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Changes in expression of putative antigens encoded by pigment genes in mouse melanomas at different stages of malignant progression

(albino locus/brown locus/slaty locus/silver locus)

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ABSTRACT **Cutaneous melanomas of Tyr-SV40E transgenic mice (mice whose transgene consists of the tyrosinase promotor fused to the coding regions of simian virus 40 early genes) strikingly resemble human melanomas in their development and progression. Unlike human melanomas, the mouse tumors all arise in genetically identical individuals, thereby better enabling expression of specific genes to be characterized in relation to advancing malignancy. The products of pigment genes are of particular interest because peptides derived from these proteins have been reported to function as autoantigens with immunotherapeutic potential in some melanoma patients. However, the diminished pigmentation characteristic of many advanced melanomas raises the possibility that some of the relevant products may no longer be expressed in the most malignant cells. We have therefore investigated the contributions of several pigment genes in melanotic vs. relatively amelanotic components of primary and metastatic mouse melanomas. The analyses reveal marked differences within and among tumors in levels of mRNAs and proteins encoded by the wild-type alleles at the albino, brown, slaty, and silver loci. Tyrosinase (the protein encoded by the albino locus) was most often either absent or undetectable as melanization declined. The protein encoded by the slaty locus (tyrosinase-related protein 2) was the only one of those tested that was clearly present in all the tumor samples. These results suggest that sole reliance on targeting tyrosinase-based antigens might selectively favor survival of more malignant cells, whereas targeting the ensemble of the antigens tested might contribute toward a more inclusive and effective antimelanoma strategy.**

Immunotherapy based on recognition by cytotoxic T lymphocytes of antigenic peptides on melanoma cells has shown promise in the treatment of cutaneous melanoma (1–9). Among the melanoma antigens thus far identified are proteins or peptides encoded by specific pigment genes—i.e., genes involved in melanin production. A major problem is that melanomas tend to become amelanotic as the malignancy progresses, and can therefore be expected to lose expression of some of these genes. The gradual emergence of increasing numbers of hypomelanotic or amelanotic cells in the primary tumors or their metastases increases the possibility that such cells will selectively escape immune destruction. The superior mitotic activity of the amelanotic cells (10) may in fact exacerbate the course of the disease after the tumor burden has been temporarily reduced by the death of melanotic cells.

This problem might be overcome by identifying changes in the antigenic profile associated with melanoma progression such that the design of a treatment protocol could encompass all the melanoma cells. Such a characterization in the case of human melanoma would be complicated by genetic differences among individuals, especially as the genetic background, as well as allelic differences at pigment gene loci themselves, can influence the expression and interactions of these genes (11, 12). The melanomas of Tyr-SV40E transgenic mice [mice whose transgene consists of the tyrosinase promoter fused to the coding regions of simian virus 40 (SV40) early genes], on the other hand, afford an opportunity to investigate the products of pigment genes in tumors arising in genetically identical (C57BL/6 inbred strain) animals and representing all stages of tumor progression (13–16). Unlike long-established cell culture lines of animal or human melanomas that have been experimentally selected for a particular pigmentary or other phenotype, the phenotypes of the transgenic mouse melanomas reflect the progression actually taking place *in vivo*.

The Tyr-SV40E transgene confers melanoma susceptibility by activation of SV40 transforming sequences under the transcriptional control of the mouse tyrosinase promoter, which is encoded at the albino locus and is specifically expressed in pigment cells (13). The transgene acts as an initiating stimulus in melanocytes, only rarely giving rise spontaneously to cutaneous melanomas. If the cells are further subjected to growth-promoting influences *in vitro* (17, 18) or *in vivo* (19), they may become tumorigenic. Cutaneous melanomas are experimentally inducible by taking advantage of the existence of separate transgenic lines of mice, each descended from a single egg injected with transgene DNA and having a characteristic level of transgene expression (13). By grafting skin from mice of a line with high expression, and therefore high melanoma susceptibility, to hosts of a low-susceptibility line, the donor skin may be exposed to growth factors and cytokines associated with wound healing. Melanomas frequently develop in the grafts and metastasize in the hosts (15), whose risk of eye melanomas, especially in the retinal pigment epithelium (20), is far below that of the donors. The histopathogenesis of these mouse skin melanomas (16) strikingly resembles that of human cutaneous melanomas (21).

