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# LONG-TERM AND RESIDUAL MELANOTROPIN-STIMULATED TYROSINASE ACTIVITY IN S91 MELANOMA CELLS IS DENSITY DEPENDENT

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## SUMMARY

Cell density is a factor that affects the capacity of Cloudman S91 melanoma cells to respond to melanotropins in monolayer culture. Continuous exposure of melanoma cells to α-melanotropin or its potent analog [Nle$^4$, *D*-Phe$^7$]-α-MSH, resulted in maximal stimulation of tyrosinase after 2 d of treatment, but the magnitude of stimulation decreased thereafter despite the continued presence of the melanotropins. However, when melanoma cells continually exposed to melanotropins were subcultured to an initial low cell density and maintained in contact with α-MSH or [Nle$^4$, *D*-Phe$^7$]-α-MSH (long-term culture), tyrosinase activity was rapidly restored and greatly enhanced. Also, when cells were seeded at initial densities ranging from 0.2 to 3.2 × $10^6$ cells/flask, and exposed for 24 h to $10^{-7}$ *M* α-MSH, only the cultures seeded at low densities (0.2 and 0.4 × $10^6$ cells/flask) exhibited maximal tyrosinase activity during the 24 h exposure to the melanotropins. Therefore, tyrosinase activity was primarily affected by cell density rather than by the duration of time the cells were in culture or by continuous exposure to melanotropin. Other flasks of various cell densities were treated with $10^{-7}$ M α-MSH or [Nle$^4$, *D*-Phe$^7$]-α-MSH for 24 h, followed by *removal* of the melanotropins from the culture medium. The magnitude and duration of the *residual* stimulation of melanoma tyrosinase activity by melanotropins were also found to be dependent on the initial cell density. These results reveal that there is a limited range of optimal cell densities at which melanoma cells can respond to melanotropins and express increased tyrosinase activity.

*Key words:* melanoma; melanotropins; tyrosinase; residual stimulation.

## INTRODUCTION

One function of α-melanotropin (α-melanocyte-stimulating hormone, α-MSH) is to regulate integumental pigmentation in many vertebrate species (8,20). This tridecapeptide, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-$NH_2$, stimulates melanogenesis within normal as well as abnormal (malignant) melanocytes (6,8,14,15). Many of the processes involved in the mechanism of action of α-MSH have been elucidated using murine S91 melanoma cells (14). α-MSH binds to specific membrane receptors, resulting in stimulation of adenylate cyclase which, in turn, increases the intracellular levels of cyclic AMP, an effect that is detectable within 20 min after exposure of melanoma cells to the hormone. Elevated cyclic AMP levels lead to stimulation of tyrosinase, the rate-limiting enzyme of the melanin biosynthetic pathway, which eventually results in increased melanogenesis (14).

[Nle$^4$, *D*-Phe$^7$]-α-MSH is a superagonist that exhibits ultraprolonged action on both normal and abnormal melanocytes (9,18,19). This α-melanotropin analog is 100-fold more effective than α-MSH in stimulating S91 melanoma tyrosinase activity (10). We have shown that the degree of tyrosinase stimulation by α-MSH and [Nle$^4$, *D*-Phe$^7$]-α-MSH is governed by at least two factors: the concentration of melanotropin and the time the melanotropin is in contact with the melanoma cells (10). Our studies involving continuous exposure of cells to melanotropins indicated that a third factor, namely, cell density, affects the magnitude of melanotropin-induced tyrosinase activity, as first reported by Fuller and Lebowitz (7). We now provide data indicating that cell density at the time of melanotropin exposure also affects the magnitude of the residual (i.e. prolonged) tyrosinase activity after the melanotropins are removed from the culture flasks.

## MATERIALS AND METHODS

*Materials.* Ham's F10 medium, horse serum, fetal bovine serum, and penicillin-streptomycin solution were purchased

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from GIBCO Laboratories (Santa Clara, CA). Radioactive *L*-3′,5′-[$^3$H]-tyrosine (specific activity 53.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA). α-MSH was obtained from Sigma Chemical Co. (St. Louis, MO). The melanotropin analog, [Nle$^4$, *D*-Phe$^7$]-α-MSH was synthesized in the laboratory of Dr. V. J. Hruby, Department of Chemistry, University of Arizona, according to previously published methods (18,19).

*Cell culture.* The murine S91 melanoma cell line, 3960, CCL 53.1, was obtained from the American Type Culture Collection Cell Repository (Rockville, MD). Periodically, cells were injected subcutaneously into syngeneic mice (DBA/2J, Jackson Laboratories, ME) and the tumors formed were then excised, reduced to single cells, and cryopreserved in liquid nitrogen. Cells from the frozen stock were maintained in monolayer culture by limited weekly subculturing for a maximum of 10 passages. Cells were grown in Ham's F10 medium, supplemented with 10% horse serum, and 2% fetal bovine serum, both heat-inactivated at 56° C for 30 min, and with 100U/ml:100 μg/ml penicillin-streptomycin, and were incubated in a humidified incubator (5% $CO_2$:95% air) at 37° C.

