

A *trans*-ACTING MECHANISM REPRESSES THE EXPRESSION
OF THE MAJOR TRANSPLANTATION ANTIGENS IN
MOUSE HYBRID THYMOMA CELL LINES

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The products of the class I H-2 genes from the K and D loci of the mouse MHC, located on chromosome 17, are found at the surface of most nucleated cells in association with β_2 -microglobulin (1, 2). Their synthesis, however, is regulated. The K and D antigens are not found on the surface of mouse preimplantation embryos (3) and embryonal carcinoma (EC)¹ cells (4), where their absence is correlated with the absence of steady-state H-2 mRNA (5, 6). Scollay et al. (7) have described adult mouse thymus K⁻,D⁺ cells, which upon intrathymic maturation become K⁺,D⁺. Certain cells in the thymus, as well as in tissues such as brain, display low or very low H-2 expression.

Quantitative variations of H-2 antigen expression have been described in tumors. They are of special interest for establishing the role of H-2 antigens in the immune response to neoplastic growth (reviewed in 8). Recent data (9-11) suggest that the metastatic potential of some tumors may be influenced, at least in part, by the expression of class I H-2 (or HLA) antigens.

We have been studying the expression of H-2 class I genes in thymomas induced by Moloney murine leukemia virus (MoMLV), some of which show abnormal H-2 gene expression (12). One thymoma, BM5R, was found to be H-2⁻ because there was no detectable steady-state mRNA, and no apparent loss or rearrangement of H-2 genes (12). Because this H-2⁻ phenotype is unusual for a differentiated cell, we have further characterized the BM5R thymoma. In cell fusion experiments with an H-2⁺ thymoma, we find that the H-2⁻ phenotype is dominant. This suggests the existence of a dominant suppressive mechanism, and our data indicate that the putative regulatory loci involved are not located on chromosome 17. We discuss these results with regard to thymocyte differen-

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¹ Abbreviations used in this paper: EC, embryonal carcinoma; Gpi, glucose-6-phosphate isomerase; MoMLV, Moloney murine leukemia virus.

TABLE I
Serological Reagents

Antibodies Reagents	Antibody specificity	Source
Monoclonal		
1	Thy-1.1	New England Nuclear (Boston, MA)
2	Thy-1.2	OLAC (Bicester, United Kingdom)
3	Ly-2.1, 2.2	Becton Dickinson & Co., (Mountain View, CA)
4	L3T4	A. Pierrès*
5	H-2K ^k , K ^q , p, r	D. H. Sachs [‡]
6	H-2K ^b , D ^b , K ^d , r, s	D. H. Sachs [§]
7	H-2D ^d	D. H. Sachs [¶]
Polyclonal		
8	TdT	F. J. Bollum [¶]
9	H-2 ^f	M. Pla**

Alloantisera and mAbs used in immunofluorescence assays to characterize the phenotypes of BM5R.9, BW5147, and the somatic cell hybrids.

* Antibody GK1-5. See Dialynas et al. (17).

‡ Prepared from the hybridoma cell line 11-4-1 established by Oi et al. (18) and characterized by Ozato et al. (19).

§ Antibody 20.8.4S (Ozato and Sachs [20]).

¶ Antibody 34.2.12S (Ozato et al. [21]).

¶ See Bollum, F. J. (22).

** Gift of M. Pla (U. 93 INSERM, Hôpital Saint-Louis, Paris, France). Anti-H-2D^fL^f made by immunizing ATFR2 mice with B10.M mice cells.

tiation, where BM5R could represent a so far undescribed intermediate in thymic lineages, and with regard to the overall regulation of H-2 gene expression.

Materials and Methods

Cell Lines. The clone BM5R.9 was derived from the BM5R thymoma isolated from an F₁ hybrid mouse, B10.M × B10.A(5R), haplotype H-2^f × H-2^b, i.e., H-2K^fD^f/K^bD^d. It is sensitive to ouabain (>0.25 mM) and grows in hypoxanthine-azaserine medium.

The HGPR⁻ thymoma, BW5147 (obtained originally from R. Hyman, the Salk Institute for Biological Studies, San Diego, CA), was isolated from an AKR mouse (haplotype H-2^k). It expresses H-2^k antigens and Thy-1.1. It is resistant to thioguanine, high concentrations of ouabain (3 mM), and dies in hypoxanthine-azaserine medium (13).

The EL4 thymoma isolated from C57BL/6 (H-2^b) expressed H-2^b antigens and served as a control for H-2K^b expression.

The cells were grown in RPMI 1640 Dutch modification (Flow Laboratories, Inc., McLean, VA) supplemented with 10% heat-inactivated FCS, glutamine, antibiotics, and 5×10^{-5} M 2-ME.

Typing of Cells. H-2, Lyt-2, L3T4, and Thy-1 antigen expression were studied by indirect immunofluorescence assessed by microscopy or flow cytofluorometry, using conventional H-2 antisera or mAbs as in Daniel et al. (14). Table I summarizes the serological reagents used. TdT was detected by indirect immunofluorescence and TdT activity was assayed as described elsewhere (15). dG sensitivity was tested in two ways: (a) we assessed the amount of [³H]thymidine (2 Ci/mM [1 μCi/well]; Amersham Corp., Arlington Heights, IL) incorporated by 5×10^4 cells incubated for 5 h in the presence or absence of 50 μM deoxyguanosine; (b) The viability of cells was assessed by the trypan blue dye exclusion test after a 24 h culture with or without dG. Glucose-6-phosphate isomerase (Gpi) was tested in cell extracts, obtained by freeze-thawing, and was run on SPE agarose gels (Paragon Electric Co., Inc., Two Rivers, WI; Beckman Instruments, Fullerton, CA). Bands containing Gpi activity were revealed as described previously (16).

Cell Hybridization. Cell fusion was performed as described by Buttin et al. (23). $\sim 3 \times 10^6$ cells of BW5147 and BM5R.9 were spun down onto a polycarbonate filter and treated with 1 ml of 45% (wt/wt) PEG 1000 (Merck & Co., Rahway, NJ) in serum-free medium for 45 s. The filter was transferred to a Petri dish, and after removing the excess PEG, 5 ml of complete nonselective medium were slowly added. ~ 6 h later, when most of the cells had come off the filter, 10^5 cells per well were distributed on a Linbro 24-well plaque. 24 h later, selective medium was added to the wells to give a final concentration of hypoxanthine 5×10^{-5} M, azaserine 10^{-5} M, and ouabain 0.5 mM. Cells were fed every 3–4 h and after 2 wk they were transferred to 25-cm² flasks.

