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Inhibition of Adenovirus DNA Replication by Vesicular Stomatitis Virus Leader RNA

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Vesicular stomatitis virus (VSV) leader RNA and a synthetic oligodeoxynucleotide of the same sequence were found to inhibit the replication of adenovirus DNA in vitro. In contrast, the small RNA transcribed by the VSV defective interfering particle DI-011 did not prevent adenovirus DNA replication. The inhibition produced by leader RNA was at the level of preterminal protein (pTP)-dCMP complex formation, the initiation step of adenovirus DNA replication. Initiation requires the adenovirus pTP-adenovirus DNA polymerase complex (pTP-Adpol), the adenovirus DNA-binding protein, and nuclear factor I. Specific replication in the presence of leader RNA was restored when the concentration of adenovirus-infected or uninfected nuclear extract was increased or by the addition of purified pTP-Adpol or HeLa cell DNA polymerase α-primase to inhibited replication reactions. Furthermore, the activities of both purified DNA polymerases could be inhibited by the leader sequence. These results suggest that VSV leader RNA is the viral agent responsible for inhibition of adenovirus and possibly cellular DNA replication during VSV infection.

The highly virulent, negative-stranded RNA virus, vesicular stomatitis virus (VSV), rapidly shuts off host cellular DNA, RNA, and protein synthesis (for a review, see reference 30). Early experiments (32, 33) showed that VSV temperature-sensitive mutants, defective in transcription at the nonpermissive temperature, do not inhibit host cell nucleic acid synthesis. VSV particles, sufficiently UV irradiated (>150,000 ergs/mm^2) to prevent detectable RNA transcription, were also reduced in their ability to inhibit host cell nucleic acid synthesis (31). These results point to the conclusion that viral transcription is required for host cell shutoff to occur.

Transcription of the VSV genome begins at its 3′ terminus, initially producing a 47-nucleotide transcript called leader RNA. The viral RNA-dependent RNA polymerase then proceeds along the genome, sequentially synthesizing each of the five monocistronic mRNAs (1). UV targeting experiments demonstrated that the relative abundance of each individual transcript is directly proportional to the gene proximity to the 3′ terminus of the viral genome (13, 14). These studies demonstrated that the viral RNA polymerase does not reinitiate within the genome and confirmed a stop-start model for VSV transcription.

UV inactivation studies, similar to those which exposed the sequential and attenuated nature of VSV transcription, identified leader RNA as the viral product most likely responsible for the inhibition of host cellular RNA synthesis (31). UV irradiation of VSV virions before infection at intensities sufficient to preclude synthesis of detectable levels of viral mRNA (12,000 ergs/mm^2) did not prevent shutoff. The length of viral transcripts synthesized at this level of UV irradiation was determined to be no greater than 150 nucleotides (9, 22). Only extremely high doses of UV irradiation sufficient to prevent all transcription or heating of the infecting virions to 50°C to inactivate the RNA-dependent RNA polymerase overcame shutoff.

The ability of VSV leader RNA to inhibit transcription has also been examined in vitro (9–11, 21). Transcription from the adenovirus early and late promoters, the simian virus 40 late promoter, and the adenovirus virus-associated (VA) genes were all inhibited to various degrees by VSV leader RNA. Defective interfering particle (DI) leader RNA at comparable concentrations had no effect. Studies that compared the VSV leader RNAs isolated from serotypes Indiana and New Jersey showed an exact correlation of their in vitro with their in vivo abilities to inhibit transcription. Additionally, the New Jersey leader RNA, the better inhibitor of transcription of the two, was present in greater numbers within infected cells.

Little has been shown of the direct effects of VSV on eucaryotic DNA synthesis, despite the fact that both RNA and DNA synthesis are drastically and rapidly inhibited in infected cells. McGowan and Wagner (22) examined the effect of VSV infection on MPC-11 and L-cell macromolecular synthesis in tissue culture. A comparison of the kinetics of inhibition of DNA, RNA, and protein synthesis of infected cells showed that the rates and extent of shutoff of DNA and RNA synthesis were almost superimposable, whereas inhibition of host protein synthesis was delayed. These data implied that VSV directly inhibits both DNA and RNA synthesis of infected cells or a parameter common to each.

