Point Mutation in Essential Genes with Loss or Mutation of the Second Allele: Relevance to the Retention of Tumor-specific Antigens

Gabriele B. Beck-Engeser,[1] Paul A. Monach,[1] Dominik Mumberg,[1] Farley Yang,[2] Sherry Wanderling,[1] Karin Schreiber,[1] Rafael Espinosa III,[3] Michelle M. Le Beau,[3] Stephen C. Meredith,[1] and Hans Schreiber[1]

[1]*Department of Pathology,* [2]*Department of Radiation and Cellular Oncology, and* [3]*Department of Medicine, The University of Chicago, Chicago, IL 60637*

Abstract

Antigens that are tumor specific yet retained by tumor cells despite tumor progression offer stable and specific targets for immunologic and possibly other therapeutic interventions. Therefore, we have studied two $CD4^+$ T cell–recognized tumor-specific antigens that were retained during evolution of two ultraviolet-light–induced murine cancers to more aggressive growth. The antigens are ribosomal proteins altered by somatic tumor-specific point mutations, and the progressor (PRO) variants lack the corresponding normal alleles. In the first tumor, 6132A-PRO, the antigen is encoded by a point-mutated L9 ribosomal protein gene. The tumor lacks the normal L9 allele because of an interstitial deletion from chromosome 5. In the second tumor, 6139B-PRO, both alleles of the L26 gene have point mutations, and each encodes a different tumor-specific $CD4^+$ T cell–recognized antigen. Thus, for both L9 and L26 genes, we observe "two hit" kinetics commonly observed in genes suppressing tumor growth. Indeed, reintroduction of the lost wild-type L9 allele into the 6132A-PRO variant suppressed the growth of the tumor cells in vivo. Since both L9 and L26 encode proteins essential for ribosomal biogenesis, complete loss of the tumor-specific target antigens in the absence of a normal allele would abrogate tumor growth.

Key words: ribosomal proteins • loss of heterozygosity • point mutation • CD4-positive T lymphocytes • tumor escape

Introduction

Tumor antigens are encoded by genes that are either normal but aberrantly expressed or overexpressed (1, 2), or altered as the result of cancer-specific somatic mutations (3–5). As an example of the latter, in a murine tumor designated 6132A, a somatic point mutation in the ribosomal protein L9 gives rise to an immunodominant $CD4^+$ T cell–recognized tumor-specific antigen (3). In some instances, a somatic mutation encoding a tumor-specific antigen is believed to contribute to the development of cancer (4, 6–8). In other cases, such a mutation has not been linked to the malignant phenotype but appears to affect a protein essential for basic cellular functions. For example, the ribosomal protein L9 is essential for protein synthesis and homozygous deletion of L9 is lethal in *Drosophila* (9). Another example is the human homologue of the yeast bet 5 gene which encodes a protein that is part of the transport protein particle involved in ER-to-Golgi transport. A mutation in this household gene causes a $CD8^+$ T cell–recognized antigen in a human melanoma (10).

Tumor antigens, whether encoded by normal or mutant genes, may be lost by more malignant variants that arise during tumor progression in mice (11–16). Indeed, this might be expected since loss of antigens may give a selective survival advantage to a loss variant. It is somewhat surprising then that certain tumor-specific antigens are retained during tumor progression. For example, it was found many years ago that certain tumor-specific antigens recognized by $CD4^+$ T cells can be retained (17), even though $CD4^+$ T cells, upon adoptive transfer, can be very effective in eliminating tumor cells (3, 18, 19); we (20) and others (21) have shown that this killing occurs by an indi-

G.B. Beck-Engeser, P.A. Monach, D. Mumberg, and F. Yang contributed equally to this work.

Address correspondence to Hans Schreiber, Dept. of Pathology, MC 3008, The University of Chicago, 5841 South Maryland Ave., Chicago, IL 60637. Phone: 773-702-9204; Fax: 773-702-9224; E-mail: hszz@midway.uchicago.edu

rect mechanism requiring IFN-γ. Since these $CD4^+$ T cells can eliminate MHC class II-negative tumor cells even in the absence of $CD8^+$ T cells, such an attack may be effective even when MHC antigens are lost by the tumor cells during tumor progression. For example, we have shown previously that $CD4^+$ T cells specific for the mutated form of L9 can eliminate in vivo MHC class II-negative tumor cells expressing this antigen, without selection for antigen loss variants (20). Tumor-specific antigens recognized by $CD8^+$ T cells can also be retained by variants during progression even though these antigens are direct targets for T cell immunity (12), and in one recent study, extensive efforts to select for antigen loss variants of a methylcholanthrene-induced sarcoma in vitro failed using $CD8^+$ T cells specific for a tumor antigen (22).

Since tumor progression and the loss of target antigens represents a major problem for the development of effective immunotherapies, it is important to understand the reasons for retention of certain tumor-specific antigens. UV-induced murine tumors offer a powerful model for studying antigen loss or retention during tumor progression. The primary UV-induced murine cancers are often rejected by normal mice but grow in T cell–deficient mice (23); these regressor (RE)* tumors give rise to heritable, more aggressive (progressor [PRO]) variants, which grow progressively to kill normal, immunocompetent mice (12, 24). Surprisingly, as many as two-thirds of these UV-induced PRO variants retain tumor-specific antigens that are detected by tumor-specific T cell clones on the parental RE tumors (12). These PRO variants usually grow faster in T cell–deficient mice than the parental RE tumors, and we have shown that mechanisms other than antigen loss or loss of MHC expression can account for this PRO phenotype (24, 25).

In this study, we have examined two independently induced murine cancers, 6132A and 6139B, in which $CD4^+$ tumor-specific antigens are retained during tumor progression. We find that the antigens in both PRO variants are caused by point mutations in genes encoding essential "household" proteins, but also that neither tumor retains a normal allele. In 6139B-PRO, we find that both alleles of the L26 gene are mutant; each is affected by a different mutation and specifies a different $CD4^+$ T cell–recognized antigen. In 6132A-PRO, in which the antigen is encoded by a mutant ribosomal L9 gene, the second allele is lost. Reintroduction of the wild-type (wt)L9 gene by transfection reversed 6132A-PRO to a RE phenotype consistent with the hypothesis that loss of the wt allele favored the PRO phenotype. Since L9 and L26 are probably essential for protein synthesis, loss of the normal alleles would explain the need for continued expression of these mutant genes encoding tumor-specific antigens in the PRO variants.

Abbreviations used in this paper: AcCN, acetonitrile; EGFP, enhanced green fluorescent protein; FISH, fluorescence in situ hybridization; HLF, heart-lung fibroblast; m, mutant; PRO, progressor; RE, regressor; RP-HPLC, reverse-phase high pressure liquid chromatography; RT, reverse transcription; SCID, severe combined immunodeficient; wt, wild-type.

Materials and Methods

Tumor Cell Lines. The derivation of tumors 6132A-RE, 6132B, 6139B-RE, 6139B-PRO-V (in vivo), and 6139B-PRO-T (in vitro) has been described previously (12, 26). 6132A-RE, 6139B-RE, and 4102-RE are rejected by normal mice, whereas the variants 6132A-PRO, 6139B-PRO (selected either in vitro [T] or in vivo [V]), and 4102-PRO grow progressively in the majority of normal mice. 6139B-PRO-T was selected in vitro by exposure to tumor-specific CTL; the other PRO variants had been selected in vivo by normal mice injected with large doses of the RE tumors (12). This selection leads to the outgrowth of heritable variants that regularly grow in the majority of normal mice. 6132A and 6132B are two UV-induced tumors that developed at different locations in the same mouse. Retention of the unique $CD4^+$ T cell–recognized 6139B antigen was tested on two different 6139-PRO variants that have lost different unique antigens recognized by $CD8^+$ T cells. 6132-heart-lung fibroblast (HLF) and 6139-HLF cells are HLFs derived from the mice in which the 6132A-RE and 6139B-RE tumors, respectively, developed originally. Tumor cells and fibroblasts were cultured in DMEM medium containing 10% fetal bovine serum. Tumor cell lysates were generated as follows: 6139B-PRO or 4102-PRO cells were washed with MEM, resuspended at a density of $6–13 \times 10^7$ cells per milliliter in MEM, and lysed by three cycles of freezing and thawing; lysates were stored at 4°C.

Generation and Analyses of $CD4^+$ T Cell Hybridomas. The hybridoma 479H-60, which is specific for the mutant (m)L9 protein of the 6132A tumor, has been described previously (3). The hybridomas 426H-64 and 425H-26 were generated analogously by fusing $CD4^+$ T cell lines, specific for 6139B or 4102 tumor cells, with BW5147. The general methods for the generation and maintenance of tumor-specific T cell lines, clones, and hybridomas have been described in detail (3). The anti-6139B T cell hybridoma was generated from immune $CD4^+$ spleen cells that had been restimulated in vitro with 6139B-PRO cell lysates. The donor of these spleen cells had rejected 6139B-RE fragments, was then treated with anti-CD8 antibody, and challenged with a lethal dose of 6139B-PRO cells. The $CD4^+$ T cell immunity remaining after the anti-CD8 treatment was apparently sufficient to reject the 6139B-PRO tumor cells. The anti–4102-PRO T cell hybridoma was generated from $CD4^+$ spleen cells of a syngeneic C3H/HeN mouse that had rejected a 4102-RE tumor challenge.

