

RNA Polymerase-associated Protein Rap94 Confers Promoter Specificity for Initiating Transcription of Vaccinia Virus Early Stage Genes*

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The association of a 94,000-Da viral polypeptide, called Rap94, with 30–40% of the multisubunit DNA-dependent RNA polymerase molecules purified from infectious vaccinia virus particles was established by immunoaffinity chromatography. The submolar amount of Rap94, relative to RNA polymerase, was confirmed by quantitative immunoblotting of total virion extracts. Only the RNA polymerase molecules containing Rap94 could functionally interact with VETF, the vaccinia virus early transcription factor, to transcribe a double-stranded DNA template regulated by a viral early stage promoter. Rap94 was required for the synthesis of short oligoribonucleotides and for the formation of stable ternary transcription complexes. With a nonspecific single-stranded DNA template, however, the Rap94-deficient polymerase had greater catalytic activity than the Rap94-containing polymerase. These data support a model in which Rap94 confers specificity to the RNA polymerase for promoters of early stage genes.

Regulated gene expression is a characteristic of the replicative cycle of DNA viruses as well as cells. In some cases, viral gene products are directly or indirectly involved in the recruitment of the host RNA polymerase to the viral genome. Poxviruses are unusual in that they encode both a DNA-dependent RNA polymerase and stage-specific transcription factors that allow gene expression to occur in the cytoplasm of the infected cell (reviewed in Ref. 1). Studies with vaccinia virus, the representative member of the poxvirus family, have revealed a programmed pattern of regulation involving the successive transcription of early, intermediate, and late stage genes. The proteins and the template needed for early gene expression are brought into the cell within the infectious virus particle, whereas *de novo* protein and DNA synthesis are required for viral intermediate and late gene expression. The three temporal classes of genes have distinguishing promoter sequences and cognate transcription factors. The vaccinia virus encoded early transcription factor (VETF) is composed of 77- and 82-kDa subunits and has promoter-specific DNA binding and DNA-dependent ATPase activities (2–6). The virus-encoded capping enzyme and at least one additional partially purified factor are required for transcription of intermediate genes *in vitro* (7–9). Genetic and biochemical experiments led to the identification of three intermediate genes required for late

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transcription (10–13). How these factors interact with the viral RNA polymerase for stage-specific transcription, remains unknown.

The RNA polymerase of vaccinia virus more closely resembles those of eukaryotes than prokaryotes with regard to subunit number and sequence (14). Of the eight unique virus-encoded RNA polymerase subunits (Rpo),¹ the two largest (Rpo147 and Rpo132) and the smallest (Rpo7) are homologous to eukaryotic RNA polymerase subunits (15–18). In addition, a 30-kDa subunit (Rpo30) is structurally related to the mammalian transcription elongation factor TFIIS (19). Recently, a virus-encoded protein with an apparent molecular mass of 85 kDa was shown to be associated with RNA polymerase isolated from infectious vaccinia virus particles (20). The protein was named RNA polymerase-associated protein of 94-kDa (Rap94) because of the coding capacity of its open reading frame and the absence of polymerase-free Rap94. A small region of similarity in Rap94 and Rap30, the small subunit of the eukaryotic transcription initiation factor TFIIF (21), was noted (20). Evidence for a functional role of Rap94 in specific transcription was suggested by the low *in vitro* activity of Rap94-deficient RNA polymerase (Rap94⁻ Pol) and by DNA transfection experiments (20). The mapping of temperature-sensitive mutations to the open reading frame encoding the RNA polymerase-associated protein, indicated that Rap94 is essential for the production of infectious virions (22). Here, we show that Rap94-containing RNA polymerase (Rap94⁺ Pol) is required for transcription initiation and for formation of a stable ternary complex with a viral early promoter template.

EXPERIMENTAL PROCEDURES

Purification of VETF and RNA Polymerase—All steps were performed at 0–4 °C. A soluble extract was prepared from 3.2×10^{13} particles of purified virions as described (2). After dialysis against buffer B (50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 0.1 mM EDTA, 0.01% Nonidet P-40, and 10% glycerol) containing 80 mM NaCl, the extract was applied to a DEAE-cellulose column for the initial separation of VETF (unbound fraction) from RNA polymerase (bound fraction). VETF was purified to apparent homogeneity by a single-stranded DNA-agarose column followed by an early promoter DNA magnetic bead affinity step (6). RNA polymerase was further purified by gel filtration on Bio-Gel A-15 m (Bio-Rad) and chromatography on phosphocellulose (P11, Whatman) as described (20). The latter enzyme was applied to a heparin-agarose (Life Technologies, Inc.) column and eluted with a linear NaCl gradient (20). The peaks of Rap94⁺ Pol and Rap94⁻ Pol eluted at approximately 350 and 450 mM NaCl, respectively. The Rap94⁺ Pol fractions were dialyzed against 0.2 M NaCl in buffer B and applied to a 2-ml Q-Sepharose column (Pharmacia LKB Biotechnologies Inc.). After

¹ The abbreviations used are: Rpo, RNA polymerase subunit; PAGE, polyacrylamide gel electrophoresis; Rap94, RNA polymerase-associated protein of 94,000 daltons; Rap94⁺ Pol, Rap94-containing RNA polymerase; Rap94⁻ Pol, Rap94-deficient RNA polymerase; VGF, vaccinia virus growth factor.

