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Vaccinia Virions Lacking the RNA Helicase Nucleoside Triphosphate Phosphohydrolase II Are Defective in Early Transcription

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Temperature-sensitive mutations (*ts*10, *ts*18, and *ts*39) of the vaccinia virus RNA helicase nucleoside triphosphate phosphohydrolase II (NPH-II) result in the production of noninfectious progeny virions at the restrictive temperature. The noninfectious mutant particles contain the wild-type complement of virion core and envelope polypeptides, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The results of Western blot (immunoblot) analysis indicate that these particles lack NPH-II, whereas other enzymatic components of the virus core are present. These components include the following: DNA-dependent RNA polymerase subunits rpo147, rpo132, rpo94, rpo35, rpo30, rpo22, and rpo18; early transcription initiation factor subunits A8 and D6; mRNA capping enzyme subunits D1 and D12; RNA cap 2′-*O*-methyltransferase; A18 DNA helicase; DNA-dependent ATPase NPH-I; and DNA topoisomerase. Although RNA polymerase is encapsidated by the mutant viruses, mRNA synthesis in vitro by permeabilized mutant virions is only 5 to 20% that of the wild-type virus, as judged by nucleoside monophosphate incorporation into acid-insoluble material. Moreover, the transcripts synthesized by the mutant particles are longer than normal and remain virion associated. Transcription initiation by mutant virions occurs accurately at an endogenous genomic promoter, albeit at reduced levels (1 to 7%) compared with that of wild-type virions. In contrast, extracts of the mutant virions catalyze the wild-type level of transcription from an exogenous template containing an early promoter. We conclude that NPH-II is required for early mRNA synthesis uniquely in the context of the virus particle. Possible roles in transcription termination and RNA transport are discussed.

Vaccinia virus early mRNAs are synthesized by virus-encoded enzymes encapsidated within the infectious particle (29). The transcription machinery is activated upon entry of the virus into the cytoplasm of the host cell. This activation process can be mimicked in vitro by treating purified virions with a reducing agent and a nonionic detergent. So-called permeabilized virions will, when provided with nucleoside triphosphates (NTPs), magnesium, and *S*-adenosylmethionine, produce functional early mRNAs that are capped, methylated, polyadenylated, and extruded from the virus core into solution. A remarkable property of this complex reaction is its absolute dependence on the hydrolysis of ATP to ADP (12, 23, 39). Two long-standing preoccupations in this field have been to define the energy-dependent steps and to ascertain which of the several virus-encoded ATPases are required for energy coupling.

The energy requirement can be recapitulated during transcription of linear DNA templates in an in vitro system consisting of partially purified vaccinia virus RNA polymerase and purified accessory factors. ATP hydrolysis is essential during transcription initiation at an early promoter (19, 25) and again during transcription termination (17). ATP hydrolysis during promoter-dependent initiation is catalyzed by early transcription factor (ETF), a heterodimer of 82- and 70-kDa subunits. The ATPase of ETF is nucleic acid dependent; duplex DNA is the preferred cofactor (4, 25). Termination of early transcription occurs downstream of a UUUUUNU termination signal in the nascent mRNA (37). Termination signaling requires two accessory factors: vaccinia virus termination factor, which is identical to the mRNA capping enzyme (27, 36), and nucleoside triphosphate phosphohydrolase I (NPH-I), which is a 68-kDa DNA-dependent ATPase (7). NPH-I and the 70-kDa subunit of ETF are both members of the DExH family of nucleic acid-dependent nucleoside triphosphatases (NTPases) (14, 24). Neither ETF nor NPH-I has been reported to possess helicase activity.

There is reason to suspect that the reconstituted in vitro system does not fully reflect the complexity of the virion transcription reaction. For example, in the reconstituted system, a single promoter directs initiation on a short naked linear DNA template, and elongation is unimpeded by DNA structure or topology. However, in the virion, a minimum of 40 genes are being transcribed simultaneously (23), in convergent or divergent orientation, from the 192-kbp DNA genome (22), which is presumably not naked but condensed and coated with DNA-binding proteins. Whereas the reconstituted system has been refined to study only a single round of transcription (17, 26), the virion system reiteratively transcribes early genes and continues to do so as long as the virus core structure remains intact and NTPs are available. Virtually nothing is known about constraints to polymerase elongation in the virus core or about how RNA polymerase molecules recycle. Similarly, it is entirely unclear how the completed transcripts are transported out of the virus core. It has been suggested that these transactions may also be coupled to NTP hydrolysis.

Consistent with the idea that additional energy-dependent steps exist during early transcription is the fact that vaccinia virus cores contain two DExH box nucleic acid-dependent NTPases beside ETF and NPH-I. One of these NTPases, the A18 protein, is a DNA-dependent ATPase (3). The other is NPH-II, a DNA- and RNA-dependent NTPase (31, 32). Both A18 and NPH-II are 3′-to-5′ helicases (35, 40). A18 unwinds short (<20-bp) regions of duplex DNA and is inactive on RNA duplexes and RNA-DNA hybrids (40). In contrast, NPH-II unwinds RNA duplexes of up to several hundred base pairs. NPH-II also unwinds RNA-DNA hybrids but does not unwind duplex DNAs (15). Although A18 and NPH-II are both essential for vaccinia virus replication in cell culture (10, 11, 30), the

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specific roles played by these proteins have not been clearly defined.

In order to assess the function of the RNA helicase NPH-II in vaccinia virus early transcription, we have exploited several temperature-sensitive (*ts*) viruses that were shown by Fathi and Condit to contain missense mutations in the gene encoding NPH-II (10). These NPH-II *ts* viruses displayed grossly normal patterns of viral protein synthesis, DNA replication, and virus assembly during synchronous infection at the nonpermissive temperature, 40°C (10, 11). We had hypothesized that the lack of infectivity of the progeny virions reflects an essential role for NPH-II during early mRNA synthesis during the next round of infection (34). We now test this idea by purifying and characterizing the virions formed by NPH-II *ts* mutants at permissive and restrictive temperatures. We demonstrate that NPH-II is not encapsidated at the restrictive temperature and that this results in defective synthesis and extrusion of early mRNAs.

MATERIALS AND METHODS

Cells and viruses. Thermosensitive mutants *ts*10, *ts*18, *ts*39, and *ts*44 were a generous gift of Richard Condit, University of Florida. Mutant viruses were propagated in BSC40 cells at 31°C (permissive temperature). The thermosensitivity of mutant virus stocks was verified at each passage by comparative titration on BSC40 cells at 31 and 40°C (nonpermissive temperature).

Purification of virions. Wild-type and mutant viruses grown at the permissive temperature were harvested from 30 culture dishes (150-cm^2) of confluent BSC40 monolayers that had been infected at a multiplicity of infection of 0.1 and maintained for 48 h at 31°C. For growth at the nonpermissive temperature, 30 culture dishes were infected at a multiplicity of infection of 2.5. Infected cells were dislodged with a Teflon scraper and recovered by centrifugation. The cells were lysed by freezing and thawing in 10 mM Tris HCl, pH 9.0. Virus particles were recovered from a postnuclear supernatant by centrifugation through a 36% sucrose cushion and then purified by sedimentation through a 25 to 40% sucrose gradient (21). Gradient-purified virions were concentrated by centrifugation and resuspended in 1 mM Tris HCl, pH 9.0. The preparations were stored at −80°C. The concentration of virus particles was determined spectrophotometrically according to the formula of Joklik (A_{260} of 1 equals 1.2×10^{10} particles) (21). The concentration of infectious virus was determined by infection of BSC40 monolayers at 31°C with serial 10-fold dilutions of each preparation.