Antigen-specific stimulation of IL-2 release from the various hybridomas was determined by measuring the proliferation of the IL-2–dependent cell line, CTLL-2, as described previously (3). Tumor cell lysates, fractions thereof (in solution or blotted on nitrocellulose), or peptides were added at indicated amounts to hybridomas cultured in the presence of normal syngeneic spleen cells (as a source of APCs) for 24 h; 0.1 ml of culture supernatant was then removed and added to CTLL-2 cells which were cultured for an additional 24 h. Proliferation of CTLL-2 cells was measured using the 1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (3). For some experiments, culture supernatants containing monoclonal antibody 10-2-16 anti–I-A^k (27) or 14-4-4S anti–I-E^k (28) were added at the beginning of culture.

Vectors and Transfections. For the expression of the various L9-enhanced green fluorescent protein (EGFP) fusion proteins or EGFP alone, wtL9 and mL9 cDNAs were fused in frame with the EGFP gene (CLONTECH Laboratories, Inc.), and these fusion genes or the EGFP gene alone were cloned into the retroviral MFG vector (29). The plasmids were transfected into the 6132A-PRO cells together with a pSV2neo plasmid using Superfect transfection reagent (QIAGEN), and G418-resistant clones

were analyzed for EGFP fluorescence by flow cytometry. Supernatants of the transfected cell lines contained virus that infected a small fraction of NIH 3T3 cells, which are highly susceptible to infection by ecotropic retroviruses. Therefore, we cannot exclude that the endogenous retrovirus from the transfected cells mobilized the mL9-EGFP, wtL9-EGFP, or EGFP-alone vectors and contributed to growth inhibition in vivo. However, this is very unlikely because the effect we observe would still have to be specific for wtL9-EGFP since mEGFP and EGFP-alone vectors were used as controls. Furthermore, all in vivo experiments were done in severe combined immunodeficient (SCID) or nude mice, so that immunogenicity due to viral protein or EGFP expression could not influence the results.

Protein Purification. Nuclei were prepared from disrupted cultured tumor cells that had been treated twice with 1% Triton X-100 in RSBI (10 mM Tris-HCl, containing 10 mM NaCl, 3 mM $MgCl_2$, 300 μg/ml leupeptin, 300 μg/ml aprotinin, and 40 μg/ml phenylmethylsulfonyl fluoride for 1 h at 4°C. The nuclei recovered by centrifugation at 2,000× *g* were incubated twice with RSBl plus 0.4 M NaCl for 1 h at 4°C, then once with RSBl plus 2 M NaCl overnight at 4°C. This last extraction was centrifuged at 200,000× *g* for 2–3 h and the supernatant retained. Ribosomes were prepared as described previously (30). In brief, the supernatant of cells disrupted with Nonidet P (NP)-40 was treated with deoxycholate, then layered on a discontinuous sucrose gradient, and centrifuged at 100,000× *g* for 24 h; the pellet, enriched in ribosomes, was saved and resuspended in 20 mM Tris-HCl, pH 7.6, 5 mM magnesium acetate, 100 mM NH_4Cl, 1 mM dithiothreitol, and 0.2 M sucrose.

To prepare a nuclear extract or ribosomal fraction for either reverse-phase high pressure liquid chromatography (RP-HPLC) or SDS-PAGE, the sample was mixed with an equal volume of UT2 (6 M urea, 10 mM Tris-HCl, pH 8.5, 2% 2-ME) and an equal volume of 3× SDS (12% [wt/vol] SDS, 150 mM Tris-HCl, pH 6.8, 15% [vol/vol] glycerol, 0.03% [wt/vol] bromophenol blue), with additional 2-ME added to a final concentration of 5% (vol/vol); this solution was then incubated at 37°C for 1 h. This unorthodox combination of denaturants was used because it was the only way of many we tried which solubilized the antigenic activity consistently. For example, high salt nuclear extracts frequently precipitated in the 0.1% trifluoroacetic acid used for chromatography.

RP-HPLC was performed on an IBM LC/9533 ternary gradient liquid chromatograph with a 4.6 mm × 25 cm C4 column (Vydac). All HPLC analyses were performed using water and acetonitrile (AcCN), both acidified with 0.1% (vol/vol) trifluoroacetic acid, at a flow rate of 1 milliliter per min; fractions of 1-min duration were collected. Routinely, a sample was loaded at 20% AcCN in several aliquots of 1 ml each; after the final loading, the concentration of AcCN was increased to 35% using a linear gradient over the first 10 min, then to 45% over the next 40 min; the sample that was used to obtain amino acid sequence was loaded at 35% AcCN and eluted, as above, with a 40-min linear gradient to 45% AcCN. Fractions were dried in a Speed Vac concentrator (Savant), resuspended in 40–80 μl of UT2, and placed at 4°C overnight before using. Fractions were tested for antigen by blotting on to nitrocellulose, then incubating with the T cell hybridoma as described previously (3).

Antigen from 6139B-PRO cells, whether from nuclei or HPLC fractions, was separated by SDS-PAGE using a Tris-tricine buffer system (31). The resolving gel was 16.5% T, 3% C, and a spacer gel with 10% T, 3% C was usually included between the resolving and stacking gels (where T = total acrylamide and C = N, N′methylene bisacrylamide). In addition, 10 mM dithiothreitol was included in the resolving and spacer gels, and 0.2% 2-ME was added to the cathode and anode buffers. An HPLC fraction in UT2 was prepared for SDS-PAGE by adding one-half volume 3× SDS and 2-ME to 5% and incubating at 37°C for 1 h. Electrophoresed proteins were transferred to nitrocellulose at 50–60 mA for 18–24 h in 25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol, and the nitrocellulose was used in the hybridoma stimulation assays as described previously (3). For obtaining amino acid sequence, proteins were transferred to PVDF (Pro-Blott; Applied Biosystems) under the same conditions. Nitrocellulose and PVDF membranes were stained with Ponceau S.

Protein Sequencing. All steps were performed at the Rockefeller University Microchemistry Facility. Protein bound to PVDF was cleaved with trypsin and the fragments eluted and separated by RP-HPLC. A prominent peak was subjected to Edman degradation, yielding the sequence.

PCR Analyses at the Transcriptional and Genomic Level. Total RNA was isolated using the Rneasy Kit (QIAGEN) according to the manufacturer's manual. For cDNA synthesis, 4 μl of RNA were reverse transcribed in a total volume of 40 μl, in the presence or absence (control samples) of 300 U Superscript reverse transcriptase. Reactions were carried out at 37°C for 90 min, followed by incubation for 10 min at 95°C to inactivate the enzyme. Genomic DNA was prepared with the blood and cell culture DNA kit (QIAGEN). All PCR reactions were carried out under linear, nonlimiting conditions, and β-actin expression was used to normalize the amounts of cDNA template. The pseudogene products served as an internal standard for the amount of genomic DNA template. Parenthetically, the fact that the pseudogene fragments were only amplified by the wt, but not by the mutant-specific PCR primers showed that the point mutation giving rise to the unique 6132A tumor antigen was not found in any of the L9 pseudogenes. PCR reactions were performed in a total volume of 100 μl containing 200 μM dNTPs, 50 pM oligonucleotide primers, and 0.3 μl Taq polymerase (Promega). The number of PCR cycles and restrictive versus nonrestrictive conditions were chosen to allow linear and specific product amplification and were determined individually for each primer combination. The following primers were used. L9 wt: 5′-ATCAACGTGGAGCTGAGTCTT-3′; L9 mut: 5′-ATCAACGTGGAGCTGAGTCAT-3′; L9 3′ cDNA: 5′-GGGAAGTGAGCGTACACAGACC-3′; L9 3′ gen.: 5′-CAGTTCCTTTCTGTACCCCACC-3′; 5′-β-actin: 5′-GGATGACGATATCGCTGCGCTG-3′; and 3′-β-actin: 5′-GTACTTCAGGGTCAGGATACCTC-3′.

For sequence analysis, PCR products were purified using the PCR purification kit (QIAGEN) and either sequenced directly or cloned into the vector pSK II Bluescript (Stratagene). Automated sequencing was performed at the University of Chicago DNA sequencing facility.

Cytogenetic Probes and Analyses. A mouse chromosome 5-specific digoxigenin-labeled painting probe was obtained from Oncor, Inc. Fluorescence in situ hybridization (FISH) was performed as described previously (32). The chromosome 5 painting probe was detected with rhodamine-conjugated, antidigoxigenin antibodies (Boehringer Mannheim). Chromosomes were identified by staining with 4,6-diamidino-2phenylindole-dihydrochloride (DAPI). The L9 probe was a 2.3-kb PCR product amplified from the murine L9 gene using the following primers: 5′-GCGAATTCGAATGGTGAGTAGATGGCTCCCC (located in the second intron); and 3′-GCGAATTCGCTCATCCGAAGCAGTCAGATGC (located in 3′ untranslated region of the mu-

rine L9 gene; unpublished data). The underlined sequence represents an EcoRI-restriction site used for cloning of the PCR-fragment. Biotin-labeled L9 probe was prepared by nicktranslation using Bio-16-dUTP (Enzo Diagnostics). For fluorescence in situ chromosomal hybridization, metaphase cells in log phase growth were prepared from 6132-HLF, 6132A-RE, and 6132A-PRO cells using standard cytogenetic techniques (33). Hybridization of the biotin-labeled L9 probe was detected with fluorescein-conjugated avidin (Vector Laboratories).

