Influenza Virus RNA Replication In Vitro: Synthesis of Viral Template RNAs and Virion RNAs in the Absence of an Added Primer

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The two steps in influenza virus RNA replication are (i) the synthesis of template RNAs, i.e., full-length copies of the virion RNAs, and (ii) the copying of these template RNAs into new virion RNAs. We prepared nuclear extracts from infected HeLa cells that catalyzed both template RNA and virion RNA synthesis in vitro in the absence of an added primer. Antibody depletion experiments implicated nucleocapsid protein molecules not associated with nucleocapsids in template RNA synthesis for antitermination at the polyadenylation site used during viral mRNA synthesis. Experiments with the WSN influenza virus temperature-sensitive mutant *ts56* containing a defect in the nucleocapsid protein proved that the nucleocapsid protein was indeed required for template RNA synthesis both in vivo and in vitro. Nuclear extracts prepared from mutant virus-infected cells synthesized template RNA at the permissive temperature but not at the nonpermissive temperature, whereas the synthesis of mRNA-size transcripts was not decreased at the nonpermissive temperature. Antibody depletion experiments showed that nucleocapsid protein molecules not associated with nucleocapsids were also required for the copying of template RNA into virion RNA. In contrast to the situation with the synthesis of transcripts complementary to virion RNA, no discrete termination product(s) were made during virion RNA synthesis in vitro in the absence of nucleocapsid protein molecules.

In influenza virus-infected cells, the eight genomic or virion RNAs (vRNAs) are both transcribed into mRNAs and replicated. A great deal is known about the mechanism of synthesis of the viral mRNAs because an in vitro system is available. Nucleocapsids isolated from purified virions catalyze viral mRNA synthesis (16). This synthesis requires priming by capped RNA fragments that are generated by a viral cap-dependent endonuclease, which cleaves capped RNAs 10 to 13 nucleotides from their 5' ends (3, 12, 16). The viral mRNA chains are then elongated until a stretch of uridine residues is reached 17 to 22 nucleotides before the 5' ends of the vRNAs are reached, where transcription terminates and poly(A) is added to the mRNAs (8, 17). The three polymerase (P) proteins (PB1, PB2, PA) that are associated with the nucleocapsids catalyze viral mRNA synthesis. Some of the roles of the P proteins have been elucidated (4, 24, 25). PB2 recognizes and binds to the cap 1 structure of primer RNAs, and PB1 most likely catalyzes the addition of each nucleotide to the growing viral mRNA chains. The three P proteins move down the elongating viral mRNA chains together as a complex. Although the PA protein is part of this complex, no specific role for PA in viral mRNA synthesis has been found.

In contrast, much less is known about the replication of influenza virus vRNAs. As the first step in replication, an alternative type of transcription of the vRNA is required that results in the production of full-length copies of the vRNAs rather than the production of viral mRNAs. The full-length copies, or template RNAs, are initiated without a primer and are not terminated at the poly(A) site used during mRNA synthesis (8, 9). In vivo, the switch from viral mRNA to template RNA synthesis requires the synthesis of one or more virus-specific proteins (1, 9). The second step in replication is the copying of the template RNAs into vRNAs.

This synthesis also occurs without a primer, since the vRNAs contain 5'-triphosphorylated ends (28). The copying of template RNAs into vRNAs is an important step of regulation during the early phase of virus infection, since the selective copying of certain template RNAs into their vRNAs determines which viral proteins are preferentially synthesized at early times of infection (8, 20, 21). As with viral mRNA synthesis (10), both steps of vRNA replication occur in the nucleus (20).

