

Purification and Identification of a Vaccinia Virus-encoded Intermediate Stage Promoter-specific Transcription Factor That Has Homology to Eukaryotic Transcription Factor SII (TFIIS) and an Additional Role as a Viral RNA Polymerase Subunit*

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Enzymes and factors, required for *in vitro* transcription of templates regulated by vaccinia virus intermediate stage promoters, are present in HeLa cells infected with vaccinia virus in the presence of an inhibitor of DNA replication. Previous studies indicated that *in vitro* transcription could be reconstituted by adding a partially purified transcription factor to the viral RNA polymerase and capping enzyme. By using an independent purification procedure, we isolated two vaccinia virus intermediate transcription factors VITF-1 and VITF-2 that were necessary for transcription of several different intermediate stage promoter templates but not for early or late stage promoter templates. VITF-1 was purified to homogeneity, and the sequences of two tryptic peptides were mapped to the fourth open reading frame within the *Hind*III E fragment (E4L) of the vaccinia virus genome, which had previously been shown to encode an RNA polymerase subunit of 30 kDa (RPO30) with homology to eukaryotic transcription elongation factor SII. Co-chromatography of VITF-1 with the E4L-derived protein was demonstrated using specific antiserum. In addition, transcriptionally active recombinant VITF-1 was made by expressing the E4L open reading frame in *Escherichia coli*. Thus, E4L encodes a multifunctional protein, serving as a RNA polymerase subunit and a stage-specific transcription factor. The stepwise binding of capping enzyme, VITF-1, and VITF-2 to a DNA/viral RNA polymerase complex was demonstrated.

The eukaryotic genome is replicated and transcribed in the nuclear compartment of the cell by a large array of enzymes and factors. Poxviruses, of which vaccinia virus is the prototype, are unlike other DNA viruses, in that they carry out DNA and RNA synthesis within the cytoplasm (1). Nevertheless, vaccinia virus mRNAs have typical eukaryotic features including a 5'-terminal methylated cap structure (2) and a 3' poly(A) tail (3). Furthermore, the expression of poxvirus genes is developmentally programmed and coupled to DNA replication. The poxviruses achieve this high degree of independence and complexity by encoding enzymes and factors that functionally,

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and in some cases structurally, resemble those of cells. Thus, vaccinia virus is well suited for combined biochemical and genetic studies of regulated mRNA synthesis (4).

Vaccinia virus genes are expressed at early, intermediate, or late stages of infection depending on their promoter sequences (5–9). The multisubunit viral RNA polymerase and factors required for transcription of the early genes are incorporated into the assembling virus particle and activated upon subsequent infection of new cells (10, 11). Specific transcription initiation can be reconstituted *in vitro* with the virion-derived RNA polymerase and associated protein (RAP94)¹ and one vaccinia virus early transcription factor (VETF) (12–14). The factors required for transcription of intermediate genes are synthesized before DNA replication (9, 15). Fractionation of cytoplasmic extracts, from cells infected in the presence of a DNA synthesis inhibitor, led to the isolation of two factors originally called vaccinia virus intermediate transcription factor-A (VITF-A) and VITF-B, in addition to the viral RNA polymerase (16). VITF-A was subsequently shown to be the capping enzyme (17), although its transcriptional activity preceded mRNA guanylylation (18). The factors required for activation of late genes include the products of three intermediate genes that are expressed after DNA replication (19), two of which have been shown to have *in vitro* transcription factor activity (20, 21). The factors specific for early transcription, VETF and RAP94, are products of late genes (13, 22, 23) as are most of the virion structural proteins. The successive synthesis of intermediate, late, and early transcription factors provides a basis for the programmed expression of vaccinia virus genes.

The purpose of the present study was to further purify and identify the factors required for transcription of intermediate genes. By using a new purification scheme, we obtained evidence for two factors, VITF-1 and VITF-2, in addition to RNA polymerase and capping enzyme. VITF-1 was purified to homogeneity and shown to be encoded by E4L, the leftward-directed, fourth open reading frame (ORF) in the *Hind*III E fragment of the vaccinia virus genome. The latter ORF was previously found to encode a viral RNA polymerase subunit of 30 kDa (RPO30) with homology to eukaryotic transcription elongation factor SII (TFIIS) (24). Thus, the E4L protein appears to have multiple roles in vaccinia virus transcription. The equally interesting finding that VITF-2, which has not

¹ The abbreviations used are: RAP94, RNA polymerase-associated protein of 94 kDa; AraC, cytosine arabinoside; DTT, dithiothreitol; E4L, fourth open reading frame in the *Hind*III E fragment of the vaccinia virus genome; G-less cassette, a segment of DNA without guanylate residues in the nontemplate strand; PAGE, polyacrylamide gel electrophoresis; ORF, open reading frame; RPO30, RNA polymerase subunit of 30 kDa; VETF, vaccinia virus early transcription factor; VITF, vaccinia virus intermediate transcription factor; TFIIS, transcription factor SII.

been completely purified, is a cellular protein will be presented elsewhere.

MATERIALS AND METHODS

Transcription Assays—Transcription reactions were performed using 2–5 μ l of RNA polymerase (0.7 mg/ml), 5 μ l of capping enzyme (0.85 mg/ml), and the amounts of VITF-1 or VITF-2 indicated in the figure legends, in a final volume of 30 μ l containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.15 mM EDTA, 5 mM MgCl₂, 1 mM ATP, 0.5 mM CTP, 0.05 mM UTP, 5 μ Ci of [α -³²P]UTP, 0.15 μ g of DNA, and 7.5% (v/v) glycerol. Transcription was carried out for 30 min at 30 °C and then 500 units of RNase T1 (Boehringer Mannheim) was added, and the incubation was continued for 10 min at 37 °C. The digestion was terminated by the addition of 0.25 ml of 150 mM NaCl and 0.3% SDS. After phenol: chloroform extraction, 0.2 μ g of tRNA was added, and the mixture was precipitated with ethanol. The RNA was resolved by polyacrylamide gel electrophoresis (PAGE) on a 4% polyacrylamide:bisacrylamide (24:4) gel containing 8 M urea and visualized by autoradiography.

DNA Templates—The vaccinia virus intermediate promoters from the G8R, A1L, A2L, and I3 ORFs were cloned separately into the plasmid (C₂AT)₁₉ (25), containing a segment of DNA without guanylate residues in the nontemplate strand (G-less cassette); the uncleaved plasmids were used for templates in the standard transcription assays and gave rise to RNAs of 400–420 nucleotides.

RNA Polymerase Assay—Nonspecific RNA polymerase assays were performed as described (26) in 135 mM Tris-HCl (pH 8.0), 80 mM NaCl, 15 μ M dithiothreitol (DTT), 7 mM MnCl₂, 2 mM ATP, 2 mM CTP, 2 mM GTP, 0.2 mM UTP, 2 μ Ci [α -³²P]UTP, and 1 μ g of M13 mp18 single-stranded DNA at 37 °C for 15 min.