Immunization and Restimulation with Peptides. Peptides were emulsified with an equal volume of CFA (H37Ra no. 3113-55; Difco). ~0.05 ml of emulsion was injected into each hind foot of C3H mice (total dose in 0.1 ml of emulsion per mouse was 40 nmol L26 H→Y, 67 nmol L26 P→S, or 3.3 nmol L9 H→T). The popliteal lymph nodes were removed 7 or 8 d later and the cells dispersed and cultured as follows: 10^6 lymph node cells and antigen (see below) in a well of a 96-well plate in 0.2 ml of DMEM supplemented with 1% fresh syngeneic mouse serum, glutamine, Hepes or MOPS, 2-ME, and penicillin and streptomycin; nonessential amino acids, nystatin (GIBCO BRL), and gentamycin (GIBCO BRL) were added (3). The specificity of $CD4^+$ T cells and T cell lines for antigen was assessed by thymidine incorporation assay. 3 d after restimulation with antigen, 1 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech) was added to each well, and 24 h later, the cultures were harvested onto glass fiber filters using a PHD cell harvester (Cambridge Technology). Radioactivity was measured by liquid scintillation counting.

Results

Retention of $CD4^+$ T Cell–recognized Antigens by PRO Variants. 6132A-RE, 6139B-RE, and 4102-RE are regularly rejected by normal mice, while the PRO variants derived from these tumors kill the majority of normal mice. PRO variants 6132A-PRO, 6139B-PRO-V, and 4102-PRO were derived from host selection in vivo, while 6139B-PRO-T was derived by selection using $CD8^+$ T cells in vitro (12, 24). Fig. 1 shows that each of three $CD4^+$ T cell hybridomas, generated from mice immunized against the three different tumor lineages, responded to lysates of the parental RE tumor cells and the PRO variants, but did not respond to tumor cells from unrelated lineages. Thus, each hybridoma recognized a unique tumor lineage-specific antigen, which was retained by the PRO variants, whether a variant was derived by host selection or by selection with $CD8^+$ T cells in vitro.

The $CD4^+$ T Cell–recognized Antigen Retained on 6139B-PRO Is Derived from L26, also a Ribosomal Protein. Our previous results have shown that the retained antigen on 6132A tumors is a mutant ribosomal protein L9. To begin to explore the generality of the molecular nature of such retained antigens, we determined the genetic origins of the $CD4^+$ T cell–recognized antigen retained on a second tumor, 6139B-PRO. The 6139B antigen was found in the nuclear and ribosomal fractions of disrupted cells and could be solubilized with 1.2 M NaCl as described in Materials and Methods. Thus, the nuclear extracts were fractionated by RP-HPLC and the fractions were individually tested for antigenicity by coculturing them with the 6139B-PRO–specific T cell hybridoma in the presence of APCs (Fig. 2 A). The antigenic activity was eluted with 24.5% acetonitrile and was fractionated further by SDS-PAGE. The antigenicity appeared to reside in a band of molecular mass of ~22 kd, as evidenced by T cell Western blot analysis (Fig. 2 B). These data were used to purify a larger sample for amino acid sequencing. Edman degrada-

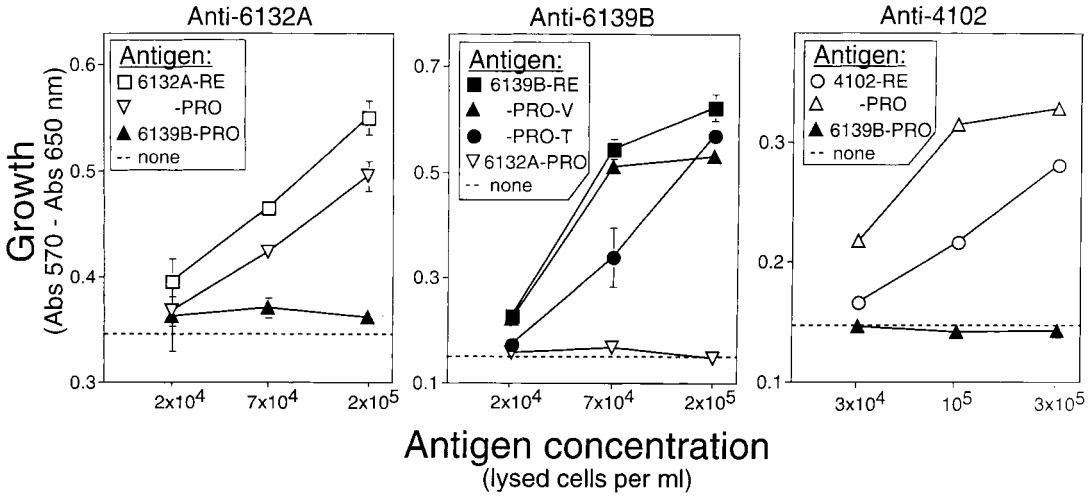

Figure 1. $CD4^+$ T cell–recognized unique tumor-specific antigens are retained on PRO variants that develop from RE tumors during tumor progression. Each lineage of RE tumors and PRO variants expresses a different unique tumor-specific antigen recognized by a $CD4^+$ T cell hybridoma. As probes for antigen expression, we used one previously described $CD4^+$ T cell hybridoma specific for a mutated form of the ribosomal protein L9 expressed by 6132A-PRO (reference 3), left panel, and two new T cell hybridomas derived from mice immunized with 6139B (center panel) or 4102 (right panel). Presence or absence of antigens was assessed by measuring the amount of IL-2 secreted by the hybridomas in response to various concentrations of lysed tumor cells (x axis) as sources of antigen. IL-2 was assayed by growth of CTLL-2 cells and quantified by MTT (y axis) as described previously (3). The anti-6132A and anti-6139B hybridoma-recognized antigens are both restricted by I-E^k, while the anti-4102 hybridoma recognized antigen appears to be I-A^k restricted (reference 3, and data not shown).

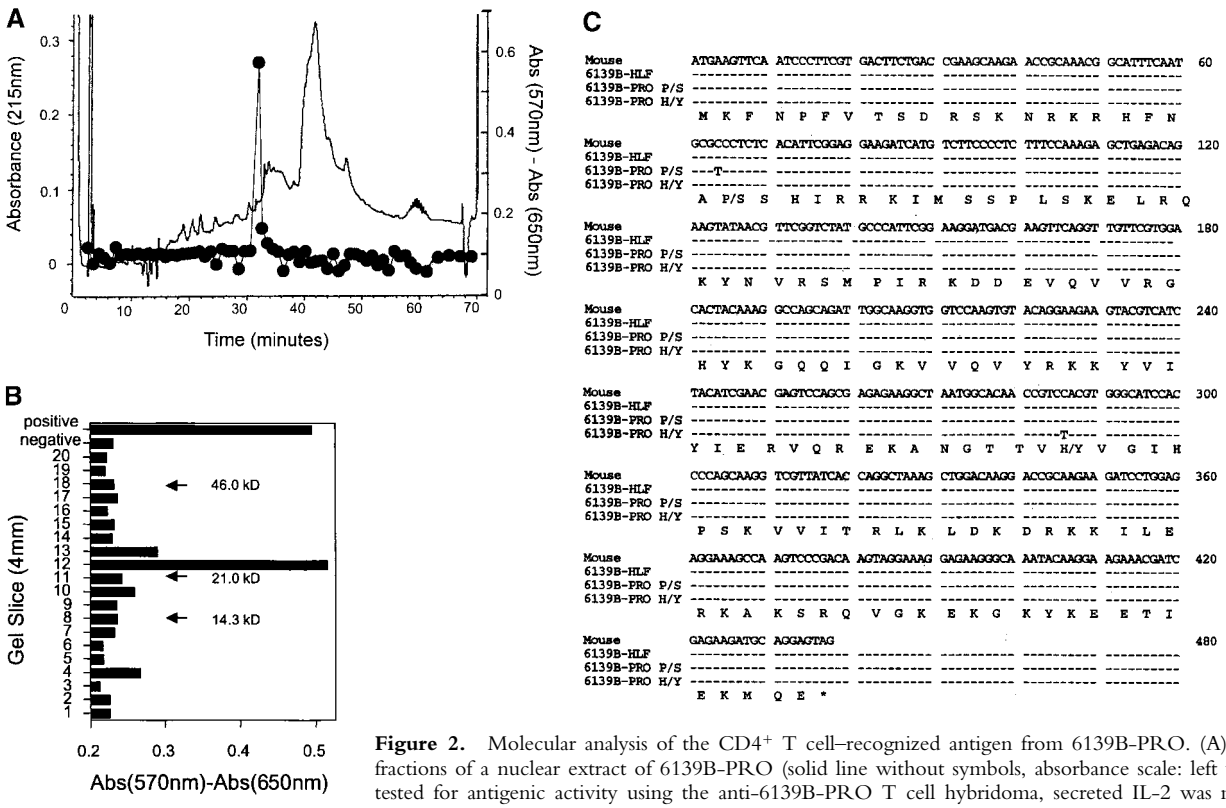

Figure 2. Molecular analysis of the CD4+ T cell–recognized antigen from 6139B-PRO. (A) RP-HPLC fractions of a nuclear extract of 6139B-PRO (solid line without symbols, absorbance scale: left y axis) were tested for antigenic activity using the anti-6139B-PRO T cell hybridoma, secreted IL-2 was measured by growth of CTLL-2 cells, quantified by MTT assay (line with filled circles, scale: right y axis). (B) The antigenic HPLC fractions were separated by SDS-PAGE and the antigenic fractions were determined by T cell Western blot analysis using the T cell hybridoma and MTT assay. Positive and negative controls are at the top of the panel, numbers within the panel show locations of molecular weight markers. (C) Two tumor-specific somatic mutations (6139B-PRO P/S, a proline to serine substitution, and 6139B-PRO H/Y a histidine to tyrosine substitution) were found, each in a different 6139B tumor cell allele; both mutations were lacking in the autologous control sequence derived from normal cells from the mouse of tumor origin (6139B-HLF).

