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Selective Inhibition of Avian Sarcoma Virus Protein Synthesis in 3-Deazaadenosine-Treated Infected Chicken Embryo Fibroblasts

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The production of B77 avian sarcoma virions was inhibited more than 90% in infected chicken embryo fibroblasts that were treated with 100 μM 3-deazaadenosine, an inhibitor of adenosylhomocysteine hydrolase and, for this reason, an inhibitor of methylation reactions. This nucleoside analog at a concentration of 100 μM inhibited the rates of overall cellular protein synthesis and polyadenylated RNA synthesis by 40 to 50%. Rates of viral protein synthesis were compared, and the results indicated that in infected cells treated with 3-deazaadenosine syntheses of both the precursor of the *gag* proteins ($pr76^{gag}$) and the precursor of the reverse transcriptase ($pr180^{gag\ pol}$) were inhibited. Synthesis of the precursor of the viral envelope glycoproteins ($pr92^{env}$) appeared to be affected less by the analog treatment. Most of the host polypeptides also continued to be synthesized in 3-deazaadenosine-treated cells. The fraction of the total RNA represented by virus-specific RNA in the 3-deazaadenosine-treated cells was approximately 40% of the fraction of the total RNA represented by viral RNA in control cells, as determined by hybridization kinetics. Therefore, there was a selective inhibition of viral RNA accumulation in the presence of 3-deazaadenosine. The amounts of genome-sized 35S and 38S RNAs were reduced compared with the amounts of 28S and 21S viral mRNA's. These results suggest that selective inhibition of the synthesis of viral proteins is due to selective decreases in the amounts of the mRNA's for these polypeptides.

Bader et al. have shown that the nucleoside analog 3-deazaadenosine, an inhibitor of *S*-adenosylhomocysteine hydrolase, reversibly inhibits the reproduction of the Bryan strain of Rous sarcoma virus and the transformation of chicken embryo cells (2). These authors postulated that this effect is due to the inhibition of methylation reactions required for virus replication. They also proposed that inhibition of virus replication and inhibition of oncogenic transformation are caused by an inhibition of 5′ cap methylations (2). However, the biochemical basis of inhibition was not investigated. We have shown previously that 2′-*O*-methyl cap methylations and internal N^6-methyladenosine methylations of cellular mRNA and viral genome RNA are inhibited more than 90% in the presence of cycloleucine, yet virus production under these conditions is inhibited only slightly (7). This suggested that the methylations blocked in the presence of cycloleucine (i.e., the 2′-*O*-methyl cap and internal N^6-methyladenosine methylations) are not essential for virus replication. We have investigated the basis for the inhibition of virus production by 3-deazaadenosine and the reasons for the differences in the effects of the two in vivo methylation inhibitors 3-deazaadenosine and cycloleucine on virus reproduction. In this work we confirmed for another strain of avian sarcoma virus (ASV), B77 ASV, the observation of Bader et al. that virus production is inhibited drastically in the presence of 3-deazaadenosine (2). We then determined the metabolic effects of 3-deazaadenosine on bulk cellular mRNA and protein syntheses and on virus-specific RNA and protein syntheses. We found that the syntheses of the internal non-glycosylated virion polypeptides (*gag* proteins) are inhibited selectively in the presence of 3-deazaadenosine. This inhibition appears to result, at least in part, from selective decreases in the amounts of viral mRNA's.

MATERIALS AND METHODS

Virus and cells. Chicken embryo cells were prepared from 10-day-old embryos and were subcultured at 3-day intervals. Cells were infected with B77 ASV at a multiplicity of infection of 2 in the presence of 2

μg of Polybrene per ml. The infected cells were passaged one or two times in order to insure that all of the cells were infected. Cells were maintained in medium 199 containing 1% (vol/vol) dimethyl sulfoxide. For experiments in which the effect of 3-deazaadenosine was tested, Earle minimal essential medium (MEM) was used, and tryptose phosphate broth was not added. The media contained a final concentration of 5% (vol/vol) calf serum.

Purification of virus. Virus was purified from the medium of infected cells by previously described techniques (21). Briefly, the virus was pelleted from the medium through a 20% (wt/vol) sucrose shelf onto a 70% (wt/vol) sucrose pad. The pelleted virus was then banded to equilibrium in a continuous 20 to 70% sucrose gradient.

Isolation of RNA. Total RNA was isolated from cells by phenol-chloroform extraction, as described previously (20). Cells were fractionated into nuclei and cytoplasm by lysis with 1% (vol/vol) Nonidet P-40–1% (wt/vol) deoxycholate, and the polysomes were isolated by discontinuous sucrose gradients as previously described (8). The fraction containing polyadenylic acid [poly(A)$^+$] was isolated by oligodeoxythymidylic acid-cellulose chromatography (20).

Determination of hybridization kinetics of viral cDNA to cellular RNA. Varying amounts of RNA from 3-deazaadenosine-treated and control cells were hybridized to ^{32}P-labeled complementary DNA (cDNA) prepared by the endogenous reverse transcriptase reaction of detergent-disrupted B77 ASV (20). Hybridization was carried out in a solution containing 0.3 M NaCl, 0.01 M Tris-hydrochloride (pH 7.5), and 0.001 M EDTA at 68°C for 18 h. The resistance of the ^{32}P-labeled cDNA to S1 nuclease was used as a measure of the extent of hybridization.

Gel electrophoresis of RNA, transfer to diazobenzoxymethyl paper, and hybridization to ^{32}P-labeled pSal^{101} DNA. Gel electrophoresis of poly(A)$^+$ RNA on 1% agarose gels containing 10 mM methylmercury hydroxide was carried out essentially by the method of Bailey and Davidson (3). The RNA in the gel was transferred to diazobenzoxymethyl paper by the procedure of Alwine et al. (1). The paper was then prehybridized for 24 h, and this was followed by hybridization with a ^{32}P-labeled nick-translated DNA probe (5.7×10^7 cpm/μg), as described by Alwine et al. (1). The ^{32}P-labeled probe used in the experiments described below was pSal^{101}, a recombinant plasmid DNA which contains the entire ASV Prague A strain genome inserted into the *SalI* site of pBR322. A stock culture of this recombinant plasmid was generously provided by J. Thomas Parsons, University of Virginia, Charlottesville. The location of the radioactivity on the paper was determined by autoradiography on Kodak BB-5 X-ray film, using a Dupont Cronex Lightning-Plus intensifying screen.

