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Immunology

Molecular analysis of an EL4 cell line that expresses H-2D^b but not H-2K^b or β_2-microglobulin

(H-2 antigens/major histocompatibility complex)

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ABSTRACT **EL4/Mar is a variant cell line that expresses H-2D^b but neither H-2K^b nor β_2-microglobulin (β_2m). Southern and RNA blot analysis and immunoprecipitation of metabolically labeled proteins established that the *B2m* gene(s), β_2m mRNA, and β_2m protein are normal in this cell line. Somatic cell hybridization showed that the defect in this cell line was in the synthesis of H-2K^b, and RNA blot analysis with an *H-2K^b* specific oligonucleotide established that the *H-2K^b* gene(s) in this cell line was not transcribed into a stable mRNA species. The apparent absence of β_2m on the surface of this cell line suggests that there may be some feature of the H-2D^b molecule that allows it to be expressed in the absence of detectable β_2m.**

The major histocompatibility complex class I molecules exist on the cell surface as noncovalently linked dimers consisting of a heavy chain of 40–45 kDa and a light chain of 12 kDa (1, 2). The light chain is referred to as β_2-microglobulin (β_2m). The locus coding for β_2m is not linked to the major histocompatibility complex, which in the mouse is known as the *H-2* complex. From studies of variant cell lines, such as Daudi or R1 TL$^-$, that are defective in the synthesis of β_2m and do not express any major histocompatibility complex class I molecules (3–5), it is currently accepted that the heavy chain of the class I molecule is not expressed in the absence of β_2m. Recently, we have described an EL4 variant cell line which expressed the H-2D^b molecule but not H-2K^b or β_2m (6). We have further characterized the nature of the defect in this variant cell line and report herein that it is a defect in the production of H-2K^b, not β_2m, that is responsible for the abnormal antigenic phenotype.

MATERIALS AND METHODS

Cell Lines. The origin of the EL4/NY cell line and of the variant EL4/Mar have been described (6).

Probes. pB2mdIII.B is a subclone of a mouse *B2m* gene obtained from J. Seidman (Harvard Medical School, Boston). This *Hin*dIII–*Bam*HI fragment extends from the long intervening sequence between the first and second exons to the exon between the third and fourth intron (Fig. 1). pB2mdIII.B was labeled by nick-translation as described (8). A 21-base oligonucleotide probe that is complementary to the *H-2K^b* sequence from codon 192 to 199 was synthesized by the phosphotriester solid-phase method. This particular region was selected because it was one in which there were several differences in sequence between *H-2K^b* and the other *H-2* class I genes that have been sequenced. The oligonucleotide was 5′ end-labeled with ^{32}P for 30 min at 37°C in the presence of T4 polynucleotide kinase and used without further purification.

Immunoprecipitation and Electrophoresis. Metabolic radiolabeling of the cell lines was achieved by culturing 10^7 cells in the presence of 200 μCi (1 Ci = 37 GBq) of [^{35}S]methionine/ml of medium (9). Cell lysates were preabsorbed three times with normal rabbit serum and sheep anti-rabbit immunoglobulin antiserum. Specific immunoprecipitates were prepared by the addition of rabbit anti-mouse β_2m antiserum followed by sheep anti-rabbit immunoglobulin antiserum. NaDodSO$_4$/PAGE (10) was in either 10% or 15% acrylamide slab gels.

RNA Blot Hybridization Analysis. Cellular RNA was prepared by the guanidinium isothiocyanate method of Chirgwin *et al.* (11). Poly(A)$^+$ RNA was prepared by chromatography on oligo(dT)-cellulose (12). RNA was electrophoresed in agarose in the presence of formaldehyde and then transferred to nitrocellulose paper (13). Hybridization with pB2mIII.B was in 5× NaCl/Cit/50% (vol/vol) formamide/1% NaDodSO$_4$/1× Denhardt's solution for 16 hr at 42°C. Washing was done sequentially in 2× NaCl/Cit/1% NaDodSO$_4$ for 10 min at room temperature; 2× NaCl/Cit/0.5% NaDodSO$_4$ for 60 min at 55°C, and 0.1× NaCl/Cit for 60 min at room temperature. Hybridization with the ^{32}P-labeled oligonucleotide was overnight at 55°C in 6× NaCl/Cit/5× Denhardt's solution; blots were washed in 6× NaCl/Cit for 45 min at room temperature and then 2 min at 55°C. NaCl/Cit is 0.15 M NaCl/15 mM sodium citrate, pH 7; Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin.

