

# Purification of a Factor Required for Transcription of Vaccinia Virus Early Genes\*

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**Steven S. Broyles, Leonard Yuen‡, Stewart Shuman, and Bernard Moss***From the Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892*

Partially purified DNA-dependent RNA polymerase from infectious vaccinia virus particles exhibits the following two activities: 1) specific transcription of double-stranded DNA templates containing vaccinia early promoters and 2) nonspecific transcription of single-stranded DNA templates. After further purification of the RNA polymerase, specific transcriptase activity was selectively diminished suggesting the loss of a transcription factor. In agreement with the latter hypothesis, transcriptase activity could be reconstituted by mixing the purified RNA polymerase with certain column fractions. A quantitative complementation assay was developed and used to locate the transcription factor during successive column chromatography steps. The factor eluted as a single peak of activity from single strand DNA-cellulose and phosphocellulose columns. An observation that the transcription factor binds specifically to vaccinia early promoter sequences was exploited in the final affinity chromatography steps. The purified factor was separated from all previously identified vaccinia enzymes and contained two polypeptides of  $M_r$  77,000 and 82,000. A DNA-dependent ATPase activity also copurified with the transcription factor. Although a single template was used for assays during isolation, the purified factor stimulated transcription of three other early genes by 20–30-fold suggesting that it has a general role in conferring promoter specificity for initiation of early transcription.

The regulation of mRNA synthesis by DNA-dependent RNA polymerases is often linked to the recognition of DNA sequences adjacent to the RNA start site. The interaction of the RNA polymerase with promoter elements can be altered by auxiliary protein factors distinct from the RNA polymerase itself. Such transcription factors can be divided into two broad categories. Factors belonging to one group can interact with free RNA polymerase to alter its affinity for the promoter. A well-known example is the prokaryotic  $\sigma$  factor (1). Factors belonging to the second group, such as the eukaryotic TATA (2, 3) and CAAT element binding proteins (4), are believed to affect transcription initiation through interaction with specific DNA sequences that usually precede the mRNA start site. Formation of a complex between the factor and the promoter may increase the frequency of transcription initiation, direct the site of initiation, or both.

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‡ Present address: National Research Council, Canada, Biotechnology Research Institute, Montreal, Quebec, Canada H4P 2R2.

Poxviruses offer a unique system for studying the regulation of transcription. These viruses replicate within the cytoplasm and transcribe their large DNA genomes in apparent autonomy of the host cell nucleus (reviewed in Ref. 5). Upon entry into the cell, synthesis of early mRNA begins. A switch in transcription from early to late genes follows initiation of DNA synthesis, but the mechanism remains unclear.

The presence of a DNA-dependent RNA polymerase within the infectious particle, a feature unique to poxviruses, is central to their nuclear independence. The RNA polymerase of vaccinia virus has been studied in some detail. The most highly purified preparations of the enzyme have at least nine subunits (6–8) that appear to be virus-encoded (9). The extensive sequence similarities between the largest subunits of vaccinia virus and cellular RNA polymerases indicate an evolutionary relationship (10). Highly purified vaccinia RNA polymerase also resembles its cellular counterparts in its inability to independently transcribe double-stranded DNA templates. The isolated enzyme will polymerize ribonucleotides only on single-stranded DNA templates using  $Mn^{2+}$  as the divalent cation.

All of the macromolecular components necessary to produce mature early mRNA are contained within the vaccinia virion. Permeabilized virions will synthesize authentic mRNA in a reaction that requires  $Mg^{2+}$ , all four ribonucleoside triphosphates, and an energy source in the form of the hydrolyzable  $\beta$ - $\gamma$  bond in ATP (11, 12). This *in vitro* reaction appears to accurately reflect early transcription as it occurs in infected cells. Furthermore, the relevant enzymes and factors can be extracted from viral cores to yield a soluble fraction that can transcribe early vaccinia genes (13, 14). The enzyme extract initiates and terminates RNA synthesis at the proper sequences on defined duplex templates (14, 15). As is the case *in vivo*, about 30 nucleotides upstream of the 5' start site are required for initiation (16, 17). Crude virus extracts have been fractionated by glycerol gradient sedimentation producing a transcriptionally competent RNA polymerase complex with the native requirements for  $Mg^{2+}$  and ATP hydrolysis preserved (18).

