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Melanoma Cells Resistant to Inhibition of Growth by Melanocyte Stimulating Hormone

(cAMP/cell division/monophenol monooxygenase)

JOHN PAWELEK*†, MARILYN SANSONE*, NORA KOCH*, GAIL CHRISTIE‡, RUTH HALABAN*, JOHN HENDEE*, AARON B. LERNER*, AND JANOS M. VARGA*

* Department of Dermatology, † Anatomy, and ‡ Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06510

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ABSTRACT **Melanocyte stimulating hormone (MSH) enhances melanization but inhibits proliferation of Cloudman S91 melanoma cells in culture. We have isolated variants of these cells that can grow in the presence of MSH. The conclusions we have reached from analyses of these cells are the following: (1) Basal tyrosinase activity (monophenol monooxygenase; monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1), i.e., the activity that is present in the absence of added MSH, is related through a common biochemical pathway to MSH-mediated control of growth. (2) MSH-inducible tyrosinase activity does not appear to be related to MSH control of growth. (3) The morphological changes that occur following the addition of MSH or cAMP are related to controls of growth and not to those of melanization.**

Melanocyte stimulating hormone (MSH) enhances melanization but inhibits the proliferation of cells from the Cloudman S91 mouse melanoma in culture (1). Both effects result from an increase in the intracellular levels of cyclic AMP which occurs within minutes after the addition of MSH to the cultures (1–3).

The concentration of cyclic AMP inside a cell is crucial to the control of proliferation of that cell (4). For example, rapidly dividing cells generally have lower levels of cyclic AMP than slowly dividing or stationary cells (5–9); some tumor viruses bring about a rapid drop in cyclic AMP in normal cells before the cells undergo a malignant transformation (10, 11); and a wide variety of transformed cells appear to revert to normal when cyclic AMP or one of its analogues is added to the medium of the cells in culture (1, 12–24), or injected into animals harboring tumors of those cells (25, 26). However, little is known of the mechanisms in the control processes. Daniel *et al.* (27) and Sibley *et al.* (28) selected for mutant mouse lymphoma cells that could grow in concentrations of cyclic AMP that were lethal to the wild-type cells. By analyzing these mutants they hope to find out more about the role of cyclic AMP in the control of growth. Similarly, we have isolated and studied clones of melanoma cells that are resistant to the inhibitory effects of MSH on growth. In this report we describe experiments which clarify the relationships of cyclic AMP to the control of both the growth and melanization of these cells.

METHODS

Isolation of MSH-Resistant Cells. Cells were cultured in a manner described previously (29) except that the culture medium contained MSH (0.2 μM). Melanotic and amelanotic clones that grew in the presence of MSH were isolated and subcloned further. Most of the data presented were obtained from the analyses of two clones. One clone was melanotic in the presence of MSH and was designated as PS1-*mel-1*. The other was amelanotic in the presence of MSH and was called PS1-*amel-1*. These clones will be referred to as *mel-1* and *amel-1*. The parental strain from which these lines were derived is designated PS1-wt (wild type) and is a pigmented subclone of the Cloudman S91 cell line.

Analytical Procedures. Tyrosinase activity (monophenol monooxygenase; monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) was measured by the Pomerantz method (30, 1). Cyclic AMP was determined by the method of Brown (3, 31). Cyclic AMP binding proteins were measured as previously described (2). Protein kinase activity (ATP:protein phosphotransferase, EC 2.7.1.37) was measured by the method of Kuo and Greengard (32). MSH receptors were detected by the method of Varga *et al.* (33). DNA was determined by the method of Burton (34).

RESULTS

Effects of MSH on the Proliferation of Cells. We compared the amount of DNA in cells blocked by MSH, blocked in metaphase by colchicine, and those growing normally (Table 1). There was about one-half as much DNA in the cells blocked by MSH as in the cells in metaphase that were blocked by colchicine. Except for the possibility that MSH reduced cellular ploidy, these findings indicated that MSH inhibits proliferation in the G-1 or early S phase of the cell cycle.

