# EFFECTS OF α-AMANITIN *IN VIVO* ON RNA POLYMERASE ACTIVITY OF CULTURED CHICK EMBRYO FIBROBLAST CELL NUCLEI: RESISTANCE OF RIBOSOMAL RNA SYNTHESIS TO THE DRUG

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## 1. Introduction

α-Amanitin, a bicyclic octapeptide from the toadstool, *Amanita phalloides* [1], inhibits DNA transcription *in vitro* by binding specifically to RNA polymerase form II of eukaryotic organisms without affecting the activity of RNA polymerase form I [2–4]. The effects of α-amanitin on RNA polymerase activity *in vitro* can be used as the basis of an assay to distinguish form I and form II polymerase activities in whole nuclei [5]. Little is known about the action of α-amanitin on growing cells. During a study of the inhibition of influenza virus replication by α-amanitin, we found that the drug, when added *in vivo*, had little effect on [$^3$H]uridine incorporation into cellular RNA, but caused a rapid, dose-dependent inhibition of RNA polymerase II activity [6]. However it has been reported that when administered to rats, α-amanitin has only a short-lived effect on RNA polymerase II activity [7], and blocks the synthesis of all types of nuclear RNA, including ribosomal precursor RNA, for several hours [7, 8]. We have now examined in more detail the effects of α-amanitin on macromolecular synthesis in chick embryo fibroblast (CEF) cells. In contrast to its effects in whole animals [7], α-amanitin when added to cells in tissue culture, causes immediate inhibition of RNA polymerase II activity, but does not inhibit RNA polymerase I activity or incorporation of [$^3$H]uridine into ribosomal RNA for several hours. A preliminary report of some of this work has appeared [9].

## 2. Materials and methods

[5-$^3$H]Uridine (27.6 Ci/mmole) and [$^3$H]UTP (10.5 Ci/mmole) were obtained from The Radiochemical Centre (Amersham, England). ATP, CTP and GTP were obtained from Boehringer (Mannheim, Germany), and 2-mercaptoethanol and calf thymus DNA from Sigma Chemical Co., London. α-Amanitin was a generous gift from Prof. Th. Wieland, or was purchased from Boehringer Ingelheim Ltd. (Isleworth, England). CEF cell cultures were prepared and maintained as previously described [6, 10].

The procedure for pulse-labelling cells, grown on Melinex pieces, with [$^3$H]uridine and subsequent measurement of incorporated radioactivity have been detailed elsewhere [6, 11]. RNA polymerase I and II activities were assayed in whole nuclei, extracted as previously described [12], by the method of Novello and Stirpe [5]. The reaction mixture for polymerase I assay contained, in 0.6 ml: 50 μmoles Tris, pH 8.0, 2 μmoles $MgCl_2$, 7 μmoles 2-mercaptoethanol, 3 μmoles NaF, 0.06 μmoles ATP, CTP and GTP, 2.5 μCi [$^3$H]UTP (0.25 nmoles), 100 μg calf thymus DNA, and about 200 μg nuclear protein. The reaction mixture for polymerase II assay was identical except that $MgCl_2$ was replaced with $MnCl_2$, and 140 μmoles $(NH_4)_2SO_4$ were present in addition. The reaction mixtures were incubated for 15 min at 37° before determining the amount of incorporation of [$^3$H]UMP into acid-insoluble material as described [12]. RNA polymerase II activity was calculated as total incorporation minus incorporation in presence of 2.5 μg of

Table 1
Effects of α-amanitin added to CEF cell cultures for 1 hr on RNA polymerase I and II activities.

| α-Amanitin added (μg/ml) | RNA polymerase I (pmoles UMP incorp./mg protein/15 min) | RNA polymerase II (pmoles UMP incorp./mg protein/15 min) |
|---|---|---|
| 0 | 9.2 | 29.9 |
| 1 | 12.0 | 19.0 |
| 5 | 13.7 | 7.3 |
| 10 | 11.0 | 5.1 |
| 20 | 13.8 | 5.7 |

Various amounts of α-amanitin were added to the medium of duplicate CEF cultures growing on 10 cm plastic petri dishes. One hr later the cells were thoroughly washed in 0.9% NaCl then nuclei isolated for determination of RNA polymerase I and II activities as in Materials and methods.

α-amanitin [5]. Protein was estimated by the method of Lowry et al. [13].