The loss of transcriptional competence during extensive purification of RNA polymerase could result either from denaturation of the complex molecule or separation of essential factors. To investigate these possibilities, we first purified the vaccinia RNA polymerase to a form that transcribed poorly and then demonstrated that a factor, containing  $M_r$  77,000 and 82,000 polypeptides, restored activity. This protein functions with all tested early promoters, binds to DNA sequences within the region known to be important for promoter function, and has an associated DNA-dependent ATPase activity.

## EXPERIMENTAL PROCEDURES

**DNA Templates**—Single-stranded DNA, prepared by phenol extraction of bacteriophage M13mp18 virions, was used to measure RNA polymerase activity (19). To determine specific transcription, a double-stranded DNA template containing the vaccinia growth factor (VGF)<sup>1</sup> gene template was prepared by EcoRI and HincII cleavage of plasmid pSC16 (18). The resulting 1.2-kilobase fragment includes the entire VGF gene as well as 235 base pairs of gene "X," an oppositely oriented gene of unknown function (20). The 7.5-kDa gene template was obtained by digestion of pGS19 with *TaqI* to produce a 1000-base pair fragment containing 245 base pairs of vaccinia DNA including the 7.5-kDa gene promoter (22). The 22-kDa RNA polymerase subunit gene template was prepared from plasmid pSB13 which contains the entire 22-kDa gene spanning the region from the *Xba*I site upstream of the RNA start site to the first *Aha*III site downstream of the termination codon of the 22-kDa gene open reading frame (18). The template was cleaved with *EcoRV* so that runoff transcripts would be observed. The 147-kDa RNA polymerase gene template was derived from plasmid pJ8 (23) by cleavage with *HincII*. All DNA fragments were purified by agarose gel electrophoresis.

**Vaccinia Virus**—HeLa S-3 cells were grown in suspension culture and infected with vaccinia virus strain WR at a multiplicity of 8 plaque forming units/cell. After 40 h cells were harvested and lysed and virions were purified twice by sucrose gradient sedimentation (24).

**RNA Polymerase Purification**—All steps were performed at 4 °C. Two-thousand  $A_{260}$  units of vaccinia virions were sedimented for 10 min at 12,000  $\times g$  and resuspended in 10 ml of Buffer A (0.25 M KCl, 0.1 M Tris-HCl, pH 8.4, 0.1 mM EDTA, 10 mM DTT). Sodium deoxycholate was added to a concentration of 0.2% (w/v). After 1 h of incubation on ice, the solution was sheared by 10 strokes in a tight-fitting Dounce homogenizer. Insoluble material was sedimented at 12,000  $\times g$  for 30 min and the supernatant was retained. The pellet was resuspended in 5 ml of Buffer A, and deoxycholate was added to a concentration of 0.1%. Following a 30-min incubation on ice, the centrifugation was repeated. The two supernatant fractions were combined and applied to a 20-ml DEAE-cellulose column (DE52, Whatman) equilibrated in Buffer A. The flow-through fractions from DEAE-cellulose column I were pooled and diluted with 1.5 volumes of Buffer B (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.01% (v/v) Nonidet P-50, 1 mM DTT, 10% (v/v) glycerol). The resulting solution was applied to a second DEAE-cellulose column (10 ml) equilibrated with 0.1 M KCl in Buffer B. Flow-through fractions from DEAE-cellulose column II were combined and retained for the purification of the transcription factor (see below). RNA polymerase was eluted with a 25-ml linear gradient of 0.1 to 0.4 M KCl in Buffer B. Fractions containing RNA polymerase activity were pooled, diluted 5-fold with Buffer B, and applied to a 40-ml single-stranded DNA-cellulose column (prepared by the method of Litman; 25) equilibrated with 50 mM NaCl in Buffer B. The column was developed with a linear gradient of 0.05–0.4 M NaCl in Buffer B.

**Purification of Transcription Factor**—The flow-through fraction from DEAE-cellulose column II (see above) was mixed with an equal volume of Buffer B and loaded onto a 40-ml volume single-stranded DNA-cellulose column. The transcription factor was eluted with a 0.05–0.6 M linear gradient of NaCl in Buffer B. Fractions that stimulated the transcription activity of DNA-cellulose-purified RNA polymerase on the VGF template were pooled, diluted to 0.1 M NaCl, and applied to a 5-ml phosphocellulose column (P11, Whatman) equilibrated with 0.1 M NaCl in Buffer B. The column was developed with a linear gradient of 0.1–0.6 M NaCl in Buffer B. Fractions containing transcription factor activity were combined, diluted to 0.1 M NaCl, and loaded onto a 10-ml DNA affinity column. An 80-ml linear gradient of 0.1–0.6 M NaCl in Buffer B was used to elute the factor. Protein concentrations were measured according to Bradford (26) using bovine serum albumin as the standard.

