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Cis-acting elements determine the locus-specific shutoff of class I major histocompatibility genes in murine S49 lymphoma sublines

(somatic cell hybrids/tumor cell recognition)

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ABSTRACT **Several tumors have been reported to down-regulate expression of their class I major histocompatibility molecules, potentially altering their immune recognition. To investigate this phenomenon, we are using various sublines isolated from the S49 lymphoma of the BALB/c mouse strain. These S49 tumor sublines were previously found to have shut off expression of their K^d, D^d, and/or L^d class I molecules in a locus-specific manner. Extensive Southern blot analyses indicated that there were no major chromosome aberrancies in these S49 sublines, and analyses of steady-state class I mRNA suggested that a form of transcriptional regulation was responsible for their variant class I expression. In this report, we characterize the nature of this locus-specific regulation of class I in S49 cells by producing somatic cell hybrids. Three phenotypically distinct S49 sublines were each fused to tumor cells with normal class I expression, and several of the resulting hybrids were analyzed. In every case, the class I molecules expressed by the hybrids were an exact composite of the two fusion partners. Thus, these fusions failed to rescue expression of the K^d, D^d, and/or L^d molecules shut off within each of the S49 tumor sublines. These findings indicate that this locus-specific shutoff of class I expression results from a cis-acting defect and not trans-acting factors. Because the analysis of each of three phenotypically different S49 cells implicated a form of cis-dominant regulation, we hypothesize that a common mechanism generating homologous mutations in class I genes is operative in S49 tumor cells.**

The highly polymorphic class I genes of the mouse are contained within the *K* and *D* regions of the major histocompatibility complex (MHC). Each MHC haplotype appears to encode either two or three functional class I molecules. For example, the BALB/c inbred mouse strain has one *K* region product designated K^d and two *D* region products designated D^d and L^d. These class I molecules are 45-kDa glycoproteins expressed on the cell surface of nucleated cells. Present dogma suggests that class I molecules function as receptors that bind foreign antigens and thereby facilitate the immune surveillance by cytotoxic T lymphocytes against infected or malignant host cells. Although the details have yet to be fully elucidated, each class I molecule appears to have a single ligand binding site that binds immunogenic peptides of ≈2 kDa (1). For example, a class I ligand could be a processed form of a viral protein, thus leading to the killing of virus-infected cells by host cytotoxic T lymphocytes (2). Alternatively, the class I ligand could be a tumor-specific immunogenic peptide, thus resulting in rejection of tumor cells by host cytotoxic T lymphocytes. Consistent with this latter notion, several studies have reported an inverse correlation between class I expression by tumor cells and their metastatic potential (cf. ref. 3). Furthermore, the high metastatic phenotype of certain tumors not expressing class I molecules was reversed by inducing the expression of class I molecules by DNA-mediated gene transfer (4–7). These and other supportive findings have led to the hypothesis that tumor cells that down-regulate their class I expression avoid immune recognition. The qualitative differences (allelic or nonallelic) among class I molecules are also thought to play a key role in tumor-cell recognition. For example, Gooding (8) reported that progressive variants of the C3H tumor V4 had shut off expression of K^k but not D^k molecules; this loss of K^k correlated with the fact that K^k is the restriction element required for cytotoxic T-cell recognition of V4 tumor cells. These and similar findings suggested that tumor cells are capable of regulating class I molecules in a locus-specific manner.

As a model to investigate class I locus-specific regulation in tumor cells, we are studying various sublines of the S49 lymphoma from BALB/c mice (9, 10). These tumor cells are unique in that several of the sublines have selectively shut off surface expression of their K^d, D^d, and/or L^d molecules (11, 12). Here we report the selection and analysis of somatic cell hybrids produced by fusion of S49 variants to tumor cells with normal class I expression. Our findings demonstrate that the variant expression of class I molecules by S49 cells is the result of cis-dominant regulatory elements.

METHODS

Cells. The S49 BALB/c mouse lymphoma sublines were provided by Gene Shearer (National Institutes of Health). All the lines are also available from the American Type Culture Collection. The S49 lines are designated in this paper according to the American Type Culture Collection, Tumor Immunology Bank (TIB) nomenclature.