*Stimulation of melanoma tyrosinase by continuous melanotropin treatment.* Flasks were seeded at a density of 0.2 × 10$^6$ cells/25-cm$^2$ flask, or at greater cell densities, in 4 ml of medium, and 24 h later (Day 2 of culture) were treated with $10^{-7}$ *M* α-MSH or [Nle$^4$, *D*-Phe$^7$]-α-MSH. Twenty-four hours before data collection, the medium in a set of control and melanotropin-treated flasks was changed and replaced with 4 ml of medium containing 1 μCi/ml [$^3$H]-tyrosine. At the end of each consecutive 24-h incubation period, cultures that were maintained in the presence of medium containing [$^3$H]-tyrosine were harvested using EDTA-containing Tyrode's solution, and the cells were counted with the aid of a hemacytometer. The labeled medium from each flask was collected daily for determination of tyrosinase activity from Days 2 to 10 in culture. To determine the effects of long-term culture and continuous melanotropin treatment, after 3 and 6 d of melanotropin contact (Days 4 and 7 in culture), some control and treated flasks were harvested and subcultured to the original initial cell density of 0.2 × 10$^6$ cells/flask and immediately treated with melanotropins in the presence of labeled or unlabeled media. Tyrosinase activity was again determined for the initial 24-h exposure period after subculturing, and daily thereafter for 2 more d.

*Residual stimulation of melanoma tyrosinase activity by melanotropins.* Cells were seeded at a density of 0.2 × 10$^6$ cells/25-cm$^2$ flask or greater, and treated 24 or 48 h later (on Day 2 or 3 in culture, respectively) with $10^{-7}$ *M* α-MSH or [Nle$^4$, *D*-Phe$^7$]-α-MSH for a 24-hr period. Then the melanotropins were removed from the culture flasks by rinsing several times with 4 ml of serum-free Ham's F10 medium until no melanotropic activity could be detected in the incubation medium by frog skin bioassay (2). Melanotropin-stimulated tyrosinase activity during the 24-h exposure period was determined ($T_0$). Daily measurements of prolonged (residual) tyrosinase stimulation began immediately after melanotropin removal by addition of 4 ml of [$^3$H]-tyrosine containing medium (1 μCi/ml) to groups of control and melanotropin-treated flasks for consecutive 24-h periods. At the end of each 24-h incubation with [$^3$H]-tyrosine the incubation media were removed and the cells were harvested and counted with the aid of a hemacytometer. The medium was changed daily in all remaining flasks.

*Tyrosinase assay.* A modification of the charcoal absorption method (6,7,24) developed by Pomerantz (16,17) was used to determine tyrosinase activity. In this modified assay, the amount of $^3H_2O$ released into the culture medium by cells growing in situ, when [$^3$H]-tyrosine was converted to L-Dopa during a 24-h period was measured. Earlier studies have shown that the release of $^3H_2O$ from cells growing in situ into the incubation medium parallels intracellular tyrosinase

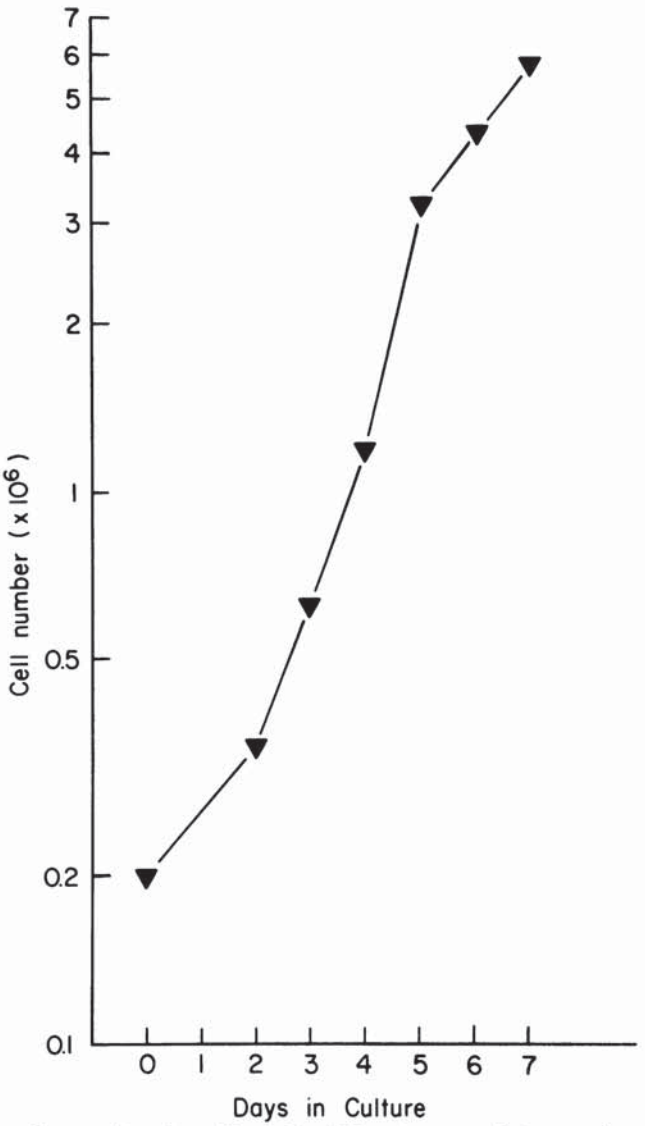


FIG. 1. Growth profile of S91 CCL melanoma cells in monolayer culture. Cells were seeded at 0.2 × 10$^6$ cells/25-cm$^2$ flask and the medium in all flasks was changed daily. Cells were counted with the aid of a hemacytometer after 48 h, and daily thereafter for 5 subsequent d. Each value represents the mean cell number of three flasks and all standard errors were less than 10% of the mean value.