We examine the expression in the mouse melanomas of four pigment genes whose protein homologues have been implicated as autoantigens in human melanoma. The products of all four genes become localized in melanosomes, the organelles in which melanin is synthesized. Three of them are known to catalyze steps in melanin biosynthesis: they are tyrosinase (EC

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Abbreviations: SV40, simian virus 40; TRP-1 and -2, tyrosinase-related protein 1 and 2, respectively.

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1.14.18.1), the rate-limiting enzyme and the product of the mouse albino locus (22–24); tyrosinase-related protein 1 (TRP-1), which functions as 5,6-dihydroxyindole-2-carboxylic acid oxidase and is encoded at the mouse brown locus (25); and tyrosinase-related protein 2 (TRP-2), which has 3,4-dihydroxyphenylalanine (DOPA)chrome tautomerase (EC 5.3.3.12) function and is encoded at the mouse slaty locus (26, 27). The fourth gene product, initially designated Pmel 17, is the product of the silver locus and is incorporated into the melanosomal matrix (28, 29); its enzymatic function, if any, remains unknown. Comparisons of melanotic and relatively amelanotic components of the mouse melanomas analyzed here reveal marked disparities within and among tumors, including cases of absent or borderline expression of some of the proteins. The exception was TRP-2, which was found in all the tumor examples analyzed.

MATERIALS AND METHODS

Melanomas. The melanomas in this study all arose in skin from Tyr-SV40E hemizygous donors of line 8, which are highly susceptible to melanoma, after discs of skin were grafted to low-susceptibility hemizygous hosts of line 12 (15). Four primary melanomas (designated P) and two metastases (designated M) were analyzed. These primary melanomas were chosen because they were zonal tumors in which a deeply melanotic and an apparently amelanotic zone were clearly evident when examined at 10–15× magnification. It should be emphasized that small hypomelanotic foci in either of the zones could have escaped detection and that the terms "melanotic" and "amelanotic" are not intended to exclude the presence of cells with an intermediate phenotype. In fact, the choice of zonal tumors was intended to represent relatively extreme examples of disparities that are likely to exist to a lesser extent among the cells in a melanoma. In three of the primary tumors (159P, 222P, and 155P), the amelanotic zone was substantially larger than the melanotic one, whereas the reverse was true in the fourth case (31P). To carry out separate analyses of the melanotic and amelanotic components, small fragments of approximately 1 mm^3 or less from the respective zones were transplanted subcutaneously by trocar into line 12 hemizygous hosts. Serial transplantation of each was continued to verify that at least two transplant generations beyond that of the tumor sample kept for analysis had retained the characteristic grossly melanotic or amelanotic phenotype. Samples were dissected in cold phosphate-buffered saline, frozen in liquid nitrogen or on dry ice, and stored at −70°C. Both melanotic and amelanotic components were analyzed from tumors 159P, 222P, and 155P; only the melanotic part was analyzed from 31P. Of the two metastases studied, one (159M), found in the lung, was melanotic and, hence, was presumably derived from melanotic rather than amelanotic cells of the skin tumor of origin. The other metastasis (183M) was an amelanotic tumor in the liver of another mouse whose skin melanoma was largely amelanotic but was not further analyzed. Thus, nine tumor samples were analyzed and comprised five melanotic and four amelanotic examples, all with the same inbred strain background and the same transgene.

Cell Lines. Three cell culture sublines of the long-established B16 mouse melanoma line (originally derived from a spontaneous tumor in a C57BL/6 mouse) were analyzed for comparison with the newly arisen Tyr-SV40E melanomas. The respective B16 examples were a darkly melanotic, a slightly melanotic, and an amelanotic subline.

