Inhibition of Ribonucleic Acid Efflux from Isolated SV40-3T3 Cell Nuclei by 3'-Deoxyadenosine (Cordycepin)

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The effect of 3'-deoxyadenosine (cordycepin) on mRNA efflux from isolated SV40-3T3 cell nuclei has been studied and compared with its effect on the nucleoside triphosphatase activity in the isolated nuclear envelope. Inhibition of mRNA efflux occurs rapidly, but is dependent on the presence of ATP. Half-maximal inhibition occurs with 40 μ M-cordycepin. The effect is not simulated by 2'-deoxyadenosine or by actinomycin D, and adenosine provides a substantial degree of protection against it. Cordycepin does not directly inhibit the nucleoside triphosphatase. The stimulation of this enzyme by poly(A) is not affected unless the poly(A) and cordycepin are incubated together with nuclear lysate in the presence of ATP; in this case the stimulation is significantly reduced. Possible interpretations of these results and their relevance for understanding the system *in vivo* for nucleo-cytoplasmic messenger transport are discussed.

Cordycepin (3'-deoxyadenosine) is capable of inhibiting RNA synthesis on the DNA template (Shiguera & Gordon, 1965; Maale *et al.*, 1975). However, it has also been shown to inhibit nucleo-cytoplasmic mRNA transport independently; it prevents the appearance of mRNA in the cytoplasm of some eukaryotic cells *in vivo* without markedly affecting the incorporation of labelled bases into hnRNA (Adesnik *et al.*, 1972; Darnell *et al.*, 1971; Mendecki *et al.*, 1972; Nakazato *et al.*, 1974). Intranuclear ATP-dependent conversion of cordycepin into its 5'-triphosphate (Klenow, 1963) is believed to occur before its incorporation into the nascent RNA chain (Maale *et al.*, 1975), or into the poly(A) sequence added post-transcriptionally (Darnell *et al.*, 1971; Mendecki *et al.*, 1972; Nakazato *et al.*, 1974), thus preventing further chain elongation. It is now clear that post-transcriptional adenylation is not a prerequisite of nucleo-cytoplasmic mRNA transport (Adesnik & Darnell, 1972; Milcarek *et al.*, 1974; Nemer *et al.*, 1975). However, the recent demonstration that poly(A) can behave as an activator of the nucleoside triphosphatase (NTPase) (Agutter *et al.*, 1977), located in the pore complexes (Yasuzumi & Tsubo, 1966), which is believed to be essential for this transport (Agutter *et al.*, 1976), led us to investigate the effect of cordycepin on the ATP-dependent efflux of mRNA from mammalian nuclei *in vitro*. The results were compared with the effect of the

RNA polymerase inhibitor actinomycin D. The incubation system *in vitro* devised by Schumm and co-workers (Schumm & Webb, 1972; Schumm *et al.*, 1973) has been shown to result almost exclusively in the efflux of messenger sequences unless cytoplasmic proteins from the same cell line as the nuclei are present in the incubation medium. In this case, ribosomal subunits are transported to the supernatant as well (Racevskis & Webb, 1974; Sato *et al.*, 1977*a,b*). These findings have been verified in this laboratory. Using a modification of the Schumm system (see the Materials and Methods section), we have found that, in the absence of ATP and cytoplasmic proteins, the RNA eluted from isolated nuclei is predominantly 4–5S material. On addition of ATP, material of 8–18S, $52 \pm 10\%$ of which contains poly(A), is also eluted. When both ATP and the cytoplasmic proteins are present, this 8–18S material again appears in the supernatant, along with 18S and 28S peaks (P. S. Agutter, unpublished work). Since the problem under investigation in this paper is primarily the effect of cordycepin on mRNA transport, cytoplasmic proteins were omitted in the experiments described.

Materials and Methods

Adenosine 5'-triphosphate (ATP, disodium salt, grade II), adenosine, 2'-deoxyadenosine, cordycepin (3'-deoxyadenosine, grade III), cordycepin 5'-monophosphate, cordycepin 5'-triphosphate, actinomycin D, spermidine, dithiothreitol, polyadenylic acid [poly(A)] and poly(U)-Sepharese were obtained

Abbreviation used: NTPase, nucleoside triphosphatase.

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from Sigma Chemical Company, London, U.K. Pancreatic ribonuclease (RNAase) and deoxyribonuclease (DNAase I) were obtained from the Boehringer Corp., Lewes, East Sussex, U.K. [$5\text{-}^3\text{H}$] Uridine (25 Ci/mmol) was obtained from The Radiochemical Laboratory, Amersham, Bucks., U.K. All other reagents used were of AnalaR grade.

