MOLECULAR PHARMACOLOGY, 22:517-523

Effects of Adenine Nucleosides on RNA Synthesis in Adenovirus-Infected Cells

9-β-D-Arabinofuranosyladenine as a Selective Inhibitor of RNA Polyadenylation

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Received July 27, 1981; Accepted May 8, 1982

SUMMARY

The effect of two adenine nucleosides, 3'-deoxyadenosine (cordycepin; 3'dAde) and 9-β-D-arabinofuranosyladenine (vidarabine; araAde), on RNA synthesized in KB cells 3–5 hr after infection with Type 5 adenovirus was investigated. AraAde had little effect on the synthesis of nuclear RNA (host or virus) at concentrations up to 25 μg/ml in the culture medium, whereas it reduced the amount of radioactively labeled RNA in the cytoplasm. Examination of discrete RNA species, including low molecular weight RNAs, showed that the major inhibitory effect of araAde was on poly(A)-containing RNA. Analysis of virus RNA by hybridization indicated that much less virus RNA was polyadenylated in araAde-treated cells than in control cells. In the nucleus, araAde treatment (50 μg/ml) resulted in a 70% reduction in those adenovirus RNAs containing the longest poly(A) tracts. Concomitantly, poly(A)-minus virus RNA accumulated in the nucleus and total virus RNA diminished in the cytoplasm of the araAde-treated cells. Another adenosine analogue, 3'dAde, also decreased the level of polyadenylated RNA in adenovirus-infected cells. However, unlike araAde, 3'dAde caused a reduction in the specific activity of both host and virus RNAs. These data suggest that araAde is a more selective inhibitor of RNA polyadenylation than is 3'dAde and raise the possibility that adenine nucleosides, particularly araAde, may alter the entry of early virus mRNA into the cytoplasm via inhibition of nuclear polyadenylation.

INTRODUCTION

The D-arabinosyl nucleoside araAde[5] is a potent antiviral and antineoplastic agent (see ref. 1). Although this drug has been used clinically for certain DNA virus infections (2), its mechanism of action has not been fully elucidated. *In vivo* araAde can be converted to the nucleoside triphosphate, araATP (3, 4), which can then compete for cellular processes requiring ATP or dATP. In particular, araATP has been shown to be a potent inhibitor of DNA polymerase *in vitro* (5, 6). Consequently, it has been postulated that the major target of this antibiotic *in vivo* is DNA synthesis. However, the observations that some of the deleterious effects of araAde can be reversed by adenosine, rather than by deoxyadenosine (7), and that araAde is incorporated (both internally and at 3'-termini) into both RNA and DNA (4) prompted us to investigate the effect of araATP on RNA-synthesizing and/or RNA-processing reactions which utilize ATP. Because the poly(A)-synthesizing reaction utilizes ATP, it seemed plausible that this reaction would be naturally susceptible to inhibition by adenosine analogues. Indeed, studies in our laboratory (8, 9) designed to test this possibility indicated that araATP selectively inhibits chromatin-associated polyadenylation of RNA *in vitro*, even when used at concentrations as high as 200 μg/ml. Although another adenosine analogue, 3'dAde, or its nucleotide, 3'dATP, inhibits initial mRNA polyadenylation *in vivo* (10) or *in vitro* (11, 12) at low concentrations, higher concentrations of this agent can inhibit synthesis of rRNA (11, 13), tRNA, and 5 S RNA (9). The emergence of araATP as a specific inhibitor of poly(A) synthesis in cell-free systems prompted us to explore the use of the parent compound, araAde, as a potential inhibitor of cellular and, more specifically, virus mRNA polyadenylation *in vivo*. We now report

This work was supported in part by United States Public Health Service Grants CA-25078 (to S. T. J.), GM-26740 (to K. M. R.), and CA-25757 (to T. H. C.)

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[5] The abbreviations used are: araAde, 9-β-D-arabinofuranosyladenine (vidarabine); 3'dAde, 3'-deoxyadenosine (cordycepin); Ad5, human adenovirus Type 5.

0026-895X/82/050517-07$02.00/0

that, prior to DNA synthesis, the adenosine analogues araAde and 3′dAde can prevent accumulation of poly(A)-containing RNA in cytoplasm of adenovirus-infected cells. Our data suggest that araAde can selectively inhibit mRNA polyadenylation with minimal effect on RNA synthesis.

