JOURNAL OF VIROLOGY, Mar. 1994, p. 1360–1370
0022-538X/94/$04.00+0

Vol. 68, No. 3

Targeting of a Multicomponent Transcription Apparatus into Assembling Vaccinia Virus Particles Requires RAP94, an RNA Polymerase-Associated Protein

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Received 19 October 1993/Accepted 18 November 1993

When expression of the vaccinia virus gene encoding RAP94 (a protein that is associated with the viral multisubunit RNA polymerase and confers transcriptional specificity for early promoters) was repressed, the infectious virus yield was reduced by more than 99%. Nevertheless, intermediate- and late-stage viral gene expression and formation of ultrastructurally mature, membrane-enveloped virions occurred under the nonpermissive conditions. The RAP94-deficient particles contained the viral genome, structural proteins, early transcription factor, and certain enzymes but, unlike normal virions, had low or undetectable amounts of the viral RNA polymerase, capping enzyme/termination factor, poly(A) polymerase, DNA-dependent ATPase, RNA helicase, and topoisomerase. The presence of these viral enzymes in the cytoplasm indicated that RAP94 is required for targeting a complex of functionally related proteins involved in the biosynthesis of mRNA.

Our understanding of transcriptional mechanisms derives largely from in vitro studies in which the individual components have been separated and purified. Nevertheless, it is likely that many enzymes and factors are physically associated. Novel approaches are needed to investigate the higher-order structure and assembly of the transcriptional apparatus. We show here that vaccinia virus can provide unique insights and experimental approaches to the investigation of this topic.

Vaccinia virus replicates in the cytoplasm of infected cells and encodes a multisubunit DNA-dependent RNA polymerase that resembles its eukaryotic counterpart; early-, intermediate-, and late-stage-specific transcription factors; a eukaryotic transcription elongation factor homolog; mRNA capping and methylating enzymes; a poly(A) polymerase; and other enzymes that may have accessory roles in transcription, including a type 1 topoisomerase, DNA- and RNA-dependent ATPases, and a protein kinase (32). The infectious virus particle can be viewed as a transcription machine, containing within itself all of the macromolecular components necessary for the synthesis and modification of specific early mRNAs upon entry into a susceptible cell. Translation products of the early mRNAs include the enzymes and factors required for viral DNA replication and the subsequent transcription of specific intermediate-stage viral genes. Intermediate genes encode factors necessary for the transcription of the late-stage viral genes which express the majority of virion proteins, including the early-stage-specific transcription factors. During assembly, each membrane-enveloped virus particle is the destination of one copy of the 200,000-bp linear double-stranded DNA genome, numerous structural proteins, and the enzymes and factors that will provide selective transcription of early genes in the next cycle of infection.

How are the appropriate proteins targeted to vaccinia virus virions? It seems unlikely that all virion-associated proteins have individual targeting signals; rather, it seems that functionally related ones are transported as complexes. Glycerol gradient sedimentation analysis of virion extracts suggested that the viral RNA polymerase is loosely associated with the vaccinia virus early transcription factor (VETF) and capping enzyme but not with the other enzymes involved in mRNA formation (7, 22). However, large complexes composed of weakly associated components might not withstand the procedures used for virion disruption and sedimentation analysis. The existence of such complexes might be revealed by analyzing the components of defective virions that assemble when putative targeting proteins are mutated or not made. In this communication, we describe studies that support this approach.

The vaccinia virus RNA polymerase contains polypeptides encoded by eight viral genes that are expressed early in infection (33). An additional RNA polymerase-associated protein of 94 kDa (RAP94) was discovered as a component of the transcriptionally active enzyme isolated from vaccinia virus virions (2). Open reading frame (ORF) H4, encoding RAP94, is expressed late in infection and has a short region similar to that of eukaryotic RNA polymerase-associated general transcription factor RAP30 (2). RAP94 is required for transcription of early genes in vitro, but on the basis of its time of synthesis in infected cells, is unlikely to be involved in either intermediate or late gene expression (2). Several temperature-sensitive viruses with mutations in H4 express viral gene products, and at least one produces normal-appearing virions under nonpermissive conditions (9, 25). Here, we describe the construction and characterization of a vaccinia virus mutant in which the H4 promoter was down regulated by a nucleotide substitution and conditionally repressed by tandem copies of the *Escherichia coli lac* operator. In vitro and in vivo studies with the mutant indicated that RAP94 has a selective role in viral early transcription and that virus particles of mature ultrastructural appearance but deficient in RAP94 could be assembled. Although these particles contained the viral structural proteins, DNA genome, VETF, and certain enzymes, they lacked the RNA polymerase and at least five additional viral enzymes with known or postulated roles in mRNA

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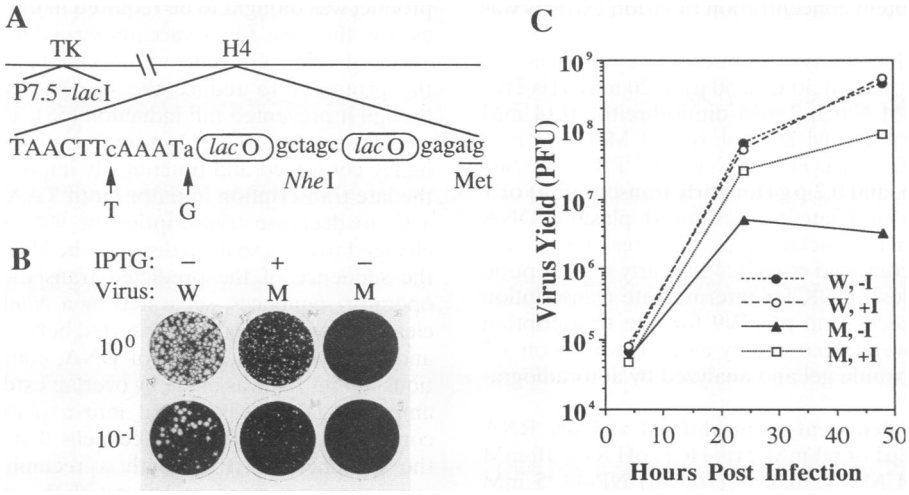

FIG. 1. Promoter mutation, plaque formation, and replication of H4 mutant virus. (A) Mutagenesis of the H4 promoter. The viral genome is represented by a line above which the locations of the thymidine kinase (TK) and H4 genes are indicated. The sequence of the proximal portion of the H4 promoter is shown, with natural and altered vaccinia virus sequences in capital and lowercase letters, respectively. The two *E. coli lac* operator sequences are represented by *lac* O. The *Nhe*I restriction endonuclease site is shown, and the translation initiation codon of the H4 ORF is underlined. (B) Plaque sizes of wild-type and mutant viruses. BS-C-1 cells were infected with approximately 300 (10^0) or 30 (10^{-1}) PFU of wild-type (W) or mutant (M) virus in the presence (+) or absence (−) of 5 mM IPTG. After 2 days, the cell monolayers were stained with crystal violet. (C) Virus yields. BS-C-1 cells were infected with 10 PFU per cell of wild-type (W) or mutant (M) viruses in the absence (−I) or presence (+I) of IPTG. Cells were harvested at the indicated times after infection, and the virus titers were determined by plaque assay in the presence of IPTG.

biosynthesis. Since the missing enzymes were present in the cytoplasm, they may compose a previously unrecognized complex that requires RAP94 for targeting into assembling virions.