activity (24). Duplicate 1-ml aliquots were taken from the medium in each flask and pipetted into glass test tubes. Each sample was then treated with 1 ml of activated charcoal (10% wt/vol in 0.2 *N* citric acid), vortexed and immediately centrifuged at 1300 × *g*. One milliliter of the supernatant was aliquoted from each tube and passed through a Dowex 50 W column, and each column was then rinsed with 1 ml of 0.1 *N* citric acid. The eluents were collected into scintillation vials, which then received 12 ml of scintillation fluid (tolune:Triton X-100, 2:1 vol/vol + 5.5 g diphenyloxazole/liter). The vials were counted in a Beckman LS-8000 scintillation spectrometer. Data were presented as counts per minute (cpm) per million ($10^6$) cells, ± SE, and also in units of nanomoles tyrosinase activity/$10^6$ cells/24 h. Treatment groups were determined to be different from control by Student's *t*-test. For some graphic presentations, data (cpm/$10^6$ cells) were expressed as a percent of control (100%), ± SE.

## RESULTS

*Melanoma cell growth in culture.* When S91 melanoma cells were grown for 7 d in culture, the cells that were seeded at 0.2 × $10^6$ cells/flask doubled once during the first 2 d in culture and grew logarithmically through Day 5 in culture, with an average doubling time of about 24 h (Fig. 1). The rate of proliferation declined after 5 d in culture as cells reached confluency in the 25-cm² flasks.

*Response of melanoma cells to continuous melanotropin exposure.* Melanoma cells were seeded at 0.2 × $10^6$ cells/25-cm² flask, and treated the 2nd d in culture and daily thereafter with $10^{-7}$ *M* α-MSH or [Nle⁴, *D*-Phe⁷]-α-MSH for 6 consecutive d, and tyrosinase activity was determined each day (Fig. 2, *solid lines*). Prolonged exposure of the cells to the melanotropins did not affect their rate of proliferation because the number of cells in melanotropin-treated flasks was comparable to the cell number in control (untreated) flasks (1,6). Tyrosinase activities increased and reached maximal levels (approximately 20 × $10^3$ cpm/$10^6$ cells) after 2 d of melanotropin treatment, with tyrosinase levels five to six-fold higher than control levels. The magnitude of both the basal and maximal tyrosinase activities was gradually diminished during Days 3 to 6 of exposure to either melanotropin (Fig. 2, *solid lines*). Days 3 to 6 of melanotropin treatment corresponded to Days 4 to 7 in culture, demonstrating that the ability to stimulate S91 (CCL 53.1) melanoma tyrosinase with melanotropins was greatest during midlogarithmic growth (Days 3 and 4 after seeding) and diminished as cells approached confluency.

*Restoration of melanoma tyrosinase responsiveness after subculture.* In the experiment described above (Fig. 2), after 3 and 6 d of continuous melanotropin treatment (Days 4 and 7 in culture), groups of control and melanotropin-treated flasks with cells that had not previously been subcultured were harvested and reseeded at a density of 0.2 × $10^6$ cells/flask, with the melanotropins immediately readded to determine the effects of long-term melanotropin treatment and reduction of cell density on tyrosinase activity. On Day 4 of melanotropin treatment, and 24 h after reducing cell density, the response of the subcultured cells (Fig. 2, *middle peak*) to the melanotropins was about double (approximately 30 × $10^3$ cpm/$10^6$ cells) that of the nonsubcultured cells (Fig. 2, *solid line*; 12 × $10^3$ cpm/$10^6$ cells). The restored tyrosinase activity gradually declined in the continued presence of the melanotropins, and on Day 6, 3 d after subculturing, the enzyme activities were again suppressed (Fig. 2, decline of the *middle peak*).

An even more dramatic enhancement of maximal tyrosinase activity was evident after some flasks with cells

