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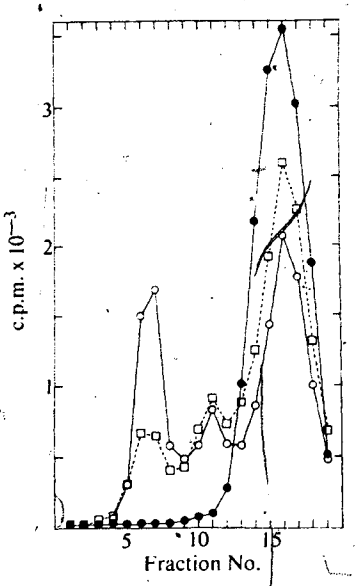

Fig. 3 Binding of the uterine 6S ^{3}H-17β-oestradiol-receptor complex with the isolated nuclear RNP particles of calf uteri and liver. Experiments were carried out as in Fig. 2, except that the uterine cytosol preparation was centrifuged at 230,000*g* for 3 h to remove the 50S particles. The E_2–R so prepared showed only the 6S peak (●—●). The E_2–R preparation was mixed with 0.1 absorbance (260 nm) unit of 80S nuclear RNP particles of calf liver (□ - - - □) or calf uterus (○—○).

particles of the types identified in electron micrographs of other mammalian cell nucleï[17], possibly related to the biochemically defined informosomes[18] or informofers[19]; or (3) a ribosome precursor particle[20,21]. It is possible that the primary role of the steroid-receptor complexes is to provide the structural requirements for the formation (including RNA synthesis), processing, and/or function of specific RNPs in the target cells. The steroid-protein complexes may remain bound to some of these particles during maturation and transport to the cytoplasm, and thus may also be able to participate in the mechanism involved in the translational controls.

In the target cells of steroid hormones, certain RNP particles may play a role in the re-cycling of steroid-receptors between cytoplasm and cell nucleus. For example, the receptor proteins may lose their ability to associate with RNP particles at different stages of maturation if the target cells are depleted of the hormones. When the hormones are replenished, the steroid-receptor complexes may reassociate with existing RNP particles so that the processing and function of these specific groups of RNP are restored. Some of the steroid-receptor complexes not bound to RNP may be re-cycled into cell nuclei for further utilization at chromatin sites.

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Control of Phenotypic Expresssion of Cultured Melanoma Cells by Melanocyte Stimulating Hormones

STUDIES of the mechanism through which MSH acts in mammals have been restricted to intact animals or to melanomas grown in animals[1,2]. We describe here a mouse melanoma cell line cultivated in monolayer which when exposed to MSH shows large increases in tyrosinase activity and melanin content, as well as changes in growth characteristics and cellular morphology. In addition, we have found that adenosine 3′,5′-monophosphate (cyclic AMP) or its analogue N^6,$O^{2'}$-dibutyryl cyclic AMP (*di*B cyclic AMP) will substitute for MSH, supporting previous evidence that MSH probably acts through cyclic AMP in mammals[1,3].

Mouse melanoma cells (Cloudman S91 NCTC 3960 (CCL 53)) were cultured with and without MSH. After 3–5 days in the presence of MSH, the cells became flattened and dendritic, and were strikingly more pigmented than those grown without hormone. Direct measurements of melanin extracted from the cells confirmed that MSH, as well as cyclic AMP, caused increased melanin deposition (Fig. 1). Cyclic GMP, cyclic UMP and cyclic CMP (Schwarz/Mann Biochemicals) had no measurable effect on melanin deposition or tyrosinase activity (data not shown).

Tyrosinase is the only enzyme known to be involved in melanin synthesis[4] and therefore tyrosinase activity by the cells was measured. Table 1 shows that ^3H_2O released to the culture medium by cells incubated with ^{3}H-tyrosine is indicative of tyrosinase activity. Non-pigmented monkey kidney cells which lack tyrosinase did not form ^3H_2O, and phenylthiourea, a specific tyrosinase inhibitor[4], prevented ^3H_2O formation by melanoma cells. Phenylthiourea was not acting as a general metabolic inhibitor since it had little or no effect on protein synthesis.

Tyrosinase activity was greatly increased when cells were incubated with either MSH, cyclic AMP, or *di*B cyclic AMP (Table 2). The concentrations of the various additives were optimal (data not shown), and MSH was more effective than either of the cyclic mononucleotides. Exposure to MSH for only 24 h followed by withdrawal of the hormone was sufficient to cause increases in tyrosinase activity for an additional 24–48 h, after which the activity returned to control levels.

