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Genetic Control of Melanization: Isolation and Analysis of Amelanotic Variants from Cultured Melanotic Melanoma Cells

(amelanotic melanoma variants/cell culture/mutagenesis/melanocyte stimulating hormone/tyrosinase)

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ABSTRACT **Melanoma cells express a phenotype that is easy to recognize: the synthesis of melanin. We used this marker to isolate clones of amelanotic variants from large populations of wild-type melanotic clones. Cloudman mouse melanoma (S91, clone M-3, CCL 53.1) was chosen as the parental line because the cells are highly pigmented, grow well as clones in soft agar, and fuse readily with Sendai virus. Subclones (10^7) of this line were screened without prior mutagenesis, and nine amelanotic variants were isolated. The mutagen ethylmethanesulfonate increased the frequency of variants by three to four orders of magnitude. Wild-type cells had both basal and melanocyte stimulating hormone-inducible tyrosinase activities. The four amelanotic variants that we have examined to date all behaved similarly: they lost basal tyrosinase (EC 1.14.18.1; monophenol monooxygenase) activity but retained melanocyte stimulating hormone-inducible activity; they contained Stage-II melanosomes but no melanized melanosomes; they exhibited growth characteristics similar to those of wild-type cells in culture but produced fewer tumors in mice.**

The understanding of genetic regulation in cells of prokaryotes has advanced rapidly in recent years, but information on regulation in complex eukaryotic cells has been more difficult to obtain because of problems in performing genetic and biochemical analyses. Techniques for working with cultured cells are now available, which should be of help in studies of genetic regulation in eukaryotes. Selective procedures are being used for the isolation of drug-resistant (1), auxotrophic (2), and conditional lethal (3–4) mutants. Human cells are being cultured from patients bearing genetic defects (5). Somatic cell hybridization is being used widely for complementation analyses (6), gene mapping (7), and analyses of factors controlling the expression of differentiated functions (8–10), nucleic acid synthesis (11), and carcinogenesis (12).

Genetic analyses of differentiated functions have largely been confined to studies of the hybrid progeny obtained from fusing cell lines of different phenotypes (10). Because of difficulties in isolating mutants, there have been relatively few analyses of mutant cells that have lost the ability to express differentiated traits (13–16).

Melanoma cells express a phenotype that is easy to recognize: the synthesis of melanin; and this characteristic has previously been utilized to isolate amelanotic variants from the melanotic Cloudman S91 mouse melanoma (17). In addition, melanoma cells have several advantages for studies of factors controlling phenotypic expression. The oxidation of tyrosine to melanin is relatively well understood; a single enzyme, tyrosinase (EC 1.14.18.1; monophenol monooxygenase), is thought to control this pathway (18, see also 19); and a sensitive assay for tyrosinase activity is available (20). Some melanoma cell lines respond dramatically to melanocyte stimulating hormone (MSH), with large increases in tyrosinase activity and melanin content (21–22). Kilogram quantities of cells can be obtained by growing tumors in animals. With these features in mind, we proceeded to develop methods to isolate variant melanoma cells in culture that lost the ability to synthesize melanin. We report here our techniques for the isolation and analysis of such variants.

Abbreviation: MSH, melanocyte stimulating hormone.

MATERIALS AND METHODS

Culture Conditions. The melanotic mouse melanoma cell line of Cloudman (S91, clone M-3, CCL 53.1) was obtained from the American Type Cell Culture Repository (23). Cells were cultured either in monolayer as described (20–21) or as clones in soft agar by the method of Chu and Fischer (24). Culture medium for cloning was Ham's F-10 nutrient medium supplemented with 2% fetal-calf serum, 15% horse serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 1.2 mg/ml of sodium bicarbonate, and 0.12% Noble Special Agar (Difco). Clone M-3 cells were chosen for genetic studies because subclones of M-3 possess a stable phenotype for pigmentation, and the cells grow well in soft agar to form large pigmented colonies within 2–3 weeks (Fig. 1). These cells fuse readily with Sendai virus. Another mouse melanoma cell line of Cloudman has been described which, when cultured in the presence of MSH, showed dramatic increases in tyrosinase activity and melanin content (22–23). But this line was not chosen for genetic studies because it grows poorly in clones, and in the absence of MSH it is light in color and has little tyrosinase activity.