tion of an internal fragment of the protein, isolated by RP-HPLC of an endoproteinase K digest, revealed the sequence YVIYIERVOREK, which is identical to the sequence of amino acids 78–89 of ribosomal protein L26. Additional RP-HPLC peaks of the digest were analyzed by mass spectrometry and had molecular masses of 1517.84, 1443.71, and 1435.71, identical to the predicted molecular masses of endoproteinase K peptides from L26 (amino acids, 17–28, 52–63, and 90–103, respectively). Together, this suggested that the ribosomal protein L26 was the recognized antigen.

The 6139B-PRO Tumor Cells Express Two Different mL26 Alleles but Lack a wtL26 Allele. Sequencing L26 cDNA from the 6139B-PRO-V revealed that there were two different mutant alleles expressed in the PRO variants but no wt sequences. One mutation was caused by a C to T nucleotide substitution at position 286 causing a histidine to tyrosine substitution at amino acid 96; the other mutation was caused by a C to T nucleotide substitution at position 64 causing a proline to serine substitution at amino acid 22 (Fig. 2 C). The wt allele was not expressed, but the absence of this allele could have been caused by either transcriptional silencing or loss of the wt allele. Therefore, we determined the genomic structure of the murine L26 gene (unpublished data) and designed a oligonucleotide primer that amplified an L26 gene fragment containing one intron. This allowed us to distinguish the active (i.e., expressed) L26 alleles from the transcriptionally silent L26 pseudogenes that are abundant in the murine genome and lack introns. The larger intron-containing L26 PCR fragment (3.2 kb) was then cloned and sequenced; again, only two different mL26 alleles and no wtL26 alleles were detected (Table I). This indicated that no wtL26 gene remained in the 6139B-PRO tumor cells. No mutations were detected in clones from two PCR reactions of cDNA of the autologous 6139-HLF fibroblasts derived from nonmalignant heart-lung tissue of the mouse of tumor origin (data not shown). This suggested that the observed mutations were of somatic tumor-specific origin. The phylogenetic conservation of the amino acids affected by the two L26 point mutations is shown in Table II. Furthermore, as could be expected, the two mutations were detected in 6139B-PRO only in the expressed L26 genes but not in L26 pseudogenes (Table I). In the parental 6139B-RE tumor, mostly the H→Y mutation was discovered (Table I). The P→S mutation was also found, but less frequently, and wt sequences were found in the expressed L26 gene as well. Since the 6139B-RE DNA used for sequencing came from an uncloned primary 14-d-old bulk culture of the original 6139B tumor, we cannot distinguish between the possibil-

Table I. *Active L26 Genes in the 6139B-PRO Tumor Are Mutant and Lack a wt Allele, but There Are no Mutations in the L26 Pseudogenes of this Tumor*

Source of sequenced L26 DNA		Number of mutant or wtL26 sequences per number of sequences analyzed		
		Mutant sequences		
Tumor	Type of DNA	H→Y	P→S	wt sequences
				%
6139B-PRO	cDNA	23/73	50/73	0/73 (0)
	active gene	7/10	3/10	0/10 (0)
	pseudogene	0/10	0/10	10/10 (100)
6139B-RE	active gene	8/11	1/11	2/11
	pseudogene	0/11	0/11	11/11 (100)

All active L26 genes in 6139B-PRO are mutant, whereas pseudogenes are not. The presence or absence of an intron allowed this distinction.

ity that some of the 6139B-RE tumor cells still retained the wt allele (as observed in 6132A-RE) and/or that some nonmalignant fibroblasts from the tumor stroma survived the adaptation and gave rise to the wtL26 sequences.

The mL26 Genes Encode Two Different CD4+ T Cell–recognized Antigens. The results of DNA sequencing of cDNA from the 6139B-PRO cells suggested that there might be two mutant ribosomal protein L26 antigens, one containing a proline to serine mutation at position amino acid 22 and the other containing histidine to tyrosine mutation at amino acid position 96. To test this, we synthesized a peptide of 25 residues centered on position 22 and containing either proline (P) or serine (S) at this point and a 25 residue peptide centered on position 96 and containing either histidine (H) or tyrosine (Y) at this position. The anti–6139B-PRO $CD4^+$ T cell hybridoma had been derived from spleen cells of a 6139B-RE immunized, CD8-depleted mouse that had rejected a subsequent lethal 6139B-PRO tumor challenge. This hybridoma specifically responded to the H→Y mL26 peptide with no detectable crossreactivity to the wt peptide or the P→S mutant peptide (Fig. 3 A). To determine whether the two mL26 peptides were similarly immunogenic, normal syngeneic mice were immunized. A series of pilot experiments (data not shown) established that the optimal amounts of antigen in vivo were 33 nmol of the P→S mL26 peptide or 20 nmol

Table II. *Phylogenetic Conservation of the Amino Acids Affected by the Two L26 Point Mutations in 6139B Tumor Cells*

Sequence	Description
HFNA**S**SHIRR	6139B tumor cells
↑	
HFNA**P**SHIRR	6139B normal fibroblasts
HFNA**P**SHIRR	human, mouse, chick
HFNA**P**SHIRR	*C. elegans*
A**P**S	*S. cerevisiae, S. pombe, Z. mays*
A**P**	*Haroarcula marismortui*
NA**P**	*Pyrococcus horikoshii*
FNA**P**	*Methanococcus jannaschii*
REKANGTTV**Y**VGIHPSK	6139B tumor cells
↑	
REKANGTTV**H**VGIHPSK	6139B normal fibroblasts
REKANGTTV**H**VGIHPSK	human, mouse, chick
REKANG+TV**H**+GIHPSK	*C. elegans*

One mutation P→S affects an extremely conserved residue and may destroy the function of the ribosomal protein L26 completely. The other mutation H→Y affects an amino acid that is less conserved, i.e., "only" down to *C. elegans*.

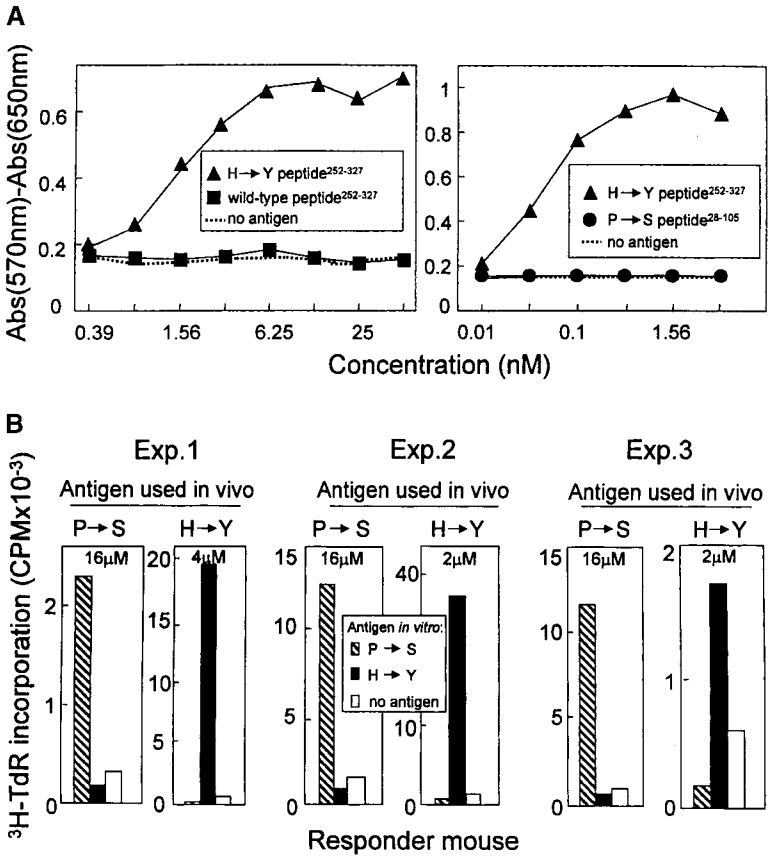

Figure 3. (A) The 6139B-specific T cell hybridoma recognizes only the H→Y mL26 peptide (filled triangles), not the wt peptide (filled squares) or the mutant P→S peptide (filled circles). Antigenicity was measured by IL-2 secretion using the MTT assay, as in previous figures. (B) Cells from mice immunized with mL26 H→Y or P→S peptide in vivo show a specific $CD4^+$ T cell response in vitro when restimulated with the same L26 peptide that had been used in vivo. Mice were injected into both hind footpads with a total amount of either 66 nmoles mL26 P→S peptide or 40 nmoles mL26 H→Y peptide emulsified in complete Freund's adjuvant. 7–8 d later mice were killed and their popliteal lymph nodes removed. Cell suspensions were cultured for 3 d in the presence of either the peptide that had been used for immunization in vivo, or the L26 peptide with the different mutation, or no peptide. Cells were pulsed on day 3 of culture with 1 μCi of [methyl-^{3}H]thymidine and harvested 24 h later, the radioactivity measured in a liquid scintillation counter. Three independent experiments are shown. The L26 P→S peptide required a 4–8-fold higher dose (100 μg/ml) for similarly effective restimulation in vitro than the L26 H→Y peptide (25 or 12.5 μg/ml).

