# Regulation of Melanocyte Stimulating Hormone Action at the Receptor Level: Discontinuous Binding of Hormone to Synchronized Mouse Melanoma Cells During the Cell Cycle

### (hormone receptor/cyclic AMP/tyrosinase)

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ABSTRACT    Melanocyte stimulating hormone coupled to Sepharose effects an increase in tyrosinase (EC 1.14.18.1; monophenol monooxygenase) activity of cultivated mouse melanoma cells. Synchronized cells are found to respond to melanocyte stimulating hormone only in the G2 phase of the cell cycle, although their response to cyclic AMP is independent of position in the cell cycle. The binding of ¹²⁵I-labeled melanocyte stimulating hormone occurs predominantly in G2. These observations are satisfied by a model in which the hormone can activate adenylate cyclase (EC 4.6.1.1) by binding to a melanocyte stimulating hormone receptor only in G2; the events distal to cyclic AMP production can occur throughout the cell cycle.

A mouse melanoma cell line has been described which responds dramatically to melanoyte stimulating hormone (MSH). This peptide hormone effects large increases in tyrosinase (EC 1.14.18.1; monophenol monooxygenase) activity, melanin content, and intracellular levels of cyclic AMP, as well as changes in growth characteristics and cellular morphology (1, 2). Cyclic AMP or its analogue, dibutyryl cyclic AMP, can substitute for MSH to produce the same responses. These findings are consistent with previous observations in mammals that MSH acts through cyclic AMP (3–5). Studies with synchronized cells reveal that the effects of MSH are localized to a specific phase of the cell cycle. Whenever MSH is added to synchronized cells, increases in tyrosinase activity and cyclic AMP levels are maximal in the G2 phase (6).

In this paper we wish to report our findings that melanocytes from a mouse melanoma respond to dibutyryl cyclic AMP throughout the cell cycle, but that the binding of labeled MSH to the cells is discontinuous and takes place only in G2.

## MATERIALS AND METHODS

*Culture Conditions.* Melanocytes of the Cloudman mouse melanoma line (NCTC 3960, CCl 53) were cultured in 30-ml Falcon tissue-culture flasks. Synchronized cells were obtained by arresting the cells in mitosis with colchicine and harvesting them at that time as described (6). Each flask was inoculated with $2 \times 10^5$ cells.

Abbreviations: MSH, β-melanocyte stimulating hormone (Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Try-Gly-Ser-Pro-Pro-Lys-Asp); cAMP, adenosine-3:5′-cyclic monophosphate; dibutyryl cAMP, $N^6,O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate; PBS, phosphate-buffered saline (0.01 M Na-phosphate–0.15 M NaCl, pH 7.4).

*Preparation of ¹²⁵I-Labeled MSH.* ¹²⁵I-labeled MSH was synthesized by the chloramine-T method (8) with the following modifications: 2.5 μg ($10^{-9}$ mol) of β-MSH was iodinated for 15 sec at room temperature with 5 mCi of carrier-free ¹²⁵I (New England Nuclear Corp.) in a total volume of 0.06 ml. The labeled peptide was separated from the other components of the reaction mixture on a 1 × 55-cm Sephadex G-10 column equilibrated with phosphate-buffered saline, pH 7.4 (PBS). The short exposure to a large excess of ¹²⁵I insures that only tyrosine (the fifth residue) is substituted, and at the same time high specific radioactivity (about 2000 Ci/mmol) is obtained. The preparation retained its original biological activity, i.e., melanoma cells exposed to the purified ¹²⁵I-labeled MSH responded with increased tyrosinase activity and melanization. Retention of biological activity was expected since the major portion of the activity resides in the middle heptapeptide of MSH, which does not contain tyrosine (9).

*Preparation of Sepharose–MSH.* MSH was coupled to CNBr-activated Sepharose (10) by the following procedures. Sepharose 4B suspension (Pharmacia, Sweden, about 1 g of dry material) was washed several times with distilled water. To a 50-ml suspension of Sepharose was added 20 ml of 5% aqueous solution of CNBr. The suspension was stirred at 20°. Four milliliters of 1 N NaOH was added a drop at a time over a 5-min period to maintain the pH at 10–10.5. The suspension was poured immediately into 5 liters of 5 mM NaHCO₃ at 4° and filtered through Whatman No. 3 on a Büchner funnel. The Sepharose cake retained by the filter was washed with 5 liters of NaHCO₃ solution. The beads were suspended in 50 ml of 0.1 M NaHCO₃, and 2.5 mg of MSH in 2.5 ml of PBS was added. The suspension was stirred very slowly overnight at 0°. The beads were separated by filtration, washed with 1 liter of 0.1 M NaHCO₃, and kept in 0.5 M β-ethanolamine at 4° for 5 hr. The suspension was filtered, and the cake was washed with 2 liters of 50 mM NaHCO₃, and stored in the same solution at 4°. All of the MSH was bound to the Sepharose in this procedure.