Establishment of Isogenic Cell Lines. Subclones of clone M-3 were obtained by growing cells in soft agar in either 13 × 100-mm screw-cap culture tubes containing 5 ml of medium, or 100-mm petri dishes containing 20 ml of medium, and incubating them at 37°C in a humidified incubator with 5% CO_2–95% air. The cloning efficiency varied from 5–20%. Several pigmented clones were isolated independently with a Pasteur pipette and dissociated with a Vortex mixer. Each pigmented clone was subcultured at least ten more times. After this procedure, the lines were considered to be isogenic. They could be distinguished from one another on the basis of gross morphology and growth characteristics.

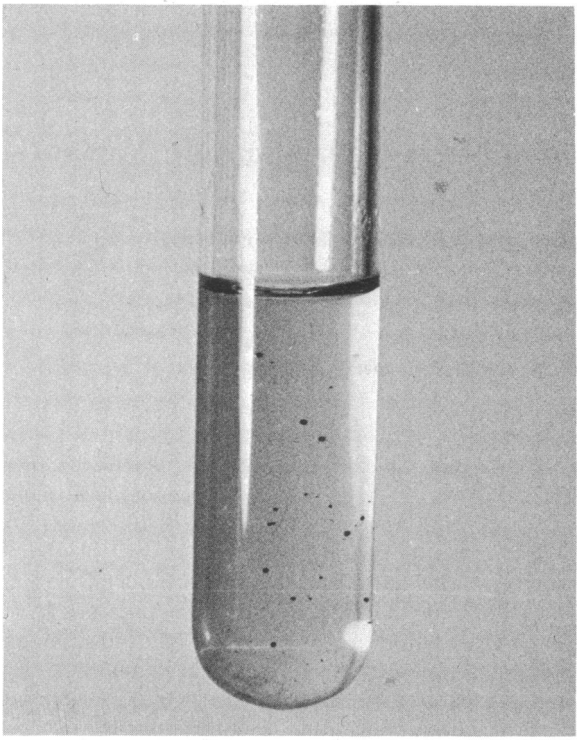

FIG. 1. Cloudman melanoma S91, clone M-3 growing in soft agar. Cells were cultured for 3 weeks. Each clone represents approximately 7000 cells.

Isolation of Amelanotic Variants. One isogenic subline of M-3, designated PS 4-wt (wild type), was selected to be screened for amelanotic variants. Approximately 5000 cells were inoculated into each 100-mm petri dish. After 3 to 4 weeks of incubation, the dishes were scanned under a dissecting microscope for amelanotic clones. These white clones were isolated, and each was subcultured at least five times before the cells were grown *en masse* in monolayer cultures for further analyses.

Tyrosinase Activity, Melanin Content, and Growth Rates. Cells were assayed for tyrosinase activity by incubating them for 24 hr with 1 μCi/ml of L-[3,5-[^{3}H]]tyrosine (20 Ci/mmol, New England Nuclear) and then measuring the ^3H_2O released into the culture medium (20–21, 25). The cells were then harvested, examined visually for the presence of melanin, and counted with a hemocytometer.

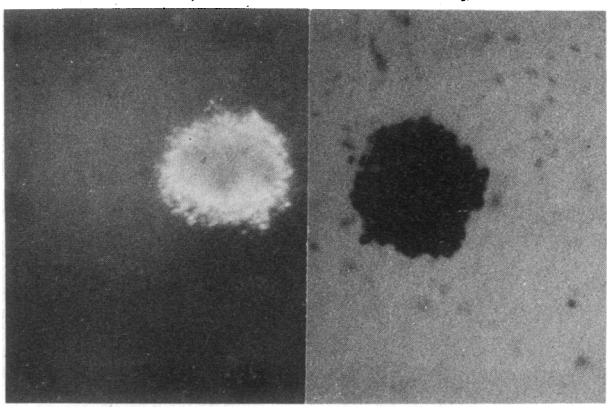

FIG. 2. Isolation of amelanotic variants. (*Left*) Amelanotic clone, PS 4-3; (*right*) wild-type clone, PS 4-wt.

RESULTS

Isolation of Amelanotic Variants. To determine the frequency of amelanotic variants, we screened clones that had not been mutagenized. Clones (10^7) were screened, and nine stable amelanotic variants were isolated (Fig. 2). The frequency of appearance of stable variants is approximately 1×10^{-6}. These variants have remained amelanotic for at least 140 generations. In addition, we isolated approximately 100 additional amelanotic variants, but these later became pigmented and were discarded.

Mutagenesis. Treatment with ethylmethanesulfonate, a potent mutagen, increased the frequency of amelanotic variants by three to four orders of magnitude (Table 1). About 30% of these variants remained stable with regard to the amelanotic phenotype. This increase in frequency provides evidence that the variants arose from mutations in genes that control melanization.