washing with 0.2 M NaCl in buffer B, the Rap94⁺ Pol was eluted with 5 ml of 0.8 M NaCl in buffer B at a flow rate of 0.5 ml/min. The Rap94⁻ Pol-containing heparin-agarose fractions were pooled, dialyzed against 0.2 M NaCl in buffer B, and applied to a column of protein A-agarose to which 2 mg of anti-Rap94 IgG (20) had been attached. (The IgG-protein A-agarose was prepared using the Affinica kit from Schleicher and Schuell; 2 mg of IgG prepared as described below was covalently bound to 2 ml of protein A beads (50% v/v) according to the directions of the manufacturer.) The unadsorbed Rap94⁻ Pol was reappplied to the IgG column, and the flow-through was chromatographed on a Q-Sepharose column as described above. The final Rap94⁺ Pol and the Rap94⁻ Pol preparations from the separate Q-Sepharose columns were dialyzed against 80 mM NaCl in buffer B and stored at -80 °C. The protein concentrations were determined with the Bradford reagent (Bio-Rad). Nonspecific RNA polymerase activity was assayed using bacteriophage M13mp18 single-stranded DNA as a template (19). One unit of activity was defined as the amount of enzyme that incorporated 1 pmol of UMP into RNA in 20 min at 37 °C.

Immunodepletion of Rap94⁺ Pol—Preimmune and immune IgG, from rabbit sera collected before or after immunization with an anti-Rap94 fusion protein (20) were purified as follows: 2 ml of serum was incubated with 4 ml of immobilized Recomb^R protein (Pierce Chemical Co.), and the column was eluted with 0.1 M Tris-glycine, pH 2.5. Tris-HCl, pH 7.5, was added to the eluate to a final concentration of 0.5 M, and the mixture was passed through a SpeedyTM (Pierce Chemical Co.) desalting column. Partially purified RNA polymerase containing Rap94⁺ and Rap94⁻ species (pooled phosphocellulose column fractions; 2,000 units polymerase activity, 2 µg of protein) was incubated with 50 µl of a 50% suspension of protein A-Sepharose beads (Immunoselect, Life Technologies, Inc.), to which 20 µg of preimmune or immune IgG had been attached, in 200 µl of buffer B containing 0.2 M NaCl. After shaking for 3 h at 4 °C, the beads were sedimented in a microfuge at 4,000 revolutions/min for 3 min and washed five times with 0.2 M NaCl in buffer B and once with 2 M NaCl in buffer B.

In Vitro Transcription—Assays were performed as described (20) using 0.1–0.2 pmol of RNA polymerase, 0.1 pmol of VETF, and 0.2 µg of a supercoiled plasmid pSB24 (23) which contained the consensus early promoter coupled to a 420-base pair DNA fragment lacking G residues in the non-coding strand.

Gel Mobility-shift Assays—Plasmid pBA7 was constructed by cloning a DNA fragment, from 50 bp upstream to 40 bp downstream of the RNA start of the vaccinia virus growth factor (VGF) gene, between the *Hind*III and *Bam*HI sites of pUC13. Protein-DNA complexes were formed as described (24), except that the 20-µl incubation mixtures contained 25 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol, 0.005% Nonidet P-40, 0.05 mg of bovine serum albumin/ml, 200 fmol of ³²P-labeled DNA (270 bp *Hind*III-*Nar*I fragment of plasmid pBA7 containing the promoter region of the vaccinia virus growth factor gene followed by 180 bp of pUC13), 20 fmol of VETF, and 20 fmol of RNA polymerase. For the formation of ternary complexes, the assays also contained ribonucleoside triphosphates (0.02–1 mM) and either labeled DNA as above or unlabeled DNA and [α -³²P]UTP. After incubation for 30 min at 30 °C, the mixtures were analyzed by electrophoresis in a non-denaturing 4% polyacrylamide gel containing 12.5 mM Tris-HCl, pH 7.9, 6.5 mM sodium acetate, and 0.1 mM EDTA at 4 °C for 2–2.5 h at a constant current of 20 mA.

RESULTS

Immunoaffinity Binding of Rap94⁺ Pol—Previous experiments had shown that Rap94⁺ Pol and Rap94⁻ Pol species of vaccinia virus RNA polymerase co-eluted through the phosphocellulose chromatography step of purification (20). To determine the relative amounts of the two species of RNA polymerase, we carried out immunodepletion experiments. The phosphocellulose purified RNA polymerase was incubated with beads to which either preimmune or anti-Rap94 IgG had been attached. After washing, the presence or absence of Rap94 and RNA polymerase subunits in the IgG-unbound and -bound fractions was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and immunoblotting with a mixture of antibodies raised to Rap94⁻ Pol (25, 26) and to a recombinant Rap94 fusion protein (20). An autoradiogram of the upper part of the membrane containing the large RNA polymerase subunits (Rpo147 and Rpo132) and Rap94 is shown (Fig. 1A). The immobilized preimmune IgG bound neither Rap94 nor RNA