Chromosome Counts. Exponentially growing cell populations were incubated in the presence of 0.4 $\mu\text{g/ml}$ of colcemid for 2 h, treated with sodium citrate solution (1%), and fixed with acetic acid/methanol (1:3). They were then spread on microscope slides and stained with a 10% Giemsa solution. At least 20 metaphases were examined for each sample.

DNA and RNA Extractions. DNA was extracted from mouse liver or cell lines as described by Gross-Bellard et al. (24). RNA was extracted from liver with lithium chloride and urea (25), and from the cell lines using the hot phenol technique (26).

Southern Analysis. 20 μg of high molecular weight DNA cleaved with Bgl II or Pvu II, as recommended by the manufacturers (Boehringer Mannheim Diagnostics, Houston, TX), was subjected to electrophoresis and transferred onto nitrocellulose as described by Southern (27). The 950 bp Pst I fragment isolated from the 3' end of the K^d gene (28) was nick-translated. The 250-bp Pst I fragment of pH-2^d-37 (29) was subcloned into the Pst I site of pSP65, and a riboprobe was prepared (30).

S1 Mapping Analysis. The Sma I fragments containing the second exon and some intron sequences of the K^b (31) and K^k (32) genes were cloned into the Sma I site of M13mp8. The β_2 -microglobulin probe was the Eco RI/Rsa I of the cDNA p β_2 -m2 (14) cloned between the Eco RI and Hinc II sites of M13mp8 (33). It contains the coding sequence from amino acids 70 to 99 plus 100 nucleotides from the 3' noncoding region. The single-stranded probes were synthesized according to Burke (34). 4 μg of total RNA were coprecipitated with 30 μg of yeast tRNA and excess single-stranded probe (about 100,000 cpm). The pellet was resuspended in 30 μl of hybridization buffer containing 0.4 M NaCl, 40 mM Pipes (pH 6.4), 1 mM EDTA, and 80% formamide. The samples were heated for 10 min at 85°C, then transferred either to 60°C (for samples containing the K^b or K^k probes) or 40°C (samples containing the β_2 -microglobulin probe), and incubated for 16 h. S1 nuclease digestion was performed for 2 h at 37°C with 5,000 U of nuclease S1 (Boehringer Mannheim Diagnostics) in 300 μl of 30 mM sodium acetate (pH 4.5) and 3 mM zinc acetate without NaCl for H-2 probes or in the presence of 280 mM NaCl for the β_2 -microglobulin probe. After phenol extraction and ethanol precipitation, the samples were resuspended in 3 μl of formamide and subjected to electrophoresis in a 6% acrylamide/7 M urea sequencing gel. Hpa II-digested pBR322 DNA, end-labeled with Klenow, was used for size markers.

Construction of the BM5R.9 Genomic Library. Total genomic DNA from BM5R.9 cell line was partially digested with Sau 3A and fractionated by centrifugation through a sucrose density gradient. Fractions containing DNA fragments 15–20 kb long were pooled and used for construction of the library. 0.5 μg of this DNA was ligated to 2.5 μg of the right and left arms of the EMBL3 λ vector (35) previously digested by Bam HI and Eco RI. After in vitro packaging (36), the recombinant phages were plated on *Escherichia coli* 803Su3r⁻m⁻. 1.4×10^6 phages were screened by in situ hybridization with the nick-translated coding region of H-2K^d cDNA (the Eco RI/Tth111 I fragment of pH-2^d-33 (37)). 80 positive recombinants were isolated and reprobbed with labeled oligonucleotides specific for the *K*, *D*, and *L* loci (38). Two and three recombinant phages reacted positively with the *D* and *L* locus-specific probes, respectively; none reacted with the *K* probe.

Results

Characterization of the BM5R.9 Cell Line. The thymoma BM5R, isolated from a mouse having the H-2 genotype K^fD^f/K^bD^d , expresses none of the correspond-

TABLE II
Characteristics of the Parental Cell Lines BM5R.9 and BW5147

Cell lines	H-2 genotype	H-2 phenotype	Thy-1	TdT	Ly-2	L3T4	dG sensitivity
BM5R.9	H-2 ^f × H-2 ^b	K ^{f-} , D ^{f-} , K ^{b-} , D ^{d-}	+	+	+	-	+
BW5147	H-2 ^k	K ^{k+} , D ^{k+}	+	-	-	-	-

ing H-2 antigens (12). For further characterization, we derived from it a cloned cell line, BM5R.9, which was used throughout this work.

BM5R.9 was analyzed for a number of markers specific for thymocytes. It expresses Thy-1 and Lyt-2 antigens, but not L3T4. It contains TdT activity, and is extremely sensitive to dG (Table II). While these data confirm its thymic origin, no thymic cells have yet been reported to exhibit this particular set of properties (39, 40).

BM5R.9 has the same H-2⁻ phenotype as BM5R where it correlates with the absence of H-2 mRNA (5). Although Southern blot experiments had not detected alterations in H-2 class I genes, these previous studies did not rigorously prove that the unexpressed *K*, *D*, and *L* genes were intact. To establish this point, we constructed a library of BM5R.9 DNA in the phage vector EMBL3. By screening 1.4×10^6 recombinant phages with a class I H-2 probe, as described in Materials and Methods, we isolated 80 positives, out of which, by use of more specific probes (38), we identified the *D^d* gene. Its restriction map was identical to the one published (41), and upon transfection into mouse L cells, we detected the *D^d* antigen with a panel of specific sera and mAbs (not shown). Another functional gene, probably *L^f*, is currently being characterized. These data show that at least two genes, and most probably the other *K*, *D*, and *L* genes of BMR.9, although unexpressed, are functional.