To determine the mechanisms of the two steps of replication and the roles of virus-specific proteins in these steps, it is necessary to establish in vitro systems that catalyze both steps of replication. Recently, we prepared infected cell nuclear extracts that were active in the antitermination event that occurs as part of the switch from mRNA to template RNA synthesis (2). These extracts, however, did not carry out the unprimed initiation of template RNAs. This deficiency was circumvented by providing the dinucleotide ApG as a primer. The viral nucleocapsids isolated from the nuclear extract synthesized only mRNA-sized RNAs and not template RNAs. Antitermination required the action of nuclear factor(s) not associated with the nucleocapsids. Because this factor was removed by depleting the nucleocapsid protein (NP) with anti-NP antibodies bound to protein A-Sepharose (PAS), we concluded that NP molecules which were not associated with nucleocapsids were required for antitermination (2). Antitermination was controlled not only by the availability of NP but also by the type of initiation. Transcripts initiated with capped RNA primers were not antiterminated in the presence of NP molecules that were active in antiterminating ApG-initiated transcripts (2).

In the present report, we prepared infected-cell nuclear extracts that catalyzed the synthesis of both template RNA and vRNA in the absence of an added primer. Using a viral temperature-sensitive (*ts*) mutant, we verified that NP was required for antitermination during template RNA synthesis.

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Antibody depletion experiments also indicated that NP molecules not associated with nucleocapsids were required for elongation during vRNA synthesis. In the absence of NP, no discrete termination product(s) was synthesized during vRNA synthesis.

MATERIALS AND METHODS

Preparation and fractionation of nuclear extracts from infected cells. Suspension HeLa cells were infected with 10 to 20 PFU of WSN influenza A virus (either the wild type or the *ts56* virus mutant) per cell as previously described (11). At the indicated times after infection, the cells were collected, and nuclear extracts were prepared as described by Dignam et al. (6). Where indicated, the nuclear extract was centrifuged to yield a supernatant fraction containing nucleocapsid-free viral proteins and a pellet fraction containing nuclear nucleocapsids (2). To prepare the supernatant, 700 μ l of extract was centrifuged at 45,000 rpm for 4.5 h in the SW50.1 rotor, and the top 200 μ l of the supernatant was collected. To prepare a fraction containing viral nucleocapsids, 5 ml of the nuclear extract was centrifuged at 45,000 rpm for 4 h in the SW50.1 rotor. The pellet was dissolved in 250 μ l of 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.6)–200 mM KCl–1 mM dithiothreitol–20% glycerol and subjected to centrifugation at 48,000 rpm for 4 h on a 20 to 50% glycerol gradient in the same buffer. The nucleocapsids were localized by assaying samples (15 μ l) of each gradient fraction for virus-specific RNA synthesis as described below.

RNA synthesis reactions. Except where indicated, reactions in a final volume of 25 μ l contained, in addition to the nuclear extract, 20 mM HEPES (pH 7.6), 1 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 5 mM Mg(OAc)₂, 2 mM dithiothreitol, 70 μ g of tRNA per ml, 10 mM creatine phosphate, 130 μ g of creatine kinase per ml, and [α -³⁵S]UTP (5 μ M, 70 mCi/ μ mol). Except where indicated, 10 μ l of unfractionated nuclear extract, or both 3 μ l of the gradient-purified nucleocapsids and 10 μ l of the nuclear supernatant, was added. Where indicated, 0.4 mM ApG was added.

Analysis of RNA products. (i) **Template RNA.** Total RNA was extracted and annealed to an excess of a vRNA-sense M13 DNA clone of the NS segment as described previously (2). After RNase T2 digestion, the RNA-DNA hybrids were collected on nitrocellulose filters. The RNA eluted from these filter-bound hybrids by heating was analyzed by electrophoresis on a 3.5% acrylamide gel containing 8 M urea.

(ii) **vRNA.** The poly(A)[−] products of in vitro RNA synthesis were annealed to filters containing an mRNA-sense M13 clone. The hybridized RNA was eluted by heating and analyzed by gel electrophoresis.

Antibody depletion experiments. Antibody depletion was carried out as previously described (2). Briefly, PAS (15 mg) was swollen in 0.5 ml of 50 mM Tris hydrochloride (pH 8)–150 mM NaCl–5 mM EDTA (buffer A) plus 0.1% Nonidet P-40. The specific antiserum (40 to 80 μ l) was incubated with the PAS for 18 h at 4°C in Eppendorf tubes. The PAS was spun out, washed three times with buffer A, and then incubated with 80 μ l of the nuclear supernatant for 2 h at 4°C. The PAS was spun out, and the resulting supernatant was removed and assayed.

