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Inactivation of Influenza and Vesicular Stomatitis Virion RNA Polymerase Activities by Photoreaction with 4'-Substituted Psoralens

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Irradiation of purified influenza virus and vesicular stomatitis virus (VSV) with long-wavelength UV light in the presence of 4'-substituted psoralens inactivated the virion-associated RNA polymerase activity. Inactivation was apparently due to psoralen modification of the viral genome RNAs, since cations that decrease psoralen binding to nucleic acids had a protective effect, and reconstitution of VSV RNA polymerase activity was inhibited by photoreaction of nucleoprotein cores but not by pretreatment of soluble fraction from dissociated virions. Partially inactivated viral particles synthesized reduced amounts of full-length RNA products in vitro without an increase in prematurely terminated transcripts. VSV leader RNA formation was relatively resistant to psoralen photoinactivation, and sequential transcription was maintained by photoreacted VSV. The all-or-none psoralen effect on virion-associated RNA polymerase activities may be due to a differential photosensitivity of promoter sites or to structural changes in modified viral genome RNAs that prevent formation of new mRNA chains.

Psoralens photoreact with nucleic acids upon irradiation with long-wavelength UV light, forming covalent monoadducts with pyrimidines and also cross-links in base-paired regions of polynucleotides (7, 14, 16, 18). As a consequence, both DNA and RNA viruses are inactivated by psoralen photoreaction (11, 12, 19-21). The potential importance of this experimental approach for studying viral genome structures in situ, as well as its practical application for vaccine production, is evident.

Previously it was shown that the inactivation of reovirus type 3 plaque-forming ability by UV irradiation with 4'-substituted psoralens was accompanied by, and possibly due to, loss of the virion-associated RNA polymerase activity (21). This enzyme transcribes one strand of each of the 10 double-stranded genome RNA segments of the virus to form the viral mRNA's (15). Reovirus particles that were partially photoinactivated synthesized reduced amounts of full-length transcripts corresponding to the large, medium, and small size classes of mRNA. Surprisingly, no prematurely terminated mRNA products were observed, and the same relative proportions of the three size classes of mRNA were produced by particles that were either slightly or highly modified (20a). The results suggested that the presence of a single covalently bound psoralen molecule prevented initiation of mRNA synthesis on the altered genome segment, whereas unmodified segments continued to function as templates for the reovirion RNA polymerase.

RNA polymerase activities are also present in purified influenza virus (6) and vesicular stomatitis virus (VSV) (3). The single-stranded genome RNA of influenza virus, like that of reovirus, consists of multiple segments that are independently transcribed to form the complementary RNAs (24, 27, 28). By contrast, the VSV genome is a single high-molecular-weight RNA molecule that is transcribed in a polar fashion by the particle-associated polymerase (1, 2). Transcription starts preferentially at the 3'-end of the VSV genome with the formation of a short leader RNA, followed by the sequential synthesis of the five viral mRNA's. These strikingly different mechanisms of influenza (24) and VSV transcription (1, 2) were deduced from UV inactivation studies. The findings made with reovirus suggested that psoralen photoreaction would be an effective alternative method to study mRNA synthesis by the influenza and VSV RNA polymerase activities. We have used this approach to analyze the different patterns of in vitro mRNA synthesis by the virion-asso-

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ciated RNA polymerase activities of these two viruses.

MATERIALS AND METHODS

Viruses. The Indiana strain of VSV was purified from infected BHK-21/13 cells as described (4). Purified WSN influenza A virus from infected MDBK cells (23) was kindly provided by Robert Krug of Sloan-Kettering Institute, New York.

Photoreaction with psoralen. Virus samples at a concentration of 250 μg of protein per ml in 10 mM Tris-hydrochloride (pH 8) containing 1 mM EDTA and the indicated 4'-substituted psoralen were irradiated at 0°C with UV light of 352-nm wavelength as described previously (21). Triton N-101 was present at concentrations of 0.2 and 0.05% for influenza virus and VSV, respectively.

