Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5' capped end

(nuclear extracts/depletion experiments/ApG dinucleotide)

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ABSTRACT The first step in the replication of influenza virion RNAs is the synthesis of full-length transcripts of these RNAs. The synthesis of these transcripts, or template RNAs, requires: (i) unprimed initiation rather than the capped RNA-primed initiation used during viral mRNA synthesis, and (ii) antitermination at the polyadenylation site used during mRNA synthesis. To determine the mechanism of template RNA synthesis, we prepared nuclear extracts from infected cells that were active in the synthesis of both template RNAs and viral mRNAs. By providing the dinucleotide ApG as primer, we circumvented the inefficient unprimed initiation catalyzed by these extracts and, as a consequence, were able to focus on the antitermination step. Antitermination, and hence template RNA synthesis, occurred when ApG but not a capped RNA was used as primer, indicating that the presence of a 5' capped end blocked antitermination at the 3' end of the transcript. Ultracentrifugation of the nuclear extract yielded a pellet fraction that contained viral nucleocapsids active in viral mRNA synthesis but not template RNA synthesis and a supernatant fraction that contained the antitermination factor. When the supernatant, which had essentially no activity by itself, was added to the pellet in the presence of ApG, template RNA synthesis was restored. Depletion experiments in which this supernatant was incubated with protein A-Sepharose containing antibodies to individual viral proteins demonstrated that the viral nucleocapsid protein was required for antitermination. The implications of these results for the control of viral RNA replication are discussed.

The synthesis of influenza virus mRNA is primed by capped RNA fragments derived from host-cell RNA polymerase II transcripts (1–3). These primers are generated by a viral cap-dependent endonuclease that cleaves RNA polymerase II transcripts 10–13 nucleotides from their 5' ends preferentially at a purine residue. Viral mRNA chains are elongated until a stretch of uridine residues is reached 17–22 nucleotides before the 5' end of virion RNA (vRNA) is reached, where transcription terminates and poly(A) is added to the mRNAs (4, 5). Viral mRNA synthesis is catalyzed by nucleocapsids from purified virions (2), and some of the roles of the constituent nucleocapsid-associated polymerase (P) proteins (PB1, PB2, and PA) have been delineated by previous experiments (6–8).

For replication to occur, an alternative type of transcription is required that results in the production of full-length copies of vRNA. These copies can then serve as the templates for new vRNA. The full-length transcripts, or template RNAs, are initiated without a primer and are not terminated at the poly(A) site used during mRNA synthesis (4, 9). *In vivo*, the switch from viral mRNA to template RNA synthesis

requires the synthesis of one or more virus-specific proteins (9, 10).

To identify these proteins and to determine their roles in the switch from viral mRNA to template RNA synthesis, it is necessary to establish an *in vitro* system that catalyzes the synthesis of template RNA as well as viral mRNA. Previously, we showed that undisrupted nuclei from infected cells synthesize *in vitro* both template RNA and viral mRNA (11). In the present report, we prepared extracts from infected cell nuclei and showed that these extracts were active in at least one of the steps involved in the switch to template RNA synthesis, the antitermination step. This enabled us to demonstrate that the viral nucleocapsid protein (NP) was required for antitermination. Further, we found that this antitermination was dependent on the absence of capped RNA-primed initiation—i.e., transcripts primed with a capped RNA were not antiterminated. The implications of these results for the control of influenza vRNA replication will be discussed.

MATERIALS AND METHODS

Preparation of Nuclear Extracts from Infected Cells. Suspension HeLa cells were infected with 10–20 plaque-forming units per cell of WSN influenza A virus as described (12). At 4 hr of infection, the cells were harvested by centrifugation, and nuclear extracts were prepared as described by Dignam *et al.* (13). Where indicated, the nuclear extract was centrifuged for 3.5 hr at 50,000 rpm in an SW 65 rotor to yield supernatant and pellet fractions. The pellet from 700 μ l of extract was resuspended in 300 μ l of 20 mM Hepes, pH 7.6/120 mM KCl/0.5 mM dithiothreitol/0.2 mM EDTA/20% (wt/vol) glycerol (buffer D).

