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Effects of Polyadenylic Acids on Functions of Murine RNA Tumor Viruses

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Single-stranded polyribonucleotides, which competitively inhibit murine RNA tumor virus reverse transcriptase in vitro, were tested as inhibitors of various virus functions in cell culture. The compounds had two concentration-dependent effects. At high concentrations (100 μg/ml), both poly(adenylic acid) [poly(A)] and poly(2'-*O*-methyladenylic acid) [poly(Am)] inhibited the uptake of radioactively labeled leukemia virus by Swiss mouse embryo cells, but neither had a similar effect on Sindbis virus adsorption. At low concentrations (10 μg/ml), poly(Am) did not inhibit the uptake of leukemia virus but did inhibit virus replication by 85%; in contrast, the replication of Sendai virus and Sindbis virus was not inhibited significantly at this concentration. Both compounds were effective only when added prior to or early during virus infection. Poly(Am) was a much more effective inhibitor than poly(A), probably due to the nuclease resistance of the former compound. Poly(Am) at 5 μg/ml also inhibited transformation of 3T3 cells by Moloney sarcoma virus. However, neither poly(A) nor poly(Am) at high concentrations inhibited the activation of endogenous leukemia virus by iododeoxyuridine in AKR mouse embryo cells. These results suggest that virus reverse transcriptase plays an essential role in both the replication of exogenous murine leukemia viruses and the transformation of cells by murine sarcoma viruses but probably has no role in the activation of endogenous leukemia virus.

Single-stranded synthetic polyribonucleotides and certain of their analogues have been found to inhibit competitively the RNA-dependent DNA polymerase of mouse leukemia viruses (2, 29). Poly(adenylic acid) [poly(A)], poly(2'-*O*-methyladenylic acid) [poly(Am)], poly(uridylic acid), and poly(vinyladenylic acid) (15) have been found to inhibit leukemia viruses in cell culture. The compounds are not active against virus in the absence of cells, and they are effective only during a relatively early stage in the virus replication cycle. Since the compounds are not cytotoxic, their effect on virus replication is compatible with inhibition of such a virus-specific function as reverse transcriptase, although an effect on uptake of virus is not excluded. This paper reports additional selective effects of poly(A) and poly(Am) on RNA tumor viruses in cell culture, related to virus adsorption and the possible role of reverse transcriptase in the expression of virus functions.

MATERIALS AND METHODS

Viruses and cell cultures. Moloney leukemia virus (MLV) and the Moloney pseudotype of mouse sarcoma virus [(M)MSV] were originally obtained from the laboratory of Wallace P. Rowe, National Institutes of Health. Stocks of each virus were prepared in secondary Swiss (NIH strain) mouse embryo cell cultures that had been grown in Eagle minimum essential medium (EMEM) containing 10% fetal calf serum. The cultures were maintained without antibiotics and were free of mycoplasma. Virus pools were prepared by collecting fluid from cultures infected at least 6 days and not more than 24 h after addition of fresh medium. Immediately after collection the virus was stored in a liquid nitrogen freezer.

Sindbis virus stocks were prepared in cultures of secondary chicken embryo cells grown in EMEM supplemented with 5% calf serum. Before virus infection the cells were treated with actinomycin D (1 μg/ml) for 1 h. Virus was added and the cultures were incubated for 20 min for adsorption. The cultures were then washed three times and given fresh medium. Fluid was harvested from the cultures 18 h after infection and stored in liquid nitrogen.

Sendai virus was obtained from Barbara Knowles, Wistar Institute, and a stock pool was prepared in embryonated eggs. Nine-day-old eggs were infected by the allantoic route, incubated for 72 h, and chilled at −20 C for 40 min. The allantoic fluid was then collected and stored in liquid nitrogen.

We used cultures of AKR mouse embryo cells (passage number 44) obtained from the laboratory of Wallace P. Rowe for leukemia virus activation (19). The cultures were grown in McCoy 5A modified medium with 10% fetal calf serum and subcultured twice a week to maintain low cell density. For the induction of virus synthesis, semiconfluent cultures were exposed to iododeoxyuridine (Schwarz/Mann) at 40 to 100 μg/ml for 24 h.

A subline of AKR mouse embryo cells derived in our laboratory was used for the preparation of radioactively labeled AKR virus. This cell line, designated AKR 130, is a high-passage culture that spontaneously initiated virus synthesis. The cell line was maintained on EMEM supplemented with 2 mM glutamine and 10% fetal calf serum.

Preparation of labeled virus. Radioactively labeled AKR leukemia virus was produced in AKR 130 cells grown to confluency in roller bottles with medium changes on alternate days. Twelve hours before labeling, the medium was changed to EMEM lacking leucine and supplemented with 1% fetal calf serum and 2 mM glutamine. The medium was then withdrawn, and fresh medium containing 50 μCi of [^{3}H]leucine per ml (53 Ci/mmol, Schwarz/Mann) was added (50 ml per bottle). The medium was removed after 12 h of incubation, and fresh medium lacking isotope and leucine was added. After an additional 12 h of incubation, the medium was again collected. This regimen yielded virus preparations that contained homogeneous, intact virus particles when analyzed by isopycnic gradient centrifugation.