MATERIALS AND METHODS

Cells and virus. HeLa S3 cells grown in suspension were used for preparation of infected-cell extracts as well as crude and purified virus stocks (12). Virions were purified twice on 25 to 40% (wt/vol) sucrose gradients. Protocols for transfection, isolation of recombinant virus, one-step virus growth, and viral plaque assay were the same as those previously described (14, 15).

Recombinant virus construction. Procedures for the use of the *E. coli lac* operator to construct inducer-dependent, conditional-lethal, mutant vaccinia viruses have been described (56–59). Four oligonucleotides, (i) ggggcatgcgagctctacagcggcc acgctttctac, (ii) gcgctcacaattccctcgagaattgtgagcgctcacaattctattt gaagttacaaacaactaggaaattggt, (iii) ggggcccgggctctggttctagttccgc attaaatgatg, and (iv) caatttcctagttgtttgtaacttcaaatagaattgtgagcg ctcacaattcgctagcaattgtgagcggataacaattgagatggactctaaagagacta ttctaattgag, together with wild-type virus genomic DNA, were used in a PCR to construct a 945-bp DNA fragment containing the authentic vaccinia virus genomic sequence upstream of the translation initiation codon of the H4 ORF, the mutated H4 promoter with consecutive copies of the 21-bp natural and 22-bp symmetrical *lac* operators (Fig. 1A), and the vaccinia virus DNA sequence downstream of the translation initiation codon. The amplified DNA segment was cloned in a plasmid that contains the *E. coli gpt* gene and recombined into the viral genome by using transient-dominant selection for virus isolation (15). The *lac* operator-containing viruses were screened by PCR (56).

SDS-polyacrylamide gel electrophoresis (PAGE). [^{35}S]methionine labeling of infected-cell proteins was carried out as described previously (56). Proteins from infected cells or purified virions were denatured at 100°C in buffer containing 2% sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (56) and resolved on a 12 or 15% polyacrylamide gel as previously described (16).

For Western blotting (immunoblotting), electrophoretically separated proteins were transferred to nitrocellulose and probed with the desired antibodies. The membranes were then incubated with ^{125}I-labeled protein A, and autoradiographs were prepared (57). The sources of rabbit polyclonal antibodies for the Western blots shown were as follows: viral RNA polymerase (24); RAP94 (2); poly(A) polymerase (19); VETF (20); nucleotide phosphohydrolase I (NPH I) (38); glutaredoxin (3); protein kinase (4); topoisomerase, a gift of S. Shuman; structural protein P4B, a gift of R. Doms; and capping enzyme subunits, a gift of N. Harris.

Preparation of infected-cell extracts and glycerol density gradient centrifugation. HeLa S3 suspension cultures infected with wild-type or mutant vaccinia virus in the presence or absence of 5 mM isopropylthiogalactoside (IPTG) were harvested after 48 h. The cells were broken with a Dounce homogenizer, and the nuclei were removed by centrifugation. The supernatant was ammonium sulfate precipitated, and the pellet was dissolved and dialyzed as described elsewhere (54). The extract was used for transcription assays or layered on an 11-ml 15 to 35% (vol/vol) glycerol gradient in buffer B (50 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 0.01% Nonidet P-40 (NP-40), 10% glycerol, 2 mM dithiothreitol) containing 100 mM NaCl. Centrifugation was for 24 h at 41,000 rpm in an SW41 rotor at 4°C. Fractions of 0.5 ml were collected and stored at −70°C.

Preparation of virion extracts. Purified vaccinia virus virions were disrupted with sodium deoxycholate, and the soluble fraction was passed through a DE52-cellulose column to remove DNA as previously described (43). The effluent was dialyzed against buffer B containing 50 mM NaCl for 16 h at 4°C, divided into aliquots, and stored at −70°C. RNA polymerase was purified and separated into RAP94$^+$ and RAP94$^-$

species (1, 2). The protein concentration of virion extracts was 40 μg/ml.

Specific transcription assays. Transcription reactions (2) were carried out for 30 min at 30°C in 50 μl of 20 mM Tris-HCl (pH 8.0), 40 to 50 mM NaCl, 2 mM dithiothreitol, 0.14 mM EDTA, 4% (vol/vol) polyvinyl alcohol, 6 mM $MgCl_2$, 1 mM ATP, 1 mM CTP, 0.02 mM UTP, 5 μCi of [α-^{32}P]UTP (3,000 Ci/mmol; Amersham), and 0.2 μg (for early transcription) or 1 μg (for intermediate and late transcription) plasmid DNA template. DNA templates lacking guanylate residues in the non-template strand consisted of pSB24 for early transcription (provided by S. Broyles), p30K for intermediate transcription (provided by R. Rosales), and pCFW9 for late transcription (54). RNA products were separated by electrophoresis on a 7 M urea–6% polyacrylamide gel and analyzed by autoradiography.

RNA synthesis by detergent-permeabilized virions. RNA was synthesized in 50 μl of 60 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol, 10 mM $MgCl_2$, 0.05% (vol/vol) NP-40, 5 mM ATP, 1 mM GTP, 1 mM CTP, 0.02 mM UTP, 5 μCi of [α-^{32}P]UTP (3,000 Ci/mmol), and the indicated amounts of purified virions. Incorporation of [α-^{32}P]UMP into materials retained on DE 81 paper was determined (8).

Nonspecific RNA polymerase activity. Nonspecific RNA polymerase activity was measured in 50 μl of 60 mM Tris-HCl (pH 8.0); 7.5 mM dithiothreitol; 3 mM $MnCl_2$; 0.5 μg of M13mp18 single-stranded DNA; 1 mM (each) ATP, GTP, and CTP; 0.1 mM UTP; 2 μCi of [α-^{32}P]UTP (3,000 Ci/mmol, Amersham); and 0.1 to 10 μl of virion extract (2). Incorporation of [α-^{32}P]UMP into materials retained on DE 81 paper was determined.

Electron microscopy. Sample preparation and electron microscopic analysis of infected cells were previously described (57).

Enzyme assays. The formation of a covalent capping enzyme-GMP complex (51) was assayed in 10 μl of 50 mM Tris-HCl (pH 8.2), 10 mM dithiothreitol, 5 mM $MgCl_2$, 0.05% NP-40, 2 μCi of [α-^{32}P]GTP (3,000 Ci/mmol), and 2 μl of infected-cell or virion extract or 0.6 A_{260} units of purified virions. The reaction was carried out for 10 min at 37°C and was analyzed on a SDS–10% polyacrylamide gel.

ATPase and GTPase activities were determined essentially as previously described (38). Reactions were carried out for 60 min at 37°C in a 10-μl volume containing 0.5 μg of heat-denatured calf thymus DNA, 100 mM morpholinopropanesulfonic acid (pH 7.0), 1 mM [α-^{32}P]ATP (10 μCi) or 1 mM [α-^{32}P]GTP (10 μCi), 1 mM $MgCl_2$, 5 mM dithiothreitol, and 0.05% (vol/vol) NP-40. In some reaction mixtures, DNA was omitted or replaced by the same amount of poly(C). A sample (1 μl) of reaction mixture was spotted on a polyethyleneimine-cellulose sheet which was subjected to thin-layer chromatography in 1 M CH_3COOH–4 M LiCl (4:1 [vol/vol]).