Transcription Reactions and Analysis of Transcription Products. Transcription reaction mixtures, 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, 2.8 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 5 μ M uridine 5'-[α -thio]triphosphate labeled at the α -thio position with ³⁵S (New England Nuclear; 70 mCi/ μ mol; 1 Ci = 37 GBq). Except where indicated, either 10 μ l of unfractionated nuclear extract or both 10 μ l of the nuclear pellet and 8 μ l of the nuclear supernatant were added. Where indicated, ApG at 0.4 mM or alfalfa mosaic virus (AIMV) RNA 4 (containing a cap 1 m⁷GpppGm 5' end) at 120 μ g/ml was added. Reactions were carried out for 60 min at 30°C. The RNA was extracted and hybridized to an excess of a vRNA-sense phage M13 DNA clone of the segment encoding

Abbreviations: vRNA, virion RNA; NP, nucleocapsid protein; NS, nonstructural protein; P proteins, polymerase proteins; AIMV, alfalfa mosaic virus; ts, temperature sensitive.

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nonstructural protein (NS) as described (11). 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.

Depletion Experiments. Protein A-Sepharose (15 mg) was swollen in 0.5 ml of 50 mM Tris-HCl, pH 8/150 mM NaCl/5 mM EDTA/0.1% Nonidet P-40 (buffer A). The specific antiserum (40–80 μ l) was incubated with the protein A-Sepharose for 18 hr at 4°C in Eppendorf tubes. The protein A-Sepharose was spun out, washed three times with buffer D, then incubated with 80 μ l of the nuclear supernatant for 2 hr at 4°C, and then spun out again; the resulting supernatant was removed and assayed.

Materials. AIMV RNA 4 was provided by John Bol, and its cap 0 structure was converted to a cap 1 structure by using a vaccinia virus enzyme (2). Monoclonal antibodies to the NP and to the NS1 protein were provided as ascites fluids by Robert Webster (14, 15), and polyclonal antiserum to the NS1 protein was supplied by Peter Palese (16).

RESULTS

Template RNA Synthesizing Activity of Infected-Cell Nuclear Extracts With and Without Primers. In our previous experiments (11), we utilized undisrupted crude nuclei from infected cells to catalyze the *in vitro* synthesis of both influenza viral mRNA and template RNA. Our goal was to prepare active extracts from these nuclei to elucidate the mechanism of the switch from viral mRNA to template RNA synthesis and to identify the viral proteins involved in this switch. We used a phage M13 single-stranded DNA specific for transcripts copied off the NS vRNA to measure the NS1 mRNA and NS template RNA synthesized by the nuclear extracts (11). This assay includes a digestion with RNase T2, which removes the poly(A) and the 5' capped primer-donated region from the NS1 mRNA. As a result, the NS template RNA is $\approx$ 20 nucleotides larger than the NS1 mRNA and, therefore, has a slower mobility than the NS1 mRNA during gel electrophoresis.

Extraction of nuclei from infected HeLa cells (4 hr after infection) by the procedure of Dignam *et al.* (13) yielded extracts capable of catalyzing both viral mRNA and template RNA synthesis. In the absence of an added primer, these extracts synthesized only low levels of NS template RNA and NS1 mRNA (Fig. 1, lane 1). Addition of the dinucleotide ApG, which has been shown to act as a primer for viral mRNA synthesis catalyzed by virion nucleocapsids (17, 18), greatly stimulated the synthesis of both NS1 viral mRNA and NS template RNA catalyzed by the nuclear extracts (Fig. 1, lane 2). Thus, ApG served as primer for template RNA as well as for viral mRNA synthesis. The ratio of template RNA to mRNA synthesis remained relatively constant over a range of nuclear extract concentrations (5–15 μ l of extract per 25- μ l assay). These results indicate that these nuclear extracts contained the factor(s) that caused antitermination at the poly(A) site used during viral mRNA synthesis but that these extracts were deficient in unprimed initiation of template RNA synthesis and in the capped primers needed for viral mRNA synthesis. With some preparations of the nuclear extract, however, higher levels of template RNA synthesis were seen in the absence of ApG, suggesting that it should be possible to prepare nuclear extracts that reproducibly initiate template synthesis in the absence of a primer. With the present nuclear extracts, we were able to focus on the antitermination step occurring during template RNA synthesis by adding ApG to the assays to initiate transcription.