To investigate the mechanism by which VSV shuts off eucaryotic DNA replication, we used an in vitro DNA replication system. Presently, there are no in vitro DNA replication systems which use eucaryotic origins. Consequently, we used the adenovirus in vitro DNA replication system (3) because it has been clearly defined and because VSV shuts off adenovirus replication in vivo (Remenick and McGowan, unpublished observations). In addition, adenovirus DNA replication does not require concomitant RNA synthesis for primer formation (8). Thus, the effects of VSV on DNA synthesis can be determined directly and not

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indirectly as a consequence of the inhibition of RNA synthesis.

VSV leader RNA was observed to inhibit adenovirus DNA replication in vitro. No other RNA tested produced inhibition at comparable concentrations. A synthetic oligodeoxynucleotide of the sequence of leader also inhibited adenovirus DNA replication. Inhibition was partially overcome by additional adenovirus-infected extract, uninfected extract, preterminal protein-adenovirus DNA polymerase complex (pTP-Adpol), or HeLa cell DNA polymerase α-primase. The polymerase activities of DNA polymerase α and Adpol were also inhibited by the leader sequence. The relationship of these results to the process of VSV infection in vivo is discussed.

MATERIALS AND METHODS

Growth and preparation of viruses. Adenovirus serotype 2 (Ad2) was propagated in suspension cultures of HeLa cells (3). Wild-type (wt) VSV (serotype Indiana) and VSV DI particle, type 011, were grown in BHK-21 cells from stocks which had been plaque purified (6). Both virus stocks were suspended at final concentrations of 2 mg/ml (10^8 to 10^{10} PFU/ml) and stored at −80°C until use.

Preparation of adenovirus-infected and uninfected HeLa cell nuclear extracts. The replication extracts were prepared (3) to final protein concentrations of 20 mg/ml. The uninfected extract was prepared in the same manner as the infected extract without the addition of virus.

In vitro transcription of VSV and isolation of wt and DI-011 leader RNA. VSV was transcribed in vitro in the presence of [α-^{32}P]UTP (specific activity of 3,000 Ci/mmol), and the plus-stranded transcription products were isolated (21). This RNA was electrophoresed through a 20% polyacrylamide, 8 M urea gel. Upon autoradiography, the position of leader RNA was determined. The gel slice containing leader RNA was removed, and the RNA was eluted by incubation at 37°C in elution buffer [500 mM NH_4CH_3COOH, 10 mM $Mg(CH_3COOH)_2$, 0.5% sodium dodecyl sulfate, 1 mM disodium EDTA] for 24 h (20). The eluate was passed through a membrane filter (pore size, 0.22 μm; Millipore Corp.), ethanol precipitated, suspended in sterile water, and quantitated spectrophotometrically. VSV mRNA was isolated from total infected HeLa cellular RNA after passage over a poly(dT) column. Torula yeast RNA was purchased from Sigma Biochemicals, Inc., as purified total cellular RNA.

Preparation of the adenovirus DNA-terminal protein template. Ad2 DNA-protein template (Ad2 DNA-prot) was isolated as previously described (15).

Adenovirus replication in adenovirus-infected nuclear extract. DNA replication in vitro was carried out as described by Challberg and Kelly (3). Briefly, the adenovirus-infected HeLa cell nuclear extract (40 μg) was incubated at 31°C for 60 min with 35 ng of *Hin*dIII-cleaved (except where noted) Ad2 DNA-prot; replication mix (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-sodium hydroxide, pH 7.5; 4 mM dithiothreitol; 3 mM ATP; 5 mM $MgCl_2$; 20 mM NaCl; 0.02% bovine serum albumin); 50 μM each dATP, dGTP, and dTTP; 50 nM dCTP; 3 nM [α-^{32}P]dCTP (specific activity of 3,000 Ci/mmol); and exogenous nucleic acid as specified in a reaction volume of 20 μl. The mixture was digested with 100 μg of proteinase K plus 1% sodium dodecyl sulfate at 37°C for 120 min and ethanol precipitated. The pellet was suspended and electrophoresed through an 0.8% agarose gel. The gel was dried and autoradiographed. The amount of replication which occurred was quantitated two ways. First, the autoradiograph was scanned with an Ortec densitometer, and the peaks produced on graph paper by the two terminal adenovirus *Hin*dIII fragments were quantitated by weighing. Second, the two terminal adenovirus *Hin*dIII fragments were removed from the gel and Cerenkov counted. The results from each method were averaged.