The culture medium was clarified in a Sorvall GSA rotor at 5,000 rpm for 10 min, and the virus was immediately concentrated by pelleting through 25% sucrose in TNE buffer [0.05 M Tris-hydrochloride (pH 7.5)—0.1 M NaCl—1 mM EDTA] at 25,000 rpm for 1 h in a Spinco SW27 rotor. After suspension in 5 ml of TNE, the virus was further purified by isopycnic banding in a Spinco SW25.1 rotor at 25,000 rpm for 12 h. Samples were removed for determination of radioactivity after gradients had been fractionated into 1-ml samples on an Isco gradient fractionator. All samples were counted in 10 ml of Aquasol (New England Nuclear Corp.) in a Nuclear Chicago scintillation counter. The virus peak which banded in 36% sucrose (~1.16 g/cm^3 at 4 C) was collected, and a sample was removed for protein determination. After the specific activity was adjusted to 3×10^7 counts per min per mg of protein, the preparation was diluted in TNE, stored at −80 C, and used immediately upon thawing. A typical virus profile, obtained by isopycnic banding as described above, is shown in Fig. 1.

Radioactively labeled Sindbis virus was produced in secondary chicken embryo cells that had been incubated in leucine-free medium for 8 h before infection. Before inoculation the cells were treated with actinomycin D (1 μg/ml) for 1 h. Virus was added (~100 PFU per cell) and allowed to adsorb for 20 min. The cells were then washed and given fresh medium containing 50 μCi of [^{3}H]leucine per ml (20 Ci/mmol). Culture fluids containing virus were collected at 14 and 18 h after infection and concentrated by centrifugation through sucrose as described previously. The virus subsequently banding at 1.18 g/cm^3 was collected, and the preparation was frozen until used.

Assay of labeled virus adsorption. Secondary Swiss mouse embryo cells were grown to confluency in 60-mm^2 plastic petri dishes (Falcon) as described above. Before the addition of labeled virus, the cells were treated as indicated. Labeled virus was then added to the cell cultures in 1.0 ml of EMEM with 2 mM glutamine but without fetal calf serum, and the cultures were incubated at 37 C. At appropriate times, sample dishes were removed and the medium was aspirated from the cells. Immediately, the monolayer was washed with three 5-ml samples of phosphate-buffered saline (PBS). The cells were scraped from the plate into 1.0 ml of PBS, transferred to a scintillation vial, and disrupted in a Raytheon sonic oscillator for 1 min (200 W, 10 kHz). Radioactivity

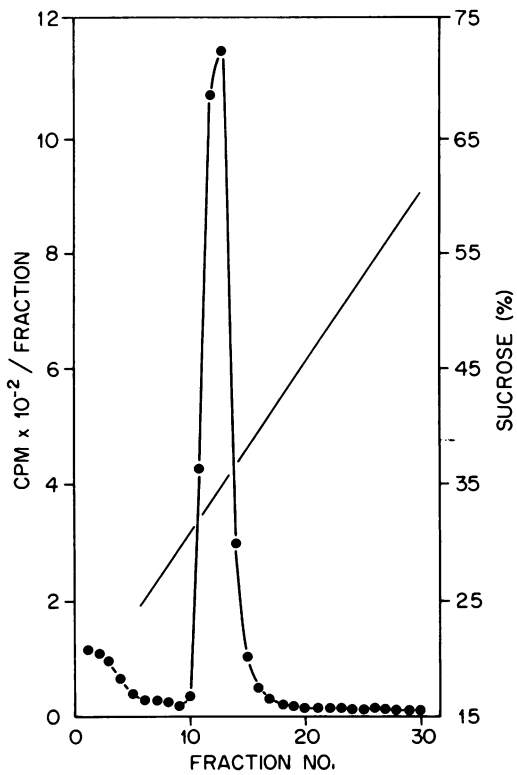

FIG. 1. *Isopycnic sucrose gradient of labeled AKR virus used in adsorption experiments. A 5-ml sample was layered on a 25-ml gradient of 25 to 60% sucrose in TNE buffer and centrifuged in a Spinco SW25.1 rotor at 25,000 rpm for 12 h. Radioactivity was determined after the gradient was fractionated into 1-ml samples.*

was determined after the addition of Aquasol. All experimental points were obtained from duplicate samples, which were generally within 5% of each other. Treatment of petri dishes as above but without cells did not result in significant adsorption of radioactivity.

Polynucleotides. Poly(A) was a product of Miles Laboratories, Inc. The synthesis of 2'-*O*-methyladenosine 5'-disphosphate was according to the following scheme: adenosine → 2'-*O*-methyladenosine (12) → 2'-*O*-methyladenosine 5'-monophosphate (32) → 2'-*O*-methyladenosine 5'-diphosphate (21; B. C. Pal and D. G. Schmidt, unpublished data). Poly(Am) (~9*S*) was prepared by a modification of the procedure of Rottman and Heinlein (18). The reaction mixture contained 0.1 M Tris-hydrochloride (pH 8.5), 1 mM NaN_3, 0.4 mM EDTA, 0.2 mg of bovine serum albumin per ml, 4 mg of polynucleotide phosphorylase per ml (P-L Biochemicals), 0.5 mM $MnCl_2$, and 20 mM 2'-*O*-methyladenosine 5'-diphosphate. The reaction was carried out at 37 C for 22 h. After removal of protein, the reaction mixture was chromatographed on a Sephadex G-150 column equilibrated with 1 mM Tris-hydrochloride (pH 7.5). Void-volume fractions were collected, dialyzed against distilled water, and concentrated by partial lyophilization. The concentration of stock solutions was determined by diluting the material into 0.1 M phosphate buffer (pH 6.8) and determining the absorbance at 260 nm. A solution (1 mg/ml) of polynucleotide was assumed to contain 20 A_{260} units/ml.

