EFFECTS OF α-AMANITIN ON MELANIN SYNTHESIS IN THE DEVELOPING CHICK RETINAL PIGMENT EPITHELIUM

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1. Introduction

During the normal development of the retinal pigment epithelium (RPE) of the chick embryo, observed rates of melanin synthesis go through two stages. The time of transition from low to high rates, at approx. 4.5 days, is linked to the withdrawal of cells from mitotic activity which begins at approx. 3.5 days [1]. These observations pose several questions directly related to the manufacture of the mRNA needed for melanosomal protein synthesis. First, while the cells are still dividing, and are making melanin at low rates, is new mRNA manufactured which is needed for maintaining the low levels of melanogenesis observed. Second, it is not known if de novo mRNA synthesis is required for the changeover to high level melanogenesis, or if an already existing but unutilized pool of mRNA is available. Alternatively, there could be a speedup in the translation rate of already extant message.

In an attempt to determine the answers to these questions, mRNA synthesis was selectively blocked with α-amanitin [2–4]. The results indicate that the mRNA needed for low level melanogenesis is present prior to the time when melanin can first be measured biochemically. Furthermore, it appears that the mRNA needed for increased melanogenesis is made shortly after the cells withdraw from mitotic activity and approximately 18–24 h prior to any measurable increase in melanin synthesis.

That these effects are not due to differences in α-amanitin uptake or effectiveness between different-aged embryos is determined on a general level. Observations on the effect of α-amanitin on the rates of total RNA and protein synthesis indicate that the rates are the same for equivalent exposure times regardless of the age of the embryo.

2. Materials and methods

2.1. *Staging and treatment of eggs*

White Leghorn eggs were staged and drugs were added as previously described [1,5]. The α-amanitin dose was 0.5 μg/egg (a sublethal dose) and well below the high dose levels usually observed to inhibit RNA polymerase activity other than polymerase 11 [3,4,6,7].

2.2. *Thiouracil assay*

Thiouracil (TU) has previously been shown to be effective in specifically measuring the rate of melanin synthesis [1,8] in embryos three days or older. Although its site of attachment is unknown at this time, it has been shown to bind to melanin only during manufacture, not to already synthesized melanin. Furthermore, its binding has been demonstrated to be specific. For this work, either 2-thio-[2-^{14}C]uracil (Amersham/Searle; 61 mCi/mmol) or 2-[^{35}S]thiouracil (Amersham/Searle; 12 mCi/mmol) was added directly to each egg. For [^{14}C]TU, the dose was 0.5 μCi; for [^{35}S]TU, the dose was 5.0 μCi. (To ensure that the observed results were not due to differences in the labelling of the compound, the experiments done with [^{14}C]TU were repeated using [^{35}S]TU.) After addition, the eggs were maintained as usual in a humidified egg incubator. At the end of the

incubation period, the eyes were removed, washed 2–4 X in cold 12% trichloracetic acid (TCA) and prepared for scintillation counting as previously described [1]. Data from 4 eyes, in duplicate, were pooled and expressed as a percentage of the control value.

2.3. *Uridine incorporation*

Eggs were treated with α-amanitin for times stated. At the end of the given time period, the eyes, and eyes from untreated embryos of the same age, were placed in 0.2 ml carrier medium [1], containing 60 μCi [^{3}H]uridine ([5,6-^{3}H]uridine; Amersham/Searle; 41.5 Ci/mmol) and for the α-amanitin treated eyes, 0.1 μg α-amanitin/ml, for 15 min at 37°C. Tissue was then washed 2X with cold 12% TCA, and prepared for scintillation counting.

2.4. *Leucine incorporation*

Eggs were treated with α-amanitin for times stated. At the end of the time period, 20 μCi [^{3}H]leucine (L-[3,4,5-^{3}H]leucine, 78.2 Ci/mmol, New England Nuclear) were added to each egg. After 4 h incubation time, the eyes were removed, washed 2X in 12% TCA and prepared for scintillation counting.

3. Results

3.1. *Manufacture of mRNA during low level melanogenesis*

There is evidence that the cells of the RPE are determined to make melanin as early as 2–2.5 days ([1], Zimmerman, Brumbaugh and Strahs, unpublished results). Therefore, eggs of 2.5, 2.75, 3.0 and 3.25 days of incubation each received 0.5 μg of α-amanitin. At 3.5 days of incubation, viable eggs received 5 μCi of [^{35}S]TU for 5 h as did a set of untreated 3.5 day-old eggs, viability being measured by a continuing heart beat.

