THE DIFFERENTIAL INHIBITORY EFFECT OF α-AMANITIN ON THE SYNTHESIS OF LOW MOLECULAR WEIGHT RNA COMPONENTS IN BHK CELLS

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1. Introduction

Vertebrate cells contain a number of low molecular weight RNA (LMW RNA) components apart from t-RNA, 5 S RNA and 5.5 S RNA (reviewed [1–3]). The major components are called D (U-1), C (U-2), A (U-3) and L. The function of these components is unknown although information is available about their metabolism [4–7], subcellular localization [4–8], occurrence in different species [9] and formation from unstable precursors [10]. Studies on the primary structures of U-1 and U-2 [11–14] show that the 5′-ends have a cap structure similar to mRNAs.

An interesting question is which polymerase synthesizes LMW RNA? We have tried to answer this question by using the differential inhibitory effect of α-amanitin. The DNA-dependent RNA-polymerases from mammalian cells can be distinguished from each other on the basis of their sensitivity towards α-amanitin. The nucleolar RNA polymerase I is insensitive to α-amanitin, the nucleoplasmic RNA polymerase II is sensitive to low concentration (0.1–1.0 μg/ml) and the nucleoplasmic RNA polymerase III is inhibited only by high concentrations of α-amanitin (about 200 μg/ml) [15–21]. When Chinese hamster ovary cells are treated with α-amanitin the synthesis of nucleoplasmic heterodisperse RNA is strongly inhibited during time periods where ribosomal RNA synthesis is not inhibited [22]. We have used similar conditions in the present investigation. The results show that α-amanitin has a differential inhibitory effect on the synthesis of the nucleoplasmic heterodisperse RNA and the low molecular weight RNA components D, C and A. It is therefore suggested that the LMW RNA components D, C and A are synthesized by RNA polymerase II.

2. Materials and methods

Baby Hamster Kidney cells (BHK-21 cells) grown in suspension cultures [6] were labelled with [^{32}P]-orthophosphate (2 μCi/ml) for 20 h. After preincubation with α-amanitin the cells were incubated for 90 min with [5-^{3}H]uridine. Ribosomal RNA and LMW RNA was extracted at 0°C with RSB (0.01 M NaCl, 1.5 mM $MgCl_2$, 0.01 M Tris–HCl, pH 7.4) and phenol containing 0.1% 8-hydroxy-quinoline [6]. The heterodisperse nuclear RNA (HnRNA) was extracted with phenol from isolated nuclei after these had been treated with proteinase K and DNAase as described in the legend to fig.3. RNA was separated on polyacrylamide gels [6], and these were scanned at 260 nm in a Zeiss spectrophotometer and the radioactivity determined [24,25]. The specific activity was determined as cpm ^{3}H incorporated from [5-^{3}H]uridine in the respective RNA component divided by the area of the absorbance peak [6].

α-Amanitin was obtained as a gift from Professor Wieland, Heidelberg and as a product from Boehringer (Mannheim).

3. Results

BHK-21 cells were preincubated with α-amanitin for different lengths of time before addition of [5-^{3}H]-

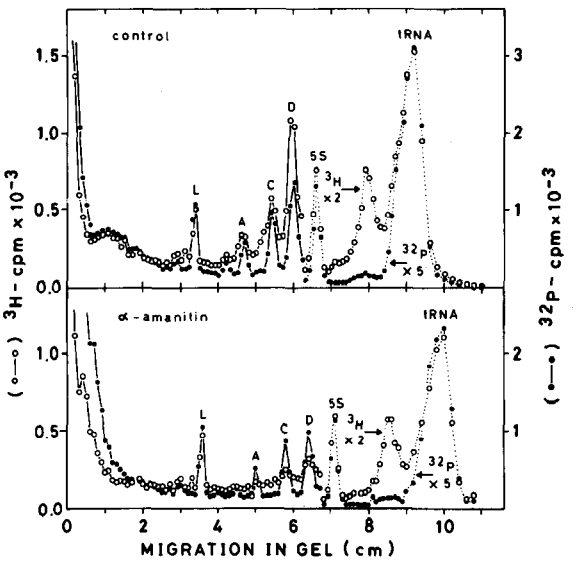

Fig.1. Inhibition of LMW RNA components D, C and A by α-amanitin. BHK cells 25 ml (5 × 10^5 cells/ml) were grown for 20 h with 50 μCi [^{32}P]orthophosphate (from Risφ, Denmark, 1.55 Ci/nmol). α-Amanitin (5 μg/ml) was added and 4 h later 150 μCi [5-^{3}H]uridine was added and the incubation continued for a further 90 min. Cell suspensions were chilled in ice and the cells were washed once with 0.15 M NaCl. RNA was extracted from whole cells with RSB and phenol at 0°C and analyzed on 10% polyacrylamide gels.