Capping Enzyme Assay—Capping enzyme activity was determined as described (27). Samples (5 μ l) of column fractions were incubated in 50 mM Tris-HCl (pH 8.2), 2 mM DTT, 5 mM MgCl₂, 2 μ Ci [α -³²P]GTP at 37 °C for 15 min. Reactions were terminated by adding 10 μ l of electrophoresis sample buffer. The GMP-95-kDa covalent complex was resolved by SDS-PAGE and visualized by autoradiography.

Purification of Transcription Components—Approximately 5 \times 10⁹ HeLa cells in 10 liters of Eagle's medium supplemented with 5% horse serum were incubated with cytosine arabinoside (AraC, Sigma) at a concentration of 40 μ g/ml for 1 h at 37 °C. The cells were collected by centrifugation and resuspended in 50 ml of AraC-containing medium and infected with 5 \times 10¹⁰ plaque-forming units of vaccinia virus (strain WR). At the end of a 1-h adsorption period, the cells were collected by centrifugation and resuspended in 10 liters of fresh medium containing horse serum and AraC and incubated for 16 h at 37 °C. The cells were collected by centrifugation and Dounce-homogenized with 25 strokes of a tight-fitting pestle. The lysate was centrifuged at 10,000 rpm in a Sorvall S34 rotor for 15 min at 4 °C. The supernatants were processed as described (18) using procedures modified from those of Manley (28). Proteins from the cytoplasmic fraction were precipitated by the addition of (NH₄)₂SO₄ (0.35 g/ml). The precipitate was resuspended in buffer C (20 mM Tris-HCl (pH 7.9), 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 25% (w/v) glycerol) containing 0.05 M KCl. Extracts (40 ml) from 10 liters of cells yielded between 150 and 200 mg of protein as determined by the method of Bradford (29). Pepstatin (5 μ g/ml), aprotinin (5 μ g/ml), leupeptin (20 μ g/ml), antipain (5 μ g/ml), and chymostatin (5 μ g/ml) were added, and the mixture was dialyzed against buffer C containing 0.05 M KCl for 4 h at 4 °C. After centrifugation for 10 min at 100,000 \times g, the supernatants were diluted to 100 ml with buffer C, and the KCl concentration was adjusted to 0.25 M. The material was applied to a 40-ml DEAE-cellulose column (DEAE-cellulose I), equilibrated with buffer C containing 0.25 M KCl, to remove nucleic acids. The flow-through material was collected and dialyzed against buffer C containing 0.05 M KCl and then loaded onto a second DEAE-cellulose column (DEAE-cellulose II) equilibrated with buffer C in 0.05 M KCl. After washing, the bound proteins were eluted with steps of buffer C containing 0.15 M, 0.5 M, and 1.0 M KCl. The DEAE 0.15 M fraction (40 mg of protein in 50 ml) and the DEAE 0.5 M fraction (35 mg of protein in 60 ml) were dialyzed against buffer C containing 0.05 M KCl.

RNA polymerase, capping enzyme, and VITF-1 were present in the DEAE 0.15 M fraction (Fig. 1), which was loaded onto a 20-ml heparin-agarose column. After extensive washing of the column with buffer C in 0.05 M KCl, the bound proteins were eluted with a 6-column volume linear 0.05–1.0 M KCl gradient followed by a wash with 1.5 M KCl to regenerate the column. After dialysis against buffer C in 0.05 M KCl, the fractions were analyzed for capping enzyme and RNA polymerase. Pooled capping enzyme (0.3–0.45 M KCl) and RNA polymerase (0.5–0.65

KCl) containing fractions each had 15–20 mg of protein. Nonidet P-40 detergent was added to a concentration of 0.1% and was maintained in all subsequent steps.

The pooled capping enzyme from the heparin column was loaded onto a 10-ml phosphocellulose column, and bound proteins were eluted with a linear 0.05–1.0 M KCl gradient. Individual fractions were assayed in specific transcription reactions; active fractions were pooled and applied to a 15-ml bed volume double-stranded DNA-cellulose column (DNA-cellulose I). After washing with 10 volumes of buffer C containing 0.1 M KCl, the bound proteins were eluted with a linear gradient of 0.1–1.0 M KCl. Based on transcription and capping enzyme assays, the gradient fractions were pooled in 0.25–0.35 M and 0.4–0.45 M KCl groups. The latter was used as purified capping enzyme. The 0.25–0.35 M KCl fraction was dialyzed against buffer C containing 0.05 M KCl and then applied to a second DNA-cellulose column (DNA-cellulose II) with a 2-ml bed volume. Bound proteins were eluted as described for the previous DNA-cellulose column. Transcriptionally active fractions from 0.35 to 0.45 M KCl were pooled and dialyzed as above to provide the purified VITF-1.

The pooled RNA polymerase fractions from the heparin column (Fig. 1) were applied to a 10-ml phosphocellulose column that was eluted as described above for capping enzyme. The active RNA polymerase fractions (0.35–0.45 M KCl) were dialyzed against buffer C containing 0.05 M KCl and loaded onto a 3-ml bed volume double-stranded DNA-cellulose column equilibrated with buffer C containing 0.05 M KCl and 0.3 M urea; the bound fractions were eluted with a linear gradient of 0.05–0.6 M KCl in the presence of 0.3 M urea. Fractions containing RNA polymerase activity (0.25–0.3 M KCl) were pooled and dialyzed as above.

The 0.5 M KCl fraction from the DEAE-cellulose II column (Fig. 1) was applied to a heparin-agarose column as described for the 0.15 M KCl fraction. The flow-through containing transcription factor activity was applied to a 20-ml Q-Sepharose column equilibrated with buffer C in 0.05 M KCl; bound fractions were eluted with a linear gradient of 0.05–1.0 M KCl. Fractions with transcription factor activity (0.35–0.45 M KCl) were pooled and dialyzed against buffer C containing 0.3 M KCl and applied to a 40-ml Sephadryl S-300 gel filtration column; fractions with transcription factor activity were dialyzed against buffer C containing 0.05 M KCl and loaded onto a 7-ml DEAE-cellulose column. After washing extensively, the bound fractions were eluted with buffer C containing 0.5 M KCl and dialyzed against buffer C with 0.05 M KCl. The latter fractions represent the purified VITF-2 fraction.

Peptide Sequencing—Approximately 3 μ g of purified VITF-1 was resolved by SDS, 10% PAGE and transferred to a nitrocellulose membrane. The protein band was detected by staining with 1% Ponceau S (Sigma) for 5 min at room temperature. The filter was washed with water to visualize the protein, and the band was cut from the filter and dried. The material was sent to the Harvard microchemistry laboratory, where the protein was digested *in situ* with trypsin, and the peptides were isolated by reverse-phase high pressure liquid chromatography on a C18 column under the direction of William Lane. N-terminal sequence analysis was done with an Applied Biosystems 477A sequencer with a 120A on-line phenylthiohydantoin-amino acid analyzer.

