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THE EFFECT OF α-AMANITIN ON THE SYNTHESIS OF POLYOMA SPECIFIC RNA

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SUMMARY

α-Amanitin inhibits the synthesis of polyoma specific RNA in cultured mouse kidney cells and in a coordinated way the synthesis of RNA stimulated by the presence of the virus. Late in infection, there is a preferential inhibition of polyoma mRNA synthesis. The results point to the involvement of host RNA polymerase II(B) in polyoma DNA transcription and suggest a possible modification of the enzyme late in infection.

INTRODUCTION

α-amanitin, a cyclic octapeptide extracted from the toadstool Amanita Phaloides, is a potent inhibitor of mammalian RNA polymerase II (1). Its use in virus infected cells has provided a means of studying the transcription apparatus involved in the in vivo transcription both of adeno virus and of simian virus 40 (SV40) DNA (2-7). With the exception of the 3T3 TK$^-$ cells used by Blasi (7) and the human embryo kidney cells (2) all these studies were carried out on whole nuclei, where de novo synthesis does not take place. In the study to be reported here the effect of α-amanitin on the synthesis of polyoma specific RNA was analyzed using baby mouse kidney cells, since the drug has a preferential affinity in vivo for the cells of the convoluted tubule (2). Using this system we have been able to show that the synthesis of polyoma specific RNA can be completely inhibited in the presence of α-amanitin. Further analysis of this effect showed that the inhibitory effect on the synthesis of viral RNA is accompanied in what appears to be a coordinated way by the inhibition of host ribosomal RNA. Late in infection, however, there appears to be a preferential inhibition of virus specific RNA. Only the synthesis of 4S RNA escapes the inhibitory effect of α-amanitin at the concentrations used in this study.

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MATERIALS AND METHODS

Large plaque polyoma virus was used in all the experiments described. Baby mouse kidney cells were infected multiplicity of infection of 10 P.F.U./cell. RNA was extracted according to Martin (8) after being labelled with ^{3}H-uridine as described for each individual experiment. RNA-DNA hybridization was carried out in 2 x SSC and 0.1% SDS for 17 hours at 67^{o}. Polyoma DNA used in these experiments was purified by the selective precipitation method of Hirt (9), followed by banding in caesium chloride gradients in the presence of ethidium bromide. Preparations of form I (superhelical) polyoma DNA were checked by analytical ultracentrifugation under neutral and alkaline conditions and by electron microscopy. Conversion of form I to form II (relaxed circular form) was achieved by X-irradiation (200 KV; 15 mAmp; 7" from source; 30 minutes) under conditions where 95% of the molecules are converted as monitored by electron microscopy. The irradiated DNA was denatured in 0.1N NaOH for 10 minutes, quickly neutralized and diluted in 50 volumes of 2 x SSC before being used to load 2.4 cm diameter nitrocellulose filters (10). From the loaded filters smaller 4 mm diameter discs were cut out and used for the hybridization assays. Efficiency of loading and of retention of DNA on the filters was monitored with ^{3}H-polyoma DNA II and found to be more than 90%. Sucrose density gradients were made from special RNAse free sucrose (Schwarz/mann) and fractionated by collecting drops from the bottom of the tube. RNA was precipitated with cold 5% TCA and the precipitate collected by filtration and washed on a glass fibre disc (Whatman) (GF./C). Radioactivity was counted in a scintillation counter in a toluene-based fluid. α-amantin was a generous gift of Professor T Wieland from Heidelberg.

RESULTS

Two sets of five 90 mm plastic plates of confluent secondary mouse embryo cells were infected and the virus allowed to absorb for two hours before fresh medium was added to one set and fresh medium containing 2 μg/ml of amanitin was added to the other. The plates were then incubated for 63.5 hours at 37^{o}C. At this time one place from each set was kept aside for haemagglutination (HA) assay and the other four were pulsed with 5μCi/ml of ^{3}H-uridine (specific activity 20 Ci/m mole) for 6.5 hours. The cells were harvested and pelleted by centrifugation. The cell pellet was washed in PBS-A (phosphate buffered saline minus divalent cations) and RNA was extracted and hybridized to polyoma DNA. Polyoma virus was titrated by HA assay in the medium of one dish from each set. The results shown in table I indicate that amanitin causes a very significant inhibition of polyoma virus production and of polyoma specific RNA synthesis. The residual virus titrated in the treated dish could have originated from non-absorbed or de-absorbed virions and represents only 2% of the