DNA topoisomerase activity of the virion extracts was measured as previously described (50) by using supercoiled plasmid pCFW9 as a substrate. The single-stranded DNase activity of the virion extract was determined as reported elsewhere (29).

RESULTS

Construction of a recombinant vaccinia virus with a mutated H4 promoter. We and others have previously constructed inducer-dependent mutants of vaccinia virus that constitutively express the *E. coli lac* repressor and have one or more copies of the *lac* operator placed just downstream of the RNA start site of a gene to be regulated (17, 41, 56). When the gene product was thought to be required in low or catalytic amounts, as was the case for a vaccinia virus late transcription factor encoded by the G8 gene, we also made point mutations within the promoter to reduce the basal level of expression even though it prevented full induction (58). The latter strategy was used to regulate the H4 promoter, as shown in Fig. 1A. The highly conserved and functionally important first T residue of the late transcription initiator motif TAAATG was changed to a C, to decrease transcription by 90% (11), and the G was changed to an A so as to displace the H4 ORF without altering the sequence of the predicted translation product. Two *lac* operator sequences, separated by a *Nhe*I restriction endonuclease site, were tandemly inserted between the RNA start site and the ORF. A segment of DNA, containing the modifications in Fig. 1A, was made by overlap extension PCR (23). The amplified DNA was cloned into a plasmid vector, and the construct was used to transfect cells that had been infected in the presence of IPTG with a recombinant *lac* repressor-expressing vaccinia virus. Virus with the operator-regulated H4 promoter was formed by homologous recombination and enriched by the transient-dominant selection method (14). Individual plaques were screened by PCR for the presence of *lac* operator DNA sequences. Positive recombinant viruses were plaque purified three additional times and propagated in BS-C-1 cells in the presence of IPTG.

Infectivity of mutant virus. The plaques formed by the recombinant virus were smaller than those of wild-type virus in the presence of IPTG and tiny in the absence of inducer (Fig. 1B). One-step growth analysis indicated that in the presence and absence of inducer, the respective yields of mutant virus were 1/6th and 1/140th those of wild-type virus (Fig. 1C). The inability of IPTG to completely restore infectivity was anticipated in view of the mutation made to decrease the basal level of expression of the H4 ORF. Nevertheless, the yield in the presence of IPTG was sufficient to propagate the mutant virus easily.

Synthesis of viral proteins by mutant virus. The DNA template and the enzymes for early gene expression are contained within the vaccinia virus particle, and consequently early viral protein synthesis can be detected soon after infection. By contrast, intermediate and late viral protein synthesis must await the replication of viral DNA and de novo synthesis of viral enzymes and factors. The pattern and timing of viral protein synthesis, as determined by pulse-labeling with radioactive amino acids and SDS-PAGE, were similar in cells infected with wild-type or mutant virus in the presence or absence of IPTG (Fig. 2A) despite a more than 99% inhibition of infectious virus formation under the latter conditions. These differences could be reconciled, since the mutant virions used for infection had been grown in the presence of IPTG and presumably packaged sufficient RAP94 for early transcription. Also, since RAP94 is itself a late protein, inhibition of its synthesis would not be expected to negatively affect viral intermediate or late gene expression during the current round of infection. The expected phenotype is, therefore, the production of RAP94-deficient particles that will exhibit reduced early transcription activity during the next round of infection.

Western blotting indicated that the amount of RAP94 was reduced by about 95% in cells infected with mutant virus in the absence of IPTG (Fig. 2B). Although IPTG stimulated RAP94 synthesis by about threefold, the level was still far below that detected in wild-type vaccinia virus-infected cells because of the H4 promoter mutation. The blots were probed with antibody to a 65-kDa viral late protein as an internal control to demonstrate that there was no general inhibition of late viral

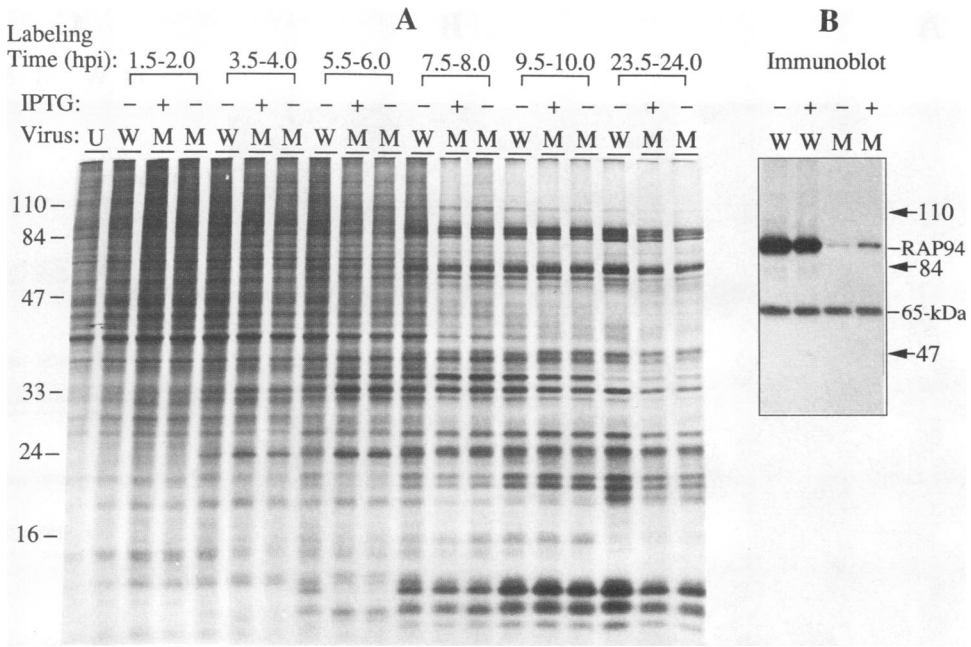

FIG. 2. Synthesis of viral proteins. (A) Autoradiogram of a polyacrylamide gel containing metabolically labeled proteins of infected cells. BS-C-1 cells that were mock infected (U) or infected with wild-type (W) or mutant (M) viruses in the absence (−) or presence (+) of IPTG were labeled with [^{35}S]methionine between the indicated hours after infection, and the proteins were analyzed by SDS-PAGE. The migration positions and molecular masses (in kilodaltons) of protein markers are indicated. (B) Lysates of BS-C-1 cells infected with wild-type or mutant virus in the presence or absence of IPTG were analyzed by SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane, incubated with antibodies against RAP94 and the 65-kDa product of the D13 gene and then ^{125}I-labeled protein A, and autoradiographed. The positions of migration and molecular masses (in kilodaltons) of protein markers are indicated by arrows.

protein synthesis and that equivalent amounts of extract had been applied to the gel (Fig. 2B).

Purification and characterization of RAP94-deficient virions. Since metabolic labeling experiments had indicated that viral late proteins were made, electron microscopy was carried out to determine whether assembly of virus particles occurred in cells infected with mutant virus in the absence of inducer. Images of thin sections of mutant virus-infected cells revealed the presence of virus particles, at all stages of maturation, that were not noticeably different in amount or appearance from those present in cells infected with wild-type virus. Photographs of typical mature-appearing mutant virions are shown (Fig. 3A and B). As the ultrastructure of wild-type virions is well documented, photographs of them are not presented.