Cell culture

Swiss mouse SV40-3T3 fibroblasts (Flow Laboratories Ltd., Irvine, Scotland, U.K.), were grown to a density of 2.5×10^8 cells/bottle in roller culture with Dulbecco's modification of Eagle's medium, supplemented with 10% calf serum. For RNA-efflux experiments, [$5\text{-}^3\text{H}$]uridine (50 μCi /bottle) was added 5 h before harvesting of the cells (Agutter *et al.*, 1976).

Isolation of nuclei

The cells were resuspended in 2 ml of homogenizing buffer (10 mM-Tris/HCl/1 mM-MgCl₂, pH 7.4) at 5°C and ruptured by 20 passages through a syringe needle of 1.1 mm internal diameter. Sucrose (60%, w/v) in homogenizing buffer was added slowly to the homogenate until the sucrose concentration was 50–52%. The suspension was then layered over 10 ml of 58% (w/v) sucrose in the same buffer and centrifuged at 130000g in the SW.27 rotor of a Beckman L2-65B ultracentrifuge for 1 h at 4°C. The pellet of nuclei was washed twice by resuspension in homogenizing buffer and centrifugation at 800g for 10 min. For RNA-efflux experiments, the pure nuclei were given a further wash in incubation buffer (see below).

Isolation of RNA-depleted nuclear envelopes

Envelopes were isolated by the method of Harris & Milne (1974), except that 0.1 mM-MgCl₂ was added during the DNAase I treatment. Before the sucrose gradient purification, the envelopes were incubated for 10 min at 22°C with RNAase (10 μg /ml) and submitted to a further two washes in 1 mM-NaHCO₃.

Measurements of RNA efflux

Nuclei were suspended to a concentration of about 10^6 /ml in incubation buffer (50 mM-Tris/HCl/25 mM-KCl/2.5 mM-MgCl₂/0.3 mM-MnCl₂/0.5 mM-CaCl₂/5.0 mM-spermidine/2.0 mM-dithiothreitol, pH 7.6) and ATP was added to a concentration of 2.5 mM. The suspension was incubated for 2–20 min at 37°C, the nuclei were pelleted by centrifugation at 1500g for 10 min at 4°C, and 0.2 vol. of 50% trichloroacetic acid was added to the supernatant. The pellet obtained by centrifugation at 3000g for 15 min was hydrolysed for 30 min at 60°C with 0.1 ml of 0.1 M-NaOH, diluted with 0.9 ml of water, and added to 15 ml of Scintillator Fluid NE 250 (Nuclear Enterprises). The amount of radioactive label was determined by using

a Packard Tri-Carb scintillation counter with the usual quench correction. In controls, the ATP was replaced by an equivalent volume of distilled water.

NTPase assay

RNA-depleted nuclear envelopes (about 50 μg of protein) were incubated with 2.5 mM-ATP/2.5 mM-MgCl₂/25 mM-triethanolamine/HCl, pH 8.0, in a final volume of 1.6 ml at 37°C for 15 min, and the inorganic phosphate released was determined by the method of Wahler & Wollenberger (1958).

Pretreatment with poly(A)

Pure nuclei (approx. 100 μg of protein) were suspended in 0.5 ml of 25 mM-triethanolamine/HCl, pH 8.0, and sonicated for 10 s at 0°C with the 0.2 cm probe of an MSE ultrasonic disintegrator set at 4 μm peak-to-peak amplitude. Poly(A) (0.5 ml; 200 μg /ml), in the same buffer with or without other reagents as described in the text below, was added and the suspension incubated at 22°C for 10 min. The poly(A) was recovered by centrifuging the suspension at 3000g for 10 min at 4°C, followed by precipitation from the supernatant with aq. 95% ethanol.

Characterization of RNA released

The trichloroacetic acid precipitate of the supernatant after the incubation of the nuclei (see above) was deproteinized with phenol/chloroform and the remaining RNA precipitated with ethanol. This RNA was redissolved in 10 mM-Tris/HCl, pH 8.0, and centrifuged on a 0–50% (w/v) sucrose gradient in the same buffer in the 3×5 ml swing-out rotor of an MSE 50 ultracentrifuge for 5 h at 5°C. Fractions (0.25 ml) of this gradient were reprecipitated with ethanol and the precipitates digested with NaOH, and the radioactivity was determined as described above. Values were expressed as percentages of total nuclear radioactivity. Pure 28S and 18S RNA and mixed tRNA (Boehringer, Mannheim, Germany) were run on the gradients as standards.