In contrast to ApG, when AIMV RNA 4 containing a m⁷GpppGm cap was added as primer, only NS1 mRNA and not NS template RNA was synthesized (Fig. 1, lane 3).

FIG. 1. Synthesis of template RNAs by infected-cell nuclear extracts with and without primers. A nuclear extract from infected HeLa cells was incubated as described in the absence of a primer (lane 1), in the presence of ApG (lane 2), or in the presence of AIMV RNA 4 containing a m⁷GpppGm cap (lane 3). Lane 4 shows a longer exposure of lane 3. The RNA products were analyzed for NS1 mRNA (M) and NS template RNA (T) as described in the text.

Darker exposure of this gel lane also did not show detectable template RNA synthesis (Fig. 1, lane 4). The amount of stimulation of viral mRNA synthesis by the capped RNA primer was similar to that obtained with ApG as primer (compare lanes 2 and 3 in Fig. 1). The lack of template RNA synthesis was not simply due to the presence of a cap-containing species in the reaction because the addition of just the cap (m⁷GpppGm) at 0.2 mM to an ApG-primed reaction stimulated rather than inhibited both viral mRNA synthesis (19, 20) and template RNA synthesis (data not shown). Thus, these results indicate that viral RNA transcripts that initiated with a capped primer were not antiterminated at the poly(A) site by the nuclear factor that antiterminated the ApG-initiated viral transcripts.

Identification of the Viral Protein Required for Antitermination. These nuclear extracts contained the factor(s) that antiterminated ApG-initiated viral transcripts. To determine whether this factor was associated with viral nucleocapsids, the nuclear extract was fractionated by ultracentrifugation to yield a supernatant fraction and a pellet fraction that contained particles larger than 20S, including viral nucleocapsids. The pellet fraction synthesized NS1 viral mRNA but little or no NS template RNA (Fig. 2, lane 1), indicating the virtual absence of the antitermination factor in the pellet. The supernatant fraction (8 μ l) by itself had little activity in either viral mRNA or template RNA synthesis (Fig. 2, lane 2). When the supernatant was added to the pellet, NS template RNA and NS1 mRNA were synthesized (Fig. 2, lane 3), indicating that the supernatant fraction contained the factor that was required for antitermination at the poly(A) site. Optimal synthesis of template RNA occurred with 10 μ l of the resuspended pellet and 8 μ l of the supernatant fraction.

To determine which viral protein was responsible for antitermination, the supernatant fraction was depleted of individual virus-specific proteins by using monospecific antisera, and this depleted supernatant was then added to the pellet fraction and assayed for its ability to antiterminate ApG-initiated transcripts. These depletions were carried out by incubating the supernatant with a specific antiserum that

FIG. 2. Separation of the factor required for antitermination from viral nuclear nucleocapsids. An infected-cell nuclear extract was separated into pellet and supernatant fractions by centrifugation for 3.5 hr at 50,000 rpm in an SW 65 rotor. In the presence of ApG, 10 μ l of the resuspended pellet (lane 1), 8 μ l of the supernatant (lane 2), or 10 μ l of the pellet and 8 μ l of the supernatant (lane 3) were incubated under RNA synthesis conditions, and the amount of synthesis of NS1 mRNA (M) and NS template RNA (T) was determined.

was bound to protein A-Sepharose. The antigen-antibody complexes bound to protein A-Sepharose were then removed from the supernatant by centrifugation. Thus, the resulting depleted supernatant was free of any residual antibody that might react with proteins in the pellet or interfere in any way with viral RNA synthesis during the assays.