To obtain sufficient amounts of virus particles for biochemical studies, a stock of infectious mutant virus that had been propagated in BS-C-1 monolayer cells in the presence of IPTG was used to infect HeLa spinner cells in the presence or absence of inducer. As a control, HeLa cells were infected with wild-type vaccinia virus. Purified wild-type and mutant vaccinia virus virions appeared similar by electron microscopy and sedimentation in sucrose and CsCl gradients (data not shown). Similar amounts of viral genomic DNA, in wild-type and mutant virions, were detected by dot blot hybridization with a labeled probe (data not shown). Even the stained polypeptide patterns obtained by SDS-PAGE, which primarily reflect the major structural proteins, were almost identical (Fig. 3C). The only differences detected in the mutant virions were diminished intensities of two bands, one above the 110-kDa marker and the other above the 84-kDa marker, corresponding in sizes to the unresolved large subunits of RNA polymerase and RAP94, respectively. Western blotting revealed a prominent RAP94 band in wild-type virions, a faint band in mutant virions that formed in the presence of IPTG, and only trace amounts in those formed in the absence of inducer (Fig. 3D).

Transcriptional activity of mutant virions. The in vitro transcriptional activities of wild-type and mutant RAP94-deficient virions were compared by incubating them with ribonucleoside triphosphates in the presence of a nonionic detergent, mimicking the early transcription from the endogenous genome that occurs after virus entry into the cytoplasm. RNA synthesis was measured at a wide range of particle concentrations because the high endogenous ATPase activity causes significant deviations from linearity. We calculated that the mutant virions had only 5% of the transcriptional activity of wild-type virions (Fig. 4A), consistent with the low amount of RAP94.

Although transcriptional activity was not directly measured in vivo, viral gene expression in cells infected with 9, 90, or 900 particles per cell of wild-type or mutant virions in the absence of IPTG was determined by pulse-labeling with [^{35}S]methionine. SDS-PAGE patterns of viral proteins similar to those in Fig. 2A were obtained with all concentrations of wild-type virus but only with the highest concentration of mutant virus, reflecting the difference in transcriptional activities of the particles (data not shown).

Absence of RNA polymerase activity in extracts of mutant virions. The low endogenous transcriptional activity of mutant virions could be ascribed solely to the low amount of RAP94, since RAP94-deficient RNA polymerase cannot interact with VETF to transcribe early viral genes (1, 2). However, we could not detect RNA polymerase activity in extracts of mutant virions by using a nonspecific single-stranded DNA template (Fig. 4B) under conditions in which RAP94-deficient RNA

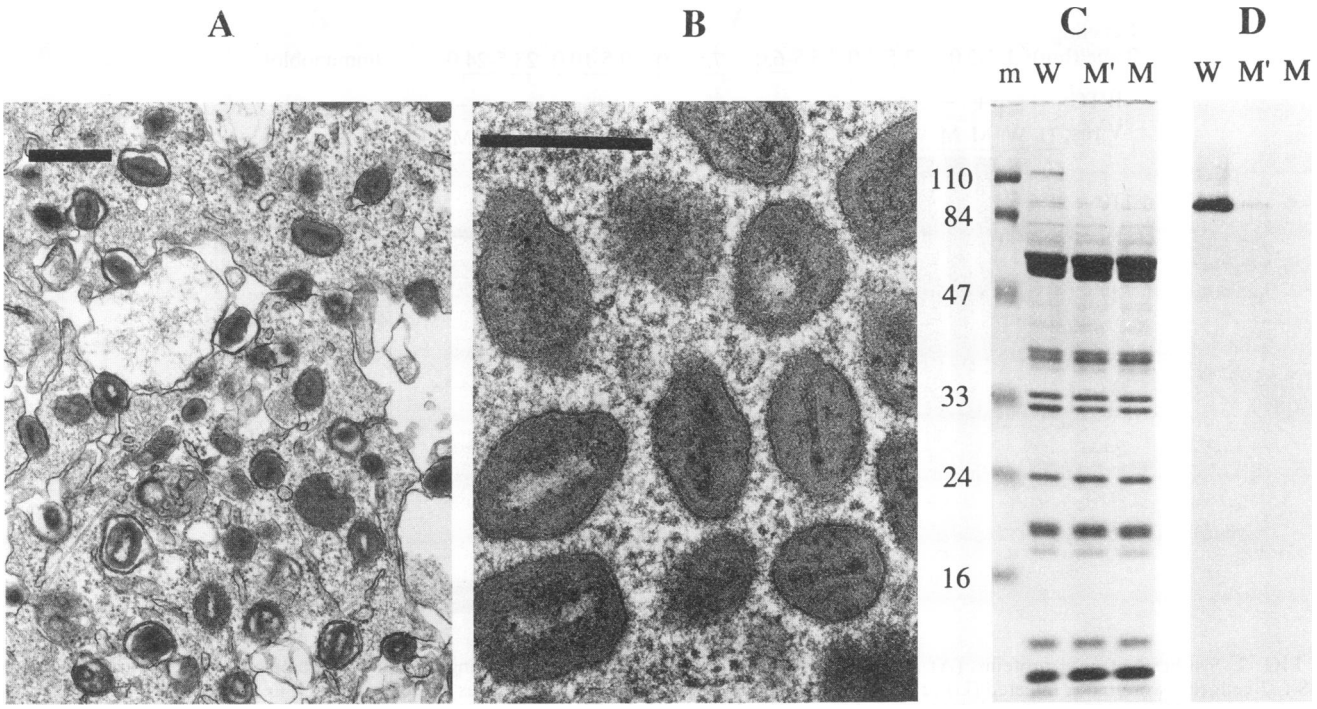

FIG. 3. Comparison of wild-type and mutant virions. (A and B) Electron micrographs of thin sections of BS-C-1 cells infected with mutant virus in the absence of IPTG. The views show mature virions of typical appearance. Many of the virions in panel A are wrapped with cisternal membranes, a normal postmaturational step leading to the exit of the virus from cells. Bars, 500 nm (A) and 250 nm (B). (C) Virions were purified from HeLa cells infected with wild-type virus (W) or mutant virus in the presence (M′) or absence (M) of IPTG, and the proteins were analyzed by SDS-PAGE. The gel was stained with Coomassie blue and photographed. Lane m, marker proteins with the indicated molecular masses (in kilodaltons). (D) Western blot of electrophoretically separated virion proteins transferred to a nitrocellulose membrane and probed with antiserum against a RAP94 fusion protein (2) and visualized with ^{125}I-labeled protein A.

polymerase is even more active than RAP94$^+$ RNA polymerase (1, 2). Therefore, the absence of nonspecific RNA polymerase activity in extracts of mutant virions must be due to a deficiency of RNA polymerase and not just RAP94.

Addition of highly purified RAP94$^+$ RNA polymerase, but not RAP94$^-$ RNA polymerase, allowed the extract of mutant virions to transcribe an early promoter-regulated template (Fig. 4C), indicating the presence of VETF in the mutant virions. Control experiments demonstrated that the purified RAP94$^-$ RNA polymerase had nearly twice the activity of RAP94$^+$ RNA polymerase with a nonspecific single-stranded DNA template and that neither polymerase by itself could transcribe the double-stranded early promoter template (1).

