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Proflavine sensitivity of RNA processing in isolated nuclei

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ABSTRACT

The intercalating agent proflavine inhibits the processing and subsequent release of preformed messenger RNA and ribosomal RNA from isolated liver nuclei to surrogate cytoplasm. The direct effect of proflavine on these processes, as monitored in a reconstituted cell-free system, supports the theory that base-paired segments (i.e. hairpin loops) in the precursor RNA's are involved as recognition sites in nuclear RNA processing.

INTRODUCTION

Eukaryotic cytoplasmic ribosomal and messenger RNA are derived from large precursor molecules within the nucleus by nonconservative processing which involves endonuclease cleavages (1,2). It is of interest that an enzyme with the specificity for double-stranded regions of RNA of E. coli RNase III (3,4) has been identified in HeLa cell nuclei and that 3% of the total nuclear RNA is double stranded (5). Post-transcriptional nuclear processing of messenger RNA also involves capping (6), limited methylation (7) and polyadenylation (8).

In earlier studies proflavine (acriflavine) was found to inhibit the degradation (processing) of prelabelled nuclear RNA in cultured KB (9) and L1210 (10) cells. The availability of a cell-free system, consisting of isolated nuclei in surrogate cytoplasm (fortified cytosol) which supports near normal RNA processing and transport (11-14) provided the opportunity to test the effects of the intercalating agents on post-transcriptional nuclear processes in a reconstituted system.

MATERIALS AND METHODS

The partial hepatectomy of rats and the procedures for purifying the nuclei and isolating the cytosol from both the 20 hour regenerating and resting liver were described previously (11-14). Before isolation the nuclei were prelabelled in vivo for 30 minutes or 120 minutes (as specified) with 40 or 15 uCi/200 gm body weight of orotic acid-6-^{14}C, respectively. As before the cytosol was dialyzed for 14 hours.

RNA release from isolated nuclei

The cell-free system which shows many of the characteristic of RNA processing and transport in the intact cell (12) has been described previously (11-14). The prelabelled nuclei (4.5 x 10^6/ml) were preincubated for 15 minutes at 0°C in fortified homologous cytosol with, or without (controls) the addition of proflavine, before assay at 30°C; the proflavine was dissolved in 5.0 mM Tris-HCl, pH 7.5 and an equivalent volume of this buffer was added to the controls.

Analysis of released labelled RNA

Following incubation the nuclei were removed at 500 x g and the RNA was precipitated from the nuclei freed medium with cold trichloroacetic acid for radioassay (11); the results are expressed as percent of nuclear cpm transported to the incubation medium as RNA.

For size distribution analysis of the transported RNA, the latter was purified from nuclei-freed incubation medium with phenol-chloroform (1:1) at 25°C in the presence of 1.0% sodium dodecyl sulfate (16), then separated on a 32 ml linear (10-30%) sucrose gradient using a Beckman SW 25.1 rotor (14).

Analysis of labelled nuclear RNA

Alternatively, the heterogenous nuclear RNA (Hn RNA) i.e, prelabelled in vivo for 30 minutes in the presence of a low dose of actinomycin D 150 µg/kg to preferentially inhibit the labelling of ribosomal RNA precursor (15), was purified from nuclei as follows. The nuclei were suspended in 0.14 MNaCl - 0.4% sodium dodecyl sulfate - 50 mM potassium acetate, pH 5.0 then treated at 16°C and again at 25°C with phenol-0.1% 8-hydroxyquinoline, also equilibrated with NaCl-sodium dodecyl sulfate buffer (cf 16). The nuclear RNA was separated on 30 ml sucrose (10-30%) gradients as above or where indicated with a 2.0 ml cushion of 55% sucrose at the bottom of the gradient. After centrifuging for 16 hours at 51,000 g.av. the gradients were fractionated into 1.0 ml fractions for radioassay; 0.1 vol aliquots of the unfractionated RNA was also radioassayed in order to determine the total recovery. Cytoplasmic marker 4S, 18S and 28S RNA was added to each gradient, to facilitate identification of RNA components.