Monoclonal Antibodies (mAbs). Culture supernatants of mAbs used for cytofluorometric analysis are as follows: Do9 (13) or MA215 (14) (γ2b), K^d specific; 34-2-12 (15) or 34-5-8 (13) (γ2a), D^d specific; 30-5-7 (16) (γ2a), L^d specific; 15-3-1 (17) (γ2a), K^k/D^k reactive; MA66 (γ2a), D^b specific (14). Also used was ascites Y-3 (18) (γ2a), K^b reactive, diluted 1:50.

Fusions. The class I-positive lymphomas used for fusion studies were obtained from the American Type Culture Collection. The BW5147.G1.4.OUAR.1 cell line is ouabain resistant, hypoxanthine/guanine phosphoribosyltransferase negative ($HGPRT^-$) and the EL4.Bu cell line is thymidine kinase negative (Tk^-). In this manuscript, the above cell lines are denoted as BW5147 and EL4, respectively. Fusions were done according to the protocol of Goldsby *et al.* (19). TIB28 cells were fused to BW5147 (ouabain resistant, $HGPRT^-$) cells and selected in medium containing hypoxanthine, aminopterin, thymidine, and ouabain (Sigma). TIB35 ($HGPRT^-$) cells were fused to EL4 (Tk^-) cells, and TIB36 (Tk^-) cells

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Abbreviations: MHC, major histocompatibility complex; RCAS, rat Con A supernatant; mAb, monoclonal antibody; HGPRT, hypoxanthine/guanine phosphoribosyltransferase; Tk, thymidine kinase.

Table 1. Surface phenotype and selection of the S49 variants

S49 subline	Surface phenotype*						Selection†
	K^d	D^d	L^d	Thy-1	CD3	Ia	
TIB33	+	+	+	+	+	−	Dexamethasone resistance
TIB28	+	+	−	+	+	−	None
TIB35	−	−	−	+	+	−	Phytohemagglutinin resistance
TIB36	+	−	−	−	+	−	Thy-1$^-$

Ia, *I*-region associated.
*Compilation of our data obtained by flow cytometry. The results obtained concerning class I expression by TIB28, TIB35, and TIB36 confirm previous findings of Joseph *et al.* (11).
†Data from refs. 9 and 10.

were fused to BW5147 (HGPRT$^-$) cells. Both latter hybrids were selected in medium containing hypoxanthine, aminopterin, and thymidine. Hybrid cells were grown in selective medium for 2 weeks and thereafter were grown in nonselective medium.

Fluorescence Labeling of Cells and Analysis on the FACS. Fluorescence analysis was performed as described in detail elsewhere (12). Cells were analyzed on a fluorescence-activated cell sorter (FACS IV, Becton Dickinson) equipped with an argon ion laser tuned to 488 nm and operating at 300 mW of power. Fluorescence histograms were generated with logarithmic amplification of fluorescence emitted by single viable cells. Each sample analyzed comprised a minimum of 10^4 cells. Cells labeled with only the fluorescein-conjugated antibody were always included as controls.

Southern Blot Analysis. Genomic DNA from tissue culture cells and mouse liver was prepared according to a modified procedure of Chang *et al.* (20), as described in detail elsewhere (12). The 3′ flanking probe (21), a 0.9-kilobase (kb) *Sst* I/*Sst* I fragment located 2.1 kb 3′ of the L^d gene, was isolated in low melting temperature agarose (Bethesda Research Laboratories) and labeled by the random hexamer technique (19).