Preparation of [^{3}H]leucine-labeled cytoplasmic extracts. Chicken embryo fibroblasts infected with B77 ASV in 100-mm petri dishes were incubated for 17 h with either 10 ml of MEM or 10 ml of MEM containing 100 μM 3-deazaadenosine. The medium was then changed to either 5 ml of MEM without leucine or 5 ml of MEM without leucine containing 100 μM 3-deazaadenosine. After a 1-h incubation, 250 μCi of [^{3}H]leucine was added to each plate, and the cells were labeled for varying intervals, as described below. The cells were then washed once with 4 ml of a solution containing 140 mM NaCl, 5 mM KCl, 0.6 mM $NaHPO_4$, 5.5 mM glucose, and 25 mM Tris-hydrochloride (pH 7.2) (TBS) at 4°C, scraped into 2 ml of a solution containing 10 mM Tris-hydrochloride (pH 7.4), 1 mM NaCl, and 1.5 mM $MgCl_2$, and swelled on ice for 10 min. After this the cell suspensions were each brought to a final concentration of 1% (vol/vol) Nonidet P-40, homogenized by five strokes of a Dounce homogenizer, and centrifuged for 30 min at 10,000 × *g* to remove nuclei and cell debris. The supernatant was removed and stored in 100-μl portions at −70°C.

Immunoprecipitation of virus-specific proteins. Formalin-fixed *Staphylococcus aureus* Cowan I strain was prepared by the method of Kessler (11). Fixed bacteria (100 μl) at a concentration of 10% (vol/vol) were sedimented and then suspended in 400 μl of a solution containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride (pH 7.4), and 0.5% (vol/vol) Nonidet P-40 (buffer A). To this suspension approximately 100 μl of [^{3}H]leucine-labeled cell extract was added, and the suspension was incubated for 30 min at room temperature. The bacteria were then pelleted, and we added to the supernatant an amount of antiserum determined to be sufficient to quantitatively immunoprecipitate protein from 10 μg of detergent-disrupted virions. The samples were incubated for 30 min at room temperature, followed by the addition of 100 μl of a 10% (vol/vol) suspension of Formalin-fixed bacteria. In some cases, varying amounts of disrupted nonradioactive virus were present during the incubation (see below). The bacteria containing the immunoprecipitated proteins were pelleted, the supernatants were discarded, and the pellets were washed three times with buffer A. The pellets were suspended in a solution containing 6 M urea and 4% (wt/vol) sodium dodecyl sulfate and heated for 3 min at 100°C. The released protein was recovered after centrifugation of the fixed bacteria and either directly counted for radioactivity or applied to polyacrylamide gels.

Polyacrylamide gel electrophoresis of proteins. Discontinuous sodium dodecyl sulfate–10% polyacrylamide slab gels were prepared essentially by the method of Laemmli (12). The samples were electrophoresed at 80 V until the tracking dye reached the bottom edge of the gel. Radioactively labeled proteins were located by fluorography essentially as described by Bonner and Laskey (4). Kodak BB-5 X-ray film was exposed to the dried gels at −70°C and then developed to visualize the bands produced by the radioactive proteins.

Materials. Embryonated gs$^-$ chf$^-$ chicken eggs were obtained from SPAFAS, Inc., Norwich, Conn. 3-Deazaadenosine was synthesized by a previously described procedure (15). L-[*methyl*-^{3}H]methionine (9 Ci/mmol), [5,6-^{3}H]uridine (41 Ci/mmol), [2-^{3}H]adenosine (22 Ci/mmol), L-[4,5-^{3}H]leucine (149 Ci/mmol), and deoxycytidine 5′[α-^{32}P]triphosphate (2,000 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, Ill. S1 nuclease was obtained from Miles Laboratories, Inc., Elkhart, Ind. Aminobenzoxymethyl paper was purchased from Schleicher & Scheull Co.,

Keene, N.H. Goat antisera directed against detergent-disrupted avian myeloblastosis virus (AMV) and p27 were obtained from the Division of Cancer Cause and Prevention, National Cancer Institute. Anti-p19 antiserum was generously supplied by Volker Vogt, Cornell University, Ithaca, N.Y.

RESULTS

Effect of 3-deazaadenosine on the production of B77 ASV virions by infected chicken embryo fibroblasts. To test the effect of 3-deazaadenosine on the production of B77 virions, plates of confluent infected chicken embryo fibroblasts were treated with either MEM or MEM containing 100 μM 3-deazaadenosine for 14 h. The radioactive precursors [^{3}H]uridine and [^{3}H]methionine (to assay for RNA and protein, respectively) were then added to the media. The media were collected after 12 h of labeling, the virus was purified, and the amounts of radioactivity in the virus bands from an equilibrium sucrose gradient were determined. Table 1 shows that both the incorporation of [^{3}H]adenosine and the incorporation of [^{3}H]methionine into virions were reduced by more than 90% in the presence of 3-deazaadenosine. These results indicated that the production of B77 virions was inhibited in the presence of 3-deazaadenosine and confirmed the results of Bader et al. (2) for a different ASV strain. The production of transformation-defective virions was also inhibited in the presence of 3-deazaadenosine (Table 1). In agreement with Bader et al., we also found that the inhibition of virus production was reversible. Upon removal of 3-deazaadenosine, the rate of virus production, as determined by incorporation of radioactivity or by reverse transcriptase activity, was restored to normal levels within 12 h (data not shown).