Western blot (immunoblot) analysis. Aliquots of purified virions (A_{260} = 0.1) were denatured in sodium dodecyl sulfate (SDS) and mercaptoethanol. The samples were electrophoresed through an 8 or 15% polyacrylamide gel containing 0.1% SDS. Proteins were transferred electrophoretically to nitrocellulose membranes, which were then blocked with TBST buffer (10 mM Tris HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) containing 5% bovine serum albumin and 1% dried milk. The membranes were incubated for 1 to 2 h at room temperature with antisera diluted in TBST buffer. After the membranes were washed with TBST buffer with 0.4% Tween 20, the immunoreactive polypeptides were visualized by using an enhanced chemiluminescence system (ECL; Amersham). The membranes were stripped and reprobed according to the instructions of the manufacturer.

Antibodies. Antisera were raised in rabbits against RNA polymerase subunits rpo147, rpo132, rpo35, rpo30, rpo22, and rpo18 by using recombinant polypeptide antigens that were produced in bacteria and purified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). (These antisera were graciously provided by Peijie Cong and Jerry Hagler.) Antiserum against DNA topoisomerase was prepared by immunization of a rabbit with native topoisomerase purified from bacteria. Antibodies generated in this laboratory to capping enzyme subunits D1 and D12, ETF subunit D6, and the rpo94 subunit of RNA polymerase have been described previously (5, 6). Other antisera were generously provided by our colleagues as follows: anti-A18 (Richard Condit, University of Florida), anti-VP39 (Paul Gershon, Texas A&M University), anti-A8 (Steven Broyles, Purdue University), and anti-NPH-I (Ed Niles, State University of New York at Buffalo).

Transcription by permeabilized virions. Reaction mixtures (0.25 ml) containing 60 mM Tris HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 5 mM ATP, 1 mM GTP, 1 mM CTP, 0.2 mM [α-^{32}P]UTP, 0.05% Nonidet P-40 (NP-40), and virus (A_{260} = 0.25) were incubated at 37°C. Aliquots (50 μl) were withdrawn at various times and mixed immediately with 0.2 ml of a solution containing 50 mM Tris HCl (pH 8.0), 10 mM DTT, 10 mM EDTA, and 0.05% NP-40. Virion cores were recovered by centrifugation for 3 min in a microcentrifuge. The supernatants containing released RNA were removed and mixed with 1 ml of 5% trichloroacetic acid (TCA). The pellets containing core-associated RNA were resuspended in 0.2 ml of 50 mM Tris HCl (pH 8.0)–0.1% SDS and then mixed with 1 ml of 5% TCA. Acid-insoluble material was collected by filtration on glass-fiber filters. [^{32}P]UMP incorporation was determined by liquid scintillation counting.

Primer extension. Transcription reaction mixtures (0.25 ml) contained 60 mM Tris HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM DTT, 5 mM ATP, 1 mM GTP, 1 mM CTP, 0.2 mM UTP, 0.05% NP-40, and wild-type or mutant virions (A_{260} = 0.25) (grown at 40°C). After incubation for 30 min at 37°C, the proteins in the samples were denatured in SDS and then extracted sequentially with phenol-chloroform-isoamyl alcohol (50:48:2) and chloroform-isoamyl alcohol (24:1). The transcription products were recovered by ethanol precipitation. The *ts*10, *ts*18, *ts*39, and *ts*44 precipitates were dissolved in 14 μl of annealing buffer (10 mM Tris HCl [pH 9.0], 250 mM KCl). The wild-type precipitate was dissolved in 70 μl of annealing buffer; 14 μl of the wild-type RNA sample was used as the template for primer extension. To each RNA sample, we added 1 pmol of a 5′ ^{32}P-labeled 24-mer oligonucleotide (5′-CATAGCAGCGAACAACAACATCAG-3′) complementary to the 5′ end of the vaccinia virus growth factor mRNA (42). The mixtures were heated at 70°C for 3 min and then incubated at 50°C for 30 min. Primer extension reaction mixtures containing 25 mM Tris HCl (pH 8.3), 25 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM spermidine, 0.25 mM (each) of the four deoxynucleoside triphosphates, 10 U of avian myeloblastosis virus reverse transcriptase (Promega), and the annealed primer-template were incubated at 50°C for 30 min. The products were ethanol precipitated, dissolved in 6 μl of 0.1 M NaOH–1 mM EDTA, and incubated for 30 min at 25°C. The samples were mixed with an equal volume of formamide, heated at 95°C, and then electrophoresed through an 8% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris-borate, 2.5 mM EDTA). Primer extension products were visualized by autoradiography and quantitated by scanning the gel with a Fujix BAS1000 BioImaging Analyzer.

Preparation of virion extracts. Purified virions (A_{260} of 2.5) were incubated on ice for 10 min in 0.8 ml of a solution containing 50 mM Tris HCl (pH 8.0), 10 mM DTT, and 0.05% NP-40. Virus cores were recovered by centrifugation and then resuspended in 150 μl of buffer B (300 mM Tris HCl [pH 8.0], 250 mM NaCl, 0.1 mM EDTA, 50 mM DTT). The samples were adjusted to 0.1% sodium deoxycholate and incubated on ice for 30 min with occasional mixing. Insoluble material was removed by centrifugation. The supernatants were applied to 150-μl columns of DEAE-cellulose that had been equilibrated with buffer B. The DEAE flowthrough fractions were collected and adjusted to 10% glycerol by adding 0.25 volume of a solution containing 200 mM Tris (pH 8.0), 8 mM DTT, 4 mM EDTA, and 40% glycerol. The enzyme fractions were stored at −80°C.

Nonspecific RNA polymerase activity. Reaction mixtures (100 μl) containing 60 mM Tris HCl (pH 8.0), 10 mM DTT, 3 mM $MnCl_2$, 1 mM ATP, 1 mM GTP, 1 mM CTP, 0.1 mM [α-^{32}P]UTP, 1 μg of single-stranded DNA from M13mp18, and virion extracts (1, 2, 4, and 8 μl of the DEAE flowthrough fraction) were incubated for 30 min at 37°C. Incorporation of [α-^{32}P]UMP into RNA was determined by TCA precipitation.