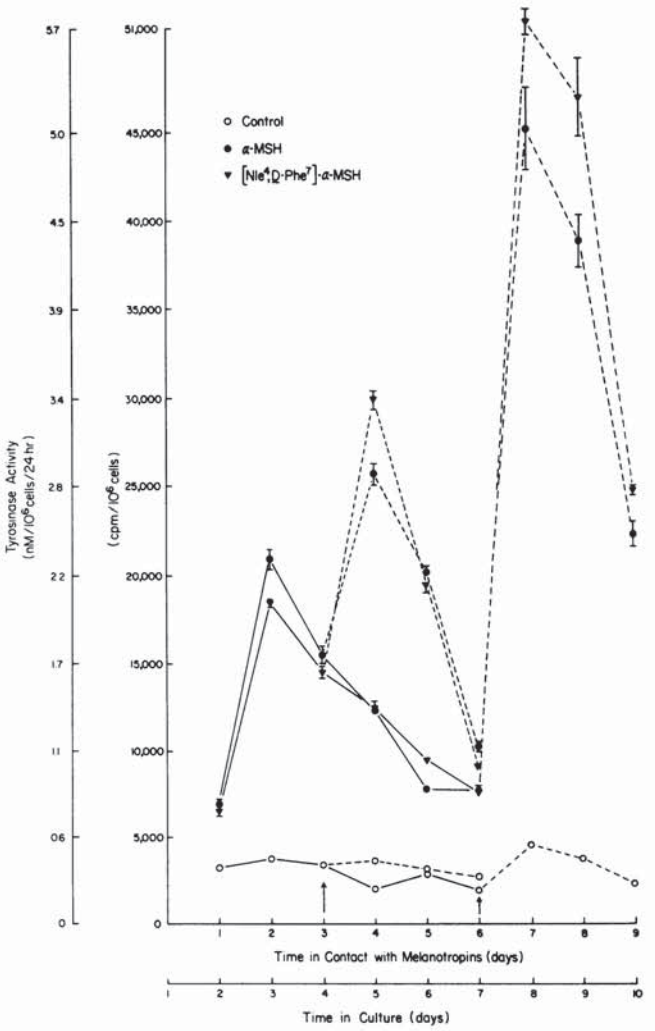


FIG. 2. Response of S91 CCL melanoma cells to continuous and long-term melanotropin exposure. Cells were seeded at 0.2 × $10^6$ cells/25-cm² flask and exposed to $10^{-7}$*M* α-MSH or [Nle⁴, *D*-Phe⁷]-α-MSH for 6 consecutive d. After the third and sixth d of contact with the melanotropins, some flasks were subcultured and the cells were reseeded at the initial low cell density of 0.2 × $10^6$ cells/flask, and immediately exposed to the melanotropins for 3 more d. *Solid lines* represent daily tyrosinase activities (mean cpm/$10^6$ cells or n*M*/$10^6$ cells/24h of 6 determinations, ± SE) of control (○) and melanotropin-treated (α-MSH, ●; [Nle⁴, *D*-Phe⁷]-α-MSH, ▼) cells that were not subcultured. *Broken lines* represent daily tyrosinase activities of control cells and melanotropin-treated cells that were subcultured after 3 or 6 d of contact with the melanotropins.

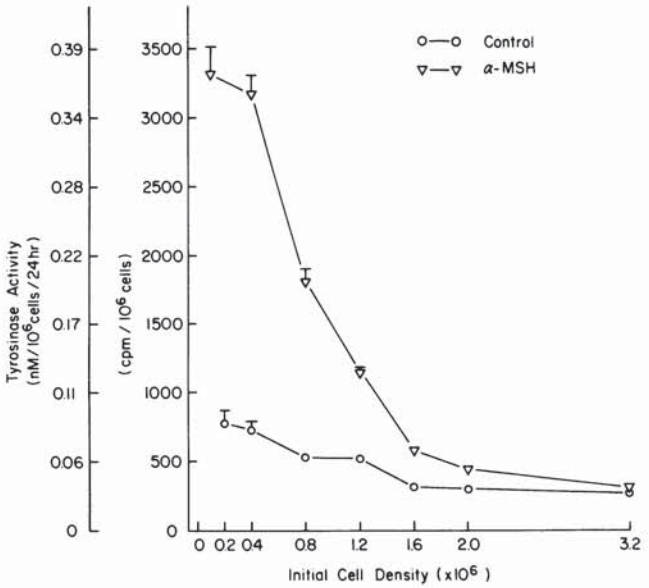


FIG. 3. The effects of initial cell density on basal and MSH-stimulated tyrosinase activities. Cells were seeded at different initial cell densities and treated the next day with $10^{-7}$ *M* α-MSH for 24 h, after which tyrosinase activity was determined. Each value represents the mean cpm/$10^6$ (or n*M*/$10^6$ cells/24 h) of 6 determinations, ± SE, for control (○) or α-MSH-treated (▽) cells.

that were maintained in continuous contact with the melanotropins for 6 d without subculturing were subcultured to 0.2 × $10^6$ cells/flask (Fig. 2, *third peak*). On Day 7 of melanotropin treatment, 24 h after reducing cell density, the subcultured cells exhibited a further increase in the magnitude of the maximally achievable tyrosinase activity (51 × $10^3$ cpm/$10^6$ cells) in the face of concurrent increases in basal enzyme activity (Fig. 2, *third peak* and corresponding *basal lines*). Again, enhanced tyrosinase activity gradually declined as cell density increased.

*Melanotropin-stimulated tyrosinase activity of melanoma cells seeded at different initial densities.* To determine whether cell density, rather than duration in continuous culture, was responsible for the decreased responsiveness of melanoma cells to the melanotropins, cells were seeded at different initial densities and exposed to $10^{-7}$ *M* α-MSH on Day 2 of culture for 24 h in the presence of [$^3$H]-tyrosine-labeled medium, and tyrosinase activity was determined at the end of this 24-h exposure (Fig. 3). α-MSH increased tyrosinase activity three to four fold above the control level in flasks initially seeded at cell densities of 0.2 and 0.4 × $10^6$ cells/flask, but the magnitude of stimulation decreased proportionately with the increase in initial seeding density. A gradual decrease in basal tyrosinase activity occured as initial seeding density increased (Fig. 3). These results suggest that the decrease in the magnitude of the melanotropin-stimulated tyrosinase activity is influenced by cell density and is not related to insensitivity of the cells due to continuous culture or prolonged treatment with melanotropins.