Adenovirus DNA replication by using purified proteins. Replication of Ad2 DNA-prot with purified proteins has been described previously (12, 23). Reaction volumes of 20 μl contained replication mix; 40 μM each dATP, dGTP, and dTTP; 4 μM [α-^{32}P]dCTP (200 cpm/pmol); and, as indicated, Ad2 DNA-prot, pTP-Adpol complex, adenovirus DNA-binding protein (DBP), and nuclear factor I. Assays were incubated for 60 min at 30°C. The amount of dCMP incorporated was determined by measurement of trichloroacetic acid-precipitable radioactivity.

Assay for pTP-dCMP complex formation. The assay conditions for pTP-dCMP complex formation have been described previously (23). The conditions employed were the same as those described for adenovirus DNA replication by using purified proteins except that dATP, dGTP, and dTTP were omitted and 0.5 μM [α-^{32}P]dCTP (1,000 cpm/fmol) was included. Products were isolated and analyzed as previously described (19).

Purification of the proteins required for adenovirus DNA replication. The pTP-Adpol complex and the adenovirus DBP were purified as previously described (7, 18). Some of the pTP-Adpol and adenovirus DBP used in these studies were provided by J. Ostrove of the National Institutes of Health, Bethesda, Md. HeLa cell nuclear factor I was purified as previously described (24). Some of the nuclear factor I was a gift from P. Rosenfeld and T. Kelly of the Johns Hopkins University, Baltimore, Md. HeLa cell DNA polymerase α-primase complex, purified by immunoaffinity chromatography, was a gift of L. Weissbachand and J. Hurwitz.

DNA polymerase assays. In vitro DNA polymerase assays measured the conversion of [^{3}H]dTTP into acid-insoluble product (27). Reaction mixtures (50 μl) contained 50 mM Tris hydrochloride (pH 8); 7.5 mM $MgCl_2$; 4 mM dithiothreitol; 400 μg of bovine serum albumin per ml; 40 μM each dGTP, dCTP, and dATP; 4 μM [^{3}H]dTTP (7,500 cpm/pmol); 0.003 U of purified enzyme; 200 μg of activated calf thymus DNA per ml; and synthetic oligodeoxynucleotide as specified. Reaction mixtures were incubated at 30°C for 60 min. Acid-insoluble radioactivity was determined by scintillation counting.

Synthetic oligodeoxynucleotides. Synthetic oligodeoxynucleotides J8 and J10 were kindly provided by J. Zon of the National Institutes of Health, Bethesda, Md. Each was high-performance liquid chromatography purified and sequenced (26).

RESULTS

VSV leader RNA inhibits adenovirus DNA replication in vitro. The adenovirus genome consists of a 36-kilobase-pair linear, duplex DNA genome which is covalently linked at each 5' terminus to a 55-kilodalton terminal protein. Replication initiates from either end by protein priming and proceeds to the opposite terminus by a strand displacement mechanism. *Hin*dIII digestion of the genome yields 13 fragments. The terminal fragments of 2.7 and 1.0 kilobase pairs represent 12% of the genome by weight. Replication of *Hin*dIII-digested Ad2 DNA-prot by adenovirus-infected

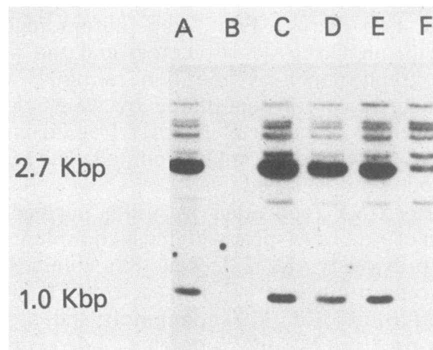

FIG. 1. The effect of various RNAs on adenovirus in vitro DNA replication. Adenovirus replication was performed in adenovirus-infected nuclear extract as described in Materials and Methods. The products were electrophoresed through a 0.8% gel and autoradiographed. All reactions contained 35 ng of *Hin*dIII-digested Ad2 DNA-prot complex and 75 ng (1.5 ng/μl) of the indicated RNA (except lanes A and F). Lanes: A, no RNA; B, wt VSV leader RNA; C, small RNA made by VSV DI particle DI-011 (DI leader RNA); D, oligo(dT)-selected VSV mRNA; E, yeast RNA; F, proteinase K-treated adenovirus DNA as template, with no RNA. Kbp, Kilobase pairs.