TABLE I: Effect of α-Amanitin on Polyoma Virus Production and on Synthesis of Polyoma Specific RNA

	HA titre/dish	RNA hyb. to Py DNA (cpm)
Infected + α-amanitin	5×10^3	33
Infected - α-amanitin	2.5×10^5	442
Inoculum	10^3	

Amount of polyoma DNA II per filter: 0.1750 µg; yields of RNA form 5 plates: amanitin treated - 60 µg; non amanitin treated - 200 µg. Specific activities of the RNA samples: amanitin treated - 5,500 cpm/µg; non amanitin treated - 7,240 cpm/µg. Input RNA for both samples - 120,000 cpm. Control filter with no DNA (same input RNA) - 14 cpm.

virus present in the non-treated dish. The two RNA preparations were then sized by centrifugation through gradients of sucrose. The results are shown in figure 1. In the α-amanitin treated sample ribosomal RNA synthesis has been practically abolished (but for very low residual synthesis) and only RNA of small molecular weight (4S) was labelled in the presence of the inhibitor. The RNA from the untreated sample on the other hand shows the size distribution expected after a long pulse with labelled uridine.

In order to try to dissociate the inhibition of synthesis of polyoma specific RNA from the inhibitory effect on host ribosomal RNA an experiment was carried out using both a lower concentration of α-amanitin (1 µg/ml) and increasing exposures to the inhibitor. The diagrammatical design of the experiment is shown in figure 2 and figure 3 shows the total RNA synthesis for the pulses at different times during infection. Total RNA synthesis in infected cells (no α-amanitin) increases as infection progresses being maximal for the pulse between 42-48 hours. Infected cultures also show a distinct increase in RNA synthesis when compared with uninfected controls for both periods studied (16-22 hours and 42-48 hours). RNA synthesis in non-infected cells

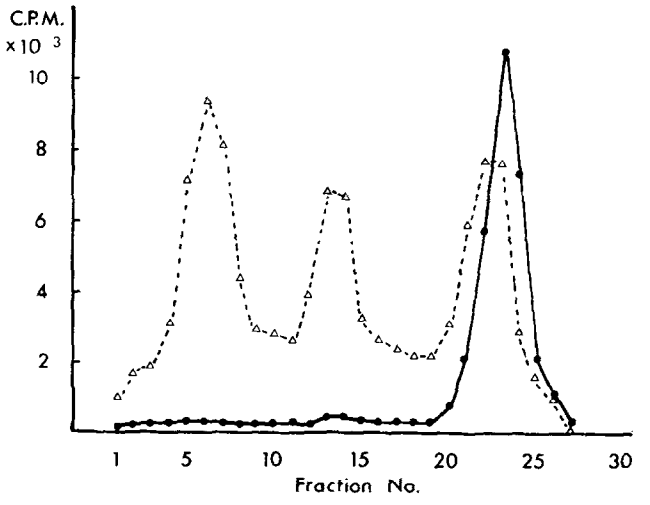

Figure 1

^{3}H-labelled RNA from mouse kidney cells infected for 70 hours with polyoma virus in the presence or absence of amanitin (2 ug/ml). RNA was pulsed between 62.5 and 70 hours of infection. The samples were made up with 50 µl of RNA buffer (10 µl of infected + amanitin sample, 43,000 cpm; 5 µl of the infected - amanitin sample, 138,000 cpm). Sucrose density gradients (5 to 20%) were made in RNA buffer (Methods) and centrifuged in the SW56 Spinaco Rotor for 3.5 hours at 48,000 rpm. Fractions were collected and TCA precipitated as described in Methods.