EXPERIMENTAL PROCEDURES

Cells and virus infection. Suspension cultures of KB cells were grown in Eagle's medium as described previously (14). Cells were centrifuged, suspended in serum-free medium at a concentration of 2–4 × 10^6 cells/ml, and infected with Ad5, 30–100 plaque-forming units per cell, obtained from H. S. Ginsberg (Columbia University, New York, N. Y.) and propagated and titered as described previously (14). After 60–90 min at 37°, cultures were diluted to 4–8 × 10^5 cells/ml in serum-free medium. Drugs were added 170 min after infection. Ten minutes later, [5,6-^{3}H]uridine (New England Nuclear Corporation, Boston, Mass.; 40–50 Ci/mmole) was added (10 μCi/ml) and labeling was allowed to proceed for an additional 2 hr at 37°. Cells were harvested 5 hr after infection by centrifugation (250 × *g* for 20 min). Centrifugation and cell fractionation were carried out at 4°. In one experiment [^{32}P]orthophosphate (New England Nuclear Corporation; carrier-free) was used to label the RNA. In this case, cells were resuspended in phosphate-free medium and isotope was added to a concentration of 50 μCi/ml.

The nucleosides araAde and 3′dAde were added to the cultures on a weight basis. Use of the molar extinction coefficient of araAde indicated that the actual nucleoside content of araAde stock solutions ranged from 50% to 75% of the theoretical value.

Cell fractionation and preparation of RNA. Procedures were essentially those reported earlier (14, 15). The sedimented cells were suspended in buffer containing 10 mM Tris-HCl (pH 7.6), 0.14 M NaCl, and 1.5 mM $MgCl_2$. Cells were lysed with 0.1% NP-40, and nuclei were separated from the cytoplasmic fraction by centrifugation at 250 × *g* for 10 min. Nuclear pellets were resuspended in buffer containing 10 mM Tris-HCl (pH 7.6), 0.14 M NaCl, 1.5 mM $MgCl_2$, and 0.2% NP-40, and centrifuged (250 × *g* for 10 min) through a cushion of buffer containing 25% glycerol, 10 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 1 mM EDTA, and 1% Triton X-100. Cytoplasmic RNA was purified by extraction with a mixture of phenol, chloroform, and isoamyl alcohol. Nuclear RNA was purified by hot phenol extraction with the addition of 0.1% 8-hydroxyquinoline to the phenol for the 65° extractions. RNA from both cell compartments was sequentially digested with DNase and pronase and re-extracted with phenol. Purified RNA was precipitated at −20° by the addition of NaCl to a final concentration of 0.3 M, followed by 3 volumes of ethanol. Precipitated RNA was collected by centrifugation and washed once with 70% ethanol at −20°. RNA concentrations were determined spectrophotometrically, using the value $\epsilon(P)_{260} = 8000$ for mammalian DNA (16) and assuming an average base molecular weight of 323 g/mol ($\epsilon_{260} = 0.023\ \mu g^{-1}\ ml^{-1}$).

Separation of poly(A)-containing RNA. Poly(U)-cellulose was prepared as described by Sheldon *et al.* (17). Poly(A)-containing RNA was separated from the poly(A)-minus RNAs essentially as reported previously (18). Oligo(dT)-cellulose (Collaborative Research, Inc., Waltham, Mass.) chromatography was performed as outlined by Gielen *et al.* (19), replacing the linear elution of poly(A)-containing RNA with seven steps of sequentially decreasing salt concentrations (0.25 M–0.008 M KCl). The elution of poly(A) of different lengths was confirmed by using commercially available poly(A) of discrete size classes. Specifically, under the conditions employed, $(Ap)_{\overline{3}}$ was not retained on the columns, and the peak fractions of $(Ap)_{\overline{25}}$, $(Ap)_{\overline{54}}$, and $(Ap)_{\overline{220}}$ eluted at 125 mM, 62 mM, and 31 mM KCl, respectively. Fractions eluting from 250 mM–125 mM, 62 mM–31 mM, and 16 mM–0 KCl were pooled and are referred to in the text as "short," "medium," and "long" poly(A) tracts, respectively.

RNA-DNA hybridization. Adenovirus DNA was prepared from purified viruses, denatured, and immobilized on cellulose nitrate membrane filters (Schleicher and Schuell B6) as described earlier (20). The filters, containing 10 μg of adenovirus DNA or *Escherichia coli* DNA (used to determine nonspecific hybridization), were soaked for at least 3 hr at 20° in Denhardt's solution (21) and then hybridized to [^{3}H]RNA in 1.5 ml of Denhardt's solution containing 0.6 M NaCl and 0.06 M sodium citrate at 67° for 1–2 days. Filters were then processed (20) and radioactivity was determined in a scintillation counter.