of the H→Y mL26 peptide in CFA. 7–8 d later, we determined the proliferative response of the lymph node cells removed from these mice and restimulated in vitro by a 3-d culture with or without the peptide antigens. Fig. 3 B shows that both peptides were immunogenic in vivo and restimulated a specific proliferative immune response in vitro. However, the P→S mL26 peptide required a several fold higher antigen concentration to induce a similar proliferation response than the H→Y mL26 peptide and was, therefore, apparently less immunogenic.

Loss of Expression of the wt Allele of L9 during 6132A Tumor Progression. One possible explanation for the retention of certain tumor antigens is that they are essential for cell survival or the malignant phenotype. Our results above show that the 6139B-PRO tumor no longer contains the wt allele of L26. Since L26 is likely to be an essential component of the ribosome (see Discussion), loss of the wt allele would necessitate retention of the mutant proteins despite their antigenicity. To explore the generality that the wt allele of the gene encoding the retained antigen is lost, we searched for loss of expression of the wtL9 allele in the 6132A tumors. Initial results using 5′ PCR primers specific for either the mL9 or the wtL9 cDNA together with a common 3′ PCR primer (Fig. 4 A), suggested that only 6132A-RE but not 6132A-PRO expressed the wt allele as mRNA. A second tumor, 6132B, that had developed independently at another skin site in the 6132 mouse, and HLFs from the same mouse expressed only the wt allele (Fig. 4 A). Direct sequencing of cDNA by PCR from 6132A-PRO tumor cells also suggested that the 6132A-PRO tumor cells did not express the wtL9 mRNA, in contrast to 6132A-RE tumor cells and 6132-HLFs (6132-HLF; data not shown). To confirm this observation, we analyzed a quantitative reverse transcription (RT)-PCR of the two L9 mRNA species (Fig. 4 B) again using 5′ PCR primers specific for either mutant or wtL9 cDNA with a common 3′ PCR primer. Again, mL9 was found to be expressed in the 6132A-RE and PRO tumor cells but not in normal 6132-HLF. In contrast, wtL9 message was found in normal 6132-HLF and at lower levels in 6132A-RE cells; the lower levels of wtL9 message in 6132A-RE may be due in part to the simultaneous expression of the mL9 mRNA in these cells, as one would expect if the total level of L9 messenger RNA (wt and mutant) per cell were unchanged. In addition, the 6132A-RE cells are mostly triploid (see below) and may therefore have two mL9 alleles but only one wt allele, thereby further reducing the wtL9 signal. In any case, the observed lack of wtL9 expression in the 6132A-PRO cells could not be accounted for by nonspecific inhibition of the L9 wt-specific PCR primers by the 6132A-PRO cDNA (Fig. 4 C). Thus, the 6132A-PRO tumor cells express only the mutant but not the wt mRNA for L9. By contrast, only wtL9 mRNA was present in PRO variant cells of the unrelated tumor 6139B-PRO (Fig. 4 B), in 6132B (Fig. 4 A) that developed as second independent tumor in the 6132 mouse, and in 6132-HLF (Fig. 4, A and

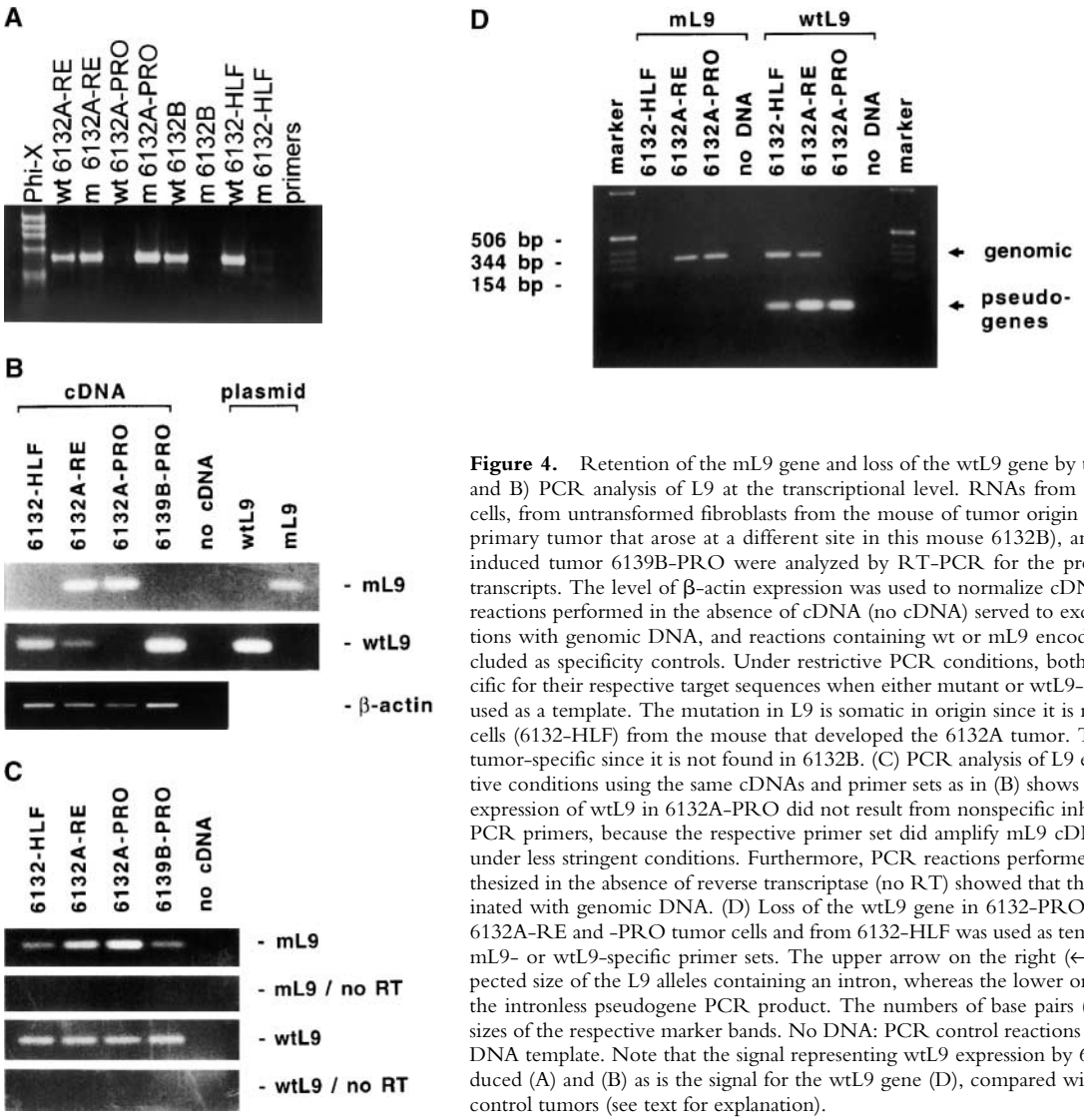

Figure 4. Retention of the mL9 gene and loss of the wtL9 gene by the 6132A-PRO variant. (A and B) PCR analysis of L9 at the transcriptional level. RNAs from 6132A-RE or PRO tumor cells, from untransformed fibroblasts from the mouse of tumor origin (6132-HLF), from a second primary tumor that arose at a different site in this mouse 6132B), and from the unrelated UV-induced tumor 6139B-PRO were analyzed by RT-PCR for the presence of mutant and wtL9 transcripts. The level of β-actin expression was used to normalize cDNA template amounts. PCR reactions performed in the absence of cDNA (no cDNA) served to exclude carry-over contaminations with genomic DNA, and reactions containing wt or mL9 encoding plasmid DNA were included as specificity controls. Under restrictive PCR conditions, both primer sets were fully specific for their respective target sequences when either mutant or wtL9-encoding plasmid DNA was used as a template. The mutation in L9 is somatic in origin since it is not found in normal control cells (6132-HLF) from the mouse that developed the 6132A tumor. The mutation is individually tumor-specific since it is not found in 6132B. (C) PCR analysis of L9 expression under nonrestrictive conditions using the same cDNAs and primer sets as in (B) shows that the apparent absence of expression of wtL9 in 6132A-PRO did not result from nonspecific inhibition of the wtL9-specific PCR primers, because the respective primer set did amplify mL9 cDNA from 6132A-PRO cells under less stringent conditions. Furthermore, PCR reactions performed in parallel on cDNA synthesized in the absence of reverse transcriptase (no RT) showed that the samples were not contaminated with genomic DNA. (D) Loss of the wtL9 gene in 6132-PRO cells. Genomic DNA from 6132A-RE and -PRO tumor cells and from 6132-HLF was used as template for PCR analysis with mL9- or wtL9-specific primer sets. The upper arrow on the right (←genomic) indicates the expected size of the L9 alleles containing an intron, whereas the lower one (←pseudogenes) refers to the intronless pseudogene PCR product. The numbers of base pairs (bp) on the left indicate the sizes of the respective marker bands. No DNA: PCR control reactions performed in the absence of DNA template. Note that the signal representing wtL9 expression by 6132A-RE appears to be reduced (A) and (B) as is the signal for the wtL9 gene (D), compared with the 6132A-HLF or other control tumors (see text for explanation).

