Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 3536–3540, April 1992
Biochemistry

RNA polymerase-associated transcription specificity factor encoded by vaccinia virus

(RNA polymerase subunit/σ factor/RAP30 RNA polymerase-binding protein/gene expression)

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Contributed by Bernard Moss, January 15, 1992

ABSTRACT **Vaccinia virus encodes a multisubunit DNA-dependent RNA polymerase (EC 2.7.7.6) that is packaged in the infectious virus particle. This polymerase was found to contain a submolar polypeptide of approximately 85 kDa in addition to the core subunits, which consist of two larger and several smaller polypeptides. The polymerase containing the 85-kDa polypeptide was separated from the core polymerase by column chromatography. Although the core polymerase actively transcribed heterologous single-stranded DNA, only the form with the associated 85-kDa polypeptide could act in conjunction with an early stage-specific factor to transcribe double-stranded DNA containing a vaccinia virus early promoter. Peptide sequencing established that the RNA polymerase-associated 85-kDa protein was derived from the vaccinia virus H4L open reading frame, which encodes a 94-kDa polypeptide that we named RAP94. RAP94 is not closely related to prokaryotic σ^{70} or eukaryotic RAP30 RNA polymerase-binding proteins, although there are short regions of sequence similarity. The specific association of RAP94 with viral RNA polymerase was corroborated with antibody raised to a recombinant fusion protein. Unlike the previously defined subunits of vaccinia virus RNA polymerase, RAP94 is synthesized exclusively late in infection, and synthesis could be prevented by a DNA replication inhibitor. The role of RAP94 in mediating specific transcription was demonstrated by using an extract from cells in which the H4L open reading frame had been transiently expressed.**

The synthesis of mRNA by DNA-dependent RNA polymerases (EC 2.7.7.6) often requires one or more accessory proteins. These factors assist the polymerase in binding to the promoter selectively and initiating transcription. In prokaryotes, this process is regulated by the σ factor protein family (1). In eukaryotes, the role of the σ subunit appears to be spread among several proteins, which can be grouped into two broad categories of general transcription factors (2). One class of factors, to which the TATA-motif-binding protein (TFIID) belongs, binds to promoter sequences and forms the initial protein–DNA complex. Factors belonging to the second category, such as RAP30/74 (also known as TFIIF), associate with RNA polymerase II or other protein factors and have no sequence-specific DNA-binding properties (3). A distant relationship between bacterial σ factors and RAP30 has been suggested on the basis of sequence similarities (4).

The poxviruses, of which vaccinia virus is the prototype, provide especially favorable systems for combining biochemical and genetic approaches to the study of transcription. These DNA viruses replicate in the cytoplasm and encode most if not all of the proteins needed for viral transcription, including a eukaryotic-like multisubunit DNA-dependent RNA polymerase and stage-specific transcription factors (reviewed in refs. 5 and 6). The early class of vaccinia virus genes is expressed immediately after infection, whereas viral DNA replication precedes the expression of the intermediate and late classes of genes. The vaccinia virus early transcription factor (VETF) is a heterodimeric protein that resembles TFIID in binding to a specific A+T-rich promoter element located upstream of the RNA start site. Here we describe a vaccinia virus-encoded protein that is tightly associated with the viral core RNA polymerase and is required for specific transcription of early genes.

