ng labelled probe (specific activity = 0.5–1.0 x 10⁶ cpm/ng DNA) were used for each assay. For competition experiments a 200 x excess of unlabelled competitor was added.

**RNAse protection experiments.** Total RNA was prepared by lysis with SDS, while cytoplasmic RNA was prepared by lysis in NP-40. The pDR5αβ plasmid, encoding the 3’ most end of HLA-DRA mRNA was used to prepare an in vitro transcribed probe in order to assess the level of mRNA present. Transcription reactions were performed with 1 µg of linearized plasmid in the presence of 100 µCi of [α-32P]UTP (Amersham, 800 µCi/mM), 2.5 mM unlabelled ribotriphosphates and 15 U of T7 RNA polymerase or SP6 polymerase (Promega), in a final volume of 20 µl. Samples were incubated for 1 hr at 38.5°C. For unlabelled RNAs, all ribotriphosphates were used at a concentration of 2.5 mM. The amount of RNA extracted from the cells was standardized by determining the amount of endogenous β-actin RNA. The amount of RNA was measured by using a RNase protection kit produced by AMBION (Austin, TX) according to the manufacturer’s specifications; 25 µg of RNA were hybridized to an aliquot of 3 x 10⁵ cpm of labelled probe.

**Competition of DNA binding by antibodies.** Rabbit anti-Jun and anti-Fos antisera, raised against the DNA binding domain of Jun and Fos, respectively, were obtained from commercial sources (CALTAG Laboratories, USA). Inhibition of binding by antibodies (1–6 µg per assay) was determined as previously reported (25); nuclear extracts were preadsorbed for 1–2 hrs with the antibodies before binding. Antisera from non-immune animals, chicken antisera and irrelevant mAbs were used as controls.

**Heparin Sepharose chromatography.** A nuclear extract (6 ml, 0.6 µg/µl) from 5 x 10⁶ cells were loaded at 4°C onto a Heparin-Sepharose column (bed volume = 6 ml) equilibrated with 20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 100 mM KCl, 0.5 mM PMSF. After extensive washing with equilibration buffer, the proteins retained were eluted by adding 10 ml a 0.1–1 M KCl gradient in Tris – HCl pH 7.4. An aliquot of each fraction was tested in band-shift assay.

**RESULTS**

**Expression of MHC class II antigens in wild type, mutant and revertant cell lines.** Raji and RJ2.2.5 cells were grown at 37°C in RPMI 1640 medium supplemented with 5% FCS. Aliquots of the cultures were withdrawn at different time intervals in order to determine the cell counts. In these conditions, the doubling time of Raji was about 17 hrs, whereas RJ2.2.5 doubled every 48 hrs. TXII, a RJ2.2.5 derivative which recovered ability to express MHC class II genes upon transfection with mouse genomic DNA (22), was grown in the same conditions and the growth rate was measured for comparison: the doubling time of this transfectant was similar to that exhibited by Raji (24 hrs).

These results suggested that either expression of MHC class II antigens onto the cell surface per se is required for optimal growth rate, or the regulatory defect in the AIR-1 locus carried by RJ2.2.5 has pleiotropic effects onto the ability of the cells to grow. To further understand this apparent correlation, a faster grower variant of RJ2.2.5 was studied. This variant appeared spontaneously in the population and was selected by its ability to overgrow the RJ2.2.5 cells in culture. Briefly, frozen RJ2.2.5 cells exhibited upon thawing a reduced viability and a longer lag before resuming growth as compared to Raji cells. A faster grower clone, which appeared at a frequency estimated to be between 1 x 10⁻⁸ and 1 x 10⁻⁹, was subcloned; the growth rate of a subclone, namely AR, was determined in comparison with Raji and RJ2.2.5. This measurement showed that AR grows considerably faster than RJ2.2.5 (doubling time = 26 hrs). Analysis of MHC class II DNA polymorphisms (not shown) using HLA-DQA1 and HLA-DRB as probes confirmed the derivation of these cells from RJ2.2.5 (see also below).

The ability of AR to express MHC class II genes was then established. Figure 1 reports a cytofluorimetric analysis of HLA-DR and HLA class I antigen expression onto the surface of Raji, RJ2.2.5 and AR cells. While RJ2.2.5 expressed, as expected, a DR-negative phenotype (mean of fluorescence = 4.35), AR was instead positive (mean = 184.82). Although DR expression was significantly higher in AR then in RJ2.2.5, it was still about 60% lower than in Raji (mean = 482.52), thus suggesting that the wild-type phenotype had been partially restored. The amount of class I antigens present in AR was similar to that exhibited by RJ2.2.5; the latter expresses a reduced amount of class I antigens onto the cell surface compared to Raji. This unexplained characteristic of RJ2.2.5 was previously reported (5,8,13,14); in this case it further confirms the derivation of AR from RJ2.2.5.

