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Influenza Virus Temperature-Sensitive Cap ($m^7GpppNm$)-Dependent Endonuclease

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The first step in influenza viral mRNA synthesis is the endonucleolytic cleavage of heterologous RNAs containing cap 1 ($m^7GpppNm$) structures to generate capped primers that are 10 to 13 nucleotides long, which are then elongated to form the viral mRNA chains. We examined the temperature sensitivity of these steps in vitro by using two WSN virus temperature-sensitive mutants, *ts*1 and *ts*6, which have a defect in the genome RNA segment coding for the viral PB2 protein. For these experiments, it was necessary to employ purified viral cores rather than detergent-treated virions to catalyze transcription, as preparations of detergent-treated virions contain destabilizing or inhibitory activities which render even the transcription catalyzed by wild-type virus temperature sensitive. Using purified wild-type viral cores, we found that the rates of endonucleolytic cleavage of capped primers and of overall transcription were similar at 39.5 and 33°C, the in vivo nonpermissive and permissive temperatures, respectively. In contrast, the activities of the cap-dependent endonucleases of *ts*1 and *ts*6 viral cores at 39.5°C were only about 15% of those at 33°C. The steps in transcription after endonucleolytic cleavage of the capped RNA primer were largely, if not totally, temperature insensitive, indicating that the mutations in the PB2 protein found in *ts*1 and *ts*6 virions affect only the endonuclease step. The temperature-sensitive defect is most likely in the recognition of the 5′-terminal cap 1 structure that occurs as a required first step in the endonuclease reaction: the cap-dependent binding of a specific capped primer fragment to *ts*1 viral cores was temperature sensitive under conditions in which binding to wild-type viral cores was not affected by increasing the temperature from 33 to 39.5°C. Thus, our results establish that the viral PB2 protein functions in cap recognition during the endonuclease reaction.

Influenza viral mRNA synthesis proceeds by the endonucleolytic cleavage of heterologous RNAs containing cap 1 ($m^7GpppNm$) structures to generate capped primers that are 10 to 13 nucleotides long, followed by the initiation of transcription via the incorporation of a GMP residue onto the primers, which are then elongated to form the viral mRNA chains (15). This entire process is catalyzed by purified viral cores (nucleocapsids) (15), which contain the virion RNA segments and four known virus-specific proteins, the nucleocapsid protein NP and the three P proteins (7, 15, 22). The NP protein, which constitutes the majority (about 92%) of the protein in viral cores, probably has primarily a structural role, as it is situated along the virion RNA chains at approximately 20-nucleotide intervals (5). The P proteins, the presumed subunits of the transcriptase, are resolved during two-dimensional gel electrophoresis into two basic species, PB1 (the larger of the two basic P proteins) and PB2 (the smaller basic P protein), and one acidic species, PA (6, 22).

Our goal has been to determine the role of each of the P proteins in the individual steps of viral mRNA synthesis. Using UV light-induced cross-linking, we have obtained evidence that PB2 is the viral protein that recognizes the 5′-terminal cap 1 structure on RNAs and that PB1 is the transcription-initiating protein which adds GMP residues onto the 3′ ends of the primer fragments (22). For the identification of the viral cap-recognizing protein, we UV irradiated endonuclease reactions carried out by viral cores in the absence of ribonucleoside triphosphates, using a primer RNA labeled with ^{32}P in its cap 1 structure. After nuclease digestion, the labeled cap was found to be cross-linked to a protein which had a mobility similar to that of the PB2

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protein in two-dimensional gel electrophoresis. The cross-linked protein and unreacted PB2 protein differed slightly in mobility, presumably because the former contained a few covalently linked nucleotides. The specificity of the cross-linking of PB2 to the labeled cap of the primer RNA was demonstrated by competition experiments in which only those unlabeled RNAs that contained a cap 1 structure blocked this cross-linking. Another group of investigators, using a photoreactive derivative of m^7GTP as an affinity label, showed that the PB2 protein binds cap structures and thus is most probably a cap-recognizing protein (1, 2).

To prove that the topographical relationship between PB2 and the cap 1 structure demonstrated by cross-linking reflects the fact that PB2 actually functions as the cap-recognizing protein during transcription, we employed temperature-sensitive (ts) mutants containing a defect in the virion RNA segment coding for the PB2 protein. Previous experiments examining the temperature sensitivity of the in vitro transcriptase activities of influenza virus ts mutants have been plagued by a major difficulty: the apparent temperature sensitivity of the transcriptase activity of wild-type (wt) virus in vitro. At the temperatures (>39°C) which are nonpermissive for ts virus mutants in vivo, the transcriptase of detergent-treated wt virions usually exhibits in vitro only about 10% of the activity seen at the optimal in vitro temperature (30 to 33°C) (12, 13). Consequently, previous investigations involved attempts to determine whether the transcriptase activity associated with a given ts virus mutant exhibited even less than 10% of the optimal activity at these higher temperatures (>39°C). For this reason, the data and the interpretation of data from previous investigations have been less than conclusive (11–13).