Supernatant that had been incubated with protein A-Sepharose containing pooled monoclonal antibodies directed against the viral NP lost its ability to antiterminate, as only NS1 mRNA and not NS template RNA was synthesized when this supernatant was added to the pellet (Fig. 3, lane 4). In contrast, antitermination activity (i.e., NS template RNA synthesis in the presence of the pellet) was retained in supernatants that had been incubated with protein A-Sepharose alone (Fig. 3, lane 3) or with protein A-Sepharose containing pooled monoclonal antibodies directed against the NS1 protein (Fig. 3, lane 5). The supernatant also retained its antitermination activity after incubation with protein A-Sepharose containing either a polyclonal antibody directed against the NS1 protein or a PB1-specific antiserum (data not shown). These results indicate that the NP protein is required for antitermination.

To verify this conclusion, we determined whether incubation of the supernatant with the protein A-Sepharose containing anti-NP antiserum actually selectively removed the NP protein. For these experiments, we used a nuclear supernatant fraction from infected cells labeled with [35 S]methionine. Analysis of the proteins in this supernatant by gel electrophoresis indicated that it contained a complex mixture of viral and cellular proteins (Fig. 4, lane 2). The pooled anti-NP monoclonal antibodies selectively immunoprecipitated NP from the nuclear supernatant (lane 4), and the pooled anti-NS1 monoclonals selectively immunoprecipitated NS1 from the nuclear supernatant (lane 8). After incubation of the nuclear supernatant with protein A-Sepharose containing the anti-NP monoclonal antibodies, essentially no NP remained as detected by immunoprecipitation (Fig. 4, lane 5), indicating that the NP had been effectively removed. Some NS1 was also removed from the nuclear supernatant (Fig. 4, lane 9). The removal of this amount of the

FIG. 3. Requirement of the viral NP protein for template RNA synthesis. The supernatant fraction from an infected-cell nuclear fraction was either untreated (lane 2), incubated with protein A-Sepharose alone (lane 3), incubated with protein A-Sepharose containing pooled NP monoclonal antibodies (lane 4), or incubated with protein A-Sepharose containing pooled NS1 monoclonal antibodies (lane 5). Each of these supernatants was incubated with the nuclear pellet in the presence of ApG, and the amount of synthesis of NS1 mRNA (M) and NS template RNA (T) was determined. Lane 1 shows the products made by the nuclear pellet alone in the presence of ApG.

NS1 protein cannot account for the loss of antitermination activity because incubation of the nuclear supernatant with protein A-Sepharose containing anti-NS1 antibody completely removed the NS1 protein (Fig. 4, lanes 10 and 11) but did not cause concomitant loss of antitermination activity (Fig. 3, lane 5). It also should be noted that the incubation of the nuclear supernatant with protein A-Sepharose containing anti-NS1 antibody did not remove any NP protein (Fig. 4, lanes 6 and 7). Thus, removal of the NP protein from the nuclear supernatant resulted in the loss of the activity that allows read-through (antitermination) at the mRNA poly(A) site and, hence, template RNA synthesis in the presence of the nuclear pellet and ApG.