Shift-up experiments with the viral *ts* mutant *ts56*. The *ts56* virus mutant was grown and titered as previously described (13, 22). Monolayer cultures of BHK-21 cells in 75-cm plastic flasks were infected with wild-type or *ts56* virus at a multiplicity of 10. The virus was adsorbed for 1 h at 4°C.

After adsorption, 5 ml of prewarmed (33°C) growth medium (reinforced Eagle medium containing 2% calf serum) was added, and the flasks were completely immersed in a water bath maintained at 33°C (permissive temperature). At 3 h postinfection, some flasks were transferred to a water bath maintained at 39.5°C (nonpermissive temperature). At this time, the medium in both the 33 and 39.5°C flasks was replaced with prewarmed (33 or 39.5°C) medium containing 1 mCi of [³H]uridine per ml. After 30 min of labeling, the cells were rinsed with RSB (10 mM Tris hydrochloride [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂) and scraped into RSB. The cells were collected by centrifugation and suspended in RSB. Total nucleic acids were obtained by incubating the cells for 45 min at 37°C with 500 μ g of pronase per ml in 100 mM Tris hydrochloride (pH 7.4)–12.5 mM EDTA–150 mM NaCl–1% sodium dodecyl sulfate–5 mM vanadyl ribonucleoside complexes, followed by phenol-chloroform (1:1) extraction. After isopropanol precipitation, the samples were digested with RNase-free DNase and reextracted with phenol-chloroform. The resulting RNAs were chromatographed on oligo(dT) cellulose to separate poly(A)⁺ and poly(A)[−] RNAs. To measure viral mRNA synthesis, the poly(A)⁺ RNA was hybridized to a filter containing a vRNA-sense M13 DNA, and the eluted RNA was analyzed by gel electrophoresis. The poly(A)[−] RNA was used to assay for template RNA and vRNA as described above.

Materials. Monoclonal antibodies to NP were generously provided as ascites fluid by Robert Webster (26), and polyclonal antisera to the NS1 and NS2 proteins were kindly supplied by Peter Palese (7, 27).

RESULTS

In vitro template RNA synthesis in the absence of a primer.

Previously, we prepared infected cell nuclear extracts that catalyzed the synthesis of template RNAs but only in the presence of a primer, the dinucleotide ApG (2). One of our goals was to obtain extracts that synthesized high levels of template RNAs in the absence of ApG. We used an M13 single-stranded DNA to measure the transcripts copied off of one of the vRNAs, NS vRNA (2). This assay includes a digestion with RNase T₂, so that the NS template RNA is about 20 nucleotides larger than the NS1 mRNA and thus has a slower mobility than the NS1 mRNA during gel electrophoresis.

The previous nuclear extracts were obtained from infected HeLa cells at 4 h postinfection (2). Because the time course of virus-specific RNA synthesis in HeLa cells was delayed as compared with that in other cells, we determined whether nuclear extracts at time points later than 4 h were more active in the absence of ApG. Maximal template RNA-synthesizing activity in the absence of ApG occurred with the extract that was collected at 6 h after infection (Fig. 1). Much lower activity was present both before and after 6 h. With other 6-h extracts, the ratio of template RNA to mRNA synthesis was higher than that shown in Fig. 1. In many 6-h extracts, template RNA was the predominant transcription product in the absence of ApG (Fig. 2). The addition of ApG to such an extract caused only a small (two- to threefold) stimulation of template RNA synthesis, whereas mRNA synthesis was strongly stimulated, so that mRNA synthesis predominated over template RNA synthesis in the presence of ApG.