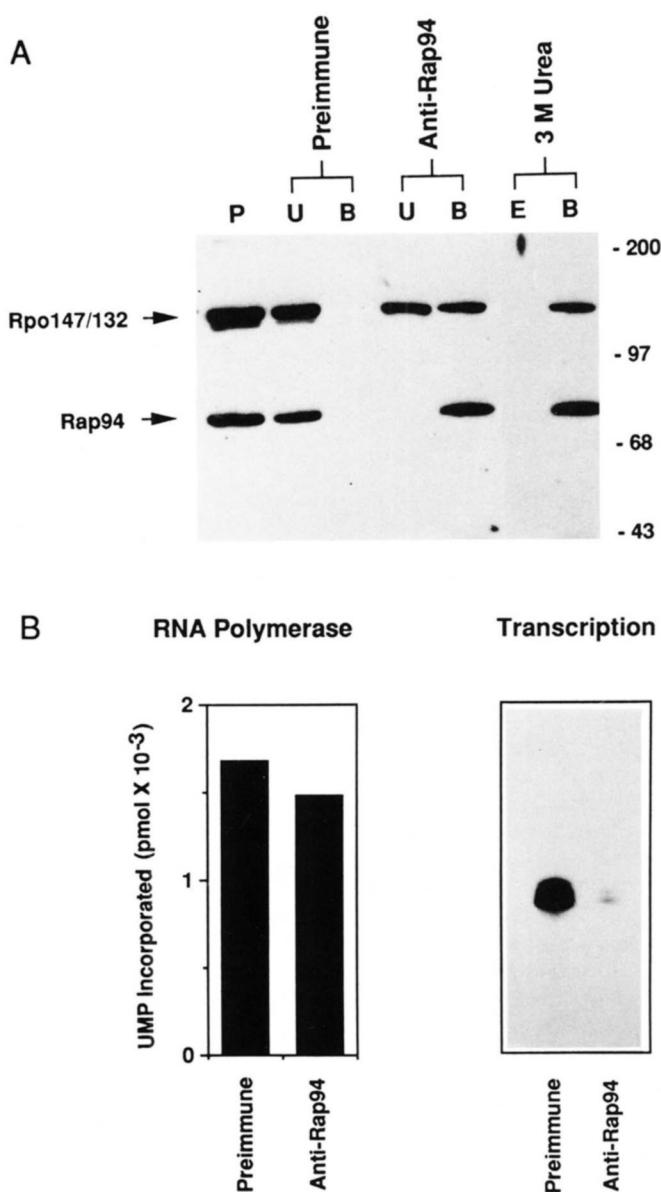


FIG. 1. Immunodepletion of early promoter-dependent transcription activity with immobilized anti-Rap94 IgG. Pooled phosphocellulose column fractions containing Rap94⁺ Pol and Rap94⁻ Pol were incubated with protein A-Sepharose beads to which IgG, from rabbits before or after immunization with recombinant Rap94, had been attached. The beads were washed five times with 0.2 M NaCl buffer and once with 2 M NaCl buffer. **A**, immunoblot analysis. RNA polymerase prior to immunoadsorption (0.5 µg, lane P), bead-bound (lanes B), and unbound supernatant (lanes U) proteins were analyzed by SDS-PAGE (8% polyacrylamide gel) followed by immunoblotting simultaneously with antibodies to Rap94⁻ Pol and to recombinant Rap94 fusion protein. Another sample of the polymerase-antibody-protein A bead complex was washed with 3 M urea and proteins in the eluate (lane E) as well as proteins that remained bound to the beads (lane B) were also analyzed. The nitrocellulose membrane was incubated with ¹²⁵I-protein A and an autoradiogram made. Only the upper part of the autoradiogram is shown. The positions of Rap94 and unresolved Rpo147 and Rpo132 are indicated by arrows on the left. The positions and molecular masses (kDa) of protein markers are indicated on the right. **B**, left panel: RNA polymerase. The unbound fractions from A were assayed for nonspecific RNA polymerase activity with a single-stranded DNA template. UMP incorporated into RNA was determined by a DEAE filter binding assay. Right panel, transcription. The unbound fractions from A were incubated with purified VETF and an early promoter double-stranded DNA template in a transcription assay. The labeled RNA synthesized was analyzed by electrophoresis in a 4% denaturing polyacrylamide gel. An autoradiogram is shown.

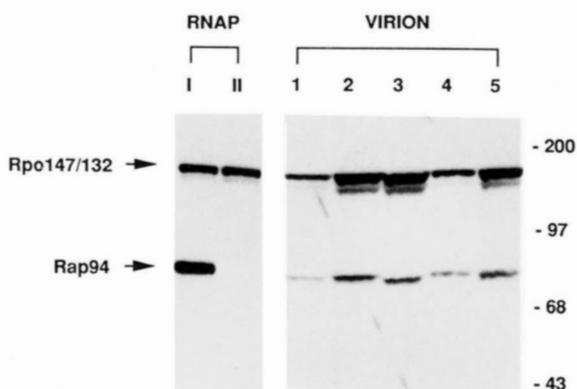


Fig. 2. Immunoblot of virion-associated RNA polymerase. Purified vaccinia virions (twice banded in sucrose gradients) from five independent preparations (*lanes 1–5*) were boiled for 3 min in 2% SDS and 0.7 M β -mercaptoethanol, and the proteins derived from 1.5 to 6.0×10^8 particles were analyzed by SDS-PAGE. The resolved proteins were transferred to nitrocellulose and probed with antibodies to RNA polymerase and Rap94 as in Fig. 1A. The first two lanes are immunobound (*I*) and unbound (*II*) RNA polymerase (*RNAP*) from Fig. 1A used as a reference for quantitation. Viral proteins and marker proteins are indicated on the *left* and *right*, respectively, as in Fig. 1A.

polymerase. However, the immobilized immune IgG bound Rap94 quantitatively but retained only a fraction of the RNA polymerase. Quantitation of the immunoblots with a Phosphor-Imager (Molecular Dynamics) indicated that 37% of the applied polymerase had been retained by the anti-Rap94 IgG beads. The retained polymerase could not be dissociated from the beads with either 0.2–2 M NaCl or 3 M urea washes (Fig. 1A). We concluded that Rap94 was tightly associated with nearly 40% of the purified viral RNA polymerase.