Immunoblotting—Samples from column fractions were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with serum from a rabbit immunized with *Escherichia coli*-expressed *rpo30* ORF (24). The blots were washed four times within a period of 20 min with phosphate-buffered saline-Tween 20 and incubated for 1 h at room temperature with ¹²⁵I-labeled protein A (Amersham Corp.) in phosphate-buffered saline containing Tween 20 and 5% milk. The blots were washed four times with phosphate-buffered saline over a 30-min period, dried, and exposed to x-ray film.

Expression of E4L ORF—The E4L ORF, with *Bam*H1 and *Nde*I restriction endonuclease sites added to the ends, was amplified by the polymerase chain reaction and ligated to the corresponding sites of the pET14b vector (Novagen, Madison, WI). The resulting plasmid was called pET14b-30. *E. coli* harboring the pET14b-30 plasmid were grown to an *A*₆₀₀ of 0.6 and induced with 5 mM isopropyl-1-thio- β -D-galactopyranoside for 3 h. The cell suspension was centrifuged, and the pellet was resuspended in 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 mM imidazole, 1 mM DTT, 10% glycerol, 0.1% Nonidet P-40, 0.1% octyl β -D-glucopyranoside (Sigma), sonicated, and resuspended with a Dounce homogenizer. The insoluble material was removed by centrifugation, and the supernatant was dialyzed against buffer C. The presence of the induced protein in the soluble fraction was verified by SDS-PAGE and Coomassie Blue staining. The recombinant protein, containing C-terminal histidine residues, was purified on a 1-ml His:Bind Ni²⁺ affinity column as described in the pET His:Tag systems protocols (Novagen).

G8R Promoter-Sepharose 4B Column—The oligonucleotide containing the G8R promoter sequence 5'-GGATCCGAACGACGCTGTTCTGCAGCCATTAACTTAAATAATTACAAAAATTAAAATGAGCAG-GATCC-3' and its complement were chemically synthesized. A 0.5-mg sample of each strand was kinased and then polymerized using T4 DNA ligase as described (30). Activated CNBr-Sepharose 4B (Pharmacia LKB Biotechnology Inc.) was resuspended in coupling buffer and incubated with the DNA as described (30). The DNA-Sepharose contained approximately 0.25–0.5 mg of DNA/ml of Sepharose, as estimated from a radioactive marker. Mixtures of proteins were incubated together in buffer C containing 0.1 M KCl for 60 min at 4 °C and loaded onto the G8R-Sepharose column. The column was washed with 50 volumes of buffer C in 0.1 M KCl, and the bound proteins were eluted with buffer C in 1.0 M KCl. Fractions were dialyzed against buffer C and analyzed by transcription and capping assays.

RESULTS

Fractionation of Infected Cell Extract into Four Transcription Components—Previously, three components required for *in vitro* transcription of an intermediate-promoter template were isolated from the cytoplasmic fraction of HeLa cells that were infected with vaccinia virus in the presence of a DNA synthesis inhibitor (16). Starting from a similarly infected cell extract, we derived a purification scheme that resulted in the isolation of two factors, VITF-1 and VITF-2, in addition to RNA polymerase and capping enzyme (Fig. 1). All four components were required for *in vitro* transcription of G-less cassette templates regulated by four different intermediate promoters (Fig. 2). The need for VITF-1 and VITF-2 extended to the I3 promoter template, which was used by Vos *et al.* (16). The greater activity of the G8R promoter, compared with the A1L and A2L promoters, is consistent with its higher activity *in vivo* (8, 9). Furthermore, templates containing point mutations in the G8R promoter that are inactive *in vivo* (8, 9) were also inactive *in vitro* (data not shown).

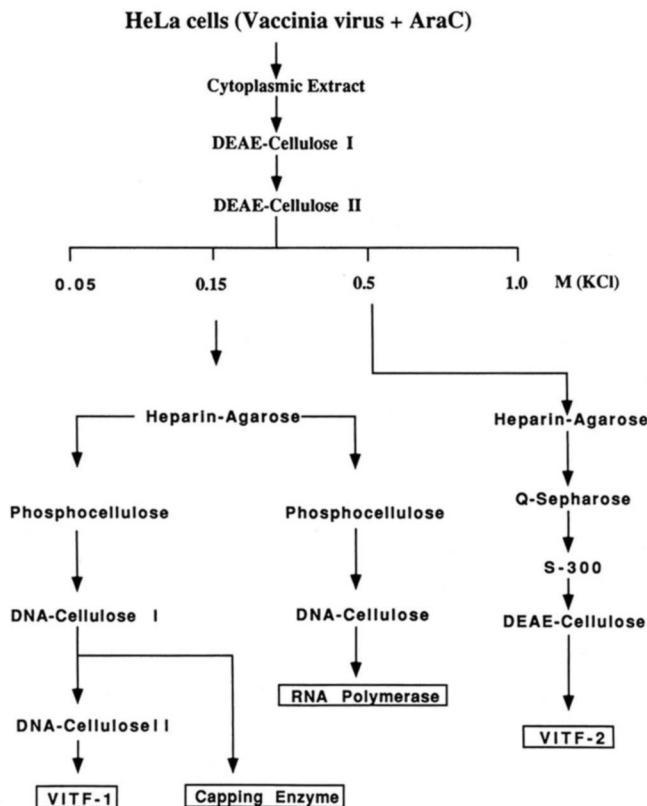


FIG. 1. Purification scheme. RNA polymerase, capping enzyme, VITF-1, and VITF-2 were purified from cytoplasmic extracts of vaccinia virus-infected AraC-treated HeLa cells. The KCl molarities used for step elution of proteins from the DEAE-cellulose II column are indicated.

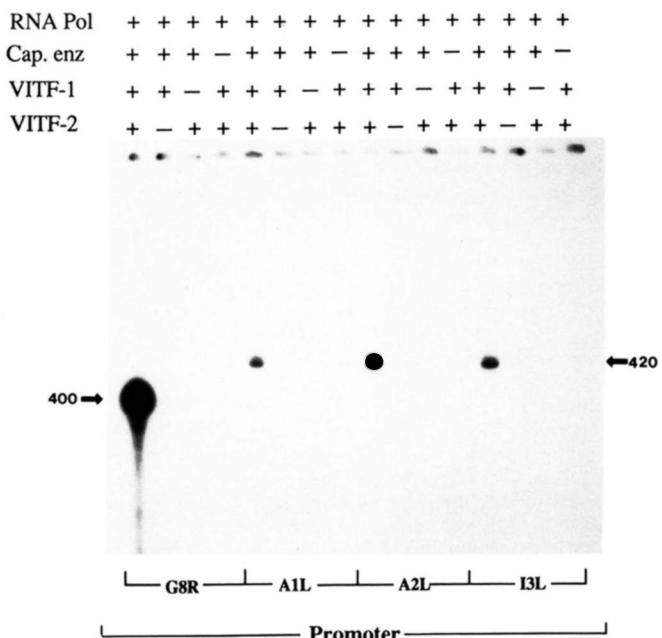


FIG. 2. Reconstitution of transcription activity. The promoter sequences of intermediate genes G8R, A1L, A2L, and I3 were cloned separately into the polylinker region of plasmid p(C₂AT)₁₉ so as to be adjacent to the G-less cassette. *In vitro* transcription reactions using these plasmid templates were carried out with (+) or without (-) the indicated components; 5 μ l of each component was used. The products were analyzed by PAGE; an autoradiogram is shown. The expected transcript sizes, 400 nucleotides for the G8R template and 420 nucleotides for the others, are indicated by arrows.