Virus assays. MLV replication and activation of endogenous AKR virus were assayed by plaque formation and immunofluorescent techniques. The plaque technique was used to determine progeny virus replication, and the immunofluorescent technique was used principally to count infected cells synthesizing virus-induced proteins. Cell transformation by (M)MSV was assayed by virus-induced focus formation.

The XC cell plaque technique was performed by the method of Rowe et al. (20). Briefly, cell cultures to be assayed for virus synthesis were exposed to UV light (1,800 ergs/mm² total dose) and overlaid with approximately 3.5×10^4 XC cells per 35-mm² culture well. After 4 days of incubation, the cultures were washed, fixed in absolute methanol for 20 min, air-dried, stained with hematoxylin for 20 min, washed, and dried, and syncytial plaques were counted.

Cell transformation and focus formation by (M)MSV were assayed on 3T3 or BALB/c 3T3 (A31) cells obtained from George Todaro, National Institutes of Health. These cells were grown in EMEM with 10% fetal calf serum and were maintained in a subconfluent state. The cells were treated with 25 μg of DEAE-dextran per ml (Pharmacia, Uppsala, Sweden) for 1 h, washed twice with PBS (pH 7.4), and infected with (M)MSV (~0.5 focus-forming units per cell). After 2 h of incubation the cells were given fresh medium and held for 4 to 6 days. Transformed foci were counted microscopically on fixed and stained cultures.

For immunofluorescent assays, cultures of secondary Swiss mouse embryo cells were grown on glass cover slips and treated with DEAE-dextran as above. After incubation with virus for 2 h the cells were washed and given fresh medium. At appropriate intervals the cover slips were collected, fixed, stained (by using rat anti-Moloney leukemia virus serum and anti-rat globulin (fluorescein-conjugated) obtained through the Office of Program Resources and Logistics, National Cancer Institute), and counted by methods described elsewhere (26).

RESULTS

Effects of poly(Am) on RNA tumor virus replication and cell division. Since any compound which delays or interferes with normal cellular functions may also have an inhibitory effect on oncornavirus replication (data reviewed in reference 3), we tested the effects of poly(Am) on mouse embryo cell cultures. As shown in Fig. 2, poly(Am) at 10 μg/ml had no inhibitory effect on cell division and no selective cytotoxicity evidenced by morphological changes. We have seen no toxic effects even at concentrations of 50 μg/ml, although the rate of cell division was not tested under those conditions. These results are comparable to those obtained with poly(A) (25), where treatment with 500 μg/ml had no effect on cell division. Treatment of virus-infected cells with poly(A) (100 μg/ml) also had no effect on the rate of cell DNA synthesis.

We then tested the effect of poly(Am) on the replication of MLV in Swiss mouse embryo cells. Poly(Am) at 10 μg/ml inhibited virus infection by 85%, as assayed by the fluorescent antibody technique 48 h after infection (Table 1). Poly(Am) was more effective than poly(A), since 500 μg of the latter per ml was required to

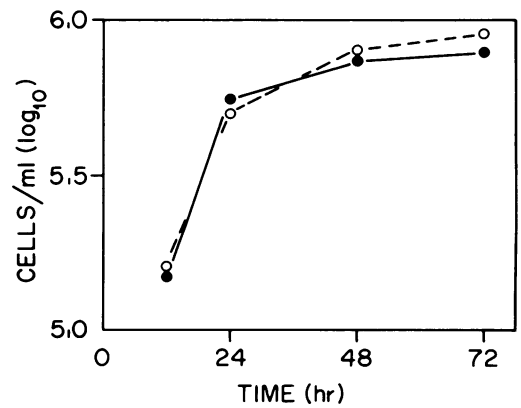

FIG. 2. *Growth of Swiss mouse embryo cell cultures in the presence* (○) *and absence* (●) *of 10 μg of poly(Am) per ml. Secondary-passage cells were plated in Linbro plates and fed with EMEM containing 10% fetal calf serum. Cell counts were determined with a Coulter counter on duplicate samples at the indicated intervals.*

TABLE 1. *Effect of poly(Am) on MLV infection*[a]

Poly(Am)	No. of fluorescent cells/total cells	Inhibition (%)
None	430/682	0
10 μg/ml	68/715	84.9
20 μg/ml	19/587	94.9

[a] NIH Swiss strain mouse embryo cells grown on glass cover slips were treated with DEAE-dextran (25 μg/ml) for 1 h at 37 C, washed twice with PBS, and incubated for 1 h with the test compound or with control medium. The cells were then infected with MLV, incubated for 2 h, and given medium containing the test compound. Forty-eight hours after infection, the cover slips were collected, fixed, and stained. The total number of cells and the number fluorescing were counted with a Zeiss fluorescent microscope system.

produce comparable inhibition (25). The following experiments describe our attempts to characterize the mechanisms of polyribonucleotide inhibition. The absence of virus-induced proteins in the treated cells suggests an effect on some early virus function(s). Although poly(A) and poly(Am) may inhibit virus reverse transcriptase in cells as well as in vitro, it is also possible that they act by other mechanisms, such as by inhibiting virus uptake.