Total nuclear RNA (mostly ribosomal) prelabelled for 120 minutes in vivo in the absence of actinomycin D, was purified with phenol at 65°C and again at 25°C, after resuspension of the nuclei in 0.14 M NaCl-0.5 M sodium acetate buffer, pH 5.1, containing 0.5% sodium dodecyl sulfate (14). The RNA was fractionated on a 10-30% sucrose gradient (SW 25.1 rotor, 16 hrs. at 51,000 x g) and 1.0 ml fractions were collected for radioassay.

RESULTS AND DISCUSSION

Previous studies using the cell-free system (13,14,17) confirmed earlier observations using the intact cell, that due to the longer nuclear processing time of ribosomal RNA precursors, over 80% of the labelled RNA transported during a 30 minute *in vitro* incubation at 30°C of nuclei prelabelled for only 30 minutes *in vivo*, is messenger-like; of the latter, over 60% contained poly (A) tracts. In contrast over 70% of the RNA released during a similar incubation of nuclei prelabelled for 120 minutes *in vivo* is ribosomal. Both the messenger and ribosomal RNA were released as the corresponding nucleoprotein particles.

Shown in Fig. 1 is the time-course of release to surrogate cytoplasm of messenger-like RNA from (a) 30 minute, or (b) 120 minute prelabelled normal liver nuclei. The control curves (i.e. no inhibitor) are typical of those published earlier (12,14) for predominantly (a) messenger and (b) ribosomal RNA release. Thus labelled messenger RNA release is completed by 30 minutes, while in the presence of sufficient energy, ribosomal RNA release continues beyond 40 minutes with linear kinetics. The smaller amount of RNA released from 120 minute as compared to 30 minute prelabelled nuclei during the early stages of the incubation is only apparent, since the former nuclei are more heavily labelled.

As indicated in Fig. 1(a), 100 µM proflavine had a minimal effect on RNA release during the initial 10 minutes of incubation; this initial release probably represents the transport of labelled messenger which had

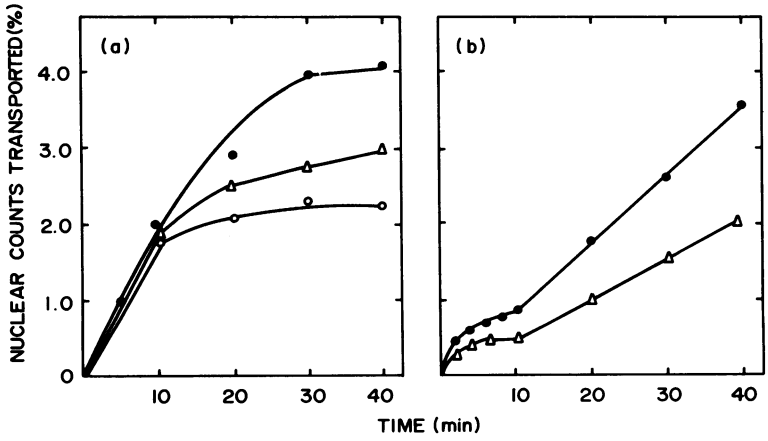

Figure 1 Time course of the release of labelled RNA from normal liver nuclei prelabelled for (a) 30 or (b) 120 minutes to homologous fortified cytosol in the absence (— ● —) or presence of 50 µM (— Δ —) or 100 µM (— o —) proflavine.

essentially matured before isolation of the nuclei. However, the intercalating agent completely inhibited the slower release which occurred during the subsequent 20 minute incubation. The labelled RNA released from the 120 minute prelabelled nuclei (Fig. 1b) was also inhibited; however, the initial RNA release was proportionately decreased, reflecting the labelled ribosomal component. This apparent discrepency in the proflavine-sensitivity of the RNA transported prior to 10 minutes incubation from the 30 and 120 minute prelabelled nuclei will be examined further below.