Identification of Viral Gene Encoding VITF-1—Although our most highly purified VITF-2 preparations still contained multiple polypeptide bands (not shown), we detected only a single 35-kDa polypeptide that co-eluted with VITF-1 activity on the final DNA-cellulose II column chromatography step (Fig. 3, A and B). The 35-kDa polypeptide was transferred from a polyacrylamide gel to a nitrocellulose membrane and digested *in situ* with trypsin. Two peptides isolated by high pressure liquid chromatography were sequenced. Comparisons with a library of vaccinia virus protein sequences revealed that both peptides (NTTPMMIQT and ATFD) had precise matches in a single 259 codon ORF designated E4L. Surprisingly, this ORF was previously shown to encode the RPO30 subunit of vaccinia virus RNA polymerase (24, 31). This result suggested that the E4L protein has different activities when free and when associated with RNA polymerase.

To confirm that VITF-1 is encoded by the E4L ORF, immunoblotting was performed on fractions from the DNA-cellulose II column (Fig. 4). The 35-kDa band, co-eluting with VITF-1 activity, reacted with a polyclonal antiserum prepared against the *E. coli*-expressed E4L ORF (24). The more rapidly migrating minor band with an apparent mass of 32 kDa was also seen when probing blots of RNA polymerase with the antiserum and probably represents translation initiation at an AUG that is 16 codons downstream of the first (24).

Extracts of *E. coli* that expressed a recombinant form of the E4L ORF with 6 histidine residues at the C terminus had VITF-1 activity, whereas control extracts had none (Fig. 5). VITF-1 activity was retained after partial purification of the recombinant protein by Ni²⁺ affinity chromatography (not shown), although the maximal activity was less than that of the native protein isolated from vaccinia virus-infected cells. Whether this difference was due to the histidine tag, *E. coli* contaminants, or other reasons has not yet been determined. Authentic VITF-1, purified from infected HeLa cells, was used for all other experiments in this study.

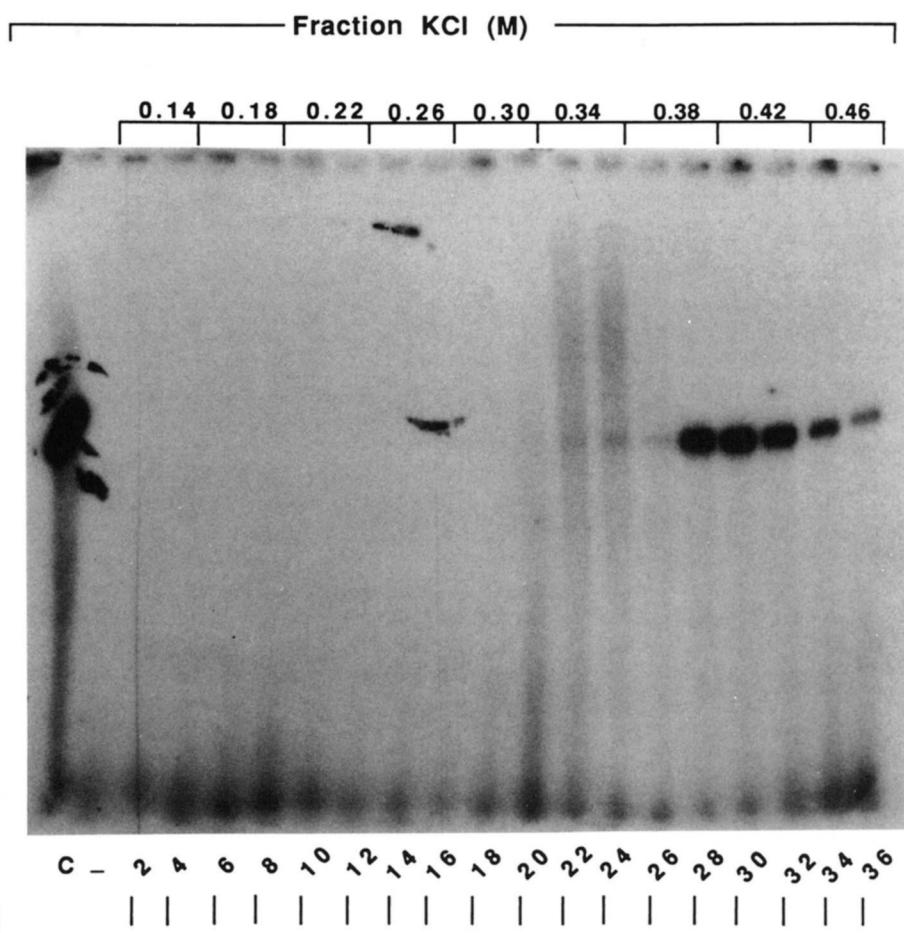
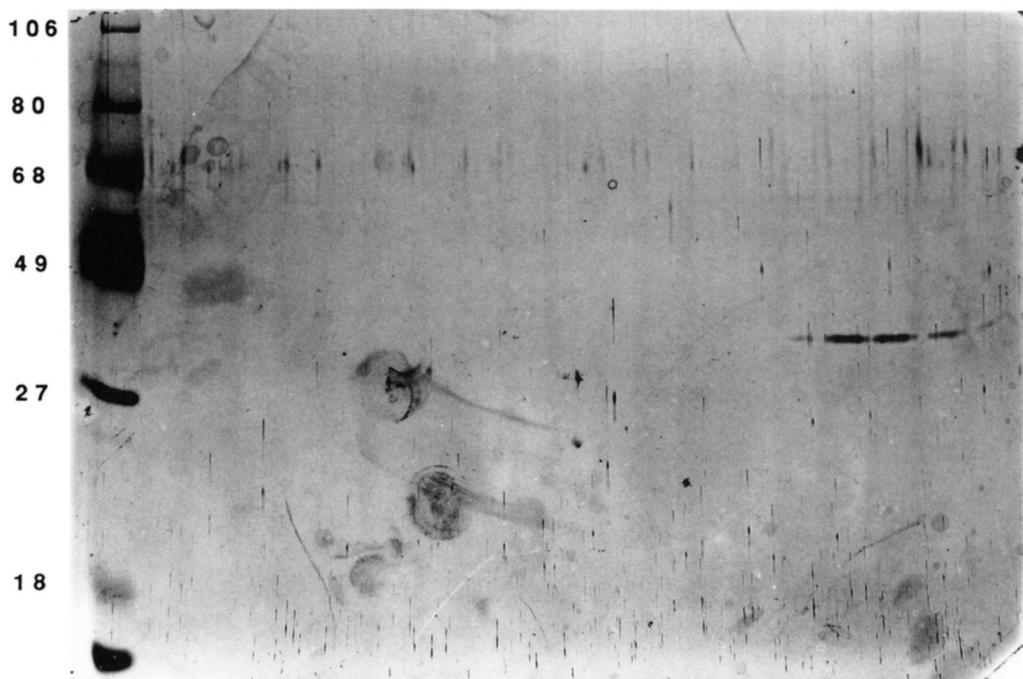
A**B**

FIG. 3. DNA cellulose chromatography of VITF-1. *A*, DNA-cellulose fractions, from the final purification step of VITF-1, were assayed in a reconstituted system using RNA polymerase, capping enzyme, VITF-2, and 10 μ l of every even numbered column fraction. *C*, positive control containing all 4 transcription components; *-*, same as *C* but without VITF-1. *B*, column fractions were analyzed by SDS-PAGE and silver staining. *Lane M*, protein markers with kDa indicated on the left.