HeLa cell nuclear extract resulted in the specific labeling of the terminal fragments (Fig. 1). These two fragments contained 95% of total incorporated isotope. In contrast, proteinase K-treated Ad2 DNA-prot only supported low levels of nonspecific incorporation (Fig. 1, lane F). Purified RNAs were tested for their ability to block the specific replication of the Ad2 DNA-prot template. wt leader RNA inhibited replication, whereas the small RNA made by the VSV DI particle DI-011 (DI leader RNA), VSV mRNA, and yeast RNA had little effect (Fig. 1, lanes B through E). Interestingly, wt leader RNA also reduced the levels of nonspecific DNA synthesis. As shown in Table 1, wt leader RNA caused 95% inhibition of the specific replication of the terminal fragments at a concentration of 1.5 ng/μl (96 nM). DI leader RNA, VSV mRNA, and yeast RNA did not inhibit adenovirus DNA synthesis even at concentrations up to 10 times higher than that of wt leader RNA.

Inhibition of adenovirus DNA replication by synthetic oligodeoxynucleotides. Synthetic oligodeoxynucleotides J8 and J10 (Fig. 2A), containing the sequences of wt and DI leader RNA, respectively, were tested for their ability to inhibit adenovirus DNA replication. J8 was as effective as wt leader RNA in producing inhibition of adenovirus DNA replication (Table 2). J10, like DI leader RNA, did not significantly affect adenovirus DNA synthesis at any concentration tested.

TABLE 1. Effect of purified RNAs on adenovirus DNA replication[a]

RNA added	Concn		Amt of adenovirus DNA synthesis[b]
	ng/μl	nM	
None			100
wt leader	0.5	31	64
	1.5	96	5
	5.0	310	1
DI leader	1.5	97	106
	5.0	323	96
	15.0	970	99
VSV poly(A)	1.5		89
	5.0		106
	15.0		104
Yeast	1.5		100
	5.0		127
	15.0		124

[a] Shown is the ability of various RNAs to inhibit adenovirus in vitro DNA replication. Replication assays were carried out and quantitated as described in Materials and Methods.

[b] Percentage of replication which occurred in the absence of exogenous RNA.

The stability of RNAs and oligodeoxynucleotides in the adenovirus DNA replication assay. The stability of wt leader RNA, DI leader RNA, J8, and J10 under in vitro replication assay conditions was examined. The stability of each was determined from aliquots which were removed at 20-min intervals, electrophoresed on a denaturing polyacrylamide gel, and quantitated by densitometric scans of the resulting autoradiogram. After 1 h of incubation, 64% of wt leader remained intact, compared with only 19% of DI leader (Fig. 3). The selective stability of wt leader RNA compared with DI leader RNA may be why only wt leader RNA inhibits adenovirus DNA synthesis. If so, the same difference of stability should be observed between J8 and J10. Both however, were equally stable (>90%), indicating that the

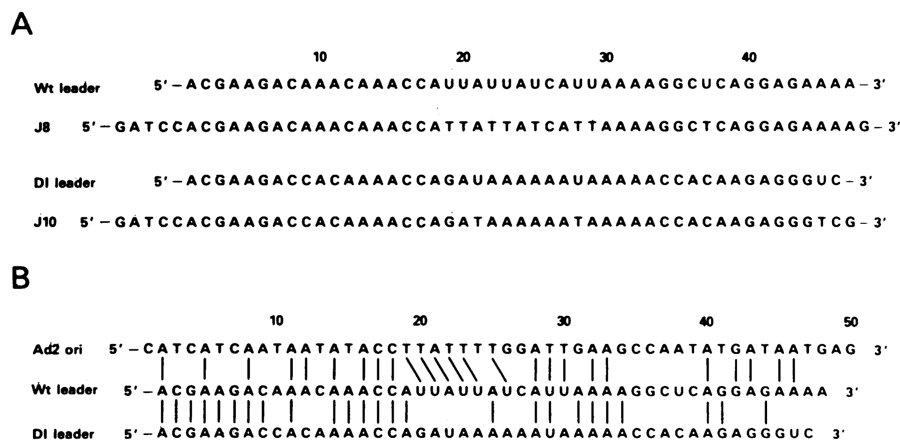

FIG. 2. Nucleotide sequences of VSV leader RNA (4), DI-011 leader RNA (28), J8 and J10 oligodeoxynucleotides, and the Ad2 replication origin (29). (A) The nucleotide sequences of wt VSV leader RNA and DI-011 leader RNA are shown along with the corresponding synthetic oligodeoxynucleotides, J8 and J10. (B) The nucleotide sequences of the Ad2 DNA replication origin, VSV leader RNA, and DI-011 leader RNA are compared. Regions of homology are indicated with vertical or diagonal lines.