Transcription reactions. Influenza virus complementary RNA (cRNA) was synthesized in incubation mixtures of 0.3 to 0.5 ml containing 30 to 50 μg of purified virus, 50 mM Tris-hydrochloride (pH 8), 0.1 M KCl, 5 mM magnesium acetate, 2 mM dithiothreitol, 0.2% Triton N-101, 0.4 mM adenylyl-(3'→5')-guanosine dinucleoside monophosphate as primer (23), 0.1 mM [α-^{32}P]CTP or [α-^{32}P]UTP (final specific activity, 0.65 Ci/mmol), and 1 mM each of the other three ribonucleoside triphosphates. Incubation was at 31°C for 1 h. For VSV mRNA synthesis, reaction mixtures of 0.3 ml containing 40 μg of purified virus, 50 mM Tris-hydrochloride (pH 8), 0.1 M NaCl, 5 mM $MgCl_2$, 4 mM dithiothreitol, 0.05% Triton N-101, 0.05 mM [α-^{32}P]UTP (final specific activity, 0.25 Ci/mmol), and 1 mM each of the other ribonucleoside triphosphates were incubated at 30°C for 2 h. Incorporation of radioactivity into acid-precipitable products was assayed as described (21).

Transcription product analysis. The influenza virus single-stranded cRNA products were analyzed by electrophoresis in 3% polyacrylamide gels containing 6 M urea (23). After hybridization of the cRNA to the virion RNA and RNase T_2 treatment, the double-stranded RNAs were resolved in 3% polyacrylamide gels without urea (23). VSV leader RNA, separated from mRNA's by sucrose gradient centrifugation, was quantitated by polyacrylamide gel electrophoresis, followed by autoradiography and tracing of the resulting film with an Ortec 4310 densitometer (8). The viral mRNA's were quantitated by hybridization to virion RNA, followed by electrophoresis in a 5% polyacrylamide gel after the single-stranded regions were trimmed by digestion with ribonuclease T_2 (10, 25). Tracings of autoradiograms were used to determine the relative amounts of the viral RNA products.

Materials. 4'-Aminomethyl-4,5',8-trimethylpsoralen (AMT) was obtained from Calbiochem-Behring Corp., La Jolla, Calif. 4'-Hydroxymethyl-4,5',8-trimethylpsoralen (HMT) was kindly provided by J. E. Hearst, University of California, Berkeley. Adenylyl-(3'→5')-guanosine dinucleoside monophosphate was obtained from P-L Biochemicals, Inc., Milwaukee, Wis., and proteinase K came from E. Merck, Darmstadt, W. Germany. [α-^{32}P]-labeled CTP and UTP (10 to 30 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Effect of psoralen photoreaction on influenza virion RNA polymerase activity. Influenza virus contains an RNA polymerase that becomes active upon detergent treatment of purified virions in vitro. The enzyme activity transcribes each of the eight genome segments into the cRNA's and is markedly stimulated by the presence of a primer dinucleotide such as adenylyl-(3'→5')-guanosine dinucleoside monophosphate (17, 22). The reaction is also stimulated by capped globin mRNA (22) and reovirus mRNA's with the incorporation of the 5' cap and 10 to 25 additional nucleotides from the primer into the cRNA products (5a). Irradiation of detergent-treated virions in the presence of AMT rapidly inactivated the adenylyl-(3',5')-guanosine dinucleoside monophosphate-primed influenza virus RNA polymerase activity (Fig. 1). Irradiation or psoralen treatment alone had little effect on cRNA synthesis. The extent of inactivation was directly related to drug concentration and time of irradiation. Consistent with the reported inhibition of psoralen binding to nucleic acids by cations (13), the presence of 0.1 M KCl partially protected against inactivation. The same level of protection was observed in the presence of 5 mM $MgCl_2$ (data not shown). Photoreaction with another 4'-substituted derivative, HMT, was about 10-fold less effective in