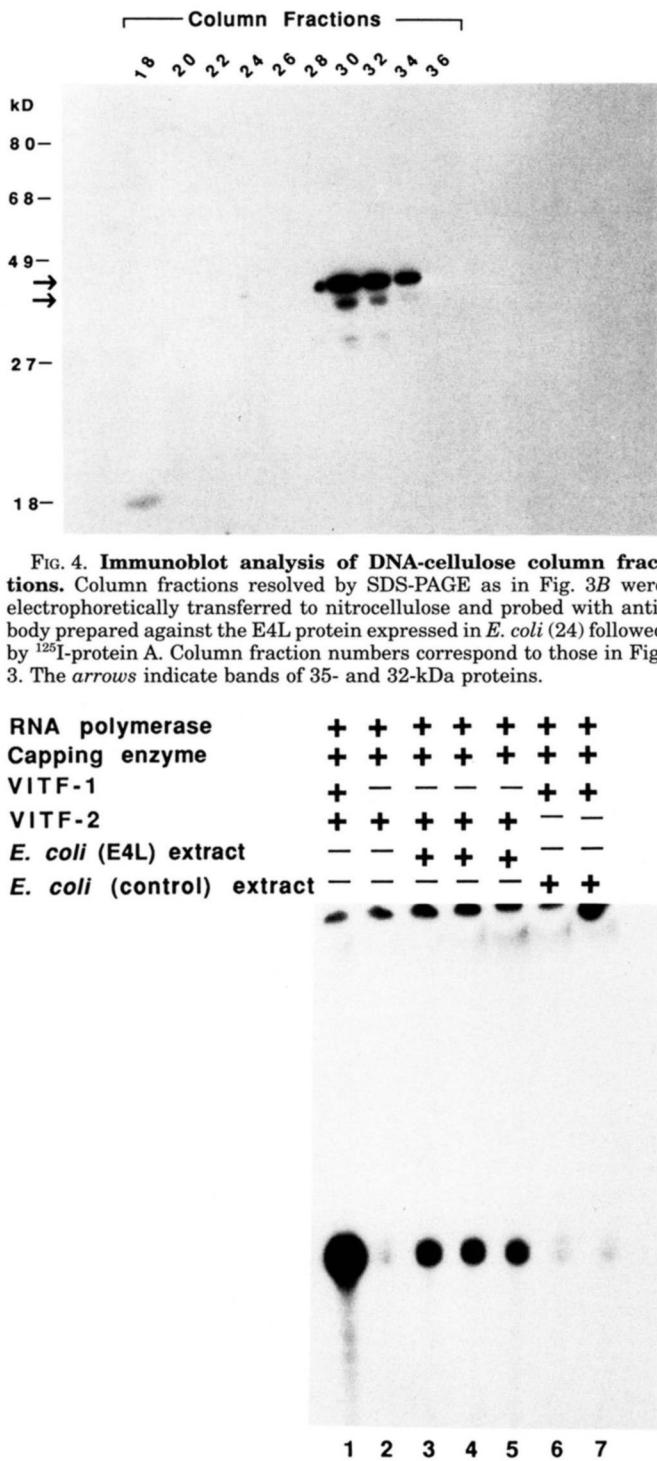


FIG. 4. Immunoblot analysis of DNA-cellulose column fractions. Column fractions resolved by SDS-PAGE as in Fig. 3B were electrophoretically transferred to nitrocellulose and probed with antibody prepared against the E4L protein expressed in *E. coli* (24) followed by ^{125}I -protein A. Column fraction numbers correspond to those in Fig. 3. The arrows indicate bands of 35- and 32-kDa proteins.

RNA polymerase	+	+	+	+	+	+	+
Capping enzyme	+	+	+	+	+	+	+
VITF-1	+	—	—	—	—	+	+
VITF-2	+	+	+	+	+	—	—
<i>E. coli</i> (E4L) extract	—	—	+	+	+	—	—
<i>E. coli</i> (control) extract	—	—	—	—	—	+	+

FIG. 5. E4L ORF expressed by *E. coli* is able to replace VITF-1 in vitro. Standard *in vitro* transcriptions were performed with (+) or without (-) RNA polymerase, capping enzyme, VITF-2, VITF-1, and 2-3 independent preparations of recombinant E4L or control *E. coli* extracts.

Stage Specificity of VITF-1—To assess stage specificity, transcription assays were performed with templates containing either an intermediate G8R or early C11R promoter (Fig. 6A). In this experiment, the appropriate RNA polymerases were used, e.g. for transcription of the intermediate promoter the RNA polymerase was isolated from cells infected in the presence of AraC (Int RNA polymerase), whereas the RNA polymerase used to transcribe the early promoter was isolated from purified virions (Virion RNA polymerase). Although intermediate

transcription was dependent on VITF-1 (*lanes 1* and *2*), early transcription was neither dependent on nor stimulated by VITF-1 (*lanes 3-5*) nor could VITF-1 substitute for VETF (*lane 6*).

We considered the possibility that the lack of VITF-1 requirement for early transcription was correlated with the use of the virion RNA polymerase. However, VITF-1 was required for intermediate transcription when the polymerase from virions or AraC-treated infected cells was used (Fig. 6B). Although the amounts of the two RNA polymerase preparations based on nonspecific activities and Western blotting were similar, higher intermediate transcription was obtained with the enzyme isolated from infected cells. The significance of this result remains to be determined.

VITF-1 and VITF-2 Are Needed to Confer Intermediate Promoter Transcription Activity to Virion Extract—Virion extracts contain all of the components needed to transcribe early promoters including VETF, viral RNA polymerase, and capping enzyme (32). Vos *et al.* (16) demonstrated that unsupplemented virion extracts could not transcribe an intermediate promoter template but that VITF-B alone could confer such activity. We therefore wished to know whether VITF-1 or VITF-2 activity is present in virion extracts. Transcription was not detected if either VITF-1 or VITF-2 were omitted, indicating that neither was present in the virion extract (Fig. 7). The absence of VITF-1 activity is consistent with the previous finding that RPO30 is present exclusively in association with RNA polymerase (24). As expected, the addition of RNA polymerase and capping enzyme was not necessary as they are present in the virion extract.