**Preparation of DNA Affinity Column**—A DNA-Sepharose column containing covalently bound nucleotides -4 to -37 of the VGF promoter was prepared essentially as described by Kadonaga and Tjian (27). The oligonucleotides CGAGCCTTAGTTATATTACT-GAATTAATAATATAAAATT and GGCTCGAATTTATATTAT-TAATTCACTAATATAAACTAA were annealed, phosphorylated, and polymerized by ligation as previously described. The products

were coupled to CNBr-activated Sepharose (Pharmacia LKB Biotechnology Inc.) according to the manufacturer's instructions.

**Enzyme Assays**—RNA polymerase assays were performed as described previously (7) with bacteriophage M13mp18 single-stranded DNA as template. One unit is defined as the amount of enzyme which incorporates 1 nmol of UMP into material retained on DE81 filters in 30 min at 37 °C (see below). Transcription reactions were conducted in a 50  $\mu$ l containing 20 mM Na-HEPES, pH 7.9, 6 mM MgCl<sub>2</sub>, 1 mM each of ATP, GTP, and CTP, 0.1 mM UTP, 1 mM DTT, 0.5  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]UTP, 0.2 units of vaccinia RNA polymerase, and 50 ng of DNA template at 30 °C for 60 min. RNA products were analyzed by electrophoresis in polyacrylamide gels containing 7 M urea (14). For quantification of transcription, reaction conditions were as described above except that the UTP was reduced to 20  $\mu$ M to increase the specific activity of labeling, and the reaction was terminated after 30 min. Reaction products were spotted onto DE81 filters (Whatman) that then were washed three times in 0.5 M Na<sub>2</sub>HPO<sub>4</sub> and once in ethanol. Filters were dried and radioactivity was determined by liquid scintillation counting. Poly(A) polymerase (28), DNA topoisomerase (18), and mRNA 2'-o-methyltransferase (29) were assayed as described previously. Guanylyltransferase (capping enzyme) was determined by formation of a covalent complex with [ $\alpha$ -<sup>32</sup>P]GTP (30). The specific activity of GTP was 1100 cpm/fmol. Labeled enzyme was analyzed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (31). Bands were excised from the gel, and radioactivity was determined by liquid scintillation counting.

ATPase activity was measured in 50 mM Tris-HCl, pH 7.5, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM ATP (except where indicated), and 0.5  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP. After specified times at 37 °C, released P<sub>i</sub> was measured by the method of Conway and Lipman (32).

**DNA Electrophoretic Mobility Shift Assay**—The VGF promoter fragment used for DNA-binding studies was produced by *Bam*HI and *Hind*III cleavage of pVGF. This plasmid contains nucleotides -48 to +23 relative to the VGF RNA start site. The promoter fragment was 3' end-labeled with either [ $\alpha$ -<sup>32</sup>P]dGTP at the *Bam*HI site or [ $\alpha$ -<sup>32</sup>P]dATP at the *Hind*III site with DNA polymerase I large fragment or by 5' end labeling with [ $\gamma$ -<sup>32</sup>P]ATP and polynucleotide kinase (21). Protein fractions were mixed with 1–2 ng of <sup>32</sup>P end-labeled VGF promoter fragment in 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT, and 5% glycerol in a volume of 20  $\mu$ l. Where indicated poly(dI-dC)·poly(dI-dC) was present at 100  $\mu$ g/ml. After 30 min at 22 °C, the solution was loaded onto a 6% polyacrylamide gel in 6.7 mM Tris-HCl, pH 7.5, 3.3 mM sodium acetate, 0.1 mM EDTA (33). Electrophoresis was at 10 V/cm for 2 h. Gels were dried and exposed to x-ray film for autoradiography.