RESULTS

Effect of nucleosides on cellular RNA species. Prior to examining the effect of the adenosine analogues, araAde and 3′dAde, on adenovirus-specific RNA, we measured the response of over-all RNA synthesis in virus-infected and uninfected KB cells to these nucleosides. Preliminary experiments indicated that, at appropriate concentrations, these analogues could reduce over-all RNA labeling and that the extent of inhibition in KB cells in the early stages (5 hr or less) of adenovirus infection was comparable to that of uninfected cells. The effect of the nucleosides on RNA labeling in the adenovirus-infected cells was then investigated. As shown in Table 1, araAde had little effect on the labeling of nuclear RNA. However, the amount of radioactively labeled RNA in the cytoplasm of these cells was reduced. For example, at an araAde concentration of 50 μg/ml (180 μM), the specific activity of the nuclear RNA was 87% that of control, whereas the cytoplasmic RNA was reduced to 56% that of the control. When the cells were exposed to the nucleoside for a longer period (1 hr instead of 10 min) prior to the addition of [^{3}H]uridine, inhibition of labeling was more pronounced (data not shown). For example, at an araAde concentration of 25 μg/ml, nuclear RNA labeling was 81% that of control when the longer exposure time was examined. Hence, in most experiments, nucleosides were added 10 min prior to radioactive label. Decreased specific activity of the RNA in araAde-treated cells was not due to defective uptake of the radioactive precursor, [^{3}H]uridine, since radioactivity in the acid-soluble fraction of the cell was unaltered by the nucleoside (data not shown).

In similar experiments, the effect of 3′dAde on RNA labeling in the nucleus and cytoplasm of adenovirus-infected cells was also measured. At a concentration as low as 7 μg/ml (28 μM), cordycepin caused a significant reduction in the specific activity of KB cell RNA. As

TABLE 1

Effect of adenine nucleosides on nuclear and cytoplasmic RNA in Ad5-infected KB cells

Nucleosides were added to Ad5-infected KB cells (at the concentration indicated) as described under Experimental Procedures. Ten minutes after addition of drug, [^{3}H]uridine was added. After a 2-hr labeling period, cells were harvested and RNA was prepared from nuclei and cytoplasm as described under Experimental Procedures. Poly(A)-containing RNA was obtained by chromatography on poly(U)- or oligo(dT)-cellulose. The specific activities (counts per minute per microgram) of the RNA fractions were obtained and expressed as percentages of control. The data represent the mean ± standard deviation of percentage values from three to five experiments. One hundred per cent values ranged from 61 to 640 × 10^3 cpm/μg and 14 to 500 × 10^3 cpm/μg for total and poly(A)-plus nuclear RNA, respectively, and from 14 to 112 × 10^3 cpm/μg and 21 to 380 × 10^3 cpm/μg for total and poly(A)-plus cytoplasmic RNA, respectively. In a typical experiment, 110 μg of nuclear RNA and 250 μg of cytoplasmic RNA were applied to oligo(dT)-cellulose; 6 μg and 20 μg of polyadenylated RNA, respectively, were recovered from the columns. Values of 2.5 × 10^6 cpm and 0.9 × 10^6 cpm nuclear polyadenylated [^{3}H]RNA were obtained from control and araAde-treated (50 μg/ml) cells, respectively. From the same experiment, 1 × 10^6 cpm and 0.5 cpm were recovered from oligo(dT)-cellulose columns of cytoplasmic RNA from control and araADE-treated cells, respectively. The wide range of specific activities was due to different amounts of [^{3}H]uridine employed for labeling. In one experiment [^{32}P]orthophosphate was used to label the RNA. Depending on the number of cells used, recovery of nuclear and cytoplasmic RNA ranged from 100 to 500 μg and 200 to 1400 μg, respectively, for individual experiments. Neither nucleoside significantly altered the recovery of RNA relative to control.

Nucleoside	Concentration	Nucleus		Cytoplasm	
		Total RNA	Poly(A)-containing RNA	Total RNA	Poly(A)-containing RNA
	μg/ml	% of control		% of control	
AraAde	25	99 ± 12	70 ± 5	89 ± 9	84 ± 3
	50	87 ± 2	40 ± 8	56 ± 19	53 ± 1
3′dAde	7	51 ± 10	35 ± 11	27 ± 10	36 ± 20

with araAde, 3′dAde had a greater inhibitory effect on the accumulation of radioactive RNA in the cytoplasm than on the labeling of the nuclear RNA. In particular, at a 7 μg/ml concentration of 3′dAde, the nuclear and cytoplasmic RNAs were decreased to 51% and 27% that of control, respectively (Table 1). It should be noted that neither araAde nor 3′dAde caused any significant changes in the recovery of unlabeled RNA.