MATERIALS AND METHODS

Purification of RNA Polymerase and VETF. All steps were carried out at 0–4°C. Proteins were extracted from purified virions and the soluble material was passed through a DEAE-cellulose (DE-52; Whatman) column, equilibrated with 250 mM KCl, to remove nucleic acids (7). The effluent was dialyzed against buffer B [50 mM Tris·HCl, pH 8.0/1 mM dithiothreitol/0.1 mM EDTA/0.01% Nonidet P-40/10% (vol/vol) glycerol] containing 80 mM NaCl and applied to a second DEAE-cellulose column that had been equilibrated with the buffer B/80 mM NaCl. The effluent contained most of the VETF, which was further purified with a single-stranded DNA–agarose column (8). The RNA polymerase, which bound to the second DEAE-cellulose column, was eluted with buffer B containing 0.3 M NaCl and applied to a Bio-Gel A-15m (Bio-Rad) column (50 ml). Fractions with polymerase activity were pooled, diluted to 0.1 M NaCl, and applied to a phosphocellulose (P11; Whatman) column, which was developed with a 0.1–0.6 M NaCl gradient in buffer B. Fractions with polymerase activity (0.24–0.3 M NaCl) were pooled, diluted to 0.2 M NaCl, and applied to a heparin-agarose (BRL-Life Technologies, Gaithersburg, MD) column, which was developed with a 0.2–1.0 M NaCl gradient in buffer B.

Assays. Protein concentrations were measured with the Bradford reagent (Bio-Rad).

Nonspecific RNA polymerase activity was determined as described (9).

Specific transcription assays were carried out for 30 min at 30°C in 50 μl containing 20 mM Tris·HCl at pH 8.0, 40–50 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 (1 Ci = 37 GBq), 0.5 μg of DNA template, 0.1 μg of partially purified VETF, and 10–20 μl of RNA polymerase fractions. The template was uncut plasmid pSB24 (a gift of S. Broyles, Purdue University), which contains the sequence AATTCAAAAAATTGA-AAAACTAGGAATTCCTTTCATAACCC related to the consensus viral early promoter (10) followed by 400 base pairs (bp) of DNA lacking G residues in the noncoding strand (11). The mixture was incubated for an additional 10 min with

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Abbreviations: ORF, open reading frame; VETF, virus early transcription factor; araC, cytosine arabinonucleoside.

50 units of RNase T1, and the RNA was separated on a 4% polyacrylamide/7 M urea gel as described (12).

Protein Sequencing. Purified RNA polymerase (100 μg) was subjected to sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE) and transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuell) for *in situ* digestion with trypsin (13). The N-terminal amino acid sequences of tryptic peptides were determined at the Harvard Microchemistry Facility under the direction of W. Lane.

Antibody Preparation. DNA corresponding to codons 1–323 of the H4L open reading frame (ORF) was amplified by polymerase chain reaction (PCR) using the cloned *Hin*dIII H DNA fragment annealed with synthetic primers TACCGGATCCCCTATGGACTCTAAAGAGACTATTCT and GTGCGAATTCCACTCTAACATCCCTTTTCCA (*Bam*HI and *Eco*RI sites underlined). The PCR product was digested with *Bam*HI and *Eco*RI and ligated to the bacterial expression vector pGEX-2T (Pharmacia). Synthesis of the fusion protein in transformed *Escherichia coli* was induced with isopropyl thiogalactoside. The SDS/PAGE-purified recombinant protein was injected into New Zealand White rabbits in Freund's complete adjuvant and at 2-week intervals in Freund's incomplete adjuvant. Serum obtained after 5 weeks reacted with the product of the H4L ORF. Rabbit antibody to the core RNA polymerase was described previously (14).

Transfection Experiments. A 3.4-kbp segment of DNA encoding ORF H4L was amplified by PCR with primers ACTTTCCATGGACTCTAAAGAGACTATTCTAATT and ATAACTGGATCCACAATAACAGGAGTTTTCACCGCCGCC (*Nco* I and *Bam*HI sites underlined) annealed with the cloned *Hin*dIII H segment of the vaccinia virus genome. The PCR product was digested with *Nco* I and *Bam*HI and ligated into the multiple cloning site of the vector pTM1 containing the bacteriophage T7 φ10 promoter (15) to form pT7-H4L. The control plasmid pT7-lacZ was a gift of Orna Elroy-Stein (National Institute of Allergy and Infectious Diseases). Monolayers of African green monkey kidney CV-1 cells were infected with 20 plaque-forming units per cell of a recombinant vaccinia virus expressing the T7 RNA polymerase (vTF7-3; ref. 16) in the presence of cytosine arabinonucleoside (araC). After 1 hr, the cells were transfected with calcium phosphate precipitated pT7-H4L or pT7-lacZ (10 μg of plasmid DNA per 10^6 cells) and incubated for 14 hr more. Cytoplasmic extracts were prepared (12) and 6–10 μg of total protein derived from about 10^6 cells was used to assay transcription activity in the presence of 0.1 μg of partially purified VETF.