RESULTS

The S49 cell lines are an interesting series of tumor sublines isolated from a single BALB/c thymoma. Several different sublines were previously selected from nonmutagenized cells by using a pharmacological agent or an antibody (8, 9). Such selections were thought to identify cell types representing distinct stages of thymic development. Unexpectedly, several of these S49 sublines were found to have aberrant class I expression (11, 12). As summarized in Table 1, four different S49 sublines were selected for further characterization of their class I locus-specific regulation. Extensive Southern blot analyses of these four sublines found them to be indistinguishable, implying there were no major deletions (12). Furthermore, analyses of mRNA implied that their defect(s) in class I expression resided at the level of transcription (12). To determine whether this defective expression was the result of cis- or trans-acting elements, cell fusion experiments were performed. The three sublines with aberrant class I expression were fused to another tumor cell that expresses a serologically distinct MHC haplotype. TIB28 and

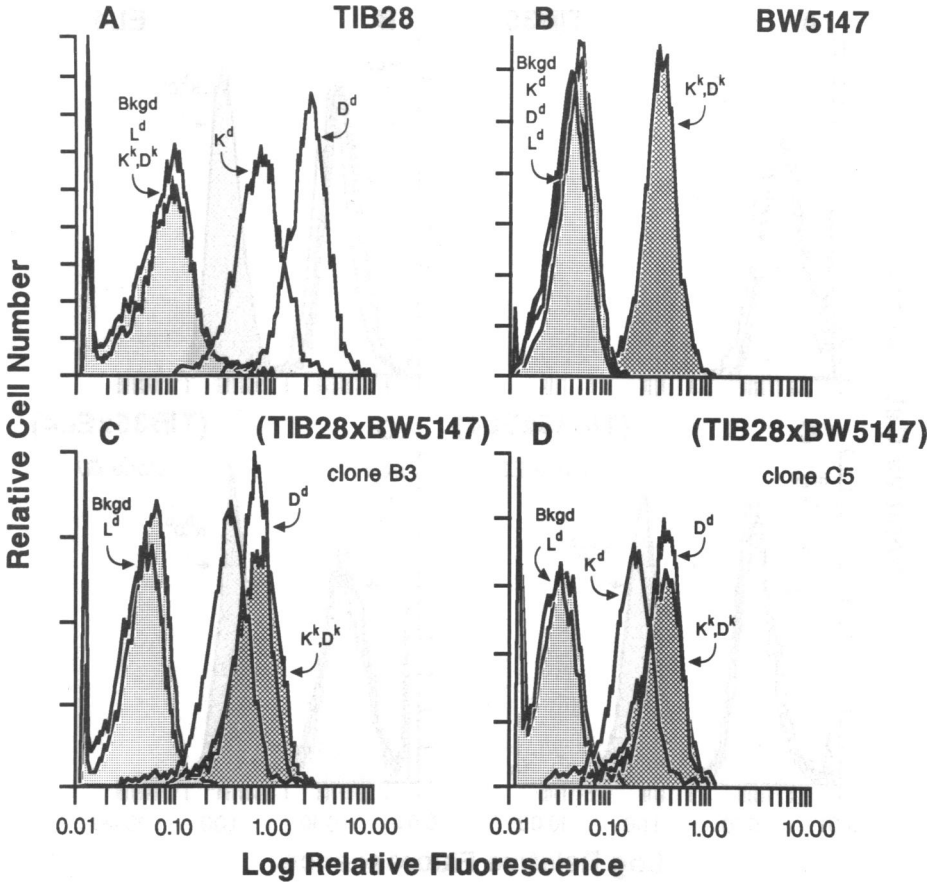

FIG. 1. Flow cytometric histograms of the class I molecules expressed by TIB28 (*A*) and BW5147 (*B*) cells compared with two representative (TIB28 × BW5147) hybrid clones—B3 (*C*) and C5 (*D*). Stippled peaks show background staining with the developing reagent; cross-hatched peaks show the positive control expression of K^k and D^k molecules. The specific mAbs used for this analysis were as follows: anti-L^d, 30-5-7; anti-D^d, 34-5-8; anti-K^d, MA215; anti-K^k D^k, 15-3-1. BW5147 cells as well as the hybrids were found to selectively express both K^k (11-4-1) and D^k (15-5-5) molecules (data not shown). The phenotypes of the hybrid clones have remained constant for 3 mo.