Outline of the Cell Fusion Experiments. To understand why H-2 genes are silent in BM5R.9 we looked for a suppressive mechanism, assuming that this mechanism would act in *trans* on other H-2 genes. Because attempts to transfect DNA into BM5R.9 by a variety of methods were unsuccessful, we undertook cell fusion experiments, using as a partner the BW5147 cell line, also derived from a thymoma but expressing H-2 antigens of a distinct haplotype (H-2^k). Several of its characteristics are listed in Table II. BW5147 is HGPRT⁻ and resistant to ouabain. After fusion, 100,000 cells were seeded per Linbro well in a medium selecting for HGPRT⁺ and ouabain-resistant cells (see Materials and Methods). The rate of reversion of the parental cell lines in the same medium was $<1.6 \times 10^{-7}$, while hybrids were formed at a frequency of at least 1 per 5×10^5 of either parental cell. From three independent experiments, we obtained a total of 68 populations, 10 of which were chosen at random for further study.

15 d after the fusion, these nonclonal populations were analyzed for H-2K^b and H-2K^k expression by indirect immunofluorescence using mAbs. All 10 populations, like BM5R.9, did not express H-2K^b. 8 out of 10 were negative for H-2K^k antigen, unlike the parental cells BW5147. We retained two populations exhibiting the K^{b-}, K^{k-} phenotype (BMW-4 and -9), and the two exhibiting the K^{b-}, K^{k+} phenotype (BMW-1 and -7), which we characterized in more detail.

TABLE III
Hybrid Cells, Clones, and Subclones and Their Characteristics

Cells	Phenotype			Presence of 17th chromosome with given haplotype		Chromosome number	gpi	Thy-1	
	K ^b	D ^d	K ^k	H-2 ^{b,d} H-2 ^k				1.1	1.2
				H-2 ^{b,d}	H-2 ^k				
Parental lines									
BM5R.9	-	-	-	+	ND	40	B2 fast	-	+
BW5147	-	-	+		+	41	A2 slow	+	-
Hybrids									
BMW-1									
1 mth	-	-	+	+	+	67	A2.AB.B2	+	-
2 mth	-	-	+	ND	ND	ND	ND	ND	ND
1-6 clone	-	-	+	+	+	ND	ND	+/-	-
BMW-7									
1 mth	-	-	+	-	+	50	A2.AB.B2	+	-
2 mth	-	-	+	ND	ND	ND	ND	ND	ND
7-2 clone	-	-	+	-	+	ND	ND	+	-
BMW-4									
1 mth	-	-	-	+	+	80	AB.B2	+	+
2 mth	-	-	+/-	ND	ND	ND	ND	ND	ND
4-10 clone	-	-	-	+	+	78-82	AB.B2	+	+
subclones									
A	-	-	+/-	ND	ND	ND	ND	ND	ND
B	-	-	-	ND	ND	ND	ND	ND	ND
C	-	-	+	ND	ND	70	ND	ND	ND
D	-	-	+/-	ND	ND	71	ND	ND	ND
E	-	-	+/-	ND	ND	ND	ND	ND	ND
BMW-9									
1 mth	-	-	-	+	+	70	A2.AB.B2	+	+
2 mth	-	-	+/-	ND	ND	ND	ND		
9-9 clone	-	-	+/-	+	+	ND	ND	+	+

The table shows, for the parental cell lines and hybrid cells: the phenotype of K^b, D^d, and K^k expression; the presence (+) or absence (-) of the 17th chromosome of either H-2^{b,d} haplotype from the BM5R.9 parent or H-2^k haplotype from the BW5147 parent, as deduced from Southern blotting experiments with appropriate probes; chromosome counts; gpi isoform (gpi is encoded in chromosome 7); and expression of Thy-1 alleles (Thy-1 is encoded in chromosome 9).

Finally, 2 mo after the fusion clones were derived from each of the populations by limiting dilution, and they were analyzed further together with some subclones that had been isolated later.

Studies on the Hybrid Populations BMW-1, -4, -7, and -9. Several markers specific for the parental cell lines were assayed in these four populations. All cells having the HGPRT⁺-ouabain resistance phenotype have therefore received the corresponding parental chromosomes. Gpi is coded by chromosome 7. Data in Table III show that the two isoforms coded in each parent are coexpressed in all four populations. We also assayed for the differentiation antigen Thy-1, encoded by chromosome 9, since BW5147 expresses the Thy-1.1 and BM5R.9 the Thy-1.2 allele. All four populations express Thy-1.1 and only two express Thy-1.2.

We also counted chromosomes and found that cells in the BMW-1, -4, and -9 populations contained almost the sum of the parental chromosomes, while BMW-7 cells had retained fewer, but still more than the diploid number ($2n = 40$) (Table III). These data confirmed that the BMW-1, -4, -7, and -9 populations were indeed composed of bona fide hybrid cells.

Three H-2 antigens (K^k , K^b , and D^d) were tested by indirect immunofluorescence, using specific monoclonal and polyclonal antibodies. 1 mo after the fusion, BMW-4 and -9 had the K^{b-}, D^{d-}, K^{k-} phenotype and BMW-1 and -7 had a K^{b-}, D^{d-}, K^{k+} phenotype. However, when tests were performed again 2 mo after the fusion, BMW-1 and -7 had retained their original phenotype, but BMW-4 and -9 displayed some heterogeneity; ~10% of the cells reexpressed the K^k antigen. This observation strongly suggested that in the BMW-4 and -9 populations, the 17th chromosome of the H-2-expressing donor (BW5147) was present but silenced, and could be reexpressed. We set up to prove this point by isolating clones with the corresponding phenotypes, and characterizing the presence or absence of genes and transcripts by using the appropriate DNA probes.

Genomic Analysis of Clones BMW-1-6, -4-10, -7-2 and -9-9. Clones isolated from the BMW-4 and -9 populations did reflect their heterogeneity since a number of them expressed K^k , as did all clones derived from BMW-1 and BMW-7. One clone from each population was selected for molecular analysis. BMW-1-6 and -7-2 had the same K^{b-}, D^{d-}, K^{k+} phenotype that the parental populations initially displayed. BMW-4-10 had the K^{b-}, D^{d-}, K^{k-} phenotype, while BMW-9-9, with a K^{b-}, D^{d-}, K^{k+} phenotype, reexpressed K^k , which was initially undetectable in the BMW-9 population.