The synthesis of template RNA in the absence of ApG suggested that at least some of these template RNAs were initiated without a primer in vitro. However, attempts to

FIG. 1. Template RNA synthesis in the absence of ApG catalyzed by HeLa cell nuclear extracts collected at various times postinfection. At the indicated times (4, 5, 6, 7, and 8 h) after infection, nuclear extracts were prepared and used to catalyze RNA synthesis in vitro in the absence of ApG. The RNA products were analyzed for NS1 mRNA (M) and NS template RNA (T) by using RNase T₂ digestion as described in the text.

prove that unprimed initiation occurred in vitro were frustrated by the presence in the nuclear extracts of enzymes that transferred the labeled γ phosphate of ATP to the α position of the four ribonucleoside triphosphates. As a consequence, with [γ -³²P]ATP or [γ -³⁵S]ATP as the precursor, most of the label was incorporated into internal positions of the NS template RNAs, and unequivocal evidence for the incorporation of pppA at the 5' ends of the NS template RNAs could not be obtained (data not shown; see Discussion).

Previously, with ApG as the primer, we showed that (i) the nucleocapsids isolated from the nuclear extract synthesized mRNA-size RNA; (ii) the synthesis of template RNA, and hence antitermination, required the addition of the nonnucleocapsid fraction (supernatant fraction) of the extract; and (iii) depletion of NP from this fraction by incubation with PAS containing anti-NP antiserum eliminated antitermination and hence template RNA synthesis (2). The same results were obtained in the absence of ApG (Fig. 3). Because anti-NP antiserum depleted antitermination activity from the supernatant, it can be concluded that the nuclear supernatant supplied the NP needed for antitermination (lanes 4 through 7). In contrast, depletion of the supernatant with anti-NS1 or anti-NS2 antiserum did not eliminate template RNA-synthesizing activity (data not shown). It had been shown previously that antibody depletion of the NS1 protein

FIG. 2. Effect of ApG on template RNA and mRNA synthesis catalyzed by a 6-h extract. A nuclear extract prepared from HeLa cells at 6 h postinfection was used to catalyze RNA synthesis in the absence (-) and presence (+) of ApG. The RNA products were analyzed for NS1 mRNA (M) and NS template RNA (T) by using RNase T₂ digestion as described in the text.

FIG. 3. Template RNA synthesis in the absence of ApG needed nonnucleocapsid NP molecules. A 6-h infected cell nuclear extract was separated into pellet (nucleocapsid-containing) and supernatant fractions by centrifugation in the SW50.1 rotor; and the nucleocapsids were isolated by subjecting the pellet fraction to centrifugation on a 20 to 50% glycerol gradient as described in the text. RNA synthesis was catalyzed by the unfractionated extract (E), the nucleocapsids alone (P), the supernatant alone (S), or the supernatant plus the nucleocapsids (S+P). Samples of the supernatant were incubated with PAS (S), PAS containing normal rabbit serum (S), or PAS containing pooled anti-NP monoclonal antibodies (S-NP). Each of these supernatant samples was added to the nuclear nucleocapsids to carry out RNA synthesis in vitro. The RNA products were analyzed for NS1 mRNA (M) and NS template RNA (T) by using RNase T₂ digestion as described in the text.

did not eliminate ApG-primed template RNA synthesis (2). The supernatant also apparently contained an inhibitor of transcription (e.g., nuclease), because the total transcription catalyzed by the unfractionated extract (lane 1) or by the nucleocapsids and supernatant combined (lane 4) was lower than that by the nucleocapsids alone (lane 2).

A *ts* mutant in NP was defective in template RNA synthesis in vivo and in vitro. Several *ts* mutants carrying a defective NP gene have been shown to have a defect in virus-specific RNA synthesis (13, 14, 19, 23). With an NP *ts* mutant of fowl plaque virus, a shift-up experiment was carried out after template RNA synthesis had already ceased (14, 18). This experiment showed that vRNA synthesis was drastically inhibited after the shift-up, indicating that the NP was required for vRNA synthesis in vivo. Our goal was to determine whether the NP was also required for template RNA synthesis in vivo. We used a WSN virus *ts* mutant, *ts*56, and performed the shift-up at a time at which the rate of template RNA synthesis was still increasing at the permissive temperature, 33°C. In cells infected with *ts*56 at 33°C, the rate of template RNA synthesis increased until a peak rate was achieved at 4 h postinfection (Fig. 4). After 4 h, the rate of template RNA synthesis decreased (data not shown). Accordingly, it was appropriate to carry out the shift-up at 3 h after infection at 33°C.