TIB36 cells were each fused with BW5147 lymphoma cells that express K^k and D^k molecules. Similarly, TIB35 cells were fused with EL4 lymphoma cells that express K^b and D^b molecules. Cell-surface expression of the class I molecules by these various hybrids were assessed by flow cytometry as shown in Figs. 1 and 2. In both of these figures, *A* and *B* show the fusion partners and *C* and *D* show two representative hybrid clones. At least 10 independent clones from each hybrid combination were tested and found to be phenotypically identical. Expression by the hybrid cells of the S49-derived class I molecules was in all cases identical to expression by the S49 parental cells. Thus, the (TIB28 × BW5147) hybrids express K^d and D^d but not L^d molecules (Fig. 1); (TIB36 × BW5147) hybrids express K^d but not D^d or L^d molecules (data not shown); and (TIB35 × EL4) hybrids do not express K^d, D^d, or L^d molecules (Fig. 2). Therefore, fusion to tumors that express other class I molecules failed to rescue expression of the K^d, D^d, and/or L^d molecules that are not expressed by the S49 variants. In addition, the shutoff of K^d, D^d, and/or L^d expression in these hybrid cells did not affect the expression of the K or D molecules derived from the BW5147 or EL4 fusion partner. Thus, these findings provide no evidence for trans-acting effects and demonstrate that the aberrant class I expression in S49 sublines results from a cis-dominant mechanism.

Interferons are known to increase levels of weakly expressed class I molecules and to induce class I expression in some negative tumors (22, 23). We therefore attempted to induce the antigens in the hybrids with interferon treatment. As a source of interferon, we used culture supernatant from Con A-treated rat spleen cells (RCAS). Although each of the expressed class I antigens increased their surface levels after treatment between 1.5- and 7-fold, none of the repressed antigens was induced (data not shown). These findings with the hybrids are consistent with our previous studies of RCAS treatment of the S49 sublines (12).

A trivial explanation for the failure to express S49 class I genes in these hybrids would be the loss of S49-derived MHC genes or perhaps the entire chromosome 17. The expression of the K^d molecules by the (TIB36 × BW5147) hybrids and the K^d and D^d molecules by the (TIB28 × BW5147) hybrids indicated that in these cell fusions at least one S49-derived chromosome 17 was retained. By contrast the (TIB35 × EL4) hybrids do not express any of the S49 class I molecules—K^d, D^d, or L^d. Thus, to verify the presence of an S49-derived chromosome 17 in these latter hybrids, a Southern blot analysis was performed with a 3′ flanking probe isolated from the L^d gene. As shown in Fig. 3, this probe shows a polymorphism between D^d and L^d genes on *Eco*RI/*Bam*HI-digested DNA from BALB/c liver. Genomic DNA from the mutant mouse strain B10.D2-$H\text{-}2^{dm1}$, which has a deletion including the relevant 3′ flanking region of the D^d gene, was used to identify the D^d-specific band (24). Similarly, genomic DNA from the mutant mouse strain, BALB/c-$H\text{-}2^{dm2}$, which has a deletion including the relevant 3′ flanking region of L^d, was used to identify the L^d-specific band (21). When tested on DNA from EL4 cells, this 3′ flanking probe hybridizes exclusively with the D^b gene, which gives a restriction fragment indistinguishable from that of the L^d gene. When DNA from the (TIB35 × EL4) hybrids was tested, two bands were detected, one coincident with L^d and/or D^b and the other coincident with D^d. Thus, these hybrids clearly contain