Labelled RNA was extracted from whole cells by a hot phenol–chloroform method [14]; the final aqueous phase (1 ml) was loaded onto a 16 ml linear gradient of 15%–30% sucrose in 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% sodium dodecyl sulphate and centrifuged in a Beckman SW27 rotor at

Table 2
Effects of α-amanitin (20 μg/ml) added to CEF cell cultures on RNA polymerase I activity.

| Time after addition of α-amanitin (hr) | RNA polymerase I activity (pmoles UMP incorp./mg protein/ 5 min) Untreated cells | α-Amanitin treated cells |
|---|---|---|
| 1 | 10.0 | 11.7 |
| 2 | 13.0 | 9.8 |
| 3 | 10.8 | 12.6 |
| 4 | 10.9 | 9.8 |
| 5 | 11.1 | 7.9 |
| 6 | 9.5 | 9.2 |

Duplicate CEF cell cultures growing on 10 cm plastic petri dishes were treated with α-amanitin (20 μg/ml), or untreated. At various times the cells were washed then nuclei extracted from treated and untreated cells in parallel before determination of RNA polymerase I activity *in vitro* as in Materials and methods.

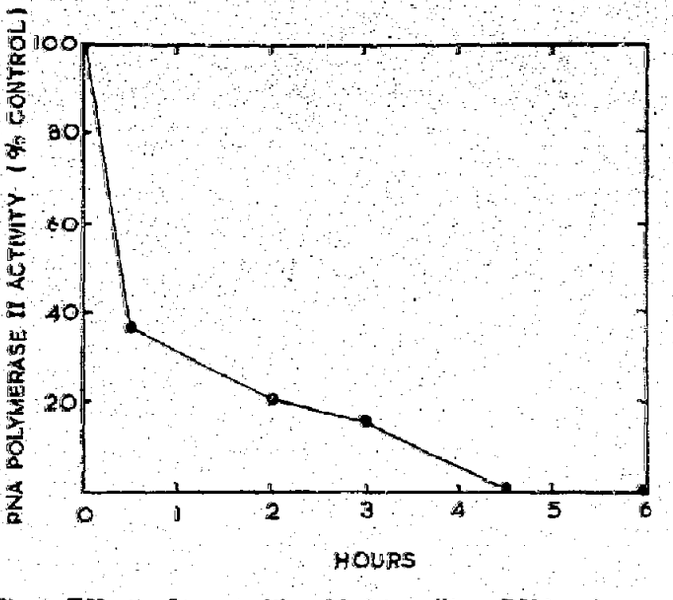


Fig. 1. Effects of α-amanitin added to cells on RNA polymerase II activity. α-Amanitin (20 μg/ml) was added to CEF cell cultures growing on 10 cm plastic petri dishes. At various times the cells from duplicate cultures were harvested and the nuclei isolated for assay of RNA polymerase II activity. Results are expressed as a percentage of untreated control cell nuclear activity of cultures harvested in parallel with the α-amanitin-treated cultures.

80,000 *g* for 16 hr at 25°. 0.6 ml fractions were collected using an ISCO fractionator attached to a recording spectrophotometer measuring optical density at 260 nm. Acid-insoluble radioactivity of the fractions was determined as previously described [12].

## 3. Results and discussion

We have previously reported the dose-dependent inhibition of RNA polymerase II activity which occurs within 1 hr after addition of α-amanitin to growing CEF cells [6]. Table 1 presents the results of an experiment in which various amounts of α-amanitin were added to the medium of CEF cells for 1 hr, following which the nuclei were extracted and assayed *in vitro* for RNA polymerase I and II activities. In contrast to the inhibition observed with RNA polymerase II, RNA polymerase I activity was slightly increased in the nuclei of treated cells.

Jacob et al. [8] have reported a continuing, and Tata et al. [7] a transient inhibitory effect of α-amanitin added *in vivo* on the RNA polymerase I level in

Table 3
Effects of α-amanitin added to cell cultures for 1 hr on [$^3$H]uridine incorporation into RNA.

| Duration of labelling with [$^3$H]uridine (min) | [$^3$H]Uridine incorporated (cpm/$10^6$ cells) Untreated cells | α-Amanitin-treated cells | (%) |
|---|---|---|---|
| 5 | 704 | 434 | 62 |
| 15 | 2242 | 2294 | 102 |
| 30 | 3866 | 3972 | 102 |
| 60 | 13520 | 12435 | 92 |