*Effect of melanoma cell density on residual melanotropin action.* The possible effect of cell density on the magnitude of residual stimulation of tyrosinase activity by melanotropins was investigated. In one experiment, cells were seeded at 0.2 × $10^6$ cells/flask and exposed on the following day (Day 2 in culture) to $10^{-7}$ *M* α-MSH for 24 h. Then both control and treated cultures were rinsed twice so that the melanotropin was completely removed from the treated flasks. Tyrosinase activity was determined right at the end of the exposure period (24 h) to melanotropins ($T_0$) and every 24 h thereafter for Days 1 to 4 after melanotropin removal (Days 3 to 7 in culture). In the latter case, tyrosinase activity per $10^6$ cells was minimally increased on Day 1 after α-MSH was removed, and returned to basal level on Day 2 (Fig. 4). Another set of flasks were seeded at the same density of 0.2 × $10^6$ cells/flask, but were treated with the melanotropin on Day 3 (rather than Day 2) in continuous culture, and residual melanotropic activity was determined daily for Days 1 to 4 after melanotropin removal (Day 4 to 8 in

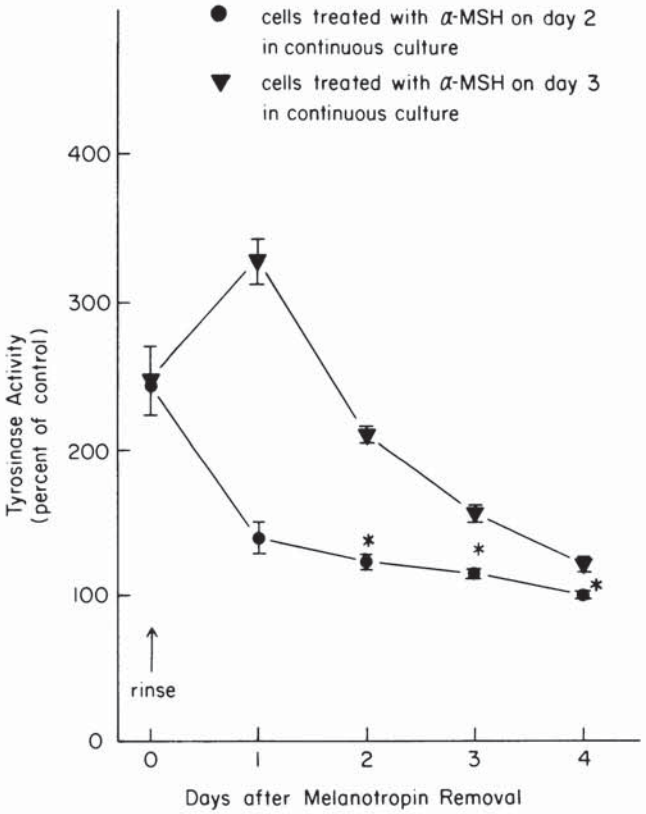


FIG. 4. Residual tyrosinase activity of cells exposed to α-MSH on day 2 or 3 in culture. Cells were seeded at 0.2 × $10^6$ cells/25-cm$^2$ flask and were treated either the next day (Day 2 in culture; ●) or after 48 h (Day 3 in culture; ▼) with $10^{-7}$ *M* α-MSH for 24 h. The melanotropin was then removed from the flasks by rinsing, and tyrosinase activity was determined for the 24-h melanotropin exposure period before melanotropin removal ($T_0$), 24 h after rinsing (Day 1), and daily thereafter for 3 more d. All data were first expressed as cpm/$10^6$ cells, then as a percent of the control with the control taken as 100%. Each value represents the mean percent of control of six determinations, ± SE. An *asterisk* indicates that tyrosinase activity is not significantly different from control, as determined by Student's *t*-test at $P < 0.05$.

culture). In both cases, melanotropin-stimulated tyrosinase activities were equal in the presence of the hormone ($T_0$ values; approximately 1.5-fold above control) whether the cells were exposed to the melanotropin on Day 2 or 3 in culture. In the latter case (treatment on Day 3 in culture), the residual effect of the hormone was quite dramatic, as tyrosinase activity was increased two to three fold above basal level and remained significantly higher than control level for all 4 d after melanotropin removal (Fig. 4).

To determine the extent to which cell density affected differences in magnitude of residual tyrosinase stimulation, cells were seeded at initial densities of 0.2, 0.5, and 0.65 $\times$ $10^6$ cells/flask, and were treated on Day 2 in culture with $10^{-7}$ *M* α-MSH or [Nle⁴, *D*-Phe⁷]-α-MSH for 24 h. On Day 1 after melanotropin removal (Day 3 in culture), tyrosinase activity per $10^6$ cells was maximally stimulated to three fold above basal level by both melanotropins when the cells were seeded at 0.5 $\times$ $10^6$ cells/flask (Fig. 5). This stimulation was greater than when the melanoma cells were seeded at either 0.2 or 0.65 $\times$ 10 cells/flask.