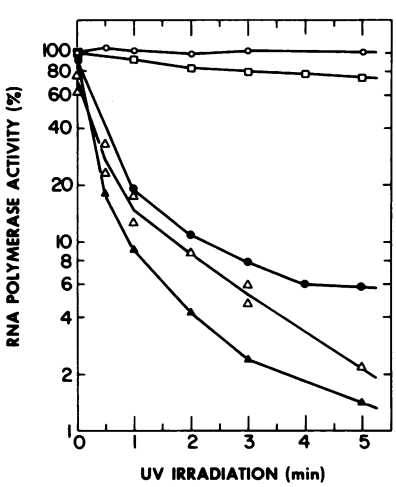

FIG. 1. *Psoralen photoinactivation of influenza virion RNA polymerase activity. Influenza virus was irradiated as described in the text in the absence* (○) *or presence of* 2.8×10^{-5} *M HMT* (□), 2.8×10^{-6} *M AMT* (●), 2.8×10^{-5} *M AMT* (▲), *and* 2.8×10^{-5} *M AMT plus 0.1 M KCl* (△). *At the times indicated, samples were taken for assay of cRNA synthesis. The 100% value for a 50-μl incubation mixture containing 9 μg of virus was 37,598 cpm or 26 pmol.*

inhibiting influenza RNA polymerase activity than with the same concentration of AMT (Fig. 1).

As observed previously for reovirus-associated RNA polymerase activity (20a), AMT photoinactivation of influenza cRNA synthesis apparently occurs at the level of initiation of polynucleotide chain formation. Total cRNA products made by the influenza virus RNA polymerase complex during a 60-min incubation were analyzed by polyacrylamide gel electrophoresis. Short running times were used to detect rapidly migrating, low-molecular-weight products that might be present as a result of premature termination of transcription on modified templates (Fig. 2A). To avoid or at least to minimize losses, 3′-polyadenylic acid was not removed from the products before electrophoresis. This resulted in low resolution (lanes 1 to 4) as compared to a preparation of cRNA that had been deadenylated by hybridization with polydeoxythymidylic acid followed by RNase H treatment (lane 5). Nevertheless, the gel patterns obtained for cRNA products, made by preparations of virion-associated RNA polymerase that had been inactivated to the extents of 0, 19, 29, and 47% by AMT photoreaction (Fig. 2, lanes 1 to 4), did not show noticeable increases in short RNA products. In agreement with the apparent lack of increased amounts of prematurely terminated transcripts, the annealed cRNA formed complete, RNase T_2-resistant duplexes with the virion RNA genome segments. Gel patterns of the duplexes obtained with products of both control and partially inactivated polymerase preparations included the three large, three medium, and two small influenza RNAs (Fig. 2B). As observed for reovirus, the synthesis of each of the size classes of influenza cRNA was affected by psoralen photoinactivation, the large segments being relatively more sensitive (Fig. 2B, compare lanes 1 and 4). Thus AMT photoinactivation of influenza RNA polymerase apparently resulted in the formation of reduced yields of full-size products without a corresponding accumulation of incomplete transcripts.