Southern Blot Analysis. Genomic DNA was obtained from cells by using published procedures (8). Genomic DNA was cleaved with various restriction endonucleases under conditions suggested by the supplier (Boehringer Mannheim). The digested DNA was subjected to electrophoresis in agarose gels. The DNA was denatured *in situ* and transferred to nitrocellulose paper. Prehybridization and hybridization with pB2mdIII.B were in 5× NaCl/Cit/50% formamide/5× Denhardt's solution/0.1% NaDodSO$_4$/25 mM sodium phosphate overnight at 42°C. Hybridization with the oligonucleotide was in 5× NaCl/Cit/10× Denhardt's solution/10% dextran sulfate/7% NaDodSO$_4$ overnight at 56°C. Blots were washed for 1 hr at 55°C in 3× NaCl/Cit/10% Denhardt's solution/5% NaDodSO$_4$ and then for 1 hr at 55°C in 1× NaCl/Cit/1% NaDodSO$_4$.

Somatic Cell Hybridization. For somatic cell hybridization, hypoxanthine/aminopterin/thymidine (HAT)-sensitive EL-4/Mar cells were isolated by selection in medium containing 6-thioguanine at 5 μg/ml. Clones that grew were tested for HAT sensitivity and used as presumptive hypoxanthine phosphoribosyltransferase minus (HPRT$^-$) cells. One such

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Abbreviations: β_2m, β_2-microglobulin; HAT, hypoxanthine/aminopterin/thymidine.

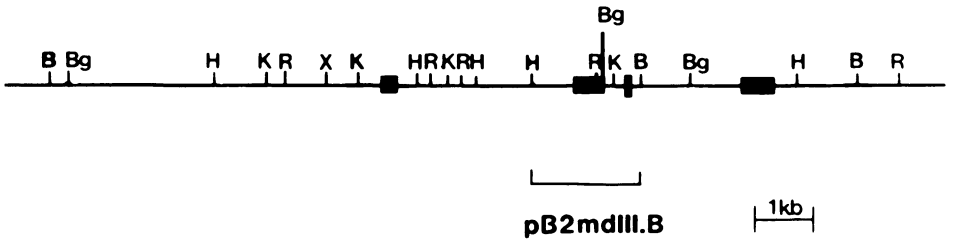

FIG. 1. Molecular map of the murine *B2m* gene showing the cleavage sites of several restriction enzymes (7): *Bam*HI (B), *Bgl* I (Bg), *Hin*dIII (H), *Kpn* I (K), *Eco*RI (R), and *Xho* I (X). Exons are represented by boxes. The *Hin*dIII–*Bam*HI fragment encoding the major part of β_2m was subcloned in pBR322 to yield pB2mdIII.B, which was used to examine *B2m* sequences in DNA and RNA. kb, Kilobases.

clone, referred to as EL4/MarTG^{r}3.1, was used for somatic cell hybridization. Mouse spleen cell suspensions were prepared and fused with EL4/MarTG^{r}3.1 by the addition of polyethylene glycol according to standard protocols (14). Hybrid cells were selected by growth in HAT medium.

RESULTS

Analysis of the *B2m* Genes in EL4/Mar. We showed previously (6) by serological criteria and by reactivity of cytotoxic T-lymphocyte clones that the surface phenotype of EL-4/Mar was H-2K^{b-} β_2m$^-$ H-2D^{b+}. To examine whether EL-4/Mar possessed intact *B2m* genes or whether the *B2m* genes of EL4/Mar had undergone deletions similar to those described for the R1 TL$^-$ cell line (7), we prepared Southern blots of genomic DNA (Fig. 2). Both EL4 cell lines contain DNA sequences that hybridize to the *B2m* probe; the sizes of these fragments were the same in both cell lines for all the enzymes used. Furthermore, the sizes of the fragments generated with all the enzymes were those predicted by the restriction map of the *B2m* gene. The results suggested that there had not been a major internal deletion in the EL4/Mar *B2m* genes.