Interactions between RNA Polymerase, Capping Enzyme, and Intermediate Transcription Factors—Attempts to show specific complex formation, between the G8R intermediate promoter and VITF-1 or VITF-2, by electrophoretic mobility shift assays were unsuccessful. As an alternative, the G8R promoter was attached to Sepharose 4B and used to examine physical interactions between the four components needed for intermediate transcription. Initial experiments indicated that under the conditions used, VITF-1, VITF-2, nor capping enzyme alone could bind tightly to the immobilized DNA, whereas RNA polymerase bound, probably in a nonspecific manner (discussed below). A different result was obtained, however, when the viral RNA polymerase, VITF-1, VITF-2, and capping enzyme were incubated together and loaded onto the G8R-Sepharose 4B column. After washing with buffer C containing 50 mM KCl, the proteins were eluted with buffer C containing 1.0 M KCl. Dialyzed column fractions were then analyzed in transcription assays and for formation of a covalent capping enzyme-GMP complex. All four components bound to the column, because the eluted proteins were sufficient to transcribe an added G8R template (Fig. 8, *upper panel*). As expected, from the latter result, capping enzyme also eluted in the same fractions (Fig. 8, *upper panel*). When VITF-1 was omitted from the incubation mixture, the addition of VITF-1 to the eluted proteins did not confer transcription activity (Fig. 8, *second panel*). Conversely, when VITF-2 was omitted, the addition of VITF-2 to the eluted proteins was sufficient to confer transcription activity (Fig. 8, *third panel*). Taken together, these results suggested that binding of VITF-1 to the complex occurred independently of VITF-2 but that binding of VITF-2 to the complex required VITF-1. The omission of the capping enzyme from the incubation mixture could not be rectified by adding the enzyme to the eluted proteins, suggesting that VITF-1 or VITF-2 had not bound. Additional experiments showed that when capping enzyme was omitted, VITF-1 and VITF-2 activity were both in the flow-through fractions of the affinity column. These results are consistent with an ordered addition of capping enzyme, VITF-1,

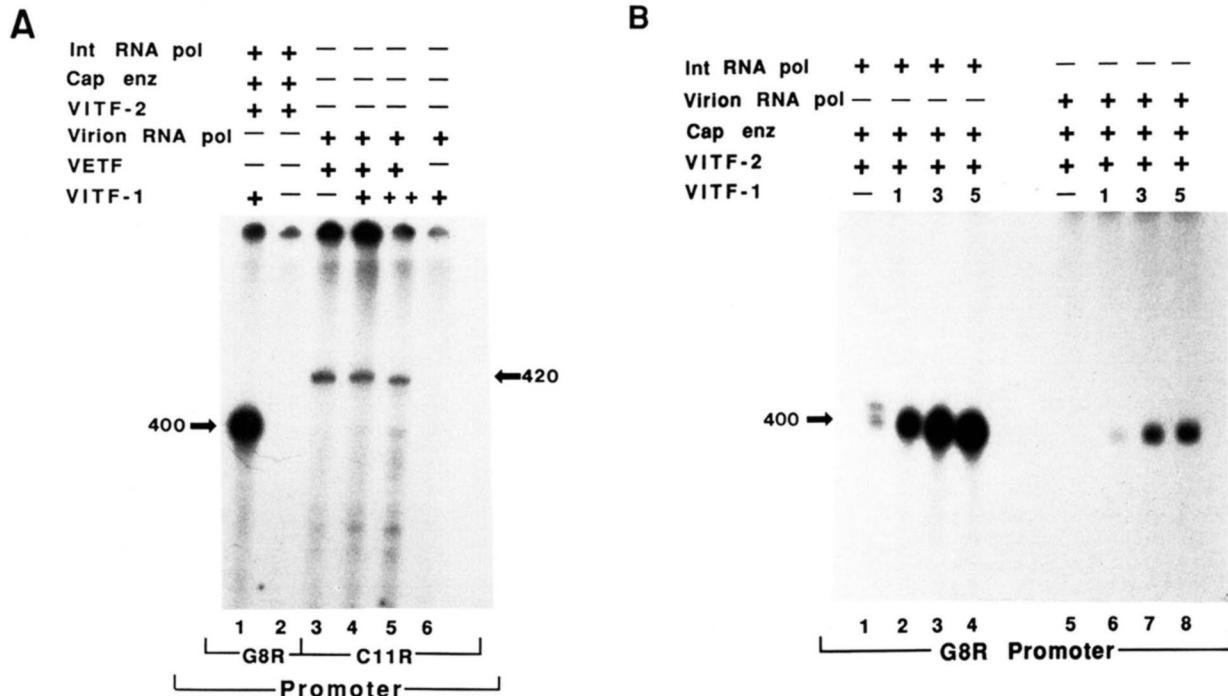


FIG. 6. Stage specificity of VITF-1. A, standard transcription assays were performed with 5 μ l of cytoplasmic viral (*Int*) RNA polymerase, 5 μ l of virion RNA polymerase, 5 μ l of capping (*Cap*) enzyme, 5 μ l of VITF-2, 3 μ l of VETF, and 5 (+) or 10 (++) μ l of VITF-1. B, equivalent amounts of intermediate or virion RNA polymerases were tested by transcription as described above. + shows the presence and - the absence of the indicated component.

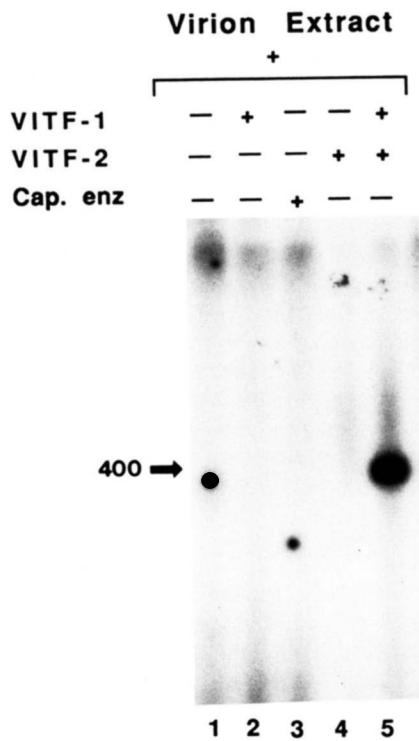


FIG. 7. Absence of VITF-1 and VITF-2 activities in virion extracts. Transcription assays were performed with 5 μ l of virion extract and 5 μ l of VITF-1, VITF-2, or capping (*Cap*) enzyme. RNA was analyzed by polyacrylamide gel electrophoresis; an autoradiogram is shown.

and VITF-2 to the immobilized RNA polymerase-DNA complex. The interactions may not depend on a specific promoter sequence, because binding of RNA polymerase, capping enzyme, VITF-1, and VITF-2 occurred using salmon sperm-DNA-Sepharose. It seems likely that the role of the DNA was to provide nonspecific binding for the RNA polymerase. Therefore, from

these experiments we cannot determine which factor(s) interact(s) specifically with intermediate promoters. It may be that intermediate promoter recognition is a property of the complex composed of the transcription factors, capping enzyme, and RNA polymerase.