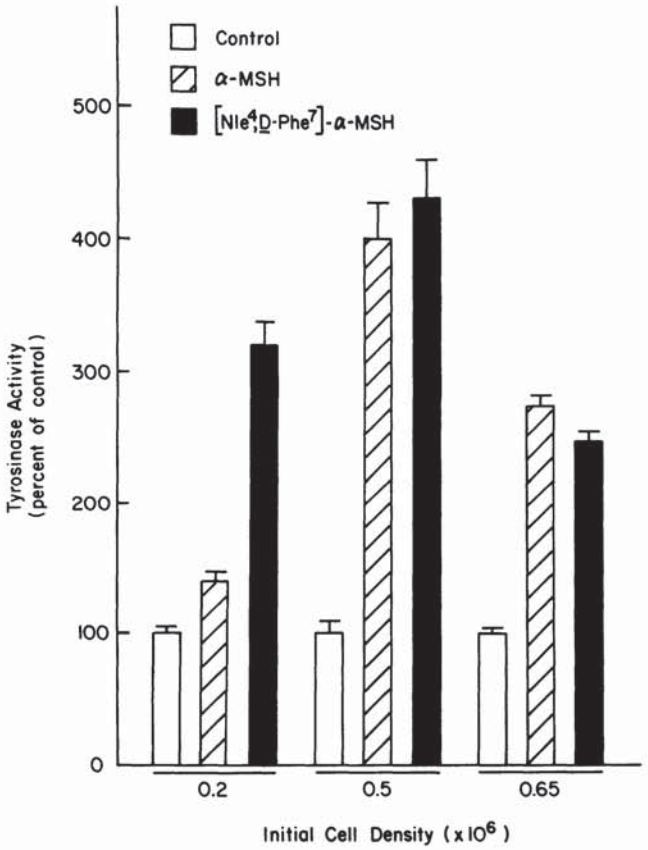


FIG. 5. Residual melanotropin stimulated tyrosinase activity of cells seeded at different initial cell densities. Cells were seeded at 0.2, 0.5, or 0.65 $\times$ $10^6$ cells/25-cm² flask, and were exposed the next day to $10^{-7}$ *M* α-MSH or [Nle⁴, *D*-Phe⁷]-α-MSH for 24 h. The melanotropins were then removed as described in Materials and Methods. Tyrosinase activity was determined for the first 24-h period after melanotropin removal. Data (cpm/$10^6$ cells) were expressed as the mean percent of control (100%) of six determinations, ± SE.

Cells initially seeded at 0.5 $\times$ $10^6$ cells/flask and treated on Day 2 in culture (Fig. 5), and cells seeded at 0.2 $\times$ $10^6$ cells and treated on Day 3 in culture (Fig. 4), attained similar final cell densities (1.33 ± 0.02 and 1.28 ± 0.04 $\times$ $10^6$ cells/flask, respectively) as well as near equivalent residual tyrosinase activity. This again suggests that it is cell density, rather than duration of time in culture, which affects the ability of the melanotropins to induce residual tyrosinase activities.

## DISCUSSION

α-MSH and [Nle⁴, *D*-Phe⁷]-α-MSH stimulate murine S91 melanoma tyrosinase activity in a dose-dependent manner (10). The rate of tyrosinase activity per million cells increases in direct proportion to time of melanotropin contact during the first 48 h of contact with α-MSH or [Nle⁴, *D*-Phe⁷]-α-MSH (10). To determine the maximal tyrosinase stimulation achievable with continuous melanotropin treatment, cells were treated with $10^{-7}$ *M* of the above two melanotropins for 6 d, and tyrosinase activity was measured daily. In response to α-MSH or [Nle⁴, *D*-Phe⁷]-α-MSH, enzymatic activity reached a maximum of five to six fold above control level after 2 d of treatment, but gradually declined thereafter to three to four fold above basal level (Fig. 2).

Similar to our findings, Fuller and Lebowitz (7) previously reported that continuous exposure of Cloudman melanoma cells to α-MSH maximally stimulated tyrosinase activity by 48 h of exposure to the melanotropin, but the magnitude of stimulation decreased thereafter despite continuous cellular contact with the hormone and a doubling of the melanotropin concentration every day to compensate for the cell population doubling that occured every 24 h. Increasing the concentration of α-MSH did not prevent the decline in the stimulated enzyme levels, suggesting that the melanotropin concentration used in our experiments ($10^{-7}$ *M*) was not limiting to the cellular response in high-density cultures. We have also demonstrated that the stimulation of tyrosinase activity by a superpotent analog [Nle⁴, *D*-Phe⁷]-α-MSH, which is resistant to inactivation by serum enzymes (3,4), is affected by cell density as are the actions of the native melanotropin, α-MSH. Thus, the present experiments confirm that the decreased tyrosinase response to melanotropin treatment was not due to a decreasing availability of hormone.