The K^{b-}, D^{d-} or K^{k-} phenotypes could be due to the loss of the corresponding 17th chromosomes. A 950-bp Pst I DNA fragment originating from the 3' end of the K^d gene, and which contains the K-specific DNA probe previously described (42), reacts with a 3.5-kb Bgl II DNA fragment in the H-2^k haplotype and with two Bgl II fragments, 2.3 and 1.8 kb long, in the H-2^b haplotype. Therefore, Southern blot experiments were performed with DNA of the four cell lines, and appropriate controls were digested by Bgl II. Results in Fig. 1 show the patterns obtained with B10.M (K^l) (Fig. 1a), AKR (K^k) (lane b), B10 (K^b) (lane i) and B10.M × B10.A(5R) (K^l/K^b) (lane c) DNAs. The data indicate that the three fragments pointed by arrows display the expected haplotype specificity. Moreover, they confirm that the K^b gene in the BM5R.9 cell line is apparently intact (Fig. 1, compare d with i).

The hybrid cell lines BMW-1-6, -4-10, and -9-9 share the characteristic fragments of the H-2^b haplotype (Fig. 1, compare e, f, and h with i), while BMW7.2 does not (lane g). Therefore, the latter probably misses the corresponding 17th chromosome. All four cell lines, including BMW-4-10, which does not express K^k , have the H-2^k-specific DNA fragment. Results in Fig. 2 illustrate this point even more clearly; we used a 250-bp Pst I fragment isolated from the cDNA of pH-2^d-37, which hybridizes only with one or two genes in the Tla region (29). In the H-2^k haplotype, this probe reacts with two Pvu II fragments of 1.7 and 0.8 kb (Fig. 2a). With this probe, we tested Pvu II-digested DNA from the H-2^f haplotype (lane b) and B10.M × B10.A(5R) liver (lane c), or from BM5R.9 DNA (lane d), where only one fragment reacts. All four hybrids (lanes e-h)

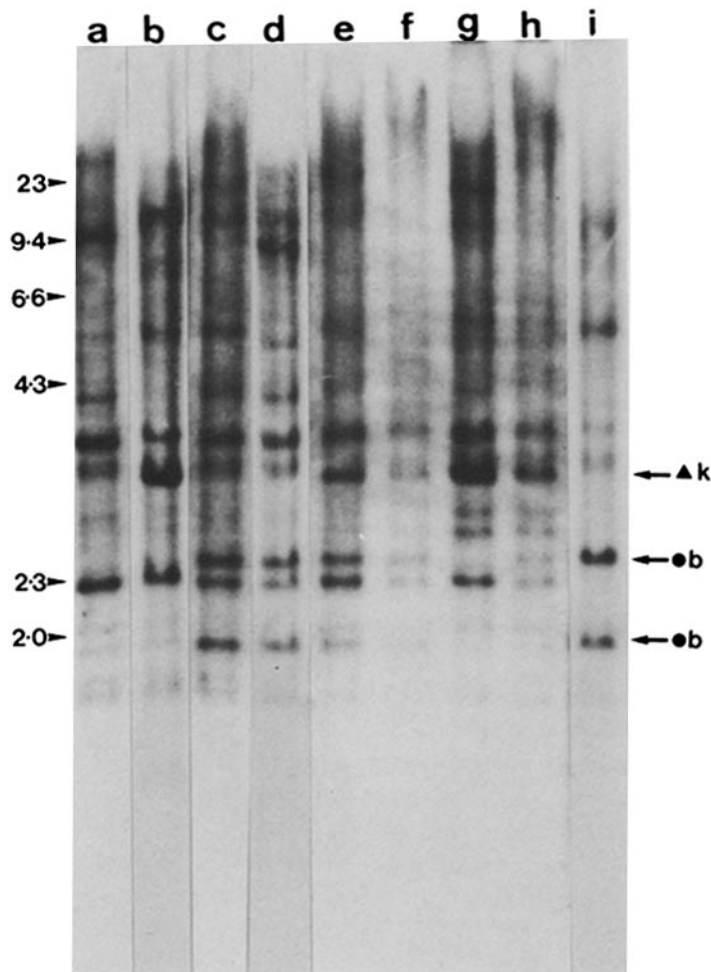


FIGURE 1. Detection of chromosome 17 present in somatic cell hybrids by Southern blot analysis. Genomic DNA samples (20 μ g) were digested with the restriction enzyme Bgl II and run in 0.7% horizontal agarose gels, transferred to nitrocellulose filters, and hybridized to the nick-translated 950-bp Pst I fragment isolated from the 3' end of the K^d gene. Lanes a-i contain, respectively, DNA from B10M liver (haplotype f), AKR liver (haplotype k), B10.M \times B10.A(5R) liver (haplotype f \times i^b), BM5R.9 (grown in vitro) (haplotype f \times i^b), BMW-1-6, BMW-4-10, BMW-7-2, BMW-9-9, and C57Bl/6 liver (haplotype b). (▲) Band characteristic of the H-2^k haplotype (~ 3.5 kb). (●) Two bands, of ~2.3 and 1.8 kb, respectively, that are characteristic of the b haplotype. The 1.8-kb fragment in lanes f and h is clearly visible on the autoradiograms. The size of marker fragments (λ DNA cut with Hind III) are indicated in kbp.

contain unequivocally the k haplotype fragments. Little is known about H-2^f genes present in BM5R.9, and we could not obtain unambiguous answers about their presence or absence in hybrids.

Transcription Analysis of Clones BMW-1-6, -4-10, -7-2 and -9-9. We wished to determine, as we had shown previously for the BM5R thymoma, whether the absence of some H-2 antigens in the hybrids is due to the absence of steady-state mRNA. The analysis of specific H-2 mRNA transcripts is complicated by the