RNAse mapping experiments confirmed that MHC class II
Figure 1. MHC class II gene expression in AR cells. A: Cytoluminometric analysis of fluorescent antibody staining of Raji, RJ2.2.5 and AR cells with an anti HLA-A,B,C mAb (B9.12.1) and with an anti HLA-DRA mAb (D1.12) or with the second antibody only (control). B: RNAse protection assay of RNA extracted from Raji, RJ2.2.5 (RJ) and AR. A cDNA fragment encompassing ca. 500 nts of the 3' portion of DRA was used to prepare the in vitro transcribed RNA probe. A cDNA probe encompassing ca 140 nts of the 5' end of β-actin was used to standardize the amounts of RNA used. Numbers on the left refer to the molecular weight standards (MW st). c = cytoplasmic RNA; t = total RNA.

mRNAs are produced by Raji and AR, but not by RJ2.2.5 cells. Figure 2 shows that the relative amount of HLA-DRA mRNA, normalized to the β-actin mRNA content, is much higher in AR cells than in RJ2.2.5, although significantly lower than in Raji.

The presence of HLA-DRB and of HLA-DQA1 mRNA in AR was also determined by Northern blot experiments (data not shown).

Characterization of DNA binding activities in nuclear extracts from Raji, RJ2.2.5 and AR. Nuclear extracts from all three cell lines were used for band shift experiments to investigate whether any correlation between transcriptional activities and DNA binding patterns could be established. The X-box element (26,27), a conserved sequence necessary for MHC class II promoter activity, was used as a probe. Figure 3 indicates that complexes exhibiting the same electrophoretic mobility were generated with all three extracts. The intensity of the band obtained when a RJ2.2.5 extract was used was much higher than that observed for Raji extracts, provided that the same protein concentrations were used; interestingly AR exhibited a binding activity lower than RJ2.5 and higher than Raji. In addition, extracts from another MHC class II negative cell line, HeLa, behaved like RJ2.2.5 extracts, thus suggesting that inability to express MHC class II genes parallels the presence of an excess of a promoter binding factor. To ascertain whether the DNA binding activities exhibited by Raji, RJ2.2.5 and AR share other properties besides a similar electrophoretic mobility, we further analyzed the characteristics of the factor(s) involved. As previously shown by others (26–30), the X-box is the target of the AP-1 factor and of other Jun and Fos containing heterodimers; accordingly we analyzed the effect of an anti-Jun antiserum onto the formation of the complex between nuclear proteins and the labelled X-box. Figure 3A shows, for example, that, both in Raji and RJ2.2.5 extracts, the formation of the complex is inhibited by titration with increasing concentrations of anti-Jun antibodies; notably the presence of a larger amount (4–10 fold) of non immune antiserum had little effect on the binding. A similar inhibition was also observed when AR extract were used (not shown). Furthermore, inhibition of the X-box binding activities of Raji, AR and RJ2.2.5 extracts by anti-Fos was demonstrated (Fig. 3B). Thus, the pattern of inhibition suggested that all the X-box binding complexes formed by these nuclear extracts contained Jun and Fos or antigenically related proteins. We also showed that all three X-box binding activities exhibited the same chromatographic
Figure 3. Inhibition of binding by anti-Jun and anti-Fos. A: Inhibition of binding of Raji and RJ2.2.5 nuclear extracts to the X-box by the indicated amounts of anti-Jun or pre-immune antiserum. B: Inhibition of the binding of AR, RJ2.2.5 and Raji nuclear extracts to the X-box by anti-Fos antiserum; binding reactions which did not contain anti-Fos (in lanes 3, 6 and 9) were supplemented with 1 μg of non-immune antiserum.

Figure 4. Heparin-Sepharose chromatography of nuclear extracts. A: Elution of adsorbed proteins from heparin-Sepharose. ——: Raji; —: RJ2.2.5 and —— •—•—•: AR. The bar indicates the fractions exhibiting the X-box binding activity. B: Band shift caused by fractions 18 to 21 of a Raji column (right) and a RJ2.2.5 column (left).
mobility when analyzed by heparin-Sepharose chromatography; in fact, overlapping elution profile were obtained in the three cases (Fig. 4A). When the corresponding fraction of two different eluates, containing the same protein concentrations (see Fig. 4A), were compared, the quantitative differences noticed in crude extracts were confirmed. For example, the intensity of the band observed when RJ2.2.5 was the source of the partially purified X-box binding activity was much higher than that found when Raji was used instead (Fig. 4B). The X-box binding activities expressed by Raji, RJ2.2.5 and AR were further characterized. For example, the effect of incubating the extracts at 26, 37 and 45°C or in the presence of 0.1–1.0 mM sodium deoxycholate at room temperature or with 0.01–1 units of alkaline phosphatase was determined; no difference in the resistance to these treatments of the binding activity of any cell line was observed (data not shown). These data suggest that the same factor, present in different amounts, is responsible for the binding of Raji, RJ2.2.5 and AR extracts to the X-box.