Growth Characteristics. The rates of growth of the different cells in the presence or absence of MSH are shown in Fig. 1a–c. MSH inhibited the growth of the wild-type cells after one or two cell divisions but had little or no effect on clones *amel-1* or *mel-1*. Dibutyryl cyclic AMP (Bt_2cAMP) also inhibited the growth of the wild-type cells (Table 2). Clone *amel-1* was not inhibited by Bt_2cAMP and clone *mel-1* was less affected than the wild-type cells.

Tyrosinase Activity. Wild-type cells had high values for basal tyrosinase activity and melanin content and both of these values were increased by MSH or Bt_2cAMP (Table 3).

Clone *amel-1* had very low basal tyrosinase activity and was amelanotic even in the presence of MSH or Bt_2cAMP. A lack of response to MSH was expected because *amel-1* was originally selected as an amelanotic clone grown in the presence of

Abbreviations: MSH, melanocyte stimulating hormone; Bt_2-cAMP, dibutyryl cAMP.

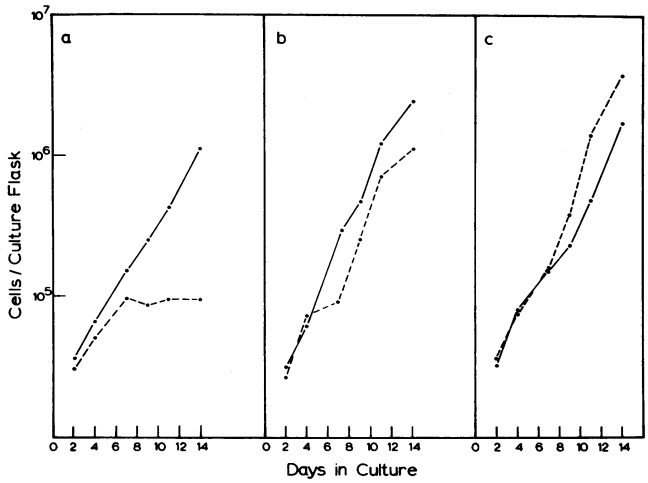

FIG. 1. Cells (5×10^4) were inoculated into 30 ml Falcon tissue culture flasks and grown in normal medium (●——●) or medium containing MSH (0.2 μM, ●- - -●). Triplicate cultures were harvested and counted in a Coulter counter. (a) Wild type; (b) clone *mel-1;* and (c) clone *amel-1.*

MSH. However, a concomitant lack of response to Bt_2cAMP had not been expected, because at least one class of mutants should be those with lesions in their MSH receptor/adenylate cyclase system. Such cells would lose the ability to react to MSH but should retain the ability to respond to an increase in the intracellular levels of cAMP.

Clone *mel-1* had very low basal tyrosinase activity and was amelanotic in the absence of MSH, but showed a striking rise in tyrosinase activity and melanin content following the addition of MSH or Bt_2cAMP to the culture medium. In this respect *mel-1* was similar to amelanotic cell lines that were described previously (29). The loss of basal tyrosinase activity and melanin content was not expected because we selected only for resistance to MSH inhibition of growth. This result implied that a relationship existed between resistance to MSH in the control of growth and loss of basal tyrosinase activity. To test whether or not such a relationship was real we examined four clones which had previously been selected for loss of basal melanization but not for MSH resistance in growth (29). Three of the four clones were MSH-resistant in growth (Table 4), although like clone *mel-1* they responded to MSH by showing an increase in tyrosinase activity and melanin content (29). Because the loss of basal activity and the acquisition of MSH resistance are both rare events, it appears unlikely that the presence of both traits in cells selected for either one or the other was coincidental. The most likely explanation for these results is that there is a biochemical relationship between resistance to MSH inhibition of growth and basal tyrosinase activity. However, since one of the four clones (PS 4-51) that was selected for loss of basal activity was still *sensitive* to MSH inhibition of growth, the relationship between growth inhibition and basal tyrosinase activity is not necessarily an obligatory one.