RESULTS

Two Forms of RNA Polymerase. Purified infectious vaccinia virus particles contain all of the enzymes and factors needed for the transcription of the early class of genes. The virion RNA polymerase consists of two large and several small polypeptides, resembling the subunit structure of the eukaryotic RNA polymerases (7, 14). Genes for eight subunits have been located in the viral genome and named *rpo147, rpo132, rpo35, rpo30, rpo22, rpo19, rpo18*, and *rpo7* according to the sizes of their predicted translation products in kDa (9, 17–25). The early transcription factor, VETF, is composed of 82- and 77-kDa polypeptides (8) encoded by separate vaccinia virus genes (26, 27).

As previously shown and confirmed here by chromatography on successive columns of DEAE-cellulose, Bio-Gel A-15m, and phosphocellulose, highly purified vaccinia virus RNA polymerase is capable of independently transcribing heterologous single-stranded DNA and of transcribing double-stranded DNA containing an early promoter in conjunction with VETF (Fig. 1*A*). (For consistency in terminology, we will refer to the former as RNA polymerase activity and

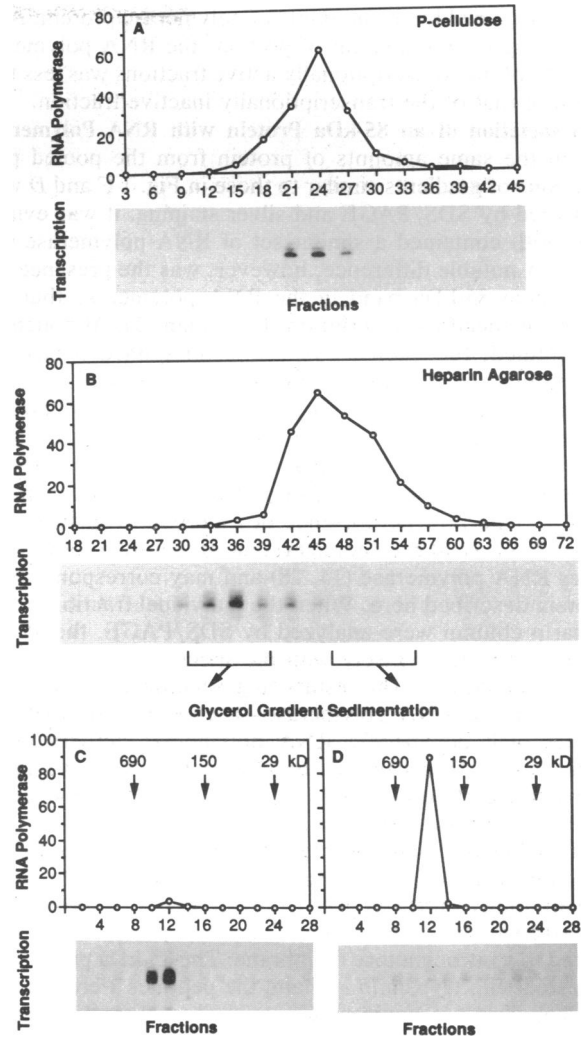

FIG. 1. Purification of RNA polymerase. (*A*) Phosphocellulose column. Fractions were assayed for nonspecific RNA polymerase activity (elution profile, pmol of UMP incorporated per 5 μl of column fraction) and early-promoter-specific transcription with added VETF (PAGE autoradiograph). (*B*) Heparin–agarose column (as in *A*). (*C* and *D*) Glycerol gradient centrifugations. Heparin column fractions 31–39 and 47–55 were pooled separately and applied to 11-ml (15–35% glycerol/0.5 M NaCl in buffer B) gradients and centrifuged at 41,000 rpm in an SW41 (Beckman) rotor for 22 hr. Fractions (0.4 ml) were collected from the bottom of the tube and assayed as in *A*. The indicated sedimentation markers were thyroglobulin (690 kDa), alcohol dehydrogenase (150 kDa), and carbonic anhydrase (29 kDa).