Effect of 3-deazaadenosine on host and viral polypeptide synthesis. One possible explanation for the inhibition of virus production by 3-deazaadenosine would be a general blockade of host protein synthesis. Therefore, we tested the effect of this drug on the overall rate of protein synthesis. Cells were treated for varying times in the absence and presence of 100 μM 3-deazaadenosine. Cell cultures were then pulse-labeled with [^{3}H]leucine for 1 h, the cells were harvested, and the incorporation of [^{3}H]leucine into hot trichloroacetic acid-insoluble material was measured (Table 2). A 35 to 50% decrease in incorporation was obtained in the presence of 3-deazaadenosine. These results agree reasonably well with those obtained by Bader et al., who reported an inhibition of approximately 20% in the rate of [^{3}H]leucine incorporation after treatment of chicken embryo cells with 100 μM 3-deazaadenosine for 24 h (2).

To test for a specific effect of 3-deazaadenosine on viral protein synthesis, ASV-infected cells were labeled with [^{3}H]leucine for 1 h, cytoplasmic extracts were prepared, and the virus-specific proteins were detected by incubation with appropriate antisera to avian retrovirus proteins, followed by adsorption of the antigen-

TABLE 2. *Rate of incorporation of [^{3}H]leucine into proteins in the presence of 100 μM 3-deazaadenosine*[a]

Time after 3-deazaadenosine treatment (h)	Radioactivity (cpm) in:		% of control
	Control	3-Deazaadenosine-treated culture	
18	18,560	12,268	66
22	21,179	10,449	49
26	15,146	8,834	58

[a] Plates (60 mm) of confluent B77 ASV-infected cells were treated with 100 μM 3-deazaadenosine in MEM for varying times. The medium was then replaced with the appropriate medium lacking leucine. After 1 h 50 μCi of [^{3}H]leucine was added, and the cells were incubated for an additional 1 h. After this, the cells were scraped from each dish and suspended in 1 ml of a buffer containing 0.1 M NaCl, 0.01 M Tris (pH 7.5), 0.001 M EDTA, 1% sodium dodecyl sulfate, and 1% β-mercaptoethanol. From these samples, 0.1 ml-portions were withdrawn into 1-ml portions of 10% trichloroacetic acid, and the samples were heated for 15 min at 90°C. The samples were then filtered and counted for radioactivity.

TABLE 1. *Inhibition of radioactive B77 virion production in the presence of 100 μM 3-deazaadenosine*[a]

Virus	Isotope	Radioactivity (cpm) in:		% Inhibition
		Control	3-Deazaadenosine-treated culture	
B77	[^{3}H]methionine	106,000	9,400	91
B77	[^{3}H]adenosine	17,000	800	95
td B77	[^{3}H]uridine	4,400	500	88

[a] Plates (100 mm) of confluent B77 ASV-infected cells were labeled with 800 μCi of [*methyl*-^{3}H]methionine per ml or 20 μCi of [^{3}H]adenosine per ml for 12 h after a 14-h treatment with 3-deazaadenosine. Cells infected with transformation-defective B77 ASV (td B77) were labeled with 20 μCi of [^{3}H]uridine per ml for 12 h. The virus was purified from the media as described previously (21), and the amount of radioactivity in the peak at a density of 1.15 to 1.16 g/ml was determined.

antibody complexes to Formalin-fixed *S. aureus* (11). The materials in the immunoprecipitates were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the relative intensities of the bands corresponding to the viral proteins were compared (Fig. 1). In the control extracts, the major bands immunoprecipitated by the AMV antiserum and detectable on the gels corresponded to p27 and the precursor of the *gag* proteins, pr76^{gag} (Fig. 1, lane 5). Figure 1 also shows that upon addition of excess competing unlabeled viral protein during the immunoprecipitation (lane 6), the relative intensities of the pr76^{gag} and p27 bands were diminished, indicating that the immunoprecipitation of these proteins was specific. Much smaller amounts of pr76^{gag} and p27 were immunoprecipitated from an equivalent amount of [^{3}H]leucine-labeled protein extract obtained from 3-deazaadenosine-treated cells (Fig. 1, lane 2). Similar results were obtained when anti-p27 antiserum was used (data not shown).

The profiles of the total labeled proteins from 3-deazaadenosine-treated and untreated cells are shown in Fig. 1, lanes 3 and 4, and the two profiles were quite similar. Since the majority of the labeled proteins are cellular, this result indicates that the synthesis of most of the cellular proteins was not inhibited selectively by the 3-deazaadenosine treatment. An exception appears to be a polypeptide with an apparent molecular weight of approximately 70,000 (designated hp), which was present in the control extract. The position of this polypeptide on the gel did not correspond to the position of any of the virion proteins. This suggested that 3-deazaadenosine may specifically inhibit the synthesis of some host cell proteins, as well as the synthesis of viral proteins.

It was possible that the viral proteins were synthesized in the same relative amounts in the 3-deazaadenosine-treated cells but that these proteins were degraded rapidly under these conditions. If this were the case, we would expect that labeling for a shorter time with [^{3}H]leucine would result in the production of relatively larger amounts of viral proteins in 3-deazaadenosine-treated cells. Therefore, the products of a 20-min pulse-labeling with [^{3}H]leucine were analyzed (Fig. 2). In these experiments we used two antisera of different specificities, anti-AMV and anti-p19. It was clear that the anti-AMV antiserum immunoprecipitated both the *gag* proteins and, to a lesser extent, the *env* proteins gp85 and gp35 (compare Fig. 2A, lane 1 [whole disrupted virions] with Fig. 2A, lane 4 [immunoprecipitate of whole disrupted virions]). The immunoprecipitation of gp85 and gp35 from vi-