Transcription of the *B2m* Gene in the EL4/Mar Cell Line. To determine whether the *B2m* genes in the EL4/Mar cell line were being transcribed, RNA from EL4/NY and EL4/Mar cell lines were subjected to blot analysis with the *B2m* probe. As shown in Fig. 3, both the EL4/NY cell line and the EL4/Mar cell line have two species of mRNA that hybridized to the probe. Of these two mRNA species, the larger is the predominant one. The sizes of β_2m mRNAs that are present in EL4 lines are the same as those identified in other cells (15).

The observation above suggested that although there was no β_2m expressed on the cell surface, there was apparently β_2m mRNA within the EL4/Mar cells. We therefore investigated whether EL4/Mar was producing β_2m that could be detected in the cytoplasm. EL4/Mar and EL4/NY cell lines were labeled with [^{35}S]methionine, and immunoprecipitates were prepared and subjected to NaDodSO$_4$/PAGE. As shown in Fig. 4, EL4/Mar produced as much immunoreactive β_2m (≈12 kDa) as the EL4/NY cell line. This observation suggested either that the β_2m produced by EL4/Mar was abnormal and thus unable to appear on the cell surface or that the absence of β_2m on the cell surface was caused by a defect elsewhere.

Rescue of β_2m by Somatic Cell Hybridization. To further establish the nature of the defect in EL4/Mar, we carried out somatic cell hybridization between EL4/Mar and normal spleen cells. After fusion of EL4/Mar (genotype *H-2K^b*, *H-2D^b*, *B2m^b*) with BALB.B (genotype *H-2K^b*, *H-2D^b*, *B2m^a*) spleen cells, 12 hybrids that grew in HAT medium were isolated and their surface phenotypes were determined by microcytotoxicity testing. All of these hybrids expressed both H-2K^b and β_2m^b (Table 1). In these hybrids, the *B2m^b* gene product could only be that produced within the EL4/Mar

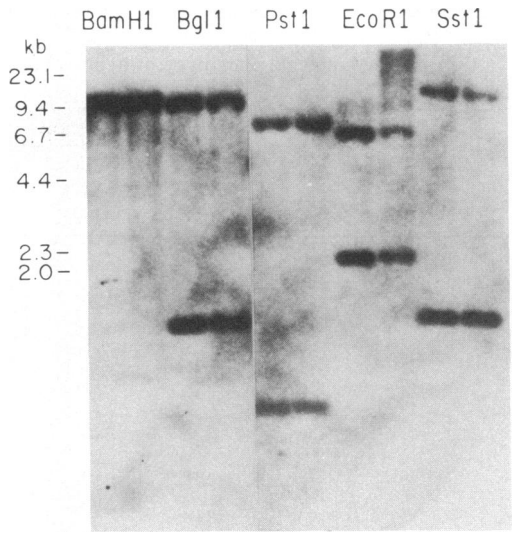

FIG. 2. Southern blot analysis of EL4/NY and EL4/Mar genomic DNA probed with pB2mdIII.B. DNA was digested with the enzyme indicated, electrophoresed on an agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled pB2mdIII.B. For each enzyme, digested EL4/NY DNA is in the left lane and EL-4/Mar DNA is in the right lane. Markers at left (in kilobases, kb) show positions of size standards from *Hin*dIII digestion of λ DNA.