RNA Isolation and Northern Analysis. These were performed as described (30). Briefly, 2.0 μg of mRNA, isolated with a total-RNA isolation kit and mRNA purification kit (Promega), were electrophoresed and then blotted to Sure-Blot nylon membranes (Oncor). The membranes were tested for hybridization with ^{32}P-labeled probes, stripped, and rehybridized with other probes as necessary. TYRS-J, a probe specific for tyrosinase, was kindly provided by H. Yamamoto and T. Takeuchi (Sendai, Japan); pMT4, which is specific for TRP-1, was provided by S. Shibahara (Sendai, Japan); and TRP-2a, which is specific for TRP-2, was a gift from I. Jackson (Edinburgh).

Melanogenic Assays. Tissues were homogenized in 1% Nonidet P-40/0.01% SDS/0.1 M Tris·HCl, pH 7.2/1 μg of aprotinin per ml/100 μM phenylmethylsulfonyl fluoride on ice by using a Potter–Elvehjem glass homogenizer, incubated for 1 h at 4°C, and then stored at −70°C until used. Following centrifugation at 12,000 × *g* for 3 min, the supernatants were used for melanogenic assays. Four assays for melanogenic catalytic activities, as described below, were carried out at pH 6.8 and 37°C for 60 min.

(*i*) Tyrosine hydroxylase activity was measured by using the [^3H]tyrosine assay (31, 32). This method specifically measures the tritiated water produced during the hydroxylation of tyrosine to DOPA. (*ii*) DOPA oxidase activity was measured on the basis of incorporation of [3-^{14}C]DOPA into acid-insoluble melanin, as described (30). (*iii*) DOPAchrome tautomerase activity was measured by HPLC (33, 34) as the disappearance of DOPAchrome substrate and the production of 5,6-dihydroxyindole-2-carboxylic acid rather than 5,6-dihydroxyindole; data were converted to pmol of products by comparison with known standards. (*iv*) Melanin production was measured by incorporation of [^{14}C]tyrosine into acid-insoluble melanin, as described (31, 32); data in the radiometric assays were converted to pmol calculated from the radioactive product.

Tyrosine and DOPA used as standards and reaction substrates in these assays were obtained from Sigma; L-[3,5-^3H]tyrosine, [3-^{14}C]DOPA, and L-[U-^{14}C]tyrosine were from New England Nuclear; and DOPAchrome was prepared with the silver oxide method originally described by Körner and Pawelek (35).

Western Immunoblot Analysis. Transgenic melanoma samples, or pelleted cultured cells of the B16 melanoma or of melanocytes cultured from skin of C57BL/6 (non-transgenic) mice, were frozen in liquid nitrogen and then extracted by homogenization in a Dounce homogenizer on ice in 50 mM Tris·HCl buffer, pH 7.5, containing 1% Triton X-100/2 mM sodium EDTA/150 mM NaCl/5 mM benzamidine/20 μg of aprotinin per ml. Protein concentration was determined with the Bio-Rad Protein Assay reagent with bovine serum albumin as the standard. Twenty micrograms of protein was denatured by the addition of SDS and 2-mercaptoethanol to 2% final concentration, boiled for 90 sec, and applied to SDS/7.5% polyacrylamide gels. Prestained marker proteins of known molecular mass (Rainbow Markers, Amersham) were included with each set of samples. Transfers to Immobilon-P membranes and immunoblotting analyses were performed as described (36). The following antisera were employed: αPEP7, rabbit antiserum against the carboxyl-terminal peptide of murine tyrosinase (37); αPEP1, rabbit antiserum against the carboxyl-terminal peptide of TRP-1 (37); αPEP8, rabbit antiserum against the carboxyl-terminal peptide of TRP-2 (34); αPEP13, rabbit antiserum against the carboxyl-terminal peptide of the silver locus protein (29); and 1D4B, a rat monoclonal antibody against murine lysosome-associated membrane protein 1 (LAMP-1) (36), which was used here as a loading control. The LAMP-1 antibody was obtained from the Developmental Studies Hybridoma Bank [Johns Hopkins University (Baltimore)/University of Iowa, Iowa City]. Enhanced chemiluminescence (Amersham) was used to visualize antigens.