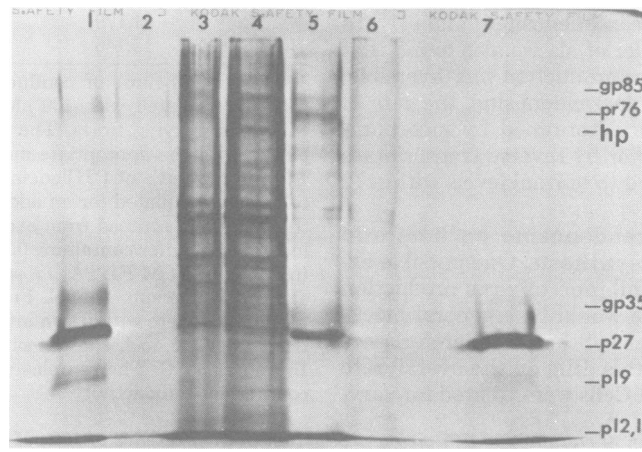

FIG. 1. *Polyacrylamide gel electrophoresis of viral proteins pulse-labeled for 1 h with [^{3}H]leucine and immunoprecipitated from extracts of 3-deazaadenosine-treated and control infected cells. [^{3}H]leucine-labeled cell extracts were prepared, and immunoprecipitation with anti-AMV antiserum to the Formalin-fixed Cowan I strain of S. aureus and elution from the bacteria were carried out as described in the text. Equal input amounts of radioactivity from the two extracts were used. Polyacrylamide gel electrophoresis was carried out on the eluted samples in discontinuous sodium dodecyl sulfate-polyacrylamide gels for 4.5 h at 80 V. The gel was prepared for fluorography by using previously described procedures (4). Lane 1, Disrupted [^{3}H]leucine-labeled B77 virions; lane 2, immunoprecipitation of proteins from 3-deazaadenosine-treated cell extract; lane 3, total proteins from 3-deazaadenosine-treated cell extract; lane 4, total proteins from control cell extract; lane 5, immunoprecipitation of proteins from control cell extract; lane 6, immunoprecipitation of proteins from control cell extract carried out in the presence of 100 μg of nonradioactive disrupted B77 sarcoma virions; lane 7, immunoprecipitation of disrupted [^{3}H]leucine-labeled B77 virions.*

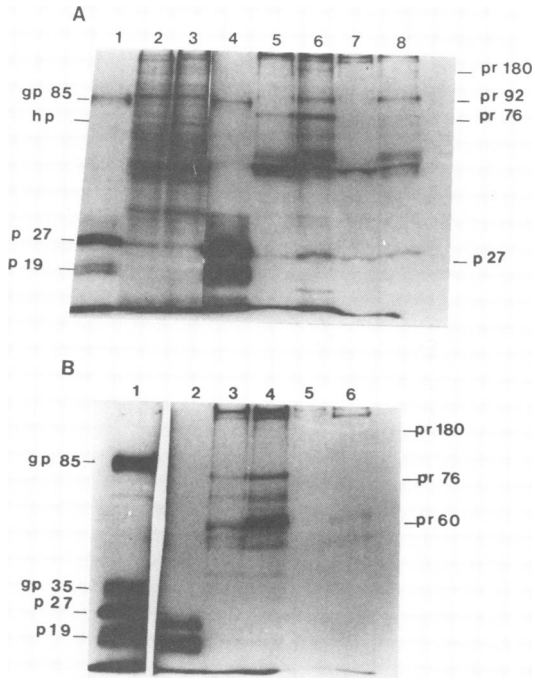

FIG. 2. *Polyacrylamide gel electrophoresis of viral proteins pulse-labeled for 20 min with [^{3}H]leucine and immunoprecipitated from extracts of 3-deazaadenosine-treated and control infected cells by anti-AMV antiserum (A) and anti-p19 antiserum (B). The experimental conditions were identical to those described in the legend to Fig. 1, except that the labeling time was 20 min rather than 1 h. (A) Anti-AMV antiserum. Lane 1, Disrupted B77 virions; lane 2, total proteins from 3-deazaadenosine-treated extract; lane 3, total proteins from control extract; lane 4, immunoprecipitation of virion proteins; lane 6, immunoprecipitation of control extract; lane 5, same as lane 6, but in the presence of 80 μg of nonradioactive disrupted B77 sarcoma virions; lane 8, immunoprecipitation of 3-deazaadenosine-treated extract; lane 7, same as lane 8, but in the presence of 80 μg of nonradioactive disrupted B77 sarcoma virions. (B) Anti-p19 antiserum. Lane 1, Disrupted B77 virions; lane 2, immunoprecipitation of virion proteins; lane 4, immunoprecipitation of proteins from control cell extract; lane 3, same as lane 4, but carried out in the presence of 80 μg of nonradioactive disrupted B77 sarcoma virions; lane 6, immunoprecipitation of proteins from 3-deazaadenosine-treated cell extract; lane 5, same as lane 6, but carried out in the presence of 80 μg of nonradioactive disrupted B77 sarcoma virions.*

rions was not obvious in the 1-h pulse shown in Fig. 2 because the exposure time of the fluorogram was not sufficiently long to detect radioactivity in these peaks. On the other hand, the anti-p19 antiserum precipitated primarily the p19 polypeptides, but it also precipitated p27 to some extent; i.e., the antiserum was contaminated slightly with antibodies directed toward p27 (Fig. 2B, lanes 1 and 2). There was no activity directed toward the *env* protein gp85.

Figure 2 also shows that in the control infected extract a number of bands were specifically precipitated with the anti-AMV antiserum (Fig. 2A, lanes 5 and 6). As expected, the antiserum precipitated pr76^{gag} and a less intense band corresponding to p27. We also detected a virus-specific polypeptide migrating at the position of pr180$^{gag\text{-}pol}$, the presumptive precursor of the reverse transcriptase (15). In addition, a polypeptide with a molecular weight of approximately 90,000 was immunoprecipitated, and this immunoprecipitation was inhibited in the presence of excess unlabeled disrupted virions.