TABLE 1. *DNA content of wild-type cells cultured under various conditions*

Culture conditions	μg of DNA/10^6 cells
Normal medium	40.5
Colchicine 36 hr	53.0
MSH 6 days	30.5

Cells were cultured in normal F10 medium, and medium containing colchicine (15 ng/ml, added after 4½ days), or MSH (0.2 μM added at time of seeding). After 6 days in normal medium or that containing MSH, cells were harvested with 5 mM EDTA. Colchicine-treated cells were harvested by manual shaking to collect that portion of the population arrested in metaphase. All cells were washed thoroughly with isotonic saline and counted in a Coulter counter, and DNA was measured by the method of Burton. Results represent averages of duplicate experiments and variations between duplicates were less than ±5%. These experiments were repeated several times with similar results each time.

TABLE 2. *Effects of Bt_2cAMP on the growth of wild-type and MSH-resistant cells*

Strain	Millions of cells/culture flask	
	Normal medium	Bt_2cAMP
PS1-*wild type*	1.4	0.2
PS1-*amel-1*	1.6	2.1
PS1-*mel-1*	2.3	1.5

Cells (10^5) are inoculated into 30 ml Falcon tissue culture flasks and grown in normal F10 medium or medium supplemented with 250 μM Bt_2cAMP. After 14 days the cells were harvested and counted in a Coulter counter. The results represent averages of triplicate culture flasks.

Morphology. MSH or Bt_2cAMP caused wild-type cells to become flattened and more dendritic in shape, but had no effect on either clone *amel-1* or *mel-1* (Fig. 2). The fact that clone *mel-1* exhibited increased melanization but no striking morphological changes in the presence of MSH indicates that the effects on morphology are related to MSH-mediated growth controls, and not to melanization. This view is supported from analyses of clones PS 4-1, PS 4-3, and PS 4-17 (ref. 29 and Table 4). These clones are MSH-resistant in growth, and respond to MSH with increased melanization, but none undergoes any striking changes in morphology in response to the hormone. Furthermore, clone PS 4-15 was MSH-sensitive in growth and also exhibited morphological changes similar to wild-type cells in response to the hormone.

TABLE 3. *Tyrosinase activity in wild-type and MSH-resistant cells*

Strain	cpm ^3H_2O production/24 hr		
	Normal medium	+MSH	+Bt_2cAMP
PS1-*wild type*	72,000	450,000	205,010
PS1-*mel-1*	2,500	150,000	100,000
PS1-*amel-1*	3,900	4,062	1,800

Cells (2×10^5) were inoculated into 30 ml Falcon tissue culture flasks in 5 ml of medium and were cultured in either normal medium, MSH (0.2 μM), or Bt_2cAMP (250 μM). After 24 hr [^{3}H]tyrosine at 1 μCi/ml and a final specific activity of 96 μCi/μmol was added to the culture medium. Twenty-four hours after the addition of [^{3}H]tyrosine 0.5 ml of medium was removed and ^3H_2O was measured. Results are averages of triplicates and represent the total ^3H_2O in 5 ml of medium.