Duplicate flasks of BHK-21 cells were infected with *ts*56 at 33°C. At 3 h postinfection, [³H]uridine was added to the cells. One flask was maintained for an additional 30 min at 33°C; the other was shifted to 39.5°C (nonpermissive temperature). In the 30 min after the shift-up the rate of synthesis of a representative viral mRNA, NS1 mRNA, at 39.5°C was nearly identical to that at 33°C (Fig. 5). In contrast, the shift to the nonpermissive temperature led to an immediate shutoff of NS template RNA and NS vRNA synthesis. This shutoff was not seen in cells infected with wild-type virus (data not shown). The synthesis of the virus-specific RNAs specific for another genomic segment, the M segment, were similarly affected by a shift-up. These

FIG. 4. Time course of template RNA synthesis in BHK-21 cells infected with the *ts56* mutant at 33°C. Infected cells were labeled with [³H]uridine for 30 min at 2, 3, or 4 h postinfection. The total poly(A)⁻ RNA of these cells was isolated and analyzed for NS1 mRNA (M) and NS template RNA (T) by using RNase T₂ digestion as described in the text.

results indicate that the NP was required for both template RNA and vRNA synthesis *in vivo*.

To verify that the defect in the NP acted directly on template RNA synthesis, we prepared a nuclear extract from HeLa cells infected by the *ts56* mutant at 33°C and determined the activity of this extract in viral mRNA and template RNA synthesis at 33 and 39.5°C (Fig. 6). In both the absence and presence of ApG, this extract synthesized template RNA at 33°C (lanes 1 and 5). In contrast, at 39.5°C this extract did not synthesize any detectable template RNA in both the absence and presence of ApG (lanes 2 and 6). No template RNA was observed in lane 6 even with this dark exposure of the gel. The synthesis of mRNA-size RNA was not decreased at 39.5°C (lanes 2 and 6). In fact, in the absence of ApG, an increased amount of mRNA-size RNA synthesis at 39.5°C was seen relative to that at 33°C (lanes 1 and 2). These results indicated that the NP made by the *ts56* mutant was inactive in antitermination at the nonpermissive temperature. However, this defect in NP did not decrease the activity at 39.5°C in mRNA synthesis catalyzed by nucleocapsids containing the same NP. In contrast to the

FIG. 5. NP was required for both template RNA and vRNA synthesis *in vivo*. BHK-21 cells were infected with the *ts56* virus mutant at 33°C. At 3 h postinfection, some of the infected cells were shifted to 39.5°C (lane 39.5), whereas other cells were maintained at 33°C (lane 33). At the same time, [³H]uridine was added. After 30 min of labeling, the cells were collected. The poly(A)⁺ RNA was analyzed for NS1 mRNA (M), and the poly(A)⁻ RNA was analyzed for NS template RNA (NS) and for vRNA as described in the text.

FIG. 6. NP was required for template RNA synthesis *in vitro*. HeLa cells were infected with the *ts56* virus mutant at 33°C. At 8 h postinfection, a nuclear extract (NPts) was prepared and used to catalyze RNA synthesis in the absence of ApG at 33°C (lane 1) and 39.5°C (lane 2) and in the presence of ApG at 33°C (lane 5) and 39.5°C (lane 6). A nuclear extract from cells infected with wild-type virus (WT) (6 h after infection at 37°C) was used to catalyze RNA synthesis in the absence of ApG at 33°C (lane 3) and 39.5°C (lane 4). The RNA products were analyzed for NS1 mRNA (M) and NS template RNA (T) as described in the text.

ts56 extracts, the nuclear extracts from cells infected with wild-type WSN virus synthesized similar amounts of template RNA at 33 and 39.5°C in the absence (lanes 3 and 4) and in the presence (data not shown) of ApG.