When the anti-p19 antiserum was used, both pr76^{gag} and a band corresponding to pr60^{gag}, a previously described intermediate (23) in the processing of pr76^{gag}, were specifically immunoprecipitated from the extract. (Fig. 2B, lanes 3 and 4). Although not visible in Fig. 2, a band corresponding to pr180$^{gag\ pol}$ was also detected in longer exposures. However, the 90,000-dalton polypeptide was not immunoprecipitated. We believe that the latter polypeptide corresponds to the envelope protein precursor (pr92^{env}) for the following reasons: (i) the polypeptide was immunoprecipitated by the anti-AMV antiserum, which contained antibodies directed against *env* proteins, but was not immunoprecipitated by the anti-p19 antiserum, which did not; (ii) the polypeptide had the approximate apparent molecular weight of the *env* precursor, as reported by other investigators (14); and (iii) the immunoprecipitation of this protein was inhibited in the presence of disrupted virions, indicating that it was related structurally to a virion protein.

In the extracts prepared from 3-deazaadenosine-treated cells, we detected little or no pr76^{gag} or the putative pr180$^{gag\ pol}$ polypeptide in either the anti-AMV immunoprecipitates (Fig. 2A, lanes 7 and 8) or the anti-p19 immunoprecipitates (Fig. 2B, lanes 5 and 6). This result was similar to the result obtained in the 1-h pulse-labeling experiment (Fig. 1) and suggested that the syntheses of these proteins were indeed inhibited in the presence of 3-deazaadenosine rather than the proteins being rapidly degraded. In contrast, the presumptive pr92^{env} polypeptide appeared to be present in the anti-AMV immunoprecipitates (Fig. 2A, lane 8). These results suggested that detectable amounts of pr92^{env} were synthesized in 3-deazaadenosine-treated cells, whereas comparable amounts of pr76^{gag} were not.

We compared the profiles of the total labeled

proteins from 3-deazaadenosine-treated and control extracts (Fig. 2A, lanes 2 and 3). As observed with the 1-h labeled extracts, the profiles appeared to be identical, except for the presence in the control extract of a heavily labeled band having a molecular weight of approximately 70,000 (band hp), which appeared to be missing in the extracts from the 3-deazaadenosine-treated cells.

Effect of 3-deazaadenosine on host and viral RNA synthesis. We next tested whether the selective inhibition of viral protein synthesis might be due to a selective inhibition of viral RNA synthesis. It has been reported previously that a 24-h treatment with 100-μM 3-deazaadenosine results in an inhibition of approximately 30% in the rate of [^{3}H]uridine incorporation into the total RNA of chicken embryo cells (2). To test the effect of the analog specifically on mRNA synthesis, cells were treated for 15 h with 3-deazaadenosine and labeled with [^{3}H]uridine for 12 h, and the total poly(A)$^+$ RNA was isolated from the cells. The amount of radioactive RNA per cell was determined. There was an inhibition in the accumulation of both poly(A)$^+$ RNA and poly(A)$^-$ RNA (34 and 22% inhibition, respectively) in the presence of 3-deazaadenosine.

To determine whether the poly(A)$^+$ RNA synthesized in the presence of 3-deazaadenosine was associated with polysomes, analog-treated and control cells were labeled for varying times with [^{3}H]uridine. After the labeling period, the cells were lysed and separated into nuclei and cytoplasm. The cytoplasmic fractions were further separated into polysomal and nonpolysomal fractions by centrifugation on discontinuous sucrose gradients. The amount of poly(A)$^+$ RNA in each fraction was then determined. Figure 3 shows that there was a decrease of approximately 50% in the rate of [^{3}H]uridine incorporation into nuclear RNA (Fig. 3A). There was a corresponding decrease in the rate of incorporation of [^{3}H]uridine into polysomal RNA (Fig. 3B). The size distributions of the polysomes were not significantly different in treated and control cells (data not shown). The decrease in the observed rate of protein synthesis shown in Table 2 was similar in magnitude to the decreased rate at which mRNA associated with polysomes. This result suggested that the inhibition of protein synthesis in 3-deazaadenosine-treated cells was due to lowered levels of mRNA in polyribosomes. In the experiments shown in Fig. 3 and Table 2, the results were not corrected for possible effects of 3-deazaadenosine on cell growth rate. However, we observed that the cell densities under the two conditions remained comparable over the time periods of the experiments.

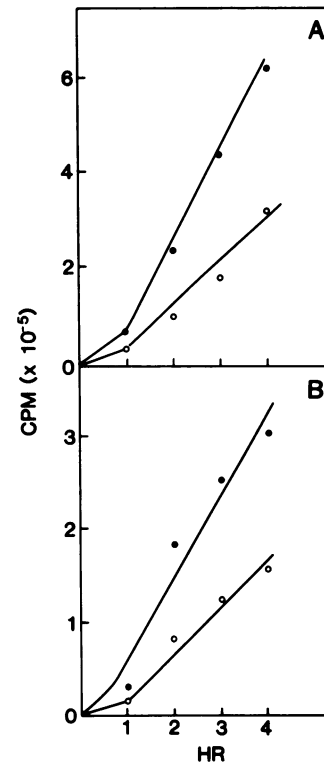

FIG. 3. *Kinetics of poly(A)$^+$ RNA synthesis in 3-deazaadenosine-treated and control infected cells. Confluent monolayers in 100-mm plastic petri dishes of B77 ASV-infected chicken embryo fibroblasts were incubated for 15 h with either MEM or MEM containing 100 μM 3-deazaadenosine. At the end of this time, the medium was removed, and fresh medium with or without 100 μM 3-deazaadenosine containing 20 μCi of [^{3}H]uridine per ml was added. The cells were harvested after varying labeling periods by first washing the plates with 5 ml of TBS and then resuspending the cells in 5 ml of TBS. The cells were collected by centrifugation, disrupted by detergent lysis, and separated into nuclear and cytoplasmic fractions. The cytoplasm was separated into polysomal and nonpolysomal fractions by discontinuous sucrose gradients as described previously (8). Polysomal and nuclear RNAs were isolated by phenol-chloroform extraction, and the poly(A)$^+$ RNA was recovered by oligodeoxythymidylic acid-cellulose chromatography. Symbols: ○, 3-deazaadenosine-treated cells; ●, control cells. (A) Nuclear RNA. (B) Polysomal RNA.*