the latter as specific transcription activity.) Unexpectedly, when the polymerase was chromatographed on a heparin–agarose column, an apparent dissociation of the two activities occurred (Fig. 1*B*). The peak of specific transcription activity eluted from the column at a NaCl concentration of 360 mM, whereas the peak of RNA polymerase activity eluted at 460 mM NaCl. To investigate this phenomenon further, heparin column fractions containing the predominant specific transcription and nonspecific RNA polymerase activities were pooled separately and applied to identical glycerol gradients. The specific transcription activity sedimented together with a low amount of RNA polymerase activity at the position expected for the multisubunit RNA polymerase (Fig. 1*C*). The RNA polymerase activity sedimented to the same position in the parallel gradient but specific transcription was barely detectable (Fig. 1*D*). The low RNA polymerase activity in the transcriptionally active fractions was only par-

tially explained by the amounts of polymerase protein. After correcting for the amount of protein, the RNA polymerase activity of the transcriptionally active fractions was less than 1/5th of that of the transcriptionally inactive fraction.

Association of an 85-kDa Protein with RNA Polymerase. When the same amounts of protein from the pooled peak fractions of gradients similar to those in Fig. 1 *C* and *D* were analyzed by SDS/PAGE and silver staining, it was evident that both contained a similar set of RNA polymerase subunits. A notable difference, however, was the presence of a prominent 85-kDa band in the RNA polymerase that was active in specific transcription (Fig. 2, lane 1). Although not quantitated, the intensity of the silver-stained bands suggested that the 85-kDa protein might be present in a stoichiometric amount relative to other polymerase subunits. In contrast, the pooled fractions with high nonspecific RNA polymerase activity had only small amounts of the 85-kDa polypeptide and slightly higher molecular mass polypeptides (Fig. 2, lane 2). The presence of a protein estimated to be about 77 kDa had been noted in some preparations of vaccinia virus RNA polymerase (14, 28) and may correspond to the protein described here. When the individual fractions of the heparin column were analyzed by SDS/PAGE, the 85-kDa band coincided precisely with the specific transcription activity (not shown). The results suggested (*i*) the existence of two forms of RNA polymerase—a core enzyme that possesses high nonspecific RNA polymerase activity and a holoenzyme that can act in conjunction with VETF to transcribe early genes; and (*ii*) an 85-kDa RNA polymerase-associated transcription specificity factor.

RNA Polymerase-Associated Protein Is Virus Encoded. To identify the gene encoding the 85-kDa protein, the transcriptionally active form of RNA polymerase was subjected to preparative SDS/PAGE and the polypeptides were transferred to a nitrocellulose membrane. The 85-kDa protein was digested with trypsin *in situ* and the peptides were resolved by high-pressure liquid chromatography. N-terminal amino acid sequences of two tryptic peptides were obtained and compared to all possible translations of the vaccinia virus genome. The peptides matched perfectly to sequences within the *Hin*dIII H fragment of the vaccinia virus genome (29, 30). The ORF designated H4L predicts a protein of 94 kDa with the same amino acid sequence in both the Copenhagen (29) and revised WR (N. B. Cole and B.M., unpublished data) strains of vaccinia virus (Fig. 3*A*).