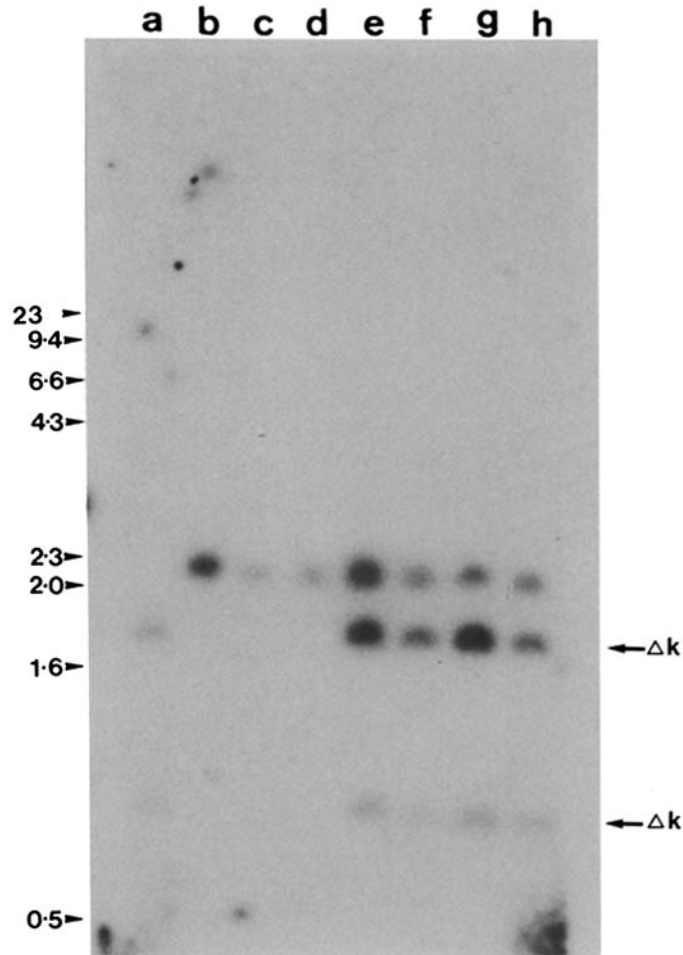


FIGURE 2. Detection of the H-2^k chromosome 17 in somatic cell hybrids by Southern blot analysis. Genomic DNA samples (20 μ g) were digested with the restriction enzyme Pvu II, run in a 1% horizontal agarose gel, then transferred to a nitrocellulose filter and hybridized to low copy probe isolated from pH-2^d-37. This probe was obtained by cloning a 250-bp Pst I fragment of pH-2^d-37 into a vector (pSP65) containing the SP6 promoter. Lanes a-h contain DNA samples from AKR liver (haplotype k) (a), B10.M liver (haplotype f) (b), B10.M \times B10.A(5R) liver (haplotype f \times i^b) (c), BM5R.9 (d), BMW-1-6 (e), BMW-4-10 (f), BMW-7-2 (g) and BMW-9-9 (h). (Δ) The two DNA fragments specific for the k haplotype.

high degree of sequence homology in the different genes. We performed an S1 mapping analysis using single-stranded probes, which could specifically detect the K^k or K^b gene transcripts. To distinguish between K^k and K^b mRNAs, we cloned the Sma I fragment that encompasses the second exon of the K^k and K^b gene in the Sma I site of the single-stranded phage vector M13mp8. This fragment was chosen because the comparison of the published sequences of the K^b and K^k genes shows that it contains numerous divergent nucleotides.

Results in Fig. 3 show that both probes yield a 270 nucleotide fragment corresponding to the entire second exon specifically protected by their cognate