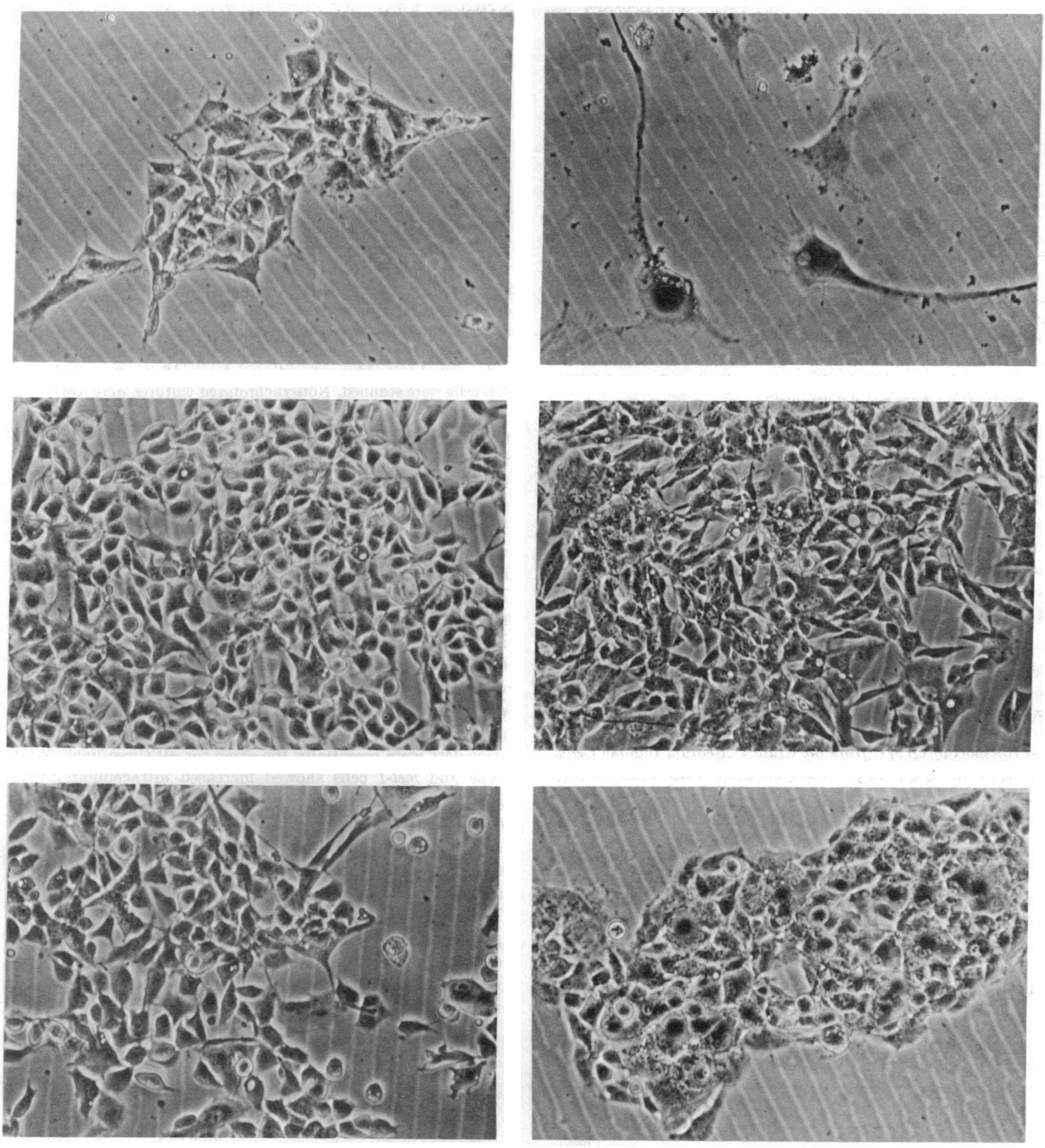

FIG. 2. Phase contrast photographs of cells growing in normal medium (left) or medium containing MSH (right). Upper: wild-type cells; middle: clone *amel-1;* lower: clone *mel-1.*

Determination of Cyclic AMP. Endogenous levels of cAMP were measured in cultures of wild-type cells as well as of clones *amel-1* and *mel-1.* In the absence of MSH all three cell lines contained approximately 2 pmol of cAMP per 10^6 log phase cells (Table 5). Wild-type cells and clone *mel-1* cells responded to MSH with a marked increase in cAMP which was potentiated by the addition of theophylline. Clone *amel-1* cells showed no increase in cyclic AMP when exposed to either MSH or theophylline. The lack of increase in cAMP in clone *amel-1* was not due to a release of cAMP into the culture medium. We conclude from these results that wild-type cells and clone *mel-1* cells have an intact MSH receptor/adenylate cyclase system while clone *amel-1* cells do not.