DISCUSSION

All of the factors required for transcription of intermediate genes are present in cells that have been infected by vaccinia virus in the presence of an inhibitor of DNA replication. From extracts of such infected cells, Stunnenberg and co-workers (16, 17) isolated three components needed for *in vitro* transcription of a template containing an intermediate promoter, the viral RNA polymerase, capping enzyme (VITF-A), and a partially purified factor (VITF-B). We confirmed those results and reported that the transcription factor activity of the capping enzyme preceded and was independent of mRNA guanylylation (18). By using an independent purification procedure, we now show that there are two intermediate transcription factors in addition to RNA polymerase and capping enzyme. The four purified components were necessary and sufficient to transcribe four different intermediate promoter-regulated templates, one of which was used by Vos *et al.* (16). The activities of both factors were stage-specific, *i.e.* they neither stimulated early transcription nor replaced the early transcription factor VETF. VITF-1 and VITF-2 also could not stimulate or replace any of the late transcription factors.² Because we are unsure of the relationship between VITF-B and the two factors described here, we have named the latter VITF-1 and VITF-2. This terminology is consistent with that used for late transcription factors.

VITF-1 was purified to homogeneity from infected cell extracts, and the sequences of two tryptic peptides within the 35-kDa protein were determined. Our finding that the peptides had perfect matches within the vaccinia virus E4L ORF was

² G. Kovacs and J. Keck, personal communication.

INTERMEDIATE TRANSCRIPTION COMPLEX FORMATION

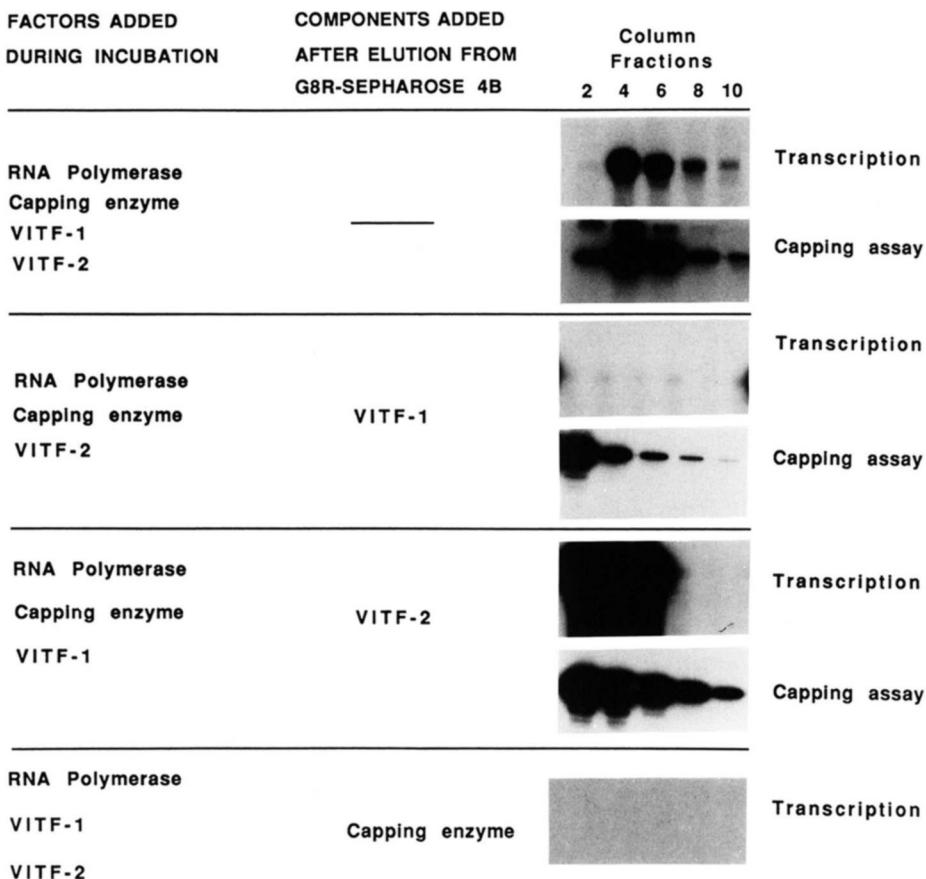


FIG. 8. Interactions between intermediate transcription factors. RNA polymerase purified from infected cells was incubated with different combinations of capping enzyme, VITF-1 and VITF-2 (*first column* in the figure) and loaded on a G8R-Sepharose 4B affinity column. After washing, the bound proteins were eluted and dialyzed, and the material in individual fractions were mixed with additional components (*second column*) and tested for the ability to transcribe an intermediate promoter template and for formation of a covalent GMP capping enzyme complex (*third column*).

unexpected, because it was previously shown to encode RNA polymerase subunit RPO30 (24, 31). Nevertheless, the early expression of E4L is appropriate for both VITF-1 and RPO30; the sizes of VITF-1 and RPO30 estimated by SDS-PAGE were identical; polyclonal antibody to a recombinant protein, derived from the E4L ORF and previously shown to bind RPO30, reacted with column fractions containing VITF-1 activity. In addition, VITF-1 activity was present in extracts of recombinant *E. coli* that expressed the E4L ORF and the activity co-purified with the histidine-tagged protein on a Ni²⁺ affinity column.

It was previously found that transcription of the *rpo30* gene is initiated from two closely spaced sites at early times and a third at late times (24). The two early species probably encode the major and minor polypeptides detected by Western blotting of VITF-1, because the factor was purified from cells infected in the presence of AraC. Post-translational modification, however, has not been ruled out. All three electrophoretic forms of the E4L polypeptide appear to be incorporated into RNA polymerase as RPO30. The existence of multiple species of RPO30 and other RNA polymerase small subunits has made it difficult to determine their stoichiometry.

The amount of E4L protein added to our transcription assays as VITF-1 was less than that added as the RPO30 subunit of RNA polymerase (determined by unpublished Western blots), strongly suggesting that the two activities are independent. It is not difficult to imagine that the tight association of RPO30 with other polymerase subunits might prevent interactions that are available to the free protein. The inability to find VITF-1 activity in extracts of virions is consistent with a previous finding that there is no free E4L protein in virions; it is found exclusively in association with RNA polymerase (24). The targeting of RNA polymerase to assembling vaccinia virions

appears to be a function of the associated protein RAP94 (33), precluding the incorporation of free VITF-1.