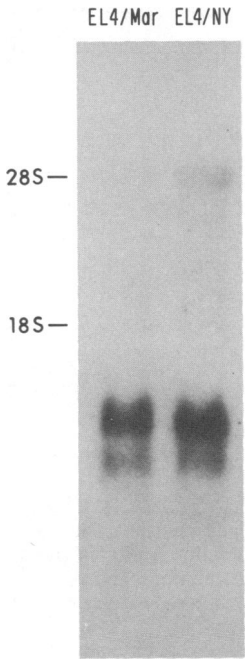

FIG. 3. Blot analysis of EL4/NY and EL4/Mar RNA probed with pB2mdIII.B. After electrophoresis, RNA was transformed to nitrocellulose paper and hybridized with ^{32}P-labeled pB2mdIII.B.

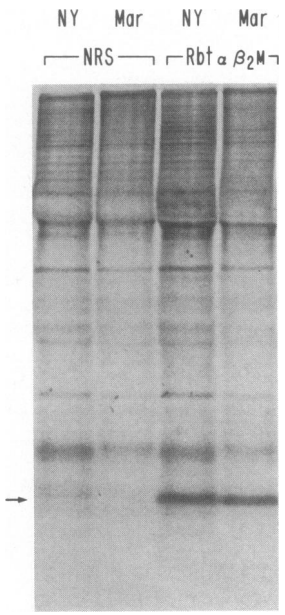

FIG. 4. NaDodSO$_4$/PAGE of immunoprecipitates from lysates of [^{35}S]methionine-labeled EL4/NY (NY) and EL4/Mar (Mar) cells. After metabolic labeling, cells were lysed in 1% Nonidet P-40 and immunoprecipitates were prepared. Samples were reduced by boiling in 2-mercaptoethanol prior to electrophoresis on 15% (wt/vol) acrylamide gels. NRS, normal rabbit serum; Rbtα β_2m, rabbit anti-mouse β_2m antiserum. Arrow at left indicates position of β_2m (12 kDa).

cell line, because the partner in the hybridization, BALB.B, is homozygous for *B2m^a*. As the expression of β_2m^b on EL-4/Mar could be rescued by fusion with *B2m^a* spleen cells, the β_2m present in EL4/Mar cytoplasm is not abnormal, and the defect in EL4/Mar most likely resides in *H-2K^b* and not *B2m^b*. This was confirmed by performing a similar fusion of EL4/Mar with B10.D2 (*H-2K^d*, *H-2D^d*, *B2m^b*) spleen cells. All 12 hybrids from the EL4/Mar × B10.D2 fusion expressed β_2m^b, H-2K^d, H-2D^d, and H-2D^b but failed to express H-2K^b (Table 2). Thus, introduction of wild-type β_2m^b from spleen cells did not overcome the inability to express K^b, thereby confirming that the defect in EL4/Mar was in *H-2K^b* and not *B2m^b*.

Examination of the *H-2K^b* Genes in EL4/Mar. To examine whether the inability of EL4/Mar to produce normal H-2K^b was caused by a deletion or rearrangement of the *H-2K^b* genes, we analyzed genomic DNA. Because the major histocompatibility complex consists of many homologous genes (16), probing with a genomic or cDNA clone generally detects 15–20 bands (17, 18). Therefore, to specifically investigate the *H-2K^b* gene, we used a single-copy 21-base synthetic oligonucleotide corresponding to the region encoding amino acids 192–199 of the H-2K^b molecule. Under the conditions used, this oligonucleotide does not hybridize to other major histocompatibility complex class I genes. As shown in Fig. 5, there was no detectable difference in the pattern of fragments of EL4/Mar and EL4/NY that hybridized with the *H-2K^b* oligonucleotide. The sizes of the fragments that hybridized with this oligonucleotide are consistent with those predicted by analysis of the published restriction map of the *H-2K^b* gene (23).