Shown in Fig. 2 is the dose-response curve for proflavine-sensitivity of release to homologous cytosol of RNA from 120 minute prelabelled normal, or regenerating liver nuclei. The regenerating liver system was selected for this experiment since a significantly higher proportion of the RNA processed and transported is ribosomal (14). During the 30 minute incubation a proflavine concentration of 100 µM inhibited RNA transport by 70% and 40% the normal and regenerating liver systems, respectively. The dose-response curves were not extended beyond 500 µg/ml of proflavine since controls involving the addition of proflavine at the end of the incubation period indicated that above this concentration a significant and increasing amount of RNA was lost with the nuclei. In subsequent experiments the effects of proflavine on RNA release was studied at a concentration of 100 µM with normal liver and 250 µM with regenerating liver nuclei.

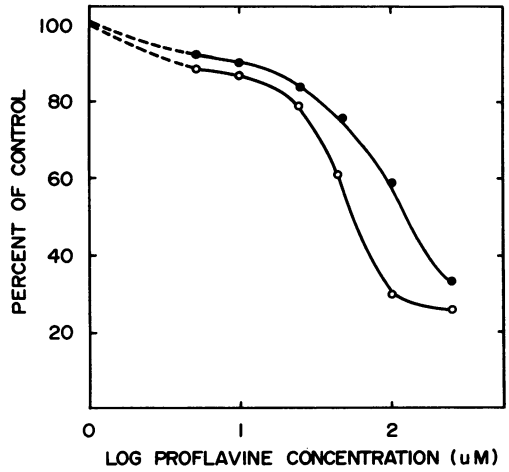

Figure 2 Dose-response curve demonstrating the effect of increasing concentrations of proflavine on the release of labelled RNA from 120 minute prelabelled normal (— o —), or regenerating (— ● —) liver nuclei to fortified homologous cytosol. The data shown are representative of a 40 minute in vitro incubation; the dose-response curves for 6 and 10 minute incubations were very similar to those shown.

That the proflavine-induced decrease is due primarily to an inhibition of nuclear RNA processing rather than to RNA transport (i.e. translocation from the site of processing through the nuclear pores) was supported by the following series of experiments.

The effect of proflavine on the intranuclear processing (i.e. size distribution) of the total nuclear RNA, representing both pre-messenger and pre-ribosomal components, is shown in Fig. 3. For this experiment regenerating liver nuclei, prelabelled in vivo for 2 hours with [6-^{14}C] arotic acid, were incubated in media containing homologous cytosol, for 40 minutes at 0°C (control) or 30°C, in the presence or absence of 250 µg/ml of proflavine. This incubation was preceded by an additional 15 minute pre-incubation of all samples at 0°C to establish optimal inhibition in those samples containing proflavine. During the in vitro incubation in the absence of proflavine (Fig. 3(a)), there is a processing of 45S pre-ribosomal RNA precursor and of the larger RNA species which probably represent the heterogeneous nuclear (pre-messenger) RNA.

The corresponding size distribution profiles of the nuclear RNA before and after incubation of the 120 minute prelabelled nuclei for 40 minutes at 30°C in the presence of 250 µM proflavine are shown in Fig. 3(b). The intercalating agent significantly inhibited the nuclear processing of high

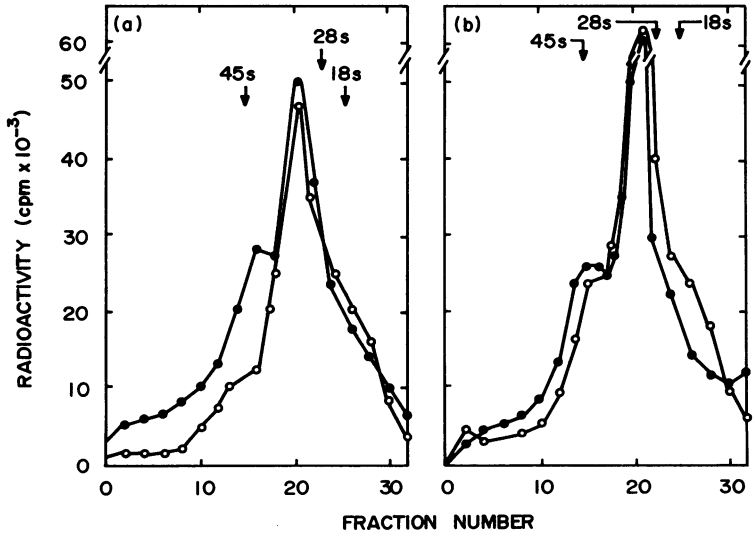

Figure 3 Size distribution of labelled RNA of regenerating liver nuclei prelabelled in vivo for 120 minutes (a) before (- ● -) and after (- o -) incubation for 40 minutes at 30°C in the absence of proflavine and (b) before (- ● -) and after (- o -) incubation at 30°C in the presence of 250 µM proflavine.