DISCUSSION

The first step in influenza vRNA replication is the switch from viral mRNA synthesis to the synthesis of the full-length copies of vRNA that then serve as templates for vRNA replication. This switch requires (i) a change from the capped RNA-primed initiation of transcription used during mRNA synthesis to unprimed initiation and (ii) antitermination at the poly(A) site used during mRNA synthesis (4, 9). We have succeeded in developing nuclear extracts from infected cells that carry out the antitermination step *in vitro*. By providing the dinucleotide ApG as a primer, we were able to circumvent the inefficient unprimed initiation catalyzed by these extracts and to focus on the antitermination step. We showed that this step requires the participation of viral NP molecules that are not associated with the viral nucleocapsids present in the pellet fraction of the nuclear extract. In the absence of these NP molecules, the nuclear nucleocapsids, like the nucleocapsids found in purified virions, catalyze the synthesis of only viral mRNAs. Thus, the nuclear nucleocapsids must contain the P proteins that catalyze viral mRNA synthesis. In addition, these nucleocapsids should contain the NP bound to the vRNA, presumably situated at the same 20-nucleotide

FIG. 4. Quantitative removal of the NP protein from the nuclear supernatant by incubation with protein A-Sepharose containing NP antibody. A nuclear extract from infected HeLa cells (labeled with [³⁵S]methionine from 3 to 4 hr after infection) was separated into pellet (lane 1) and supernatant (lane 2) fractions, and the labeled proteins in these two fractions were analyzed by electrophoresis on a 14% polyacrylamide gel. The supernatant was divided into four equal aliquots. One aliquot was immunoprecipitated with either normal rabbit antiserum (lane 3), pooled NP monoclonal antibodies (lane 4), or pooled NS1 monoclonal antibodies (lane 8). The second aliquot was incubated with protein A-Sepharose containing pooled NP monoclonal antibodies, and the resulting supernatant was immunoprecipitated with either pooled NP monoclonal antibodies (lane 5) or pooled NS1 monoclonal antibodies (lane 9). The third aliquot was incubated with protein A-Sepharose containing pooled NS1 monoclonal antibodies, and the resulting supernatant was immunoprecipitated with either pooled NP monoclonal antibodies (lane 6) or pooled NS1 monoclonal antibodies (lane 10). The fourth aliquot was incubated with protein A-Sepharose containing a polyclonal NS1 antibody, and the resulting supernatant was immunoprecipitated with either pooled NP monoclonal antibodies (lane 7) or pooled NS1 monoclonal antibodies (lane 11). The positions of the NP, membrane (M), and NS1 proteins are indicated on the left.

intervals along the vRNA as in virion nucleocapsids (21). Initial analysis of the nuclear pellet indicates that mRNA-synthesizing activity and also template RNA-synthesizing activity (after supplementation with the nuclear supernatant) was, as expected, associated with nucleocapsids containing the P proteins and large amounts of NP (L. Meyers, A.B., B. Broni, R.K., unpublished data). Consequently, there are two populations of NP molecules, one associated with nucleocapsids and one free of nucleocapsids, and the latter population is required for the antitermination step.

The requirement for NP to synthesize template RNA is consistent with the phenotype of a WSN influenza virus temperature-sensitive (ts) mutant, ts56, with a defect in the NP protein. Early experiments indicated that this viral mutant most likely has a ts defect in some step of viral RNA synthesis other than viral mRNA synthesis (22). Using phase M13 clones to assay for the individual steps in viral RNA synthesis in infected cells, we have found recently that shifting ts56-infected cells from the permissive to the nonpermissive temperature leads to the immediate shutoff of

template RNA and of vRNA synthesis but not of mRNA synthesis (G. Shapiro and R.K., unpublished experiments). Most likely, the mutant NP molecules that were not associated with nucleocapsids were unable to antiterminate at the nonpermissive temperature, resulting in no template RNA synthesis and, hence, no vRNA synthesis after the temperature shift-up. It is also possible that NP might have a direct role in vRNA synthesis as well as in template RNA synthesis. Apparently the mutant NP molecules that were already in the nucleocapsids catalyzing viral mRNA synthesis did not cause a rapid inhibition of viral mRNA synthesis after the temperature shift-up.