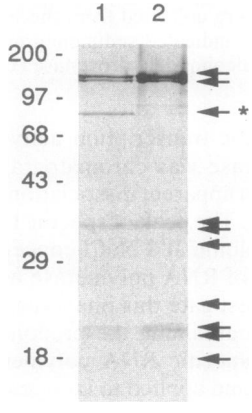

FIG. 2. Polypeptide composition of forms of RNA polymerase that are active and inactive in specific transcription. Silver-stained gradient gel of 8–18% polyacrylamide containing 100 ng of polymerases from the peak fractions of glycerol gradients similar to those in Fig. 1*C* (lane 1) and Fig. 1*D* (lane 2). Core subunits and the 85-kDa protein are indicated by arrows; the latter also has an asterisk. Shown on the left are positions of protein markers in kDa.

A

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MDSKETILIEIIPKIKAYLLDANISPKSYDDFISRNKNIF  40
VINLYNVSTITEEDIRLLYTTIEQNIDADDQTLVAIFSYI  80
GYKFEQAVKEEISTSLSFNDKNTTDEMTYNLYDLFFNTLD 120
MYLRQKKISILVNDDVRGDVIVSYKNSDLVSSFNAELEPE 160
IKKIPFNMKNLLPYLEKNLDQLRFSKKYLDFAYLCRHIGI 200
PISKKKYNVRYVFLYKIDGLSIPIIIKDFLDVKYVYLENT 240
GKIYKNSFSEDHNNSLSDWGKVIIPLLKDRHLYSYIFLSS 280
YHLHSYYTDLIARDEPVFVKRKKLDIIEIDEPEAWKRDVR 320
VEFAPCEHQIRLKEAMKVDANYFTKINNFANEFIYYEDGV 360
AYCRVCGINIPIFNLDAADVIKNTVIVSTFNKTIFLSEPY 400
SYFVHSQRFIFNIIMSFDNIMKSQTWVMKYNINRLILNFL 440
IDINSRRQEYEKKFSSEIKRGLFFLRLSANLFESQVSSTE 480
LFYVSKMLNLNYIVALVIILNSSADFIVSYMTSKNKTVEE 520
STLKYAISVVIYDFLVKTRICEKGSLDTIVLFTDVYTSIM 560
PEELDLHFQRITLELRKLVSIQRSALEPNYDVESRGEELP 600
LSALKFFDTSTIIVKTMAPVHTCVEQKIVAPTPSVEPTDA 640
SLKNFKELTCDEDIKILIRVHDTNATKLVIFPSHLKIEIE 680
RKKLIIPLKSLYITNTLKYYYSNSYLYVFRFGDPMPFEEE 720
LIDHEHVQYKINCYNILRYHLLPDSDVFVYFSNSLNREAL 760
EYAFYIFLSKYVNVKQWIDENITRIKELYMINFNN*      795
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B

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vaccinia LIRVHDTNATKLVIFPSHLKIEIERKK 683
         | :|  ||  | |    :  || ||||
   RAP30 LDKVVTTN-YKPVA-NHQYNIEYERKK 169
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FIG. 3. Identification and analysis of the gene encoding the 85-kDa protein. (*A*) Predicted amino acid sequence of the H4L ORF (29), with the two determined peptide sequences of the 85-kDa protein underlined. (*B*) Alignment of regions of vaccinia virus H4L and RAP30 gene sequence. Vertical lines and colons indicate identical and conserved amino acids, respectively. Two gaps (hyphens) were inserted in the RAP30 gene sequence for optimal alignment.

Data base searches with the FASTA program (31) did not reveal any proteins closely related to H4L. Because of the association with the core RNA polymerase, we compared the H4L sequence to that of *E. coli* σ^{70} and human RAP30. A program based on the algorithm of Needleman and Wunsch (32) aligned a 27-amino acid sequence of H4L with a 25-amino acid sequence of RAP30 to give a 44% identity (Fig. 3*B*). The N-terminal half of the same RAP30 segment was previously considered to be related to the core-binding domain (subregion 2.2) of *E. coli* σ^{70} (4). The biological significance of the H4L/RAP30 alignment, which scored between 3 and 4 standard deviations over the mean score of 100 random shufflings of the same amino acids, is uncertain.