In the present study, we overcame this difficulty by using purified viral cores rather than detergent-treated virions to catalyze transcription. We found that with purified viral cores the cap-dependent endonuclease and the entire transcriptase reaction of wt WSN influenza virus exhibited essentially the same activity at 39.5°C (nonpermissive temperature) as at 33°C (permissive temperature). This enabled us to demonstrate that two WSN virus ts mutants (*ts*1 and *ts*6) with a defect in the virion RNA segment coding for the PB2 protein (14) possess a ts cap-dependent endonuclease, with the ts defect most likely being in cap recognition.

MATERIALS AND METHODS

Virus. The procedures for the growth of WSN wt virus in MDBK cells have been described previously (9). Stocks of the WSN *ts*1 and *ts*6 virus mutants were kindly provided by Peter Palese. In initial experiments, these two ts mutants were grown in MDBK cells at 33°C (permissive temperature), using plaque-purified virus as the inoculum, as previously described (21). Subsequently, we found that with these two ts virus mutants it was possible to grow large amounts of virus which retained unaltered ts characteristics by using as an inoculum plaque-purified virus which had been passaged one time in MDBK cells. A virus stock was prepared by inoculating about 5×10^7 MDBK cells with the virus present in a single plaque (from a 33°C plaque assay) and incubating the infected cells for 40 h at 33°C. This stock was used to infect a larger number of MDBK cells at a multiplicity of 10^{-2} to 10^{-3} PFU (33°C) per cell for 40 h at 33°C. The infectivities of all ts virus yields were determined by plaque assays in MDBK cells at 33 and 39.5°C. All ts virus preparations used in this study had titers at 39.5°C which were 10^{-3} to 10^{-5} those at 33°C, whereas the wt virus had approximately equal titers at 39.5 and 33°C. The wt and ts virus yields were purified as described previously (9); this involved two successive bandings in sucrose-D_2O density gradients as the final steps in purification.

Purification of viral cores. Viral cores were prepared by treating purified virus with lysolecithin, followed by sedimentation in glycerol gradients to separate the cores from membrane components (18, 22). As described previously, viral cores purified in this manner contain essentially only four proteins, the viral NP protein and three P proteins (22). The protein concentrations in viral core preparations were determined by using Coomassie brilliant blue (19).

Assays for viral endonuclease and for transcription. The viral endonuclease was assayed by incubating either viral cores or detergent-treated virus with alfalfa mosaic virus (AlMV) RNA 4 containing ^{32}P in its cap 1 structure in the absence of ribonucleoside triphosphates (15). Incubation in a final volume of 50 μl was at 33 or 39.5°C for the times indicated below. The reaction mixture was extracted with phenol, and the labeled cleavage products were separated from the uncleaved AlMV RNA 4 by electrophoresis on 20% acrylamide–7 M urea gels. Transcription with either viral cores or detergent (lysolecithin)-treated virus was performed in a 50-μl reaction mixture containing the four ribonucleoside triphosphates, using as a primer AlMV RNA 4 containing a cap 1 structure (2 μg) (3, 4). The labeled precursor was [α-^{32}P]CTP at a concentration of 30 μM, and incubation was at 33 or 39.5°C, as indicated below. The amount of radioactivity incorporated was determined by precipitating the RNA with trichloroacetic acid and counting the precipitated RNA on glass fiber filters in a scintillation counter. Where indicated, the RNA products were extracted with phenol-chloroform at pH 9, the polyadenylic acid-containing RNA was selected on oligodeoxythymidylic acid cellulose columns, and this RNA after enzymatic deadenylylation was analyzed by electrophoresis on 3% acrylamide–6 M urea gels (16).

Assay for the binding of primer fragments to viral cores. The ^{32}P-cap-labeled fragment of AlMV RNA 4 cleaved at the adenosine residue 13 nucleotides from the cap (A13 fragment) was prepared as described previously (22). This fragment was incubated with viral cores at 33 or 39.5°C for the times indicated below. As indicated below, the reaction mixture either contained no ribonucleoside triphosphates, contained

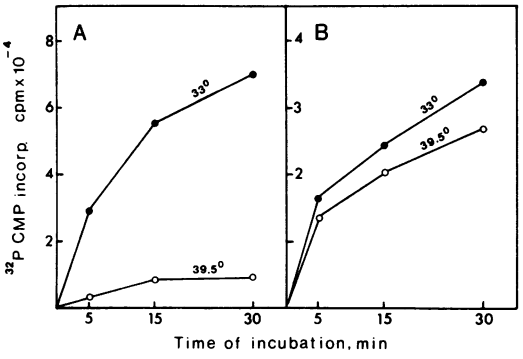

FIG. 1. Effect of temperature on in vitro transcription catalyzed by wt virus, using detergent-treated virions (A) or purified viral cores (B). Detergent-treated virus (20 μg of protein) or purified viral cores (4 μg of protein) were incubated for different times at 33°C (●) or 39.5°C (○) under the transcription conditions described in the text, using [α-^{32}P]CTP (6 mCi/μmol) as the labeled precursor. The amount of acid-precipitable radioactivity incorporated per 50-μl reaction mixture is shown.

only 0.5 mM GTP, or contained ATP, GTP, and CTP (each at a concentration of 0.5 mM). The reaction mixture was subjected to centrifugation on a 15 to 40% glycerol gradient to separate viral cores from unbound primer fragments, and the amount of radioactivity in each gradient fraction was determined, as described previously (22). After phenol extraction, the free and bound primer fragments were analyzed by electrophoresis on 20% acrylamide–7 M urea gels.