We also measured tyrosinase activity in cell homogenates.

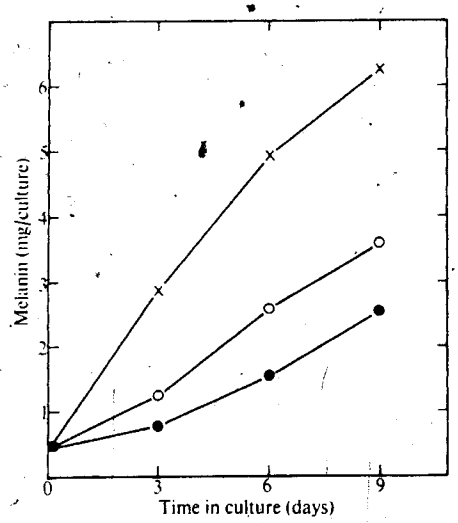

Fig. 1 Effect of α-MSH and cyclic AMP on melanin deposition by melanoma cells. 10^6 cells were cultured with either no additions (●—●), 10^{-4} M cyclic AMP (○—○), or 2×10^{-7} M α-MSH (×—×). At the indicated times cells were harvested from the culture flasks and the melanin was measured. Cells were lysed by freezing and thawing in distilled H_2O, precipitated with cold 10% perchloric acid (PCA), washed three times with cold PCA, heated for 15 min at 80° C in PCA, and centrifuged. Melanin, present in the pellet, was dissolved in 1 M sodium hydroxide at 100° C, and measured by the method of Whittaker[10]. Melanin standards were prepared by incubating DOPA with purified frog skin tyrosinase in 0.01 M potassium phosphate, *p*H 6.8, at room temperature. These experiments were repeated several times with similar results each time.

There was far greater tyrosinase activity in homogenates of cells grown in the presence of MSH, than in its absence, paralleling observations made with cells growing *in situ* (Fig. 2).

The roles of genetic transcription and translation in the response to MSH were investigated. MSH was added to culture medium for 24 h and then withdrawn. Actinomycin D or cycloheximide was present during hours 0–24 or 24–48, and tyrosinase activity was measured during both intervals (Table 3). Actinomycin D inhibited tyrosinase activity if

Table 1 *In situ* Tyrosinase Assay

Cell type	^3H_2O formation (c.p.m.)	^3H-Leucine incorporation (c.p.m.)
Culture medium only	1,600	Not done
Monkey kidney	1,700	Not done
Mouse melanoma	17,900	330,000
+phenylthiourea	1,940	300,000
+cycloheximide	Not done	1,675

2×10^6 cells were cultured for 24 h in 4 ml. medium containing either 0.8 μCi ml.$^{-1}$ L-tyrosine-3,5-^3H (New England Nuclear), dried twice from distilled H_2O with a stream of nitrogen to evaporate any residual ^3H_2O, or 1 μCi ml.$^{-1}$ ^3H-leucine (New England Nuclear). 0.4 ml. medium was measured for ^3H_2O formation by the charcoal absorption method of Pomerantz[8]. To measure ^3H-leucine incorporation into protein, cells were harvested with a rubber policeman, lysed, precipitated with trichloroacetic acid, and collected on a 'Millipore' filter. Phenylthiourea (10^{-3} M) (Eastman Organic Chemicals) or cycloheximide (10^{-6} M) (Sigma) were added at zero time. Each number represents an average of duplicate culture flasks; variation between duplicates was less than ±10%. No corrections were made for c.p.m. obtained from culture medium incubated without cells. Cloudman S91 NCTC 3960 (CCL 53) mouse melanoma cells were obtained from the American Type Culture Collection Cell Repository. Vero green monkey kidney cells were obtained from Dr. Sherman Weissman. Cells were grown in 30 ml. tissue culture flasks in 4 ml. Ham's nutrient mixture F10 supplemented with 10% horse serum (GibCo), 2% foetal calf serum (GibCo), 100 U ml.$^{-1}$ penicillin, 100 μg ml.$^{-1}$ streptomycin, and 1.2 mg ml.$^{-1}$ sodium bicarbonate.