The decreased cellular responsiveness to melanotropins may be attributed to one or more of the following factors: (a) melanotropin receptor down regulation due to prolonged exposure of the cells to melanotropins; (b) increased cell density with days in culture; or (c) the duration of the time the melanoma cells were in culture, or both (b) and (c). To determine if the gradual loss of responsiveness to melanotropins was due to down regulation of the melanotropin receptor, cells exposed to α-MSH or [Nle⁴, *D*-Phe⁷]-α-MSH for 3 or 6 consecutive d were subcultured and seeded at 0.2 $\times$ $10^6$ cells/flask, and kept in *continuous* contact with the melanotropins. This resulted in an immediate restoration and enhancement of

tyrosinase activity (Fig. 2). We have shown that as Cloudman S91 melanoma cultures approach confluency the proliferative rate slows (Fig. 1), possibly because of increased cell cycle time or because of the arrest of some cells in $G_0$ phase. It has been proposed that S91 melanoma melanotropin receptors are only expressed during the $G_2$ phase of the cell cycle (22). If this is the case, then subculturing of high-density cultures to a low density may allow the cells to traverse the cell cycle and to gain express melanotropin receptors, thus regaining their responsiveness to melanotropins. Fuller and Lebowitz (7) noted that melanoma cells maintained at high density were refractory to a prostaglandin, a methylxanthine, and even to dibutyrl cyclic AMP, which suggests that the block to enhanced tyrosinase activity is localized to a point distal to cyclic AMP production, rather than at the melanotropin receptor.

It has been demonstrated that some strains of S91 melanoma produce a tyrosinase inhibitor (5). Hence, it has been suggested that cells in high-density cultures produce a factor that inhibits tyrosinase expression. It is possible that such a factor may be diluted out when cell density is decreased, resulting in the restoration of responsiveness to melanotropins by cells in long-term culture, as seen in the present studies.

One interpretation for the incremental increases in tyrosinase activity achieved after a reduction of cell density with continued exposure to the melanotropins is that the cells may have increased the synthetic machinery required for enhanced enzyme activity (e.g., endoplasmic reticulum, RNA) that can only be expressed when cell density is decreased. In one experiment, cells were seeded at different densities and were treated the next day with $10^{-7}$ *M* $\alpha$-MSH for 24 h (Fig. 3). Similar to the results previously reported by Fuller and Lebowitz (7), maximal tyrosinase activation (four to five fold above basal level) was achieved in cultures seeded at 0.2 to 0.4 $\times$ $10^6$ cells/flask, and at higher seeding densities the magnitude of the cellular response diminished (Fig. 3). The results clearly demonstrate that cell density, rather than duration of time in culture is responsible for the decreased responsiveness of S91 melanoma cells to melanotropins, indicating that there is an optimal density at which melanoma cells can respond to melanotropin treatment with increased tyrosinase activity.

We have frequently observed that basal tyrosinase activity gradually decreased as the cells were maintained in continuous culture. The decrease in enzyme activity became evident after 4 to 5 d of maintenance in monolayer culture, as the cells approached plateau phase of logarithmic growth (Figs. 1 and 2), which fits the Type I model of melanogenesis described by Oikawa et al. (12). This decrease in basal tyrosinase activity was also related to cell density, rather than the duration of time in culture. This was evident because cells seeded into flasks at different initial densities, showed basal tyrosinase activities that were inversely proportional to the seeding density (Fig. 3)(13). In contrast to the S91 melanoma cell line utilized in our studies, two clones of B16 melanoma cells were reported to exhibit a progressive increase in enzyme activity during logarithmic growth and to achieve a maximum level at confluency (23), which fits the Type II model of melanoma cultures (12).

When the prolonged actions of $\alpha$-MSH were determined, a remarkable increase in the magnitude and duration of residual tyrosinase stimulation was observed when cells were treated on Day 3, rather than on Day 2 in culture (Fig. 4). $T_0$ values were the same for cells treated on Day 2 or 3 in culture, yet the decline of tyrosinase activity of the cells exposed to $\alpha$-MSH on Day 2 in culture was very rapid. This indicates that although the cells were able to respond equally in the presence of the melanotropin, their ability to maintain the response after the melanotropin was removed was a function of cell density.

To determine whether cell density rather than duration of time in culture was responsible for the observed differences in the magnitude and duration of residual stimulation, cells were seeded at different densities, treated after 24 h with $10^{-7}$ *M* $\alpha$-MSH or [Nle$^4$, *D*-Phe$^7$]-$\alpha$-MSH, and the prolonged effects of the melanotropins were determined 24 h after melanotropin removal (Fig. 5). The results obtained indicate that cell density is a factor that also influences the magnitude of residual tyrosinase activation.

It has previously been documented that basal tyrosinase activity of melanoma cells, as well as enzyme activity stimulated by various melanogenic factors, such as cyclic AMP, theophylline, prostaglandin $E_1$, and $\alpha$-MSH, are a function of cell density (7,12,13,23). The present study also emphasizes the importance of cell density as a factor that determines the immediate responsiveness of S91 melanoma cells to melanotropin stimulation, as well as a factor that influences the residual stimulation of tyrosinase activity by melanotropins. The present results may have implications for related studies on the response of other cell types to endocrine or related stimulation. For example, aside from the effect that cell density had on the phenotypic expression of S91 melanoma cells, plating density has also been shown to affect the proliferation of these cells and of human tumor cells in soft agar (11,21). In the bilayer agar culture system the size of colonies formed by Cloudman melanoma cells was inversely related to the initial number of cells plated, indicating that there was an optimal range of plating densities at which maximal plating efficiency and melanotropin stimulation of proliferation (large colony formation) were evident (1).