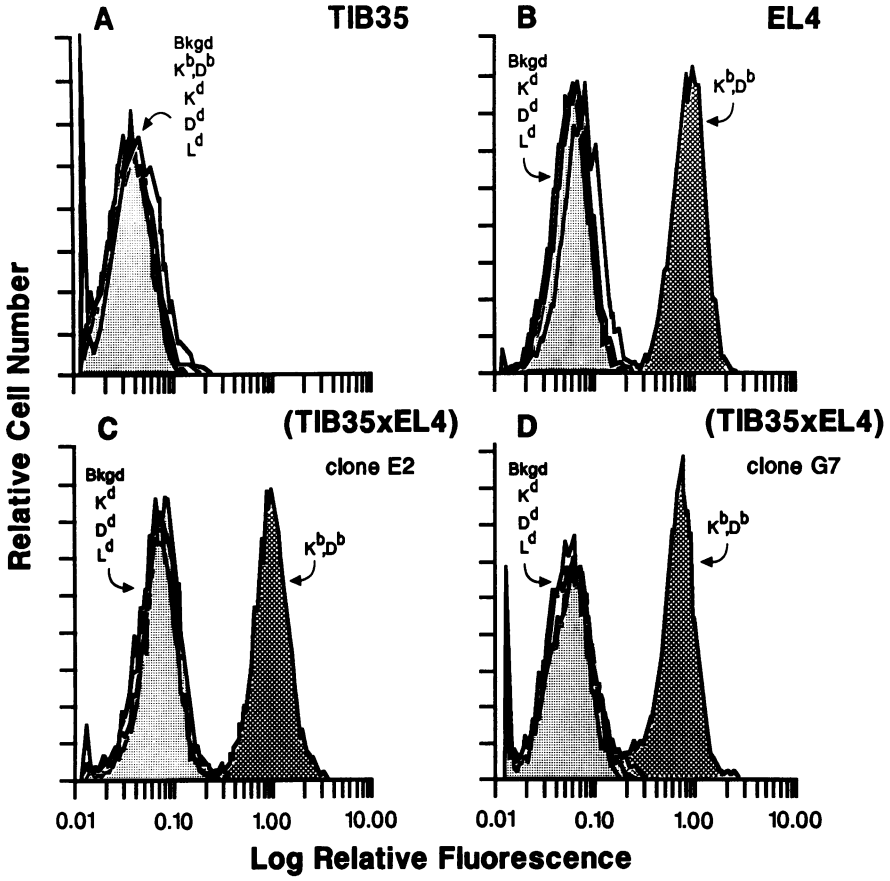

FIG. 2. Flow cytometric histograms of the class I molecules expressed by TIB35 (*A*) and EL4 (*B*) cells compared with two representative (TIB35 × EL4) hybrid clones—E2 (*C*) and G7 (*D*). The experimental details are the same as indicated in the legend of Fig. 1 except antibodies 28-14-8 and Y3 were used to detect D^b and K^b, respectively. For the data shown here, a mixture of both 28-14-8 and Y3 was used; however, they have been tested individually in other experiments to confirm the expression of both D^b and K^b molecules on EL4 cells as well as (TIB35 × EL4) hybrid clones.

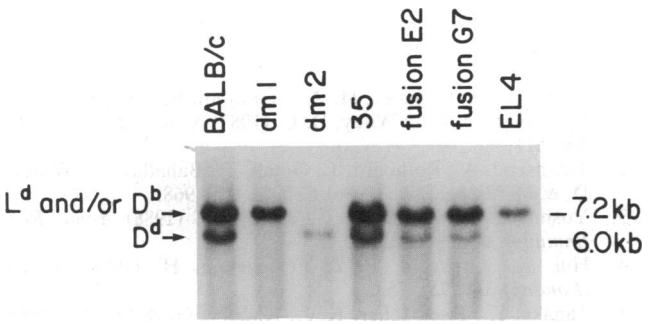

FIG. 3. Southern blot analysis of *Eco*RI/*Bam*HI-digested genomic DNA probed with an *Sst* I fragment located 2.1 kb 3′ of the L^d gene. This probe distinguishes a polymorphism between the L^d and D^d genes as indicated by its hybridization on dm1 (D^d negative) versus dm2 (L^d negative) DNA (21). The D^b gene, being highly homologous to L^d, gives the same 7.2-kb fragment.

DNA encoding the D^d gene derived from the TIB35 parental cell. Therefore, in all three fusion combinations, markers were detected indicating the presence of the S49-derived MHC. Furthermore, these findings coupled with our extensive Southern blot analyses of the class I genes of the TIB28, TIB35, and TIB36 cell lines (12) indicate that selective loss of class I expression in these cell lines cannot be attributed to large genetic deletions.