The phosphocellulose-purified RNA polymerase was active in a nonspecific assay using a single-stranded DNA template and in an early promoter-specific transcription assay using a double-stranded DNA template in the presence of VETF. The activities remaining in the immune IgG-unbound fraction, which lacked Rap94⁺ Pol, were compared with those present in the preimmune IgG-unbound fraction, containing both forms of polymerase. Although 37% of the total RNA polymerase protein was depleted when Rap94⁺ Pol was removed (Fig. 1A), the nonspecific RNA polymerase activity was reduced by only 15% (Fig. 1B). This result suggested that the Rap94[−] Pol was actually more active than Rap94⁺ Pol in the nonspecific assay. By contrast, removal of the Rap94⁺ Pol almost completely depleted the early promoter-specific transcription activity (Fig. 1B). That the low promoter-specific activity was due to removal of Rap94⁺ Pol, rather than to the presence of an inhibitor, was confirmed by mixing experiments. These data suggested that Rap94[−] Pol is competent to synthesize RNA nonspecifically from a single-stranded DNA template but that Rap94⁺ pol is needed, together with VETF, for early promoter-dependent transcription of a double-stranded DNA template.

Rap94 Is Present in Submolar Amounts Relative to RNA Polymerase.—The isolation of Rap94[−] Pol in the above experiments suggested that Rap94 is present in submolar quantities relative to RNA polymerase in vaccinia virions. Another possibility, however, was that Rap94 was incompletely extracted from virions or was dissociated from RNA polymerase during purification. To investigate these alternatives, the relative amounts of Rap94 and large RNA polymerase subunits in purified virions were determined. Five independent virion preparations were dissociated and analyzed by SDS-PAGE and immunoblotting (Fig. 2). Immunopurified Rap94⁺ Pol and Rap94[−] Pol were run alongside as standards. The amounts of ¹²⁵I-protein A bound were determined with a PhosphorImager. As-

signing a value of 1 for the ratio of Rap94 to large RNA polymerase subunits in immunoaffinity purified Rap94⁺ Pol, we calculated ratios of 0.32–0.43 for whole virion preparations. These values are consistent with the binding of 37% of the partially purified RNA polymerase to anti-Rap94 IgG, as determined in the preceding section, and argue against loss of Rap94 during enzyme extraction or purification. Thus, vaccinia virions contain both Rap94[−] and Rap94⁺ forms of RNA polymerase.

Transcription Activities of Rap94⁺ Pol and Rap94[−] Pol.—In a preceding section, we demonstrated that early promoter-specific transcription activity was lost upon selective immunodepletion of Rap94⁺ Pol from a preparation containing both forms of polymerase. The activity of the immunopurified Rap94⁺ Pol was not determined, however, since it could not be eluted from the anti-Rap94 IgG beads without denaturing agents. To compare the activities of Rap94⁺ Pol and Rap94[−] Pol, the two forms of the enzyme present at the phosphocellulose purification step described above were separated by chromatography on a heparin-agarose column. The ratio of Rap94 to Rpo147/132 in the pooled Rap94⁺ Pol heparin-agarose column fractions, as determined by immunoblotting, approached that of the anti-Rap94 IgG-bound enzyme described above. However, the heparin-agarose fractions enriched in Rap94[−] Pol contained some Rap94⁺ Pol. The latter was removed by passage of the mixture through a column of immobilized anti-Rap94 IgG. The purified Rap94⁺ Pol and Rap94[−] Pol preparations were concentrated using a Q-Sepharose column and analyzed by SDS-PAGE. Coomassie Blue-stained gels, shown in Fig. 3 (*left panel*), indicated that the polymerases were of similar purity though only one contained detectable Rap94. Neither RNA polymerase preparation alone was able to transcribe a double-stranded DNA template regulated by an early gene promoter (Fig. 3, *center panel*). Addition of affinity purified VETF conferred transcriptase activity to Rap94⁺ Pol but not to Rap94[−] Pol. However, the activity of Rap94[−] Pol was 1.85 times greater than Rap94⁺ Pol using a nonspecific single-stranded DNA template (Fig. 3, *right panel*) in agreement with the prediction of the immunodepletion experiment in Fig. 1B. Rap94[−] Pol also actively transcribed a double-stranded DNA template that had a single-stranded poly(dG) 3'-extension to allow nonspecific initiation (not shown). Taken together, these data indicated that Rap94 is required for specific transcription of early promoter templates rather than for catalytic activity associated with RNA synthesis *per se*.