*Binding of ¹²⁵I-Labeled MSH to Synchronized Cells.* At various times after the cells were released from colchicine, the medium was poured off and the cells were rinsed gently with 30 ml of Hank's balanced salt solution at 37°. The salt solution was poured off immediately, the flasks were drained for 1 min, and 80,000 cpm of ¹²⁵I-labeled MSH in 10 μl of PBS was pipetted onto a marked area of each flask. The flasks were

closed and incubated at 37° for 15 min. The unbound $^{125}$I-labeled MSH was removed by rinsing the cells three times with 30 ml of PBS at room temperature. The flasks were then drained for 2 hr and dried in a vacuum dessicator at room temperature overnight. The marked area of the flask where $^{125}$I-labeled MSH had been applied (1 inch$^2$) was cut out with a red-hot razor blade, and the side to which the cells were attached was marked with radioactive ink to permit later alignment of the cells with the autoradiographs. The plates were pressed against x-ray film with the cells facing the film for 1–3 days. After the exposure was completed the plates were crushed and the radioactivity was determined in a gamma-counter.

[$^3$H]*Thymidine Incorporation.* The S-phase was determined by measuring the incorporation of [$^3$H]thymidine into DNA (11).

## RESULTS

*Location and Number of MSH-Binding Receptors.* MSH, linked to Sepharose beads by covalent bonds, stimulated both tyrosinase activity and melanization (Table 1). These findings showed that MSH reacted with receptors at the cell surface.

After the cells were labeled in the G2 phase in the culture flask with $^{125}$I-labeled MSH, and the cellular components separated by sedimentation in a sucrose density gradient (12), a major part of the MSH-binding activity was found to be in the plasma membrane fraction. $^{125}$I-labeled MSH binding to immobilized G2 phase cells gave the kinetics of a reaction of the saturation type. The process could almost be eliminated by the addition of saturating amounts of unlabeled MSH. A preliminary estimate of the association constant gave a value of $K_0 = 3 \times 10^8$ liters/mol. The $^{125}$I-labeled MSH concentration required for 50% saturation of binding sites is approximately the same as that required to elicit half of the maximal stimulation of tyrosinase activity by unlabeled MSH. At saturation with $^{125}$I-labeled MSH, the number of receptors per cell was calculated to be in the order of $10^4$ (Varga *et al.*, in preparation).

*Binding of MSH During the Cell Cycle.* Very little binding of $^{125}$I-labeled MSH occurred in G1 phase; there was measurable binding in S, but by far the major binding of the hormone occurred as S terminated and the cells entered G2 (Fig. 1). The low level of binding in G1 was not a consequence of washing the cells away; the cells remained attached to the plastic substrate (Fig. 2B). At the beginning of mitosis (45–50 hr after release from colchicine), a decline in MSH binding was seen in several experiments. The precise end of the MSH-binding period could not be located at this time because the cells were no longer synchronized.

*Binding of MSH to Synchronized and Nonsynchronized Cells.* Synchronized cells were highly homogeneous in their binding characteristics. In G2, virtually all of the cells were labeled (Fig. 2A), but in G1 practically none were (Fig. 2B). As expected from these results, nonsynchronized cells were heterogeneous in their binding, some of the cells being labeled and others not (Fig. 2C).

*Sensitivity of MSH Binding to Proteolytic Treatment.* One explanation for the absence of the binding of MSH in G1 and S would be that the availability of the MSH receptor is related to the configuration of the cell, which changes through the
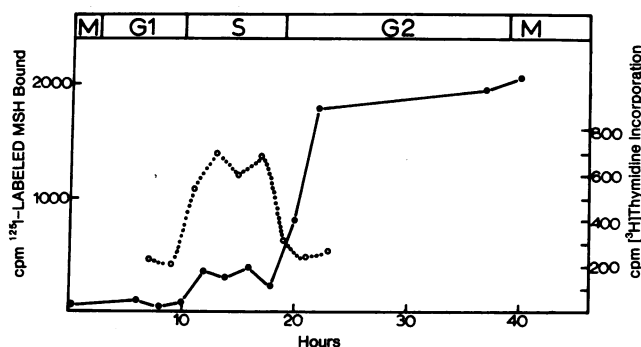