RESULTS

NPH-II *ts* mutant viruses. In agreement with the results of Fathi and Condit (10, 11), we found that four *ts* mutants of vaccinia virus WR (*ts*10, *ts*18, *ts*39, and *ts*44), each bearing a single missense mutation in the I8 gene encoding NPH-II, displayed normal patterns of viral protein synthesis during synchronous infection of BSC40 cells at 40°C (not shown). All four *ts* mutants could be restored to temperature-independent growth by marker rescue with a plasmid containing the wild-type NPH-II gene (not shown). Marker-rescued *ts*10, *ts*18, and *ts*39 viruses formed wild-type-size plaques when plated at 40°C. However, marker-rescued *ts*44 produced smaller plaques at 40°C. We suspect that *ts*44 contains a weak *ts* mutation outside the I8 gene. This second mutation (which remains unmapped) is likely to account for the more severe biochemical phenotype described below for *ts*44 virions assembled at the restrictive temperature.

Mutant virions formed at 40°C are noninfectious. Progeny virions were purified by sucrose gradient sedimentation from cells infected at 31 and 40°C with either wild-type or *ts* mutant virus. The purified virus preparations were titrated at 31°C. The virus titer (PFU) was then normalized to the concentration of virus particles, as determined by UV absorbance (21). The particle/PFU ratio provides a quantitative index of virus infectivity. The wild-type and *ts* mutant viruses grown at 31°C had similar particle/PFU ratios (Table 1). As expected, the infectivity of wild-type virus grown at 40°C was the same as that of virus (wild type and mutant) grown at 31°C. In contrast, the particle/PFU ratios of the *ts* mutants grown at the restrictive temperature were much higher by a factor of at least 300 for

TABLE 1. Infectivity of mutant virions formed at 40°C

Virus	Particle/PFU ratio for virions grown at:	
	31°C	40°C
Wild type	120	96
*ts*10	75	1.5×10^6
*ts*18	65	2.1×10^4
*ts*39	93	2.1×10^5
*ts*44	106	4.1×10^5

*ts*18 and by as much as 4 orders of magnitude for *ts*10 (Table 1).

Polypeptide composition of mutant virions. The proteins in aliquots containing equal amounts of purified virions were denatured in SDS and analyzed by SDS-PAGE. The polypeptides detected by Coomassie blue staining represent the major structural components of the virion, the most abundant being proteins 4a and 4b, which migrated as a doublet of ~60 kDa (Fig. 1A). The polypeptide compositions of wild-type and *ts* mutant virions grown at 31°C were indistinguishable at this level of resolution. Similarly, the polypeptide compositions of the noninfectious *ts*10, *ts*18, and *ts*39 virions grown at 40°C were similar to that of wild-type virus. This was not true of mutant *ts*44 which, when grown at the restrictive temperature, formed virus particles that contained smaller amounts of structural proteins 4a and 4b (Fig. 1A).

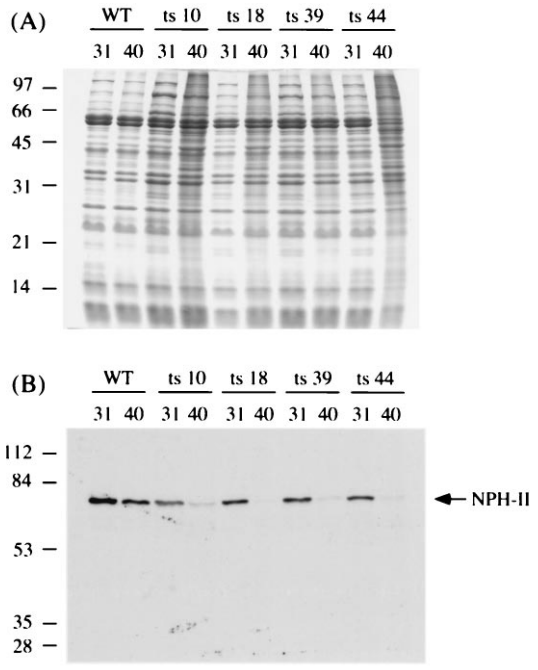

FIG. 1. Noninfectious mutant virions lack NPH-II. (A) The polypeptide compositions of wild-type (WT) and *ts* mutant virions grown at either 31 or 40°C were analyzed by SDS-PAGE. Gradient-purified virus (A_{260} = 0.1) was applied to each lane. Polypeptides were visualized by Coomassie blue staining. The positions and sizes (in kilodaltons) of coelectrophoresed marker proteins are indicated on the left. (B) The amount of virion-associated NPH-II was analyzed by Western blotting using anti-NPH-II antibody. All lanes of the gel contained equal amounts (A_{260} = 0.1) of the indicated preparations of gradient-purified virions. The chemiluminescence of membrane-bound antibody was detected by exposure to X-ray film. The polypeptide corresponding to NPH-II is denoted by an arrow on the right. The positions and sizes (in kilodaltons) of marker proteins are indicated on the left.

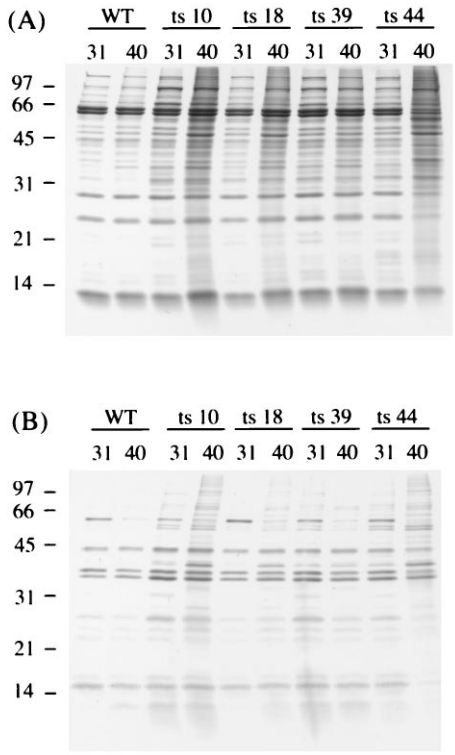

FIG. 2. Polypeptide compositions of the virus core (A) and envelope (B) fractions. Wild-type (WT) and *ts* mutant virions were grown at either 31 or 40°C. Equal amounts of purified virions (A_{260} = 0.1) were incubated on ice for 30 min at 37°C in 20 μl of a solution containing 50 mM Tris HCl (pH 8.0), 50 mM DTT, and 0.5% NP-40. The samples were then separated by centrifugation into core (pellet) and envelope (supernatant) fractions. The samples were denatured and analyzed by SDS-PAGE. Polypeptides were visualized by staining with Coomassie blue dye. The positions and sizes (in kilodaltons) of marker proteins are indicated on the left.

Mature vaccinia virions consist of a core particle surrounded by a membrane envelope. The two compartments are defined biochemically as follows: the envelope fraction is the soluble material released from virions by treatment with DTT and NP-40, and the core is the insoluble particle that resists extraction by DTT and NP-40. Virion polypeptides partitioned into envelope and core in a characteristic fashion, as shown in Fig. 2. Wild-type cores contained proteins 4a and 4b, plus other prominent structural polypeptides migrating at 28, 24, and 11 kDa. The composition of the wild-type core did not vary with growth temperature (Fig. 2A). Each of these proteins was present at the wild-type level in the core fractions of *ts*10, *ts*18, and *ts*39 grown at either the permissive or restrictive temperature (Fig. 2A). *ts*44 cores, which had a normal polypeptide composition when grown at 31°C, were deficient in proteins 4a and 4b at 40°C. The cores of *ts*44 virus grown at 40°C apparently had normal amounts of the 11-kDa structural protein (Fig. 2A).