Formation of Transcription Complexes.—Non-denaturing gel electrophoresis has been used to characterize binary complexes composed of VETF and early promoter sequences (2, 5, 24) and ternary complexes which contain DNA, VETF, RNA polymerase, and nascent RNA (27, 28). As shown in *lane 2* of Fig. 4, VETF retarded the electrophoretic mobility of a labeled early promoter-containing DNA fragment. Alone, neither Rap94⁺ Pol nor Rap94[−] Pol formed a stable complex with the DNA fragment (Fig. 4, *lanes 3 and 4*) nor did they alter the mobility of the VETF-DNA complex (Fig. 4, *lanes 5 and 6*). Abundant slower mobility complexes were formed, however, provided the promoter DNA fragment, VETF, and Rap94⁺ Pol were incubated with ATP, UTP, and CTP (Fig. 4, *lane 11*). Incubation with all four ribonucleoside triphosphates, decreased the average mobility and autoradiographic intensity of the complexes slightly (Fig. 4, *lane 13*) presumably due to transcript elongation and release, respectively. ATP alone was ineffective in promoting the formation of slow mobility complexes (Fig. 4, *lane 7*) and only traces of slow mobility complexes were formed with ATP and UTP (Fig. 4, *lane 9*). Significantly, the slow mobility complexes did not form under any circumstances when Rap94⁺ Pol was replaced by Rap94[−] Pol.

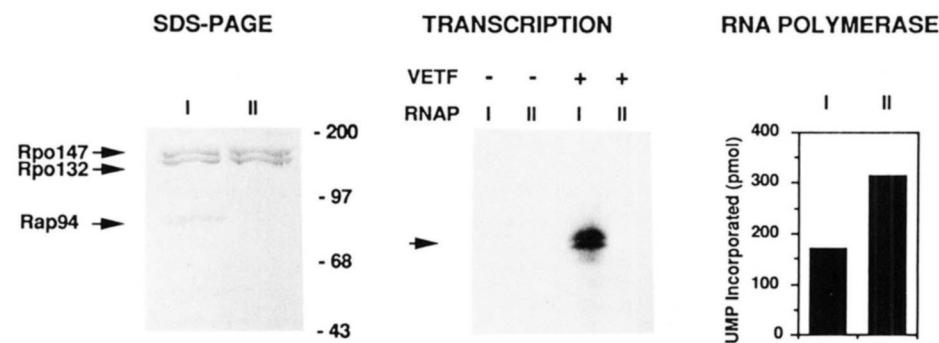


FIG. 3. Nonspecific and early promoter-dependent transcription activities of purified Rap94⁺ Pol and Rap94⁻ Pol. Samples (0.5 µg each) of purified Rap94⁺ Pol (lanes I) and Rap94⁻ Pol (lanes II) were analyzed by SDS-PAGE and stained with Coomassie Blue (left panel). The upper part of the gel is shown with the viral proteins and size markers indicated as in Fig. 1A. Specific transcription assays were carried out with 50 ng of each enzyme in the absence or presence of VETF and early promoter template as in Fig. 1B. An autoradiogram of the ³²P-labeled RNA products analyzed in a denaturing 4% polyacrylamide gel is shown in the center panel. The nonspecific RNA polymerase activity of 50 ng of each enzyme was determined and the data shown as UMP incorporated per 20 min in the right panel.

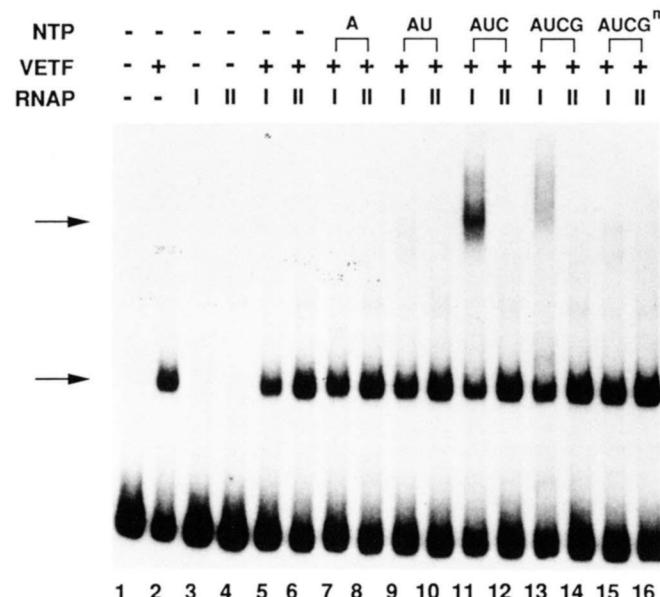


FIG. 4. Ternary transcription complex formation. Rap94⁺ Pol (RNAP I) and Rap94⁻ Pol (RNAP II) were incubated with a ³²P-end-labeled double-strand DNA template (270-bp *Hind*III-*Nar*I fragment) containing the VGF gene sequence (-50 to +40 relative to the RNA start site) in the presence of VETF and ribonucleoside triphosphates (NTP) as indicated. The reactions were analyzed by electrophoresis in a non-denaturing 4% polyacrylamide gel. An autoradiogram is shown. Arrows indicate protein-DNA complexes. A, U, C, G, and G^m indicate 1 mM ATP, 0.2 mM UTP, 0.2 mM CTP, 0.2 mM GTP, and 0.2 mM 3'-O-methyl GTP, respectively.