Presence of viral RNA polymerase in the cytoplasm under nonpermissive conditions. The RNA polymerase deficiency of mutant virions could be explained by diminished synthesis, assembly, or packaging of the multisubunit enzyme during infection. To investigate these possibilities, we analyzed remaining portions of the infected-cell extracts that had been used to purify the wild-type and mutant virions. Western blotting indicated similar amounts of viral RNA polymerase subunits, although there was a 20-fold difference in the amounts of RAP94 (data not shown). The cytoplasmic extracts of infected and uninfected HeLa cells were sedimented through glycerol gradients, and fractions were assayed for RNA polymerase activity and analyzed by SDS-PAGE. Insignificant amounts of polymerase activity were obtained from the cytoplasm of uninfected cells (Fig. 5A), whereas substantial amounts were obtained from the cytoplasm of cells infected with wild-type (Fig. 5A) or mutant (Fig. 5B) virus in the absence of IPTG. Western blots of material from wild-type and mutant virus-infected cells confirmed the presence of viral RNA polymerase subunits in the enzymatically active glycerol gradient fractions (Fig. 5A and B). The association of RAP94 with RNA polymerase from wild-type virus-infected cells but not from mutant virus-infected cells was confirmed as well (Fig. 5A and B). We concluded, therefore, that the absence of RNA polymerase from mutant virions was not due to diminished synthesis or assembly of the subunits.

Transcriptional activities of cytoplasmic extracts. Although vaccinia virus-infected HeLa cells synthesize predominantly late viral proteins in vivo at 48 h after infection, cytoplasmic extracts can transcribe templates regulated by all three types of promoters in vitro. The deficiency of RAP94 therefore provided us with a unique opportunity to directly compare the role of this protein for in vitro transcription of early, intermediate, and late promoter-regulated templates. Extracts of cells infected with mutant virus in the absence of IPTG were unable to transcribe early promoter templates, whereas the extracts of wild-type virus-infected cells could do so (Fig. 5C). There was early transcription activity, however, when the extracts from mutant virus-infected cells were supplemented with purified RAP94$^+$ RNA polymerase but not with RAP94$^-$ RNA polymerase (Fig. 5C). Transcription of intermediate promoter templates by extracts of mutant and wild-type virus-infected cells was similar and relatively unaffected by RNA polymerase addition (Fig. 5C). The much greater transcription of the late promoter template by the extract of mutant virus-infected cells compared with the extract of wild-type virus-infected cells was unanticipated (Fig. 5C). The extract of wild-type virus-infected

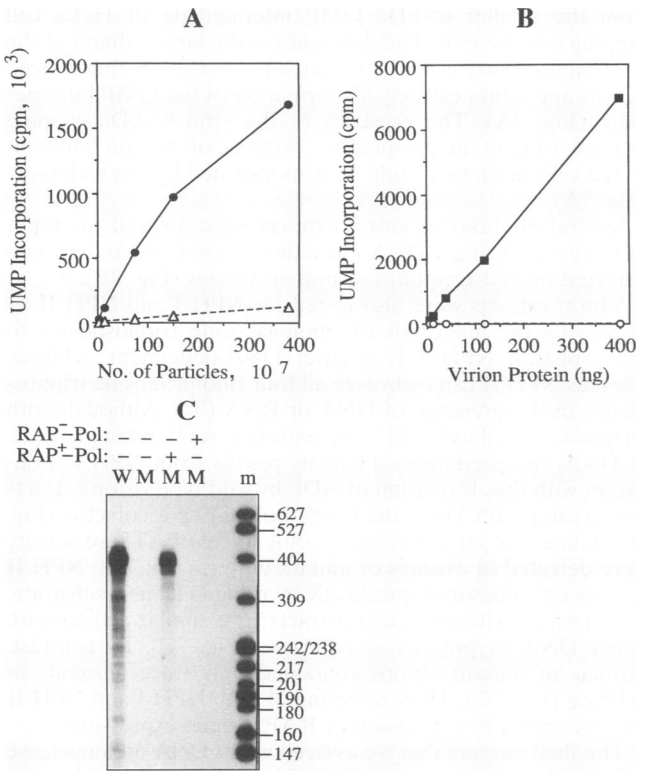

FIG. 4. Specific and nonspecific RNA polymerase activities of wild-type and mutant virions. (A) Viral RNA synthesis by permeabilized wild-type virions (●) and mutant virions formed in the absence of inducer (△). Purified virions were incubated with nonionic detergent and ribonucleoside triphosphates, and incorporation of [α-^{32}P]UMP into RNA was measured. (B) Nonspecific RNA polymerase activity extracted from purified wild-type (■) and mutant (○) virions. Virion extracts were incubated with M13 single-stranded DNA and ribonucleoside triphosphates; incorporation of [α-^{32}P]UMP into RNA was determined. (C) Specific transcription of early promoter template with wild-type (W) or mutant (M) virion extracts. The incubation mixtures were supplemented with RAP94^{+} (RAP^{+}) or RAP94^{-} (RAP^{-}) RNA polymerase as indicated. Lane m, DNA markers, the lengths of which are indicated (in nucleotides).

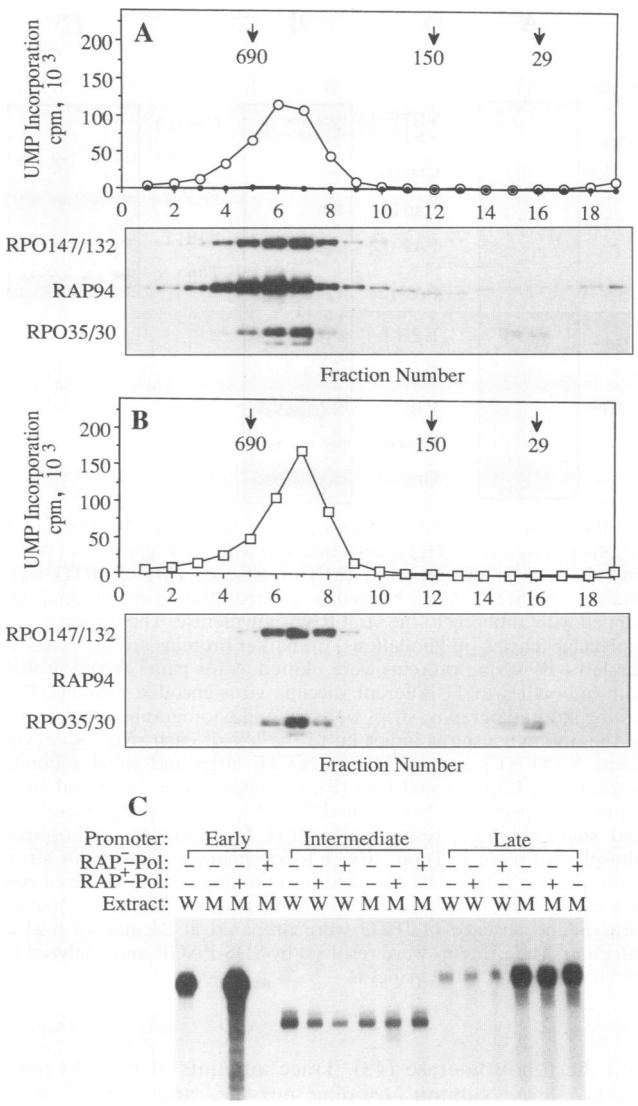

FIG. 5. RNA polymerase and specific transcription activities of cytoplasmic extracts. (A and B) Glycerol gradient sedimentation of cytoplasmic extracts of HeLa S3 suspension cells infected with wild-type or mutant virus in the absence of inducer. Fractions were tested for nonspecific RNA polymerase activity (●), uninfected; ○, wild type virus infected; and □, mutant virus infected). The sedimentation positions of markers of known molecular mass (in kilodalton) are indicated by arrows. SDS-PAGE and Western blots were made of fractions from gradients containing extracts of wild-type (A) and mutant (B) virus-infected cells. The blots were probed with antibody to the viral RNA polymerase and RAP94 and then by ^{125}I-protein A. Autoradiographic strips of the large (RPO147 and -132) and middle (RPO35 and -30) RNA polymerase subunits and RAP94 are shown. (C) In vitro transcription, in the presence (+) or absence (−) of added RAP94^{+} RNA polymerase or RAP94^{-} RNA polymerase, of early, intermediate, and late promoter-regulated DNA templates by extracts from HeLa S3 cells infected with wild-type (W) or mutant (M) virus. The RNA products labeled with [α-^{32}P]UTP were analyzed by PAGE, and an autoradiograph is shown.