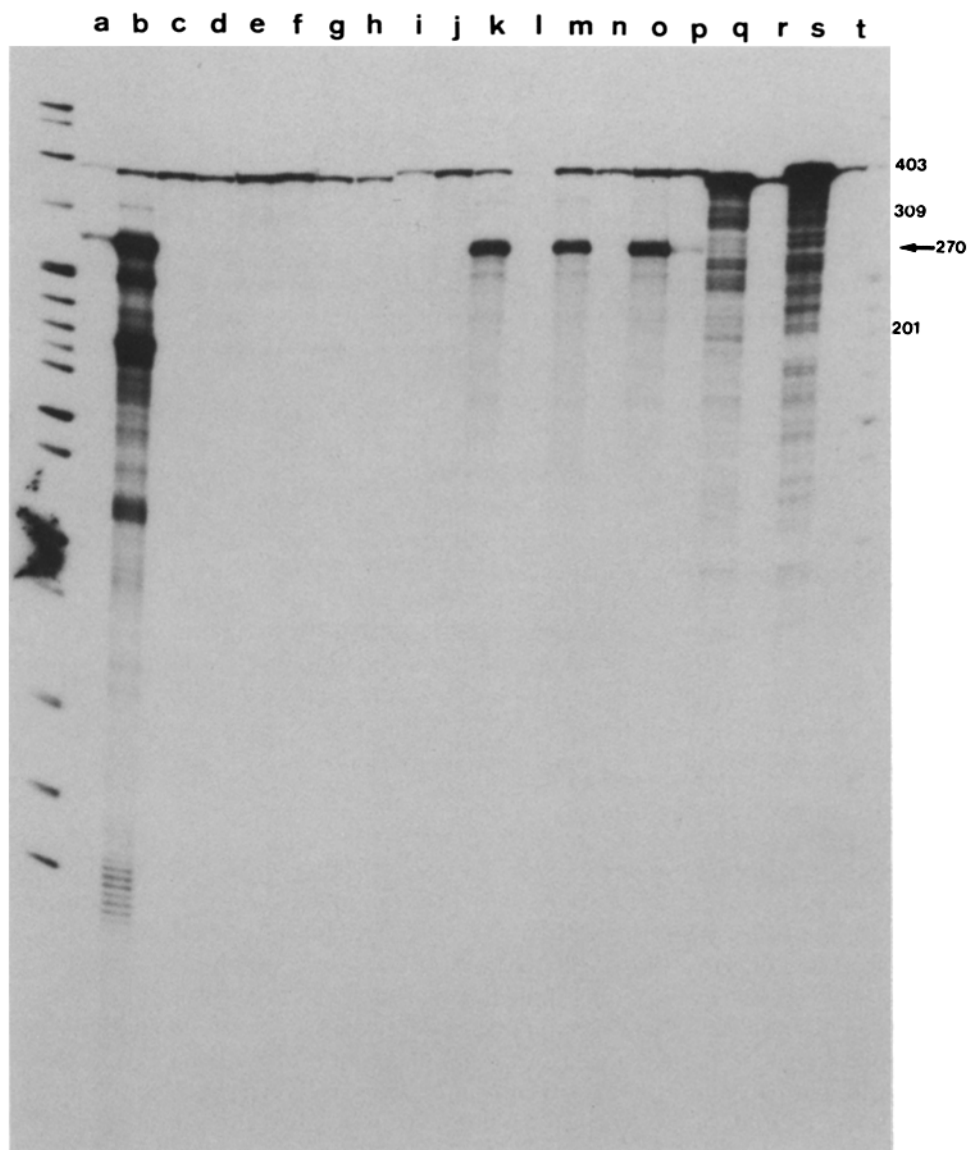


FIGURE 3. Detection of K^b and K^k transcripts in somatic cell hybrids. 4 μ g of total RNA were hybridized with 100,000 cpm of either the K^b or K^k single-strand probes, as described in Materials and Methods. The products obtained after S1 nuclease treatment were separated on a 6% polyacrylamide-urea gel. The gel was dried and autoradiographed. Lanes *a-h* were hybridized with the K^b probe. Lanes *i-p* were hybridized with the K^k probe. RNAs were from the EL4 cell line (haplotype b) (*a* and *i*), B10.M \times B10.A(5R) liver (haplotype $f \times i^5$) (*b* and *j*), BW5147 (haplotype k) (*c* and *k*), BM5R.9 (*d* and *l*), BMW-1-6 (*e* and *m*), BMW-4-10 (*f* and *n*), BMW-7-2 (*g* and *o*), BMW-9-9 (*h* and *p*). Lanes *q* and *s*, *r*, and *t* contain, respectively, the probes K^b and K^k alone in the absence or presence of S1 nuclease. The 270-bp, protected fragment is indicated by an arrow. The markers are pBR322 DNA cut with Hpa II.

mRNAs (Fig. 3*a*, *b*, and *k*). The K^b probe was not protected by K^k mRNA and vice versa (compare lanes *b* with *c*, and *i* with *k*). It is seen in lane *b* that the K^b

probe yields additional protected fragments (about 240, 190, and 130 nucleotides in length) when hybridized with B10.M \times B10.A(5R) RNA. The RNAs responsible for this protection have not been identified, but protection of the two smaller fragments is observed upon hybridization with RNA isolated from B10.M mouse (H-2^f haplotype). Results in lanes *e-h* show that all the hybrid lines that are phenotypically K^{b-} lack detectable quantities of K^b mRNA, and do not contain detectable D^d transcripts (data not shown). Hybrid lines with K^{k+} phenotype (BMW-1-6, -7-2, and -9-9) contain K^k mRNA (Fig. 3 *m, o, and p*), while BMW-4-10 that is K^{k-} has no detectable K^k mRNA (lane *n*). It was verified that the RNA isolated from BMW-4-10 was not degraded. In summary, the phenotype of K^k, K^b, and D^d expression is directly correlated with the presence or absence of steady-state mRNA.

It was important to check for the presence of β_2 -microglobulin mRNA in these hybrids. Fig. 4 shows that all hybrid cell lines contain significant amounts of β_2 -microglobulin mRNA, including BMW-4-10 (lane *e*); therefore the absence of H-2 antigens is unlikely to be due to the lack of β_2 -microglobulin.

While these studies were in progress the BMW-4-10 clone, after 4 mo of passaging, showed, in turn, some heterogeneity. Five subclones were isolated (BMW-4-10-A to E), some with a K^{b-}, K^{k+} the others with a K^{b-}, K^{k-} phenotype. S1 analyses showed the presence of K^k, but not K^b mRNA in the former subclones (Figs. 5 and 6, lanes *i* and *k*). As a control, we included RNA from the initial hybrid populations (BMW-1, -4, -7, and -9) extracted 1 mo after the fusion, where neither K^b nor K^k mRNA was detectable. These results confirm that the phenotype instability of K^k expression is reflected at the level of K^k mRNA.

Discussion

We have further investigated the properties of a thymoma, BM5R, which we had shown earlier to be deficient in the expression of the major class I transplantation antigens. We have derived from it a cloned cell line, BM5R.9, with the same H-2^f phenotype. We have tested BM5R.9 for a number of markers of thymocyte differentiation, and found that it has many of the characteristics of immature thymocytes. However, it expresses L γ t-2 in a so far undescribed configuration of markers. Does it represent an immortalized version of a minor class of immature cells, either cortical or medullary thymocytes? The phenotype of in vitro transformed cells often closely reflects that of normal stages of differentiation, as illustrated by studies of Abelson-transformed B and T cells and human T cell leukemias (43-45). However, the interesting question of whether BM5R.9 identifies a new type of thymocytes cannot be answered without a direct search in a normal population, for the corresponding, presumably minor class of thymocytes.

What causes the H-2^f phenotype of BM5R.9? Earlier analysis of BM5R tumor cells had revealed no alteration in H-2 genes that could be detected in Southern analyses. To rule out that H-2 genes were inactivated by mutations, we prepared a genomic library with BM5R.9 DNA out of which the *H-2D^d* gene was isolated and shown to be expressed upon transfection into mouse L cells. Being confident that genes in BM5R.9 were silent but not inactivated by mutation, we wanted to