Inspection of the promoter/template sequence (*pBA7*, Fig. 5C), suggests that in the presence of ATP, UTP, and CTP, an RNA chain of up to six nucleotides could be synthesized. However, when the retarded complexes were eluted from the gel and analyzed, RNA chains of about 30–200 nucleotides in length were resolved by electrophoresis in a denaturing polyacrylamide gel (data not shown), evidently because commercially available ribonucleoside triphosphates are contaminated with traces of other nucleotides or deamination products (28, 29). (With all four ribonucleoside triphosphates, the 240 nucleotide run-off transcript was predominant although smaller chains also were detected upon analysis of the complex.) When extension beyond position 7 was prevented by addition of the chain terminator 3'-O-methyl GTP, stable ternary complexes were not detected (Fig. 4, lane 15) suggesting that the longer RNA chains were indeed required for their formation.

To more precisely determine the RNA chain length requirement for stable ternary complex formation with purified

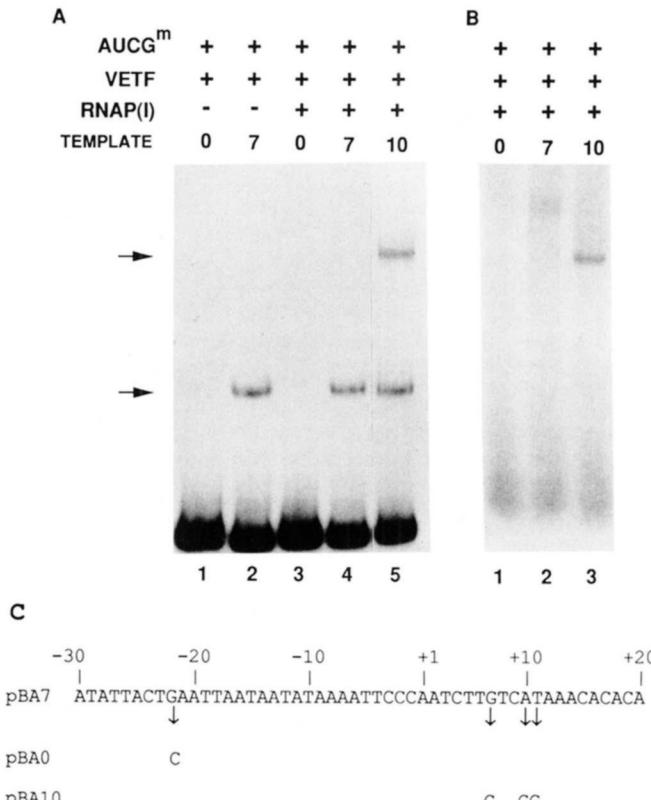


FIG. 5. Effect of RNA length on ternary transcription complex formation. A, Rap94⁺ Pol (RNAPI) was incubated with ³²P-labeled VGF templates derived from pBA0, pBA7, or pBA10 (abbreviated as 0, 7, and 10, respectively) plus VETF, ATP, UTP, CTP, and 3'-O-methyl GTP as indicated. The reactions were analyzed by electrophoresis on a non-denaturing 4% polyacrylamide gel and an autoradiogram was made. The lower and upper arrows on the left indicate the binary and ternary complexes, respectively. B, same as A, except that unlabeled DNA templates and 20 µM [α -³²P]UTP (10 µCi) were used. C, the sequence in plasmid pBA7 from -30 to +20 relative to the RNA start site of the vaccinia virus growth factor gene is shown. The sequence -28 to -13 is critical for VETF binding and promoter activity. pBA0 and pBA10 are derivatives of pBA7 with a G to C mutation in the VETF-binding site and 3 residues altered downstream of the RNA start site, respectively.

Rap94⁺ Pol and VETF, additional templates were prepared. In pBA10, the G residue at the +7 position of the VGF gene sequence was converted to C and the AT at +10 and +11 was converted to GG (Fig. 5C) in order to allow a transcript of 10 nucleotides instead of 7 to form in the presence of the chain terminator 3'-O-methyl GTP. Another template, pBA0, with a

G to C transition at a nucleotide critical for VETF binding (24) was constructed for use as a promoter specificity control (Fig. 5C). As expected VETF-DNA complexes formed when the template was derived from pBA7 or pBA10 but not pBA0 (Fig. 5A). In the presence of ATP, UTP, CTP, and 3'-O-methyl GTP, a low mobility complex was resolved only with the template from pBA10. Evidence that the pBA10-derived complex contains nascent RNA was obtained by the formation of a labeled complex with equivalent mobility when [α -³²P]UTP was used and the DNA was unlabeled (Fig. 5B). These data suggested that RNA chains of at least 8–10 nucleotides are required for stabilization of the ternary complexes, in agreement with results of Hagler and Shuman (28).