The decrease in radioactivity of RNA from KB cell cultures treated with araAde and cordycepin was further investigated by examining the labeling of specific classes of RNA. To ascertain whether these adenine nucleosides altered the polyadenylation of mRNA precursors in the nucleus and/or the appearance of newly synthesized mRNA in the cytoplasm of adenovirus-infected KB cells, the effect of araAde and 3′dAde on poly(A)-containing RNA was next examined. The poly(A)-containing RNA from control and treated cultures was separated from the bulk of the nuclear and cytoplasmic RNA by chromatography on oligo(dT)-cellulose or poly(U)-cellulose columns. As indicated in Table 1, relative to control cells, the specific activity of the poly(A)-containing RNA in the nucleus and the cytoplasm of the drug-treated cells was reduced. For example, values for specific activity of poly(A)-containing RNA in the nucleus of araAde-treated cells were 70% and 40% that of control at araAde concentrations of 25 μg/ml and 50 μg/ml, respectively. The reduction in specific activity of the nuclear poly(A)-containing RNA in araAde-treated cells was significantly ($p \leq 0.02$, Student's t-test) greater than the reduction in the labeling of the total nuclear RNA in these cells. Considerable variability in the degree of inhibition of nuclear RNA was observed when concentrations of nucleoside greater than 50 μg/ml were used (data not shown), possibly due to a defect in transport of araAde into the cell or in its conversion to the nucleoside triphosphate. The accumulation of poly(A)-containing RNA in the cytoplasm was also decreased by araAde treatment. The inhibitory effect of araAde on the specific activity of the poly(A)-containing RNA in the cytoplasm was similar in magnitude to that of the total cytoplasmic RNA. AraAde appeared to exert a greater inhibitory effect on polyadenylated RNA in the nucleus as compared with the cytoplasm (70% versus 84% and 40% versus 53% of control at 25 and 50 μg/ml, respectively). Since araAde is incorporated internally (see ref. 4), it is possible that the turnover of the polyadenylated RNA in araAde-treated cells is somewhat slower than that in untreated cells, which may account for the differential inhibition of nuclear and cytoplasmic RNAs by the nucleoside. The decreased labeling of poly(A)-containing RNA was specific for the adenosine analogue, since the cytosine arabinoside (25 μg/ml) had no effect on the labeling of this class of RNA either in the nucleus or in the cytoplasm (data not shown).

The effect of 3′dAde on the specific activity of the poly(A)-containing RNA of the Ad5-infected KB cells was next investigated. As shown in Table 1, the specific activities of poly(A)-containing RNAs in the nucleus and cytoplasm of 3′dAde-treated (7 μg/ml) cells were approximately 35% and 36% those of control cells, respectively. However, unlike araAde, the inhibition of nuclear poly(A)-containing RNA was not significantly greater than the inhibition of the total nuclear RNA.

The effect of araAde and 3′dAde on nonpolyadenylated RNAs in Ad5-infected cells was also investigated. Thus, rRNA from control and drug-treated cultures was separated from the bulk of other cytoplasmic RNAs by sucrose gradient centrifugation. After treatment with araAde (25 μg/ml), the specific activities of 18 S and 28 S RNAs were 84% and 66% that of control, respectively (data not shown). The addition of 3′dAde at a similar concentration to the culture medium resulted in barely detectable levels of labeled rRNA in the cytoplasm. The synthesis of rRNA (45 S) and mRNA nuclear precursors was measured in experiments in which the labeling time was reduced to 5 min. Under these conditions, the addition of araAde (50 μg/ml) to the culture medium (10 min prior to the addition of [^{3}H]uridine) resulted in a 15% reduction (average of two experiments) in the specific activity of high molecular weight (>30 S) nRNA relative to control (data not shown).

The effect of araAde and 3′dAde on low molecular weight RNAs of Ad5-infected KB cells was next measured. Preliminary experiments indicated that araAde did not alter the labeling of the small RNAs (7 S and less) in the nucleus or in the cytoplasm of Ad5-infected KB cells

when added to the culture medium at a concentration of 25 μg/ml. Consequently, the concentration of this nucleoside was increased to 100 μg/ml. Figure 1 shows the polyacrylamide gel pattern of low molecular weight [^{3}H]RNAs in the cytoplasm of Ad5-infected KB cells. As indicated in the fluorograph, even at a concentration of 100 μg/ml, araAde had minimal effect on the labeling pattern of the small RNAs in the cytoplasm. Specifically, analysis of the gel pattern by densitometry demonstrated that the most abundant RNA species, 4 S and 5 S, were 84–96% of control. In contrast, at the low concentration of 7 μg/ml, 3′dAde had a significant inhibitory effect on the low molecular weight RNAs. At 25 μg/ml, 3′dAde reduced the labeling pattern of all the small RNAs to 25% or less of control (not shown). More detailed analysis of the low molecular weight RNAs other than 4 S and 5 S was undertaken by examination of these RNAs in the nucleus. Synthesis of U_1, U_2, U_3, and 7 S cellular low molecular weight RNAs and VA I and VA II adenovirus RNAs was not affected by araAde at a concentration of 100 μg/ml (data not shown).