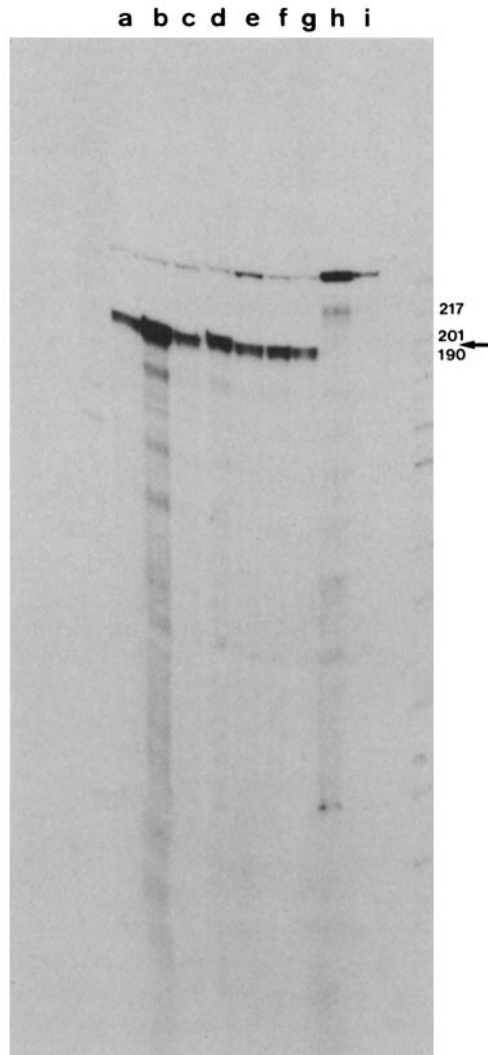


FIGURE 4. Detection of β_2 -microglobulin transcripts in somatic cell hybrids. 4 μg of total RNA from the different hybrids and controls were hybridized with 90,000 cpm of a single-stranded β_2 -microglobulin probe. The β_2 -microglobulin probe protects a fragment of ~ 198 nucleotides. Lanes *a-g* contain total RNA from BW5147 (*a*), B10.M \times B10.A(5R) liver (*b*), BM5R.9 (*c*), BMW-1-6 (*d*), BMW-4-10 (*e*), BMW-7-2 (*f*), BMW-9-9 (*g*). Lanes *h* and *i* contain the probe alone in the absence or presence of S1 nuclease. The protected fragment (~ 195 nucleotides) is indicated with an arrow.

know whether a suppressive mechanism would operate in BM5R.9 cells, and postulated that it could act in *trans*. Accordingly, we performed a cell fusion experiment.

Fusion experiments with another thymoma with an H-2^{k+} phenotype strongly suggest that such a mechanism exists. Bona fide hybrids were formed, and most had a H-2⁻ phenotype. The analysis of hybrid populations, clones, and subclones

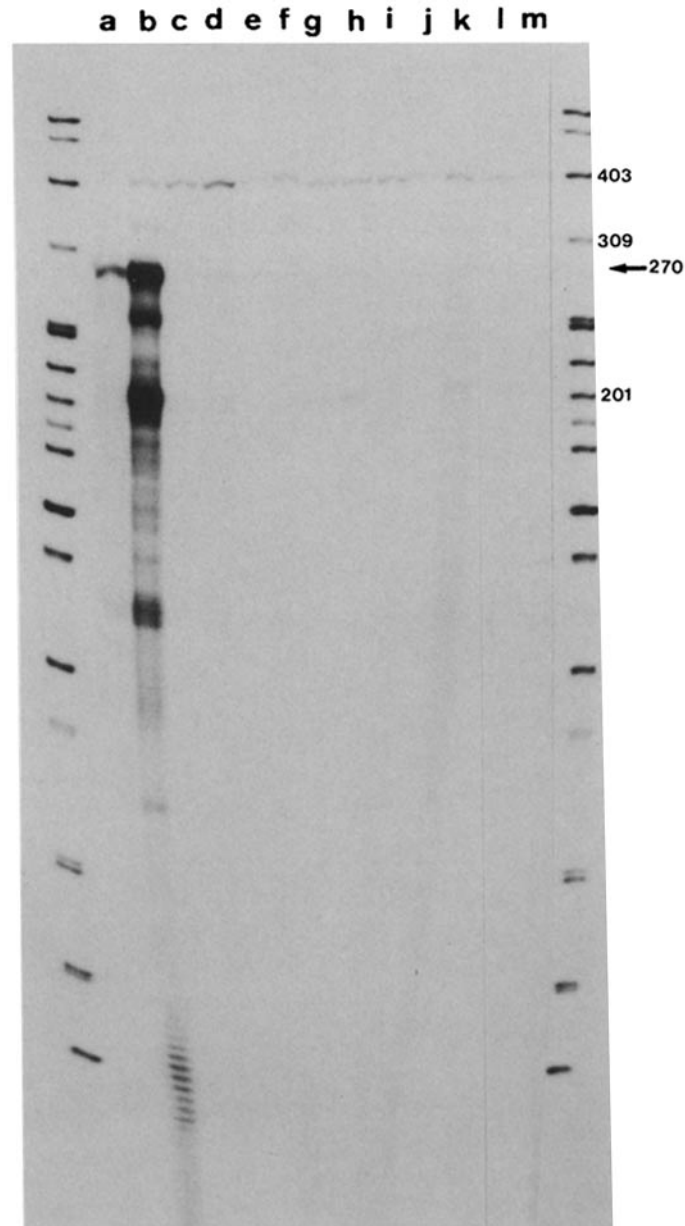


FIGURE 5. Absence of K^b transcripts in the initial hybrid cell populations and subclones derived from BMW-4-10. S1 mapping was performed as in Fig. 3. Lanes *a-m* contain 4 μ g total RNA hybridized with the K^b probe (100,000 cpm) from EL4, B10.M \times B10.A(5R) liver, BW5147, BM5R.9, BMW-1, BMW-4, BMW-7, BMW-9, BMW-4-10A, -4-10B, -4-10C, -4-10D, and -4-10E, respectively. The K^b transcript protects a fragment of 270 bp, indicated by an arrow.

derived from them is summarized in Table III. The major conclusions, backed by analysis of genomic DNA and gene transcripts with available specific probes, are as follows: (a) the H-2⁻ phenotype of BM5R.9 is dominant, since H-2K^k and

