Modified Messenger Ribonucleic Acid Release from Isolated Hepatic Nuclei After Inhibition of Polyadenylate Formation

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Treatment of rats with Cordycepin (3'-deoxyadenosine), an inhibitor of nuclear poly(A) synthesis, selectively decreased the energy and cytosol-dependent release of a portion of the mRNA species from the isolated hepatic nuclei in a cell-free system by approximately 40%. An analysis of the differential binding of the transported mRNA containing poly(A) tracts to nitrocellulose filters, and to cellulose or poly(U)-Sephadex columns suggested that the poly(A) tracts in the mRNA transported from the isolated hepatic nuclei of Cordycepin-treated rats, are decreased in size. This size decrease was confirmed through an analysis of the average size of the poly(A) tracts, released from the messengers by ribonuclease activity, on sucrose density gradients.

Controls involved in the nuclear processing and nucleocytoplasmic transport of RNA have become more amenable to investigation with the development of cell-free systems (Yu *et al.*, 1972; Schumm & Webb, 1972; Schumm *et al.*, 1973) which consist of isolated nuclei in fortified cytosol. Release of rRNA as ribosomal subunits (Yu *et al.*, 1972) and mRNA as informosomes (Schumm & Webb, 1972; Schumm *et al.*, 1973) from nuclei incubated in these cell-free systems is both temperature- and energy-dependent and is closely regulated by macromolecules in the cytosol. This RNA release is dependent upon processing and transport mechanisms, but not on concomitant RNA synthesis.

The operation of post-transcriptional controls within the nucleus was suggested by the observation that a significant portion of the RNA transcribed within the nucleus never reaches the cytoplasm of the normal eukaryotic cell (Aronson & Wilt, 1969; Harris, 1970; Cooper & Gibson, 1971; Rizzo & Webb, 1972). These, as yet unidentified, controls appear to be modified in the transformed cell (Shearer & Smuckler, 1972). The addition of poly(A) to a portion of the potential mRNA (i.e. heterogeneous nuclear RNA) has been implicated (Edmonds *et al.*, 1971; Brawerman *et al.*, 1972; Adesnick *et al.*, 1972; Rizzo *et al.*, 1972; Butcher *et al.*, 1972) as one of the post-transcriptional events in the processing of mRNA in the nucleus. Most, but not all, eukaryotic messengers are transported in combination with poly(A) tracts (Adesnick *et al.*, 1972).

Cordycepin (3'-deoxyadenosine) has been a very useful tool in the study of the involvement of poly(A) in nuclear processing since in the intact cell it has little effect on the synthesis of heterogeneous nuclear RNA, but causes a strong inhibition of

poly(A) and a moderate inhibition of rRNA synthesis (Penman *et al.*, 1970). The inhibition of rRNA synthesis is apparently due to the premature termination of the 45S ribosomal precursor RNA by the analogue rather than to a general inhibition of RNA synthesis (Darnell *et al.*, 1971). The suppression of labelling of cytoplasmic mRNA in the presence of Cordycepin, on the other hand, appears to be due to an inhibition of mRNA processing and transport as a result of the inhibition of poly(A) synthesis (Darnell *et al.*, 1971). In the present study Cordycepin is used in conjunction with a cell-free system consisting of regenerating liver nuclei in autologous cytosol, to evaluate further the involvement of poly(A) in mRNA processing and transport, and the necessity of these tracts for RNA processing and transport. The results, although compatible with the theory that poly(A) is necessary for processing or transport (i.e. release to the incubation medium) of the portion of messenger containing poly(A) tracts, also suggest that the size of the tracts may be considerably decreased before transport is inhibited.

Materials and Methods

The nuclei were prepared from the regenerating liver of male rats (250g) of the Sprague-Dawley strain 19h after the surgical removal of two-thirds of the liver (Rizzo *et al.*, 1971). The nuclear-isolation procedure, which is that of Muramatsu & Busch (1968) with minor modifications, involves the homogenization of minced liver in 2.3M-sucrose (1:15, w/v) containing 3.3mM-calcium acetate, followed by sedimentation of the nuclei at 40000g for 1h. The nuclear pellet is resuspended in 1.0M-sucrose (1.5ml/g of liver) containing 1.0mM-calcium acetate, then

centrifuged at 2000g for 5min to give a pellet of purified nuclei. When prepared by this procedure there is little loss of heterogeneous nRNA. The nRNA was pre-labelled by injecting interperitoneally 40 μ Ci of [6-¹⁴C]orotic acid/250g 30min before the removal of the regenerating liver remnant. Where indicated the rats also received 20mg of Cordycepin/kg (interperitoneally) 30min before the injection of the isotope.