Table 2 Tyrosinase Activity in Melanoma Cells under Various Culture Conditions

Days in culture	^3H_2O formation day^{-1} (c.p.m.) No additions	MSH	Cyclic AMP	*di*B cyclic AMP
1	300	2,600	900	700
2	1,750	10,500	4,500	4,300
3	2,250	36,250	10,200	10,000
4	7,500	54,000	21,750	21,000
5	14,300	68,000	36,500	34,000

2×10^5 melanoma cells were cultured in 4 ml. medium containing no additions, α-MSH (5×10^{-7} M), cyclic AMP (10^{-4} M), or *di*B cyclic AMP (10^{-6} M). Medium contained 0.8 μCi ml.$^{-1}$ ^3H-tyrosine. Old medium was replaced with fresh medium every 24 h, at which time the ^3H_2O formed in 0.4 ml. old medium was measured as in Table 1. All numbers have been corrected by subtracting 1,100 c.p.m. obtained when medium containing ^3H-tyrosine was incubated under identical conditions without cells. Each number represents an average of duplicate culture flasks; variation between duplicates was less than ±10%. These experiments were repeated at least 3 times with similar results each time. Highly purified α-MSH was prepared by Drs. G. Upton, A. Lerner and S. Lande. Cyclic AMP and *di*B cyclic AMP were obtained from Schwarz/Mann Biochemicals.

present during the early time but had no effect if present during the later time, even though RNA synthesis was inhibited greater than 90% during both intervals. In contrast, cycloheximide inhibited tyrosinase activity during both intervals. Cycloheximide inhibition of protein synthesis was greater than 95%.

There were fewer cells and less DNA per culture flask in the presence of MSH and cyclic AMP than in their absence (Fig. 3). Therefore, the increases in melanin deposition and tyrosinase activity were greater per cell than per culture flask. Similar changes in growth characteristics have been reported for the effects of ACTH and cyclic AMP on adrenal tumour cells[4], and for the effects of *di*B cyclic AMP on Chinese hamster ovary cells[5] and transformed fibroblasts[6,7].

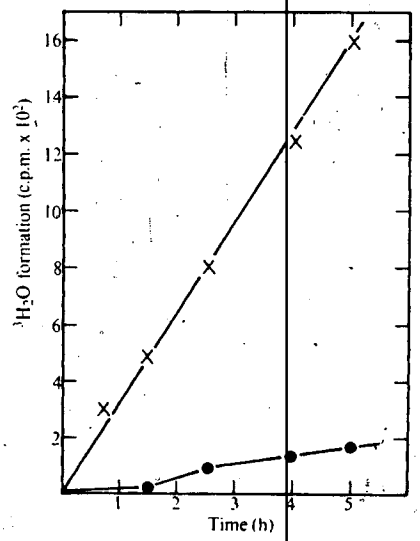

Fig. 2 Tyrosinase activity in cell homogenates obtained from cells cultured for 4 days in the presence (×—×) or absence (●—●) of 2×10^{-7} M α-MSH. In each case 4×10^6 cells were broken in a dounce homogenizer in 1 ml. 5 mM sodium phosphate, *p*H 6.8, and incubated at 37° C with 0.8 μCi ml.$^{-1}$ ^3H-tyrosine. At the times indicated 0.1 ml. aliquots were withdrawn and ^3H_2O was measured. Counts were corrected by subtracting 450 c.p.m. which were the average (±10%) counts obtained at each time point for ^3H-tyrosine incubated in buffer only. The radioactivity was measured in a scintillation counter having 5-fold lower counting efficiency than the one used for the other experiments presented in this paper. These experiments were repeated several times with similar results each time.

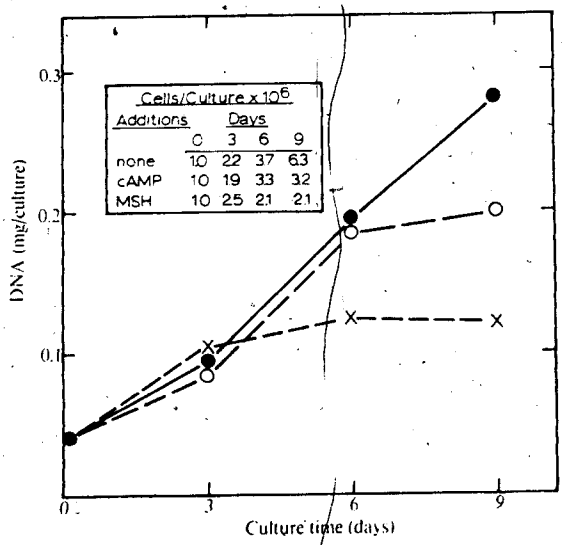

Fig. 3 Effects of MSH and cyclic AMP on growth characteristics of cultured melanoma cells. 10^6 cells were cultured with either no additions (●—●), 10^{-4} M cyclic AMP (○—○), or 2×10^{-7} M α-MSH (×—×). At the indicated times cells were harvested from the culture flasks, counted in a haemocytometer, and DNA was measured. Cells were lysed and treated as in Fig. 1 and the hydrolysed DNA in the supernatant was measured by Burton's modification of the diphenylamine reaction[11]. Deoxyadenosine standards were used. These experiments were repeated three times with similar results each time.