Transcription of *K^b* Genes. To determine whether the *H-2K^b* gene(s) detected in the EL4/Mar cell line is transcribed into mRNA, total cellular RNA and polyadenylylated RNA were prepared, electrophoresed, transferred to nitrocellulose, and probed with the oligonucleotide specific for *H-2K^b* (Fig. 6). In total RNA from the EL4/NY cell, the probe hybridized specifically with a 17S RNA species (lane 1). RNA from the EL4/Mar cell line did not hybridize to the *H-2K^b* specific oligonucleotide. A similar result was also obtained using a *H-2K^b* specific oligonucleotide to a sequence in the α_2 domain (data not shown). The failure of the oligonucleotides to hybridize with the EL4/Mar RNA was not due to degradation of this RNA preparation, as the same preparation hybridized with the β_2m (Fig. 3). Analysis of poly(A)$^+$ RNA confirmed that EL4/NY but not EL4/Mar contained

Table 1. Cell surface antigenic phenotype of hybrids between EL4/Mar and BALB.B spleen cells

	Cell surface phenotype		
	H-2K^b (Y-3,* EH144)	H-2D^b (28-14-8, H141/51)	β_2m^b (Lym11)
Parental partners			
EL4/Mar	−	+	−
BALB.B	+	+	−
Hybrid clones (EL4/Mar × BALB.B)			
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+
11	+	+	+
12	+	+	+

Antigenic phenotypes were determined by microcytotoxicity using the monoclonal antibodies (given in parentheses) at an optimum concentration (1:200 dilution) and rabbit complement at a final dilution of 1:12. +, >90% lysis after 60 min at 37°C; −, <5% lysis after 60 min at 37°C.

*Monoclonal antibodies were obtained from S. G. Nathenson, Albert Einstein College of Medicine (Y-3); D. Sachs, National Cancer Institute (28-14-8); G. Hammerling, German Cancer Research Center, Heidelberg (H141/51); and U. Hammerling, Sloan–Kettering Cancer Center (Lym11).

Table 2. Cell surface antigenic phenotype of hybrids between EL4/Mar and B10.D2 spleen cells

	Cell surface phenotype		
	H-2K^b (Y-3, EH144)	H-2D^b (28-14-8, H141/51)	β_2m^b (Lym11)
Parental partners			
EL4/Mar	−	+	−
B10.D2	−	−	+
Hybrid clones (EL4/Mar × B10.D2)			
1	−	+	+
2	−	+	+
3	−	+	+
4	−	+	+
5	−	+	+
6	−	+	+
7	−	+	+
8	−	+	+
9	−	+	+
10	−	+	+
11	−	+	+
12	−	+	+

See legend to Table 1.

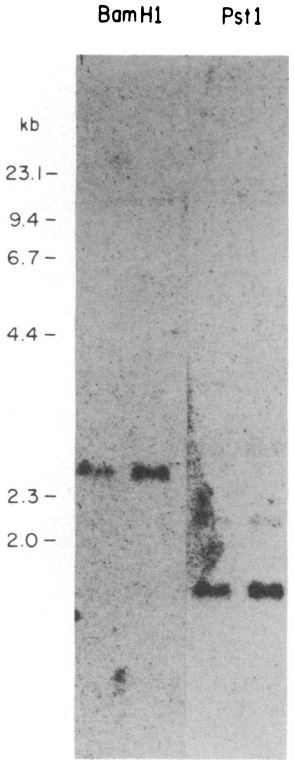

FIG. 5. Southern blot analysis of EL4/NY and EL4/Mar genomic DNA probed with the *H-2K*^{b}-specific oligonucleotide. DNA was digested with the enzyme indicated, electrophoresed in an agarose gel, transferred to GeneScreen paper, and hybridized with ^{32}P-labeled oligonucleotide. For each enzyme, EL4/NY DNA is in the left lane and EL4/Mar DNA is in the right lane. kb, Kilobases.

mRNA that hybridized to the *H-2K*^{b} oligonucleotide (data not shown).