## References

1. Abdel Malek, Z. A.; Hadley, M. E.; Bregman, M. D., et al. Actions of melanotropins on melanoma cell growth in vitro. JNCI in press; 1985.
2. Castrucci, A. M. de L.; Hadley, M. E.; Hruby, V. J. Melanotropin bioassays; *in vitro* and *in vivo* comparisons. Gen. Comp. Endocrinol. 55:104–111; 1984.
3. Castrucci, A. M. de L.; Hadley, M. E.; Sawyer, T. K., et al. Enzymological studies of melanotropins. Comp. Biochem. Physiol. 78B:519–524; 1984.
4. Castrucci, A. M. de L.; Hadley, M. E.; Yorulmazoglu, E. I., et al. Synthesis and studies of superpotent melanotropins resistant to enzyme degradation. Bagnara, J.; Klaus, S. N.; Paul, E., et

al., eds. Biological, Molecular and clinical aspects of pigmentation. Pigment Cell 1985. Tokyo: University of Tokyo Press; 1985: 145–151.
5. Chian, L. T. Y.; Wilgram, G. F. Tyrosinase inhibition: its role in suntanning and in albinism. Science 155:198–200; 1967.
6. Fuller, B. B.; Viskochil, D. H. The role of RNA and protein synthesis in mediating the action of MSH on mouse melanoma cells. Life Sci. 24:2405–2416; 1979.
7. Fuller, B. B.; Lebowitz, J. Decay of hormone responsiveness in mouse melanoma cells in cultures as a function of cell density. J.Cell. Physiol. 103:279–287; 1980.
8. Hadley, M. E. The melanotropins. In: Endocrinology. New Jersey: Prentice-Hall, Inc; 1984:160–178.
9. Hadley, M. E.; Abdel Malek, Z. A.; Marwan, M. M., et al. [Nle$^4$, *D*-Phe$^7$]-$\alpha$-MSH: a superpotent melanotropin that "irreversibly" activates melanoma cell tyrosinase. Endocrin. Res. in press; 1985.
10. Marwan, M. M.; Abdel Malek, Z. A.; Kreutzfeld, K. L., et al. Stimulation of S91 melanoma tyrosinase activity by superpotent $\alpha$-melanotropins. Mol. Cell. Endocrinol. 41:171–177; 1985.
11. Meyskens, F. L.; Thomson, S. P.; Hickie, R. A., et al. Potential biological explanation of stimulation of colony growth in semi-solid agar by cytotoxic agents. Br. J. Cancer 48:863–868; 1983.
12. Oikawa, A.; Nakayasu, M.; Claunch, C., et al. Two types of melanogenesis in monolayer cultures of melanoma cells. Cell Differ. 1:149–155; 1972.
13. Oikawa, A.; Nakayasu, M.; Nogara, M. Tyrosinase activities of cell-free extracts and living cells of cultured melanoma cells. Dev. Biol. 30:198–205; 1973.
14. Pawelek, J. Factors regulating growth and pigmentation of melanoma cells. J. Invest. Dermatol. 66:201–209; 1976.
15. Pawelek, J. M.; Körner, A. M. The biosynthesis of mammalian melanin. Am. Sci. 70:136–145; 1982.
16. Pomerantz, S. H. The tyrosine hydroxylase activity of mammalian tyrosinase. J. Biol. Chem. 241:161–168; 1966.
17. Pomerantz, S. H. L-tyrosine-3,5-$^3$H assay for tyrosinase development in skin of new born hamsters. Science 164:838–839; 1969.
18. Sawyer, T. K.; Sanfilippo, P. J.; Hruby, V. J., et al. 4-Norleucine, 7-D-Phenylalanine $\alpha$-melanocyte stimulating hormone: a highly potent melanotropin with ultralong biological activity. Proc. Natl. Acad. Sci. USA 77:5754–5758; 1980.
19. Sawyer, T. K.; Hruby, V. J.; Wilkes, B. C., et al. Comparative biological activity of highly potent active-site analogues of $\alpha$-melanotropin. J. Med. Chem. 251:1022–1027; 1982.
20. Sawyer, T. K.; Hruby, V. J.; Hadley, M. E., et al. $\alpha$-Melanocyte stimulating hormone: chemical nature and mechanism of action. Am. Zool. 23:529–540; 1983.
21. Thomson, S. P.; Moon, T. E.; Meyskens, F. L., Jr. Kinetics of clonogenic melanoma cell proliferation and the limits on growth within a bilayer agar system. J.Cell. Physiol. 121:114–124; 1984.
22. Varga, J. M.; DiPasquale, A.; Pawelek, J., et al. Regulation of melanocyte stimulating hormone action at the receptor level: discontinuous binding of hormone to synchronized mouse melanoma cells during the cell cycle. Proc. Natl. Acad. Sci. USA 71:1590–1593; 1974.
23. Wade, D. R.; Burkart, M. E. The role of adenosine 3′,5′-cyclic monophosphate in the density-dependent regulation of growth and tyrosinase activity of B-16 melanoma cells. J. Cell. Physiol. 94:265–274; 1978.
24. Wong, G; Pawelek, J. Control of phenotypic expression of cultured melanoma cells by melanocyte stimulating hormones. Nature 241:213–215; 1973.