The role of the E4L protein as a transcription factor is consistent with its structural similarity to eukaryotic TFIIS. Computer alignment of the vaccinia virus and eukaryotic ORFs indicated that 23% of the amino acids were identical over a 180-amino acid overlap of the C-terminal regions (24), comparable with the degree of similarity in sequence of the two large subunits of vaccinia virus and eukaryotic RNA polymerases. Furthermore, both the E4L and TFIIS ORFs have similar zinc finger motifs in the same locations, suggesting that they interact with nucleic acids in a similar way. TFIIS is about 4 kDa larger than E4L, however, and contains unrelated N-terminal sequences. Purified TFIIS, a kind gift of Danny Reinberg (UMDNJ-RW), was unable to replace VITF-1 and did not stimulate intermediate transcription. This is not surprising in view of the substantial sequence differences between E4L and TFIIS and the primary role of the latter in transcription elongation.

We have evidence for the ordered addition of capping enzyme, VITF-1 and VITF-2 to an immobilized DNA/RNA polymerase complex. Thus, in the absence of capping enzyme, neither VITF-1 nor VITF-2 bound, and in the absence of VITF-1, VITF-2 did not bind. Nevertheless, VITF-2 was not required for the binding of VITF-1. This assay, however, was not promoter-specific and therefore only provides information regarding protein-protein interactions. Therefore, we cannot determine which factor(s) interact(s) specifically with intermediate promoters. It may be that intermediate promoter recognition is a property of the complex composed of the transcription factors, capping enzyme, and RNA polymerase. Vos *et al.* (16) have reported that VITF-B mediates promoter commit-

ment and melting in the absence of RNA polymerase. Whether these reactions can be carried out by VITF-1 or VITF-2 remains to be determined.

The present data suggest that VITF-1 is involved in an early transcriptional step but does not preclude an additional role in elongation, as suggested by its homology with TFIIS. TFIIS is required for the 3' shortening of nascent transcripts (34), and a similar role for RPO30 has been suggested (35). Genetic studies to determine the functions of the E4L protein may be complicated because of its dual nature as a transcription factor and as an RNA polymerase subunit. The finding of vaccinia virus proteins with multiple roles in mRNA synthesis is not unprecedented, however. For example, the capping enzyme is a RNA triphosphatase, guanylyltransferase, and a guanine-7-methyltransferase (36, 37) as well as an early transcription termination factor (38) and an intermediate transcription initiation factor (17, 18). The small subunit of poly(A) polymerase (39) is also a cap-specific 2'-O-methyltransferase (40).

The discovery that VITF-1 is encoded by a viral gene that is expressed early in infection is consistent with the cascade model of vaccinia virus gene regulation. Studies to be described elsewhere, however, suggest that VITF-2 is a cellular protein (41). This unprecedented finding has important implications for poxvirus/host interactions.

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REFERENCES

- Moss, B. (1990) in *Virology* (Fields, B. N., Knipe, D. M., Chanock, R. M., Hirsch, M. S., Melnick, J., Monath, T. P., and Roizman, B., eds) pp. 2079–2112, Raven Press, New York
- Wei, C. M., and Moss, B. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72**, 318–322
- Kates, J., and Beeson, J. (1970) *J. Mol. Biol.* **50**, 19–23
- Moss, B. (1990) *Annu. Rev. Biochem.* **59**, 661–688
- Davison, A. J., and Moss, B. (1989) *J. Mol. Biol.* **210**, 749–769
- Davison, A. J., and Moss, B. (1989) *J. Mol. Biol.* **210**, 771–784
- Hirschmann, P., Vos, J. C., and Stunnenberg, H. G. (1990) *J. Virol.* **64**, 6063–6069
- Baldick, C. J., Keck, J. G., and Moss, B. (1992) *J. Virol.* **66**, 4710–4719
- Baldick, C. J., Jr., and Moss, B. (1993) *J. Virol.* **67**, 3515–3527
- Kates, J. R., and McAuslan, B. R. (1967) *Proc. Natl. Acad. Sci. U. S. A.* **58**, 134–141
- Munyon, W. E., Paoletti, E., and Grace, J. T., Jr. (1967) *Proc. Natl. Acad. Sci. U. S. A.* **58**, 2280–2288
- Broyles, S. S., Yuen, L., Shuman, S., and Moss, B. (1988) *J. Biol. Chem.* **263**, 10754–10760
- Ahn, B.-Y., and Moss, B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 3536–3540
- Ahn, B.-Y., Gershon, P. D., and Moss, B. (1994) *J. Biol. Chem.* **269**, 7552–7557
- Vos, J. C., and Stunnenberg, H. G. (1988) *EMBO J.* **7**, 3487–3492
- Vos, J. C., Saskar, M., and Stunnenberg, H. G. (1991) *Cell* **65**, 105–114
- Vos, J. C., Saskar, M., and Stunnenberg, H. G. (1991) *EMBO J.* **10**, 2553–2558
- Harris, N., Rosales, R., and Moss, B. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2860–2864
- Keck, J. G., Baldick, C. J., and Moss, B. (1990) *Cell* **61**, 801–809
- Keck, J. G., Kovacs, G. R., and Moss, B. (1993) *J. Virol.* **67**, 5740–5748
- Wright, C. F., Keck, J. G., and Moss, B. (1991) *J. Virol.* **65**, 3715–3720
- Gershon, P. D., and Moss, B. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 4401–4405
- Broyles, S. S., and Fesler, B. S. (1990) *J. Virol.* **64**, 1523–1529
- Ahn, B.-Y., Gershon, P. D., Jones, E. V., and Moss, B. (1990) *Mol. Cell. Biol.* **10**, 5433–5441
- Sawadogo, M., and Roeder, R. G. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 4394–4398
- Baroudy, B. M., and Moss, B. (1980) *J. Biol. Chem.* **255**, 4372–4380
- Shuman, S., and Moss, B. (1990) *Methods Enzymol.* **181**, 170–180
- Manley, J. L. (1987) in *Transcription and Translation: A Practical Approach* (Hames, B. D., and Higgins, S. J., eds) pp. 72–73, IRL Press, Oxford
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Kadonaga, J. T., and Tjian, R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 5889–5893
- Broyles, S. S., and Pennington, M. J. (1990) *J. Virol.* **64**, 5376–5382
- Rohrmann, G., and Moss, B. (1985) *J. Virol.* **56**, 349–355
- Zhang, Y., Ahn, B.-Y., and Moss, B. (1994) *J. Virol.* **68**, 1360–1370
- Izban, M. G., and Luse, D. S. (1992) *Genes & Dev.* **6**, 1342–1356
- Hagler, J., and Shuman, S. (1993) *J. Biol. Chem.* **268**, 2166–2173
- Martin, S. A., and Moss, B. (1975) *J. Biol. Chem.* **250**, 9330–9335
- Venkatesan, S., Gershowitz, A., and Moss, B. (1980) *J. Biol. Chem.* **255**, 903–908
- Shuman, S., Broyles, S. S., and Moss, B. (1987) *J. Biol. Chem.* **262**, 12372–12380
- Gershon, P. D., Ahn, B. Y., Garfield, M., and Moss, B. (1991) *Cell* **66**, 1269–1278
- Schnierle, B. S., Gershon, P. D., and Moss, B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 2897–2901
- Rosales, R., Sutter, G., and Moss, B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, in press