B) derived from the mouse that gave rise to the 6132 tumors. This pattern would be expected for a ubiquitously expressed gene and a somatic mutation that caused a unique (i.e., individually distinct) tumor-specific antigen.

The Gene Encoding the wt Allele of L9 Is Lost During Tumor Progression in the 6132A Lineage. The absence of wtL9 transcripts in the 6132A-PRO tumor cells could be due to either transcriptional silencing or loss of the wt gene. To distinguish between the two alternatives, we analyzed an allele-specific PCR using genomic DNA from 6132A-RE and PRO cells and from 6132-HLF. We determined the genomic structure of the murine L9 gene (unpublished data) and designed a 3′-oligonucleotide primer that amplifies an L9 gene fragment containing an intron. This allowed us to distinguish the expressed L9 alleles from transcriptionally silent L9 pseudogenes present in the murine genome. As shown in Fig. 4 D, the mL9 gene was not found in normal 6132-HLF, but was present in the 6132A-RE and PRO tumor cells. The wtL9 allele was detected in the 6132-HLF cells and the 6132A-RE cells. As observed already for the mRNA signal in Fig. 4, A and B, the intensity of the genomic wt signal was also weaker in the 6132A-RE tumor cells compared with 6132-HLF, for the same possible reasons as listed above. In any case, no genomic wtL9-PCR fragment was amplified from 6132A-PRO tumor cells, even though the same PCR primers efficiently amplified the product from L9 pseudogenes (which is smaller because it lacks introns; Fig. 4 D), thereby excluding a nonspecific inhibition of the PCR reaction in these samples. Thus, the 6132A-PRO tumor cells have no intact wt allele of the L9 gene.

Loss of wtL9 Allele by 6132A-PRO Cells Is due to its Deletion from Chromosome 5. The absence of the wtL9 allele in the 6132A-PRO cells could be due to either somatic recombination resulting in loss of the wt gene through replacement by a second mutant copy, or loss of the gene as a result of a chromosomal abnormality, such as chromosome loss or deletion. To distinguish between these two possibilities, we analyzed FISH of metaphase cells from the 6132-HLF, 6132A-RE, and 6132A-PRO lines. We first mapped

the L9 gene to mouse chromosome 5, bands E1-E5 using the human-mouse homology map (NCBI, and reference 34). To determine the number of chromosome 5 homologues and copies of the L9 gene, we sequentially hybridized metaphase spreads with the L9 probe (reactive with both wtL9 and mL9) and the chromosome 5-specific painting probe. A minimum of 20 metaphase cells were examined from each cell line. The 6132A-PRO cell line contained two populations of cells. One population (8/20 cells) was diploid-hyperdiploid, each with 40–48 chromosomes and two chromosome 5 homologues per cell. The second population (12/20 cells) was hypotetraploid-tetraploid, and each cell contained four chromosome 5 homologues. By FISH analysis with the L9-specific probe, only one of the two chromosome 5 homologues was labeled in the near-diploid population, whereas only two of four homologues were labeled in the tetraploid cells (Fig. 5). Thus only 50% (32/64) of the chromosome 5 homologues of the 6132A-PRO tumor had the L9-specific signal. By contrast, nearly all (52/58, 90%) of the chromosome 5 homologues of the control 6132-HLF fibroblasts had the L9-specific signal. Similarly, 85% (91/107) of all chromosome 5 homologues analyzed in 6132A-RE cells had the L9 signal. The absence of a signal in minor fractions of chromosome 5 from control fibroblasts and 6132A-RE is most likely due to low hybridization efficiency resulting from the relatively small probe (a 2.3-kb genomic clone) and not due to loss of the wt allele, as the fraction is similar (90 vs. 85% labeled chromosome 5 homologues) in 6132 control fibroblasts and 6132A-RE malignant cells.

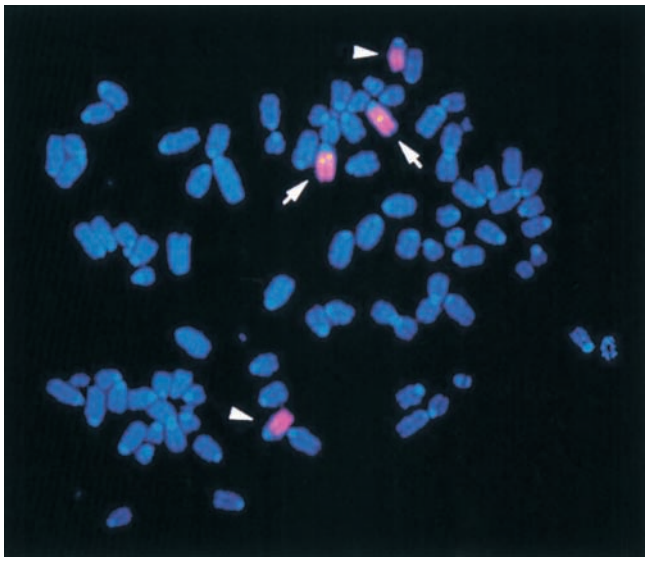

Figure 5. In situ hybridization of a genomic L9 probe and a chromosome 5-specific painting probe to 6132A-PRO cells. Only two of the four chromosome 5 homologues present in near-tetraploid cells from 6132A-PRO cells show a hybridization signal for L9 (arrows); the unlabeled homologues are identified with arrowheads. Hybridization of the biotin-labeled L9 probe was detected with fluorescein-conjugated avidin (yellow-green signal), and the digoxigenin-labeled chromosome 5 painting probe was detected with rhodamine-conjugated, anti-digoxigenin antibodies (red signal). Images were obtained using a Zeiss Axiophot microscope coupled to a cooled-charge, coupled device camera. Separate images of DAPI-stained chromosomes, the L9 hybridization signal, and the painting probe signal were merged using image analysis software (NU200 and Image 1.57).

Cytogenetic analysis of the 6132-HLF cell line revealed a hypertriploid-tetraploid karyotype with 63–75 chromosomes. The majority of cells (14/20) had three chromosome 5 homologs, and the L9 hybridization signal was observed on each of these; the remaining six cells had two of these homologs labeled (five cells) or one of two homologs labeled (one cell). The 6132A-RE cell line was characterized by increased karyotypic variability, with chromosome numbers ranging from hyperdiploid to polyploid (45–131 chromosomes per cell). The number of chromosome 5 homologs ranged from 2–9 per cell, but the majority had three (16/29 cells) or four (8/29 cells) copies. Of note was that each homolog contained hybridization signal for the L9 probe in the majority of cells (22/29). The absence of signal on one of the chromosome 5 homologs in the remaining seven cells was most likely due to poor hybridization efficiency.

These data suggest that loss of the wtL9 allele by 6132A-PRO cells is the result of a small interstitial deletion encompassing the L9 gene. Combined with the data from genomic PCR analysis (Fig. 4 D), these findings indicate that 6132A-PRO has lost the wtL9 allele without loss of the entire chromosome 5 homologue.

The wt but Not the mL9 Gene Suppresses the Growth of 6132A-PRO In Vivo. Since loss of heterozygosity during tumor progression is a hallmark of genes that are functionally involved in the malignant process, loss of the wtL9 gene might have played an essential role in the progression from 6132A-RE to 6132A-PRO. To test the hypothesis that wtL9 acts as a tumor growth suppressor, we transfected expression vectors containing wtL9 into 6132A-PRO cells using mL9 as a control. Using conventional expression vectors for initial attempts, we were never able to achieve the high levels of expression characteristic of ribosomal proteins. Therefore, we expressed both ribosomal proteins as fusions with the EGFP in order to select transfectants with high and comparable protein expression levels as assessed by flow cytometry (Fig. 6 A). Examination of these transfectants by fluorescence microscopy showed that the ribosomal–EGFP fusion protein, but not EGFP alone, localized to the nucleoli, where ribosomes are assembled (Fig. 6 B). In other experiments, cells transfected with cDNA for an wtL9-EGFP were lysed, the ribosomes isolated by sucrose gradient ultracentrifugation, and analyzed by Western blot analysis using an anti-EGFP antibody. These Western blot analysis clearly demonstrated incorporation of the fusion protein into ribosomes (Spiotto, M., unpublished data). Most of the 6132A-PRO transfectants had a similar or somewhat faster growth rate than untransfected 6132A-PRO cells in vitro (data not shown). To assess growth rates in vivo independent of immune responses, we injected transfected cells into SCID mice. We have previously shown that when particular numbers of tumor cells are injected, 6132A-RE cells grow very slowly in T cell–defi-