cells was apparently not deficient in RNA polymerase, since additions had no effect. Perhaps some other factor, targeted to assembling virions in wild-type virus-infected cells, remains available for late transcription in extracts of mutant virus-infected cells. Most importantly, these in vitro experiments demonstrated the stage-specific transcriptional specificity of RAP94.

Mutant virions exhibit selective enzyme deficiencies. The presence of viral RNA polymerase activity in cytoplasmic extracts but not in mutant virions suggested a deficiency in enzyme packaging. Western blotting of SDS-disrupted virions confirmed this interpretation. Antibody to the viral RNA polymerase revealed the three major size groups of incompletely resolved subunit polypeptides in wild-type virions but not in mutant virions (Fig. 6A).

At this stage in our investigation, we suspected that RAP94 might be required for the targeting of other enzymes as well as RNA polymerase, and we therefore collected specific antibodies to probe Western blots. Appropriate strips, excised from autoradiograms of the blots, were compiled to form Fig. 6B. The following virus-encoded enzymes were detected in SDS-disrupted wild-type virions but were present in low or undetectable amounts in mutant virions: large and small capping enzyme subunits (31, 36), the large (VP55) subunit of poly(A) polymerase (19), DNA-dependent ATPase (NPH I) (6, 40),

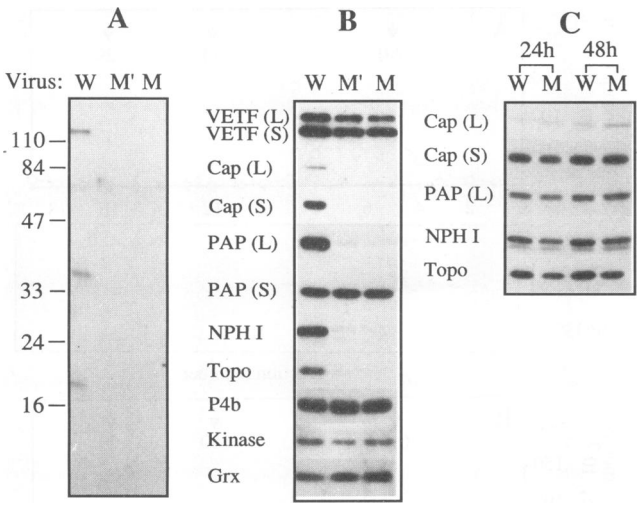

FIG. 6. Western blot analysis of virion proteins. (A) Proteins of virions purified from HeLa cells infected with wild-type virus (W) or mutant virus in the presence (M′) or absence (M) of IPTG were analyzed by SDS-PAGE, blotted to a nitrocellulose membrane, and probed with antibody to the viral RNA polymerase. The positions and molecular masses (in kilodaltons) of marker proteins are indicated on the left. (B) Virion proteins were blotted as for panel A and probed with antibodies to 11 different vaccinia virus-encoded proteins. Following autoradiography, strips were cut and photographed. The target of the antibody used is indicated to the left of each strip. Abbreviations: VETF (L) and VETF (S), VETF large and small subunits, respectively; Cap (L) and Cap (S), capping enzyme large and small subunits, respectively; PAP (L) and PAP (S), poly(A) polymerase large and small subunits, respectively; NPH I, nucleoside triphosphate phosphohydrolase I; Topo, type 1 topoisomerase; P4b, major structural protein; Kinase, B1-encoded protein kinase; Grx, glutaredoxin. (C) Cytoplasmic extracts of cells infected with wild-type or mutant virus in the absence of IPTG were prepared at 24 and 48 h after infection. The proteins were resolved by SDS-PAGE and analyzed by Western blotting as for panel B.

and the topoisomerase (48). Trace amounts of poly(A) polymerase large subunit, capping enzyme, and NPH I were detected in mutant virions upon prolonged exposure of the autoradiograms, and in those cases the bands were more intense in particles produced in the presence of IPTG than in its absence (data not shown). As may be seen in Fig. 6B, the following proteins were present in similar amounts in wild-type and mutant virions: both VETF subunits (20), the small (VP39) subunit of poly(A) polymerase which also has mRNA nucleoside-2′-methyltransferase activity (19, 46), the B1R protein kinase (4, 27, 39), glutaredoxin (3), and the major core protein P4b (44).

To determine whether the enzymes missing from mutant virions had been synthesized, cytoplasmic extracts from the same cells used to purify the virions were subjected to Western blotting. Normal amounts of capping enzyme subunits, DNA-dependent ATPase, poly(A) polymerase, and topoisomerase were detected in the cytoplasm (Fig. 6C) despite their absence from virions.

We also assayed the mutant virions or virion extracts for several enzymatic activities either as an alternative to Western blotting (e.g., capping enzyme, topoisomerase, and NPH I) or because we did not have good antibodies (e.g., for NPH II and DNA endonuclease). The large subunit of the vaccinia virus capping enzyme forms a 95-kDa covalent intermediate with GMP when incubated with GTP (49) which is distinguishable from the smaller 65-kDa GMP intermediate of HeLa cell capping enzyme (53). The deficiency in the large subunit of the viral capping enzyme was confirmed by assaying permeabilized virions and virion extracts for formation of the GMP intermediate (Fig. 7A). The presence of the viral 95-kDa capping enzyme subunit in cytoplasmic extracts of mutant virus-infected cells, however, could be demonstrated by this technique (Fig. 7A).

Several dilutions of virion extracts were assayed for topoisomerase by using a DNA unwinding assay (50). Activity was detected in wild-type but not mutant virions (Fig. 7B).

Virion extracts were also tested for NPH I and NPH II by measuring the hydrolysis of ribonucleoside triphosphates to diphosphates. NPH I is a strict DNA-dependent ATPase, whereas NPH II can hydrolyze all four ribonucleoside triphosphates in the presence of DNA or RNA (37). Although both enzymes, as well as VETF, can hydrolyze ATP in the presence of DNA, the predominant activity results from NPH I. Consistent with this, formation of ADP by wild-type virion extracts was greater with DNA than with poly(C) as a cofactor (Fig. 7C). Under the same conditions, only traces of ATPase activity were detected in extracts of mutant virions (Fig. 7C). NPH II activity was measured specifically by using GTP as a substrate. As expected, wild-type virus extracts gave similar activity with either DNA or poly(C) as cofactors (Fig. 7C). By contrast, extracts of mutant virions contained only trace amounts of GTPase (Fig. 7C). Thus, targeting of both NPH I and NPH II was prevented by repression of RAP94 gene expression.