MSH Receptors. The following points were established from several experiments on MSH binding activity: (*1*) wild-type, *amel-1*, and *mel-1* cells all had receptors for MSH; (*2*) the association constant was approximately 3×10^8 liters/mole

TABLE 4. *Effect of MSH on growth of cells that were selected for loss of basal melanization*

Strain	Millions of cells/culture flask	
	Normal medium	+MSH
PS 4-*wild type*	1.8	0.3 (growth arrested)
PS 4-1	1.3	1.3
PS 4-3	1.7	1.1
PS 4-15	1.5	0.2 (growth arrested)
PS 4-17	3.2	2.3

Cells (10^5) were inoculated into 30 ml Falcon tissue culture flasks and grown in normal medium or medium supplemented with MSH (0.2 μM). After 14 days the cells were harvested and counted in a Coulter counter. The results represent averages of duplicate culture flasks. These cell lines were described in a previous publication (29) but in that study the effects of MSH on proliferation were not determined.

in each case; (*3*) the number of receptors was of the same order in each case but, *amel-1* and *mel-1* cells consistently had about two times more receptor activity than wild-type cells; and (*4*) when Bt_2cAMP was added to the culture medium, receptor activity was increased in wild-type and *mel-1* cells but was usually decreased in *amel-1* cells. Bt_2cAMP did not change the percentage of receptor-bearing cells in the population (Table 6).

Protein Kinase Activity and Cyclic AMP Binding Proteins. Cells from all three lines had about the same levels of cAMP-dependent protein kinase activity and proteins that bound cAMP (Table 7).

Transport of cAMP from the Culture Medium into the Cells. Cyclic AMP was transported from the culture medium into the cells at the same rate by all three lines. Each line transported [^{3}H]cAMP at the rate of 6.5 pmol/hr per 10^5 cells.

Fine Structure. Cells from all three lines had melanosomes. Wild-type cells contained heavily melanized stage-III melanosomes, whereas clones *amel-1* and *mel-1* contained stage-II melanosomes without melanin.

DISCUSSION AND CONCLUSIONS

The appearance of MSH-resistant clones in the population is a rare event and we do not yet know its frequency. Our findings are as follows: MSH or Bt_2cAMP caused growth inhibition and morphological changes in wild-type cells but not in *amel-1* or *mel-1* cells. Wild-type cells had high basal tyrosinase activity which was stimulated further by MSH or Bt_2cAMP. *amel-1* cells had very low basal tyrosinase activity which was not stimulated by MSH or Bt_2cAMP. *mel-1* cells also had very low basal tyrosinase activity but retained MSH or Bt_2cAMP inducible activity. There was somewhat more MSH receptor activity in *amel-1* and *mel-1* cells than in wild-type cells. Bt_2cAMP caused an increase in MSH receptor activity in *mel-1* and wild-type cells but usually decreased the activity in *amel-1* cells. All three lines contained melanosomes. Endogenous cAMP levels, cAMP-dependent protein kinase activity, cAMP binding proteins, and cAMP transport were essentially the same for all three lines. Wild-type and *mel-1* cells showed increased intracellular cAMP levels in response to MSH and/or theophylline; *amel-1* cells did not.

Our major conclusions are the following: Tyrosinase activity that is present in the absence of added MSH is related through a common biochemical pathway to MSH control of growth. However, MSH-inducible tyrosinase activity does not appear to be related to MSH control of growth. The morphological changes that occur following the addition of MSH or cAMP to melanoma cells are related to growth of the cells and not to melanization.