Rap94 Is Required for Transcription Initiation—The failure of nascent transcripts shorter than 8–10 nucleotides to be stably associated with both prokaryotic or eukaryotic RNA polymerases is correlated with abortive initiation (30, 31). The possibility existed, therefore, that Rap94 was required for the transition from unstable to stable transcription elongation complexes rather than for initiation. To evaluate these alternatives, experiments were designed to analyze the formation of short oligoribonucleotides by Rap94⁺ Pol and Rap94⁻ Pol in the presence of VETF. Transcription reactions, in which pBA0, pBA7, and pBA10 were used as templates, were applied directly to high resolution polyacrylamide gels for electrophoresis under denaturing conditions. Because of the mutated promoter, the pBA0 template served as a specificity control. In the presence of ATP, [α -³²P]UTP, CTP, and 3'-O-methyl GTP, Rap94⁺ Pol catalyzed the synthesis of RNA chains of about 7–10 and 10–13 nucleotides with pBA7 and pBA10 templates, respectively (Fig. 6, panel I). In the presence of ATP and [α -³²P]UTP, the RNAs varied in size from about 4 to 7 nucleotides. Notably, when Rap94⁻ Pol was used, no radioactive products longer than those seen with the mutated pBA0 template were detected (Fig. 6, panel II), suggesting that Rap94 is required at the initiation step of transcription.

The basis for the length heterogeneity of the oligoribonucleotides, formed with Rap94⁺ Pol, was considered. The site of RNA initiation within the two adjacent A residues (Fig. 5C) was previously established *in vitro* and *in vivo* (32, 33). Significant initiation at the upstream C residues was unlikely because initiation with purines is preferred and the concentrations of ATP and CTP used in the assay were 1 and 0.02 mM, respectively (28). Moreover, labeled oligonucleotides were not detected in the presence of ATP and [α -³²P]CTP, whereas products as short as four nucleotides were made by ATP and [α -³²P]UTP (Fig. 6, panel I). Heterogeneity at the 3' end also seemed unlikely since it was not reduced by use of a 3' chain terminator. Furthermore, multiple RNAs of the same sizes were formed when excess unlabeled ribonucleoside triphosphates, including GTP, were added to chase the transcripts into longer species which were then digested with RNase T1 (data not shown). Slippage of RNA polymerase at the initiation site resulting in the incorporation of a variable number of additional A residues at the 5'-end of the RNA remains a strong possibility. The vaccinia virus RNA polymerase is known to slip *in vivo* and *in vitro* when the RNA initiation site contains three consecutive A residues in the non-template strand (34), although slippage has not been described at two As.

DISCUSSION

The present study was undertaken to determine the structural and functional implications of the recent finding of Rap94 associated with transcriptionally active vaccinia virus RNA polymerase (20). IgG raised against a recombinant fusion protein derived from the viral open reading frame encoding Rap94 was immobilized on beads. When an RNA polymerase prepa-

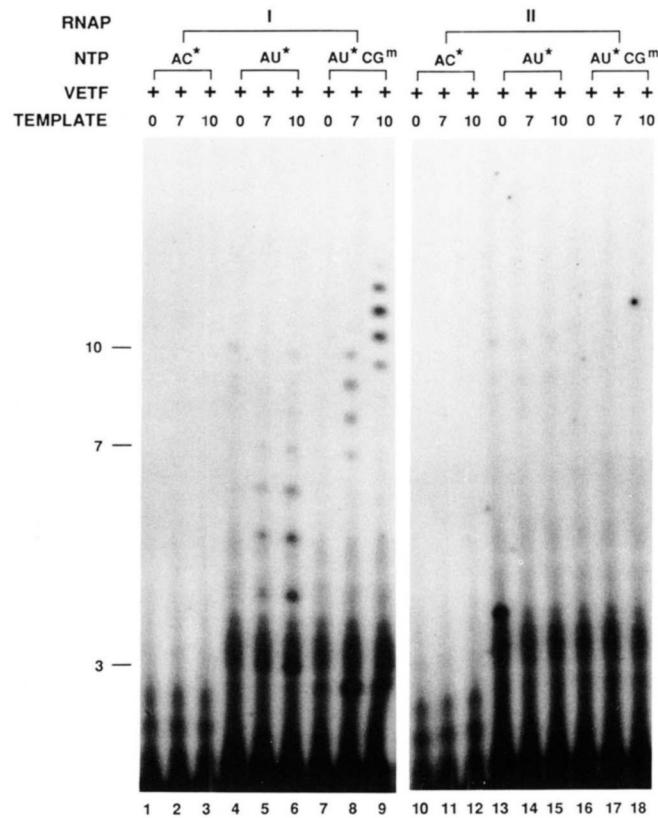


FIG. 6. Analysis of oligoribonucleotide transcription products. Rap94⁺ Pol (RNAP I) and Rap94⁻ Pol (RNAP II) were incubated with VETF and templates derived from pBA0, pBA7, and pBA10 (abbreviated as 0, 7, and 10, respectively) in the presence of VETF and indicated ribonucleoside triphosphates. The asterisks indicate 20 μ M α -³²P-labeled ribonucleoside triphosphates (10 μ Ci). Final concentrations of other nucleotides were 1 mM ATP, 20 μ M CTP and UTP, and 0.1 mM 3'-O-methyl GTP. Transcription was stopped by addition of formamide gel loading buffer followed by boiling for 2 min. Aliquots were directly applied to a 25% polyacrylamide gel containing 7 M urea. Electrophoresis was performed at 2,000 V for 3 h, and an autoradiogram was made from the wet gel. Shown at the left are the sizes estimated from synthetic 8-mer and 20-mer oligodeoxynucleotide markers analyzed in the same gel.