RESULTS

Melanogenesis in Melanotic vs. Amelanotic Melanoma Components and Metastases. The specific activities of three

enzymes representing progressive steps in the melanogenic pathway in relation to melanin formation for five samples of transgenic melanomas are shown in Table 1. It is of interest to compare the results for visibly melanotic and amelanotic samples within and between the two primary tumors tested and between one of the primary tumors and its metastasis. It should be noted that visible pigmentation reflects turnover, accumulation, and distribution of melanin over a relatively long time frame, whereas the assay for melanin formation only measures melanin production over a 1-h time period. Moreover, homogenization of tumor cells in preparing the extracts could release any inhibitors present in amelanotic areas that might suppress melanin formation in the extracts.

While the melanotic zones from both primary tumors (159P-Mel and 222P-Mel) appeared black on visual inspection, much more melanin was produced by 159P-Mel in the assay. However, this was not unequivocally reflected in the specific activities of any of the three melanogenic enzymes measured. Melanin formation in the melanotic lung metastasis (159M-Mel) is also noteworthy, as it was substantially lower than in the melanotic zone of the skin melanoma of origin (159P-Mel). Samples originating within the two primary tumors (159P and 222P) confirm that melanin formation was higher in the melanotic portion than in the amelanotic portion of each. In addition, they document a much greater disparity in melanin formation between melanotic and amelanotic parts of tumor 159P than of tumor 222P, due both to relatively lower melanin production in 222P-Mel and to higher melanin production in 222P-Amel than in the corresponding zones of 159P. A further notable aspect of the results was the relatively high DOPAchrome tautomerase activity in all transgenic melanoma samples, including the amelanotic ones, with only a 2-fold difference between the extremes. DOPA oxidase activity showed a 3-fold difference between the extremes with measurable activity, and was undetectable in one amelanotic case. In contrast to those enzymes, tyrosine hydroxylase showed a 14-fold difference between extreme cases and was very low in one.

Transcription of Tyrosinase-, TRP-1-, and TRP-2-Encoding Genes. Northern blot analysis of expression of these genes was carried out on parts of the same five transgenic melanoma examples as in Table 1. The bands seen in Fig. 1 were densitometrically scanned and quantitated by the IMAGEQUANT program, with corrections for density made by taking the signal for GAPDH and arbitrarily setting it as 100%. The results revealed that tyrosinase was only marginally expressed (0.1%) in 159P-Amel (Fig. 1, lane 2). Reverse transcription–PCR analysis of the same amelanotic tumor derivative documented a very low level of tyrosinase expression (S. Kelsall, N. Le Fur, and B.M., unpublished data). Thus, while none of the five melanoma samples completely failed to express any of the genes tested, their expression varied among the tumors. There

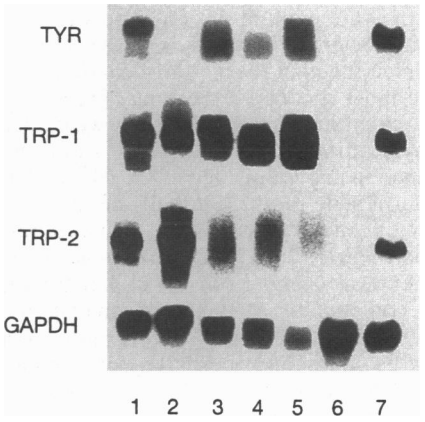

FIG. 1. Northern blot analysis of melanogenic/melanosomal gene expression in transgenic mouse melanomas. The same blots were repeatedly hybridized to probes specific for tyrosinase (TYR), TRP-1, or TRP-2, or for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. RNAs were extracted from melanotic or apparently amelanotic components of primary (P) or metastatic (M) melanomas in the cases of 159P-Mel (lane 1), 159P-Amel (lane 2), 159M-Mel (a lung metastasis from 159P-Mel) (lane 3), 222P-Mel (lane 4), and 222P-Amel (lane 5); from normal mouse muscle (lane 6); and from a slightly melanotic cell culture subline of the B16 mouse melanoma (lane 7).

appears to be only a moderate level of expression (36%) of tyrosinase in tumor 222P-Mel (Fig. 1, lane 4). In relation to hybridization results for the GAPDH loading control, tumor 222P-Amel in lane 5 (Fig. 1) had reduced but appreciable (49%) expression of TRP-2.