Results

The uridine ^3H label incorporated into the nuclei was largely present in the RNA (Agutter *et al.*, 1976) at $12 \pm 2 \times 10^3$ d.p.m./ 10^6 nuclei. After 20 min incubation in the medium used, the amount of label present in the supernatant was 40 ± 8 d.p.m./ 10^6 nuclei in the absence of ATP, and 345 ± 50 d.p.m./ 10^6 nuclei in the presence of ATP. Thus efflux over the 20 min time-course amounted to about 2.5% of the total nuclear RNA.

Fig. 1 demonstrates the inhibitory effect of cordycepin on RNA efflux from isolated nuclei. This effect is clearly not simulated by 2'-deoxyadenosine at the same concentration or by actinomycin D at

Fig. 1. Inhibition of RNA efflux from isolated nuclei

Time-course of appearance of trichloroacetic acid-precipitable ^3H radioactivity in the supernatant during incubation of nuclei. ○, Control (i.e. difference between radioactivity counts released in the presence and the absence of 2.5 mM-ATP); ●, with 0.2 mM-cordycepin added; □, with 0.2 mM-2'-deoxyadenosine added; ■, with actinomycin D (1 $\mu\text{g}/\text{ml}$) added. The results are means $\pm$ 1 S.E.M. for three experiments.

Fig. 2. Effects of cordycepin concentrations on inhibition of efflux

ATP-dependent release of trichloroacetic acid-precipitable ^3H radioactivity from isolated nuclei during a 10 min incubation in the presence of various concentrations of cordycepin. The results are means $\pm$ 1 S.E.M. for three determinations.

ATP. Fig. 2 shows the dependence of inhibition of efflux on cordycepin concentration; the concentration for half-maximal inhibition is approximately 40 μM (10 $\mu\text{g}/\text{ml}$), which is lower than that required for significant inhibition of RNA polymerase activity (Levey & Brinster, 1977).

In another series of experiments, the nuclei were preincubated with cordycepin in the presence and absence of ATP, or with cordycepin 5'-triphosphate. The nuclei were recovered by centrifugation at 800g and washed three times with fresh incubation medium; RNA-efflux rate was then measured as before. The results (Fig. 3) suggest that ATP is necessary for the inhibitory effect of cordycepin.

Fig. 4 shows that the inhibition by cordycepin 5'-triphosphate is less rapid than that by cordycepin alone during ATP-stimulated efflux, possibly because of the relatively slow entry of the triphosphate into the nucleus (see the Discussion section). Cordycepin 5'-monophosphate, however, acts approximately as quickly as the free nucleoside.

Fig. 5 shows that inclusion of a 20-fold molar excess of adenosine over cordycepin in the incubation medium greatly reduces the inhibitory effect of the latter. The protective effect of adenosine was found to be correspondingly less marked when it was present in 10- or 5-fold excess. In less than 5-fold excess its effect was not significant.

1 $\mu\text{g}/\text{ml}$. Preincubation of the nuclei for 10 min with actinomycin D at 1 $\mu\text{g}/\text{ml}$ concentration before addition of ATP to the medium had an effect on RNA efflux not significantly different from that shown in Fig. 1; that is, retardation of efflux did not become evident until 12–15 min after addition of

Fig. 3. Effects of preincubation with cordycepin on RNA efflux

ATP-dependent RNA release from nuclei preincubated for 10 min at 37°C with: ●, 0.2 mM-cordycepin; □, 0.2 mM-cordycepin + 1 mM-ATP; ■, 0.2 mM-cordycepin 5'-triphosphate. The control nuclei (○) were preincubated in the incubation medium alone for 10 min at 37°C. Between the preincubation and the determination of ATP-dependent RNA efflux the nuclei were washed thrice with fresh incubation medium (see the text). The results are means $\pm$ 1 s.e.m. for three experimental observations.

Fig. 4. Inhibition of RNA efflux by cordycepin mono- and tri-phosphate

ATP-dependent RNA efflux was measured as before, but 0.2 mM-cordycepin (●), 0.2 mM-cordycepin 5'-monophosphate (□) or 0.2 mM-cordycepin 5'-triphosphate (■) was added after 5 min incubation. To the controls (○) a similar volume (one-fifth of total incubation volume) of fresh incubation medium was added at the same time. The results are means $\pm$ 1 s.e.m. for three experiments.

Fig. 5. Protection against the effects of cordycepin by adenosine
Time-course of RNA efflux in the presence of: ●, 0.1 mM-cordycepin; □, 2 mM-adenosine; ■, 0.1 mM-cordycepin + 2 mM-adenosine. ○, Control. The results are means ± 1 S.E.M. for three experiments.