For comparison of the release of RNA from the nuclei of normal and Cordycepin-treated rats, the corresponding nuclei were incubated at 30°C in an optimum cell-free system containing autologous cytosol (dialysed 105000 g supernatant) fortified with spermidine, ATP, an energy-regenerating system and appropriate buffers and salts as described by Schumm & Webb (1972). The time-course of the release of RNA from the nuclei was followed by precipitating the released RNA from the nuclei-free incubation medium with cold 5% (w/v) trichloroacetic acid, then counting the hyamine-solubilized precipitate for radioactivity in liquid scintillant (Yu *et al.*, 1972). A size-distribution analysis of the released RNA was performed by centrifuging the RNA, purified from the nuclei-free incubation medium with phenol-chloroform (1:1, v/v), through 10–30% (w/v) sucrose density gradients as described by Schumm *et al.* (1973). The proportion of transported labelled RNA that contained poly(A) tracts was estimated by measuring the percentage of purified labelled RNA that adsorbed to cellulose columns or Millipore filters in the presence of 500mM-KCl (Brawerman *et al.*, 1972; DeLarco & Guroff, 1973), or to poly(U)-Sephacrose columns (Lindberg & Persson, 1972). The ability of the RNA containing poly(A) tracts (i.e. that which was released from normal isolated nuclei and which adsorbed to cellulose columns) to stimulate amino acid incorporation was tested in a cell-free protein synthesizing system. The latter system consisted of purified rat liver ribosomes and initiation factors in a reconstituted system identical with that described by Uenoyama & Ono (1972).

The size of the poly(A) tracts released from nuclei in the cell-free system was estimated as follows. The nRNA in the regenerating liver of control rats and those pretreated for 30min with 20mg of Cordycepin/kg was labelled *in vivo* for 30min with 20 μ Ci of ¹⁴C-labelled adenosine before isolation of the nuclei. The RNA released from the nuclei during a 20min incubation in homologous cytosol was purified by phenol-chloroform extraction, precipitated with ethanol, then dissolved in 2×SSC. After digestion with pancreatic ribonuclease (10 μ g/ml) for 45min at 37°C, carrier yeast RNA was added and the digested material was re-extracted with phenol-chloroform; the ethanol-precipitated RNA was dissolved in 0.01M-Tris, pH7.6, 0.1M-KCl, 0.001M-EDTA and a sample was analysed on a

5–20% (w/v) sucrose gradient made with the same buffer. The gradients were centrifuged for 18h at 22000rev./min in a SW 25.1 rotor of a Beckman ultracentrifuge. One gradient contained marker tRNA. Fractions (1 ml) were collected for radioactivity counting in liquid scintillant.

The [6-¹⁴C]orotic acid (sp. radioactivity 323 μ Ci/mg) was purchased from the New England Nuclear Corp., Boston, Mass., U.S.A. Cordycepin (3'-deoxyadenosine) and deoxyribonuclease I, were obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A., and from the Worthington Corp., Freehold, N.J., U.S.A., respectively. The filters used for the poly(A) binding assay were purchased from the Millipore Filter Corp., Bedford, Mass., U.S.A. Poly(U) and Sepharose 4B were purchased from Miles Laboratories Inc., Kankakee, Ill., U.S.A. and Pharmacia Fine Chemicals Inc., Piscataway, N.J., U.S.A., respectively.

Results

As indicated by Rizzo & Webb (1972), electron micrographs of the purified nuclei showed normal ultrastructure including an intact double nuclear membrane; they were essentially free of cytoplasmic membranes with associated ribosomes. Further, when the nRNA was pre-labelled *in vivo* for 10min then the nuclei were isolated, less than 5% of the total nuclear acid-precipitable radioactivity was lost to the homogenizing medium, indicating that there was little loss of heterogeneous nRNA during the purification procedure (D. E. Schumm, unpublished work).