Materials. [α-^{32}P]CTP was purchased from New England Nuclear Corp. and Amersham Corp. AlMV RNA 4 was kindly provided by John Bol.

RESULTS

wt virus: lack of temperature sensitivity of transcription enzymes in purified viral cores. Our first goal was to determine conditions under which the transcription enzymes of wt WSN influenza virus exhibited similar activities at 39.5 and 33°C, the in vivo nonpermissive and permissive temperatures, respectively. When detergent-treated wt virions are used to catalyze transcription, the rate of viral RNA transcription at 39.5°C is only about 10% of the rate at 33°C (Fig. 1A), as previously observed by others (12, 13). In contrast, when viral cores purified from wt virions are used, the rates of viral RNA transcription are similar at 39.5 and 33°C (Fig. 1B). In several experiments, the rate of transcription catalyzed by wt viral cores at 39.5°C ranged from 70 to 110% (average, 95%) of the rate at 33°C. This lack of temperature sensitivity was observed with all amounts of wt viral cores tested, from 0.6 to 10 μg of protein per 50-μl reaction mixture. These results strongly suggest that the apparent temperature sensitivity of the transcriptase activity of detergent-treated wt virions is due to the presence of destabilizing or inhibitory activities associated with the viral membrane. We have found that detergent-treated wt virions contain an undefined nuclease which is more active at 39.5 than at 33°C and which degrades the primer fragments generated by the viral cap-dependent endonuclease (data not shown). Little or none of this undefined nuclease activity was detected in purified wt viral cores, indicating that it was largely eliminated during purification of the cores.

ts mutants with a defect in the PB2 protein have a ts cap-dependent endonuclease. Because viral cores from wt virus exhibit little or no temperature sensitivity in in vitro transcription, we could meaningfully examine the effect of temperature on the rate of the transcription reaction catalyzed by viral cores purified from viral ts mutants, specifically the two WSN virus ts mutants *ts*1 and *ts*6, each of which contains a defect in the virion RNA segment coding for PB2 (14). As shown in Fig. 2, in vitro transcription catalyzed by *ts*6 viral cores was markedly temperature sensitive: the rate and extent of transcription at 39.5°C were about 15 to 20% of the rate and extent of transcription at 33°C. This temperature sensitivity was observed with 5 μg or less of viral core protein per 50-μl reaction mixture (i.e., in the concentration range at which the rate and extent of transcription at 33°C was directly proportional to the amount of ts [or wt] viral cores added to the reaction mixture). Similar results were obtained with *ts*1 viral cores.

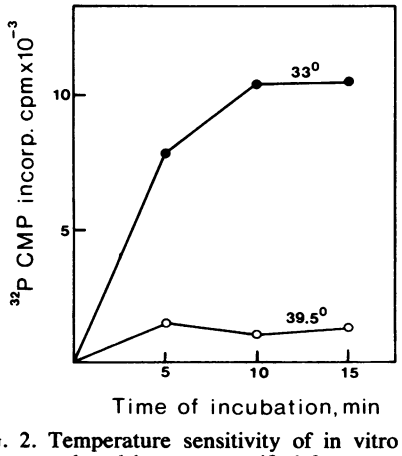

FIG. 2. Temperature sensitivity of in vitro transcription catalyzed by cores purified from *ts*6 virus. Purified *ts*6 viral cores (2.5 μg of protein) were incubated for different times at 33°C (●) or 39.5°C (○) under transcription conditions, using [α-^{32}P]CTP (6 mCi/μmol) as the labeled precursor. The amount of acid-precipitable radioactivity incorporated per 50-μl reaction mixture is shown.

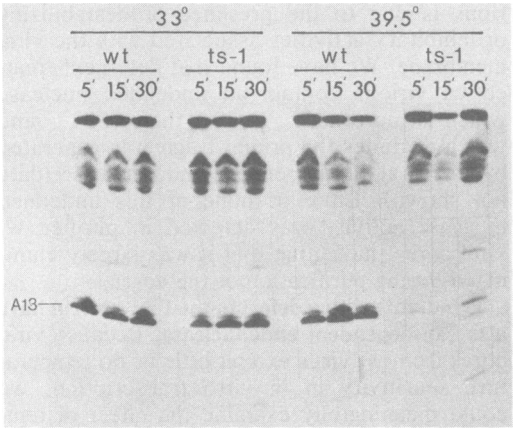

FIG. 3. Effect of temperature on the endonuclease reaction catalyzed by purified viral cores from wt and *ts*1 viruses. Viral cores (3.5 μg of protein) from wt of *ts*1 virus were incubated with ^{32}P-cap-labeled AlMV RNA 4 (5,000 cpm) for different times at 33 or 39.5°C under endonuclease assay conditions (in the absence of ribonucleoside triphosphates). The labeled cleavage products were analyzed on 20% acrylamide–7 M urea gels. The position of the A13 fragment is indicated. The labeled RNAs that migrated between intact AlMV RNA 4 (which remained at the origin) and the A13 cleavage product are the partial degradation products of AlMV RNA 4 present in the unreacted ^{32}P-labeled AlMV RNA 4 preparation. These RNAs are also substrates for the endonuclease, as they along with the full-length AlMV RNA 4 were cleaved when an excess of wt viral cores was used in the reaction.