The envelope fraction of wild-type virus contains prominent polypeptides of 44, 38, 36, and 15 kDa (Fig. 2B). Each of these polypeptides was present at the wild-type level in the envelope fractions of *ts*10, *ts*18, *ts*39, and *ts*44 grown at either the permissive or restrictive temperature (Fig. 2B). A 60-kDa species that was detected in the envelope fraction of wild-type and *ts* virions grown at 31°C was missing from the envelopes of virions grown at 40°C. (The identity of this polypeptide remains to be determined.)

These results indicated that the lack of infectivity of the *ts*10, *ts*18, and *ts*39 mutants grown at 40°C was not caused by defective encapsidation of any of the major structural proteins of either the envelope or core. *ts*44, on the other hand, displayed a thermosensitive defect in the encapsidation of core proteins 4a and 4b. As discussed above, we believe that this defect reflects the existence of a second *ts* mutation in this virus.

Noninfectious mutant virions lack NPH-II. NPH-II and other enzymes of the virion are less abundant than the major structural proteins and are therefore difficult to visualize on a stained SDS-polyacrylamide gel of total virion protein. In order to determine whether the noninfectious *ts* mutant particles contained NPH-II, we subjected virion samples to SDS-PAGE, transferred the resolved polypeptides to membranes, and probed the blots with anti-NPH-II antibody. A single 72-kDa immunoreactive polypeptide corresponding to NPH-II was detected in wild-type virions grown at 31 or 40°C (Fig. 1B). Similar amounts of NPH-II were encapsidated by *ts* virions grown at 31°C. However, *ts* virus particles grown at 40°C contained little or no NPH-II (Fig. 1B). Thus, these noninfectious particles are, to a first approximation, null mutants of NPH-II.

Immunoblot analysis of total protein from cells harvested at various times during synchronous infection revealed a significant reduction in the steady-state level of NPH-II within *ts* virus-infected cells at 40°C from that of wild-type-virus-infected cells or *ts* virus-infected cells at 31°C (not shown). This reduction may account for the failure to encapsidate mutant NPH-II protein at the nonpermissive temperature.

Noninfectious mutant virions contain RNA polymerase and other virus-encoded enzymes. Western blots of purified virions were probed with antisera against seven of the nine known subunits of vaccinia virus RNA polymerase. These antibodies were raised against recombinant versions of the rpo147, rpo132, rpo94, rpo35, rpo30, rpo22, and rpo18 subunits that were expressed individually in bacteria. (The subunits are named according to their molecular masses [in kilodaltons], calculated from their amino acid sequences.) All antibodies, except anti-rpo30, reacted with a single virion polypeptide of the appropriate size (Fig. 3). Two forms of rpo30 were detected (Fig. 3); this heterogeneity was described previously and attributed to the use of alternative translation start sites within the rpo30 gene (1). The immunoblots in Fig. 3 show that each of the noninfectious *ts* virions grown at 40°C contains all seven RNA polymerase subunits in amounts comparable to those of wild-type virions grown at 40°C.

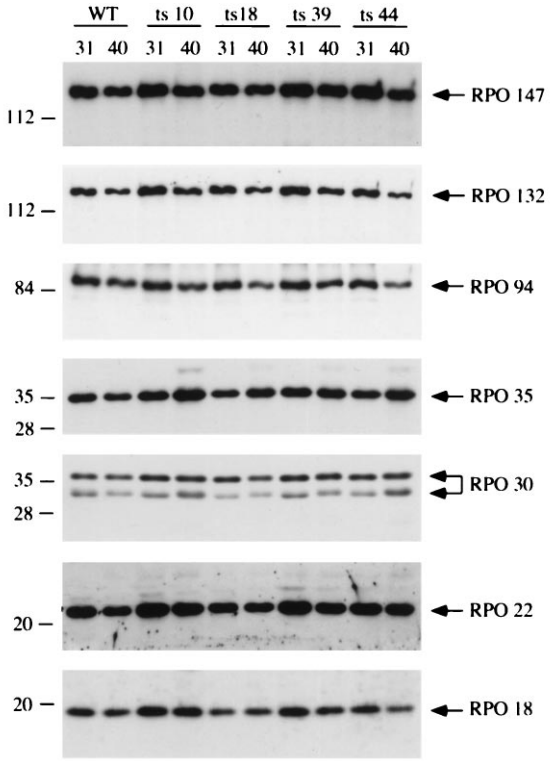

FIG. 3. Noninfectious mutant virions contain RNA polymerase. The amount of virion-associated RNA polymerase was analyzed by Western blotting using antibodies to individual polymerase subunits. All lanes of the gels contained equal amounts (A_{260} = 0.1) of gradient-purified wild-type (WT) or *ts* mutant virions grown at either 31 or 40°C, as indicated above the lanes. The chemiluminescence of membrane-bound antibody was detected by exposure to X-ray film. The polypeptide corresponding to the immunoreactive rpo subunit is denoted by an arrow(s) to the right of each panel. The position and size (in kilodaltons) of at least one marker protein for each immunoblot indicated on the left of each panel.

Initiation of transcription of early genes by RNA polymerase requires ETF, a heterodimeric protein of 82- and 72-kDa subunits encoded by the A8 and D6 genes, respectively (25). Transcription termination requires the vaccinia virus capping enzyme, which is a heterodimer of 95- and 33-kDa subunits encoded by the D1 and D12 genes (27). Termination in vitro also requires NPH-I, a 66-kDa DNA-dependent ATPase (7). We found by Western blotting that noninfectious *ts*10, *ts*18, and *ts*39 virions encapsidated the two ETF subunits (A8 and D6), the two capping enzyme subunits (D1 and D12), and NPH-I to the same extent as that of wild-type virus grown at 40°C (Fig. 4). These three mutants also packaged wild-type amounts of the virus-encoded type I DNA topoisomerase and VP39 [a bifunctional protein that catalyzes 2-O methylation of the cap ribose sugar and also serves as a processivity factor for the vaccinia virus poly(A) polymerase (33)] (Fig. 4). The *ts*44 virions, which contained wild-type levels of capping enzyme and NPH-I, encapsidated subnormal amounts of topoisomerase, VP39, and the A8 subunit of ETF (Fig. 4). Thus, this virus mutant displayed a *ts* defect in packaging some of the virion enzymes that paralleled its *ts* defect in packaging some of the virion structural proteins. Remarkably, the *ts*10, *ts*18, *ts*39, and *ts*44 viruses grown at 40°C all contained larger amounts of the A18 DNA helicase than did wild-type virions or mutant virions grown at 31°C (Fig. 4). This phenomenon was unique to A18. (It is tempting to speculate that the two vaccinia virus helicases, NPH-II and A18, may bind at common or overlapping sites in the virus core during virus assembly, such that the lack of NPH-II permits the uptake of greater than normal levels of A18).