A fusion protein containing the N-terminal 41% of H4L was used to make antibodies in order to confirm the association of the H4L protein with the transcriptionally active form of RNA polymerase. Western blot analyses indicated that all of the immunoreactive protein from lysates of vaccinia virions cosedimented with RNA polymerase and that none was detected in a free form (Fig. 4). Immunoblotting also showed that the H4L product coeluted with the specific transcription activity from the heparin–agarose column of Fig. 1*B* (data not shown). In addition, H4L-specific antibody attached to agarose beads depleted approximately 80% of the specific transcription activity but only 5–10% of the RNA polymerase activity from soluble virus extracts. In that experiment, core RNA polymerase subunits coprecipitated with the 85-kDa protein. In view of the complete and tight association of the H4L product with RNA polymerase, the protein was named vaccinia virus RNA polymerase-associated protein of 94 kDa (RAP94).

Synthesis of RAP94. The time of synthesis of RAP94 was determined by immunoblot analysis of proteins from vaccinia virus-infected HeLa cells that were harvested at various times after infection. The blot was probed first with H4L-specific antibody and then with antibody to the core RNA polymerase. Autoradiographs were made after the first and second probes, but only the latter is shown. RAP94 was detected at 4 hr after infection and increased steadily in amount thereafter (Fig. 5). The inability to detect RAP94 at

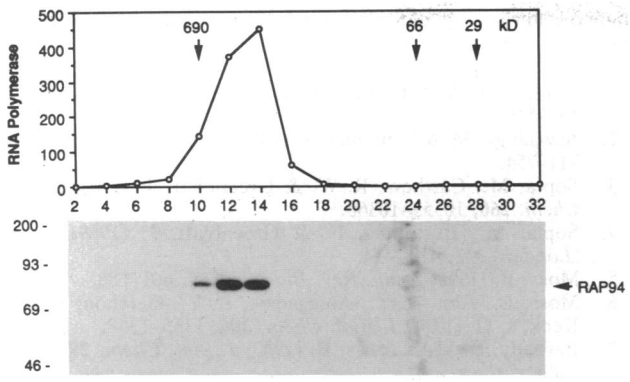

FIG. 4. Association of the product of the H4L ORF with RNA polymerase. Approximately 0.5 mg of soluble nucleic acid-free virion extract was applied to a glycerol gradient as described for Fig. 1, except that 0.2 M NaCl buffer was used, centrifugation was for 18 hr, and 0.33-ml fractions were collected. RNA polymerase assays (*Upper*) and SDS/PAGE immunoblots (9) with antiserum to the H4L product (1:1000) and ^{125}I-labeled staphylococcal A protein (autoradiograph, *Lower*) were made with 5-μl and 40-μl aliquots, respectively.

2 hr after a normal infection or at 16 hr after infection in the presence of araC, an inhibitor of DNA replication, suggested that expression was regulated by an intermediate or late promoter. In contrast, synthesis of the previously identified core RNA polymerase subunits (as exemplified by the large subunits in Fig. 5) were detected at the earliest time examined and in the presence of araC, consistent with regulation by an early promoter. The late synthesis of RAP94 is in agreement with previous studies demonstrating a late transcriptional start site preceding ORF H4L (called H5L in ref. 30).