molecular weight RNA, in particular those species sedimenting in the 45S region. However there also appears to be a greater accumulation of RNA precursor molecules sedimenting in the lighter region of the gradient during processing in the presence of proflavine. Whether this accumulation is a result of decreased RNA transport is not clear.

Comparisons similar to those shown in Fig. 3 were carried out using normal liver nuclei prelabelled for 30 minutes in vivo with [6-^{14}C] orotic acid after administration of 150 µg/kg of actinomycin D, a dosage shown earlier to differentially inhibit the synthesis (labelling) of precursor ribosomal RNA. This inhibition, observed earlier in tissue culture systems (18) allowed an analysis of the effect of proflavine on the processing and transport of messenger-like RNA. Data obtained from profiles such as those shown in Fig. 3 are summarized in Fig. 4, for both 120 minute prelabelled and 30 minute prelabelled nuclei. The ratio of the radioactivity (cpm) in each 1.0 ml fraction following incubation at 0°C to that incubated at 30°C is plotted as a function of the relative size of the RNA species for both control and proflavine treated nuclei. Ratios greater than 1.0 are

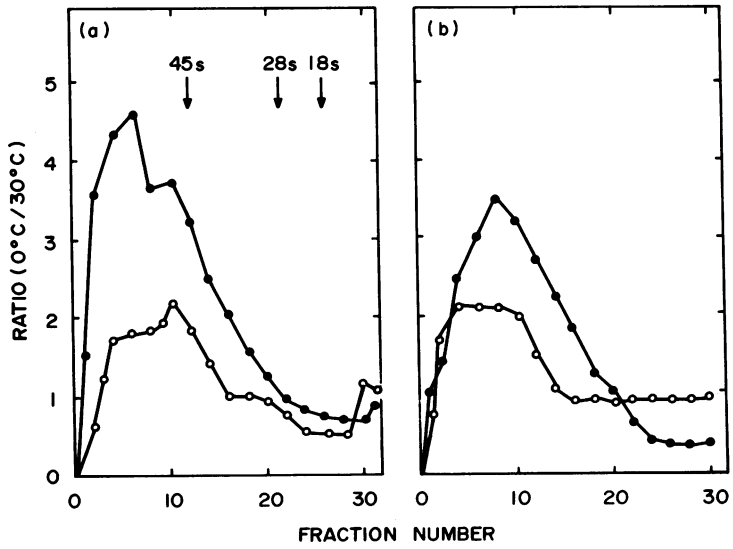

Figure 4 Ratio of radioactivity (cpm) in various sized RNA species of (a) regenerating liver nuclei prelabelled for 120 minutes in vivo or (b) normal liver nuclei prelabelled for 30 minutes in vivo in the presence of actinomycin D, for aliquots (21.6 x 10^6 nuclei/ml) held at 0°C (control), or at 30°C for 40 minutes in the absence (— ● —), or presence (—○—) of proflavine. In (a) the RNA was separated on 10-30% linear sucrose gradients and in (b) on 10-30% linear sucrose gradients with a 2.0 ml cushion of 55% sucrose to prevent pelleting of the heavier species of heterogeneous nuclear RNA.

indicative of the degree of RNA processing and therefore a decreased nuclear concentration of the larger RNA species; similarly a decrease in the ratio below 1.0 in the lighter region of the gradient would indicate an accumulation of the products of RNA processing. The results in Fig. 4(a) reflect the marked inhibition of the heavier RNA, particularly the 45S ribosomal precursor RNA shown in Fig. 3. That the proflavine also inhibits the processing of heterogeneous nuclear RNA (i.e. having nuclear RNA prelabelled within 30 minutes in vivo in the presence of 150 µg/kg of actimomycin D) which is the putative precursor of at least some messenger RNA, is shown more clearly in Fig. 4(b).