Table 1. Nucleoside triphosphatase activity and its stimulation by poly(A) in the presence of cordycepin
NTPase activities in isolated nuclear envelopes were assayed in the presence and the absence of poly(A). Cordycepin and cordycepin 5'-triphosphate were present at 0.2mM concentration. Cordycepin 5'-triphosphate was itself hydrolysed by the enzyme at approximately the same rate as ATP; the K_m value for each substrate was 0.27 ± 0.04 mM (Agutter *et al.*, 1976). The results quoted here are mean values (μ mol of ATP/h per mg of protein) ± 1 S.E.M. for four experiments.

	Without poly(A)	With 50 μ g of poly(A)/ml
Control	5.1 ± 1.3	11.4 ± 1.8
+ Cordycepin	4.7 ± 0.8	11.8 ± 1.5
+ Cordycepin 5'-triphosphate	5.6 ± 1.1	12.7 ± 2.1

These results may be taken to suggest that the observed inhibition of RNA efflux (a) is not attributable to any effect of cordycepin on RNA polymerase activity and (b) requires phosphorylation of the nucleoside, and that (c) adenosine competes with the cordycepin for its target site.

Table 1 summarizes results demonstrating that cordycepin and cordycepin 5'-triphosphate do not inhibit the pore complex NTPase and have no significant effect on the stimulation of this enzyme by 50 μ g of poly(A) (Agutter *et al.*, 1977). The stimulatory effect of the poly(A) was significantly reduced ($P < 0.001$ by analysis of variance) on preincubation with sonicated nuclei at 37°C for 10 min in the presence of 0.1 mM-cordycepin + 1 mM-ATP (Table 2). The pretreatment of the poly(A) in this series of experiments was performed as described in the Materials and Methods section, the ethanol-precipitated polynucleotide being redissolved to a concentration of 2.0–2.5 A_{260} units in the triethanolamine buffer used for the NTPase assay. The cordycepin + ATP could be replaced by 0.1 mM-cordycepin 5'-triphosphate in this experiment. If the cordycepin or the ATP was omitted during the preincubation, stimulation of the enzyme was not significantly different from that obtained with untreated poly(A).

Fig. 6 shows that cordycepin inhibits the efflux of relatively high-molecular-weight RNA (predominantly 8–18S), which is predominantly messenger material (P. S. Agutter, unpublished work). There is no inhibition of the efflux of low-molecular-weight material (4–5S), which occurs in the absence as well

Table 2. *Poly(A) stimulation of nucleoside triphosphatase after preincubation with cordycepin and ATP*

Poly(A) was preincubated with sonicated nuclei as described in the Materials and Methods section and its stimulatory effect on nuclear envelope NTPase was examined. Cordycepin and cordycepin 5'-triphosphate were present at 0.1 mM concentration; ATP and adenosine were at 1 mM concentration. For the blank, poly(A) was preincubated in triethanolamine/HCl buffer alone, and was recovered as described in the text. Values quoted are the mean enzyme activities (μmol of ATP/h per mg of protein) $\pm$ 1 S.E.M. for three or four experiments. The last column shows the probability (by analysis of variance) that the diminution of stimulation of the enzyme was due to chance.

Reagents present	NTPase activity	P
(Blank)	10.8 $\pm$ 1.4	—
—	11.3 $\pm$ 1.8	—
Cordycepin	12.1 $\pm$ 2.3	>0.2
Cordycepin+ATP	8.2 $\pm$ 1.7	<0.001
Cordycepin 5'-triphosphate	7.1 $\pm$ 1.5	<0.001
ATP	11.5 $\pm$ 1.6	>0.5
Cordycepin+ATP+adenosine	10.2 $\pm$ 1.3	>0.1

Fig. 6. *Sucrose density gradient of eluted RNA*

Distribution of trichloroacetic acid-precipitable label in sucrose gradient (fraction 1: 0%, w/v; fraction 20: 50%, w/v) after centrifugation at 100000g for 5 h. Δ , Nuclei incubated with no ATP; $\triangle$, nuclei incubated in the presence of ATP; $\bullet$, nuclei incubated in the presence of ATP and 0.1 mM-cordycepin. Values are means of three determinations. The half-error bars represent 1 S.E.M.

as in the presence of ATP. It is noteworthy that under the conditions used no significant amounts of mature rRNA or of high-molecular-weight species such as the 45S ribosomal precursor escape from the nuclei.

Discussion

The nuclei and nuclear envelopes prepared by the methods described here show well-preserved morphological integrity and are substantially free of plasma membrane, lysosomal and mitochondrial marker enzymes.