TABLE 2. Effect of synthetic oligodeoxynucleotides on adenovirus DNA replication[a]

Synthetic oligodeoxynucleotide added	Concn		Amt of adenovirus DNA synthesis[b]
	ng/μl	nM	
None			100
J8	0.75	42	41
	1.5	84	7
	5.0	280	1
J10	0.75	43	104
	1.5	85	110
	5.0	285	109

[a] Shown is the ability of two synthetic oligodeoxynucleotides to inhibit adenovirus DNA replication in adenovirus-infected nuclear extracts. J8 contains the sequence of wt VSV leader RNA, and J10 contains the sequence of DI-011 leader RNA (Fig. 2A). Replication assays were carried out as described in Materials and Methods.

[b] Percentage of replication which occurred in the absence of added RNA.

inhibition of adenovirus in vitro DNA replication by leader RNA is not due to the greater stability of wt leader RNA in the assay. The greater stability of wt leader RNA in the extract may be produced by the formation of a specific RNA-protein complex which protects the RNA from nucleases present in the extract.

Inhibition of the initiation of adenovirus replication by J8. The initiation of adenovirus DNA replication occurs by the covalent attachment of the 80-kilodalton adenovirus pTP with dCMP, the 5′-terminal nucleotide of the adenovirus DNA genome. Lichy et al. (19) developed an assay to measure the initiation (pTP-dCMP complex formation) of adenovirus DNA replication in vitro. Ad2 DNA-prot was incubated with the pTP-Adpol complex, the adenovirus DBP, and nuclear factor I in the presence of [α-^{32}P]dCTP.

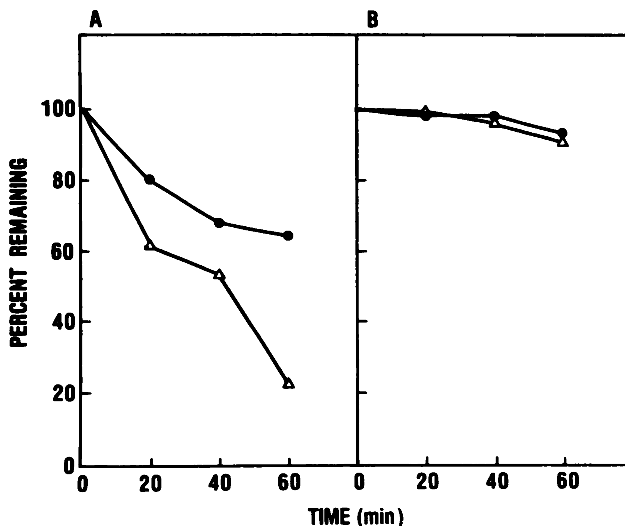

FIG. 3. The stability of VSV RNAs and oligodeoxynucleotides in the adenovirus DNA replication assay. Shown are comparisons of the stabilities of (A) 2 ng each of ^{32}P-labeled wt VSV leader RNA (●) with DI leader RNA (△) and (B) 1 ng each of ^{32}P-labeled J8 (●) with J10 (△) in adenovirus-infected nuclear extract under replication assay conditions. Reactions were performed as described in Materials and Methods without the addition of free radionucleotide. Aliquots were removed at 20-min intervals and electrophoresed on an 8 M urea–20% polyacrylamide gel, and the amount of full-length nucleic acid remaining was determined by densitometrically scanning the resulting autoradiogram.

After incubation, pTP-dCMP complex formation was assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of reaction mixtures. Figure 4 shows that formation of the radiolabeled, 80-kilodalton pTP-dCMP complex is dependent on the presence of nuclear factor I. J8 inhibited pTP-dCMP complex formation by 90%. In contrast, J10 had no effect on the initiation of adenovirus DNA replication.