To determine whether the temperature sensitivity of overall transcription reflected a ts endonuclease, we carried out endonuclease assays at 33 and 39.5°C in the absence of ribonucleoside triphosphates, using ^{32}P-cap-labeled AlMV RNA 4 as the substrate. As shown in Fig. 3, wt viral cores generated approximately the same amount of the specific A13 cleavage product (the capped fragment cleaved at the adenosine residue 13 nucleotides from the cap) at each time point at 33 and 39.5°C, indicating that the cap-dependent endonuclease in wt viral cores is not temperature sensitive. In contrast, *ts*1 viral cores clearly catalyzed very little specific cleavage at 39.5°C. Similar results were obtained with *ts*6 viral cores. Thus, both *ts*1 and *ts*6 viral cores have a ts cap-dependent endonuclease.

If the temperature sensitivity of the endonuclease is the cause of the temperature sensitivity of the overall transcription rate, then we would expect that preincubation of ts viral cores with a primer RNA at 33°C in the absence of ribonucleoside triphosphates would generate enough primer fragments so that a subsequent transcription reaction (in the presence of the four ribonucleoside triphosphates) at 39.5°C would proceed at a rate approaching the rate observed at 33°C. As shown in Fig. 4, preincubation at 33°C did relieve most of the inhibition of transcription observed at 39.5°C. The rate of transcription during the first 5 min after the shift-up to 39.5°C was similar to the rate in the reaction maintained at 33°C after the addition of triphosphates. Subsequently, the rate of transcription at 39.5°C decreased relative to the rate at 33°C. We presume that this was due to the fact that further specific cleavage of the primer RNA was greatly reduced during the transcription reaction after the shift-up to 39.5°C, but would have continued at a high rate in the reaction maintained at 33°C throughout. The viral RNA transcripts synthesized by either ts or wt viral cores after a shift-up to 39.5°C and the addition of triphosphates contained polyadenylic acid, and after enzymatic deadenylylation these transcripts distributed into the eight viral mRNA species during gel electrophoresis (data not shown). These results indicate that the transcription steps after cleavage of the primer RNA catalyzed by these two

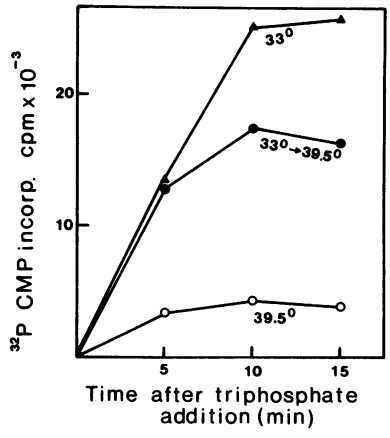

FIG. 4. Effect of a prior endonuclease reaction at 33°C on subsequent transcription at 39.5°C catalyzed by *ts*6 viral cores. *ts*6 viral cores (3 μg) were preincubated with unlabeled (cap 1-containing) AlMV RNA 4 (2 μg) for 15 min at 33 or 39.5°C under endonuclease assay conditions (in the absence of ribonucleoside triphosphates). To the 39.5°C endonuclease reaction mixture (○) and to one 33°C endonuclease reaction mixture (▲), the four ribonucleoside triphosphates (1 mM ATP, 1 mM GTP, 1 mM CTP, and 30 μM [α-^{32}P]UTP [6 mCi/μmol]) were added, and incubation was continued at the same temperature. A second 33°C endonuclease reaction mixture was first shifted up to 39.5°C, the four ribonucleoside triphosphates were rapidly added, and incubation was continued at 39.5°C (●). At different times after the addition of ribonucleoside triphosphates, the amounts of acid-precipitable radioactivity in samples of each reaction mixture were determined. The amount of incorporation per 50-μl reaction mixture is shown.

virus mutants are largely, if not totally, temperature insensitive.

ts defect is most likely in cap recognition. The ts defect in the viral cap-dependent endonuclease could reflect a ts defect in cap recognition or in some other step in the endonuclease reaction (e.g., in the cleavage of the primer RNA at a position 10 to 13 nucleotides from the cap). To differentiate between these possibilities, we supplied the viral cores with an already cleaved primer fragment, the A13 fragment of AlMV RNA 4. This primer fragment labeled with ^{32}P in its cap 1 ($m^7G\overset{*}{p}ppGm$) structure was incubated with ts or wt cores at 33 or 39.5°C in the absence of ribonucleoside triphosphates, and the fraction of the labeled fragment that cosedimented with the viral cores in glycerol gradients was determined. We have shown previously that the binding of such a fragment to viral cores requires the presence of a fully methylated cap structure in the fragment (22).