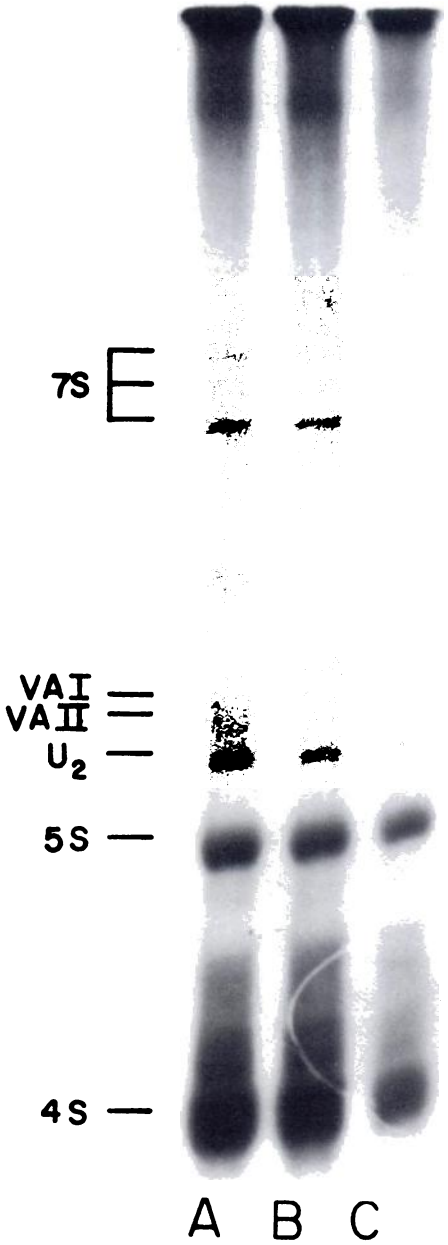

FIG. 1. *Sensitivity of Ad5 and KB cell low molecular weight RNAs to adenosine analogues*

KB cells were infected with Ad5 as described under Experimental Procedures. AraAde (100 μg/ml) or 3′dAde (7 μg/ml) was added 10 min prior to the 2-hr labeling period with [^{3}H]uridine. Cells were harvested 5 hr after infection, and cytoplasmic RNA was extracted as delineated under Experimental Procedures. RNA (40 μg/lane) was subjected to polyacrylamide gel electrophoresis using the Tris-borate (pH. 8.3) buffer system (containing 0.5% sodium lauryl sarcosine) described by Mathews and Pettersson (22). [^{3}H]RNA was detected by fluorography according to the protocol of Bonner and Laskey (23). *Tracts A, B,* and *C* represent RNA obtained from control, araAde-treated, and 3′dAde-treated cells, respectively. Identification of 7 S, 5 S, 4 S, and U_2 RNAs was made by comparing the labeled pattern of KB cell RNA with the A_{260} profile of rat liver RNAs run under similar conditions. VA I and VA II were designated by their relative migration according to ref. 22.

Effect of nucleosides on Ad5 RNA. The data presented in Table 1 and Fig. 1 suggested that the primary effect of araAde was on the labeling of polyadenylated RNA. The presence of large quantities of nonmessenger RNA in the poly(A)-minus fractions makes it difficult to demonstrate directly a change in the proportion of nonpolyadenylated mRNA. However, such changes can be ascertained in the case of adenovirus RNA, which is predominantly mRNA and can be measured by hybridization to the virus DNA. Prior to investigation of polyadenylated virus RNA, the effect of araAde and 3′dAde on early adenovirus RNA was measured. As indicated in Table 2, araAde (50 μg/ml) did not significantly affect the incorporation of radioactivity into total or nuclear virus RNA when it was added to the cultures 10 min prior to labeling (Experiments 1 and 2). However, as observed with cellular RNA,

TABLE 2

Effect of adenosine analogues on early Ad5 RNA in the nucleus and cytoplasm of KB cells

KB cells were infected with Ad5, and treated with araAde (50 μg/ml) or 3′dAde (7 μg/ml) as described under Experimental Procedures. Superscripts refer to individual experiments. In Experiments 1 and 2, [^{3}H]uridine was added 10 min after the addition of nucleoside (130-min total exposure to nucleoside). In Experiments 3–5, radioactive label was added 1 hr after drugs (180-min total exposure to nucleosides).