Growth in Culture. Four of the amelanotic variants designated PS 4-1, PS 4-3, PS 4-15, and PS 4-17, which were obtained without mutagenesis, are now growing in monolayer culture. Wild-type cells and amelanotic variants had similar growth rates (Fig. 3). The generation time was approximately 48 hr. The cells reached confluency by day 10, but they showed no contact inhibition at this point and continued to divide for another week before reaching stationary phase.

Tyrosinase Activity. Wild-type cells had a basal level of tyrosinase activity and an MSH-inducible level of activity; both levels were highly dependent upon cell density (Fig. 4*a*, see Fig. 3 for growth rates). Exposure to MSH before each assay stimulated tyrosinase activity throughout the culture period. In both MSH-treated and control cultures, however, specific activity of tyrosinase was low in cultures of low density, increased sharply as the density increased, and decreased again as the cultures reached confluency. If the cells were subcultured more frequently to maintain them at low density, they lost their pigment and tyrosinase activity remained low, even though they were in the logarithmic phase of growth.

TABLE 1. *Mutagenesis with ethylmethanesulfonate**

Ethylmethanesulfonate concentration (mg/ml of culture medium)	Total clones	Amelanotic variants	Relative survival (%)
0	6110	0	100
0.02	6400	0	105
0.05	2292	11 (0.5%)	38
0.10	1846	51 (2.7%)	31
0.20	1438	27 (1.9%)	24
0.50	100	5 (5%)	1.7
1.0	0	0	0

* Cells were incubated with ethylmethanesulfonate for 2 hr, harvested, washed free of ethylmethanesulfonate by centrifugation, and cloned.

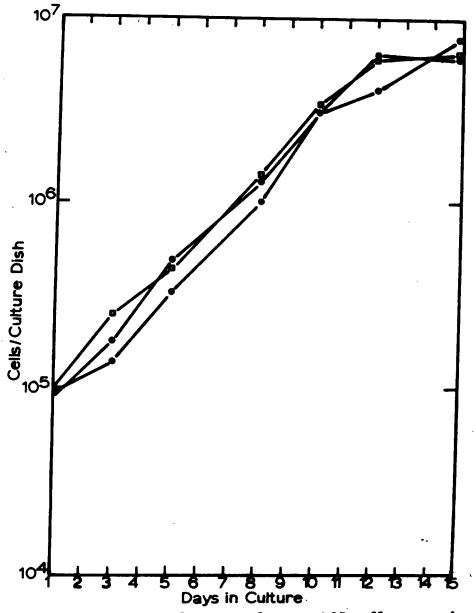

FIG. 3. Growth in monolayer culture. 10^5 cells were inoculated into Falcon 30-ml tissue culture flasks. Duplicate cultures were harvested and counted with a hemocytometer. Results are presented here for wild-type clone, PS 4-wt (●), and amelanotic variants PS 4-1 (○) and PS 4-17 (□). The other amelanotic variants had essentially the same growth characteristics. Points represent averages of duplicates.

These results indicated that the increase in the specific activity of tyrosinase was a function of cell density rather than growth phase.

All four amelanotic variants behaved in a similar fashion. They retained the density-dependent, MSH-inducible tyrosinase activity, but they lost the basal activity (Fig. 4*b*). Specific activity of tyrosinase was maximal in the midlogarithmic phase of growth, but only in the presence of MSH. In the absence of the hormone, there was little or no measurable activity.

Melanin. Melanin content paralleled tyrosinase activity. In the midlogarithmic phase of growth, wild-type cells were dark brown in the absence of MSH and jet black in the presence of the hormone. Without MSH, amelanotic variants were white throughout the culture period. Variants treated with MSH became dark brown in the midlogarithmic phase of growth. Wild-type or variant cells were refractory to MSH when they were confluent.

Fine Structure. Wild-type cells, as well as four amelanotic variants cultured in the absence of MSH, were examined with an electron microscope. Wild-type cells contained large numbers of heavily melanized Stage-III (26) melanosomes (Fig. 5*a*). All four variants contained numerous well-developed Stage-II melanosomes, which have no melanin, but no Stage-III melanosomes (Fig. 5*b*). No other qualitative differences in the fine structure between wild-type cells and amelanotic variants were observed. It appeared that the synthesis of melanosomes was not the defective step of melanization in the amelanotic variants.

Tumor Formation. The tumor-forming ability of the wild-type cells and variants was tested by injecting cells subcutaneously into DBA/2J mice. The variants formed tumors with lower frequency than the wild-type cells (Table 2).