It will be important to establish the mechanism by which NP causes antitermination. Most likely, the NP protein acts by binding to the viral RNA transcript rather than by binding to the P protein complex catalyzing transcription. It has been shown that template RNAs in infected cells are in the form of nucleocapsids containing NP (4, 23), and we presume that the templates synthesized *in vitro* also become coated with NP to form nucleocapsids. Indeed, with another negative-strand RNA virus, vesicular stomatitis virus, the NP protein is also required for antitermination *in vitro* (24), and the resulting RNAs are in the form of nucleocapsids (24–26). In this case, there is only a single vRNA template that has a termination signal near its 3' end. In the absence of NP protein, RNA synthesis terminates, yielding a small 47-nucleotide-long RNA, and the transcriptase then reinitiates at the cap site of the first downstream mRNA sequence. However, most likely as a consequence of the binding of NP to a sequence in the nascent 47-nucleotide-long RNA, antitermination occurs and a full-length template RNA is made (26). This binding site is also apparently the site for the initiation of nucleocapsid assembly (27). With influenza virus, there are eight vRNA templates, all of which have termination signals at their 5' ends rather than their 3' ends (4, 9). One possibility is that, as with vesicular stomatitis virus, the NP initially binds to the nascent transcripts at a sequence close to the site of termination, both causing antitermination and initiating nucleocapsid assembly. However, the eight viral RNA transcripts do not have a common sequence in this region. An alternative possibility is that NP binds at, or close to, the common 12-nucleotide-long sequence at the 5' ends of the nascent transcripts. Subsequent addition of NP molecules to the growing chains would allow read-through when the termination site is reached.

The latter hypothesis would provide an explanation for our observation that influenza viral RNA transcripts initiated with a capped primer were not antiterminated in the presence of the NP molecules that were active in the antitermination of ApG-initiated transcripts. Perhaps the 5'-terminal cap structure and/or the primer-donated sequence preceding the common 5' sequence of the viral transcripts blocks the binding of the NP protein. An alternative explanation would be that a P transcription complex that initiates with a capped RNA primer might be different from the complex found after unprimed or ApG-primed initiation and that this different structure might not allow recognition of the antitermination signal. In any case, it is clear that the type of initiation used by the transcriptase largely determines whether termination of transcription occurs, which is not the case for vesicular stomatitis virus. The termination of all capped RNA-primed transcripts at the poly(A) site likely ensures that these transcripts, which contain host sequences at their 5' ends, are used only as mRNAs and not as templates for vRNA replication. It is conceivable that if these transcripts had copies of the 5' ends as well as of the 3' ends of the vRNAs, they might inadvertently be recognized as templates by the replicating enzymes. *In vitro*, the uncapped (i.e., ApG-initiated) transcripts were either terminated at the mRNA poly(A) site or were read through to the 5' end of the vRNA

to produce template RNAs, but it is quite likely that *in vivo* essentially all the uncapped (i.e., unprimed) transcripts become template RNAs. The efficiency of antitermination of uncapped transcripts is most probably controlled largely by the amount of available NP protein. Since transcription initiation by ApG *in vitro* is extremely efficient (similar to that by capped RNAs), there is probably insufficient NP in the nuclear supernatant to cause antitermination of all these transcripts, resulting in the production of both mRNA and template RNA. In contrast, unprimed transcription *in vivo* is probably much less efficient, and the amount of NP available in the infected cell may be sufficient to antiterminate all these transcripts.

Our results indicate that the NP protein is required for antitermination but do not rule out the possibility that other viral proteins are also involved. The data do make it unlikely that the NS1 protein participates directly in antitermination. Removal of all detectable NS1 protein from the nuclear supernatant using protein A-Sepharose containing various anti-NS1 antisera did not eliminate antitermination activity. Because the amount of the NP protein was not reduced by this incubation, it is not likely that a stable NP-NS1 complex exists and is involved in antitermination. The removal of some NS1 protein from the nuclear supernatant by the incubation with protein A-Sepharose containing anti-NP is probably due to the stickiness of the NS1 protein and might also occur with other antisera. It is conceivable, however, that NS1 might act indirectly in antitermination (e.g., by modifying the NP protein so that it is then active in antitermination). We have not determined whether the NS2 protein, which has been reported to be in the nucleus (28), has a role in antitermination.