Addition	Ad5 RNA			
	Total, cpm (% of control)	Nucleus, cpm (% of control)	Cytoplasm, cpm (% of control)	Cytoplasm, % total (% of control)
None	103,238 (100)[1]	73,998 (100)	29,240 (100)	28 (100)
	15,447 (100)[2]	8,037 (100)	7,410 (100)	48 (100)
	41,799 (100)[3]	18,543 (100)	23,256 (100)	56 (100)
	28,793 (100)[4]	16,813 (100)	11,980 (100)	42 (100)
	14,261 (100)[5]	6,300 (100)	7,961 (100)	56 (100)
AraAde	97,031 (94)[1]	75,217 (102)	21,814 (75)	22 (79)
	20,160 (130)[2]	14,964 (186)	5,196 (70)	26 (54)
	15,083 (36)[3]	10,892 (59)	4,191 (56)	27 (48)
	13,845 (48)[4]	9,461 (56)	4,384 (37)	32 (76)
	4,659 (33)[5]	2,919 (46)	1,740 (22)	37 (66)
3′dAde	25,912 (25)[1]	21,214 (28)	4,698 (16)	18 (64)
	12,006 (77)[2]	10,032 (124)	1,974 (26)	16 (33)
	18,789 (45)[3]	12,399 (68)	6,390 (27)	34 (61)

araAde reduced the accumulation of Ad5 RNA in the cytoplasm (to 79% and 54% that of control cells in Experiments 1 and 2, respectively). In three other experiments, araAde was added 1 hr prior to [^{3}H]uridine. Even though in these experiments the nucleoside caused a reduction in the labeling of Ad5 RNA, the percentage of newly synthesized RNA in the cytoplasm was also significantly reduced (to 48%–76% that of control cells). 3′dAde (7 μg/ml) inhibited the labeling of the virus RNA, as well as the level of Ad5 RNA in the cytoplasm (33%–64% that of control cells).

Effect of nucleosides on poly(A) size. The decreased recovery of radioactive polyadenylated cellular RNA species in the nucleus and in the cytoplasm, the lack of inhibition of over-all nuclear RNA labeling, and the reduced accumulation of AD5 RNA in the cytoplasm suggested that the primary target of araAde might be the polyadenylation of mRNA precursors rather than their synthesis. If this were the case, araAde treatment might result in redistribution of mRNA precursors from larger to smaller poly(A) size classes and from poly(A)-plus to poly(A)-minus mRNAs. To investigate such a possibility, a technique was employed which can separate poly(A)-containing RNAs on the basis of the size of their poly(A) tracts. This method consists of RNA fractionation on oligo(dT)-cellulose and elution of poly(A)-containing RNAs by a gradual decrease in salt concentration (19). Under such conditions, those poly(A)-containing RNAs with lesser affinity for oligo(dT)-cellulose, i.e., with shorter poly(A) tracts, are released prior to those with longer poly(A) sequences. Thus, nuclear and cytoplasmic RNA were prepared from control and nucleoside-treated, virus-infected cells and subjected to sequential fractionation on oligo(dT)-cellulose columns. Fractions were collected and analyzed for labeled cellular and Ad5 RNA.

As indicated in Table 3, the nuclear RNAs with the longest poly(A) sequences were affected the most by araAde; in both experiments the virus RNAs with poly(A) tracts greater than 200 nucleotides were reduced to 30% that of control cells. Intermediate inhibition (45%–98% those of controls) of those nuclear virus RNAs with shorter poly(A) sequences (less than 200 adenylate residues) was observed after araAde treatment. These data suggested that a shift in the distribution of poly(A) of Ad5 mRNA was occurring after araAde treatment. This trend was evident when the data were expressed as percentages of poly(A)-containing RNAs with short, medium, or long poly(A) sequences (Table 3). After araAde treatment, the percentage of nuclear polyadenylated Ad5 RNA with long poly(A) tracts was reduced to 63% (average) that of control. The relative proportions of Ad5 RNAs with medium and short poly(A) sequences were actually increased relative to control (average of 128% and 187% those of controls, respectively). Simultaneously, the amount of labeled Ad5 RNA in the poly(A)-minus fraction also increased (average of 191% that of control; see Table 3). Nuclear RNA of the cells also showed a shift toward shorter poly(A) tracts after araAde treatment. Labeling of the nuclear RNAs with the longest poly(A) tracts was decreased more than that of RNAs with medium and short poly(A) sequences. As with Ad5 mRNA, the distribution of polyadenylated cellular mRNA was shifted to shorter poly(A) lengths (Table 3). Specifically, the percentage of cellular mRNA with long poly(A) tracts was reduced to 78% (average) that of control, whereas the percentage of mRNAs with medium poly(A) tracts was 98% (average) that of control and the percentage of those with short poly(A) stretches was 160% (average) that of control.