Reconstitution of adenovirus DNA replication in the presence of wt leader RNA with HeLa cell extracts. Previous reports (10, 21, 22, 33) have focused on the ability of VSV leader RNA to inhibit nucleic acid synthesis. Reconstitution of an inhibited assay, however, is essential to identify the factor targeted by leader RNA which is responsible for inhibition.

Reconstitution of VSV leader RNA-inhibited adenovirus DNA replication was attempted by the addition of increasing amounts of adenovirus-infected and uninfected HeLa cell nuclear extract (Table 3). The addition of more adenovirus-infected or uninfected extract partially overcame the inhibitory effects of leader RNA.

The known host cell proteins required for adenovirus DNA replication are HeLa cell nuclear factors I and II. The addition of a 25-fold excess of purified nuclear factor I over inhibitor concentration, however, did not restore adenovirus DNA replication (Table 3). The preparation of nuclear factor I was determined to be active by its ability to stimulate pTP-dCMP complex formation in vitro. Nuclear factor II is not required for this assay (8).

Inhibition and reconstitution of adenovirus DNA replication in vitro by using purified proteins. Adenovirus DNA replication was performed in vitro with purified proteins. Ad2 DNA-prot was incubated with the pTP-Adpol complex, the

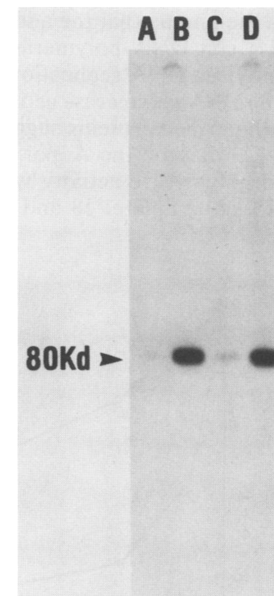

FIG. 4. The effect of VSV oligodeoxynucleotides on pTP-dCMP complex formation. pTP-dCMP complex formation reactions were performed as described in Materials and Methods with the following modifications: lane A, in the absence of nuclear factor I; lane B, complete; lane C, complete plus 1.5 ng of J8 per μl; and lane D, complete plus 1.5 ng of J10 per μl. The complete reaction contained Ad2 DNA-prot (300 pmol as nucleotides), pTP-Adpol complex (0.005 U), adenovirus DBP (1.0 μg), and nuclear factor I (0.01 μg). Reaction products were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. 80 Kd, Apparent molecular mass in kilodaltons.

TABLE 3. Reconstitution of VSV leader RNA-inhibited adenovirus DNA replication reactions

Addition(s) to extract[a]	Amt of replication (%)
None	100
wt RNA	6
wt RNA + infected extract (μg)	
20	33
40	75
wt RNA + uninfected extract (μg)	
20	21
40	65
wt RNA + nuclear factor I (μM)	
1.0	7
2.5	9

[a] Adenovirus DNA replication was performed in adenovirus-infected nuclear extract (40 μg) as described in Materials and Methods. A moderate level (96 nM) of wt leader RNA (wt RNA) was added to produce a significant degree of inhibition (94%) while allowing for the possibility of reconstitution of replication with reasonable amounts of additional adenovirus-infected nuclear extract (infected extract).

adenovirus DBP, and nuclear factor I (Table 4). DNA synthesis was specific for adenovirus DNA replication, as can be seen by its dependence on nuclear factor I. Consistent with previous observations using crude extracts, titration of J8 into replication reactions by using purified proteins resulted in nearly complete inhibition of adenovirus DNA synthesis. At similar concentrations, J10 had only a small inhibitory effect.

Inhibition of adenovirus DNA replication and pTP-dCMP complex formation by J8 suggested that a possible target of inhibition may be the pTP-Adpol complex. Consistent with this possibility was the finding that the addition of either the pTP-Adpol or HeLa cell DNA polymerase α-primase partially restored adenovirus DNA replication (Table 4).

The effects of J8 on DNA polymerase activities in vitro. The results obtained with purified proteins suggest that the target of J8 (or leader RNA) may be the Adpol. The effect of the leader sequence on polymerase activity was tested directly with activated DNA as template. J8 and J10 were titrated into reaction mixtures containing pTP-Adpol or HeLa cell DNA polymerase α-primase (Fig. 5). J8 inhibited the polymerase activity of pTP-Adpol complex by 50% at approximately 70 nM. J10 also had an inhibitory affect on Adpol activity but required six- to sevenfold higher concentrations of oligodeoxynucleotide to achieve similar levels of inhibition. The effect of the VSV leader sequence on host DNA polymerase activity was more dramatic. J8 at 30 nM inhibited DNA polymerase α activity by 50%, whereas J10 had only a slight inhibitory effect even at the highest concentration tested. J8 and J10 had no effect on the activity of *Escherichia coli* DNA polymerase I (data not shown).