Effects on virus adsorption. Infection of cells in culture by oncornaviruses is potentiated by treatment of the cells with DEAE-dextran (28), and we have found that this treatment also enhances, by about twofold, inhibition of leukemia virus replication by poly(A) and poly(Am). Thus, it is possible that the inhibition could be due to competition between the polynucleotides and the virus for sites on the cell surface that bind DEAE-dextran.

To examine the effects of polynucleotides on mouse oncornavirus adsorption, we assayed their effects on the uptake of labeled virus. The effects of various treatments known to influence virus adsorption were tested with AKR leukemia virus labeled with [^{3}H]leucine in Swiss mouse embryo cells (Fig. 3). Untreated cells adsorbed radioactive virus at a linear rate over a 4-h period, whereas cells treated with DEAE-dextran or polybrene (Abbott Laboratories) (28) before or at the time of addition of virus increased the initial rate of adsorption by seven- and threefold, respectively. These values are comparable to the increases in infectivity observed as a result of such treatments. Exactly analogous results were observed with virus labeled with [^{3}H]uridine or ^{32}P.

The amount of labeled virus adsorbed to a constant number of cells (Fig. 4) was linear over a 10-fold concentration; thus, under these conditions the virus was limiting and not all of the potential sites on the cell were saturated. Also, the linearity of adsorption over a 10-fold concentration of virus suggests adsorption at a single class of sites, since a broken curve would be expected if two or more sites with different affinity constants were available. The actual number of particles adsorbed per cell can be estimated, from the cell number and specific activity of the virus (estimating 3×10^{11} particles/mg of protein), at 1 to 10 particles per cell over the 10-fold concentration range. The ratio of infectious virus to total virus in these preparations was approximately 1:1,000, based on the observation that, at a viral concentration of 100 particles per cell, 12% of the cells synthesized virus-induced protein by 48 h after infection as determined by immunofluorescent staining. Although these numbers suggest that we cannot observe the adsorption of an infective particle, we assume that noninfective particles attach to the cell in a similar manner. The percentage of labeled virus adsorbed relative to the amount added was approximately 30%, independent of the type of radioactive label. Since the virus preparations contained 90 to 95% of the label in intact virus, as determined by isopynic sucrose gradient sedimentation or

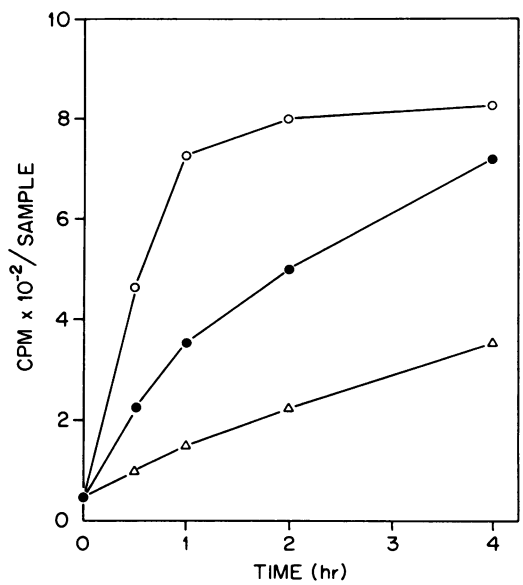

FIG. 3. *Adsorption of [^{3}H]leucine-labeled AKR virus (2,000 counts/min) in the presence and absence of compounds known to affect virus adsorption. Radioactivity was measured in cells, as described in Materials and Methods. Polybrene (25 μg/ml) added with virus (●), with DEAE-dextran (25 μg/ml) pretreatment (○), and in untreated cultures (△).*

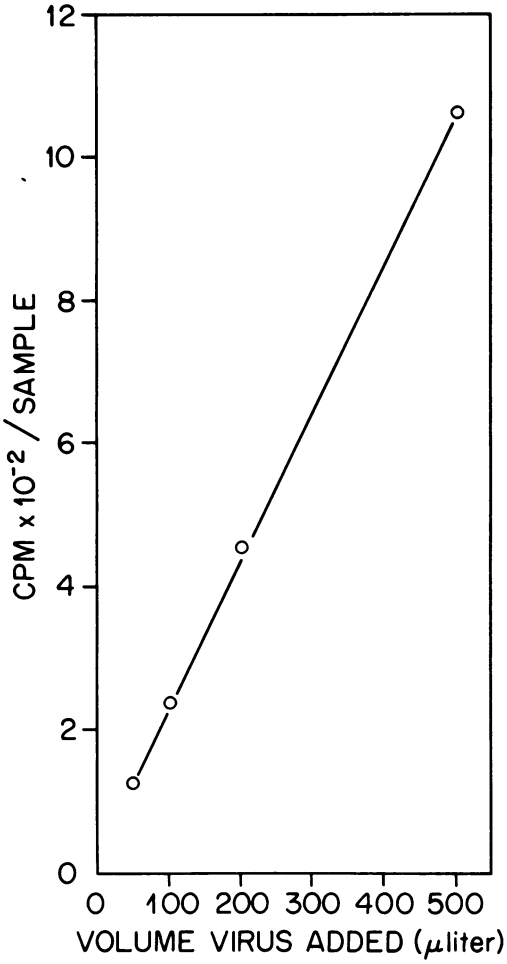

FIG. 4. *Adsorption of [³H]leucine-labeled AKR virus as a function of virus concentration. Cells were treated with DEAE-dextran for 1 h, rinsed twice, and infected with increasing amounts (600 to 6,000 counts/min) of radioactive virus. After 3 h the cells were processed as described in Materials and Methods.*

radioimmune precipitation (14), the inability of all of the virus particles to be adsorbed to the cells is unexplained.