Δ-------Δ infected - amanitin; ●——● infected + amanitin.

decreases by 50% between 16-22 hours and 42-48 hours after mock infection reflecting the decrease in general metabolic activity of cells that have become confluent. The addition of α-amanitin causes significant inhibition of RNA synthesis. This effect is observed in infected cells at 16-22 hours (30% inhibition), 24-30 hours (38%), but is particularly marked in the 42-48 hour pulse (83.5%). A lesser degree of inhibition is also seen in the shorter pulses of α-amanitin followed by replacement with fresh medium (experiments 8 and 11 in figure 2) when compared with the controls (figure 3). The short pulse between 0-5 hours caused no depression of RNA synthesis between 16-22 hours. In fact there is a slightly increased synthesis when compared to the control (no α-amanitin) reflecting either no inhibition or complete recovery between the addition of fresh medium and the beginning of the pulse. In the

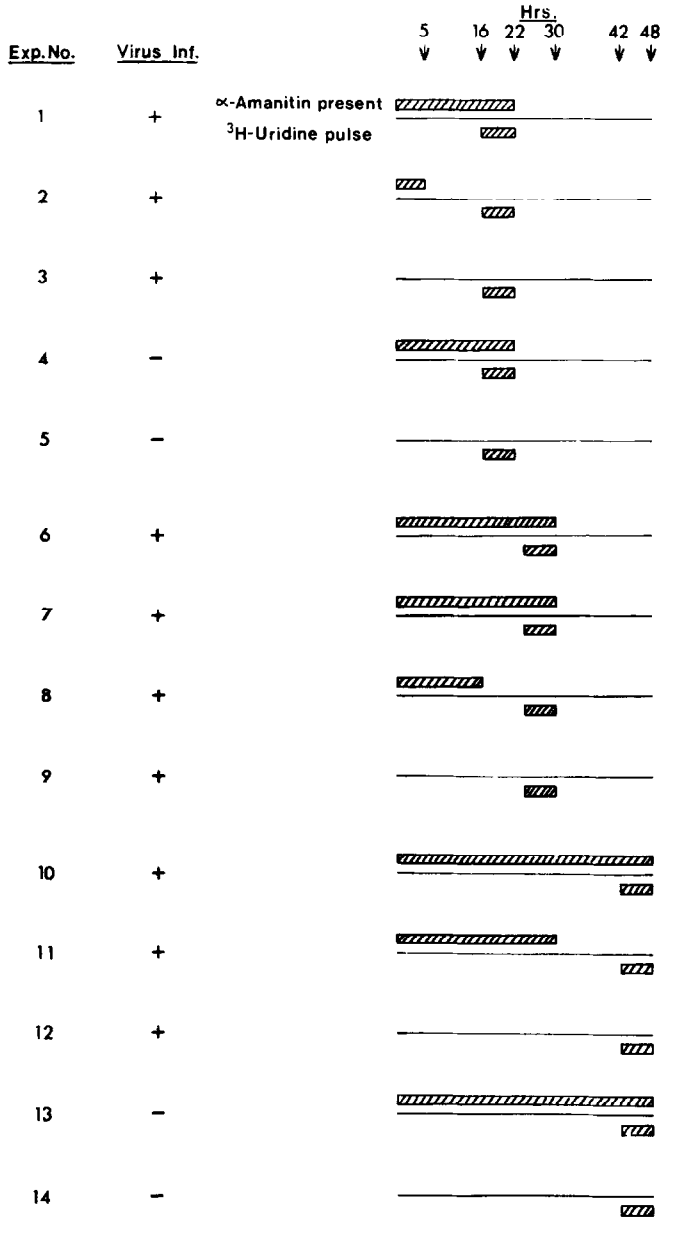

Figure 2

Effect of amanitin on polyoma specific RNA synthesis. Diagrammatic description of the experiment. Infection time is 0.