We next tested whether all or part of the action of 3-deazaadenosine on virus production was due to selective inhibition of viral RNA synthesis. The fractions of virus-specific RNA in analog-treated and control cells were compared by determining the kinetics of annealing of a viral cDNA probe to RNA isolated from infected cells (Fig. 4). The $C_rt_{1/2}$ values for the RNAs

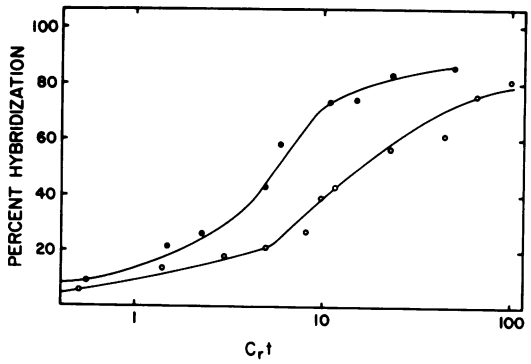

FIG. 4. *Kinetics of RNA from 3-deazaadenosine-treated and control infected cells hybridized to viral cDNA. Confluent monolayer cultures of B77-infected cells were incubated with either MEM or MEM containing 100 μM 3-deazaadenosine for 18 h. After this, [^{3}H]adenosine (final concentration, 20 μCi/ml) was added, and the incubation was continued for an additional 12 h. Total RNA was isolated from the cells by phenol-chloroform extraction, DNase digestion, LiCl precipitation, and oligodeoxythymidylic acid-cellulose chromatography, according to previously described techniques (21). Hybridization of RNA at varying concentrations to approximately 1,000 cpm of ^{32}P-labeled cDNA was carried out in a solution containing 0.3 M NaCl, 0.01 M Tris-hydrochloride (pH 7.5), and 0.001 M EDTA for 18 h at 68°C. The extent of hybridization was assayed by the sensitivity of the ^{32}P-labeled cDNA to S1 nuclease digestion. Symbols: ○, 3-deazaadenosine-treated cells; ●, control cells.*

from the control and 3-deazaadenosine-treated cells were 4.7 and 11.5 mol·s/liter, respectively. This result indicated that the fraction of the total RNA represented by virus-specific RNA in 3-deazaadenosine-treated cells was 40% of the fraction of virus-specific RNA present in control cells. Therefore, there appeared to be a selective decrease in the amount of viral RNA in the analog-treated cells. Since the overall poly(A)$^-$ RNA synthesis was inhibited somewhat less than the overall poly(A)$^+$ RNA synthesis, we also directly tested the poly(A)$^+$ RNA from 3-deazaadenosine-treated cells for the fraction of virus-specific RNA by hybridization kinetics. In this case we obtained a value of 50% of the control value (data not shown). We concluded that there was a selective decrease in the steady-state levels of viral RNA in the 3-deazaadenosine-treated cells.

We then analyzed the RNA in polyribosomes for individual species of virus-specific mRNA. Chicken embryo fibroblasts infected with ASVs reportedly contain subgenomic 28S and 21S mRNA's, as well as 38S genomic RNA (10, 24). The 28S and 21S mRNA's are thought to serve as messengers for the precursor of the envelope proteins gp85 and gp35 and for the protein required for transformation (pp60^{src}), respectively (17–19). On the other hand, at least part of the 38S genomic RNA serves as the mRNA for the *gag* protein presursor (pr76^{gag}) and the presumptive reverse transcriptase precursor (pr180$^{gag\ pol}$) (16). The 28S and 21S mRNA's probably arise from the 38S RNA by a process in which sequences in the 3′ half of the genome RNA are spliced to a 5′-terminal leader sequence (6, 13, 20). To test for the presence and relative amounts of the various mRNA species, we electrophoresed poly(A)$^+$ RNAs from the polysomal fractions of 3-deazaadenosine-treated and untreated cells on agarose gels containing 10 mM methylmercury hydroxide. The separated RNA species were transferred to diazobenzoxymethyl paper by the procedure of Alwine et al. (1), and the virus-specific species were detected by hybridization of the blot to a plasmid preparation containing the entire ASV Prague A genome (pSal^{101}) which had been labeled with ^{32}P by nick translation (Fig. 5). Qualitatively, it appeared from the gels that the intensities of the 38S and 35S genome RNA bands in the polysomal RNA from 3-deazaadenosine-treated cells were reduced relative to the amounts of 28S and 21S mRNA's when compared with equal amounts of polysomal RNAs from the control cells. (The 35S RNA was derived from the presence of transformation-defective deletion mutants which contaminated the B77 ASV stock used in this experiment). To approximate the relative amounts of the various virus-specific RNA species present in the 3-deazaadenosine-treated and control infected cells, the autoradiograms were scanned with a densitometer, and the areas under the peaks were compared. The sizes of areas under the peaks corresponding to the 21S, 28S, and 35S-38S RNAs were 47, 40, and 12% of the sizes of the areas under the same peaks when equal amounts of control RNA were used. These differences were observed when the gels were exposed for several different intervals. Therefore, there appeared to be a relatively larger decrease in the amount of 35S-38S RNA than in the amounts of the subgenomic 28S and 21S mRNA species in the 3-deazaadenosine-treated cells.