TABLE 4. Adenovirus DNA replication by using purified proteins[a]

Addition(s) to reaction mixture[b]	dCMP incorporated (pmol)	% of replication
None	9.4	100
J8 (nM)		
140	5.6	60
420	4.0	43
700	1.8	19
J10 (nM)		
143	10.2	109
429	8.7	93
715	6.6	70
J8 (700 nM) + pTP-Adpol (0.05 U)	13.4	143
J8 (700 nM) + HeLa pol (0.8 U)	6.4	68
HeLa pol (0.8 U)	7.8	83

[a] Adenovirus DNA replication reactions were performed as described in Materials and Methods, using purified proteins.

[b] All reactions contained Ad2 DNA-prot (60 ng), pTP-Adpol complex (0.05 U), adenovirus DBP (1.1 μg) and nuclear factor I (50 ng). HeLa pol, HeLa DNA polymerase α-primase.

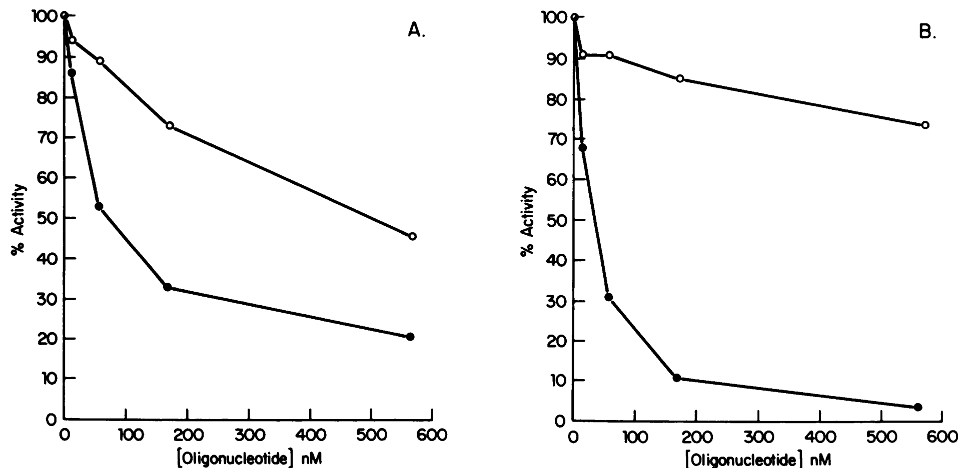

FIG. 5. Effect of VSV oligodeoxynucleotides on DNA polymerase activities. DNA polymerase activity was assayed with activated DNA as a template as described in Materials and Methods. pTP-Adpol complex (A) and HeLa cell DNA polymerasel α-primase complex (B) were assayed in the presence of increasing amounts of the synthetic oligodeoxynucleotide J8 (●) or J10 (○). The amount of polymerase activity is expressed as the percentage of incorporation which occurred in the absence of added oligodeoxynucleotide, with 100% equal to 1.8 (by pTP-Adpol) or 3.0 (by HeLa polymerase α-primase) pmol of dTMP incorporated in 60 min at 30°C.

DISCUSSION

Death of VSV-infected cells is preceded by the rapid inhibition of host cellular DNA, RNA, and protein synthesis (30). Presumably, this inhibition is the reason for the ultimate destruction of the host cell, although a direct cause-and-effect relationship has yet to be demonstrated. Previous experiments have shown that VSV inhibits RNA synthesis in vivo and in vitro and protein synthesis in vivo (2, 9, 10, 21, 33). Despite these data, the precise mechanism of inhibition has remained elusive and it has been unclear whether upon infection VSV directly inhibits eucaryotic DNA synthesis or whether the inhibition observed is the indirect consequence of the shutoff of RNA and protein synthesis.