In summary, the immunoblotting experiments suggest that the *ts*10, *ts*18, and *ts*39 mutant virions were noninfectious because they lacked NPH-II, not because of a global defect in encapsidating the transcription apparatus. The outstanding issue is whether NPH-II deprivation affected the function of the virion transcription system. This issue was addressed in a series of experiments described below in which we compared the abilities of wild-type and mutant virions to synthesize early mRNAs in vitro.

Noninfectious mutant virions are defective in RNA synthesis and RNA release in vitro. Transcription in vitro by wild-type and mutant virions was measured by [^{32}P]UMP incorporation into TCA-insoluble material. Aliquots of the transcription reaction mixtures were withdrawn after 5, 10, 20, and 30 min of incubation at 37°C, quenched immediately by the addition of EDTA, and then separated by centrifugation into particulate (core) and soluble (released) fractions. These samples were

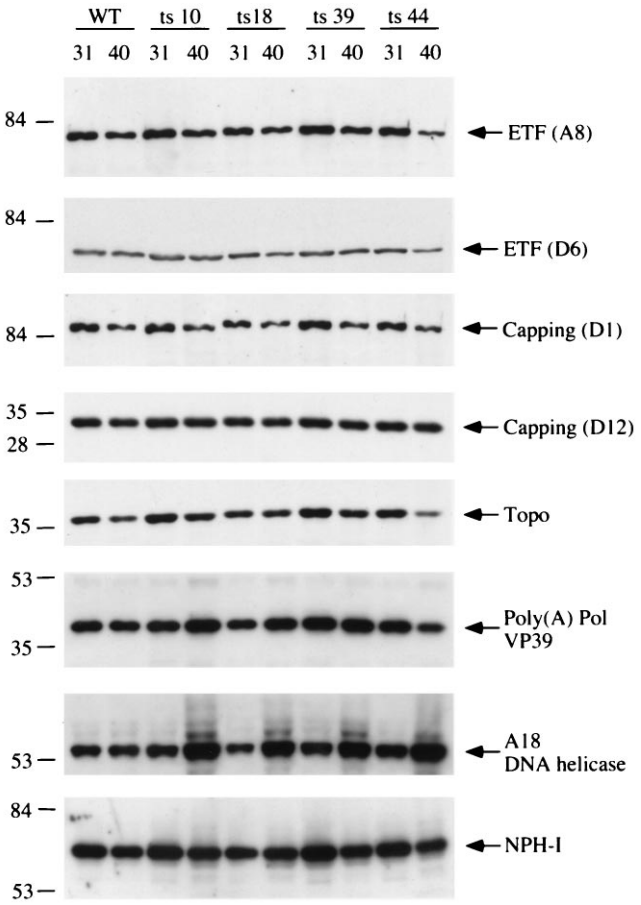

FIG. 4. Encapsidation of virion-associated enzymes by NPH-II *ts* mutants. Western blots of total virion protein were probed with the antisera raised against the subunits of ETF and capping enzyme, topoisomerase (Topo), the VP39 subunit of poly(A) polymerase [Poly(A) Pol], A18 DNA helicase, and NPH-I. All lanes of the gels contained equal amounts (A_{260} = 0.1) of gradient-purified wild-type (WT) or *ts* mutant virions grown at either 31 or 40°C, as indicated above the lanes. The identities of the immunoreactive polypeptides are denoted by an arrow to the right of each panel. The position and size (in kilodaltons) of at least one marker protein for each immunoblot is indicated at the left of each panel.

precipitated with TCA. The kinetics of accumulation of core-associated and released RNA are plotted in Fig. 5. (Similar results were obtained when the in vitro transcription reactions were carried out at 31°C [data not shown]).

Infectious wild-type and mutant virions (grown at 31°C) displayed the characteristic accumulation of core-associated RNA at early times after activation of the transcription apparatus. Core RNA reached a steady-state level by 10 min. Released RNA appeared after a time lag and continued to accumulate for at least 30 min, at which time 75 to 90% of the RNA was released (Fig. 5A).

The mutant virions grown at 40°C were defective in both RNA synthesis and release compared with either wild-type virions grown at 40°C or with the corresponding mutant virions grown at the permissive temperature (Fig. 5). The extents of UMP incorporation (core-associated plus released RNA) in 30 min by the *ts*10, *ts*18, *ts*39, and *ts*44 virions grown at 40°C were 9, 17, 20, and 5%, respectively, of the level of UMP incorporation by wild-type virions grown at 40°C. Moreover, whereas 70% of the RNA made by wild-type virions grown at 40°C was released in 30 min, the majority of the transcripts synthesized by the noninfectious NPH-II mutant virions remained core associated (Fig. 5B). Attempts to complement the transcription defect of these mutant virions by adding back purified recombinant NPH-II protein were unsuccessful.

Noninfectious mutant virions synthesize longer RNAs. UMP-labeled RNAs synthesized in vitro by wild-type and noninfectious mutant virions were analyzed by electrophoresis through a formaldehyde-containing agarose gel. In the experiment shown in Fig. 6, total RNA was recovered after a 30-min transcription reaction programmed by equivalent amounts of virus. For the reaction mixtures containing *ts* mutant virions, the entire sample was applied to the gel. One-fifth of the wild-type RNA was analyzed in parallel. As noted previously (38), the majority of the transcripts produced by wild-type virions were between 0.5 and 2 bp long. In contrast, all of the mutant viruses grown at 40°C synthesized much longer RNAs (Fig. 6).

A finer kinetic analysis was performed with the wild-type, *ts*10, and *ts*39 virions. Total RNA was isolated from aliquots of the transcription reaction mixtures that had been withdrawn and quenched after 5, 10, 20, and 30 min of incubation. These samples were analyzed by agarose gel electrophoresis (Fig. 7). Although the total amount of RNA synthesized by wild-type virions increased steadily with time, the size distribution did not change between 5 and 30 min. This suggests that the virion RNA polymerase catalyzed multiple rounds of transcription. The average length of wild-type RNA in this experiment was ~1.2 kb (Fig. 7).

Abnormally long transcripts were synthesized by the *ts*10 and *ts*39 virions as early as 5 min (Fig. 7). The size distribution of *ts*10 and *ts*39 RNAs did not appear to increase between 10 and 30 min. This suggests that these virions also engage in multiple rounds of transcription, although obviously not as many as the wild-type virions. The synthesis of longer chains was presumably caused by a reduced efficiency of transcription termination. We think it unlikely that increased RNA size and retention of transcripts in the core were caused by a complete failure to terminate transcription, because that molecular phenotype is characterized by a steady increase in RNA chain length over the course of the transcription reaction (38). It was conceivable that the *ts*10 and *ts*39 transcripts terminated normally but acquired extremely long 3′ poly(A) tails, on the order of several thousand adenylates. To test this hypothesis, we compared [α-^{32}P]AMP incorporation into acid-insoluble material with [α-^{32}P]UMP incorporation. We predicted that if very long poly(A) tails were being made, then the ratio of AMP to UMP incorporation during transcription by mutant virions should be higher than the wild-type ratio. We found that this was not the case (data not shown).