The results of treatment of cells with various concentrations of poly(A) (Fig. 5A) show that 100 μg of the compound per ml effectively blocked all virus attachment. Lower concentrations were progressively less effective, and no inhibition was seen at 10 μg/ml. The effects of poly(Am) (Fig. 5B) were similar: 100 μg/ml effectively inhibited all adsorption, with less inhibition at lower concentrations. Likewise, poly(Am) at 10 μg/ml did not inhibit adsorption. Since the 2'-*O*-methyl polymers are highly resistant to nuclease, the lack of inhibition at 10 μg/ml should not be due to enzymatic degradation and probably reflects a true concentration dependency for inhibition of adsorption.

We examined the specificity of the effects of polynucleotides on virus adsorption by testing their effects on attachment of [³H]leucine-labeled Sindbis virus (Fig. 6). Neither poly(A) at 100 μg/ml nor poly(Am) at 10 μg/ml inhibited Sindbis adsorption, but poly(Am) at 100 μg/ml inhibited adsorption by about 40%. In view of the complete inhibition of AKR leukemia virus adsorption at 100 μg/ml, these results suggest

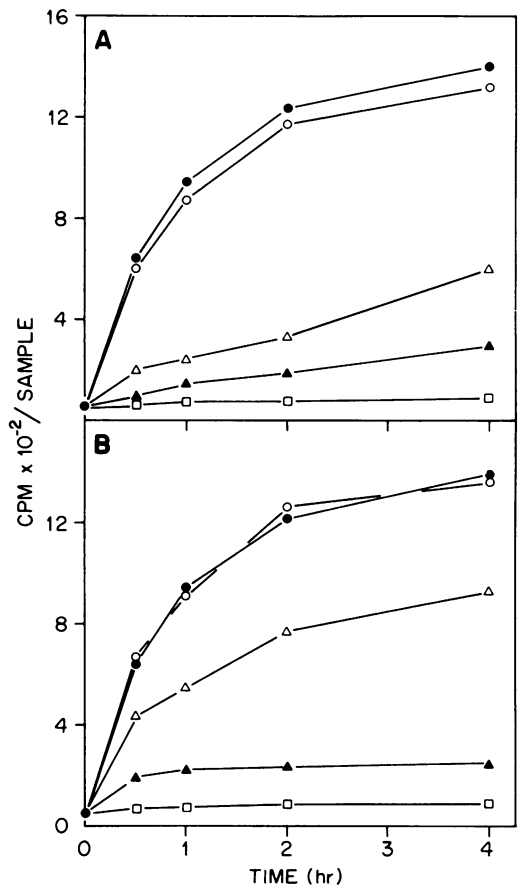

FIG. 5. *Effects of poly(A) and poly(Am) on adsorption of [³H]leucine-labeled AKR virus (5,000 counts/min) by Swiss mouse embryo cells. Cells were treated with DEAE-dextran (25 μg/ml) for 1 h, rinsed twice with EMEM, and treated with polynucleotide at the concentration shown for 1 h. The cells were then washed twice with EMEM and virus was added. At zero time, 0.5, 1, 2, and 4 h, the cells were processed as described in Materials and Methods. A, Untreated (●); poly(A) at 10 μg/ml (○), 25 μg/ml (△), 50 μg/ml (▲), and 100 μg/ml (□). B, Untreated (●); poly (Am) at 10 μg/ml (○), 25 μg/ml (△), 50 μg/ml (▲), and 100 μg/ml (□).*

that the adsorption of murine oncornaviruses is selectively inhibited.

Effects on Sindbis and Sendai virus replication. To explore further the specificity of the effects of the polynucleotides, we tested various concentrations of poly(A) and poly(Am) on Sindbis virus replication in mouse embryo cells. The results of several experiments (Table 2) show that poly(A) at 100 μg/ml or poly(Am) at 10 μg/ml had no inhibitory effect on Sindbis virus multiplication. Poly(Am) at 50 μg/ml had an inhibitory effect, possibly related to the previously described effects on adsorption. Incubation of Sindbis virus in the absence of cells with either polymer at 50 μg/ml had no effect on virus infectivity.