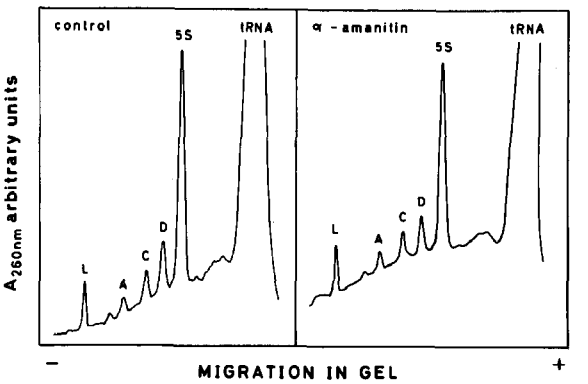

Fig.2. Absorbance profile of LMW RNA from α-amanitin-treated cells. BHK cells were grown without and with α-amanitin (5 μg/ml) for 4 h. The cells were harvested and RNA extracted and separated as indicated in fig.1 legend.

uridine and incubation was continued for a further 90 min. RNA was extracted from whole cells with phenol at 0°C and analyzed on 2.2% and 10% polyacrylamide gels, respectively. The radioactivity profiles of LMW RNA synthesized in control cells and in cells preincubated with α-amanitin for 4 h are shown in fig.1. The synthesis of component A, C and D is profoundly inhibited, whereas the synthesis of t-RNA, 5 S RNA is not affected by α-amanitin. Component L is slightly affected (see below). The synthesis of 28 S and 18 S RNA is unaffected (not shown).

The absorbance profile of LMW RNA as shown in fig.2 was used for calculation of the specific activity of the components. From this figure and from gel profiles of ribosomal RNA it can be concluded that no degradation of any of the RNA components occurs after preincubation with α-amanitin for 4 h. This was confirmed by comparing the ratio ^{32}P cpm/A_{260} for the different peaks. The ratios for 28 S, 18 S, 5 S and t-RNA were identical in control and α-amanitin-treated cells.

The specific activity of the different RNA components was determined after preincubation with α-amanitin for 2 h, 3 h, 4 h, 5 h, 7 h and 17 h (table 1). These results show that the synthesis of A, C and D are inhibited to about 50% of the control after 2 h or 3 h of preincubation with α-amanitin and that this treatment has no effect on the synthesis of 28 S, 18 S, L, 5 S or t-RNA. Preincubation for 4 h or 5 h inhibits A, C and D to 10–15% control without affecting the synthesis of 28 S, 18 S, 5 S or t-RNA but with a slight inhibition of L. After preincubation for 7 h the synthesis of A, C and D is completely blocked. The synthesis of 28 S and 18 S is inhibited to about 70% and L to 25% control but 5 S and t-RNA are not inhibited. Preincubation for 17 h with α-amanitin inhibits the synthesis of 28 S and 18 S to 6% control but does not further inhibit the synthesis of L or 5 S RNA.

The concentration of α-amanitin (5 μg/ml) used in the present experiments completely inhibits growth of BHK cells in suspension cultures whereas conc. 0.5 μg/ml only slightly inhibits growth after about 24 h incubation (E. G. J. et al. unpublished results). When BHK cells were incubated with α-amanitin at 0.5 μg/ml for 4 h under conditions as described in the legend for fig.1., only the synthesis of D, C and A was inhibited although to a somewhat lesser degree. Synthesis was not significantly inhibited at 0.1 μg/ml.

In experiments with 0.5 μg/ml α-amanitin the inhibition of D, C and A is less profound than in fig.1 and

Table 1
The effect of α-amanitin (5 μg/ml) on the synthesis of different RNA components

Preincubation	Specific activity in % control							
(h)	28 S	18 S	L	A	C	D	5 S	t-RNA
2	–	–	93	37	31	52	89	80
3	110	89	92	50	47	49	100	110
4	100	95	88	14	10	17	84	93
5	115	101	63	10	12	16	129	81
7	82	56	25	0	0	0	108	121
17	6	7	27	0	0	0	83	43

The cells were incubated and RNA extracted as described in fig.1 except for the length of preincubation with α-amanitin. Ribosomal RNA was separated on 3% polyacrylamide gels and LMW RNA on 10% gels. Specific activity was determined from the ^3H cpm in the peak divided by the area of the corresponding absorbance peak

precursor-A and precursor-C [10] can be seen together with the mature products. Thus it is concluded that the inhibitory effect of α-amanitin on the synthesis of D, C and A is not due to an inhibition of the processing of the precursors.