Accurate transcription initiation by noninfectious *ts* virions. A primer extension assay was employed to determine whether the noninfectious mutant virions initiated transcription accurately at a specific early promoter on the endogenous viral DNA genome. We assayed initiation at the vaccinia virus growth factor (VGF) promoter because the site of transcription initiation in vivo has been mapped (42). Total RNA was isolated after 30 min of RNA synthesis by wild-type and mutant virions. The RNA samples were annealed to a 5′-end-labeled DNA oligonucleotide complementary to the 5′-proximal portion of the VGF mRNA. Extension by reverse transcriptase of primer annealed to wild-type RNA yielded a cluster of five DNA species with single-nucleotide spacing (Fig. 8, lane WT). The primer extension products were electrophoresed in parallel with a dideoxy sequence ladder formed by annealing the end-labeled primer on a VGF DNA template; in this way we localized the 3′ ends of the primer extension products to within the sequence CCCAAT (data not shown). These sites corre-

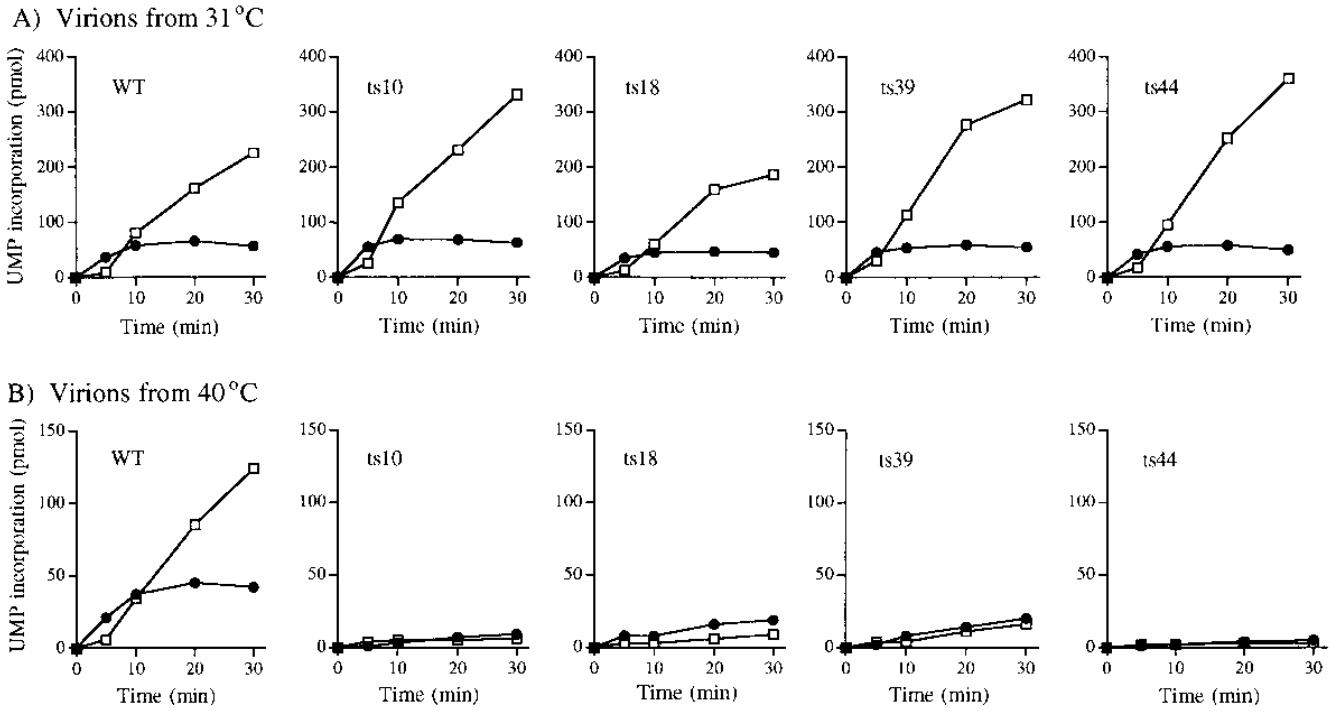

FIG. 5. Kinetics of RNA synthesis and release by permeabilized wild-type (WT) and mutant virions. Transcription reactions were performed as described in Materials and Methods. The amounts of acid-insoluble [^{32}P]UMP (in picomoles) recovered in the core-associated (closed circles) and released (open squares) RNA fractions are plotted as a function of time for each virus. RNA synthesis by virions purified from cells infected at 31°C (A) or 40°C (B).

spond to the transcription initiation region defined previously for the VGF gene (42).

An identical cluster of extension products was formed during reverse transcription of primer annealed to RNA synthesized by *ts*10, *ts*18, *ts*39, and *ts*44 virions (Fig. 8). A control primer extension was performed by using nucleic acid recovered from a reaction mixture containing wild-type virions but no NTPs or magnesium. In this case, no primer extension products were detected (Fig. 8, lane C). This result shows that primer extension required de novo mRNA synthesis. We surmise that mutant virions lacking NPH-II were able to initiate accurately at an early promoter, albeit less efficiently than wild-type virions. The yield of the primer extension products (determined by scanning the gel with a PhosphorImager) provided a measure of the relative levels of transcription initiation at the VGF promoter by the mutant virions (compared with those of wild-type virions). (Note that only 20% of the RNA synthesized by wild-type virions was used in the primer extension reaction shown in Fig. 8). These values were 2% for *ts*10, 6% for *ts*18, 7% for *ts*39, and 1% for *ts*44. Initiation frequency was depressed to a lower level in the mutant virions grown at 40°C than was total UMP incorporation; this is consistent with each initiation event resulting in the synthesis of a longer RNA chain.

Promoter-dependent transcription by extracts of noninfectious mutant virions. The reduced rates of transcription initiation in vitro by the mutant virions grown at 40°C were at variance with their normal content of immunoreactive RNA polymerase and ETF. This raised the question of whether the encapsidated enzymes were defective per se in promoter-dependent transcription in vitro. To address this issue, we extracted wild-type, *ts*10, and *ts*39 cores with sodium deoxycholate. After removal of residual insoluble material by centrifugation, the soluble extracts were freed of endogenous nucleic acid by

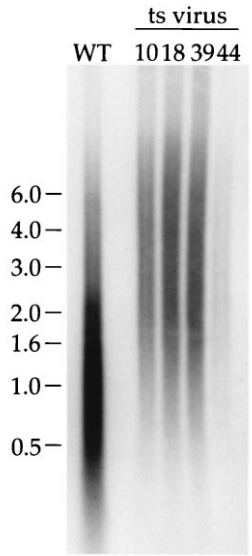

FIG. 6. Mutant virions synthesize longer RNA chains. Reaction mixtures (50 μl) containing 60 mM Tris HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM DTT, 5 mM ATP, 1 mM GTP, 1 mM CTP, 0.2 mM [α-^{32}P]UTP, 0.05% NP-40, and equal amounts of gradient-purified virus (A_{260} = 0.05) (grown at 40°C) were incubated for 30 min at 37°C. The reactions were quenched by adding 0.2 ml of a solution containing 50 mM Tris HCl (pH 8.0), 10 mM DTT, 10 mM EDTA, and 1% SDS. The proteins were extracted sequentially with phenol-chloroform-isoamyl alcohol (50:48:2) and chloroform-isoamyl alcohol (24:1). RNA was recovered by ethanol precipitation. The RNA samples were denatured in formamide and electrophoresed through a 1.25% agarose gel containing 6% formaldehyde, 20 mM morpholinepropanesulfonic acid (MOPS), 10 mM sodium acetate, and 1 mM EDTA. The gel was dried under vacuum, and labeled RNAs were visualized by autoradiography. Note that only 20% of the 40°C wild-type (WT) transcription reaction was loaded on the gel. The RNA samples from the mutant viruses *ts*10, *ts*18, *ts*39, and *ts*44 are identified above the lanes. The positions and sizes (in kilobases) of denatured end-labeled DNA markers are indicated on the left of the gel.