Further evidence for a decrease in the relative amount of the 35S-38S RNA in 3-deazaadenosine-treated cells compared with control cells was obtained by hybridization of nuclear and polysomal RNAs from glycerol gradient fractions to radioactive viral cDNA (Fig. 6). Clearly, the resolution of RNA species in these gradients was not nearly as good as in the methylmercury hydroxide gels shown in Fig. 5. However, the data obtained from this hybridization of the

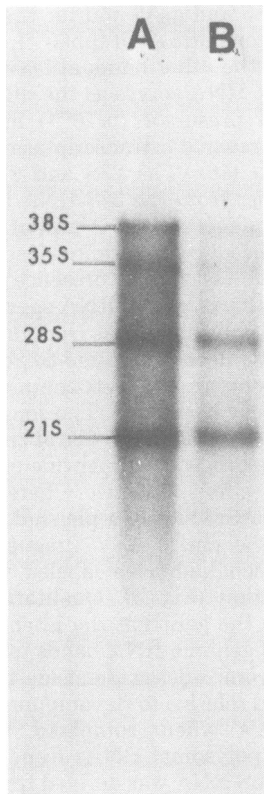

FIG. 5. *Electrophoresis of viral RNA on 1% agarose gels containing 10 mM methylmercury hydroxide. The fractionation of cytoplasmic extracts into polysomes and postpolysomal cytoplasm was carried out as described in the text. Approximately 15 μg of RNA from each of the polysome fractions was electrophoresed on 1% agarose gels containing 10 mM methylmercury hydroxide for 6 h at 50 V. The locations of the virus-specific RNA bands were determined by procedures described in the text. The exposure time was 24 h. Lane A, Polysomal RNA from control ASV-infected cells; lane B, polysomal RNA from 3-deazaadenosine-treated ASV-infected cells.*

RNAs from the gradient fractions allowed an independent quantitative comparison of the relative amounts of genomic and subgenomic RNAs. Hybridization values in the range of 0 to 40% can be assumed to be roughly proportional to C_rt and, therefore, for a constant hybridization time proportional to viral RNA concentration (9). These data are summarized in Table 3. We observed that there was a decrease in the ratio of genomic RNA to subgenomic RNA in both the nuclear RNA and the polysomal RNA from 3-deazaadenosine-treated cells. Therefore, these data support the data in Fig. 5 and suggest that the relative concentration of the 35S-38S RNA

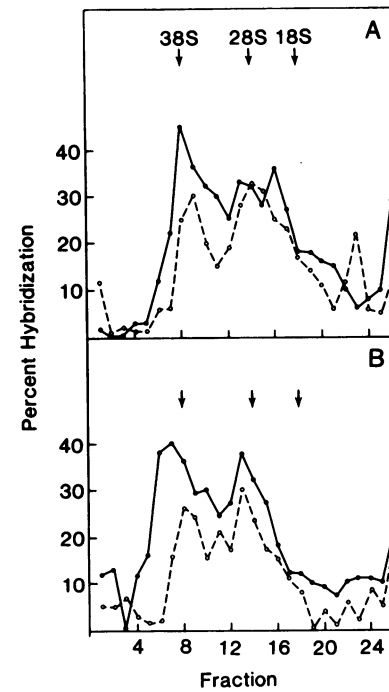

FIG. 6. *Glycerol gradient sedimentation of virus-specific nuclear RNA (A) and polysomal RNA (B) from 3-deazaadenosine-treated and control cells. Four 100-mm plates of confluent cells infected with B77 ASV were treated with either MEM or MEM containing 100 μM 3-deazaadenosine for 19 h. The cells were fractionated into nuclei and cytoplasm, and the polysomes were isolated on discontinuous sucrose gradients. The RNAs from the nuclear and polysomal fractions were purified as described previously (8). The poly(A)$^+$ RNA was isolated by oligodeoxythymidylic acid-cellulose chromatography. Approximately equal amounts of RNA were applied to 5 to 30% glycerol gradients. The gradients were sedimented by centrifugation at 24,000 rpm for 16 h in a Beckman SW41 rotor. The gradients were fractionated, and the RNA in each fraction was precipitated with 2 volumes of 95% ethanol. The precipitates were collected and dissolved in sterile distilled water (100 and 50 μl for the polysomal RNAs from the control and 3-deazaadenosine-treated cells, respectively; 50 and 25 μl for the nuclear RNAs from the control and 3-deazaadenosine-treated cells, respectively). Samples (10 μl) from each fraction were hybridized for 42 h at 68°C to ^{32}P-labeled B77 ASV cDNA. A gradient containing 38S viral genome RNA and 28S and 18S rRNA's was run in parallel, and the positions of the peaks are marked on the figure. Symbols: ○, 3-deazaadenosine-treated cells; ●, control cells.*

was indeed reduced in 3-deazaadenosine-treated cells.

DISCUSSION

We show in this paper that, in the presence of 100 μM 3-deazaadenosine, there is a selective

TABLE 3. *Distribution of virus-specific RNA in genomic and subgenomic size classes from 3-deazaadenosine-treated and control cells*[a]

Fraction	Control			3-Deazaadenosine-treated culture		
	% of virus-specific RNA in:		G/SG	% of virus-specific RNA in:		G/SG
	G	SG		G	SG	
Nuclei	57	43	1.33	33	67	0.49
Polysomes	55	45	1.22	36	64	0.56

[a] The values for percent hybridization obtained from the results in Fig. 6 were assumed to be proportional to concentration. This is only an approximate relationship and is valid between values of 0 and 50% hybridization (9). Genomic (G) and subgenomic (SG) RNAs were defined as the material sedimenting between approximately 32S and 40S and between 18S and 30S, respectively.

inhibition of the synthesis of the ASV proteins $pr76^{gag}$ and $pr180^{gag\ pol}$. The synthesis of a polypeptide presumed to be $pr92^{env}$ appears to be affected less. In general, host cell protein synthesis is also less sensitive to inhibition. It appears that the inhibition of viral *gag* protein synthesis, coupled with a decrease of 30 to 50% in the rate of overall protein synthesis, could explain the inhibition of more than 90% in the production of virions previously observed by Bader et al. (2) and confirmed by us here.