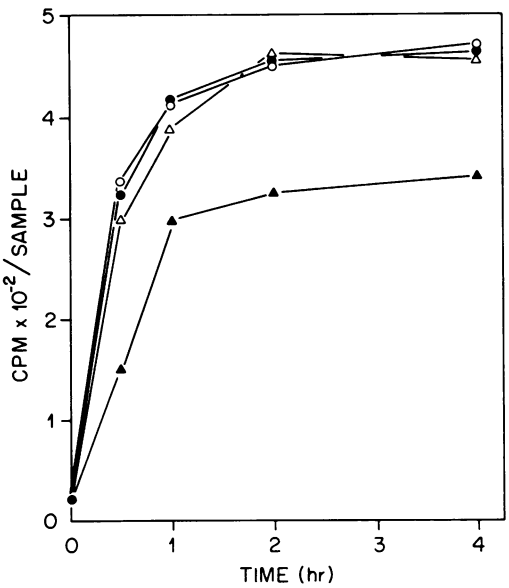

FIG. 6. *Effects of poly(A) and poly(Am) on adsorption of [³H]leucine-labeled Sindbis virus (3,000 counts/min) by Swiss mouse embryo cells. Cells were treated with DEAE-dextran (25 μg/ml) for 1 h, rinsed twice with EMEM, and treated with polynucleotide at various concentrations for 1 h. The cells were then washed twice with EMEM and virus was added. At zero time, 0.5, 1, 2, and 4 h, the cells were processed as described in Materials and Methods. Control (●), poly(A) at 100 μg/ml (○), and poly(Am) at 10 μg/ml (△) and 100 μg/ml (▲).*

No inhibition was observed in the replication of Sendai virus in $LLMK_2$ monkey kidney cells treated with poly(A) at 500 μg/ml (Table 3). Treatment of the cells with poly(Am) at 10 μg/ml resulted in an apparent enhancement of virus replication, and this effect may be related to the stimulation in vitro of Sendai virus RNA polymerase by poly(A) and other polyanions (23).

Effects of time of poly(Am) treatment. We investigated the time dependency of the effects of poly(Am) on MLV replication by adding 10 μg of the polymer per ml 1 h before and at various intervals after infection. The number of cells initiating virus synthesis by 48 h after infection was assayed by the fluorescent antibody technique. The compound was most effective when it was added before or at the time of infection (Fig. 7). However, virus synthesis was inhibited by 75% and by 40 to 50% when poly(Am) was added at 1 or 2 h, respectively, after infection. These results show that the inhibition involves an event in the replication cycle that occurs early after adsorption.

Effects of poly(Am) on cell transformation by (M)MSV. Whereas the results presented above show that poly(Am) inhibits leukemia virus replication, it was also of importance to determine its effect on (M)MSV-induced transformation. (M)MSV-induced focus formation was inhibited significantly by poly(Am) at 5

TABLE 2. *Effects of poly(A) and poly(Am) on Sindbis virus multiplication*[a]

Virus and treatment	PFU/ml			
	Time after infection (h)			
	0	12	24	48
Sindbis	1.5×10^5	1.5×10^6	2.0×10^6	7.0×10^7
Sindbis + 100 μg of poly(A) per ml	6.0×10^5	1.0×10^6	2.0×10^6	5.0×10^7
Sindbis	2.5×10^2	3.8×10^5	1.0×10^7	5.2×10^7
Sindbis + 10 μg of poly(Am) per ml	1.5×10^3	8.0×10^5	9.6×10^6	1.0×10^7
Sindbis + 50 μg of poly(Am) per ml	2.5×10^2	2.2×10^4	2.6×10^6	1.1×10^7

[a] In separate experiments, Swiss mouse embryo cell cultures were treated with DEAE-dextran (25 μg/ml for 1 h) and washed, and medium containing poly(A) or poly(Am) was added for 1 h. Cells were infected by adding one of two lots of Sindbis virus (~10 to 100 PFU/cell) to the medium and allowing 30 min for adsorption, after which the cultures were washed twice and given fresh medium containing the same concentration of polynucleotide. At the indicated intervals medium was collected and frozen. Virus multiplication was assayed by the plaque technique on chicken embryo cell cultures.

TABLE 3. *Effect of poly(A) on Sendai virus replication*[a]

Time (h)	$TCID_{50}$/0.1 ml	
	Sendai	Sendai + 500 μg of poly(A) per ml
0	<2	<2
24	5.8	5.8
48	4.0	≥6.0
72	3.5	5.8
96	3.8	≥6.0

[a] The effects of poly(A) at 500 μg/ml were tested on Sendai virus grown in $LLMK_2$ monkey kidney cells (obtained from Microbiological Associates, Inc.). Prior to infection cells were treated with DEAE-dextran (25 μg/ml) for 1 h and washed twice before addition of poly(A) to one group. After 2 h the cells were infected with Sendai virus (2-h adsorption period), and one group was held in medium containing poly(A). At the indicated intervals cultures were collected and frozen. Virus concentration in the respective samples was assayed in green monkey kidney cell cultures by Sue Cross, Microbiological Associates, Inc., using a 10-day cytopathic end point. Titers are expressed as 50% tissue culture infectious units ($TCID_{50}$).

μg/ml (Table 4), and essentially complete inhibition could be achieved with 10 μg/ml. The inhibition of focus formation was comparable whether poly(Am) was added 1 h before or 2 h after infection (Table 5).