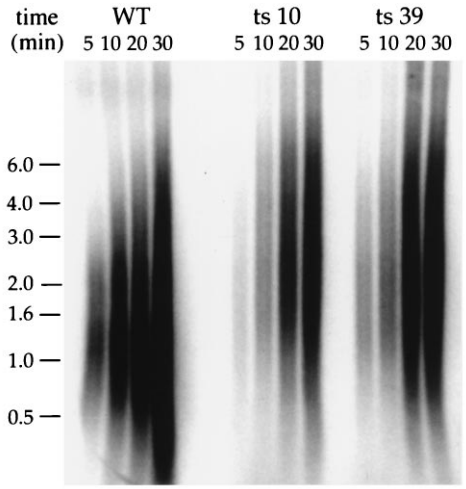

FIG. 7. Longer RNAs accumulate at early times during transcription in vitro by mutant virions. Transcription reactions contained purified wild-type (WT), *ts*10, or *ts*39 virions grown at 40°C. Aliquots were withdrawn at the times indicated over the lanes. RNA isolation and size analysis were performed as described in the legend to Fig. 6. (Only 20% of each wild-type RNA sample was loaded on the gel). The positions and sizes (in kilobases) of denatured end-labeled DNA markers are indicated on the left.

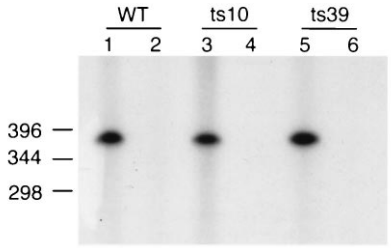

FIG. 9. Promoter-dependent transcription by mutant virion extracts. Reaction mixtures (60 μl) containing 40 mM Tris HCl (pH 8.0), 4 mM DTT, 12 mM $MgCl_2$, 1 mM ATP, 1 mM CTP, 10 μM [α-^{32}P]UTP, 50 ng of *Sma*I-cut plasmid DNA template, and virion extracts as indicated over the lanes (WT, wild type) (between 1 and 4 μl of the DEAE flowthrough fraction, containing equivalent amounts of nonspecific RNA polymerase activity) were incubated for 15 min at 30°C. The reactions were quenched by adding 0.24 ml of a stop solution containing 5 M urea, 12.5 mM EDTA, 0.625% SDS, and 90 μg yeast tRNA per ml. After phenol-chloroform extraction and ethanol precipitation, the transcription products were electrophoresed through a 4% polyacrylamide gel containing 7 M urea in TBE. Labeled RNA was visualized by autoradiography of the dried gel. The positions and sizes (in nucleotides) of denatured DNA markers are indicated on the left. The reaction mixtures in lanes 1, 3, and 5 were programmed by pSB24 DNA, which contains a vaccinia virus early promoter fused to a 382-nucleotide G-less cassette. The reaction mixtures in lanes 2, 4, and 6 contained pC_2AT DNA, which contains the same G-less cassette but lacks the viral promoter.

DEAE-cellulose chromatography. RNA polymerase and other soluble enzymes were recovered in the DEAE flowthrough fractions. Nonspecific RNA polymerase activity in the DEAE preparation was measured by nucleoside monophosphate incorporation into TCA-insoluble material in reaction mixtures containing a single-stranded DNA template and manganese (39). After the values were normalized for nonspecific polymerase activity, the extracts were tested for their ability to transcribe a duplex DNA template in the presence of magnesium. The requirement for a vaccinia virus early promoter during in vitro transcription was assessed by using two different plasmid DNA templates, pSB24 and pC_2AT. pSB24 contains a vaccinia virus early promoter fused to a 382-nucleotide G-less cassette (26). pC_2AT contains the identical G-less cassette but has no early promoter. The plasmid DNAs were linearized at the end of the G-less cassette by treatment with endonuclease *Sma*I and then used as templates for in vitro transcription. The reactions were carried out without the addition of GTP; this limited transcription to the G-less cassette.

pSB24 directed the synthesis of a 390-nucleotide RNA, consistent with transcription of the entire G-less cassette from the promoter-driven initiation site to the end of the linear molecule. Similar amounts of the runoff transcript were synthesized by extracts of wild-type, *ts*10, and *ts*39 virions (Fig. 9, lanes 1, 3, and 5). No radiolabeled RNA was detected in reactions programmed by pC_2AT (Fig. 9, lanes 2, 4, and 6). Thus, the RNA polymerase encapsidated in the noninfectious mutant virions retained specificity for a viral early promoter and was just as active on an exogenous template as polymerase extracted from wild-type virus. These results suggest that NPH-II plays a unique role during transcription in the virion that is not evident in the soluble in vitro system.

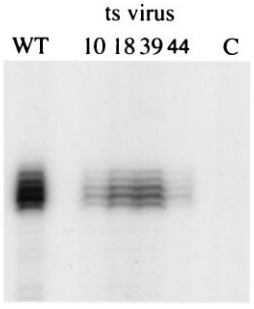

FIG. 8. Primer extension analysis of transcription initiation by mutant virions. Primer extension was performed as described in Materials and Methods with RNA template isolated from in vitro transcription reaction mixtures containing wild-type (WT) or mutant virions (*ts*10, *ts*18, *ts*39, and *ts*44) grown at 40°C, as specified above the lanes. Wild-type primer extensions were performed by using 20% of the annealed primer-template. The ^{32}P-labeled extension products were resolved by denaturing PAGE. An autoradiogram of the gel is shown. Lane C, control primer extension, using nucleic acid recovered from a reaction mixture containing wild-type virions but no NTPs or magnesium.

DISCUSSION

Conditional mutations of NPH-II result in the production of noninfectious virions at the restrictive temperature. Three different mutants, *ts*10, *ts*18, and *ts*39, packaged little or no NPH-II protein during virus assembly at 40°C. Despite this, the polypeptide compositions of the envelope and core were otherwise unperturbed, and the viral RNA polymerase, its accessory factors, and various other enzymes were encapsidated normally. NPH-II is apparently not required to recruit the transcription apparatus to the core but is instead essential for proper execution of early mRNA synthesis by the core enzymes.