Fig. 1. Binding of $^{125}$I-labeled MSH to melanocytes during the cell cycle. Radioactivity of cells exposed to $^{125}$I-labeled MSH at various times after inoculation was determined as described in the *text*. A background of 450 cpm was subtracted from each point. The values were averages of two measurements. Three culture flasks for one point were used to determine [$^3$H]thymidine incorporation. These experiments were repeated three times with similar results each time.

cycle (13). It is possible that such masking of the receptor might be selectively eliminated by mild proteolytic treatment. In mouse melanoma cells such treatment completely abolished MSH binding. At various stages in the cell cycle, cells were incubated for 5 min with 10–50 μg/ml of trypsin or chymotrypsin. Afterwards, the enzymes were removed and $^{125}$I-labeled MSH was added. There was no binding in G1, S, or G2. Since the receptors were sensitive to proteolysis, we could not determine whether "masked" receptors were present when the cells were not in G2.

*Effects of Dibutyryl Cyclic AMP on Tyrosinase Activity in Synchronized Cells.* It has previously been demonstrated that either cyclic AMP (1) or its analogue, dibutyryl cyclic AMP (1, 4), can substitute for MSH in eliciting increased tyrosinase activity and melanin content in mouse melanoma cells. It has also been shown that increased tyrosinase activity and cyclic AMP content in response to MSH are seen only in the G2 phase of the cell cycle (6). It is now clear that the reason for the localization in G2 is that the cells can bind MSH only in this phase of the cycle. It remained to be seen whether the response to cyclic AMP was also restricted to the G2 phase. Results in Fig. 3 show that the response of synchronized cells

TABLE 1. *Stimulation of tyrosinase activity in melanoma cells by MSH covalently linked to Sepharose*

| Treatment | cpm of $^3$H$_2$O production |
|---|---|
| None | 86 |
| β-MSH (0.14 μM) | 3546 |
| β-MSH linked to Sepharose (0.23 μM) | 3274 |

Melanoma cells (2 × 10$^5$) were inoculated into 30-ml Falcon tissue-culture flasks in 5 ml of culture medium. Tyrosinase activity was measured (1) after 48 hr of treatment with either culture medium only, free β-MSH, or β-MSH linked to Sepharose beads. Tyrosinase activity was expressed as cpm of $^3$H$_2$O released into 0.5 ml of culture medium per 24 hr. The numbers represent averages of duplicate culture flasks within the same experiment. The variation between duplicates was less than 10%. This experiment was repeated three times with similar results each time.
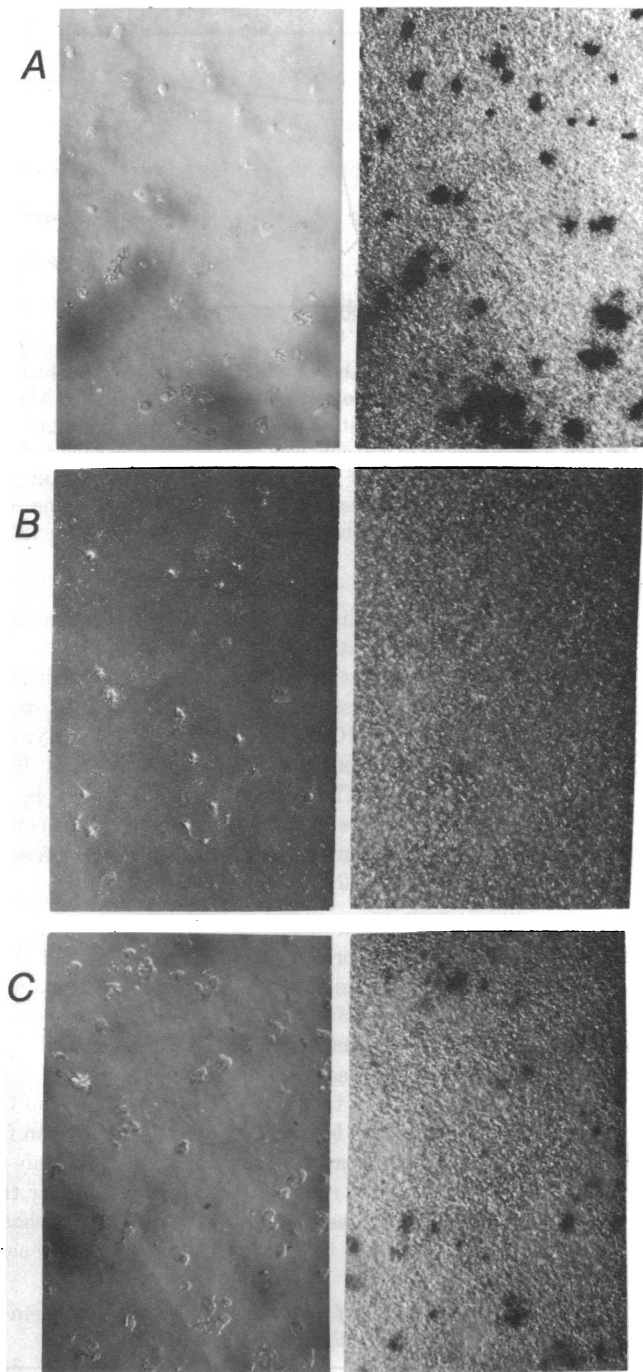
FIG. 3. Response of synchronized melanoma cells to dibutyryl cyclic AMP. Cells were synchronized and seeded at $5 \times 10^5$ cells per flask in 5 ml of culture medium. Dibutyryl cyclic AMP (1 mM) and $6 \times 10^{-4}$ M theophylline were added 8 hr before the first point for each curve. Each point represents duplicate flasks; the difference between tyrosinase activities at each point was less than 5%. Tyrosinase activity is expressed as $^3H_2O$ released into 0.5 ml of culture medium per 8 hr.