TABLE 5. *cAMP levels in wild-type and MSH-resistant cells*

Strain	pmol of cAMP/10^6 cells			
	Normal medium	+Theophylline	+MSH	+Theophylline and MSH
PS1-*wild type*	2.2	5.4	28.0	32.0
PS1-*mel-1*	2.4	10.0	22.0	29.0
PS1-*amel-1*	2.0	2.2	2.2	2.0

Cells (2×10^6) were inoculated into 100 mm diameter Falcon petri dishes. After 24 hr MSH (0.2 μM), or theophylline (2 mM), or both were added to duplicate cultures for 30 min. cAMP was extracted by the "rapid extraction technique" (see Table 2 of ref. 3). These experiments were repeated several times with similar results each time.

TABLE 6. *Binding of MSH to wild-type and MSH-resistant cells*

Strain	cpm ^{125}I-labeled MSH bound per 10^4 cells		Percentage labeled cells	
	Normal medium	+Bt_2cAMP	Normal medium	+Bt_2cAMP
PS1-*wild type*	745	1,450	22	18
PS1-*mel-1*	1,263	1,462	11	15
PS1-*amel-1*	1,320	810	19	20

Cells were seeded into 30 ml Falcon tissue culture flasks and 48 hr later MSH receptor activity was determined as previously described (33). The values for cpm ^{125}I-labeled MSH bound are average values from two experiments with three parallel points taken in each experiment. The values for the percentage labeled cells were obtained by autoradiography, and for each point 600 to 700 cells were scanned. Nonsynchronized cultures were used.

TABLE 7. *Protein kinase activity in wild-type and MSH-resistant cells*

Strain	cpm ^{32}P incorporated into acid-precipitable material		
	Complete reaction mixture	Complete mix minus cAMP	Complete mix minus histones
PS1-*wild type*	6,030	2,810	730
PS1-*mel-1*	7,450	2,890	910
PS1-*amel-1*	8,800	2,975	875

Cells (10^6) were lysed and incubated with [^{32}P]ATP (gamma labeled) under conditions described by Kuo and Greengard (32). Numbers represent averages of duplicate experiments. These experiments were repeated several times with similar results each time.

The MSH-resistant cell lines had as much or more MSH receptor activity as wild-type cells, and their resistance to the G-1 restriction cannot be explained by a lack of receptors. Receptor activity was increased with the addition of Bt_2-cAMP. A similar observation with another melanoma cell line was made earlier (A. DiPasquale, J. M. Varga, and J. Pawelek, unpublished data). There may be a system to amplify the appearance of receptors on the cell membranes once a rise in cAMP occurs after the addition of MSH (compare ref. 35).

Of major interest to us is an observation that MSH controls proliferation of melanoma cells in culture but not when the cells are grown as tumors in mice. We inoculated mice with Cloudman S91 melanoma cells and found that their growth was not retarded by daily injections of MSH. Similar findings have been reported for the B-16 melanoma (36).

Varga and Pawelek (manuscript in preparation) proposed an explanation for this phenomenon based upon the observations that cultured Cloudman melanoma cells do not exhibit MSH receptor activity throughout the cell cycle but rather restrict the availability of receptors to the G-2 phase (34). We could explain how cells escape the proposed "G-1 restriction point common to normal nonproliferating-cells" (37). Perhaps MSH receptor activity is discontinuous throughout the cycle so that intracellular cAMP levels are low during periods of DNA synthesis and high during periods of melanization. Thus, *cells could escape specific restriction points by the discontinuous reception of signals.*

However, the reason melanoma cells in culture become blocked after one or two cell divisions remains unclear (Fig. 1a). Perhaps the cells in culture are unable to release the MSH once it is bound to them in the G-2 phase and they become restricted when they traverse the cycle to G-1 again.

It is obvious from the analyses of these cells that the control of their phenotypic expression is of a high order of complexity. However, we believe that the further isolation and analysis of mutants, and particularly the achievement of genetic complementation between them should be of great use in understanding some of the control mechanisms that are involved.

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