Unexpectedly, not only with the ts (either *ts*1 or *ts*6) viral cores but also with the wt viral cores, the fraction of the capped A13 fragment bound at 39.5°C was reduced relative to the fraction bound at 33°C (Fig. 5A and B). The ratio of the amount bound at 39.5°C to the amount bound at 33°C was approximately the same for wt and ts viral cores. This ratio increased with increasing amounts of viral cores added to the reaction mixture, so that at 15 μg of wt viral core protein per 50-μl reaction mixture the amount bound at 39.5°C was 80% of the amount bound at 33°C (Fig. 5C and D). The only significant difference that we detected between wt and ts viral cores was that per microgram of protein the wt cores had about 30% higher binding activities than ts cores at both 33 and 39.5°C. This assay actually measures the equilibrium state (or steady state) between the binding of capped fragments to cores and the dissociation of these fragments from cores. The assay detects a smaller fraction of the capped A13 fragments bound to wt cores at 39.5 than at 33°C not because there is less binding at 39.5°C, but because there is increased dissociation at 39.5°C. This was observed most clearly in experiments in which intact ^{32}P-cap-labeled AlMV RNA 4 was added to wt viral cores at 33 or 39.5°C in the absence of ribonucleoside triphosphates. The wt viral cores cleaved the AlMV RNA 4 at the same rate at 33 and 39.5°C (Fig. 3), indicating that binding and cleavage of AlMV RNA 4 by wt cores occur at about equal rates at these two temperatures. However, most (about 75%) of the resulting A13 fragment remained bound to wt cores (6 μg) at 33°C, whereas only about 20% remained bound at 39.5°C. The ratio of the amounts of the A13 fragment which remained bound to the wt cores at these two temperatures was similar to the ratio observed when the isolated A13 fragment was added directly to the same amount (6 μg) of wt viral cores (Fig. 5C).

Consequently, to determine whether there is a ts defect in the binding of capped primer fragments to ts viral cores, it was necessary to modify the binding assay to reduce the dissociation of primer fragments from wt viral cores at 39.5°C to the same low level that occurred at 33°C. The increased dissociation of capped primer fragments from wt viral cores at 39.5°C detected in the absence of ribonucleoside triphosphates (Fig. 5) almost certainly did not occur when binding, endonucleolytic cleavage, and chain elongation were coupled during tran-

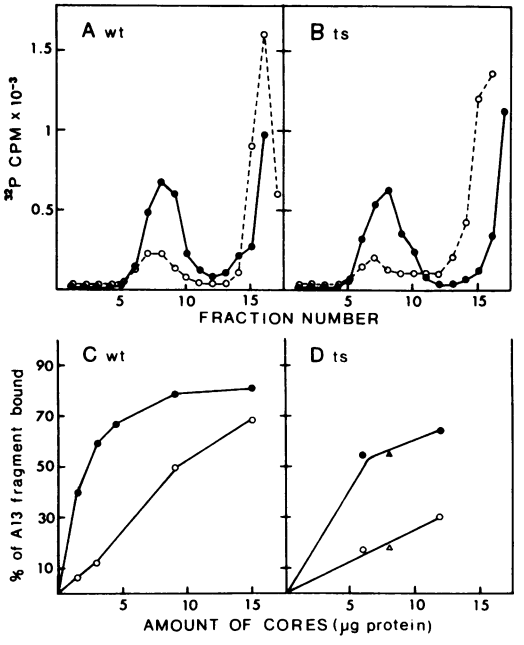

FIG. 5. Effect of temperature on the amount of the A13 fragment of AlMV RNA 4 bound to wt and ts (*ts*1 and *ts*6) cores under endonuclease conditions (in the absence of ribonucleoside triphosphates). The ^{32}P-cap-labeled A13 fragment of AlMV RNA 4 was incubated for 15 min at 33 or 39.5°C with wt or ts (*ts*1 or *ts*6) cores, and the reaction mixture was subjected to gradient centrifugation to separate viral cores (i.e., gradient fractions 5 to 11 [A and B]) from unbound A13 fragments (top of the gradient). Sedimentation was from right to left. (A and B) Results obtained with one level of wt (4 μg) and *ts*6 (6 μg) viral cores. Symbols: ●, 33°C; ○, 39.5°C. (C) Precentage of the A13 fragment bound to increasing amounts of wt viral cores at 33°C (●) or 39.5°C (○). (D) Percentages of the A13 fragment bound to increasing amounts of *ts*6 (○ and ●) and *ts*1 (△ and ▲) at 33°C (● and ▲) and 39.5°C (△ and ○). Approximately the same amount of 32P-cap-labeled A13 fragment of AlMV RNA 4 was added to each binding assay.