In vitro synthesis of virion RNAs. The nucleus is also the site of vRNA synthesis (20). To determine whether the nuclear extracts synthesized vRNAs, the poly(A)⁻ products of *in vitro* synthesis were hybridized to filters containing mRNA-sense M13 DNA specific for either M or NS vRNA. The hybridized RNA was eluted and analyzed by gel electrophoresis. Both M and NS vRNAs were synthesized *in vitro* by nuclear extracts collected at 6, 7, and 8 h postinfection (Fig. 7). In this particular experiment, the rate of NS vRNA synthesis was highest with the 8-h extract and the rate of M vRNA synthesis was highest with the 6-h extract. However, the results of several experiments indicated that extracts collected at 6, 7, and 8 h were similar in their activities in M and NS vRNA synthesis (data not shown). In contrast (Fig. 1), template RNA-synthesizing activity decreased markedly with extracts collected later than 6 h postinfection. These results reflect the situation *in vivo*,

FIG. 7. vRNA synthesis catalyzed by HeLa cell nuclear extracts collected at various times postinfection. At the indicated times (6, 7, and 8 h) after infection, nuclear extracts were prepared and used to catalyze RNA synthesis *in vitro* (in the absence of a primer). The poly(A)⁻ RNA products were analyzed for NS or M vRNA as described in the text.

FIG. 8. vRNA synthesis needed nonnucleocapsid NP molecules. A 7-h infected HeLa cell nuclear extract was separated into nucleocapsids and a supernatant fraction as described in the text. RNA synthesis was catalyzed by the unfractionated extract (E), the nucleocapsids alone (P), the supernatant alone (S), or the supernatant plus the nucleocapsids (S+P). Samples of the supernatant were incubated with PAS containing normal rabbit antiserum (S), pooled NP monoclonal antibodies (S-NP), NS1 antiserum (S-NS1), or NS2 antiserum (S-NS2). Each of these supernatant samples was added to the nuclear nucleocapsids to carry out RNA synthesis in vitro (lanes 5, 6, 7, and 8, respectively). The poly(A)⁻ RNA products were analyzed for NS vRNA as described in the text.

where template RNA synthesis shut down soon after its peak rate of synthesis was achieved whereas vRNA synthesis continued at essentially maximal rate (20).

In contrast to the situation with the synthesis of transcripts complementary to vRNA, the nucleocapsids present in the nuclear pellet fraction did not synthesize a vRNA-sense RNA of discrete size in the absence of the supernatant fraction (Fig. 8, lane 2). A heterogeneous array of RNAs of small size was made, as detected by analysis of the products on high-percentage gels (data not shown), and this array varied between experiments. The supernatant also lacked activity (lane 3). When the nucleocapsids in the pellet were combined with the supernatant, vRNA synthesis was restored (lane 4). Antibody depletion experiments indicated that NP molecules in the supernatant were required for vRNA synthesis. Thus, supernatant that had been incubated with PAS containing pooled monoclonal antibodies directed against the viral NP lost its ability to support vRNA synthesis in the presence of the nucleocapsids in the pellet fraction (lane 6). In contrast, activity was retained in supernatants that had been incubated with PAS containing normal rabbit antiserum (lane 5), anti-NS1 antibody (lane 7), or anti-NS2 antibody (lane 8). In the presence of supernatant lacking the NP (lane 6), a vRNA-sense RNA of discrete size was not made, as was the case with the pellet alone (lane 2). Again, shorter RNAs of consistent sizes were not detected by analysis on high-percentage gels (data not shown). Thus, in the absence of NP, vRNA-sense RNA(s) of discrete size(s) was not made.

DISCUSSION

The two steps in influenza virus RNA replication are, first, the synthesis of template RNAs, i.e., full-length copies of the vRNAs, and, second, the copying of these template RNAs into new vRNAs. The synthesis of template RNAs and of vRNAs is initiated without a primer, since the template RNAs and vRNAs made in infected cells contain 5' (p)ppA termini (9, 28). We have prepared infected cell nuclear extracts that catalyzed the in vitro synthesis of both template RNAs and vRNAs in the absence of an added primer. These extracts clearly carried out the elongation of both template RNA and vRNA chains in vitro. Whether some of these template RNA and vRNA chains were also initiated in vitro could not be determined with certainty. The nuclear extracts contained enzymes that transferred the labeled γ phosphate of ATP to the α position of the four ribonucleoside triphosphates. Therefore, essentially all of the radioactivity from a [γ -³²P]ATP or [γ -³⁵S]ATP precursor was incorporated into internal positions of the template RNA and vRNA chains. Clearly, it will be necessary to use a better assay for initiation and/or to purify the components needed for template RNA and vRNA synthesis so that they are free of the phosphate exchange enzymes.