The time-course of the energy-dependent release of labelled RNA from nuclei labelled *in vivo* for 30min with [6-¹⁴C]orotic acid is shown in Fig. 1(a). Less than 0.5% of the total nuclear radioactivity was released in the absence of the energy source. The latter not only further confirms the ATP-dependence (cf. Yu *et al.*, 1972; Schumm & Webb, 1972) of the release reaction but also demonstrates the stability of the nuclei in the system *in vitro*. In the presence of an energy source and an energy-regenerating system the time-dependent release of labelled RNA is biphasic; this biphasic release is predictable from a consideration of the labelling time and the known differences in the processing time for rRNA and mRNA (Rizzo & Webb, 1972). Earlier RNA-DNA hybridization studies indicated (Schumm & Webb, 1972) that the RNA released during the first 20min of incubation was predominantly messenger, whereas that occurring in a second burst of RNA release, beginning after approximately 35min of incubation, was predominantly ribosomal. Further, it was shown that the messenger and ribosomal RNA species were released as ribonucleoprotein particles, i.e. as informosomes and ribosomal subunits, respectively.

Fig. 1. Labelled RNA released from the isolated nuclei of regenerating rat liver after 30min prelabelling *in vivo* with 40 μ Ci of [6-¹⁴C]orotic acid

(a) Time-course of the release of RNA from the nuclei of control (●) and Cordycepin-treated (○) rats. (b) Size distribution of the labelled RNA transported during a 20min incubation of the nuclei from control (●) or Cordycepin-treated (○) rats. In (a) the rapidly labelled RNA released to the medium was estimated by radioassay after trichloroacetic acid precipitation from samples of the nuclei-free medium. In (b) the labelled RNA transported over a 20min period was purified from samples of the medium by use of phenol-chloroform (1:1, v/v), then separated by size on sucrose density gradients. The top of the gradient is to the right. Each 10ml of incubation medium contained approximately 5×10^6 nuclei with a total of approximately 26000 c.p.m. as RNA. Further experimental details are given in the Materials and Methods section. Bars indicate $\pm$ s.d.

Rizzo *et al.* (1972) also showed that Cordycepin at a dose of 10mg/kg inhibited the accumulation of ribosomes in rat liver cytoplasm with 2.5 h of administration. This effect was attributed to a premature termination of the transcription of the 45S ribosomal precursor in the presence of the analogue. Although Cordycepin at a concentration of 10 or 20mg/kg did not appear to affect the synthesis of heterogeneous RNA in rat liver, it appeared to decrease the poly(A) content by 50% as measured by the ability of the latter to bind to Millipore nitrocellulose filters in the presence of 0.5M-KCl (Brawerman *et al.*, 1972), and inhibited the corticosteroid-mediated induction of tyrosine aminotransferase by 60%. This inhibition appeared to be due to interference with poly(A) tract formation rather than to inhibition of synthesis of the precursor mRNA. As shown in Fig. 1(a), pretreatment of rats with Cordycepin before the administration of [6-¹⁴C]-orotic acid did not modify the biphasic nature of the RNA release; however, the amount of labelled RNA transported during the first 20min was decreased by 40%. In contrast the amount of RNA released after a 60min incubation, was comparable with that released from the nuclei of untreated rats. Only after pretreatment with Cordycepin for more than 1 h was the synthesis of rRNA adversely affected (Rizzo *et al.*, 1972).

As shown previously for the nuclei from normal and regenerating rat liver (Schumm & Webb, 1972; Schumm *et al.*, 1973), the labelled RNA released from the nuclei of Cordycepin-treated rats after a 20min incubation *in vitro* was present in ribonucleo-

protein particles with a buoyant density of 1.47 and a sedimentation size range of 20S to greater than 100S, the major portion sedimenting at approximately 40S. This was followed after 30min incubation by the appearance of labelled 40S and 60S ribosomal subunits with buoyant densities in CsCl of 1.54 to 1.61 (D. E. Schumm, unpublished work).