scription in the presence of the four ribonucleoside triphosphates, as overall transcription catalyzed by wt viral cores occurred at essentially the same rate at 33 and 39.5°C (Fig. 1B). This suggested the possibility that dissociation of primer fragments from wt (and ts) viral cores at 39.5°C could be reduced to the level occurring at 33°C by the transcriptase-catalyzed addition of a few nucleotides to the 3′ end of the primer fragment. To test this possibility, we carried out a binding assay with the capped A13 fragment and wt viral cores at 33 and 39.5°C under the following conditions: in the absence of ribonucleoside triphosphates; in the presence of only GTP; and in the presence of ATP, GTP, and CTP (no UTP) (Fig. 6A and B). In the presence of only GTP, one or a few guanine residues are added to the 3′ end of the primer fragment (15, 22), and in the reaction lacking UTP the viral mRNA chains are elongated only up to the first adenosine residue in the virion RNA templates (12 to 19 nucleotides from the 3′ end of the vRNA [17, 20]) (data not shown). Under both of the latter two conditions, the amounts of the A13 fragment bound to wt viral cores at 33 and 39.5°C were the same (the results with GTP alone are not shown in Fig. 6), indicating that by adding a few nucleotides to the 3′ end of the A13 primer fragment the binding of this fragment to wt viral cores at 39.5°C was stabilized so that dissociation was no greater than at 33°C.

In contrast to wt viral cores, the amount of the A13 primer fragment bound to *ts*1 viral cores in the presence of ATP, GTP, and CTP at 39.5°C remained substantially lower than the amount bound at 33°C (Fig. 6D). This most likely reflected the fact that there was less binding of primer fragments by *ts*1 viral cores at 39.5 than at 33°C. However, it first had to be established that, as is the case with wt viral cores, the transcriptase-catalyzed addition of a few nucleotides by *ts*1 viral cores at 39.5°C was capable of effectively reducing the dissociation of primer fragments at 39.5°C. To establish this, the capped A13 fragment was incubated with *ts*1 viral cores at 33°C, the temperature was shifted up to 39.5°C, and ATP, GTP, and CTP were then rapidly added (Fig. 7). The amount of binding was compared with the amount that occurred in reactions maintained at 33 or 39.5°C before and after the addition of ATP, GTP, and CTP. Approximately the same amount of bound A13 fragment (binding carried out at 33°C) was found whether the subsequent addition of a few nucleotides in the presence of ATP, GTP, and CTP occurred at 33 or 39.5°C (Fig. 7A and B). Without the addition of these three ribonucleoside triphosphates, a substantial fraction (about 60 to 80%) of the primer fragments bound at 33°C dissociated after the shift-up to 39.5°C (data not shown). The same requirement for triphosphate addition to inhibit dissociation after a shift-up to 39.5°C was observed with wt viral cores. Consequently, the relative amounts of the capped A13 fragment bound to *ts*1 viral cores at 33 and 39.5°C in the presence of ATP, GTP, and CTP (Fig. 6D) can be taken to represent the relative binding efficiencies of this primer fragment to *ts*1 viral cores at these two temperatures. Thus, this binding is

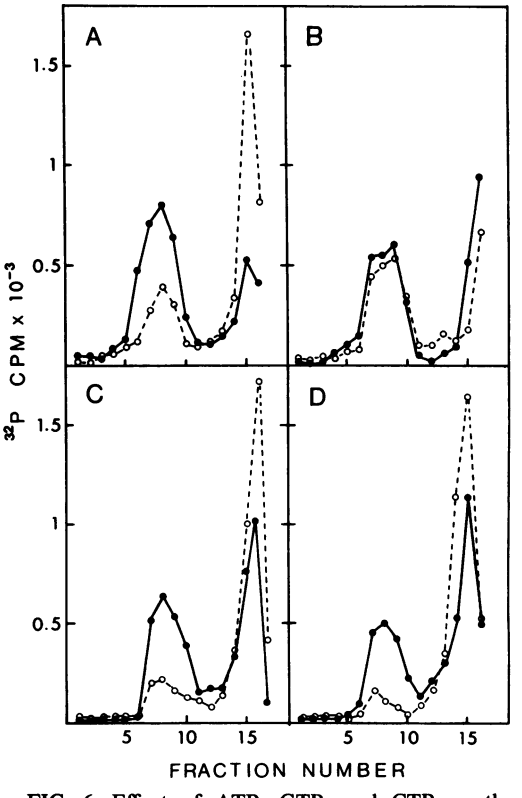

FIG. 6. Effect of ATP, GTP, and CTP on the amount of the A13 fragment of AlMV RNA 4 bound to wt and *ts*1 viral cores at the permissive (33°C) and nonpermissive (39.5°C) temperatures. Purified wt viral cores (4 μg) were incubated with ^{32}P-cap-labeled A13 fragment for 15 min at 33°C (●) or 39.5°C (○) in the absence of ribonucleoside triphosphates (A) or in the presence 0.5 mM ATP, 0.5 mM GTP, and 0.5 mM CTP (B). *ts*1 viral cores (3 μg) were incubated similarly at 33°C (●) or 39.5°C (○) in the absence of ribonucleoside triphosphates (C) or in the presence of ATP, GTP, and CTP (D). The reactions were analyzed on glycerol gradients as described in the text. Sedimentation was from right to left. A gel electrophoretic analysis (20% acrylamide–7 M urea) of the capped fragments bound to wt and ts cores in the presence of ATP, GTP, and CTP (B and D) indicated that most of the fragments were elongated (i.e., about 10 to 20 nucleotides larger than the input A13 fragment); the capped fragments remaining at the top of the gradient were predominantly the input A13 fragment.