Melanogenic and Melanosomal Proteins. Immunoblot analyses were carried out on nine transgenic tumor samples. Five samples were derivatives of the same tumor zones used to measure melanogenic enzyme activities, melanin formation (Table 1), and RNA transcripts (Fig. 1). The four others were 155P-Mel and 155P-Amel, from the respective zones of another largely amelanotic primary tumor; 31P-Mel, from the melanotic zone of a largely melanotic primary tumor; and 183M-Amel, a liver metastasis from a mainly amelanotic primary tumor. Three reference materials were also included: cultured melanocytes from nontransgenic C57BL/6 skin; and two cell culture sublines of the B16 melanoma, one melanotic and one amelanotic (whereas the B16 subline shown in Table 1 and Fig. 1 was slightly melanotic).

All the samples were assayed for proteins encoded by the same wild-type genes at the mouse albino, brown, and slaty loci whose transcripts were assayed in Fig. 1, as well as by the wild-type gene at the mouse silver locus (Figs. 2–4). Lysosome-associated membrane protein 1 protein served as a positive

Table 1. Melanogenic enzyme activities and melanin formation

Sample*	Visible pigment	Tyrosine hydroxylase	DOPA oxidase	DOPAchrome tautomerase	Melanin formation
159P-Mel	++++	20.2 ± 0.3	6.7 ± 13.2	25.9	37.1 ± 0.6
159P-Amel	−	1.8 ± 0.1	0.0 ± 0.0	40.1	0.1 ± 0.1
159M-Mel	+++	25.5 ± 0.9	20.6 ± 3.4	36.0	10.8 ± 0.9
222P-Mel	++++	21.7 ± 2.1	20.6 ± 2.8	42.0	9.3 ± 0.9
222P-Amel	±	11.4 ± 0.9	6.4 ± 0.7	57.0	1.6 ± 0.1
B16 cell line†	+	3.9 ± 0.3	24.4 ± 8.1	33.8	0.8 ± 0.2
Mouse muscle	−	1.4 ± 0.2	1.2 ± 2.1	0.0	0.0 ± 0.0

All enzyme activity assays were performed at least in triplicate except the DOPAchrome tautomerase assay, which was performed in duplicate. Results are presented as the means ± SEM in pmol per μg of protein per h. Visible pigment represents relatively long-term melanin accumulation; melanin formation represents amount produced in 1 h.

*P, primary melanoma; M, metastasis; Mel, melanotic; and Amel, amelanotic. The 159M lung metastasis was presumably derived from melanotic rather than amelanotic cells of the primary tumor (159P-Mel).

†This B16 subline was slightly melanotic.

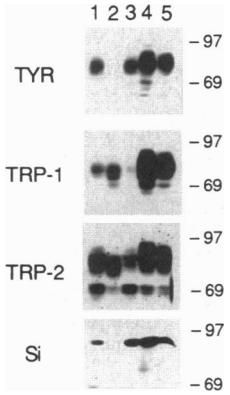

FIG. 2. Western immunoblot analysis of levels of the same melanogenic/melanosomal proteins described in Fig. 1 and of the silver (Si) protein. Equal quantities of sample protein were applied to SDS/polyacrylamide gels. Samples were analyzed from primary (P) or metastatic (M) transgenic mouse melanomas whose melanotic and apparently amelanotic components were separately tested in the cases of 159P-Mel (lane 1), 159P-Amel (lane 2), and 159M-Mel (a lung metastasis from 159P-Mel) (lane 3); from a melanotic cell culture subline of the B16 mouse melanoma (lane 4); and from melanocytes cultured from skin of nontransgenic C57BL/6 mice (lane 5). The migration of markers of known molecular mass is shown at the right.

control and yielded a strong signal of similar intensity in all lanes (data not shown).