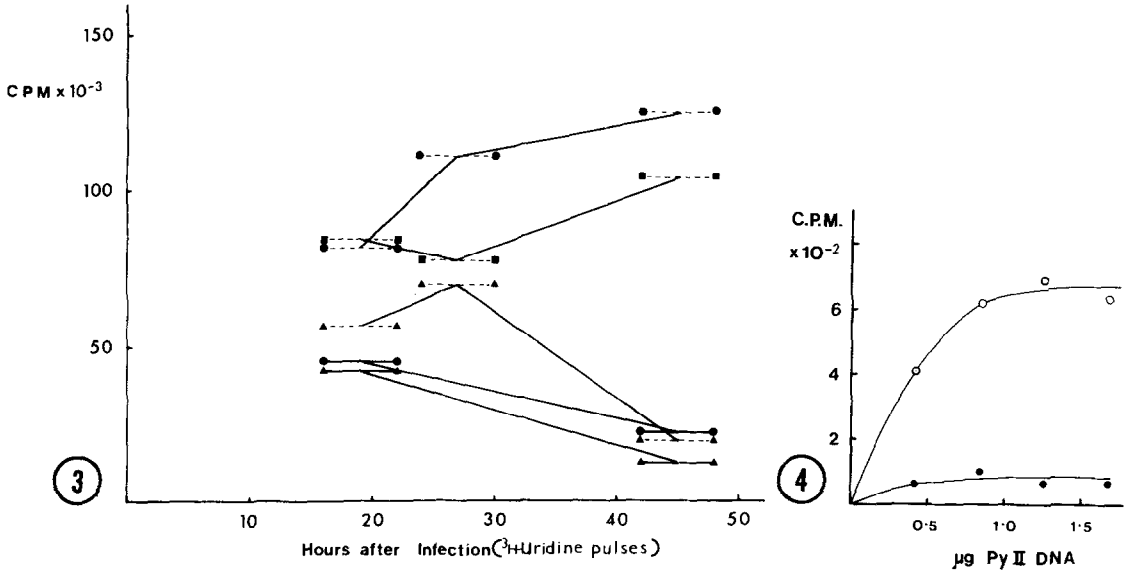

Figure 3

RNA synthesis during polyoma virus infection. Abcissa represents the length of the ^{3}H-uridine pulse. Ordinate represents the amount of ^{3}H-uridine labelled RNA per 10^{6} cells. Broken line (-------) indicates the infected samples; O-------O : no α-amanitin; Δ-------Δ : α-amanitin treated; □-------□ : short pulse of α-amanitin, medium replaced by fresh medium. Full line (———) indicates non-infected samples; O———O : no α-amanitin; Δ———Δ : α-amanitin treated. Other details as depicted in figure 2.

Figure 4

DNA-hybridization of ^{3}H-RNA from samples 10 and 12 in experiment described in figure 2. Inputs of RNA were 62,000 cpm for the untreated (O———O) sample and 14,500 cpm for the amanitin treated (●———●). Blank filter: 20 cpm. Background was not subtracted.

non-infected cells the addition of α-amanitin does not seem to depress RNA synthesis between 16-22 hours after mock infection. At 42-48 hours however RNA synthesis is depressed (44%) reflecting a possible delay in achieving an effective intracellular concentration of the inhibitor in the rather quiescent cells.

To elucidate the effect of α-amanitin on the synthesis of polyoma specific RNA, saturation hybridization of polyoma specific RNA from the various pulses was carried out. RNA samples from the experiment described in figure 2 were

therefore annealed with increasing amounts of DNA (obtained by increasing the number of microfilters per hybridization assay from 1 to 4).

Figure 4 shows the saturation hybridization data for polyoma specific RNA pulsed with ^{3}H-uridine between 42-48 hours in the presence and in the absence of α-amanitin. The results clearly indicate a pronounced inhibition of viral specific RNA during this period in the presence of the inhibitor. When expressed as percentage of the total RNA input (table II) it is seen that there appears to be a preferential depression of viral RNA synthesis during this period in contrast to what was seen during the earlier pulses, where viral specific RNA content (expressed as percentage of input RNA) is similar in treated and untreated samples. In the sample where α-amanitin was added between 0-5 hours followed by a change of medium and a ^{3}H-uridine pulse between 16-22 hours an increased viral RNA content was found when compared to the untreated sample. This finding appears to indicate that virus specific RNA synthesis recovers faster from the short early addition of α-amanitin than the bulk of host RNA.