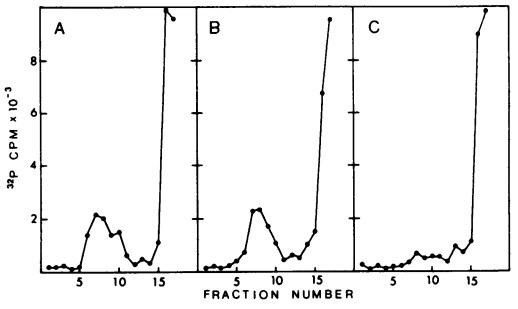

FIG. 7. Reduction of primer fragment dissociation from *ts*1 viral cores at 39.5°C by the transcriptase-catalyzed addition of a few nucleotides by *ts*1 cores at 39.5°C. Purified *ts*1 viral cores (3 μg) were preincubated with the ^{32}P-cap-labeled A13 fragment of AlMV RNA 4 for 15 min at 33°C (A and B) or 39.5°C (C) in the absence of ribonucleoside triphosphates. To the 39.5°C reaction mixture (C) and to one of the 33°C reaction mixtures (A), 0.5 mM ATP, 0.5 mM GTP, and 0.5 mM CTP were added, and incubation was continued at the same temperature for 15 min. The second 33°C reaction mixture (B) was first shifted up to 39.5°C; then ATP, GTP, and CTP were rapidly added, and incubation was continued for 15 min at 39.5°C. The reactions were analyzed on glycerol gradients, with sedimentation from right to left. A gel electrophoretic analysis indicated that most of the capped fragments bound to *ts*1 cores were elongated and most of the capped fragments reamining at the top of the gradient were the input A13 fragment.

temperature sensitive, in contrast to the binding observed with wt virus (Fig. 6B and D).

DISCUSSION

The two WSN influenza virus ts mutants used in the present study (*ts*1 and *ts*6) have been shown to have a ts defect in the genome RNA segment coding for the PB2 protein (14). By our analysis of the temperature sensitivity of the in vitro transcription reaction catalyzed by viral cores from these two ts virus mutants and from wt virus, we have established that the viral PB2 protein is a functional constituent of the cap-dependent endonuclease, the enzyme which generates the short capped primers that initiate viral mRNA synthesis. The activity of the endonuclease of ts viral cores at 39.5°C is only about 15% of the activity at 33°C, whereas the endonuclease activities of wt viral cores are essentially the same at 39.5 and 33°C. With the ts viral cores, the steps in transcription after the endonucleolytic cleavage of the capped RNA primer are largely, if not totally, temperature insensitive, indicating that the mutations in the PB2 protein found in *ts*1 and *ts*6 virions affect only the endonuclease step in transcription.

In fact, our results strongly suggest that the PB2 protein functions in the recognition of the 5′-terminal cap 1 structure that occurs as a required first step in the endonuclease reaction. Specifically, we have shown that the cap-dependent binding of a specific primer fragment (the A13 fragment of AlMV RNA 4) to ts viral cores is temperature sensitive under conditions in which binding to wt viral cores is not affected by increasing the temperature from 33 to 39.5°C. These experiments were complicated by the observation that there was a large increase in the dissociation of primer fragments from wt viral cores during binding assays carried out in the absence of ribonucleoside triphosphates as a result of increasing the temperature from 33 to 39.5°C. Therefore, it was necessary to devise a modified binding assay in which the dissociation of primer fragments from wt (and also ts) viral cores at 39.5°C was reduced to the low level occurring at 33°C. This was accomplished by allowing the transcriptase to add a few nucleotides to the 3′ end of the primer fragments by providing either only GTP or three ribonucleoside triphosphates (ATP, GTP, and CTP) to the binding assay. Under these conditions (i.e., in the presence of ATP, GTP, and CTP), the dissociation of primer fragments from both wt and *ts*1 viral cores at 39.5°C was effectively reduced to the level occurring at 33°C. Consequently, the relative amounts of the A13 fragment bound to wt and ts viral cores at 33 and 39.5°C in the presence of ATP, GTP, and CTP should indicate the binding efficiencies of this fragment to wt and ts cores at these two temperatures. The binding of the A13 primer fragment to *ts*1 viral cores, but not to wt viral cores, is temperature sensitive under these conditions. Because the binding of primer fragments to viral cores is completely dependent on the presence of a cap 1 structure (22), the temperature sensitivity in primer fragment binding exhibited by *ts*1 viral cores most likely reflects temperature sensitivity in recognition of the cap 1 structure. Our experiments did not address the question of the temperature sensitivity of the cleavage reaction, so that it is not yet known whether PB2 not only recognizes the cap 1 structure on the primer RNA but also cleaves this RNA 10 to 13 nucleotides from the cap.