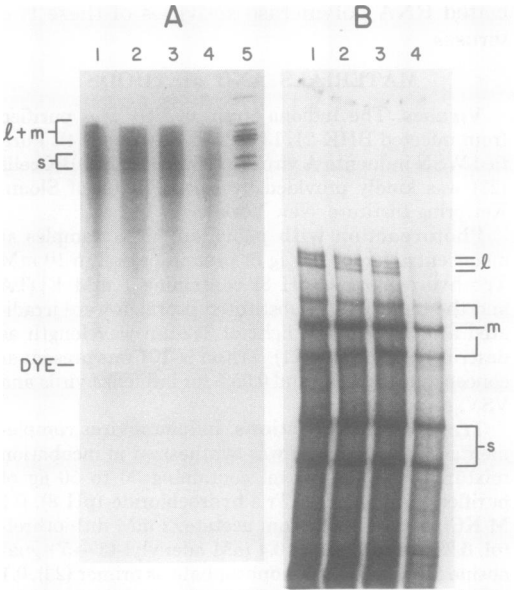

FIG. 2. *Polyacrylamide gel electrophoresis of cRNA synthesized by partially photoinactivated influenza virus. Purified virus (50 μg) was irradiated in 2.8×10^{-5} M AMT for 2.5 s (lane 2), 5 s (lane 3), and 10 s (lane 4). These samples and an untreated control virus suspension (lane 1) were incubated in the dark under conditions of cRNA synthesis in 0.33-ml incubation volumes. The radioactive products were extracted with phenol after digestion by proteinase K (0.5 mg/ml) in 0.5% sodium dodecyl sulfate for 1 h at 37°C. Unincorporated precursors were removed by gel filtration in Sephadex G-50 and ethanol precipitation. RNAs were analyzed (A) by electrophoresis for 3 h at 75 V in a 3% polyacrylamide slab gel containing 6 M urea or (B) for 17 h at 30 mA in a 3% polyacrylamide gel after hybridization to virion RNA and RNase T_2 treatment (23). Gels were dried, and autoradiographs were prepared. Dye, Bromophenol blue. Lane 5 in (A) shows the profile of deadenylated, single-stranded cRNA. l, m, and s, large, medium, and small species of influenza RNAs.*

Photoinactivation of VSV RNA polymerase activity. Irradiation of detergent-treated, purified VSV with long-wavelength UV light had no effect on the particle-associated RNA polymerase activity (Fig. 3). The addition of AMT without irradiation diminished RNA synthesis by the virion polymerase by 20% or less. However, irradiation for 2 min in the presence of 2.8×10^{-4} M AMT resulted in >98% loss of enzyme activity. Smaller decreases occurred at lower concentrations of AMT. As observed for the reovirus and influenza virus-associated polymerases, the activity was partially protected by the presence of mono- or divalent cations which inhibit dark (noncovalent) binding of psoralens to nucleic acids.

4′-Substituted psoralens modify both RNA and DNA, but photocatalyzed covalent attachment to proteins has not been observed. The inactivation of the VSV RNA polymerase by AMT presumably results from alteration of the genome RNA template. VSV RNA polymerase activity was reconstituted from AMT-treated and untreated soluble and core fractions prepared by dissociating purified virions with detergent at high ionic strength (9). As previously shown (9), the high-salt-soluble fraction, which

contains the L, NS, M, and G proteins released from the genome RNA, had little RNA-synthesizing activity (Table 1). The cores derived from virions by high-salt treatment contained the genome RNA and N protein and retained a low level of RNA polymerase activity. Addition of high-salt-soluble fraction, either untreated or after photoreaction with AMT, increased the residual polymerase activity of untreated high-salt cores by two- to threefold. However, the residual RNA polymerase activity was not increased when soluble fraction was added to the AMT-reacted cores. These results are consistent with inactivation of the VSV RNA polymerase activity by psoralen alteration of the genome template RNA.

Analysis of the VSV RNA products. Previous studies have shown that transcription of the VSV genome RNA in vitro by the virion-associated RNA polymerase is a sequential process (1, 2). Transcription begins at the 3′ end of the template with the synthesis of a 48-nucleotide leader sequence (8) that is followed by reading of the N, NS, M, G, and L genes. The products can be partially resolved by sucrose gradient centrifugation into the low-molecular-weight leader RNA, which remains at the top of the gradient, a 12S peak that includes the M and NS messages, a 14.5-17S peak comprising the N and G mRNA's, and the 31S mRNA for the L protein (5). Photoreaction with AMT under conditions that diminished overall VSV RNA synthesis by 47, 69, and 83% decreased the yields of each of the peaks of mRNA, but synthesis of the low-molecular-weight leader RNA that remained at the top of the gradient appeared to be relatively unaffected (Fig. 4). Furthermore, as in the case of influenza and reovirus in vitro RNA synthesis, there was no indication of abortive transcript formation even in the most extensively inactivated VSV preparation. To test directly for the apparent absence of premature termination and the relative resistance of leader RNA synthesis to AMT photoinactivation, the low-molecular-weight products corresponding to fractions 23 to 27 in Fig. 4 were analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 5 (lane A), the small products synthesized by untreated VSV included the leader RNA (arrow), mRNA products that were incomplete but larger than leader, and at least two other low-molecular-weight RNAs migrating faster than the 48-nucleotide leader RNA (see Discussion). Partially inactivated particles synthesized a diminished yield of the same products, but the predominant material made by the most extensively photoinactivated virus migrated in the position of leader RNA (Fig. 5, lane D). Analysis of tracings of the autoradiographs confirmed that synthesis of the leader RNA was differentially resistant to AMT photoreaction, i.e., treated samples that retained 53, 31, and 17% of