FIG. 2. Binding pattern of synchronized and nonsynchronized cell populations. The plastic plates carrying the dehydrated cells were aligned on a microscope with their autoradiographed replica by the help of the visible radioactive mark made on the plastic plates before autoradiography was set up. Pictures were taken of a synchronized culture 31 hr after release from colchicine (*A*) and 7 hr after release from colchicine (*B*), and of a 2-day-old nonsynchronized culture (*C*), with the microscope focussed first on the cells (*left*) and then on the corresponding autoradiograph (*right*).

to cyclic AMP was independent of their position in the cell cycle. The cells are continuously prepared to respond to cyclic AMP, but their intracellular levels of cyclic AMP remain relatively low unless they are exposed to MSH in the G2 phase.
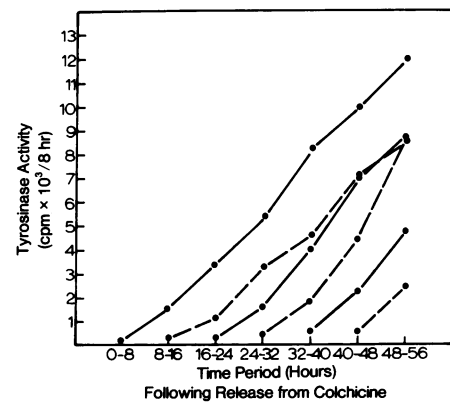
## DISCUSSION

We have demonstrated that MSH acts at the cell membrane and that the binding of MSH to the membrane of cultured melanoma cells is discontinuous and occurs primarily in the G2 phase of the cell cycle. The simplest explanation for this observation is that specific cell-surface receptors for MSH are available only in G2. It is likely that phenotypic expression of melanoma cells is regulated by the oscillation of the availability of these receptors. The reasons for localizing reception of binding to G2 and thereby restricting phenotypic expression are obscure, because the cells are able to respond to cyclic AMP throughout the cycle.

When cells are exposed to MSH in G2, cyclic AMP levels rise within 10 min; however, there is a lag period of 8–10 hr before increased tyrosinase activity is seen (6). Cells exposed to dibutyryl cyclic AMP at any time in the cell cycle exhibit the same 8 to 10-hr lag period (Fig. 3). This period is of interest because events leading to increased tyrosinase activity must occur during this time. We do not know whether the increased tyrosinase activity is due to synthesis of new tyrosinase molecules or activation of preexisting ones.

A major question that emerges from these experiments is whether the MSH receptors appear in G2 from *de novo* synthesis or whether they are somehow masked during other phases of the cycle. The experiments with proteolytic digestion are inconclusive because even though we failed to "unmask" a silent receptor in G1 and S, we also destroyed receptor activity in G2. The extreme sensitivity of the receptors to proteolytic treatment indicates that proteins play a role in receptor activity. Recently, we found that MSH-receptor activity increases as the plasma membranes are purified. This finding is consistent with the possibility that a blocking moiety is lost during purification of the receptors.

Evidence from many sources has implicated cyclic AMP as the intracellular mediator for the response of melanocytes to MSH (1–6, 15, 16). We have shown that melanoma cells regulate their response to MSH by making MSH receptors available at a particular time in the cell cycle. Since the cell can respond to cyclic AMP at any time in the cycle, we believe

it is likely that the sole role for MSH is to promote increased cyclic AMP concentrations within the cells. The melanocytes then respond to the higher levels of cyclic AMP with increased tyrosinase activity and melanin content, and changes in growth characteristics and morphology.

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