The results are shown in table 1 (the amount of label incorporated in the untreated 3.5 day embryos is used as 100% in order to normalize the data). The results clearly indicate that at no time in the 24 h preceding 3.5 days was melanin synthesis blocked by adding α-amanitin. (Possible reasons for the apparent stimulation of melanin synthesis are discussed below.) There are several possible ways by which this could occur. First, there may be a long-lived mRNA present throughout the whole time period, making it unnecessary to continually manufacture new message and thus, the α-amanitin treatment time was too short to be effective. The second possibility is that it takes longer than 24 h to get from synthesis of the message to biochemically measurable product, again making the α-amanitin-treatment time too short. By examining the effect of α-amanitin when new message should be needed for the transition from low to high rates, some idea of which of these two possibilities is correct may be discerned.

Table 1
Effect of α-amanitin on low level melanogenesis

Age (days) at α-amanitin addition	% Control value melanin synthesis
2.50	170
2.75	193
3.00	242
3.25	135
3.50	100

α-Amanitin added at the age indicated. At 3.50 days all eggs and one set of untreated eggs receive labeled TU. At the end of 5 h, the tissue was processed for scintillation counting as previously described [1]. Data are expressed as percent of incorporation of 3.50 day untreated tissue, average of 2 experiments

3.2. *Manufacture of mRNA necessary for increased melanogenesis*

At approximately 4.5 days of development, there is a rapid increase in the rate of melanin synthesis in the chick RPE. This increase is linked to a withdrawal of the cells from mitotic activity at approx. 3.5 days of development, and shifts in withdrawal have been shown to result in concomitant shifts in melanogenic rates [1]. This suggests that those factors which control the mRNA necessary for this increase (i.e., its synthesis, release from a pool or increased translation rate) come into play after withdrawal, at 3.5 days or later.

In accordance with this, α-amanitin was added at 3.5, 3.75, 4.0 and 4.25 days of development. At 4.5 days, all of the above, plus an untreated set of eggs, received labeled TU (either ^{14}C or ^{35}S, depending upon the run) for 8 h. The averaged results for all four runs are shown in table 2, column 2. (There was no

Table 2
Effect of α-amanitin during high level melanogenesis

Age (days) at α-amanitin addition	Measurement at 4.5 days			Measurement at 5.0 days
	Melanin synthesis	[^{3}H]Uridine	[^{3}H]Leucine	melanin synthesis
3.5	51.5	105.8	105.8	165.0
3.75	81.3	–	–	156.5
4.0	102.7	70.7	100.0	208.5
4.25	97.5	–	–	151.0
4.5	100.0	100.0	100.0	–
5.0	–	–	–	100.0

α-Amanitin added at age indicated. At either 4.5 or 5.0 days of incubation, melanin synthesis measured or total RNA or protein synthesis measured (see Materials and methods, table 3). Data expressed as percent (α-amanitin treated)/(control). Melanin synthesis at 4.5 days average of 4 experiments, all others average of 2 experiments

notable difference between the use of [^{35}S]- and [^{14}C]TU.) It is clear from the results that the addition of α-amanitin at 3.5 days results in a significant decrease in the amount of melanin synthesized at 4.5 days. The addition of α-amanitin later than 3.75 days has no discernible effect on the rate of melanin synthesis.

In order to insure that this lack of effect is not due to a failure of α-amanitin to enter, or act upon, older embryos, 3.5 and 4.0 day eggs received α-amanitin. (These points were chosen as representatives of those times at which α-amanitin affects melanin synthesis and those times it does not.) At the end of the given times (table 3), both treated and untreated eyes received either [^{3}H]leucine or [^{3}H]uridine. The results show that α-amanitin acts on both ages in a similar fashion. Total RNA synthesized after 4 h is reduced by about 50%, while after 24 h the rate of total RNA synthesis is at approximately the same levels as the controls. Protein synthesis remains in the 90–100% range for all times measured.