Aaronson and Rowe (1) derived a line of BALB/c 3T3 cells transformed by the Kirsten mouse sarcoma virus which are negative for virus production. These nonproducer cells replicate the sarcoma virus when infected with mouse leukemia virus, and the sarcoma virus can be assayed by overlaying the nonproducing cells with indicator cells and counting the transformed foci that develop. Poly(Am) at 5 μg/ml inhibited focus formation by 50% when it was added 1 h before infection with MLV and the nonproducer cells were held in the presence of poly(Am) until the indicator cells were added.

Effects of poly(A) and poly(Am) on AKR virus activation. Leukemia virus can be activated in normal AKR mouse embryo cells by treatment with iododeoxyuridine (19). We tested the effects of poly(A) and poly(Am) on virus activation in AKR embryo cells by adding the compounds immediately after iododeoxyuridine treatment and maintaining the treatment until the cells were assayed for virus-induced protein. There was no inhibition of induction of virus synthesis in the presence of 100 μg of poly(A) per ml or 10 μg of poly(Am) per ml (Table 6).

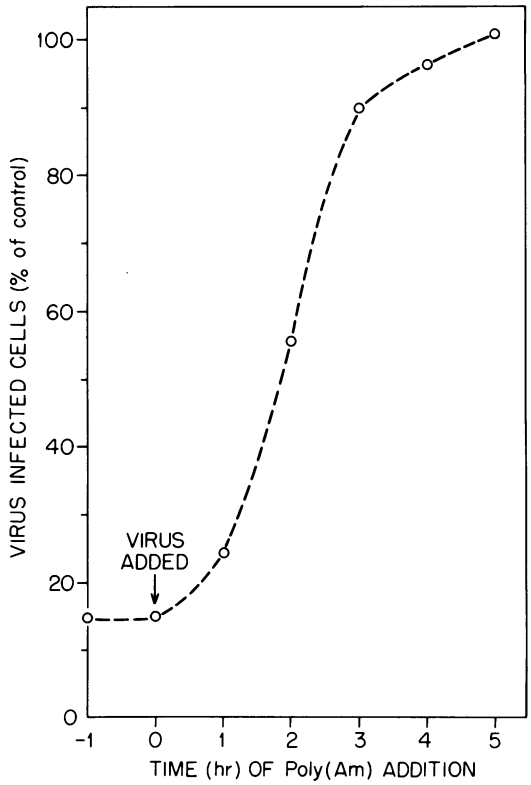

FIG. 7. *Effects of poly(Am) addition at various stages of infection. Secondary Swiss mouse embryo cells were infected with MLV as described in Table 1. Poly(Am) (10 μg/ml) was added to separate culture wells at the times before or after virus infection indicated in the figure. Twenty-four hours after infection the cover slips were collected and stained, and fluorescent cells were counted.*

TABLE 4. *Effect of poly(Am) on cell transformation by (M)MSV*[a]

Poly(Am)	Virus dilution			
	1:20		1:50	
	Mean no. of foci	% of control	Mean no. of foci	% of control
None	115		45	
1 μg/ml	117	100	47	100
5 μg/ml	25	22	9	20
10 μg/ml	5	4	0.6	2
20 μg/ml	3	2.6	0	0

[a] Cells were treated with DEAE-dextran (25 μg/ml) for 1 h and washed twice with PBS. They were then treated with poly(Am) for 1 h and subsequently infected with (M)MSV (~0.5 PFU/cell). After 2 h of incubation the cells were given fresh medium and held for 6 days, after which transformed foci were counted.

TABLE 5. *Effect of poly(Am) treatment sequence on cell transformation by (M)MSV*

Poly(Am) treatment (10 μg/ml)	Mean no. of foci[a]		Inhibition (%)
	Control	Poly(Am)	
1 h before infection[b]	18 ± 2	6 ± 1	67
1 h before and during infection[c]	17 ± 1	6 ± 1	65
2 h after infection[d]	19 ± 2	2 ± 1	90

[a] Mean of three replicates per group plus standard error.

[b] Experiment carried out as in Table 4, except that poly(Am) was removed during infection.

[c] Experiment carried out as in Table 4.

[d] No poly(Am) added until 2 h after infection.

TABLE 6. *Effects of poly(A) and poly(Am) on activation of virus from AKR cells by iododeoxyuridine*[a]

Treatment	Fluorescent cells/total cells	Activation (%)
None	0/≥2000	0
IdUrd	52/1392	3.7
IdUrd + 100 μg of poly(A) per ml	58/1745	3.3
IdUrd + 10 μg of poly(Am) per ml	56/1590	3.5
None	0/≥2000	0
IdUrd	26/315	8.2
IdUrd + 100 μg of poly(A) per ml	24/260	9.2
IdUrd + 10 μg of poly(Am) per ml	23/272	8.5

[a] In the first set of experiments, 2×10^5 to 3×10^5 cells were plated into 35-cm² wells containing duplicate cover slips. Eighteen hours later all wells were incubated with DEAE-dextran (25 μg/ml) for 1 h and washed twice with PBS. The cells were then incubated with control medium or medium containing the appropriate polyribonucleotide. After 2 h, iododeoxyuridine (IdUrd) (100 μg/ml) was added to the appropriate wells and incubated for 24 h. After incubation, all samples were washed twice with PBS and reincubated in fresh medium containing polyribonucleotide where necessary. Forty-eight hours later the medium was removed and the cover slips were fixed in cold (4 C) acetone for 20 min and stored at −20 C until they were stained for the presence of virus-induced proteins. The total number of cells and the number fluorescing were counted with a Zeiss fluorescent microscope system. The second set of experiments was identical to the first, with the exception that the initial inoculum contained only one-third the number of cells plated. All incubations in both sets of experiments were carried out at 37 C.