The final enzyme that we assayed was a DNA endonuclease activity that nicks superhelical DNA (5, 45). Activity was present in extracts of mutant as well as wild-type virions (Fig. 7D), indicating that targeting of this enzyme occurred independently of RAP94.

DISCUSSION

During the last phase of vaccinia virus replication, late genes are expressed while the components necessary for transcription of early genes are selectively packaged in assembling virus particles. If RAP94 conferred early promoter specificity to the viral RNA polymerase, we considered that it might also have a role in targeting the polymerase into virions. To investigate the requirement for RAP94 in transcription and packaging, we devised a method of inhibiting the synthesis of RAP94. The consequences of this genetic manipulation exceeded our initial expectations and provided evidence for previously unrecognized interactions between enzymes involved in synthesis and modification of mRNA and enzymes only suspected of having such roles.

Our goal was to make an IPTG-dependent mutant by inserting *E. coli lac* operator sequences downstream of the RNA start site of H4, the gene encoding RAP94. However, as catalytic proteins are frequently made in excess and our past experience indicated that it is difficult to obtain complete repression, we also decreased the basal transcription of the H4 gene by promoter mutagenesis, knowing that this might prevent complete induction. We preferred to achieve severe repression in the absence of inducer as long as there was sufficient induction to propagate the mutant virus. The mutant virus was constructed by homologous recombination and isolated in the continuous presence of inducer. The mutant had a small plaque phenotype in the presence of IPTG and produced tiny plaques in the absence of inducer. The yields of infectious mutant virus were 17 and 0.7% of normal with and without 5 mM IPTG, respectively.

We had anticipated that mutant virus grown in the presence

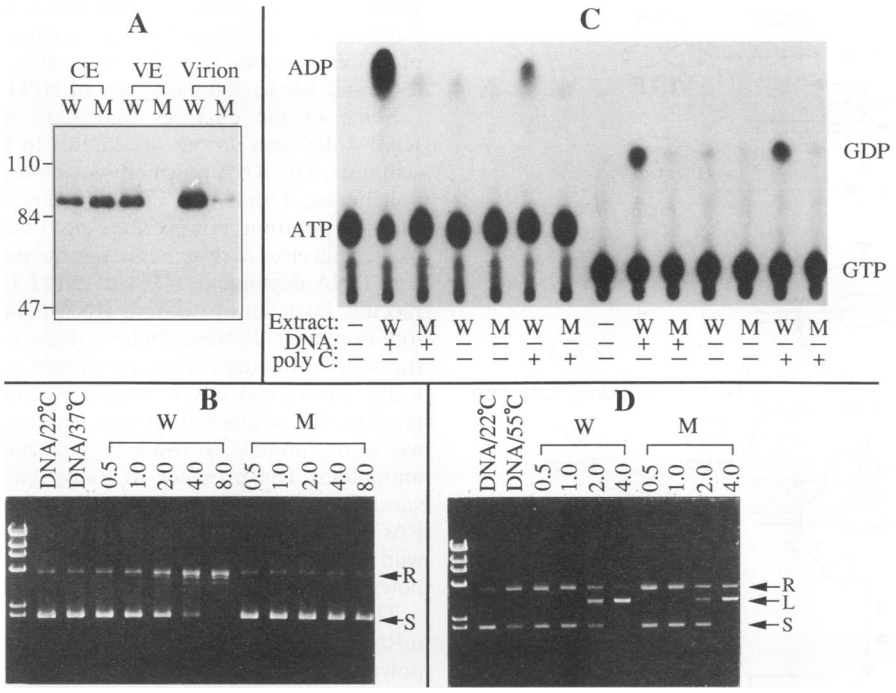

FIG. 7. Enzymatic activities of wild-type and mutant virions. (A) Covalent capping enzyme-GMP complex. [α-^{32}P]GTP was incubated with cytoplasmic extracts (CE), with extracts of purified virions (VE), or with permeabilized virions (Virion). Proteins were resolved by SDS-PAGE, and the covalent capping enzyme-GMP complex was visualized by autoradiography. In all panels, W and M represent wild-type and mutant virus, respectively. The molecular masses (in kilodaltons) of marker proteins are indicated on the left. (B) DNA topoisomerase activity. Supercoiled plasmid DNA was incubated with indicated amounts (in microliters) of extracts of wild-type (W) or mutant (M) virions at 37°C. Samples were then subjected to electrophoresis in a 1.5% agarose gel. The leftmost (unmarked) lane contains *Hin*dIII-cleaved phage lambda DNA markers. The next two lanes contain plasmid incubated in buffer at the indicated temperatures. The arrows point to relaxed (R) and supercoiled (S) forms of DNA. (C) NPH I and NPH II activities. [α-^{32}P]ATP or GTP was incubated with extracts of wild-type (W) or mutant (M) virions in the presence (+) or absence (−) of denatured calf thymus DNA or poly(C). The formation of ribonucleoside diphosphates was determined by thin-layer chromatography and autoradiography. The migration positions of ATP, ADP, GTP, and GDP are shown. (D) Endonuclease activity. The ability of virion extracts to nick supercoiled DNA was measured at 55°C. Abbreviations and markings are similar to those for panel B. L, linearized DNA molecules.

of IPTG would contain sufficient RAP94 so that initial expression of early genes would occur upon infection of cells even in the absence of inducer. Moreover, the subsequent expression of intermediate and late genes might proceed if RAP94 were not needed at these stages. Precisely these results were obtained consistent with a role for RAP94 as an early-stage-specific transcription factor. This interpretation was confirmed by making cytoplasmic extracts of wild-type and mutant virus-infected cells. The extracts of wild-type virus-infected cells could transcribe templates regulated by all three classes of viral promoters. The RAP94-deficient extracts, however, were unable to transcribe early genes in vitro unless supplemented with RAP94$^+$ RNA polymerase. Nevertheless, the unsupplemented extracts were competent to transcribe intermediate and late promoter templates at normal and higher than normal rates, respectively.

In addition to synthesis of viral late proteins, morphologically mature virions were assembled in cells infected with mutant virus in the absence of inducer. This phenotype was similar to that caused by a temperature sensitivity mutation in the H4 gene (25). To obtain sufficient amounts of our mutant virions for characterization, we scaled up their production in HeLa cell suspension cultures. The purified RAP94-deficient particles appeared normal by electron microscopy and contained viral DNA and a full complement of structural proteins as determined by SDS-PAGE. Nevertheless, the particles had low or undetectable amounts of the multisubunit RNA polymerase, capping enzyme/termination factor, catalytic subunit of poly(A) polymerase, DNA-dependent ATPase (NPH I), RNA-dependent nucleoside triphosphatase (NPH II), and topoisomerase. Except for RAP94, these viral proteins were abundant in the cytoplasm of mutant-infected cells. The exclusion from mutant virions of these six enzymes, comprising at least 15 polypeptides, was specific, since VETF as well as other viral enzymes including the B1R protein kinase, glutaredoxin, an endonuclease, and the VP30 subunit of poly(A) polymerase were present.