CEF cell cultures were grown on 18 × 16 mm Melinex strips. Half the cultures were treated with α-amanitin (20 μg/ml) for 1 hr, then [$^3$H]uridine (2 μCi/ml) was added to each culture for the indicated periods. After labelling, acid-insoluble radioactivity of the cultures was determined as in Materials and methods. Each value is the mean obtained from triplicate cultures.

rat liver nuclei. 20 μg/ml α-amanitin were added to the medium of CEF cell cultures which were harvested at various times up to 6 hr thereafter and nuclei assayed for RNA polymerase I activity (table 2). No significant inhibition of polymerase I activity was observed at any time; in a parallel experiment using the same concentration of α-amanitin, RNA polymerase II activity was completely suppressed by 4.5 hr after adding the drug, and there was no evidence of recovery (fig. 1).

The results of these experiments suggest, in contrast to the anomalous reports concerning administration of α-amanitin to rats [7, 8], that when added to tissue culture cells the drug has a similar action on RNA polymerases *in vivo* as *in vitro*. However Rott and Scholtissek [15] reported that up to 50 μg/ml α-amanitin did not affect [$^3$H]uridine incorporation into RNA of CEF cells during 1 hr pulses for several hours, and we confirmed this finding using 15 min [$^3$H]uridine pulses [9]. With shorter labelling periods (5 min), inhibition of RNA synthesis could be detected as early as 1 hr after adding α-amanitin to the culture medium. Monolayer cultures of CEF cells grown on 18 × 16 mm Melinex strips were treated with 20 μg/ml α-amanitin for 1 hr, then pulse-labelled with [$^3$H]uridine (2 μCi/ml) for 5, 15, 30 or 60 min, following which the amount of acid-insoluble radioactivity incorporated by drug-treated and control cultures were determined (table 3). No significant differences in total [$^3$H]uridine incorporation were detected during pulses of 15 min or longer, although there was a 40% reduction in the amount of radioactivity incorporated by α-amanitin-treated cells during a 5 min pulse. The latter result is not surprising, since RNA polymerase II is almost certainly responsible for the synthesis of rapidly-labelled RNA in the cell [16], and treatment with 20 μg/ml α-amanitin for 1 hr inhibited this enzyme activity almost completely (table 1). However, the results obtained with longer periods of labelling indicate that α-amanitin causes no overall inhibition of the bulk of RNA synthesis, in agreement with previous reports for cell cultures [9, 15], but contrary to results obtained in rats, where it was found that synthesis of all species of nuclear RNA, including ribosomal precursor RNA, remained blocked for several hours after α-amanitin administration [7].

The effect of α-amanitin on RNA synthesis in CEF cells was further investigated by extracting RNA labelled during a 15, 30 or 60 min pulse of [$^3$H]uridine and analysing the distribution of radioactivity following sedimentation through sucrose density gradients in comparison with control cells. Monolayer CEF cultures grown on 10 cm plastic petri dishes, untreated or treated for 1 hr with 20 μg/ml α-amanitin, were labelled with [$^3$H]uridine (2 μCi/ml) and at 15, 30 or 60 min thereafter the cells were extracted with hot phenol–chloroform and the final aqueous phase of the extraction applied to 15–30% sucrose density gradients (fig. 2). The patterns observed during 15, 30 or 60 min labelling were identical in α-amanitin-treated and control cultures; in particular, there was no evidence of any inhibition of labelling in 45 S nucleolar precursor RNA. This result supports the conclusion that in CEF cells RNA polymerase I activity is not sensitive to α-amanitin added *in vivo*, and agrees with previous reports in which RNA polymerase activities of whole nuclei [16, 17] or purified enzymes [2–4] have been studied.

The different effects of α-amanitin on CEF cells and on rat liver might perhaps be explained by metabolism and/or detoxication of the drug in the whole animal system. This would account for the transient nature of the inhibition of RNA polymerase II activity [7]. Ribosomal RNA synthesis was unaffected for at least 2 hr after the addition of α-amanitin to CEF cells; this was not the case in rats where inhibition of ribosomal RNA synthesis was