RAP94 Is Required for Transcription. The studies described above established that RAP94 is associated with transcriptionally active RNA polymerase. However, the absence of naturally occurring free RAP94 and our inability to overexpress the protein in *E. coli* (B.-Y.A., unpublished results) prevented us from directly investigating its role in transcription. As an alternative, we developed a transfection approach that took advantage of the fact that RAP94, in contrast to the core RNA polymerase subunits, is not made in the absence of DNA replication (Fig. 6*A*). In the presence of araC, cells were infected with a recombinant vaccinia virus

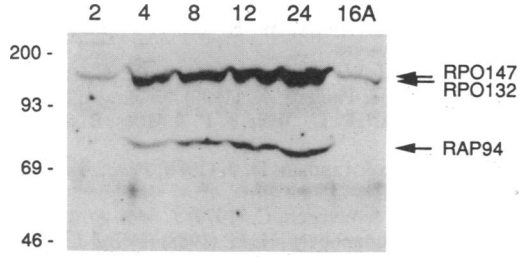

FIG. 5. Time course of RAP94 synthesis. HeLa cell suspensions were infected with 20 plaque-forming units of vaccinia virus per cell. Where indicated, araC was included in the medium at 40 μg/ml during infection. Cells were harvested at 2, 4, 8, 12, and 24 hr in the absence or 16 hr in the presence of araC, respectively, as indicated above the consecutive lanes. Cytoplasmic fractions were prepared as described (12) and the proteins (120 μg per lane) were analyzed by immunoblotting as in Fig. 4. A single band of approximately 85 kDa was detected by autoradiography (not shown). The membrane was then probed with antibody to the core RNA polymerase, again treated with ^{125}I-labeled staphylococcal A protein, and autoradiographed. The upper part of the autoradiograph is shown. The positions of unresolved RPO147 and RPO132 core RNA polymerase subunits and RAP94 are shown on the right (arrows); positions of molecular mass markers in kDa are shown on the left.

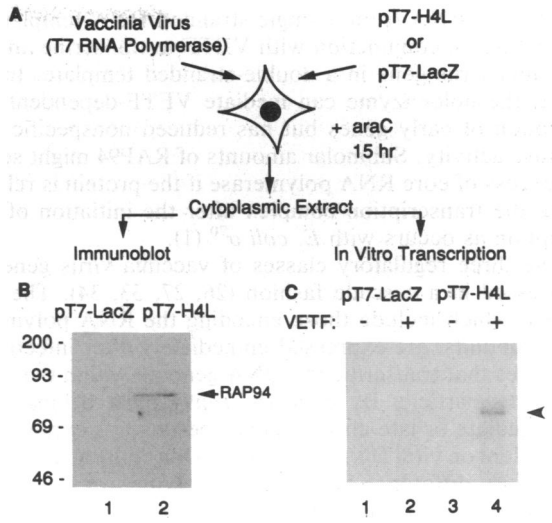

FIG. 6. Requirement for RAP94 in transcription. (*A*) Infection/transfection scheme. CV-1 cell monolayers were infected with a recombinant vaccinia virus expressing the T7 RNA polymerase (vTF7-3) in the presence of araC. After 1 hr, the cells were transfected with a plasmid containing the H4L ORF (pT7-H4L) or *lacZ* (pT7-lacZ) under control of the T7 ϕ10 promoter and incubated for 14 hr more. (*B*) Cytoplasmic extracts (24 μg of total protein), from cells transfected with pT7-lacZ (lane 1) or pT7-H4L (lane 2) were analyzed by SDS/PAGE and immunoblotting with H4L antibody. (*C*) Cytoplasmic extracts from cells transfected with pT7-lacZ (lanes 1 and 2) or pT7-H4L (lanes 3 and 4) were assayed for transcription activity in the absence (−) or presence (+) of VETF.

that constitutively expresses the bacteriophage T7 RNA polymerase (16). The cells were then transfected with a plasmid containing H4L under control of a T7 promoter to produce RAP94 in the absence of other late viral proteins. Replicate infected cell cultures were transfected with a plasmid containing the *lacZ* gene as a control. Immunoblotting confirmed that only the cells that were transfected with H4L DNA synthesized RAP94 (Fig. 6*B*). Extracts from the transfected cells were tested for the ability to transcribe a viral early gene template with the endogenous RNA polymerase and added VETF. VETF-dependent transcription occurred only with extracts from the H4L-transfected cells (Fig. 6*C*), indicating that RAP94 is a transcriptional specificity factor. The requirement for added VETF was consistent with previous reports (26, 27) that this transcription factor is also under late promoter control and consequently not made in the presence of araC. Further analyses indicated that the H4L protein had been assembled into the RNA polymerase complex in the transfected cells (data not shown).