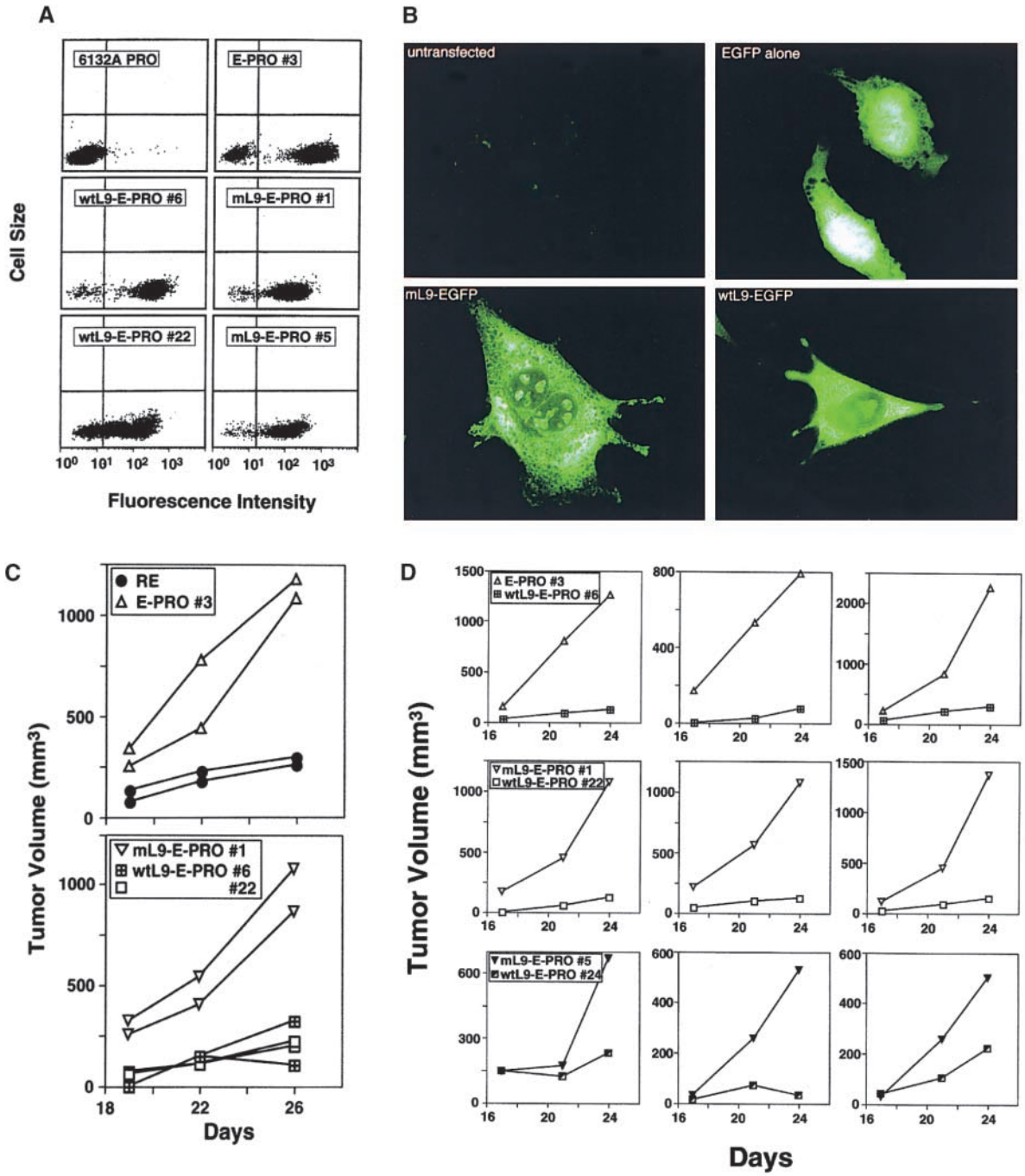

Figure 6. Reduced growth of 6132A-PRO cells that have been transfected to reexpress the wtL9 gene. (A) Expression levels of the transfected wt or mutant fusion genes as analyzed by flow cytometry. Fluorescence of EGFP is shown on the x axis for tumor cells either untransfected (6132A-PRO) or transfected with EGFP alone (E-PRO) or fusions of EGFP with wtL9 (wtL9-E-PRO) or mL9 (mL9-E-PRO). (B) Microscopic analysis of 6132A-PRO cells transfected with the EGFP gene alone or with a fusion gene of EGFP and mutant or wtL9. Fusing EGFP to L9 (bottom left and bottom right photographs) leads to a cellular distribution of the fusion protein that is characteristic for a ribosomal protein (i.e., nucleoli are stained), whereas unfused EGFP (top right photograph) does not show a specific localization. (C) 6132A-PRO cells transfected to reexpress wtL9 (wtL9-E-PRO) (bottom panel) grow as slowly as untransfected 6132A-RE cells (top panel) (RE) in C3H SCID mice. 6132A-PRO transfected with either EGFP alone (E-PRO) or (top panel) mL9-EGFP (mL9-E-PRO) (bottom panel) both have a more rapid growth rate. All cell lines shown were tested concurrently but are shown in the two panels to reduce overlap of lines. SCID mice were inoculated subcutaneously with 3×10^5 tumor cells. Tumor volume was determined using a caliper and the formula for the volume of an ellipsoid ($\pi/6 \times$ abc, where a, b, c are orthogonal diameters). (D) Similar results were consistently obtained using independent transfectants and experiments. Growth of 6132A-PRO cells expressing mL9-EGFP, injected subcutaneously into opposite flanks of the same SCID mouse was compared. Each panel represents results of one mouse. Even though individual SCID mice differ in their relative ability to support the growth of tumor inocula, the wtL9-expressing tumor cells always formed tumors much more slowly than did those expressing mL9-EGFP.

cient mice, whereas 6132A-PRO cells grow quickly in such mice (24). Fig. 6 C shows that 6132A-PRO cells transfected to express wtL9-EGFP grew in SCID mice as slowly as 6132A-RE cells tested concurrently, while 6132A-PRO cells transfected to express EGFP alone or mL9-EGFP grew at a significantly higher rate, and this was observed in several independent experiments using independent transfectants (Fig. 6 D). When threefold to 100-fold lower tumor cell numbers were used for challenge, 6132A-PRO cells transfected to express wtL9-EGFP failed to form tumors. Likewise, no tumors were obtained after inoculating the SCID mice with 6132A-RE cells at this tumor cell number. In marked contrast, SCID mice inoculated with 6132A-PRO cells transfected to express EGFP had already formed large tumors at a comparable time point (Table III). Thus, expression of wtL9 specifically inhibited or prevented the growth of 6132A-PRO cells in vivo. These observations suggest that loss of the wtL9 allele during tumor progression contributed to the transition from 6132A-RE to -PRO. Since this growth inhibition in vivo is apparent in SCID mice, it is independent of functional T or B cell immunity and also not influenced by the immunogenicity of EGFP.

Discussion

In this paper, we have studied two tumor-specific antigens, recognized by $CD4^+$ T cells, that were retained by tumors as they evolved from RE to PRO in vivo (12, 24). In both of the UV light-induced tumors analyzed (6132A and 6139B), the retained antigens are point-mutated ribosomal proteins (L9 and L26, respectively). In both tumors, the wt alleles have been lost during tumor progression. In the case of 6132A, the wtL9 allele was lost in the transition from RE to PRO by an interstitial deletion in chromosome 5, as shown by loss of a signal for the gene by FISH. In the case of 6139B, the two alleles of L26 have different point mutations (position 22P→S and position 96H→Y), with no wt allele remaining; these changes may already be complete in the parental RE tumor. The aggressive growth phenotype of 6132A-PRO could be reversed to that of the parental RE by transfection with wtL9-EGFP, but not by transfection of mL9-EGFP or EGFP alone. We will argue below that these findings (mutant ribosomal proteins and loss of the wt alleles) have implications relevant to elucidating the reasons that some highly immunogenic mutations are retained: first, the mutant proteins are likely to be essential for cell growth and function and loss of the normal alleles makes the associated antigens difficult to lose. Second, the observations of allelic loss are consistent with the possibility that the wtL9 and L26 genes serve as tumor growth suppressors, mutation or loss of which contributes to tumor progression, and this is suggested by our results with reintroduction of wtL9 into 6132A-PRO.