Steady-state message was not detected for any of the class I molecules not expressed on the surface of the S49 sublines, suggesting a transcriptional defect(s). To demonstrate this was also the case for the fusions; mRNA from (TIB28 × BW5147), (TIB36 × BW5147), and (TIB35 × EL4) hybrid cells was compared in an RNase protection assay. For this analysis, a genomic probe from the $\alpha 3$ exon of the L^d gene was used that could differentiate most, but not all, of the relevant messages. Cells were treated for 3 days with RCAS to maximize the level of class I-specific mRNA. No D^d- or K^d-specific message was detected in the (TIB35 × EL4) hybrids, whereas no D^d-specific message was detected in the (TIB36 × BW5147) hybrids (data not shown). Thus, these findings with the hybrid cells are entirely consistent with our more extensive investigations of mRNA from the S49 sublines (12), suggesting a defect(s) at the level of transcription.

DISCUSSION

A series of different sublines were previously isolated from the S49 BALB/c thymoma by various *in vitro* selection regimens (9, 10). Each subline was selected from nonmutagenized cells to represent different stages of thymic development using either a drug or a specific antibody as indicated in Table 1. Unexpectedly, several of these S49 sublines were found to have shut off expression of certain of their class I molecules (11, 12). Furthermore, this aberrant class I expression was found to be stable during *in vivo* passage or *in vitro* culture (11) and was shown to be nonreversible by treatment with γ interferon (12). Four different phenotypes represented among the S49 sublines were characterized at the molecular level: wild-type [$(K^d\ D^d\ L^d)^+$], null [$(K^d\ D^d\ L^d)^-$], and two intermediates [$(K^d)^+\ (D^d\ L^d)^-$ and $(K^d\ D^d)^+\ (L^d)^-$]. Comparisons were made of the steady-state levels of mRNA from these S49 variants by using either specific oligonucleotide probes in a Northern blot analysis or a class I-specific probe in an RNase protection assay. No L^d-, D^d-, and/or K^d-specific transcripts were detected in each of the sublines for the respective class I molecules not expressed on their cell surface (12). These findings suggested that the defective class I expression by S49 sublines resides at the level of transcription, although gross disparities in message lability could not be excluded. Extensive Southern blot comparisons of DNA from each of these sublines indicated there were no major deletions, insertions, or chromosome rearrangements in or around their K^d, D^d, and L^d class I genes (12). These findings suggested to us that these S49 variants may contain defective regulatory elements, resulting in a locus-specific shutoff of class I expression. For example, the variant class I genes could contain mutations in 5′ regulatory sequences affecting the binding of relevant nuclear-binding proteins. Such a defect would be expected to be cis-acting. Alternatively, the mutations in these S49 variants could be in genes encoding regulatory proteins controlling class I expression and thus would be expected to be trans-acting. To distinguish between cis- and trans-acting forms of regulation in these S49 tumor variants, extensive fusion analyses were performed.

Three phenotypically distinct S49 lines (TIB28, -35, and -36) were each fused to a class I-expressing thymoma of a haplotype serologically distinct from $H\text{-}2^d$. Several hybrids were obtained from each fusion and the surface class I phenotypes were compared by flow cytometry. Our findings were remarkably consistent such that in every case the hybrids were found to express an exact composite of the class I molecules expressed by each of the fusion partners. Thus, the nonexpressed class I molecules of S49 variants were not rescued by the fusion nor were expressed class I molecules in the fusion partner shut off. These findings suggested that cis- and not trans-acting elements were operative in shutting off class I genes in these S49 variants.