The accumulation of RNA species less than 28S was small both in the presence and absence of proflavine, due to the fact that most of the conserved RNA is transported to the medium. The inhibitor effect of proflavine was further confirmed by examining the labelled RNA transported from prelabelled nuclei in the absence and presence of the intercalating agent. Prominent among the labelled RNA species transported from the 120 minute prelabelled nuclei (Fig. 5a) are 18S and 28S ribosomal components together with components sedimenting at values less than 18S, with a peak centered at 4S. As shown previously (14, 17) labelled RNA sedimenting at values less than 18S will include 4S transfer 5S ribosomal and messenger RNA.

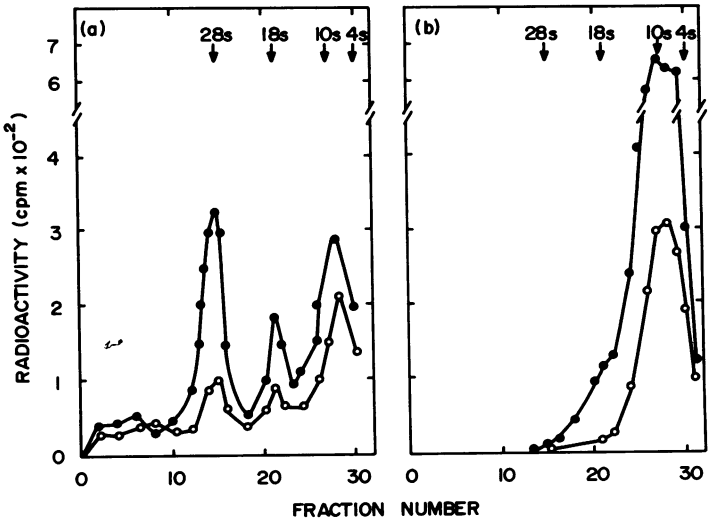

Figure 5 Size distribution of RNA transported from (a) regenerating liver nuclei prelabelled for 120 minutes in vivo or (b) normal liver nuclei prelabelled for 30 minutes in vivo in the presence of actinomycin D (150 µg/kg), then incubated for 40 minutes at 0°C or 30°C in vitro without (—●—) or with (—o—) proflavine.

Proflavine caused a depressed release of all classes of RNA, at least in part a result of decreased processing.

The effect of proflavine on the release of messenger RNA is shown more clearly in Fig. 5b, the nuclear RNA being prelabelled in the presence of 150 µg/kg of actimomycin D (cf Fig. 4b) to inhibit the labelling of ribosomal precursors. In this particular experiment 91% of the transported RNA contained poly (A) tracts characteristic of messenger RNA as determined by affinity for poly (dT)-cellulose (13). The decreased release of messenger-like RNA in the presence of proflavine can be attributed at least in part to decreased processing of the precursor RNA; 89% of this RNA had poly (A).

The results of the present study utilizing a cell-free system offers further support for the theory that base-paired regions in the nuclear precursor RNA species are involved in RNA processing. Double stranded hairpin loops may serve as recognition sites for endonucleases specific for single stranded RNA, or endonucleases specific for double stranded RNA may cleave the RNA at this site. The report (19) that mature messenger RNA contains sequences hybridizable to double stranded nuclear RNA is consistent with the cleavage of the messenger RNA precursor at the double stranded region.

While the results of the present study do not rule out completely the possibility that the intercalating agent affects nuclear RNA transport, it is clear that a significant fraction of the inhibition can be attributed interference with nuclear RNA processing. The lack of stoichiometry between messenger and ribosomal precursor RNA and products due to non-conservative processing and actual wastage of potential messenger and ribosomal RNA (i.e. nuclear RNA restriction), precludes strict analysis of the data. The fact that regenerating liver nuclei appear more resistant to proflavine than do normal liver nuclei may be due to their larger size and higher content of precursor ribosomal RNA.

ACKNOWLEDGEMENT

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