Apart from the many minor quantitative variations seen in Figs. 2–4, the most cogent result was that some of the proteins were undetectable, or only marginally detected, in one or another component of some of the transgenic melanomas. This occurred most frequently in the case of tyrosinase protein, which was not apparent in three of the transgenic tumor samples (159P-Amel, 222P-Amel, and 183M-Amel). The TRP-1 protein, although present in all samples, had a relatively very low representation in three of them: 159M-Mel, 222P-Mel, and 183M-Amel. The silver protein was not detected in 159P-Amel and was very faintly positive in 222P-Mel and 183M-Amel. In contrast, TRP-2 protein was clearly present in all samples. It should be pointed out that results for the B16 mouse melanoma cell lines do not mirror the extent or nature of the variability found in the transgenic mouse melanoma model.

DISCUSSION

The genetically identical background of all the melanomas in the transgenic mouse model offers the special advantage that independent tumors from different animals or at different stages of progression can be compared. This enables the extent of variability in expression of specific genes to be clarified and changes in gene expression that are associated with malignant progression to be identified.

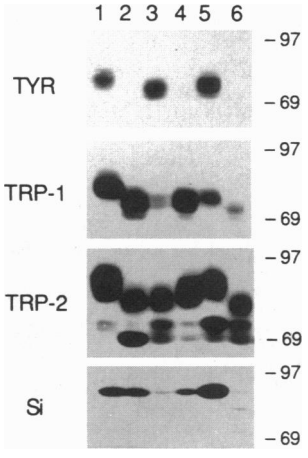

FIG. 3. Western immunoblot analysis of protein levels as described in the legend to Fig. 2. Samples were from melanocytes cultured from skin of nontransgenic C57BL/6 mice (lane 1), an amelanotic cell culture subline of the B16 melanoma (lane 2), and melanotic or amelanotic components of transgenic mouse melanomas in the case of 222P-Mel (lane 3), 222P-Amel (lane 4), 31P-Mel (lane 5), and 183M-Amel (an amelanotic liver metastasis from another primary tumor) (lane 6).

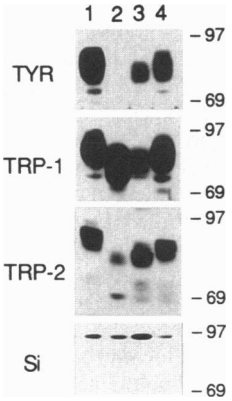

FIG. 4. Western immunoblot analysis of protein levels as described in the legend to Fig. 2. Samples were from melanocytes cultured from skin of nontransgenic C57BL/6 mice (lane 1), an amelanotic cell culture subline of the B16 melanoma (lane 2), and melanotic vs. relatively amelanotic components of a mouse melanoma in 155P-Mel (lane 3) and 155P-Amel (lane 4), respectively.

Peptides derived from melanogenic proteins encoded by pigment genes have recently been detected among the antigens capable of mediating immune destruction of human melanoma cells by the patient's cytotoxic T-lymphocytes (1–9). Melanomas characteristically become less melanized with advancing malignancy, although melanotic tumors may already be invasive and metastatic. Moreover, melanotic tumors or tumor zones are likely to harbor some less melanized cells, while amelanotic tumors may contain some hypomelanotic or melanotic cells. Thus, the dynamic nature and common occurrence of pigmentary change in melanoma progression require that variations in expression of pigment genes be taken into account in any immunotherapeutic strategy directed against products of these genes.

The transgenic mouse melanomas exhibit a trend toward decreasing melanization comparable with that of the human tumors. As in human melanomas (10), the amelanotic state in the mouse melanomas is correlated with greater mitotic activity (W.K.S., A. J. P. Klein-Szanto, and B.M., unpublished data). With the long-range objective of using this experimental animal model to define and test therapeutic possibilities based on antigens encoded by pigment genes, we have examined, in examples of transgenic melanomas, the expression of four pigment genes whose homologues encode some of the antigenic products found in human melanoma. These are the wild-type alleles at the albino, brown, slaty, and silver loci. Tumors with contrasting melanotic and amelanotic zones occur among both human and transgenic mouse melanomas. The use of such zonal tumors in the present investigation provides the opportunity to learn whether these pigment genes exhibit a uniform pattern of expression within areas of relatively extreme pigmentary phenotypes.