ration was applied, the beads retained all of the Rap94 and approximately 37% of the polymerase molecules as determined by immunoblotting. The bound RNA polymerase could not be dissociated from the Rap94-IgG complex with NaCl concentrations of 0.2–2.0 M or with 3 M urea, indicating a tight association. To determine whether the absence of Rap94 from more than 60% of the extracted RNA polymerase molecules represented the saturation state of the enzyme in virions or the loss of Rap94 during enzyme preparation, several samples of purified virions were disrupted with SDS and the proteins applied directly to a polyacrylamide gel. After electrophoresis, the ratio of Rap94 to the large RNA polymerase subunits was determined by quantitative immunoblotting with Rap94 and RNA polymerase-specific antisera. Using immunopurified Rap94⁺ Pol as a standard, the ratio of Rap94 to the RNA polymerase large subunits in virions was calculated to be between 0.32 and 0.43. This submolar estimate for whole virions was consistent with the relative amounts of Rap94⁺ Pol and Rap94⁻ Pol isolated and the absence of free Rap94 in virion extracts. The submolar amount of Rap94 relative to RNA polymerase is puzzling; perhaps an excess of Rap94 would adversely affect late transcription. Experiments to test this possibility are planned. We note, however, that *Escherichia coli* RNA polymerase also has submolar amounts of sigma⁷⁰. The *in vivo* sigma⁷⁰ level is usually 0.3–0.4 relative to the core polymerase but varies with

the bacterial strain, growth conditions, and harvest time (35). Rap94⁺ Pol and Rap94⁻ Pol elute from heparin-agarose columns at slightly different salt concentrations (20). By choosing appropriate column fractions, we obtained an RNA polymerase preparation with nearly stoichiometric amounts of Rap94 relative to the large RNA polymerase subunits. The RNA polymerase fractions deficient in Rap94 were passed through an anti-Rap94 immunoaffinity column to remove residual Rap94⁺ Pol. Alone, neither Rap94⁺ Pol nor Rap94⁻ Pol could transcribe a double-stranded DNA template regulated by an early vaccinia virus promoter. Addition of VETF to Rap94⁺ Pol led to efficient transcription whereas no transcription occurred on addition of VETF to Rap94⁻ Pol. By contrast, Rap94⁻ Pol had nearly twice the activity of Rap94⁺ Pol with a nonspecific single-stranded DNA template.

The finding that Rap94⁺ Pol is less active than Rap94⁻ Pol with nonspecific single-stranded DNA templates is reminiscent of properties relating to site selection conferred to *E. coli* RNA polymerase by sigma⁷⁰ (36) and to eukaryotic RNA polymerase II by rat liver β , γ -factor (37), or human Rap30 (38). In this regard, we previously noted that a 27-amino-acid region of Rap94 could be aligned with Rap30 to give a 44% identity (20). Interestingly, the N-terminal half of the same Rap30 segment was previously considered to be related to the core RNA polymerase-binding domain of *E. coli* sigma⁷⁰ (39, 40). Further studies on the nonspecific DNA binding properties of Rap94⁺ Pol and Rap94⁻ Pol are warranted.

Although both Rap94 and VETF are required for transcription of early genes, Rap94 is tightly associated with the viral RNA polymerase whereas VETF is only weakly associated with the polymerase and is readily isolated in a free form (2, 41). Both Rap94 (20, 42) and VETF (4, 6) are synthesized late in infection at the time of virion assembly. This contrasts with the RNA polymerase core subunits, which are made early and continue to be synthesized throughout infection. Conditional lethal viral mutants that have been mapped to VETF and Rap94 display no defects in late gene expression under non-permissive conditions consistent with exclusive roles in early transcription (4, 6, 22, 43, 44).

Our finding that Rap94 confers promoter specificity to the viral RNA polymerase implies that it acts at or prior to the initiation of transcription. Nevertheless, we could not demonstrate Rap94⁺ Pol binding to VETF-promoter complexes by gel shift assays in the absence of ribonucleoside triphosphates. Indeed stable complexes of RNA polymerase, VETF, and promoter DNA were not detected unless the nascent RNA was about 10 nucleotides long. Our results regarding the nascent RNA requirements for stabilization of ternary complexes are similar to those reported for *E. coli* by Krummel and Chamberlin (45) and for vaccinia virus by Hagler and Shuman (28). Although Li and Broyles (23) reported the detection of preinitiation complexes by gel electrophoresis assays, the amounts were very low with respect to ternary complexes. Unable to demonstrate stable RNA polymerase-containing preinitiation complexes, we looked to see whether Rap94 was needed for the formation of short oligoribonucleotides. We determined that in the presence of VETF, Rap94⁺ Pol but not Rap94⁻ Pol could catalyze the synthesis of short oligoribonucleotides from templates with an early promoter. Thus, it seems likely that Rap94 is required at or before the initiation step of transcription. Like bacterial sigma⁷⁰ (36) and eukaryotic Rap30 (46), Rap94 might be involved in the recruitment of the RNA polymerase into the preinitiation complex as well as in altering the conformation of

the template DNA at the RNA start site. An interaction with promoter-bound VETF could account for the stage specificity of Rap94. Whether Rap94 remains in the elongation complex as reported for Rap30 (47) or dissociates as does sigma⁷⁰ (48) remains to be determined. Recent studies indicate that Rap94 is also required for the targeting of the transcription complex into assembling vaccinia virions (49). The specificity and relative simplicity of the vaccinia virus transcription system encourages further mechanistic studies.

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