Therefore, the lack of α-amanitin effect on 4.0 day or older animals, or, if the mRNAs are the same, in 2.5–3.25 day animals, probably cannot be attributed to a failure of the drug to affect RNA synthesis in those animals. Furthermore, the similar effect, after a given time period, in both 3.5 and 4.0 day animals on RNA and protein synthesis indicates that α-amanitin is acting similarly upon them.

The above results suggest the existence of long-lived

Table 3
α-Amanitin effect on total RNA and protein synthesis

Age (days)	[^{3}H]Uridine (Exposure time to α-amanitin)		[^{3}H]Leucine (Exposure time to α-amanitin)	
	4 h	24 h	4 h	12 h
3.5	49.9	105.8	99.9	87.7
4.0	48.7	119.7	90.7	100.0

3.5 and 4.0 day eggs received α-amanitin for indicated time. At the end of that time: (1) the eyes were removed and they and the control eyes were incubated in [^{3}H]uridine containing medium for 15 min or (2) the eggs received [^{3}H]leucine for 4 h. After the exposure to label, the eyes were prepared for scintillation counting. Data are expressed as percent (α-amanitin treated)/(control)

mRNA. It may be tested for by blocking mRNA manufacture and waiting a period of time sufficient to significantly decrease the proportion of short-lived mRNA present; this should favor the translation rate of the long-lived mRNA population. Then, by extending the period of time of α-amanitin exposure, one should increase the percentage of melanin manufactured relative to the controls.

The results shown in table 2, column 5, represent a 36 h maximum exposure to α-amanitin prior to measurement. In comparing columns 2 and 5, it is clear that where addition of α-amanitin at 3.5 days and measurement at 4.5 days results in a decreased manufacture, relative to controls, an extension of 12 h, i. e., measurement at 5 days, results in an increased level of activity.

4. Discussion

The results presented in this paper support the idea that transcriptional control of new mRNA is, in fact, involved in the change from low to high melanogenic rates, and that this occurs between 3.5 and 3.75 days (table 2). (That this does not continue to occur later is suggested by the failure of α-amanitin treatment at 4.0 days to decrease the rate of synthesis at 5.0 days.) Treatment of eggs with α-amanitin at 3.5 days results in a marked decrease in the ability of the RPE to produce melanin at 4.5 days. Treatment with α-amanitin at later times results in a decreasing inhibition when measured at 4.5 days.

The results presented in table 1 of this paper suggest that all the mRNA needed for low level melanogenesis is in existence prior to 2.5 days of development. This requires the existence of a long-lived mRNA or a transcription-to-finished-product time greater than 24 h. Assuming that the mRNA involved in the results in tables 1 and 2 are the same would rule out the possibility that the transcription-to-measurement time is greater than 24 h. This leaves the idea that the mRNA involved in melanin synthesis is long-lived, which in light of the findings concerning, for example, possible myeloma mRNA [9] and fibroin mRNA [10], is not surprising.

The idea of a long-lived mRNA is further supported by the fact that the data indicate the possibility of a selective effect of α-amanitin in reducing the short-lived mRNA population and so effectively increasing the rate at which long-lived mRNA is read and translated (table 2, column 5). Furthermore, the data presented in table 2, columns 3 and 4, add additional support to this idea. In these columns total RNA and protein synthesis were measured at the same time as melanin synthesis was measured, and thus after different times of exposure to α-amanitin. Protein synthesis again remains unaffected by exposure to α-amanitin. Total RNA however seems to vary inversely with melanin synthesis. If total RNA synthesis is a measure of melanin related mRNA synthesis, then this effect is inconsistent with melanin mRNAs being short-lived. If they were short-lived then the decrease shown in total RNA for the 4.0 day embryos should be reflected as a decrease in melanin synthesis, while exactly the opposite should be the case for the 3.5 day embryos. That this is not true is consistent with the idea that long-lived mRNA is involved and that it is synthesized prior to 4.0 days of development. (If total RNA synthesis is not a measure of melanin related mRNA synthesis, then the data need only be examined as it relates to table 3.)

These results and discussion do not preclude the possible existence of a pool of mRNA synthesized but unutilized during low level melanogenesis, whose release is somehow stimulated by α-amanitin, but at least make it unlikely since during high level melanogenesis α-amanitin initially blocks the production of normal levels of melanin and this suggests a requirement for synthesis of new message. To come to definitive conclusions on these points requires the isolation of melanosomal proteins and their mRNA.

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