The labelled messenger-like RNA transported during the first 20min of incubation was purified from the incubation medium by use of phenol-chloroform, a method shown (Ono *et al.*, 1971) to preserve intact the poly(A) portion of the mRNA. The size-distribution pattern of the RNA in sucrose density gradients (Fig. 1b) indicates that pretreatment of the rats with Cordycepin decreases the amounts of heavy (i.e. 20S and 26S) messenger-like RNA species which were shown (Schumm & Webb, 1972) to be restricted to regenerating tissue. The concentrations of the main mRNA component of regenerating liver (which is also the main component in normal and neoplastic liver) centred at approximately 10S, was also significantly decreased by the Cordycepin treatment. The 10S, 20S and 26S RNA peaks were identified as mRNA by analysis of poly(A) content and by DNA-RNA competition-hybridization studies (Schumm *et al.*, 1973). The proportion of the 20S and 26S RNA which bound to Millipore filters, was much higher than that of the 10S peak and this fact could account for the high sensitivity of the former to Cordycepin. All three RNA components could also be extracted from ribonucleoproteins that had the same density in CsCl as that characteristic of informosomes (Schumm *et al.*, 1973). The

Table 1. *Effect of Cordycepin on the transport of mRNA from isolated nuclei*

The rapidly labelled RNA released to the incubation medium over a 20min incubation period was estimated by radioassay after trichloroacetic acid precipitation from samples of the nuclei-free medium. Transported mRNA was also purified from samples of the latter by use of phenol-chloroform mixture and the proportion containing poly(A) tracts was estimated from the degree of absorption to nitrocellulose filters, or to cellulose or poly(U) columns. Other conditions were identical with those in Fig. 1.

Pretreatment <i>in vivo</i>	Released labelled mRNA			
	Total trichloroacetic acid-precipitable radioactivity (c.p.m.)	% containing poly(A)		
		Nitrocellulose filters	Cellulose column	Poly(U) column
None (controls)	11290 $\pm$ 600	38 $\pm$ 3	40 $\pm$ 3	41 $\pm$ 3
Cordycepin	7423 $\pm$ 350	6 $\pm$ 2	22 $\pm$ 3	45 $\pm$ 3

decreased transport of the 20S and 26S RNA from regenerating nuclei after Cordycepin treatment does not appear to be due to enhanced nuclease activity as these components were also transported when nuclei from untreated rats are incubated in cytosol from Cordycepin-treated animals. Also the ratio of trichloroacetic acid-soluble to insoluble radioactivity remained constant during a 20min incubation irrespective of whether the nuclei were derived from a normal or Cordycepin-treated rat; only the total number of radioactive counts released to the incubation medium varied with the source of the nuclei. However, after 60min of incubation a slight increase occurred in the proportion of acid-soluble radioactivity released from the Cordycepin-treated nuclei, perhaps owing to breakdown of RNA that was not transportable (D. E. Schumm, unpublished work).

Brawerman *et al.* (1972) showed that mRNA from eukaryotic cells with poly(A) tracts of approximately 200 nucleotides bind to Millipore filters in 0.5M-KCl; however, messengers with tracts containing less than approximately 70 adenylic acid residues do not bind under these conditions (Eaton & Faulkner, 1972). On the other hand, Sheiness & Darnell (1973) showed that messengers can contain poly(A) tracts of less than 50 nucleotides, and from investigations of RNA-RNA interactions (Niyogi, 1969), it is predicted that tracts containing as few as 12 nucleotides will form stable helices with and therefore bind to poly(U) columns. Thus differential adsorption to nitrocellulose filters and poly(U) columns provides a rapid and efficient method of estimating the general size of the poly(A) tracts in messengers and consequently of detecting any drastic changes in the size of these tracts. This method has been used to detect possible changes in the size of the poly(A) tracts in the released mRNA as a result of Cordycepin pretreatment.