In order to characterize further the effect of α-amanitin on cellular RNA synthesis the inhibition of HnRNA synthesis was determined. HnRNA was obtained from cells incubated as described in legend to fig.3. After incubation for 5 h with α-amanitin (5 μg/ml), actinomycin D (0.06 μg/ml) was added to block the synthesis of ribosomal RNA [6]. The nuclear RNA was extracted from detergent-purified nuclei after treatment with proteinase K and DNAase, then electrophorezed on 2.2% polyacrylamide gels; figure 3 shows the profile of nuclear RNA extracted at 0°C. α-Amanitin has no effect on the synthesis of the ribosomal RNA precursors whereas actinomycin D completely inhibits the synthesis of these precursors. HnRNA synthesized in the presence of actinomycin is inhibited to 30–40% control in the presence of α-amanitin (lower part fig.3). Similar results were obtained when nuclear RNA was extracted from enzyme-digested nuclei by the acetate–phenol procedure at 55°C [26] or when cells were labelled for 30 min with [5-^3H]uridine instead of 1 h.

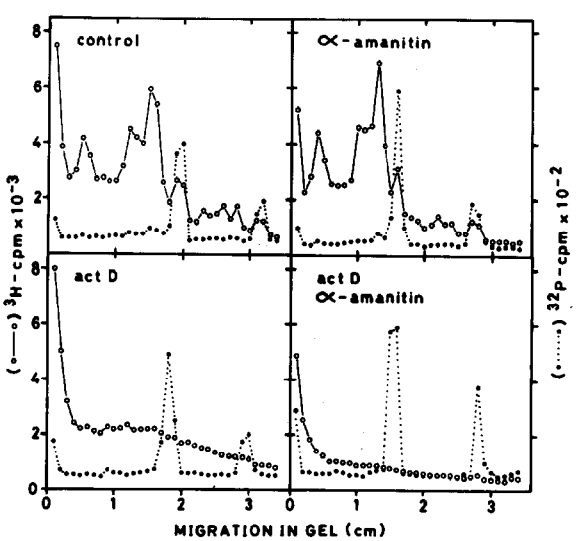

Fig.3. Inhibition of HnRNA by α-amanitin. BHK cells, 100 ml (4.2×10^5 cells/ml) were grown for 18 h with 200 μCi [^{32}P]orthophosphate and then divided into four flasks. α-Amanitin (5 μg/ml) was added to two of the flasks and incubation was continued for 5 h. Actinomycin D (0.06 μg/ml) was then added to one culture with α-amanitin and to one without α-amanitin. After 10 min incubation 300 μCi [5-^3H]-uridine was added to all of the cultures and incubation continued for further 1 h. The cells were harvested and washed with 0.15 M NaCl. The nuclei obtained by Dounce-homogenization were purified by Tween–DOC treatment [23]. The nuclei were then treated with proteinase K (100 μg/ml) in RSB with 1% SDS for 30 min. The nucleic acids were precipitated by addition of 3 vol. ethanol. The pellet was suspended in 0.8 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris–HCl, pH 7.4 and treated for 5 min with RNAase-free DNAase (20 μg/ml). The RNA was finally extracted with phenol–RSB at 0°C and separated on 2.2% polyacrylamide gels.

4. Discussion

The present data show that α-amanitin can profoundly inhibit the synthesis of LMW RNA components D, C and A and also the synthesis of HnRNA under conditions which leaves the synthesis of ribosomal RNA, 5 S RNA, t-RNA and component L undisturbed. The effect of α-amanitin on the synthesis of ribosomal RNA and HnRNA confirms results obtained with CHO cells [22] although the inhibitory effect in BHK cells described here seems to occur somewhat faster than in CHO cells. In liver cells α-amanitin has a more rapid and less specific effect [27–29]. In a recent paper [30] it is shown that in rat liver cells α-amanitin inhibits rRNA, U-1 (D), U-2 (C) as well as 5 S RNA and t-RNA. The effect on U-3 (A) was not determined.

The results presented in this paper strongly suggest that RNA polymerase II catalyzes the synthesis of LMW RNA components D, C and A since α-amanitin inhibits the synthesis of these components and HnRNA under conditions which do not inhibit the products formed by RNA polymerase I and III. It is interesting that all three LMW RNA components are synthesized by RNA polymerase II since D and C are localized in the nucleoplasm where RNA polymerase II is also localized, whereas component A is localized in the nucleolus [4–8].

RNA synthesis has been studied in isolated nuclei [21] and in nuclear suspensions with added RNA polymerase III [31] and it was suggested that this enzyme catalyzes the synthesis of LMW RNA as well as 5 S and t-RNA. Using different inhibitors of RNA synthesis it has, however, also been found that A, C and D are synthesized by RNA polymerase I [32]. The conclusion obtained in the present paper is that RNA polymerase II catalyzes the synthesis of A, C and D and this conclusion is supported by results obtained with actinomycin D and 3′-deoxyadenosine [6]. Using several different concentrations of these drugs it was shown that the synthesis of A, C and D is less inhibited than ribosomal RNA but more inhibited than the synthesis of 5 S and t-RNA [6]. Recent results obtained in this laboratory with α-amanitin-resistent CHO cells likewise support the conclusion that RNA polymerase II catalyzes the synthesis of LMW RNA components D, C and A.

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