We have now proven that NP molecules not associated with nucleocapsids are required during template RNA synthesis for antitermination at the poly(A) site used during viral mRNA synthesis. Nuclear nucleocapsids both in the absence (present study) and in the presence of ApG (2) synthesized mRNA-sized transcripts. Antitermination and hence template RNA synthesis required the addition of supernatant factor(s), and the activity of the supernatant was eliminated by incubation with PAS containing anti-NP antibodies (2; present study). Consequently, NP molecules and/or protein(s) that were associated with NPs were implicated in antitermination. The experiments presented here with the viral *ts56* mutant containing a defect in NP proved that NP molecules were indeed required. Temperature shift experiments with this mutant showed that NP was required for template RNA synthesis in vivo. In addition, nuclear extracts prepared from mutant virus-infected cells were defective in antitermination at the nonpermissive temperature (39.5°C). Template RNA was synthesized at 33°C (permissive temperature) but not at 39.5°C in the absence or presence of ApG, whereas the synthesis of mRNA-sized transcripts was not decreased at 39.5°C. Now that it has been established that NP molecules are required for template RNA synthesis, it will be important to determine whether NP is sufficient by itself or, as is the case with vesicular stomatitis virus (5, 15), whether another viral protein is also required.

NP molecules not associated with nucleocapsids are also required for the copying of template RNA into vRNA. In the absence of these NP molecules, namely, with nuclear nucleocapsids alone or with nuclear nucleocapsids supplemented with supernatant depleted of NP, no discrete vRNA-sense RNA was synthesized in vitro. Thus, elongation of vRNA chains most likely ceased at any point at which NP was not available, causing termination at multiple sites. Presumably, these terminations also occurred at sites close to the 5' ends of the vRNA chains, so that it is conceivable that initiation of vRNA would be effectively blocked in the absence of NP molecules. Indeed, the 13-nucleotide-long sequence that is found at the 5' ends of the eight influenza vRNAs is a likely candidate for the site at which NP molecules initially bind to nascent vRNA chains.

In apparent contrast, during the synthesis of transcripts complementary to vRNA, discrete RNA species, i.e., mRNA-size transcripts, are made in the absence of nonnucleocapsid NP molecules (2; present study). These mRNA-sized transcripts terminate about 20 nucleotides before the 5' ends of the vRNA templates. In the presence of NP molecules, some of the transcripts do not terminate at this site, yielding full-length copies or template RNAs. Clearly, NP is required for this antitermination step. However, it is likely that NP actually first binds to the nascent template RNAs at or close to their 5' ends. The only sequence common to the eight influenza virus complementary RNAs is the 12-nucleotide-long sequence at their 5' ends. In addition, transcripts initiated with a capped primer fragment cannot be antiterminated in the presence of NP (2), strongly suggesting that the capped primer sequence preceding the common 5' sequence of the viral transcripts blocks the binding of NP. If it is indeed necessary for NP to bind initially to the 5' ends of transcripts that will become full-length template RNAs, then it is likely that, as is the case with nascent vRNA chains, elongation of template RNAs would cease at any point at which NP was not available, resulting in termination at multiple sites when NP was depleted. A different enzyme system, i.e., a different set of nucleocapsids with their associated P proteins, which is independent of nonnucleocapsid NP molecules, would then be responsible for the synthesis of mRNA-size transcripts, which consequently would not be direct precursors to the encapsidated template RNAs. Because we have shown that discrete vRNA-sense RNAs are not synthesized in the absence of nonnucleocapsid NP, an enzyme system that is independent of nonnucleocapsid NP molecules apparently does not exist for the copying of template RNA into vRNA-sense RNA.

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