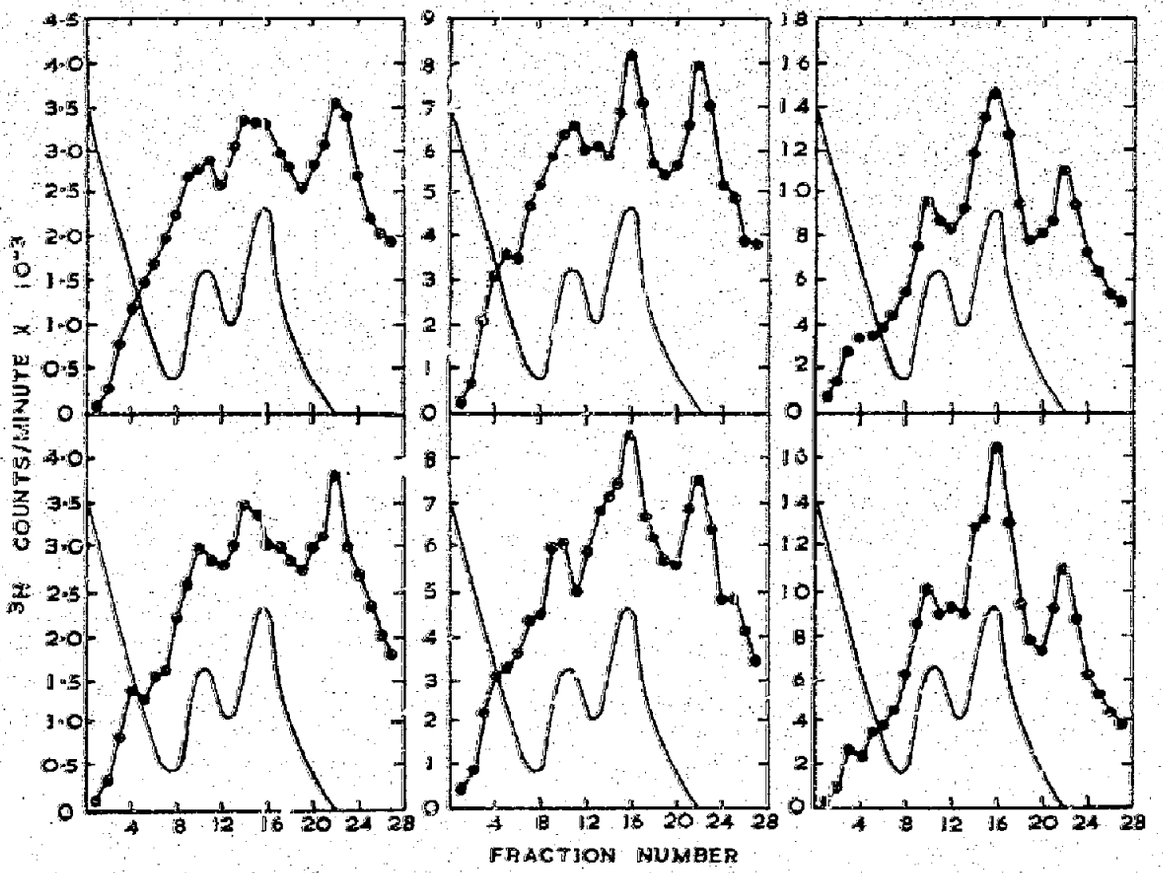


Fig. 2. Sucrose density gradient analysis of [$^3$H]uridine-labelled RNA extracted from α-amanitin-treated and control cultures. Duplicate sets of CEF cultures in 10 cm plastic petri dishes were labelled for 15, 30 or 60 min with [$^3$H]uridine (2 μCi/ml). ml). One set of cultures was treated with α-amanitin (20 μg/ml) 1 hr before labelling. The cells were harvested at the end of the labelling period and the total cell RNA extracted and loaded onto a 16 ml gradient of 15–30% sucrose which was sedimented at 80,000 *g* for 16 hr as described in Materials and methods. Left to right: 15, 30 and 60 min labelling. Upper profiles, untreated cells; lower profiles, α-amanitin-treated cells. The continuous line shows the optical density tracing at 260 nm. The direction of sedimentation is from left to right.

an immediate effect of the drug [8]. Taken together, our results suggest that in CEF cells α-amanitin acts for several hours on RNA polymerase II activity alone.

The replication cycles of three viruses, adenovirus [18], influenza [6, 15] and Rous sarcoma [19] have previously been found sensitive to the presence of α-amanitin in the culture medium. The conclusion reached in each case, that this reflects a specific requirement for transcription by RNA polymerase II of the host cells, would seem justified.

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