As indicated by the data in Table 1, the amount of labelled RNA transported is decreased approximately

40% when the nuclei are derived from Cordycepin-treated rats. Further, in contrast with the labelled RNA transported from the nuclei of control rats, the fraction of RNA released from the nuclei of Cordycepin-treated rats which contained poly(A) tracts, depended markedly on the type of assay used. For example, estimates of the percentage of mRNA containing poly(A) tracts released from the nuclei of Cordycepin-treated rats as compared with control rats was 13, 55 and 100% as estimated by the Millipore filter, cellulose column and poly(U)-Sephadex column assay, respectively. No such variation was observed when the three assays were applied to the mRNA released from the nuclei derived from the liver of control rats. It is concluded that Cordycepin decreases the size of the poly(A) tracts in the transported mRNA in addition to inhibiting the release of a fraction of the RNA transported from normal nuclei. Thus the data in Table 1 suggest that Cordycepin decreases the average length of the poly(A) tracts in the transported mRNA, whereas the proportion of molecules that contain some poly(A), however short that tract may be, remains essentially constant.

That the size of the poly(A) tract on the messenger-like RNA transported from nuclei of Cordycepin-treated rats is shorter than the corresponding RNA transported from the nuclei of control rats in the cell-free system was confirmed by separation of the poly(A) tracts on sucrose density gradients. Fig. 2 shows the size-distribution patterns of the poly(A) fragments released by use of ribonuclease from the mRNA transported from liver nuclei of normal and Cordycepin-treated rats. The rats were pretreated for 30min with Cordycepin then the poly(A) tracts were labelled *in vivo* for 30min with [14 C]adenosine before isolation of the nuclei. It is clear that the results of the gradient analysis agree with the results of the cellulose and poly(U) chromatography in that the size of the poly(A) fragment is markedly smaller as a result of Cordycepin treatment. These results

Fig. 2. A size-distribution analysis by using sucrose density gradients of the poly(A) tracts released in the cell-free system from liver nuclei of normal (○) and Cordycepin-treated (■) rats

The poly(A) tracts prelabelled with [^{14}C]adenosine were released from the messenger-like RNA with ribonuclease before density-gradient analysis. The top of the gradient is to the right and the position of marker 4S tRNA in the gradient is indicated.

agree with those of Sarkar *et al.* (1973) who demonstrated that the poly(A) tract isolated from embryonic chick neural retina is decreased in size in response to Cordycepin treatment. It is noteworthy that the size distribution of the poly(A) tracts released from the nuclei of the regenerating liver of untreated rats is similar to that isolated from the messengers of mouse sarcoma 180 ascites cells (Lee *et al.*, 1971) and from murine leukemia-sarcoma virus (Ross *et al.*, 1972).

Discussion

The present study was undertaken to investigate the relationship between the poly(A) tracts and the transport (release) of mRNA from nuclei by using the cell-free system. In addition to the use of isolated nuclei, advantage has been taken of the report (Darnell *et al.*, 1971) that Cordycepin inhibits processing and/or transport of mRNA, but not the synthesis of the mRNA precursors in cells in culture; this drug has also been shown (Adesnick *et al.*, 1972) to inhibit poly(A) synthesis in tissue-culture cells. The results reported above indicate that there is a significant inhibition of the release of RNA from from the nuclei of Cordycepin-treated animals. This applies, in particular, to the two classes of the

high-molecular-weight messenger-like RNA of high poly(A) content which are released from the nuclei of regenerating, but not normal (resting) or neoplastic rat liver (Schumm *et al.*, 1973).

Although it is still not clear whether poly(A) is obligatory for the release of mRNA from isolated nuclei, and consequently the regulated processing and nucleocytoplasmic transport *in vivo*, the present data do suggest that the transport of a fraction of the mRNA is inhibited. The results also indicate that energy and cytosol-dependent transport (release) of a fraction of the mRNA from the isolated nuclei can occur under circumstances where the size of the covalently linked poly(A) tracts has been decreased. Coincidentally, the results also confirm that Cordycepin, an adenosine analogue, acts primarily by inhibiting poly(A) synthesis and that the effects observed *in vivo* are not primarily the results of interference with energy supply of the cell, or to other non-specific effects. Further, the present results are in general agreement with studies *in vivo* (Adesnick *et al.*, 1972; Penman *et al.*, 1970) which indicate that Cordycepin both inhibits the appearance of mRNA in polyribosomes and interferes with poly(A) tract formation.

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