The slight and delayed effect of actinomycin D on

RNA efflux observed (Fig. 1) might be explained as follows. In the presence of ATP, RNA synthesis proceeds, albeit slowly, in isolated nuclei; this is inhibited by actinomycin D. This reagent therefore causes a more rapid depletion of nuclear RNA by efflux, such that the nuclear RNA concentration falls after 12–15 min to below the 'gating' threshold for transport (Nägel & Wunderlich, 1977). The failure of a 10 min preincubation of the nuclei with actinomycin D to alter the time-course shown in Fig. 1 excludes the possibility that the delay in its effect is attributable to slow diffusion into the nucleus. Certainly, the results indicate that under certain

experimental conditions, such as those used here, cordycepin has an effect which cannot be simulated by a well-established general inhibitor of RNA polymerases (cf. Levey & Brinster, 1977).

The onset of inhibition was slower with cordycepin triphosphate than with cordycepin itself (Fig. 4), though the inhibition finally achieved was no less complete (Fig. 3). The lag observed with cordycepin triphosphate may be explained in terms of its relative slowness in entering the nucleus. The possibility that the nuclear envelope is a significant diffusion barrier to nucleoside triphosphates is controversial. The above explanation is consistent with the belief of E. Racker (personal communication), but inconsistent with the observations of Siebert (1978), concerning nuclear envelope permeability. The latter work was performed with nuclei isolated in organic solvents, which, although undoubtedly helping to retain RNA and DNA polymerase activities, might be expected to alter the properties of the envelope. The results of Paine *et al.* (1975) on envelope permeability also suggest that, on the basis of molecular weight alone, nucleoside triphosphates should enter the nucleus freely. However, the high negative charge density on these molecules might interfere with free diffusion, particularly in view of the evidence that fixed positive charges are present around the pore complex at physiological pH (Feldherr, 1974).

The results indicate that in these experiments cordycepin acted on some post-transcriptional stage in the processing of ribonucleoproteins in the same molecular-weight range as mature mRNA. That the site of action is not the stage of transport through the nuclear envelope itself is indicated by the finding that, unlike the RNA transport inhibitors described previously (Agutter *et al.*, 1976), cordycepin does not inhibit the pore complex NTPase (Table 1). The rapidity of onset of the inhibition (Fig. 4) suggests that the cordycepin acted at an advanced stage in RNA maturation, more or less immediately before transport out of the nucleus. On the assumption that poly(A) added to nuclear envelopes during NTPase assay interacts functionally with the transport system in a manner resembling that of mature RNA *in vivo* (Agutter *et al.*, 1977; Agutter & Ramsay, 1979), the results shown in Table 2 suggest that cordycepin, after 5'-phosphorylation (Klenow, 1963), prevented such functional interaction. Thus the most likely implication of the findings is that phosphorylated cordycepin prevents the functional interaction of mature RNA with the envelope transport system. Unphosphorylated cordycepin, on the basis of the results shown in Table 2 and Fig. 3, appears to have no such effect.

The molecular basis of this effect is not obvious from the results, but two alternative explanations may be advanced in the light of current information about cordycepin.

(1) As in the inhibition of RNA polymerase (Shiguera & Gordon, 1965), cordycepin may be incorporated as a 3'-deoxy terminus into a polynucleotide. This would account for the inhibition, if a 3'-OH group on the transported RNA were necessary for functional interaction with the transport system. However, there seems to be no evidence for this assumption. Moreover, incubation of poly(A) with nuclear lysate, ATP and tritiated cordycepin (cf. Table 2), followed by recovery of the poly(A) on poly(U)-Sephadex, did not result in the retention of more than 10 d.p.m. of label above background on the column after exhaustive washing.

(2) As in the stimulation of translation *in vitro* described by Leinwand & Ruddle (1977), cordycepin may alter the secondary structure of the mature RNA and thus prevent its functional interaction with the transport system. A recent demonstration in this laboratory (Agutter & Ramsay, 1979) that heat denaturation reversibly diminishes the stimulation of pore complex NTPase activity in isolated nuclear envelopes by poly(G) and other RNA species, is consistent with this interpretation. It is noteworthy, however, that Leinwand & Ruddle (1977) found a similar stimulation of translation *in vitro* when they used actinomycin D instead of cordycepin, and again interpreted this effect in terms of an alteration of mRNA secondary structure.

Although details of the explanation of the effect remain obscure, the results demonstrate that cordycepin inhibits RNA efflux from isolated nuclei by some mechanism unrelated to the inhibition of RNA synthesis. It seems likely that cordycepin will therefore provide a useful tool for further studies of RNA transport *in vitro*.

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