We chose to examine the temperature sensitivity of the binding of the A13 fragment of AlMV RNA 4 to viral cores because this fragment is an actual intermediate in the transcriptase reaction (15) and hence would be expected to bind to the site(s) on the viral cores normally used during the transcription occurring in the presence of a capped RNA primer. Consistent with this expectation, the A13 fragment is elongated to form completed viral mRNA chains in the presence of the four ribonucleoside triphosphates (22). Also, the A13 fragment binds to wt

viral cores at 30 to 33°C with high efficiency, comparable to the efficiency observed in experiments in which intact AlMV RNA 4 is added to the reaction mixture and cleavage is coupled to binding (22). In contrast, the cap 1 structure alone ($m^7GpppGm$) or the cap 1 structure containing only one or two additional nucleotides (e.g., $m^7GpppGmpUp$) binds to detergent-treated wt virions or wt viral cores with low efficiency at 30 to 33°C (at least 100-fold less than the efficiency observed with the A13 fragment) (8, 22), although these smaller cap-containing species also bind to the PB2 protein (1, 2). At 30 to 33°C, about seven nucleotides attached to the cap are needed for efficient binding to wt viral cores (22). This effect of chain length probably reflects a phenomenon similar to the phenomenon observed in the present study, namely the reduction in the extent of dissociation of cap-containing species from wt viral cores by increased chain length. At 30 to 33°C, seven or more nucleotides attached to the cap are apparently needed to inhibit dissociation, whereas at 39.5°C more than 13 nucleotides are required. The increase in length of the primer fragment beyond 13 nucleotides involves the action of other P proteins (certainly PB1, which adds the initial GMP residue onto the primer fragment [22], and possibly PA), and it is likely that it is the interaction with one or both of these other P proteins that at 39.5°C effectively reduces the dissociation of the capped primer fragment from its interaction with PB2 in wt and ts viral cores.

It was necessary to employ purified viral cores rather than detergent-treated virions as a source of transcription enzymes in order to establish the ts transcription defect of the *ts*1 and *ts*6 virus mutants. Preparations of detergent-treated virions contain destabilizing activities or inhibitory activities or both, which render even the transcription catalyzed by wt virus temperature sensitive. When detergent-treated virions are used, the cap-dependent endonuclease and overall transcription activities of wt virus at 39.5°C are only 10 to 15% of the activities at 33°C. We have found that detergent-treated wt virions contain an undefined nuclease which is more active at 39.5 than 33°C and which degrades the primer fragments generated by the viral cap-dependent endonuclease. This nuclease activity and other destabilizing activities or inhibitory activities or both are largely, if not entirely, removed during the purification of viral cores, as purified wt viral cores exhibit essentially the same cap-dependent endonuclease and overall transcription activities at 39.5 and 33°C. Consequently, only experiments in which purified viral cores are used can yield interpretable results about the temperature sensitivity of in vitro transcription catalyzed by ts virus mutants.

The results of our experiments with the *ts*1 and *ts*6 virus mutants verify one of the conclusions from our previous UV cross-linking experiments (22). These experiments showed that the PB2 protein is closely associated with, and hence capable of being cross-linked to, the labeled cap 1 structure of a primer RNA during the endonuclease reaction. This close association was competitively inhibited by preincubating the viral cores with an excess of an unlabeled capped RNA, but not by preincubating the cores with an excess of an unlabeled RNA lacking a cap. Based on these results, we concluded that PB2 functions as the viral cap-recognizing protein during transcription. This conclusion was verified by the demonstration in the present study that a ts mutation in the PB2 protein results in a ts cap-dependent endonuclease, most likely reflecting a ts defect in cap recognition. This verification gives us confidence in the ability of UV cross-linking experiments to discern the functions of proteins in RNA synthesis and hence indirectly provides support for the other major conclusion of our previous UV cross-linking experiments, namely, that PB1 catalyzes the initiation of transcription via the incorporation of a GMP residue onto the 3′ end of the primer fragment (22).

The *ts*1 and *ts*6 WSN virus mutants also behave as ts transcription mutants in vivo (10). Little or no viral mRNA synthesis was detected in infected cells maintained at 39.5°C throughout infection. When cells infected by these mutants were shifted up to 39.5°C after several hours of incubation at 33°C, net viral mRNA synthesis immediately ceased. The in vivo and in vitro results are thus in close agreement. It will be very interesting to establish the mechanism by which these mutants complement other transcriptase mutants (e.g., mutants defective in the PB1 protein) in vivo at 39.5°C (10, 21). In order for complementation to occur, viral mRNAs must be produced at 39.5°C by both of the input ts virus mutants at 39.5°C. This could conceivably involve reciprocal exchange of PB1 and PB2 proteins between the two input transcriptase complexes in the infected cells.

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