Finally, it should be emphasized that the two NS proteins might be involved in the other step of template RNA synthesis, unprimed initiation. Also, one of the P proteins, PA, may participate in this step. Early experiments indicated that a WSN influenza virus ts mutant, ts53, with a defect in PA, has a phenotype like that of the NP ts56 mutant, namely a defect in some step in viral RNA synthesis other than mRNA synthesis (22). Our recent experiments indicate that the defect is in template RNA synthesis and/or in the subsequent copying of this template into vRNA (G. Shapiro and R.K., unpublished experiments). The determination of the participation of these proteins in unprimed initiation of template RNA synthesis awaits the development of an *in vitro* system that catalyzes this initiation more efficiently.

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1. Bouloy, M., Plotch, S. J. & Krug, R. M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4886-4890.
2. Plotch, S. J., Bouloy, M., Ulmanen, I. & Krug, R. M. (1981) *Cell* **23**, 847-858.
3. Krug, R. M. (1981) *Curr. Top. Microbiol. Immunol.* **93**, 125-150.
4. Hay, A. J., Lomniczi, B., Bellamy, A. H. & Skehel, J. J. (1977) *Virology* **83**, 337-355.
5. Robertson, J. S., Schubert, M. & Lazzarini, R. A. (1981) *J. Virol.* **38**, 157-163.
6. Ulmanen, I., Broni, B. B. & Krug, R. M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7355-7359.
7. Ulmanen, I., Broni, B. & Krug, R. M. (1983) *J. Virol.* **45**, 27-35.
8. Braam, J., Ulmanen, I. & Krug, R. M. (1983) *Cell* **34**, 609-618.
9. Hay, A. J., Skehel, J. J. & McCauley, J. (1982) *Virology* **116**, 517-522.
10. Barrett, T., Wolstenholme, A. J. & Mahy, B. W. J. (1979) *Virology* **98**, 211-225.
11. Beaton, A. R. & Krug, R. M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 4682-4686.
12. Katze, M. G., Chen, Y.-T. & Krug, R. M. (1984) *Cell* **37**, 483-490.
13. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475-1489.
14. van Wyke, K. L., Bean, W. J., Jr., & Webster, R. G. (1981) *J. Virol.* **39**, 313-317.
15. Brown, L. E., Hinshaw, V. S. E. & Webster, R. G. (1983) *Virology* **130**, 130-143.
16. Young, J. F., Desselberger, U., Palese, P., Ferguson, B., Shatzman, A. R. & Rosenberg, M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 6105-6109.
17. McGeoch, D., Fellner, P. & Newton, C. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3045-3049.
18. Plotch, S. J. & Krug, R. M. (1977) *J. Virol.* **21**, 24-34.
19. Bouloy, M., Plotch, S. J. & Krug, R. M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3952-3956.
20. Penn, C. R. & Mahy, B. W. J. (1984) *Virus Res.* **1**, 1-13.
21. Compans, R. W., Content, J. E. & Duesberg, P. H. (1972) *J. Virol.* **10**, 795-800.
22. Krug, R. M., Ueda, M. & Palese, P. (1975) *J. Virol.* **16**, 790-796.
23. Krug, R. M. (1972) *Virology* **50**, 103-113.
24. Patton, J. T., Davis, N. L. & Wertz, G. (1984) *J. Virol.* **49**, 303-309.
25. Patton, J. T., Davis, N. L. E. & Wertz, G. (1983) *J. Virol.* **45**, 155-164.
26. Peluso, R. W. & Moyer, S. A. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3198-3202.
27. Blumberg, B. M., Leppert, M. & Kolakofsky, D. (1981) *Cell* **23**, 837-845.
28. Greenspan, D., Krystal, M., Nakada, S., Arnheiter, H., Lyles, D. S. & Palese, P. (1985) *J. Virol.* **54**, 833-843.