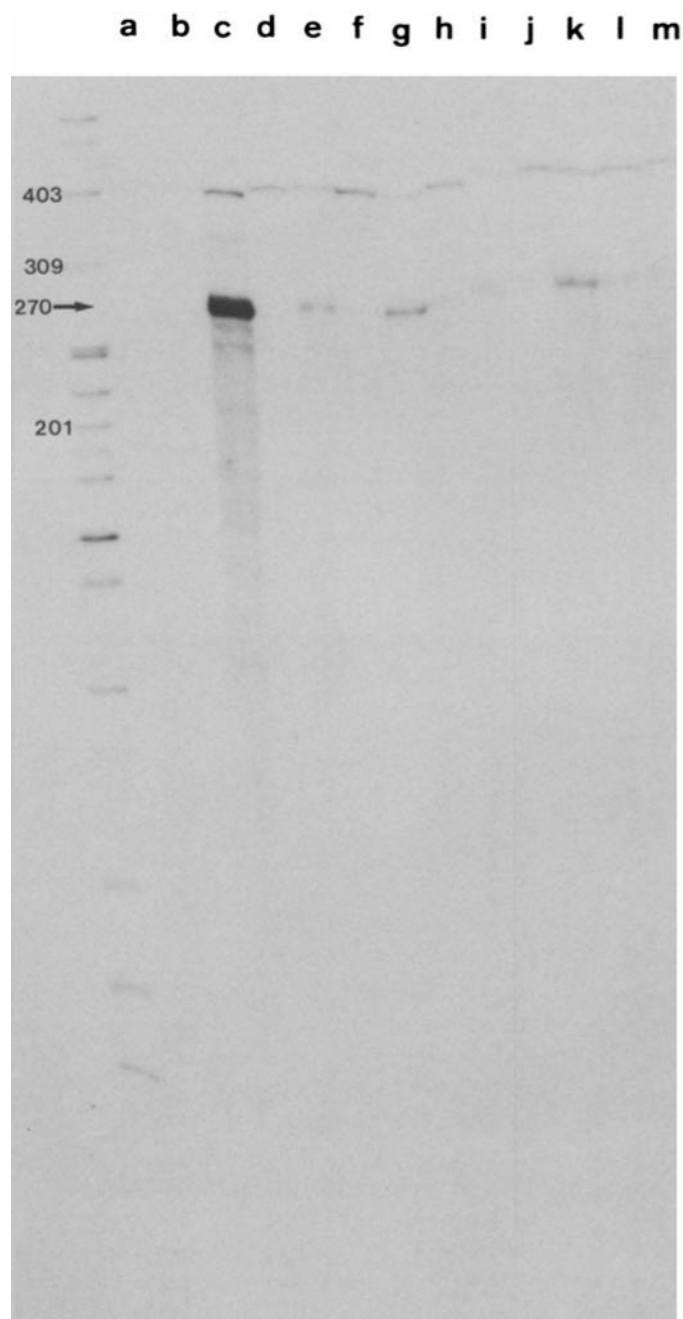


FIGURE 6. Detection of K^k transcripts in the initial hybrid populations and subclones derived from BMW-4-10. S1 mapping was performed as described in Fig. 3. Lanes *a-m* contain, respectively, 4 μ g total RNA from EL4, B10.M \times B10.A(5R) liver, BW5147, BM5R.9, BMW-1, BMW-4, BMW-7, BMW-9, BMW-4-10A, -4-10B, -4-10C, -4-10D, and -4-10E hybridized with 100,000 cpm of K^k probe. The protected fragment is indicated by an arrow.

H-2D^k expression is turned off in most hybrids; (b) lack of expression of K^k antigen in hybrids is not due to the absence of the corresponding 17th chromosome, and is correlated with the lack of steady-state K^k mRNA; (c) the H-2^{k-} phenotype is unstable, and upon passaging of cell populations and cloned cell lines, H-2K^{k+} cells are found together with H-2K^k mRNA; (d) however, H-2 genes from BM5R.9, particularly H-2K^b and H-2D^d, are never expressed, and no K^b or D^d mRNA is ever found, although in most instances the corresponding chromosome is present.

The simplest interpretation of these experiments is that a suppressive mechanism acting in *trans* at the transcriptional level operates to turn off H-2^k genes normally expressed in the other parental cell line. Furthermore, the gene(s) responsible for this suppression are likely to be located on a chromosome distinct from chromosome 17, which is occasionally lost by segregation in successive passages. This would explain why the suppressed phenotype shows some instability. Indeed, upon passaging, our hybrids cells lost chromosomes, as evidenced by chromosome counts (Table III). However, since detailed karyotype analyses have not been performed we cannot assess whether the proposed explanation is consistent with the loss of one or several specific chromosomes from the BM5R.9 parent.

We are, however, faced with a paradox: we looked for a suppressive mechanism to explain the silence of H-2 genes in BM5R.9; We found such a mechanism acting in *trans*, but the H-2 genes from the BM5R.9 parental chromosomes are never reexpressed, even when those from the BW5147 chromosomes are, presumably after loss of the chromosome(s) that are responsible for suppression. Therefore, we must formulate an additional assumption. We propose that these ever silent H-2 genes behave this way after suppression because there is a secondary block in their expression, such as DNA methylation or packaging into inactive chromatin. In this view, the H-2 genes in BM5R.9 would be considered undetermined, in the terminology of Brown (46), whereas H-2 genes in BW5147 would be considered "determined."

The existence of a suppressive mechanism in the BM5R.9 thymoma cell line raises the question of whether the same or a similar mechanism could operate in other cells with an H-2⁻ phenotype. To address this point, similar cell fusion experiments have previously been performed, but have not been confirmed by molecular analyses, as our results have been. For example, H-2⁻ phenotype of the Ehrlich tumor is dominant upon fusion with fibroblasts, although recessive with other tumors (47, 48). In an AKR thymoma, a dominant *trans*-acting, haplotype-dependent, suppressive mechanism has been suggested (49). The situation with H-2⁻ EC cells is not clear; a number of experiments have suggested the existence of a *trans*-acting suppressive mechanism (48-51), others have not (52, 53). We have recently shown that an enhancer sequence is located upstream of the H-2K^b gene and is inactive in EC cells (54). This strongly suggests that the lack of activation of this enhancer is at least in part responsible for the nonexpression of H-2 genes in those cells. But we have also shown that this enhancer is not the target of the well-documented inhibition of H-2 gene expression observed in primary cells transformed by adenovirus 12 (55). Altogether, it appears likely that the genes coding for the major transplantation antigens are regulated both

positively, through enhancer activation, and negatively, perhaps through the suppressive mechanism found in the present study. This, however, remains to be established at the molecular level, and is the goal of studies we are now performing.

Summary

We have fused an H-2⁻ thymoma (BM5R.9) with an H-2⁺ thymoma (BW5147) and have found that many of the resulting hybrids exhibit an H-2⁻ phenotype. In several hybrids that were analyzed in detail, this phenotype is related to the absence of steady-state H-2 mRNA and shows some instability, possibly related to the loss of chromosomes in segregants. We conclude from our studies that BM5R.9 cells display a *trans*-acting mechanism that can repress the expression of H-2 antigens, and that the gene(s) causing the repression are not located on chromosome 17. This mechanism is not sufficient to explain the H-2⁻ phenotype of BM5R.9, for which an additional, *cis*-acting process, must be postulated. We discuss these results in the context of the regulation of expression of the major class I transplantation antigens.

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