DISCUSSION

Previous *in vitro* studies indicated that at least two viral components are needed for transcription of vaccinia virus early genes: a complex multisubunit RNA polymerase and VETF, a heterodimeric promoter-binding factor. In this report, we identify an RNA polymerase-associated protein RAP94 and provide evidence of its requirement for specific transcription of early genes *in vitro*. The protein was named according to the size of the viral genomic ORF, which is somewhat larger than the 85 kDa estimated by SDS/PAGE.

Analysis of the proteins extracted from vaccinia virions suggested that RAP94 is present in submolar amounts, relative to the core RNA polymerase subunits, and does not exist in a free form. Consequently, there are two separable forms of RNA polymerase that differ with regard to the presence or absence of RAP94. The core enzyme is fully competent to

synthesize RNA from a single-stranded DNA template but cannot act in conjunction with VETF to transcribe an early vaccinia virus gene in a double-stranded template. In contrast, the holoenzyme can mediate VETF-dependent transcription of early genes but has reduced nonspecific polymerase activity. Submolar amounts of RAP94 might service an excess of core RNA polymerase if the protein is released from the transcription complex after the initiation of transcription as occurs with *E. coli* σ^{70} (1).

The three regulatory classes of vaccinia virus genes are expressed in a cascade fashion (26, 27, 33, 34). The early genes, which include those encoding the RNA polymerase core subunits, are expressed immediately after infection by enzymes that transcribe the DNA genome within the infecting virus particle. By contrast, *rap94* must belong to the intermediate or late class of genes because its expression is dependent on viral DNA replication. One consequence of this regulatory difference is that RNA polymerase made in the presence of a DNA synthesis inhibitor lacks RAP94 (B.-Y.A., unpublished data) which is, therefore, unlikely to be required for transcription of intermediate genes. RNA polymerase isolated at late times of infection does contain RAP94 (J. G. Keck and B.-Y.A., unpublished data) and a role in late transcription is therefore possible. The late synthesis of RAP94 is appropriate for packaging in virus particles and is similar in this respect to VETF.

Our inability to obtain free RAP94 from either native or recombinant sources (unpublished results) precluded attempts to reconstitute the holoenzyme *in vitro*. Nevertheless, by making extracts from transfected cells that were infected with vaccinia virus in the presence of araC, we could demonstrate that expression of the RAP94 gene was required for VETF-dependent *in vitro* transcription of early genes.

Bacterial σ factors fulfill promoter recognition and RNA polymerase binding functions. These activities may be divided between vaccinia virus-encoded VETF and RAP94, as has been proposed for RAP30/74 and other eukaryotic general transcription factors. RAP94, however, is not closely related to RAP30, although the two could share a functional domain. The apparently more stable association of RAP94 with vaccinia virus RNA polymerase, compared to RAP30/74 with RNA polymerase II, may reflect in part the added virion packaging requirement for the viral proteins. A similar explanation was given for the association with the viral RNA polymerase of a vaccinia virus-encoded protein that has sequence similarities to eukaryotic transcription elongation factor SII (9). The role of RAP94 requires further investigation; possibly it mediates the binding of the core RNA polymerase to VETF.

We are grateful to Norman Cooper for cells and virus; Jerry Sisler for oligonucleotides; Bernard Amegadzie, James Keck, Michael Merchlinsky, and Paul Gershon for suggestions and discussions; Rafael Blasco for helping with the computer analysis of the sequences; and Kuan-Teh Jeang and Carl Wu for their comments on the manuscript. A similarity between amino acids 677–683 of RAP94 and amino acids 163–169 of RAP30 was brought to our attention by Paul Gershon.

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