In several previous studies in which fusions were made by using tumors with aberrant class I expression, trans-acting control was implicated. For example, Baldacci *et al.* (25) fused the class I-negative thymoma BM5R with BW5147 cells and found that many of the resulting hybrids exhibited a class I-negative phenotype. Furthermore, they showed that this trans-acting shutoff of class I was determined by a non-MHC-linked gene(s). Paradoxically, hybrid cells that lost this putative non-MHC regulatory gene(s) regained only expression of their BW5147-derived and not their BM5R-derived class I molecules. Thus, cis mechanisms were also implicated. It is also important to note that these investigators found considerable variability between different hybrids and that expression in the presence of γ interferon was not tested (25). In a separate study, Salter *et al.* (26) reported the results of fusing two human class I-deficient cell lines. Results obtained with these hybrid cells suggested the existence of two complementary trans-acting factors. Although these aforementioned factors are non-locus specific, other reports have postulated the existence of locus-specific trans-acting factors. For example, Gmur *et al.* (27) fused teratocarcinoma cells with normal mouse spleen cells or BW5147 cells and assayed the hybrids for class I expression before and after differentiation. In hybrid clones that were morphologically undifferentiated (i.e., resemble embryonal carcinoma cells), class I expression was negative. However, as these hybrids "aged," *K* and *D* locus products were separately induced, with D molecules being expressed first. These authors interpreted their findings as evidence for trans-acting factors that independently regulated expression of *K* and *D* locus products. In the S49 studies reported here, we cannot formally rule out regulatory factors that are both haplotype and allele specific. However, this possibility appears extremely unlikely, given the high level of homology between the nonexpressed S49 loci and the expressed alleles in the fusion partner. For example, in the (TIB35 × EL4) hybrids, the L^d gene of the S49 subline remained shut off despite the expression of the highly homologous D^b gene. Indeed, DNA sequence comparisons, including 5′ upstream regulatory regions, have shown the L^d and D^b genes to be strikingly similar (28).

Other cell fusion studies of class I repression by tumors have postulated cis-acting mechanisms regulating expres-

sion. For example, Andrews and Goodfellow (29) fused the class I-deficient murine embryonal carcinoma cell pcC4 ($H\text{-}2^b$) with C3H ($H\text{-}2^k$) thymocytes. Analyses of two resulting hybrids found that K^k molecules of thymocyte origin continued to be expressed, while D^b molecules of pcC4 origin were not reexpressed. Although this result was interpreted as a form of cis control (29), it remains possible that these hybrids merely lost the MHC genes of the teratocarcinoma parent cell type. In another apparent example of cis control, Benham *et al.* (30) produced a hybrid cell line by introducing an *HLA* chromosome into the mouse embryonal carcinoma pcC4. They found that even though the H-2 antigens of mouse origin remained negative, the HLA class I antigens were fully expressed. Although this result also suggested cis control, their conclusion could be obfuscated by the failure of murine regulatory proteins to act on human genes. In contrast to these previous analyses of hybrids involving class I-deficient tumor cells, the findings with S49 cells provide a thorough and unambiguous indication of cis control. Furthermore, recent technological advances permit molecular characterization of the locus-specific defects in these S49 variant cell lines. Thus, these S49 tumor variants represent an ideal model system to precisely define the genetic basis for down-regulation of class I molecules by tumor cells. Another pertinent study indicating a form of cis-dominant regulation in tumor cells was recently published by Green and colleagues. They reported that the low level of K^k antigen expression by AKR tumor SL3 was not inducible by γ interferon, whereas the normal expression of D^k antigen by these tumors was fully inducible (31). Using cell fusion experiments, these investigators further demonstrated that this differential class I regulation in SL3 cells resulted from a form of cis-dominant regulation (32). It is possible that a common mechanism is responsible for the defective class I expression in both SL3 and S49 tumor cells. However, in the case of S49 cells, the defect is clearly not γ interferon dependent and the shutoff expression is qualitative and not quantitative as seen in SL3 cells. Molecular comparisons of the aberrant class I expression in SL3 and S49 tumor cells should prove to be very interesting.

It is intriguing to speculate on the molecular basis of the defective class I expression by the S49 subline variants. Their cis-acting defects are probably not the result of methylation since the demethylating agent 5-azacytidine had no effect on class I expression by these S49 sublines (unpublished observation). Alternatively, their defective class I expression could be related to chromatin structure. However, the proximity and independent regulation of the L^d and D^d genes might suggest that if chromatin structure were involved it is secondary to changes in primary sequence. Therefore, we favor the hypothesis that the aberrant class I genes in the S49 sublines contain relatively small mutations undetectable by the Southern blot analyses. Since each of these putative mutations in the various S49 sublines is cis-dominant, we further speculate that there may be a common mechanism propagating homologous mutations among the class I genes in these tumor cells.

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