Relatively little is known about the precise roles of the >50 proteins comprising about one-third of the mass of the ribosome, but it is clear that assembly of new ribosomes and thus synthesis of ribosomal proteins is an essential first step after growth factor stimulation to allow proliferation (35). Ban et al. (36) proposed, based on 2.4A resolution crystal structure, that ribosomal proteins function as "mortar," filling cracks and gaps between RNA "bricks," thereby principally serving to stabilize RNA structure. Even though RNA, not protein, appears to be responsible for the catalytic activity of the ribosome (36), complete deproteination destroys this activity (37), so these building materials appear to be essential. In addition, there is evidence that L9 and L26 also have important regulatory functions. Bacterial L6, the prokaryotic homologue of L9, is one of the most highly conserved ribosomal proteins (38) and is located at the binding site of elongation factor (EF)-2, which contains the sarcin-ricin loop. EF-2 and the ricin

Table III. *Reversion to a RE Phenotype of 6132A-PRO Cancer Cells After Reexpression of the wtL9 Protein*

		Size of tumor cell inoculum			
		10^3 cells		3×10^3 cells	
Tumor cells	Transfected gene	Tumor incidence	Tumor volume	Tumor incidence	Tumor volume
			(cm^3)		*(cm^3)*
6132A-PRO	EGFP	3/3	1.4 ± 0.7	3/3	2.0 ± 0.7
	wtL9-EGFP	0/3	0	0/3	0
6132A-RE	EGFP	0/3	0	0/3	0

6132A-PRO tumor cells were transfected with a vector encoding the EGFP or EGFP fused to the wtL9 protein (wtL9-EGFP). 6132A-RE cells transfected with the gene encoding EGFP only were used as control representing cells of the RE phenotype. The three cell lines were injected into three different dorsal subcutaneous locations of C3H SCID mice at one of the two different doses as indicated. Final tumor volumes were calculated using the formula $\pi/6 \times abc$, where a, b, and c are orthogonal diameters using measurements at day of killing, 28 d after injection with 10^5 cells or 52 d after injection with 3×10^3 tumor cells. All tumors that were detected at time of sacrifice were isolated and found to have retained expression of EGFP as analyzed by flow cytometry.

A chain have been cross-linked to eukaryotic L9 (39, 40). Phosphorylation of eukaryotic S6, which follows stimulation of cells with nutrients or growth factors (35, 41, 42) results in the translational upregulation of 5′ track of pyrimidines (TOP) mRNAs (43) which encode most ribosomal proteins and elongation factors. L9 is conformationally changed after phosphorylation of S6 (44) and this change may be essential for the formation of new ribosomes. mL9 may act as a constitutively active form permitting ribosome neogenesis in the absence of growth factors whereas the inactive wt configuration may not allow this, thereby acting as a growth suppressor. Interestingly, a somatic tumor-specific point mutation in EF-2 was found to be the target of a $CD8^+$ T cell response to a human squamous cell lung carcinoma (45), and it is tempting to speculate that this mutation contributed to tumor growth by affecting a similar pathway as mutation of L9 in our study.

The prokaryotic homologue of L26, L24 is one of the two assembly initiator proteins of the large ribosomal subunit (46) and appears to play a key role in the folding and organization of the 23S rRNA early in ribosome assembly (47), an essential first step for any cell proliferation. Prokaryotic L24 has also been shown to encircle the exit of the polypeptide exit tunnel of the large ribosomal subunit (36). The location of eukaryotic L26 in the large subunit has not yet been reported; earlier cross-linking studies had suggested that both eukaryotic L26 and L9 bound to the same protein, L4, but this is not supported by the crystallographic studies of the prokaryotic ribosomal subunit (36). Thus, we have less of a hint about possible growth regulation by L26; such a function need not occur in the ribosome, however, as other ribosomal proteins have been shown to have other functions elsewhere in the cell (48–56).

Mutation of one allele of a regulatory gene, followed by inactivation of the second allele by loss (as we observe with L9) or mutation (as we observe with L26), is a pattern characteristic of the alteration in tumor suppressor genes that occurs during development of cancer, the "two hit" hypothesis proposed by Knudson (57). Our finding that the reintroduction of wtL9 into 6132A-PRO cells causes reversion to the growth characteristics of the RE tumor identifies L9 as a tumor growth suppressor. In contrast to classic tumor suppressor genes, however, wtL9 appears to slow the rate of tumor growth, likely through interaction with host factors (the slower growth being apparent only in vivo, in the absence of T cell immunity), rather than suppressing malignancy per se. The growth suppressive effect of reexpressing the wtL9 by the transfected 6132 cells may be detectable in vivo only because in vivo growth factors are limiting and the tumor cells may be more susceptible to this growth regulation than tumor cells growing in vitro where FCS provides for an abundance of growth factors. In any case, the conclusions that can be reached by our tumor suppressor experiments are only preliminary because of the possibility that fusing EGFP to the wt protein might alter the function of the L9 molecule. More detailed examination of this function will require "knockout" and "knock-in" of L9 alleles, to determine the phenotype of 6132A-RE cells in which either wtL9 or mtL9 is eliminated, as well as exploration of the effects of different ratios of normal and mL9 expression.

Independent of the specific roles of wt or mL9 and L26 in ribosomal function, several lines of evidence suggest that their expression is essential to the function of any cell. Both proteins or their respective prokaryotic homologues L6 and L24 are found in ribosomes throughout evolution. Homozygous disruption of the L9 gene in *Drosophila* is lethal, and heterozygous disruption is associated with a *minute* phenotype (9). *Minutes* are a group of >50 phenotypically similar mutations in *Drosophila*, characterized by generally retarded development, infertility, and recessive lethality (58). Mapping studies suggest that these mutations affect genes for ribosomal proteins; where the disrupted loci have been characterized in detail, all have indeed been found to encode ribosomal proteins. Further evidence suggests a similar importance for ribosomal alleles in mammals, including humans. First, it has been proposed that the Turner Syndrome (monosomy X) in humans, characterized by short stature, developmental abnormalities of the heart, kidney, and skeleton, infantile genitalia, and infertility, may be caused in part by loss of an allele of the X chromosome–encoded isoform of ribosomal protein S4, analogous to the heterozygous *minute* phenotype in *Drosophila* (52, 59). Second, the effects of conditional homozygous deletion of a ribosomal protein in adult liver tissue have been tested in mice; deletion of S6 did not immediately kill liver cells, but it prevented the formation of new ribosomes and abrogated any regenerative proliferation of the hepatocytes after partial hepatectomy (35).

These data argue strongly that a cell, even a tumor cell, could not lose expression of a ribosomal protein and continue to be able to proliferate. In 6132A-PRO and 6139B (-RE or -PRO), the tumors express only mutant alleles of the L9 and L26 proteins, respectively. Since the mutations are immunogenic, these tumors contain antigens that cannot be lost, short of highly improbable reversion point mutations to the wt (which may also reinstate a tumor growth suppressor effect), as a means of escaping from host immunity. If analogous antigens were to be found on human tumors, they would serve as ideal targets for immunotherapy. Although the existence of such antigens in humans is hypothetical, other classes of tumor-specific point mutations originally found in mice, such as mRNA helicases, have subsequently been found also in human cancers (60). Our findings thus support a search for mutant ribosomal proteins in human cancers. In the meantime, the generalizability of some of our findings is suggested by the recent discovery that a $CD4^+$ T cell recognized antigen on the methylcholanthrene-induced tumor MethA is a point-mutated L11 ribosomal protein (61). MethA was induced in 1962 and is, by comparison with our UV-induced tumors, already highly aggressive. Variants representing stages of malignant progression are, therefore, not available. Since the wtL11 allele was retained, MethA cells might be able to lose the mL11 antigen unless it played a role in maintaining

malignant behavior. A broader search for immunogenic ribosomal proteins could be undertaken by immunizing mice with ribosomes from a variety of tumors.

The UV-induced RE tumors such as 6132A-RE or 6139B-RE that are rejected by syngeneic normal recipients grew in the primary hosts because UV is not only carcinogenic but also immunosuppressive in mice (62). This conclusion is supported by the fact that the RE tumors can grow, at least slowly, in UV-irradiated and T cell–deficient hosts (24). Other factors, such as an inherent growth advantage that primary tumors have over transplanted tumors and the advanced age of the host developing primary UV-induced RE tumors, may also be essential and contribute. Of course, the importance of the observation that an antigen is retained during tumor progression depends on its role in immunity. Our previous studies have shown that $CD4^+$ cells are dispensable for rejection of either 6132A or 6139B by normal mice, whereas $CD8^+$ cells are essential (12, 26). However, we have subsequently found that $CD4^+$ cells can be essential to resist a tumor cell challenge, that $CD4^+$ clones and lines are highly effective in eliminating 6132A-PRO cells in vivo in the absence of $CD8^+$ cells, and that immunization with purified L9 antigen affects the subsequent growth of 6132A in normal mice (3, 20, and unpublished data). Antigens retained on the 6132A- and 6139B-PRO tumors can still be rejection antigens. 6132A-PRO and 6139B are still immunogenic tumors, growing progressively in only ~50% of normal mice even when transplanted as tumor fragments, and essentially never when implanted as cultured cells (while growing regularly when implanted as cells or fragments into T cell–deficient mice). The ability of 6132A-PRO to grow progressively at all in immunocompetent mice, and therefore its difference from 6132A-RE, appears to depend on a greater sensitivity of the PRO variant to growth stimulation by host cells (24, 25). In many other models, loss of MHC class I antigens has also been shown to be an effective way for a tumor to escape from immunity (63). Such a finding could be devastating for the development of durable immunotherapy of cancer, because cancer cells can lose MHC expression without perturbation of cell function. However, we (20) and others (18, 19) have shown that tumor cell killing by $CD4^+$ cells in vivo is independent of expression of MHC class II molecules by the tumor cells. Thus, the existence of antigens that cannot be easily lost, due to their essential functions in the physiology of the cancer cell, is particularly relevant when considering antitumor immunity of $CD4^+$ T cells.

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