**DISCUSSION**

Studies concerning the characterization of the regulatory defect carried by the MHC class II negative B-cell lymphoma RJ2.2.5 mutant were previously presented and the segregation pattern of somatic cell hybrids obtained by fusion of the mutant cells with mouse spleen B-cells was reported (13,14). These experiments led to the conclusion that a recessive defect in the AIR-1 locus of RJ2.2.5 prevents expression of MHC class II genes by abrogating a transcriptional activator (10,13). Further studies have shown that RJ2.2.5 may lack a factor required for the activity of a MHC class II specific enhancer function (18,19); in particular, it was reported that a conserved cis-acting regulatory signal of the MHC class II promoters, the X-box, enhances MHC class II expression in Raji, but not in RJ2.2.5 (19). However, absence of a specific DNA binding activity could not be demonstrated using nuclear extracts of RJ2.2.5 (2,3,18). In addition, it was reported that MHC class II promoter occupancy in vivo is the same in the class II-positive Raji and in class II-negative RJ2.2.5 cells (21). The defect of this mutant may thus reside in a factor that modulates transcription without directly interacting with the promoter elements of the MHC class II genes.

As, in comparison with Raji, the RJ2.2.5 mutant exhibits a slower growth rate, it was possible to isolate a revertant cell line in which the ability to express the MHC class II genes is partially restored (Fig. 1). Notably, suppression of the AIR-1 phenotype by transfection with heterologous DNA (22) also caused a reduction of the doubling time, suggesting that either class II antigen expression affects the growth of the cell or that the AIR-1 mutations have pleiotropic effects. As lack of MHC class II antigens on the cell surface does not adversely affect the ability of cells to grow (35), it is possible that pleiotropism is implicated. Characterization of the binding activities present in the nucleus of the wild-type, the mutant and the revertant cells has shown that there is an inverse correlation between the amount of class II mRNA expressed and the level of a nuclear complex formed with the X-box. The accumulation of a nuclear factor having a negative effect onto transcription, rather than loss of an activator, may thus cause the lack of gene expression observed in RJ2.2.5. The recessivity of the RJ2.2.5 phenotype, however, contrasts with the possibility that lack of MHC class II expression may be due to de novo synthesis of a repressor. We propose that in Raji cells the accumulation of a repressor is prevented, thus allowing gene expression. RJ2.2.5 cells would carry a mutation affecting this prevention mechanism. Such a model is compatible with the results of the segregation analysis of RJ2.2.5 proving the recessivity of the AIR-1 defect (13,14). The hypothesis that MHC class II gene expression in RJ2.2.5 cells is suppressed because of the presence of a repressor, does not exclude that activation is also required. The requirement for a transcriptional activator has in fact been documented in several instances (see Ref. 2 and 3 for a review). We have shown, for example, that treatment of Raji with a protein synthesis inhibitor, cycloheximide, blocks class II mRNA transcription, thus indicating that a positive factor is required for MHC class II gene expression in these cells (23).

We found that the X-box binding activities exhibited by Raji, RJ2.2.5 and AR have the same electrophoretic and chromatographic properties and all contain Jun/Fos or antigenically related proteins (Fig. 3–4); therefore they seem to differ only quantitatively, although differences in their composition, not detectable by our approach, cannot be excluded. Most experimental evidence suggests that Jun or Fos containing factors that bind to the X-box are required for the activation of MHC class II promoters (26,28–30), although a negative role was suggested for the Jun/Fos heterodimer (27). Accordingly besides being implicated in activation, a negative role has been reported for the X-box (31,32). It is thus tempting to suggest that the same X-box binding complex may act both as an activator and as a repressor. In other cases too, the ability of Jun/Fos containing factors to act both in a negative and in a positive fashion has been proposed (33).

In several circumstances it has been reported that the ability of a DNA binding factor to modulate transcriptional activity can be controlled by the interaction with other factors. For example, blocking of activator factors by specific inhibitors, such as the I-xB inhibitor of NF-xB (33) and the IP-1 inhibitor of AP-1 (25) has been reported. These inhibitions are relieved by a number of treatments which interfere with the inhibitor functions. In the case of I-xB, the inhibition of NF-xB can be sedated by selectively inactivating the inhibitor at high temperature. In other cases, treatment with non-ionic detergents, phosphatase or DTT interferes with the interaction of DNA binding proteins with their cognate modulating factors. We have therefore examined the possibility that the amount of DNA binding complex formed by Raji extracts with MHC class II promoter elements be increased by removal of an inhibitor by any of these treatments. In no case significant effects onto the amount of DNA binding activity were observed. Another mechanism by which the amount of repressor can be kept low in the nucleus involves the possibility that the repressor be confined in the cytoplasm; we were thus prompted to test the nucleo-cytoplasmic ripartition of the X binding activity in Raji and RJ2.2.5. Again the ratio of DNA binding activity found in the nucleus to that found in the cytoplasm was constant in the two cell lines (M. N. Ombra, unpublished results). Furthermore mixing experiments based on combinations of extracts from Raji and RJ2.2.5 excluded the presence of an inhibitor of binding in Raji nuclear extracts (M. N. Ombra, unpublished results). We conclude that it is unlikely that the increase of X-box binding factor observed in RJ2.2.5 is due to any of the mechanisms discussed above.
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