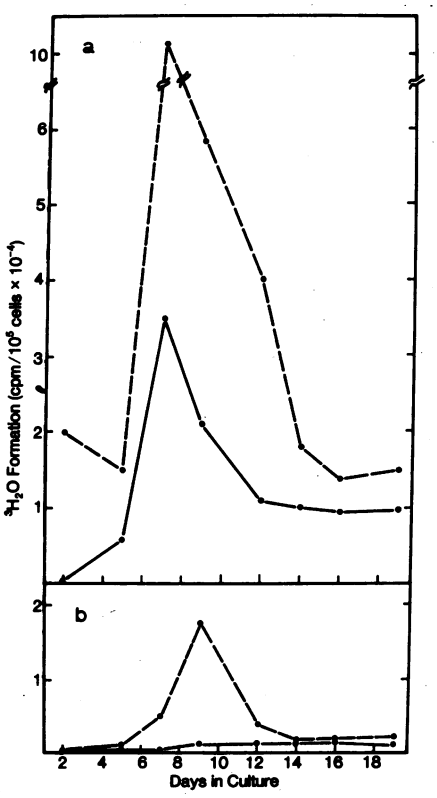

FIG. 4. Tyrosinase activity. Activity was measured *in situ*. 10^5 cells were inoculated into 30-ml Falcon flasks, and at various times duplicate cultures were incubated for 24 hr with [^{3}H]-tyrosine at 1 μCi/ml and a final specific activity of 96 μCi/μmol in the culture medium. 0.5 ml of medium was removed and ^3H_2O was measured (19). Controls (●——●); MSH-treated (○- - -○). Points represent averages of duplicates. (*a*) Wild-type cells PS 4-wt; (*b*) amelanotic variant cells, PS 4-1. Similar results were obtained for the other variants mentioned in the text. cpm have been multiplied by 10^{-4}.

DISCUSSION AND CONCLUSIONS

We have developed simple procedures for isolating lines of amelanotic melanoma cells from large populations of isogenic melanotic cells. The four amelanotic variants analyzed to date behaved similarly: they lost basal tyrosinase activity but retained density-dependent, MSH-inducible activity; they contained Stage-II nonmelanized melanosomes but no Stage-III melanosomes; they exhibited growth characteristics similar to those of wild-type cells in culture but produced fewer tumors in mice. Initial attempts to achieve genetic com-

TABLE 2. *Tumor formation by wild-type and variant cells*

Cell line	No. of mice injected	Tumors
PS 4-wt (wild type)	20	18
PS 4-1	10	3
PS 4-3	10	4
PS 4-15	10	0
PS 4-17	10	1

DBA/2J mice were injected subcutaneously with 5×10^6 cells. Palpable tumors were recorded after 5 weeks.