TABLE 1. *Effect of AMT photoreaction on reconstitution of VSV RNA polymerase activity*[a]

Viral components	RNA synthesis (cpm incorporated)
Untreated soluble fraction	790
Untreated core fraction	3,980
Untreated soluble + untreated core	10,450
Treated soluble + untreated core	10,200
Untreated soluble + treated core	2,960

[a] Soluble and core fractions were prepared by treating 100 μg of purified VSV with 0.8 M NaCl and 1.88% Triton X-100 as described (9). Samples of each were irradiated for 1 min in the presence of 0.1 M NaCl and 2.8×10^{-4} M AMT. Soluble and core fractions or the reconstituted mixtures were incubated in 10 mM Tris-hydrochloride (pH 8), 0.1 M NaCl, 1 mM dithiothreitol, and 10% glycerol for 30 min at 30°C. Ribonucleoside triphosphates, including [^{3}H]UTP (final specific activity, 660 cpm/pmol) and 5 mM $MgCl_2$, were then added as described in the text. After further incubation for 1 h at 30°C, the mixtures were assayed for acid-precipitable radioactivity.

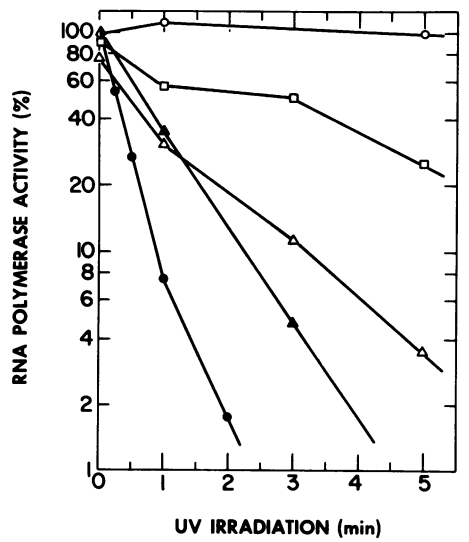

FIG. 3. *Effect of AMT and UV irradiation on VSV-associated RNA polymerase activity. Purified VSV was irradiated in the absence* (○) *or presence of* 2.8×10^{-5} *M AMT* (▲), 2.8×10^{-5} *M AMT plus 0.1 M NaCl* (□), 2.8×10^{-5} *M AMT plus 0.002 M* $MgCl_2$ (△), *or* 2.8×10^{-4} *M AMT* (●). *At the times indicated, samples were taken for assay of RNA synthesis as described in the text. The 100% value for a 200-μl reaction mixture containing 40 μg of virus was 52,368 cpm or 95 pmol.*