Poly(A)-containing Ad5 RNA in the cytoplasm was also reduced by araAde treatment. As in the nucleus, inhibition of those virus RNAs with the longest poly(A)

TABLE 3

Effect of adenine nucleosides on poly(A) size

KB cells were infected with Ad5 and treated with araAde (50 μg/ml), or 3′dAde (7 μg/ml) was labeled with [^{3}H]uridine as described under Experimental Procedures. RNA, extracted from nuclei or cytoplasm, was subjected to oligo(dT)-cellulose chromatography as described under Experimental Procedures. Short, medium, and long poly(A) tracts correspond to poly(A) lengths in the range of 5–30, 30–200, and more than 200 nucleotides, respectively. Superscripts refer to separate experiments and correspond to Experiments 1 and 2 in Table 2. The proportions of nuclear and cytoplasmic RNAs retained by oligo(dT)-cellulose were similar and ranged from 22% to 28% of the total RNA in each cell compartment in control cells. Poly(A)-minus RNA was expressed as percentage of control (poly(A)-minus RNA cpm from treated cells/poly(A)-minus RNA cpm from untreated cells × 100). Counts per minute recovered from the column, corresponding to the different poly(A) sizes, were expressed as percentage of control. Values in parentheses were expressed relative to total polyadenylated RNA, which was computed by summing the radioactivity (counts per minute) obtained from individual fractions from oligo(dT)-cellulose columns. Counts per minute in each size-class of poly(A) were divided by the total counts per minute retained on the column and then expressed as percentage of control (untreated cells).

Cell fraction	Poly(A)-minus RNA			Poly(A)-containing RNA according to Poly(A) size					
	Addition	Cell	Virus	Short		Medium		Long	
				Cell	Virus	Cell	Virus	Cell	Virus
		% control		*% total polyadenylated RNA*					
Nucleus	AraAde	95[1]	131	66 (216)	98 (250)	30 (95)	45 (109)	20 (63)	30 (73)
		98[2]	228	96 (103)	71 (123)	92 (100)	86 (146)	84 (92)	30 (52)
	3′dAde	39[1]	34	49 (192)	91 (350)	27 (97)	37 (128)	19 (67)	13 (45)
		51[2]	149	74 (153)	168 (323)	43 (87)	57 (110)	27 (56)	14 (28)
Cytoplasm	AraAde	39[1]	79	61 (122)	79 (150)	54 (100)	65 (106)	53 (92)	52 (83)
		82[2]	81	47 (78)	55	62 (106)	28	63 (104)	
	3′dAde	15[1]	21	58 (288)	40 (500)	20 (96)	8 (94)	8 (39)	7 (86)
		37[2]	33	50 (133)	27 (173)	45 (115)	12 (75)	22 (56)	11 (72)

sequences was greater than that of those with shorter or no poly(A) tracts (Table 3). Specifically, in the cytoplasm of araAde-treated cells, labeled Ad5 RNAs with long, medium, and short poly(A) stretches were reduced to 52%, 65%, and 79% those of controls, respectively, in one experiment. In another experiment, the cytoplasmic AD5 RNAs with medium and short poly(A) tracts were 28% and 55% those of control cells, respectively. In contrast to its effect on Ad5 RNA in the nucleus, araAde treatment resulted in a decrease in cytoplasmic poly(A)-minus Ad5 RNA. Polyadenylated cellular RNA was also reduced in the cytoplasm of araAde-treated cells, and values ranged from 47% to 65% those of control cells for the various poly(A) lengths (Table 3). The distribution of cellular polyadenylated RNA in the cytoplasm of araAde-treated cells was not significantly different from that in the cytoplasm of control cells (Table 3).

The effects of 3′dAde were analogous to araAde in several ways; that is, nuclear mRNAs with the longest poly(A) sequences, either cellular or viral, were decreased to a greater extent than those with shorter poly(A) tracts in the 3′dAde-treated cells. This resulted in a redistribution of nuclear polyadenylated RNAs with a shift toward smaller poly(A) tracts. This trend was also evident in the cytoplasm of cells exposed to cordycepin (Table 3).

DISCUSSION

The present studies demonstrate that the adenine nucleoside araAde decreases the amount of polyadenylated RNA in KB cells with minimal effect on RNA synthesis. In particular, exposure of adenovirus-infected KB cells to araAde (a) did not significantly alter the specific activity of cell or virus nuclear RNA after 2 hr; (b) reduced the level of poly(A)-containing RNA in both nucleus and cytoplasm; (c) decreased the average length of the poly(A) tract of the cell and virus RNA in the nucleus; and (d) prevented the exit of virus mRNA from nucleus to cytoplasm. These findings confirm and extend our previous observation that araATP can selectively inhibit cellular RNA polyadenylation *in vitro* (8). In contrast to AraAde, 3′dAde inhibited labeling of all types of RNAs. The general inhibitory effects of 3′dAde may have been due to its RNA chain-terminating properties and the ability of 3′dATP, but not araATP, to inhibit other cellular reactions.[6]

The lack of inhibition of RNA synthesis in cell culture by araAde substantiates earlier studies (7) and may partially explain the low cytotoxicity of this antibiotic in intact animals. The slight reduction in the specific activity of cytoplasmic 18 S and 28 S rRNA observed after araAde treatment could be a reflection of decreased stability and/or transport of rRNA, possibly as a consequence of reduced methylation of the rRNA precursors. Ribose methylation, an important reaction in the processing of rRNA, requires *S*-adenosylmethionine and, at least in cell culture, is sensitive to inhibition by adenosine analogues, including 3′dAde (24). The finding that the specific activity of 28 S RNA is reduced by a larger percentage than that of 18 S RNA after araAde treatment is consistent with a decrease in ribose methylation, since 28 S RNA is the more highly methylated of the two rRNAs.