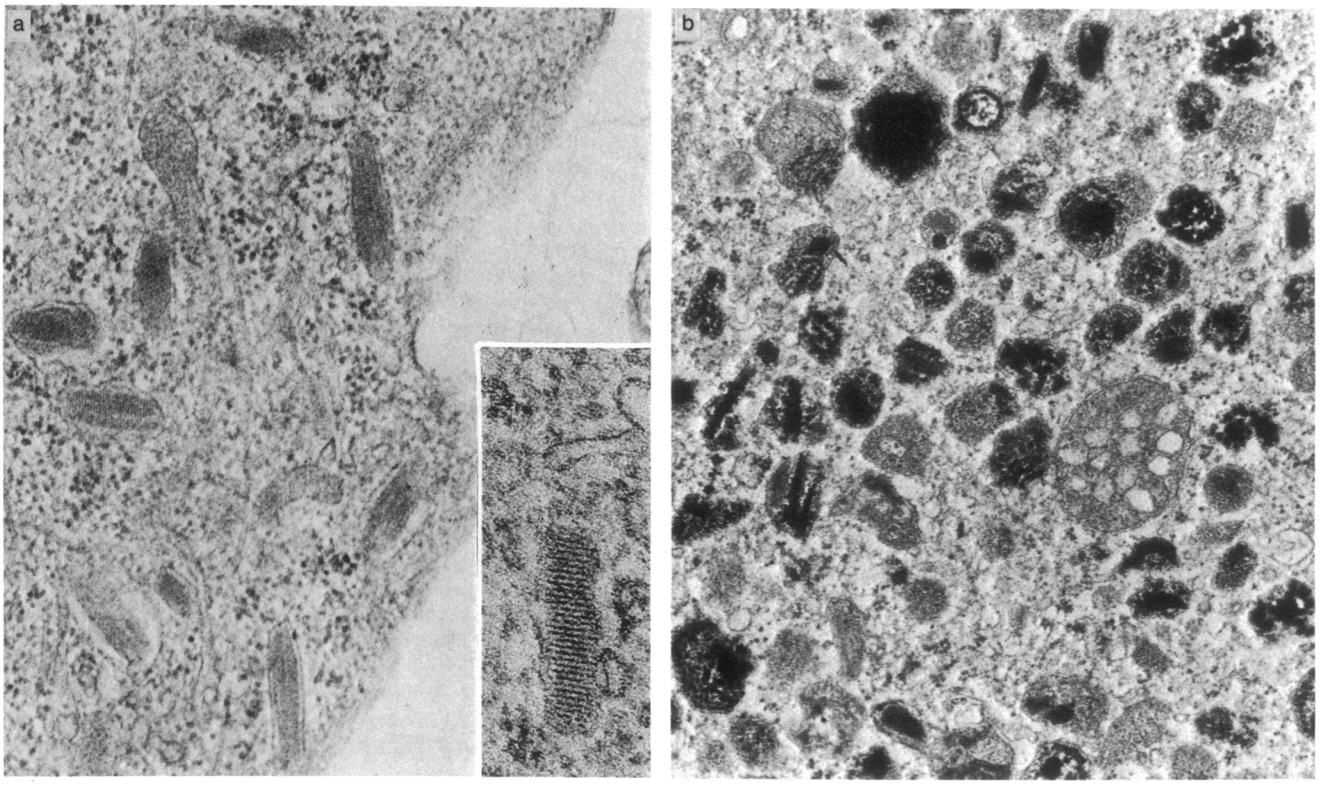

FIG. 5. Electron micrographs of typical segments of (*a*) a cell from amelanotic clone PS 4-1, and (*b*) a cell from wild-type clone PS 4-wt. Cells were harvested, centrifuged, and fixed in buffered solutions of 3% glutaraldehyde followed by 1% osmium tetroxide. The cells were embedded in epoxy resin and sectioned on an LKB Ultrotome III. Ultrathin sections were stained with uranyl and lead ions and examined in a Hitachi HU-11B electron microscope.

The extensive internal lattice matrix of melanosomes in amelanotic variant cells does not contain melanin. Melanosomes are in Stage-II. See *insert* for morphological detail. Many of the melanosomes of wild-type cells, however, are moderately to heavily melanized. Magnifications: ×30,000; *insert:* ×99,000.

plementation between the four variants have been unsuccessful. Together, these findings suggest that the four independently isolated variants might have gone through the same alteration to become amelanotic, perhaps acquiring mutations at the same genetic locus. It is also possible that the four variants are sibling progeny of a single amelanotic parent. If the latter were true, the frequency of spontaneous amelanotic variants in the population would be somewhat lower than 1×10^{-6}. Further isolation and analyses of variants, particularly those that have been subjected to mutagenesis, will be necessary to determine whether or not this is a common phenotypic change.

The patterns of density-dependent tyrosinase activity shown in Fig. 4 are similar to those reported for hamster melanoma RPMI 3460 (27) and mouse melanoma B16 (25) and, therefore, may represent typical behavior of melanoma cells in culture. We do not understand the density-dependent phenomenon. However, factors such as changes in the cell surface or metabolite concentration in the culture medium could be responsible for the dependence.

Treatment of cells with ethylmethanesulfonate increased the frequency of appearance of variants by three to four orders of magnitude. This finding suggested that the variants had acquired mutations that affected melanization. The frequencies of appearance of amelanotic variants seemed quite high when one considers that the cells have a heteroploid karyotype. In the heteroploid state, most recessive mutations would be masked by wild-type alleles on homologous chromosomes. However, others have found the "mutation" frequency among somatic cells in culture was higher than expected (13–14, 16, 28–30). The observed frequencies may be explained by one of the following possibilities: (*a*) we might have selected for dominant mutations; (*b*) through random loss of chromosomes the cells might have become haploid for the chromosomes bearing the gene in question (28); (*c*) a single recessive gene may have acquired a mutation, and amelanotic segregants were produced by somatic cell crossing-over; (*d*) a large number of genes might be involved in melanization so that a mutation in any one of them could interrupt the pathway, thereby increasing the "target size" for the mutational event; or (*e*) variations might have arisen by "stable shifts in phenotypic expression rather than changes in genetic information," as proposed by Harris (30). These possibilities are not mutually exclusive. Consistent with the first three points was our observation that, on occasion, mixed clones containing both melanotic and amelanotic cells appeared.

One of our major goals is to isolate enough amelanotic variants so that we can perform a genetic complementation analysis of the melanization pathway. Such an analysis should provide information as to the number and nature of genes governing the expression of this differentiated phenotype.

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