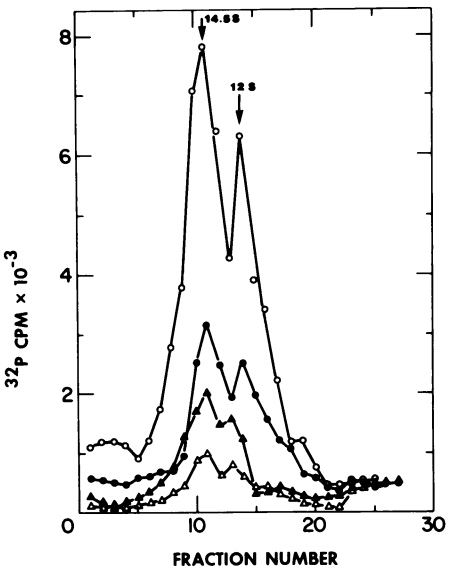

FIG. 4. *Sedimentation analysis of RNA products synthesized by partially photoinactivated VSV. Purified VSV (40 μg) was irradiated in the presence of 2.8 × 10^{-5} M AMT for 15 s (●), 30 s (▲), and 60 s (△) and then incubated together with an untreated control virus sample (○) in the dark under conditions of RNA synthesis. Products were extracted by phenol in the presence of 0.5% sodium dodecyl sulfate and analyzed by centrifugation (SW40, 33,000 rpm, 17 h, 23°C) in 15 to 30% sucrose gradients over a cushion of 0.6 ml of 60% sucrose, containing 0.5% sodium dodecyl sulfate, 0.1 M NaCl, 10 mM Tris-hydrochloride (pH 7.4), and 1 mM EDTA. Fractions of 0.48 ml were collected, and samples were assayed for acid-precipitable radioactivity.*

the total initial RNA synthesis activity synthesized 93, 70, and 69%, respectively, of the amount of leader RNA made by the untreated preparation under the same conditions.

Irradiation of VSV at a wavelength of 254 nm inhibited differentially the in vitro synthesis of the five VSV mRNA's, and only the 3'-proximal N gene had a target size consistent with the molecular weight of its transcript (1, 2). The other genes had considerably larger target sizes that were cumulative, indicative of a compulsory order of gene transcription (1, 2). To determine the relative sensitivity of the VSV genes to AMT photoinactivation, the mRNA products (corresponding to fractions 1 to 22 in Fig. 4) were annealed with viral genome RNA. The hybrids were treated with ribonuclease T_2 to trim the single-stranded portions (10, 25), and the duplexes were resolved by polyacrylamide gel electrophoresis. Gene N transcription was the least affected by AMT photoinactivation (Fig. 6). These results are similar to those obtained previously with UV-inactivated VSV (1, 2).

DISCUSSION

The recently synthesized 4'-substituted psoralens, AMT and HMT, effectively inactivated the infectivity of VSV (12) and reovirus (21) by photoreacting with the viral genome RNAs. Modification of the viral RNAs in situ also abol-

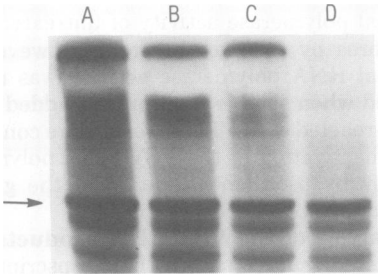

FIG. 5. *Analysis of the leader RNAs synthesized in vitro by photoreacted VSV. The fractions containing the leader RNAs (fractions 23 to 27 in Fig. 4) were pooled, and the ethanol-precipitated RNAs were analyzed by electrophoresis in a 20% polyacrylamide slab gel containing 7 M urea as described previously (8). Electrophoresis was for 15 h at 140 V, followed by autoradiography. Lanes A, B, C, and D contain the leader RNAs synthesized by VSV that was photoreacted with AMT for 0, 15, 30, and 60 s, respectively. The arrow indicates the leader RNA band.*