One explanation for the data is that many of the enzymes involved in the biosynthesis of vaccinia virus early mRNAs are simultaneously targeted by RAP94 to developing virus particles, perhaps through previously unrecognized physical associations. How could RAP94 target such a complex to virions? The model depicted in Fig. 8 is based partly on the detection of normal amounts of viral DNA and the heterodimeric early transcription factor VETF in RAP94-deficient virions. VETF is the only known vaccinia virus-encoded sequence-specific DNA-binding protein (8, 55). We suggest that VETF binds to early promoter sequences in the genomic DNA and then acts as the receptor for RAP94 and the associated RNA polymerase. The additional enzymes could interact with RAP94 itself or as depicted with other RNA polymerase subunits either before (step 2) or after (step 3) association with the

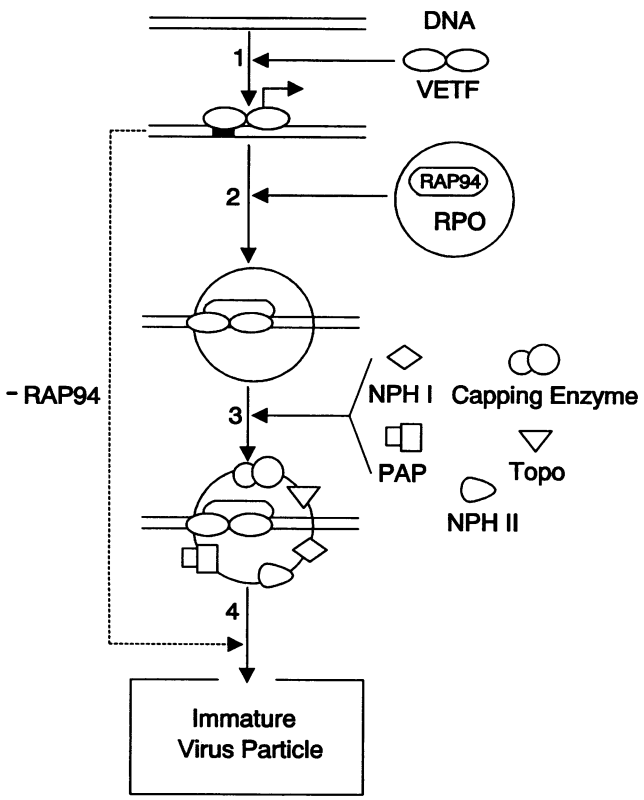

FIG. 8. Model for RAP94-dependent targeting of the transcription complex. Viral DNA is represented by parallel lines. The sequence-specific binding site for VETF is shown by a filled box. The arrow with a 90°C bend on the VETF-DNA complex represents an early gene RNA start site. RPO, RNA polymerase. Abbreviations for other enzymes are given in the legend to Fig. 6. Numbers indicate steps in targeting the complex to assembling virions.

genome occurs. A second model (not shown) is that VETF and RAP94$^+$ RNA polymerase initiate the transcription of early genes within the immature virions and that additional enzymes are recruited to the complex. In this regard, Roening and Holowczak (42) detected ^{32}P-labeled RNA in purified vaccinia virus virions, although it was not possible at the time to determine whether it resulted from accidental inclusion of late RNA or specific synthesis of early RNA. Perhaps the recently reported late reactivation of transcription of certain early genes (18) is related in some way to packaging. A prediction of either of our models is that repression of the genes encoding VETF will prevent targeting of the enzyme complex into virions.

The proposed models raise questions regarding the stoichiometry between early promoters, VETF, RAP94, RNA polymerase, and the associated enzymes. One molecule of RAP94 may be sufficient to target two to three RNA polymerase molecules, since only 30 to 40% of the RNA polymerase isolated from purified virions has an associated molecule of RAP94 (1). This apparent discrepancy could occur if the association of RAP94 with RNA polymerase is a dynamic one or if there are associations between multiple RNA polymerase molecules so that submolar amount of RAP94 is sufficient. No estimates of the amount of VETF or RNA polymerase in virions have been made, although Kates and Beeson (26) suggested that there is a minimum of 40 RNA chain-growing points per virus particle. Calculations, on the basis of protein purification, suggested that virions contain about 90 molecules of capping enzyme (28), 100 molecules of poly(A) polymerase (34), and 100 to 300 molecules of NPH I (37).

Some of the enzymes that were not incorporated into RAP94-deficient virions are known to be involved in mRNA synthesis, e.g., RNA polymerase, capping enzyme, and poly(A) polymerase. Other enzymes that were dependent on RAP94 for incorporation, however, are not essential for transcription of vaccinia virus early genes under the usual in vitro conditions, e.g., DNA-dependent ATPase (NPH I); NPH II, which has recently been shown to have RNA helicase activity (47); and topoisomerase I. Nevertheless, these enzymes may enhance transcription under certain conditions, such as with a topologically constrained DNA template within the virus core. It is possible that additional proteins are targeted by RAP94, since we were limited by available enzyme assays and specific antibodies. The presence of more than 100 polypeptides in vaccinia virus virions has been suggested by two-dimensional PAGE (13). A similarly detailed comparison of mutant and wild-type virions might reveal additional RAP94-targeted polypeptides that could have roles in mRNA biosynthesis.

The behavior of VP39, a protein that has cap-specific mRNA (nucleoside-2′-)methyltransferase (46) and poly(A) polymerase stimulatory activities (19), was contrary to that of other enzymes involved in viral mRNA biosynthesis. VP39 exists both as a monomer and as a heterodimer in association with VP55, the catalytic subunit of poly(A) polymerase. In wild-type virions, the ratio of VP39 to VP55 is approximately 5:1 (19). VP39 was present in RAP94-deficient virions but VP55 was not, indicating that VP39 must be targeted separately and apparently by a mechanism that is inadequate to transport the heterodimer. Possibly, VP39 interacts with an alternative targeting protein through the same site that binds VP55.

What is known regarding the assembly of vaccinia virus virions has come primarily from electron microscopy (10, 52). In brief, the first highly characteristic structures are spicule-coated membrane arcs that develop into spheres containing a single eccentrically placed electron-dense body known as a nucleoid that is thought to contain DNA (21, 35). Some electron microscopic images suggest that the nucleoid is preassembled and inserted into the immature particle prior to complete membrane closure (30). Nucleoids were detected in cells infected with mutant virus in the absence of inducer, suggesting that their formation is not dependent on an association with the complete early transcription complex. More information on the protein composition of the nucleoids, obtained either by physical isolation or immunoelectron microscopy, is needed.

In conclusion, inhibiting the synthesis of RAP94 had specific and profound effects on the targeting of enzymes with known or suspected roles in mRNA biosynthesis. Although the data can be interpreted as resulting from a defect in the assembly or targeting of a previously unrecognized complex of functionally related proteins and/or prevention of transcription initiation within assembling particles, there is little available biochemical data on this subject. Indeed, the difficulty in obtaining such information highlights the significance of our approach. Nevertheless, there is now an obvious need to test the models proposed here and provide physical evidence for the putative complex and/or a role for transcription initiation in virion assembly.

ACKNOWLEDGMENTS

We thank S. Broyles, R. Condit, R. Doms, P. Gershon, N. Harris, E. Niles, E. Paoletti, P. Traktman, S. Shuman, and G. Smith for kindly providing antibodies to viral proteins. P. Gershon, N. Harris, and M. Merchlinsky gave helpful advice regarding thin-layer chromatography, capping enzyme assays, and DNase determinations, respectively. M. Challberg, K.-T. Jeang, G. Kovacs, and P. Gershon provided helpful commentary on the manuscript.

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