DISCUSSION

Two cell lines have been described in the literature in which a primary defect in β_2m synthesis results in nonexpression of H-2 or HLA (human major histocompatibility complex) antigens on the cell surface. In the case of the HLA$^-$ variant cell line Daudi, the defect has been shown to be due to both chromosome loss and production of inactive mRNA species (19). In contrast, the defect in the R1 TL$^-$ variant is due to deletions and possibly to an insertion in both copies of the *B2m* genes (7). In this report, we have characterized a cell line which is unusual on two counts. First, although the cell line is β_2m$^-$ on the cell surface, it continues to express one H-2 antigen, namely H-2D^b; second, the primary defect appears not to be in the synthesis of β_2m. Our analysis of this cell clearly indicates that the *B2m* gene is present and is transcribed into mRNA which is translated into β_2m protein. Furthermore, this β_2m protein is immunologically reactive with both a monoclonal antibody and a xenoantiserum specific for β_2m and can appear on the cell surface in somatic cell hybrids, provided other H-2 antigens are synthesized in the hybrid cell. Thus, in the somatic cell hybrids with the BALB.B spleen cells, the provision of normal *H-2K*^{b} gene product results in the appearance on the cell surface of β_2m from the EL4/Mar cell line. Thus, the β_2m pathway in this cell would appear to be intact and the failure of β_2m to appear on the cell surface must be attributed to a defect elsewhere. The molecular defect in this cell appears to be in the synthesis of the *H-2K*^{b} gene product. At least one copy of the *H-2K*^{b} gene is present in the cell line, as shown by the presence of a DNA fragment that can hybridize specifically with the *H-2K*^{b} synthetic oligonucleotide. However, this gene is not transcribed into an RNA species.

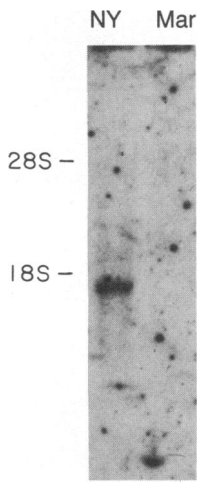

FIG. 6. Blot analysis of EL4/NY (NY) and EL4/Mar (Mar) RNA probed with the *H-2K*^{b}-specific oligonucleotide. After electrophoresis, RNA was transferred to nitrocellulose paper and hybridized with ^{32}P-labeled *H-2K*^{b}-specific oligonucleotide.

Nonexpression of a gene in a homozygous cell line raises the question of the precise mechanism by which defects arise to inactivate two functional copies of the gene. Southern blot analysis of EL4/Mar DNA with the oligonucleotide probe implies that there is at least one intact copy of the *H-2K*^{b} gene. They would also imply that large deletions have not occurred in any copy of the *H-2K*^{b} gene. If significant deletions of either the 5′ end or the 3′ end have occurred in any copy of the *H-2K*^{b} gene in the cell line, we would expect to see an anomalously migrating fragment. We did not observe this. However, a deletion that included the entire sequence corresponding to the probe in one copy of the *H-2K*^{b} gene would result in absence of signal from that particular copy of the gene. It is entirely possible that the *H-2K*^{b} gene(s) in the EL4/Mar cell line has undergone separate, distinct, but small deletions making the gene(s) nonfunctional, but that these deletions are too small to be resolved by the relatively crude technique of Southern blot analysis.

Another question that these studies raise is if β_2m production is normal in this cell line, why is β_2m not expressed on the cell surface in association with D^b? One possibility is that the *H-2D*^{b} gene product is inserted into the plasma membrane without any association with β_2m. If this is the case, it raises some questions about the properties of the H-2D^b molecule that apparently set it apart from most of the major histocompatibility complex class I gene products. As we noted in our previous publication (6), the H-2D^b molecule has an additional carbohydrate side chain compared to the H-2K^b molecule (20), and this additional glycosylation site is on the α_3 domain of the molecule which has been suggested to be the β_2m binding region (21). It is therefore tempting to speculate that the third carbohydrate chain may replace β_2m on D^b. An alternate possibility is that H-2D^b and β_2m do associate and are brought to the cell surface together, but this complex readily dissociates, with β_2m being shed into the cell supernatant or culture medium leaving naked H-2D^b molecules on the surface. Although it has recently been shown that cells cultured in the presence of fetal calf serum may express bovine β_2m (22), we feel that exchange of mouse β_2m for bovine β_2m does not account for the complete absence of detectable murine β_2m on EL4/Mar, because culture of these cells in the absence of serum does not restore expression of murine β_2m on EL4/Mar (data not shown).

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