Table 3 Effects of Actinomycin D and Cycloheximide on Tyrosinase Activity

Additions	C.p.m. ^3H_2O formation/24 h Hours 0–24	Hours 24–48
No additions	3,800	5,800
MSH 0–24 h	17,300	30,500
MSH 0–24 h + actinomycin D 0–24 h	4,000	Not done
MSH 0–24 h, then actinomycin D 24–48 h	17,300	30,600
MSH 0–24 h + cycloheximide 0–24 h	1,200	Not done
MSH 0–24 h, then cycloheximide 24–48 h	17,300	3,450

3×10^5 melanoma cells were cultured as indicated. Concentrations of additions were as follows: α-MSH (2×10^{-7} M), actinomycin D (0.05 μg/ml.), (Nutritional Biochemicals), cycloheximide (10^{-6} M). Old medium was removed after each 24 h interval, at which time the ^3H_2O formed in 0.4 ml. old medium was measured as in Table 1. All numbers have been corrected by subtracting 1,300 c.p.m. obtained when medium containing ^{3}H-tyrosine was incubated under identical conditions without cells. Each number represents an average of duplicate culture flasks; variation between duplicates was less than ±10%. These experiments were repeated three times with similar results each time.

During the preparation of this manuscript Johnson and Pastan reported that *di*B cyclic AMP increases pigmentation and causes morphological changes in the Cloudman melanoma cell line[3]. Our findings confirm this work. It is likely that MSH exerts its effect on mammalian melanocytes by causing increased intracellular levels of cyclic AMP[1,2]. It remains to be seen whether this is the only mechanism through which MSH acts.

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Radiolabelling of *Drosophila* Embryos

THE potential of *Drosophila melanogaster* for the study of embryonic development is unparalleled by any higher organism owing to the availability of numerous mutations that modify embryogenesis[1–3]. Knowledge about biochemical events during embryogenesis is limited, however, owing to the small size of the embryo and its impermeability to exogenous radioisotopically labelled precursors and inhibitors of DNA, RNA, and protein synthesis.

Oocytes of King's stage 14 are permeable[4] and have been shown to incorporate large molecules when prematurely oviposited[5]. However, they normally acquire a waterproofing waxy layer on the surface of the vitelline membrane on leaving the ovariole[6] and a protective chorion, which is secreted by the follicle cells[7]. Thus, the newly oviposited mature egg, a closed nutritional system, is rendered impermeable. We have developed a technique whereby *Drosophila* embryos can be collected in gram lots, dechorionated and dewaxed, and made to assimilate radioisotopically labelled precursor molecules in aqueous media without significant loss of viability.

The Oregon-R strain of *D. melanogaster* is maintained in a 12 × 12 × 24 inch plastic population cage on standard cornmeal–yeast–sugar–agar medium. Embryos of known developmental age are obtained by replacing the 5 × 8 inch food trays and allowing the females to oviposit for one hour. Embryos are loosened from the surface of the food with a light camel hair brush and suspended in a 40% sucrose solution. They are then washed with several volumes of *Drosophila* Ringer's solution[8] in a small separatory funnel.

The chorion is removed by treatment with 4.6% sodium hypochlorite for 2 min, followed by several washes with Ringer's solution. The embryos are then suspended in 5 ml. of Ringer's solution made to 0.5% (v/v) with 'Triton X-100' and containing the exogenous substance to be incorporated. The suspended embryos are incubated at 22° C for 2 h in a 30 ml. disposable plastic culture flask on a reciprocating rotator (Eberbach) at 180 r.p.m. At the end of the incubation period, the embryos are collected on 106 μm pore 'Nitex' nylon bolting cloth, placed on a moist filter paper in Petri dishes and allowed to develop at 25° C.

Embryos that have been dechorionated and dewaxed develop into apparently normal flies. The viability of the embryos with this technique is quite high (> 90% of controls) as long as the concentration and time of exposure to 'Triton X-100' are not exceeded. The 'Triton X-100' (an anionic detergent) adequately emulsifies the waxy layer in 2 h, and if longer exposure to exogenous substances is desired, the embryos must be transferred to a solution lacking 'Triton X-100'. Rotation of embryos is required as long as the embryos are suspended in aqueous media.