VSV has no nuclear function, but leader RNA accumulates in the nucleus of cells shortly after infection (17). At 2 h postinfection, when the most significant inhibition of nucleic acid synthesis takes place, there occurs the largest accumulation of leader RNA molecules within the nucleus. In more recent studies, the 3′-terminal noncoding regions of VSV genomes isolated from two independent, persistently infected L-cell populations were sequenced and found to contain single-base mutations in the regions which code for leader RNA (34). Leader RNA failed to accumulate rapidly in the nucleus of these cells, as would be expected with wt leader RNA, and the fast rate of inhibition of cellular DNA synthesis was not observed. Similarly, in cultures of VSV-infected *Drosophila melanogaster* cells, wt leader RNA was not transported to the nucleus and host cell shutoff did not take place (5). Indirect evidence, however, is not conclusive. Here we present the first direct evidence for the ability of VSV leader RNA to inhibit DNA synthesis.

In our study, wt VSV leader RNA inhibited the replication of the adenovirus DNA genome in vitro. Other RNAs tested, including DI leader RNA which is 61% homologous in sequence to wt leader RNA, did not produce detectable inhibition at comparable concentrations. Synthetic oligodeoxynucleotides of the same sequence as wt or DI leader RNA produced identical results. The wt leader oligonucleotide inhibited the initiation of adenovirus DNA replication, which requires the pTP-Adpol complex, the adenovirus DBP, and nuclear factor I. The addition of purified pTP-Adpol complex or HeLa cell DNA polymerase α-primase to inhibited replication reactions restored adenovirus DNA synthesis, whereas additional nuclear factor I had no effect. Furthermore, the leader sequence inhibited the activities of both purified DNA polymerases in vitro. These findings suggest that the target of leader RNA in our assay is the pTP-Adpol complex. The striking homology between wt VSV leader RNA and the 5′-terminal sequence of Ad2 (Fig. 2B) suggests that leader RNA may associate with a protein or proteins which also recognize the terminal sequence of the adenovirus genome. This region of the genome is believed to be recognized by the pTP-Adpol complex and nuclear factor I (24, 25), and is highly conserved among many strains of adenovirus (29). It is well established that leader RNA does bind to at least one host cell factor, la protein (16). Although the function of la protein is as yet unknown, it is interesting that anti-la antibodies also inhibit adenovirus DNA replication in vitro (8).

VSV leader RNA-induced inhibition of adenovirus DNA replication was overcome by the addition of increased amounts of uninfected HeLa cell nuclear extract. The inhibition of HeLa DNA polymerase activity (Fig. 5) implies that leader RNA may bind to the HeLa DNA polymerase α, supplied by the additional extract, thereby allowing the viral DNA polymerase to initiate and complete adenovirus DNA replication. Gel retardation experiments have shown that the leader sequence specifically associates with at least one protein present in immunopurified HeLa DNA polymerase α-primase complex (J. Remenick and J. J. McGowan, unpublished observations).

Our results imply that the in vivo target of leader RNA may be the host cell DNA polymerase α-primase complex. If so, the nearly identical kinetics of inhibition of DNA and RNA synthesis observed in VSV-infected cells (22) would represent the direct effect of VSV on each cellular process. VSV may take advantage of a similarity which may exist between DNA and RNA polymerases, inhibiting both upon infection.

To more assiduously analyze shutoff, perhaps the inhibitory ability of leader RNA could be mutated without interfering with its essential functions to the virus. The first 18 nucleotides of leader RNA are highly homologous between wt and DI leader RNAs (Fig. 2B) and most likely essential for polymerase recognition of the viral genome and packaging of newly formed virus particles (30). Therefore, mutational analysis should focus on the central and 3′-terminal regions of wt leader RNA. The ability of oligodeoxynucleotides to substitute for VSV leader RNA should facilitate these studies. Such future experiments would also provide additional data on the nature of persistence in VSV-infected cells. Similar studies with small oligodeoxynucleotides representing portions of wt leader RNA identified the AU-rich sequence (nucleotides 18 to 24) as the region required for inhibition of in vitro transcription and concluded that the presence of additional flanking sequences enhanced this ability (11). However, it should be noted that the inhibitory properties of the RNA and its oligodeoxynucleotide analog, although apparently identical, may act through different mechanisms.

Finally, the inhibitory properties of VSV leader RNA must be examined in vivo. This can be accomplished through the use of an expression vector that produces leader RNA in the absence of VSV infection. Analysis of protein and nucleic acid synthesis in cells containing this vector should answer definitively whether VSV leader RNA is the viral agent responsible for shutoff of host cell macromolecular synthesis.

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