DISCUSSION

Our results show that α-amanitin (2.0 µg/ml) when added to polyoma virus infected baby mouse kidney cells suppresses both the production of new virions and the synthesis of virus specific RNA. Similar findings have been reported for adenovirus and simian virus 40 (2-6) and more recently in respect to the production of new virions in polyoma infected 3T3 cells and 3T3 x BHK hybrids (7). The observed inhibition of virus specific RNA was found to be accompanied by inhibition of synthesis of host ribosomal RNA but did not affect the synthesis of 4S RNA. In experiments devised to dissociate the effect on virus specific RNA from the suppression of host RNA synthesis it was found that using a lower concentration of the inhibitor (1 µg/ml) a preferential effect on virus specific RNA can be demonstrated in late infection. These findings and the fact that RNA polymerase II is sensitive to α-amanitin whereas form I is

TABLE II: Py RNA Content of RNA Samples from α-Amanitin Treated Cells and Controls

^{3}H-uridine pulse (hrs P.I.)	α-Amanitin	Viral RNA as % of input
16-22	-	0.7
16-22	+	0.8
16-22	+ (0-5 hrs)	2.0
24-30	-	0.8
24-30	+	0.9
24-30	+ (0-16 hrs)	Not done
42-48	-	1.1
42-48	+	0.3
42-48	+ (0-30 hrs)	1.0

Polyoma RNA contents of ^{3}H-RNA samples from experiment described in figure 2. DNA-RNA hybridization was performed in the presence of saturating amounts of DNA (1.6 µg).

resistant and III is only sensitive to much higher concentrations (6) clearly indicate that a class of polymerase II is involved in the transcription of polyoma DNA *in vivo*. Inhibition of host ribosomal RNA synthesis by α-amanitin has previously been noted by other authors (11-15) and it has been suggested that a newly synthesised protein might be required for transcription of ribosomal genes by polymerase I (11).

Under our experimental conditions infection by polyoma virus stimulates overall RNA synthesis of baby mouse kidney cells. The stimulatory effect appears to be coordinated with the synthesis of viral RNA and inhibition of virus specific RNA by α-amanitin (1 µg/ml) is accompanied by the inhibition of the increase in RNA synthesis associated with the presence of the virus, as can be seen by comparing treated, untreated and uninfected samples for the 16-24 hours and 42-48 hours pulses (figure 3).

Further analysis of the virus specific RNA by saturation hybridization confirms these findings for the first two pulses (16-24 hours and 30-36 hours) showing an identical content (as percentage of input) of viral specific RNA in α-amanitin treated and untreated samples. However for the late pulse between

42-48 hours a preferential inhibitory effect is seen on polyoma mRNA synthesis as shown by the reduced content (27%) of viral RNA sequences when α-amanitin treated and untreated samples are compared (table II). Here again the increased host RNA synthesis has been suppressed by the presence of the inhibitor as shown when treated, untreated and uninfected samples are compared (figure 3). The fact that this preferential effect can only be seen after 30 hours of incubation with the inhibitor at a concentration of 1 μg/ml indicates that the synthesis of classes of viral RNA during late infection are more susceptible to α-amanitin inhibition than those synthesised during early infection. A similar effect was also suggested for nucleoside analogs in the case of SV40 (16) and could be of importance in elucidating the functions required for the expression of the transformed state in cells transformed by these viruses. A preferential effect on late viral RNA synthesis could be explained either by the presence of a new (or modified) RNA polymerase or else as a result of increased accessibility of the drug to the RNA polymerase engaged in the transcription of newly synthesized free viral DNA molecules.

The inhibition of host ribosomal RNA synthesis appears to suggest that there may be a regulatory mechanism by which the amount of ribosomal RNA synthesis is adjusted to the level of protein synthesis taking place. The observation that normal amounts of polymerase I are present in the presence of the inhibitor (15) suggests the interference by a viral induced (or coded) factor (6). The finding that small molecular weight RNA is spared by amanitin confirms reports by others (2,17). This RNA has been identified as transfer RNA (18) and appears to be synthesized by polymerase III (17).

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