Three features define the transcriptional phenotype of NPH-II-deficient virions. (i) Initiation occurs accurately at early promoters, albeit at reduced frequency. (ii) The RNA products are abnormally long. (iii) RNA is inefficiently released from the core. The production of accurately initiated but longer transcripts implies that the efficiency of transcription termination is reduced in virions lacking NPH-II. The transcripts that are synthesized are retained in the core, either because the lack of NPH-II directly affects the poorly understood process of RNA extrusion or because the transcripts are not accessible to the extrusion machinery (e.g., they remain bound to the template or to some component of the transcription apparatus).

Along the same lines, NPH-II deficiency may also affect the recycling of RNA polymerase for subsequent rounds of transcription initiation. If our hypothesis is correct that NPH-II acts during 3′-end formation, RNA trafficking, and/or polymerase recycling, the reduced efficiency of initiation by mutant virions may be viewed as a secondary effect.

The mutant phenotype likely reflects a requirement for the activity of NPH-II in catalyzing NTP hydrolysis and duplex nucleic acid unwinding during mRNA synthesis by virus cores. Genetic experiments support this view, insofar as the NPH-II *ts* viruses cannot be rescued by NPH-II alleles containing point mutations that inactivate the NTPase (13, 14, 16). This raises two important questions, the first being Is the NPH-II ATPase involved directly in the synthesis of the mRNA chain, i.e., is it required for initiation or termination of transcription?

Our experiments using crude extracts suggest that NPH-II is dispensable for initiation but do not address its role in termination. We find that promoter-dependent initiation by extracts of NPH-II-deficient virions is comparable to that seen with wild-type extracts; this implies, at the very least, that NPH-II activity is not rate limiting in this system. Assaying signal-dependent transcription termination in crude extracts was not pursued, because detection of discrete terminated RNAs was hindered by the presence of poly(A) polymerase. A more definitive answer can be marshaled on the basis of experiments in this laboratory using a transcription system composed of purified virion enzymes. We argue that NPH-II is not involved directly in chain initiation or termination because of the following observations. (i) Initiation and termination can be achieved with purified virion enzymes (or with virion polymerase and ETF plus recombinant termination factors) that contain no detectable NPH-II activity and no detectable immunoreactive NPH-II protein (7, 16). (ii) The addition of purified recombinant NPH-II has no effect on the level of initiation or termination in the reconstituted in vitro system (7, 16). (iii) NPH-II hydrolyzes any of the common NTPs, whereas transcription in the virion and in the reconstituted system is coupled specifically to the hydrolysis of ATP or dATP (12, 17, 18, 25, 31, 36, 38, 39).

A second key question is How would NPH-II activity facilitate transcription in the virion context? NPH-II is an NTP-dependent helicase that catalyzes unidirectional unwinding of 3′-tailed duplex RNAs in the presence of a divalent cation and any NTP (34, 35). It is proposed that nucleic acid unwinding occurs by unidirectional translocation of NPH-II along the single strand to which it initially binds (35). RNA-DNA hybrids with a 3′ RNA tail are unwound by NPH-II (15). Hybrids containing a 3′ DNA tail are also displaced, albeit less effectively. In contrast, we find that NPH-II does not displace a 3′-tailed double-stranded DNA substrate of identical sequence, even though it binds to the double-stranded DNA molecule with the same affinity as that of the RNA-DNA hybrid (15). These properties suggest that NPH-II acts either on RNA strands or RNA-DNA hybrids during mRNA synthesis by virus cores.

If NPH-II is not required for termination on linear DNA templates, how might its absence affect termination in the virion? We suggest that NPH-II facilitates transcription termination in the virion by preventing R-loop formation behind the elongating RNA polymerase. It has been shown that transcription termination in the reconstituted in vitro system is suppressed when the UUUUUNU signal base pairs to a complementary sequence in the nascent RNA. This result has led to the conclusion that the signal must be in the single-strand form to be recognized by vaccinia virus termination factor (28). We expect that the signal would also be masked by formation of a base-paired hybrid between nascent RNA and the template DNA strand (an R loop). These structures are not formed when transcription is carried out in solution with linear DNA templates containing a single transcription unit. However, R-loop formation is favored by the negative superhelicity generated during transcription of divergently oriented genes (8), as is the case with early transcription by vaccinia virions. Although the encapsidation of a DNA topoisomerase might relieve some of this superhelical tension and thereby limit R-loop formation, it is plausible that a backup mechanism exists to disrupt R loops after they are formed. Disruption of R loops by NPH-II could occur by either of two pathways involving: (i) NPH-II movement in a 3′ to 5′ polarity along the nascent RNA strand or (ii) NPH-II movement in a 3′ to 5′ direction along the template strand of the DNA. In the former case, the helicase is moving away from the elongating polymerase, whereas in the latter view the helicase moves in the same direction as that of the transcription elongation complex. The observation that purified NPH-II unwinds hybrids more effectively when moving along the RNA strand might favor the first pathway. However, the rate of elongation of vaccinia virus RNA polymerase (20 to 50 nucleotides per s) (18) may be slow enough to allow disruption of R loops by NPH-II moving along the DNA template strand.

According to this hypothesis, when NPH-II is missing from virions, the probability of the nascent RNA forming an R loop would be higher than that for RNA from wild-type virions. This would decrease the likelihood of termination at the first signal encountered, resulting in the synthesis of longer transcripts that terminate at TTTTTNT sequences located further downstream. Note that even during wild-type early mRNA synthesis, termination downstream of the first available UUUUUNU signal occurs with variable efficiency. Polymerase molecules that transcribe through a signal without terminating will continue elongating all the way through the downstream gene; these polymerases can then terminate in response to a terminator at the 3′ end of the distal gene (2, 9, 20).

The R-loop hypothesis also offers a parsimonious explanation for the observed reduction in mRNA extrusion even after termination has occurred, i.e., if a segment of the terminated transcript remains hybridized to the template DNA, then the RNA will be recovered with the pelleted cores. The present findings do not exclude the earlier suggestion that NPH-II might directly facilitate the extrusion of newly completed mRNA chains out of the virion core by acting as an energy-coupled RNA transporter (35).

The transcriptional defect of virions lacking NPH-II is similar in several respects to that produced by a conditional null mutant of the vaccinia virus DNA-binding protein VP8 (41). Under nonpermissive conditions, this VP8 mutant forms noninfectious virions that lack VP8 but contain an apparently normal complement of core enzymes. The virions are defective in mRNA synthesis and transport in vitro, although interestingly, the RNA size was not affected (41). Extracts of the VP8-deficient virions initiated transcription as efficiently as wild-type extracts did (41). Thus, NPH-II and VP8 define a class of virion core components that either facilitate or regulate the early transcription reaction in the virion but are not required for transcription outside the core.

In conclusion, our results uphold the long-held presumption that NPH-II plays an accessory role in early transcription by the virus-encapsidated RNA polymerase. We speculate that NPH-II may disrupt RNA-DNA hybrids that form during the simultaneous transcription of early genes from a single genomic DNA template. Further insights into the function of NPH-II should emerge as new approaches are developed to

probe the spatial arrangement of the enzymes in the core, as well as the state of the DNA template, at discrete stages of the early transcription reaction.

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