DISCUSSION

The single-stranded synthetic polyribonucleotides poly(A) and poly(Am) have been reported to be effective inhibitors of the virion reverse transcriptase of murine oncornaviruses (2, 15, 29). The results presented here show that the inhibition of leukemia virus replication in cell culture has two components. Both compounds at concentrations greater than 10 μg/ml inhibit leukemia virus adsorption but have significantly less effect on adsorption of Sindbis virus. Since treatment of cells with DEAE-dextran potentiates both virus adsorption and the inhibitory effects of poly(A) and poly(Am), it is possible that inhibition of virus uptake is related to the anionic nature of these polymers and their competition for virus attachment sites. For example, DEAE-dextran may facilitate adsorption by binding to the cell and providing a highly positively charged site with which virus interacts ionically. The ability of the polyribonucleotides to block adsorption could thus be explained by charge neutralization or masking of these sites. This conclusion is also suggested by the observation that poly(vinyladenine), which is uncharged (15), at 100 μg/ml did not inhibit leukemia virus adsorption (data not shown). Since inhibition of virus uptake by charged compounds is apparently a selective effect on oncornavirus adsorption, this may provide a useful approach for exploring the mechanism of infection.

The second component of the polynucleotide inhibition appears to be related to an effect on an early viral function. First, poly(Am) is an effective inhibitor of virus replication and transformation at concentrations which have no effect on virus adsorption. Pitha et al. (15) also reported an inhibitory effect of poly(A) on MLV replication in mouse embryo cells. They report that poly(A) is able to inhibit virus replication at concentrations as low as 10 μg/ml. We observed no inhibition at that concentration, but this may be due to differences in the source or molecular weight of our polymer. The greater effectiveness of poly(Am) is most probably related to the strong nuclease resistance conferred by methylation (18). Second, poly(A) and poly(Am) are only effective when added within 4 h after infection. This observation is compatible with results reported by others (4, 5) on the apparent formation of a DNA intermediate catalyzed by reverse transcriptase and required for virus replication. The evidence for the role of the virion reverse transcriptase in this process has been reviewed (11, 24) and will not be reiterated. Third, the effects of poly(A) and poly(Am) are apparently selective for the murine oncornaviruses. The replication of Sen-

dai and Sindbis viruses, which are nononcogenic RNA viruses, was unaffected by concentrations of the polynucleotides which inhibited mouse leukemia virus. Pitha et al. (15) reported similar results for vesicular stomatitis virus and Sindbis virus. Thus, a nonspecific effect of poly(A) and poly(Am), such as induction of interferon, is apparently excluded. Single-stranded polynucleotides have been shown to be poor interferon inducers (8), and the action of interferon, which is not virus specific, would be expected to be equally effective against Sindbis and Sendai viruses. Some other nonspecific effect of treatment with polynucleotides, such as activation of a cell RNase, are also possible but would be expected to also effect the replication of other RNA viruses such as Sindbis. The evidence indicates, then, that the inhibition of murine oncornavirus replication in cell cultures by low concentrations of polyribonucleotides and by postinfection treatment with the polynucleotides is due to the inhibition of viral reverse transcriptase.

The experiments presented here confirm the results obtained with rifampin derivatives and streptovaricins (6, 7, 9, 16, 17, 27, 30), which indicate an obligate role for DNA polymerase in the replication of exogenous murine oncornavirus. The inhibitory effect of poly(Am) on transformation of mouse embryo cells by (M)MSV also suggests a requirement for reverse transcriptase function. However, (M)MSV is defective and requires leukemia virus to provide a helper function for sarcoma virus replication (13). Since (M)MSV replication and spread perpetuate the focus of transformed cells, the role of reverse transcriptase may be involved mainly in providing the helper virus function. Although the effect of poly(Am) may be principally related to inhibition of helper virus replication, the initial (M)MSV inoculum contains virus competent to induce transformation (10, 22). The size of the transformed foci which arise from 3T3 cells would depend mainly on cell proliferation and would be expected to develop at a slower rate. Since we observed no significant difference in the size of the new foci in the treated cultures, it appears that poly(Am) inhibits the number of initial foci formed by the (M)MSV component of the inoculum. Also, although poly(A) inhibited the rescue of (M)MSV from transformed nonproducer cells, we saw no effect on the transformed characteristics of the cells; this suggests that reverse transcriptase function is not required for maintenance of the transformed state.

Finally, poly(A) or poly(Am) treatment had no effect on the activation of endogenous virus in AKR mouse embryo cells. It appears, therefore, that reverse transcriptase is not required for the process of activation of virus by iododeoxyuridine. The role of reverse transcriptase in the biology of the murine oncornaviruses thus appears to involve principally the activity of exogenous leukemia and sarcoma viruses, which obligately catalyze the formation of a DNA intermediate or provirus (3, 24) required for the expression of viral functions. Since virus activation proceeds in the presence of polynucleotides, it appears that the endogenous virus exists in cells as a stable DNA intermediate (31).

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