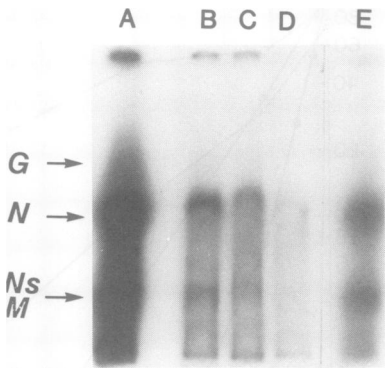

FIG. 6. *Polyacrylamide gel electrophoresis of the VSV mRNA products synthesized in vitro by partially photoinactivated VSV and hybridized to virion RNA. Fractions 1 to 22 in Fig. 4 representing the 12–18S region and containing the N, NS, G, and M protein mRNA's (5) were pooled, and the RNA was ethanol precipitated. Portions of the RNA samples were then annealed with VSV genome RNA, followed by digestion with RNase T_2 as described previously (25). The resulting RNA duplexes were analyzed by electrophoresis in a 5% polyacrylamide slab gel. Electrophoresis was for 18 h at 50 V, followed by autoradiography. The positions of the mRNA duplexes are shown by letters on the left. Lanes A to D represent mRNA's synthesized by VSV that was photoreacted with AMT for 0, 15, 30, and 60 s, respectively. Lane E is the same as lane A but exposed for a shorter time.*

ished their ability to function as templates for the RNA polymerase activity associated with these virions (21; Fig. 3). Similarly, influenza RNA polymerase activity was lost after psoralen photoreaction of purified virions (Fig. 1). It seems clear that the primary lesion is in the virion RNA (16, 18; Table 1), although direct effects on the proteins may also occur, especially at high AMT levels or long times of photoreaction. The main effect on the virion RNA polymerase activities appeared to be at the initiation step of transcription, since AMT photoinactivation was not accompnied by an increased production of prematurely terminated transcripts. Their absence suggests that transcription initiation is diminished by structural effects on the RNA-protein complexes in psoralen-photoreacted particles. This might occur, for example, by a twisting of the strands of the duplex genome RNA of reovirus or a similar unwinding of the helical nucleocapsids of influenza virus by psoralen intercalation. Alternatively, the RNA polymerase promoter sites may be more susceptible to psoralen adduct formation than other regions of the template RNAs, or modifications in the promoter region may block initiation while alteration at internal sites may allow continued elongation at a reduced rate of nucleotide incorporation.

The observations with psoralen-photoreacted purified reovirus and influenza virus contrast sharply with findings made with VSV. It has been shown previously that partial inactivation of VSV by irradiation with UV light at a wavelength of 254 nm apparently induced pyrimidine dimers randomly in the viral genome RNA (1). Their presence caused termination of RNA synthesis by the virion polymerase at the modified sites, resulting in the formation of a large amount of products that were heterogeneous in size and not 3'-polyadenylated. If psoralen adducts, like pyrimidine dimers, form at random sites on the viral genome RNAs, prematurely terminated transcripts would be expected. However, psoralen-reactive sites on the VSV genome RNA in situ appear to be specific, since the products made by AMT-photoreacted VSV did not include increased amounts of prematurely terminated transcripts (Fig. 4). On the other hand, sequential transcription was apparently maintained in AMT-reacted VSV, since leader RNA synthesis was highly resistant to inactivation (Fig. 5) and N gene transcription was affected less than the other viral genes (Fig. 6), as was also observed with UV-inactivated VSV (1, 2).

In addition to leader RNA, the products of untreated and psoralen-treated VSV included rapidly migrating, distinct bands of low-molecular-weight RNA (Fig. 5). It was recently shown that they are not incomplete leader RNA molecules but correspond to 5'-terminal sequences of VSV mRNA species. The small RNAs contain the unblocked 5'-sequence (p)ppA-A-C-A-G, as compared to the capped 5'-sequence G(5')ppp(5')A-A-C-A-G, present in full-length VSV mRNA's (26). The results indicate that VSV mRNA synthesis in vitro is in fact mediated by multiple initiation processes (D. Testa, P. K. Chanda, and A. K. Banerjee, submitted for publication). Thus, the formation of VSV leader RNA and mRNA apparently occurs by independent processes. From Fig. 5 it can be seen that the synthesis of the low-molecular-weight RNAs, like leader RNA, is resistant to psoralen photoinactivation. The findings suggest further that a site which is highly sensitive to psoralen modification may be located 5'-distal to the leader template sequence, beyond the promoter region of N gene transcription. Further work on the precise location of psoralen binding sites in viral genomes in situ should help to elucidate the mechanisms of gene regulation.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Independent transcription of individual influenza mRNA species has also been reported by G. Abraham (Virology **79**:177–182, 1979).

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