We found that at least part of the selective effect of 3-deazaadenosine treatment on viral protein synthesis is due to a selective decrease in the relative amounts of viral mRNA's. The $C_rt_{1/2}$ values for the rate of hybridization of viral cDNA to infected cell RNA indicated that the concentration of viral RNA in treated cells was to approximately 40% of the concentration in control cells. If the inhibition of the rate of synthesis of radioactively labeled cellular RNA (≅40 to 50%) is also reflected in a decreased steady-state level of cellular RNA in 3-deazaadenosine-treated cells, the absolute amounts of viral RNA may be reduced to levels which are as little as 20% of the amounts in control cells. It should be stated that the cDNA used in the hybridization kinetics experiments (Fig. 4) was not a representative copy of the viral genome, and therefore we cannot be certain from this experiment whether the concentrations of all viral sequences are reduced equally. From analyses of the viral RNA species on agarose gels containing methylmercury hydroxide and on glycerol gradients, it appeared that in 3-deazaadenosine-treated cells, there were relatively larger decreases in the concentrations of the genome 38S and 35S mRNA's than in the concentrations of the 28S *env* and 21S *src* mRNA's. These relative decreases in the levels of the putative mRNA's for $pr76^{gag}$ and $pr180^{gag\ pol}$ may explain the greater effect of the 3-deazaadenosine treatment on the synthesis of these polypeptides compared with the presumptive $pr92^{env}$ polypeptide (Fig. 2).

The mechanism by which the steady-state levels of viral RNA are reduced in 3-deazaadenosine-treated cells remains obscure. Such a decrease could be caused either by a preferential decrease in the rate of virus RNA synthesis or by a preferential increase in the turnover of viral RNA. Bader et al. have proposed that since 3-deazaadenosine is an inhibitor of *S*-adenosylhomocysteine hydrolase and since the intracellular concentration of *S*-adenosylhomocysteine increases dramatically in treated cells, methylation reactions are inhibited. In particular, the failure to methylate viral RNA may render it nonfunctional (2). We have shown previously that another in vivo inhibitor of methylation, cycloleucine, inhibits the internal N^6-methyladenosine and 2′-*O*-methyl ribose cap methylations of both viral genome RNA and cellular mRNA's by 80 to 90%. However, the production of ASV under these conditions is inhibited only slightly. This led us to propose that the methylations inhibited by the cycloleucine treatment are not essential for virus replication (7). Therefore, if the effects on viral RNA levels are due to inhibition of RNA methylations, we would expect that those methylations which are not blocked in the presence of cycloleucine (i.e., the 5′-terminal N^7-methylguanosine methylations) would be affected. We have tested for such an effect on the bulk cellular poly(A)$^+$ RNA from 3-deazaadenosine-treated ASV-infected chicken embryo fibroblasts and have found little or no inhibition of N^7-methylguanosine methylations (Stoltzfus, unpublished data). We have not yet tested for a possible specific effect on the N^7-methylguanosine methylation of the various viral mRNA's. However, it would be surprising if such were the case, since cellular methylating enzymes are presumably used to methylate viral RNA.

As an alternative hypothesis, we propose that 3-deazaadenosine may selectively inhibit the synthesis of viral RNA. It has been shown that the production of avian retrovirions is extremely sensitive to the effects of the transcription inhibitor actinomycin D. Even at concentrations of actinomycin D as low as 0.1 μg/ml, virus production is inhibited more than 90% (22). Under these conditions, the synthesis of host poly(A)$^+$ RNA is inhibited only about 30% (Stoltzfus, unpublished data). Whether this sensitivity to low concentrations of actinomycin D is due en-

tirely to effects on viral RNA synthesis has not been established yet. We have also obtained preliminary evidence that treatment of ASV-infected cells with α-amanitin (a specific inhibitor of RNA polymerase II) at a concentration of 10 μg/ml inhibits cellular poly(A)$^+$ RNA synthesis by approximately 30%, whereas virion production is inhibited by about 70% (Stoltzfus, unpublished data). For both inhibitors, the effect on virus production and presumably the effect on viral RNA transcription are considerably greater than the effect on the synthesis of the bulk cellular mRNA. We have shown that 3-deazaadenosine inhibits bulk cellular poly(A)$^+$ RNA synthesis by 40 to 50%. Therefore, it would not be surprising if the 3-deazaadenosine inhibition of viral RNA levels were due to a selective inhibition of viral RNA synthesis. Additional studies on the kinetics of viral RNA synthesis in 3-deazaadenosine-treated cells will be required to test this hypothesis.

The reason for the selective decrease in the amount of 35S-38S RNA relative to the amounts of 28S and 21S mRNA's in 3-deazaadenosine-treated cells is also not clear. It is thought that the 38S RNA is a precursor of the 28S and 21S mRNA's and that these subgenomic RNAs are derived by a splicing process (6, 13, 20). If this is indeed the case and if a constant amount of viral 38S RNA is spliced per unit time, a decrease in the rate of transcription of 38S RNA would result in a larger proportion of spliced RNA and an increase in the ratio of subgenomic RNA to genomic RNA. Therefore, the alteration in the steady-state levels of the various viral RNA species observed in 3-deazaadenosine-treated cells may reflect the effect of the analog on the rate of viral RNA transcription.

Finally, we point out that the partial reversion of cells infected with the Bryan strain of Rous sarcoma virus from a transformed phenotype to a more normal phenotype upon treatment with 3-deazaadenosine, as reported by Bader et al. (2), could also be explained by inhibition of viral RNA transcription. This would result in the production of less *src* mRNA and, therefore, presumably would lead to a reduction in the intracellular levels of the protein which is required for transformation, pp60^{src} (5). We have not yet determined the rate of synthesis of pp60^{src} in 3-deazaadenosine-treated cells. Based on a comparison of the relative amounts of the putative 21S *src* mRNA in 3-deazaadenosine-treated and control cells (Fig. 5), we anticipate that the reduction in the amount of pp60^{src} is not as profound as the reduction in the amount of pr76^{gag}.

To our knowledge, 3-deazaadenosine is the only reported example of a substance that exhibits selectivity in the inhibition of retroviral RNA and protein syntheses. For this reason, it may be a useful tool for altering the intracellular levels of retroviral mRNA's and proteins and determining the consequences of these changes on cellular and viral functions.

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