The effects of araAde and 3′dAde in cell culture are remarkably similar to those obtained *in vitro* employing isolated nuclei or chromatin preparations. Specifically, araATP was shown to inhibit the initial polyadenylation of mRNA precursors (8), but not RNA synthesis (8), including that of tRNA and 5 S RNA (9). 3′dATP also inhibited the initial polyadenylation reaction (11, 12) and, to a lesser extent, the synthesis of rRNA (11), tRNA, and 5 S RNA (9). It should be pointed out that the decrease in poly(A) synthesis resulting from treatment with either araATP or 3′dATP was restricted to polyadenylation on the chromatin. The poly(A) extension reaction catalyzed by soluble poly(A) polymerase was relatively insensitive to these nucleotides (8, 11). Specifically, with the use of the chromatin preparation, the apparent K_i values for araATP and 3′dATP were 4 μM and 1.3 μM, respectively. When solubilized enzyme was used, the apparent K_i values for araATP and 3′dATP were 60 μM and 40 μM, respectively. Failure to detect the deleterious effects of araATP (25) by other investigators can be explained by use of solubilized rather than chromatin-associated poly(A) polymerase as enzyme.

Although it is difficult to compare the data obtained in isolated systems with those in cell culture, it is not unreasonable to assume that the effects of araAde and 3′dAde are mediated via their triphosphates since both compounds are rapidly phosphorylated in culture (4, 26). Since deaminases can convert the adenosine derivatives to the hypoxanthine counterparts, the total nucleoside complement is not available for the phosphorylation pathway. For example, in mouse fibroblast cells incubated in 0.1 mM araAde, araATP was the predominant nucleotide product and was present at 20 μM (4). If a similar situation exists in KB cells, one might anticipate that cultures incubated with an araAde concentration of 50 μg/ml (180 μM) might accumulate araATP in the range of 5–10 μg/ml (18–36 μM). This concentration is of the same order of magnitude as the K_i of araATP, and inhibition of polyadenylation should be (and was) observed. The similarity of the relative potency of araAde/araATP and 3′dAde/3′dATP in inhibiting polyadenylation *in vitro* and in cell culture (the deoxynucleoside/nucleotide being 3 times more potent in both systems) is striking and lends credence to the assumption that data obtained in appropriate *in vitro* systems is compatible with those obtained from whole cell studies.

Examination of adenovirus RNA synthesis and polyadenylation allows analysis of a specific group of mRNAs. Measurements made during the early phases of virus infection are particularly important because virus RNA synthesis at this time occurs in a relatively unperturbed cellular environment (27, 28). Furthermore, because early virus transcription occurs before virus DNA replication, the effects of the antibiotics on RNA synthesis and processing can be differentiated from those on DNA synthesis. Finally, early adenovirus RNA synthesis and processing are required for virus DNA synthesis (29); thus any deleterious effects at this time by the antiviral agents may influence virus replication. In aggregate, the effects of araAde and 3′dAde on adenovirus RNA are

[6] K. M. Rose and S. T. Jacob, unpublished observations.

similar to those on cellular RNA. The results observed with 3′dAde are in general agreement with an earlier report by Philipson *et al.* (30). Both araAde and 3′dAde cause a decrease in the extent of polyadenylation of virus RNAs in the nucleus. In contrast to araAde, 3′dAde reduces the over-all recovery of virus mRNA and the low molecular weight virus RNAs, VA I and VA II. The ability of araAde, but not 3′dAde, to decrease the extent of nuclear polyadenylation of adenovirus mRNA and its transport without altering the synthesis of virus RNA indicates that the former nucleoside is more selective for post-transcriptional events. The observation that poly(A)-minus virus RNAs are specifically retained in the nucleus after treatment with araAde, coupled with our previous observation that araATP can inhibit polyadenylation *in vitro* (8) where transport is not required, prompts us to suggest that polyadenylation of adenovirus mRNA may be closely linked to its transport.

ACKNOWLEDGMENT

The authors wish to acknowledge the support and advice of Dr. Samson T. Jacob, in whose laboratory this project was initiated.

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