Melanogenic assays (Table 1), Northern analysis of mRNAs (Fig. 1), and Western immunoblot analysis of proteins (Figs. 2–4) all document considerable variability in activities or contributions attributable to these pigment genes. Differences are seen between melanotic and amelanotic components of individual tumors and also among melanotic or amelanotic components of separate tumors. Such differences at the level of tissue samples are highly likely to encompass the presence of many gradations of variability at the cellular level. This would not be surprising in view of the cellular heterogeneity found in human melanomas with respect to expression of other determinants (38, 39). Metastases may also differ from the primary melanoma of origin, as seen in 159M-Mel. Possibly the lower level of melanin formation in this lung metastasis (Table 1) reflects either its origin from a more advanced subset of cells in the grossly melanotic part of the primary melanoma from which it arose (159-Mel) or its subsequent progression in the lung.

In the mouse melanomas examined, the variability was greatest in expression of the albino locus. While tyrosinase protein was found in all five melanotic melanoma samples tested in immunoblots, it was absent or undetectable in three of the four amelanotic samples (Figs. 2–4). Of the five tumor samples analyzed for both mRNA and protein, the presence of some tyrosinase mRNA in 222P-Amel (Fig. 1, lane 5), while the protein was not detected (Fig. 3, lane 4), may seem paradoxical. A likely explanation, reinforced by tyrosinase detection in the assay for enzyme activity (Table 1), is that the cytoplasmic carboxyl tail that serves as the epitope for the αPEP7 antibody may have been cleaved by endogenous protease(s) without loss of catalytic function.

Protein products of the brown and silver loci were expressed, respectively, in all samples or in most, although each was only marginally present in at least one melanotic or one amelanotic case (Figs. 2–4). The presence of the slaty locus protein (TRP-2) in all nine transgenic tumor samples is noteworthy when compared with the very low levels or absence of contributions from the other genes in some of the samples. Whether this protein will prove to be common in a larger number of mouse melanomas or in human melanomas remains to be determined. Its more widespread occurrence in the tested mouse melanomas may simply reflect its much earlier appearance in melanocyte development than that of the other members of the tyrosinase family, tyrosinase and TRP-1 (40). Hence, malignant pigment cells may more readily retain expression of genes ordinarily associated with the early rather than the fully differentiated state of normal pigment cells.

The proteins detected in these melanoma analyses describe a potential rather than an actual source of anti-melanoma immunogens. Antibodies used to detect the antigens in our Western blots are specific for the carboxyl termini of the proteins; therefore, they do not recognize all possible peptides which those proteins might generate. As suggested above, in a given tumor with a high level of proteases, the carboxyl end may be cleaved off so that the antibody would not react with other peptides. It is now known that intracellular proteins may be subject to proteolytic processing and the peptides brought to the cell surface, presented as epitopes associated with class I major histocompatibility gene products, and recognized by cytotoxic T lymphocytes (41). While all the epitopes may not be effective in activating cytotoxic T lymphocytes, the host might be experimentally sensitized against them, such that their immunogenicity could be significantly increased.

Despite these unresolved questions, the mouse melanoma analyses demonstrate that even in genetically identical animals the pigment gene products differ widely in their representation and therefore in their potential to contribute immunologically useful antigens. It is evident that tyrosinase epitopes are not sufficient to eradicate all melanomas if amelanotic cells are present, as those cells may have a tyrosinase-null phenotype. However, a mixture of antigens derived from proteins of a collection of pigment genes may enable all the melanoma cells to be targeted. Such an approach might also allow adjustments to be made that would be less injurious to cells of the retinal pigment epithelium, whose loss would result in blindness.

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