## RESULTS

**Isolation of Transcription-defective RNA Polymerase**—Although purified vaccinia RNA polymerase will synthesize RNA only on single-stranded DNA templates, previous studies showed that extracts of vaccinia virions or partially purified RNA polymerase can accurately initiate transcription from double-stranded templates containing viral early promoters (13, 14, 18). These observations suggested the existence of one or more transcription factors that separated from the polymerase during extensive purification. As a first step in the search for the putative factors, we sought a simplified method of preparing a transcription-defective form of RNA polymerase. For clarity and consistency in terminology, we distinguish RNA polymerase activity and transcription activity by the use of nonspecific single-stranded DNA templates and Mn<sup>2+</sup> to assay the former and a double-stranded DNA template containing an early vaccinia viral gene and Mg<sup>2+</sup> for the latter. Fractionation of virus extracts by glycerol gradient sedimentation (18) or by batch elution from DEAE-cellulose (34) yielded RNA polymerase activity that still retained transcription activity. Because DEAE-cellulose chromatography separates the vaccinia RNA polymerase from the major portion of the other known vaccinia enzymes and is amenable to large scale preparation, we chose this as our first purification step. When virion extracts were chromatographed on DEAE-cellulose in 0.1 M KCl, the RNA polymerase was retained quantitatively on the column and was eluted with a salt

<sup>1</sup> The abbreviations used are: VGF, vaccinia growth factor; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

gradient (Fig. 1A). The polymerase was released at about 0.15 M KCl, as previously observed (7). Assessment of transcription capability was made using the VGF gene template (15). The DEAE-cellulose column II-purified RNA polymerase preparation produced a 530-nucleotide VGF transcript indicative of accurate initiation and termination of transcription (Fig. 2). The bands of 1000 and 235 nucleotides corresponded to the nonterminated runoff VGF RNA and the runoff gene X RNA, respectively.

The RNA polymerase was subsequently applied to a single-stranded DNA-cellulose (Fig. 1B), and 34% of the activity, measured with a single-stranded DNA template, was recovered in the flow-through fraction. This fraction resembled DEAE-cellulose-purified enzyme in that it also was able to initiate and terminate transcription (data not shown). The bound RNA polymerase was eluted with a NaCl gradient and was released from the column with 0.075–0.20 M NaCl. The chromatographic profile of RNA polymerase activity is bi-

phasic in shape. Both peaks of RNA polymerase exhibited similar low levels of transcription activity but differed in termination ability. The peak at fraction 50 produced primarily runoff VGF transcript (Fig. 2), while fraction 58 gave rise to significant amounts of terminated VGF transcript (data not shown). When equal units of DEAE-cellulose and DNA-cellulose-purified RNA polymerase were used in a transcription assay, the latter had only 3–5% of the activity of the former as determined by densitometry of the autoradiograph. Under standard assay conditions, approximately 65 fmol of VGF transcript was produced with the DEAE-cellulose purified enzyme. The apparent separation of transcriptionally competent RNA polymerase in the DNA-cellulose flow-through fraction from a transcription-deficient enzyme bound to DNA-cellulose is suggestive of heterogeneous populations of RNA polymerase molecules. The appearance of RNA polymerase in the flow-through fraction in Fig. 1B is not the result of saturation of binding sites on the DNA-cellulose. Rechromatography of this enzyme on a second DNA-cellulose column in 50 mM NaCl showed that less than 12% of the applied activity was recovered in the bound fraction (data not shown).

**Reconstitution of Transcription Activity**—The fact that RNA polymerase purified by DNA-cellulose chromatography was incapable of catalyzing transcription efficiently suggested that a necessary factor had been lost. Nevertheless, attempts to reconstitute transcription activity by mixing the polymerase with other fractions from the same column were not successful. We considered, however, that excess free factor might have been resolved from RNA polymerase at earlier steps in the fractionation of the virus extract. The flow-through fraction from DEAE-cellulose column II is an abundant source of non-polymerase protein since all known soluble enzymes from vaccinia virions (except RNA polymerase) are found in this fraction. Mixing the DEAE-cellulose flow-through material with the DNA-cellulose-purified RNA polymerase did not appreciably affect single-stranded template-dependent RNA synthesis but did restore the ability of the polymerase to efficiently transcribe the VGF template (Fig. 2). However, because of the large amounts of poly(A) polymerase in this fraction, the transcripts were large and heterogeneous in length. This problem was eliminated with further purification of the factor.

**Quantitative Assay for Transcription**—In order to use a complementation assay to measure transcription factor activity, we needed a more convenient and quantitative procedure than polyacrylamide gel electrophoresis. The latter method was especially difficult because of the heterogeneity in transcript length resulting from variable polyadenylation and termination efficiencies. A simple DEAE-filter-binding assay for RNA synthesis that measures incorporation of [ $\alpha$ -<sup>32</sup>P]UMP into RNA chains was linear with respect to time and amount of transcription factor added (data not shown). Although the template used here contains two separate oppositely oriented promoters, one of which is followed by a terminator sequence, this was not a significant source of variability in quantification of transcription factor. Experiments using the 22-kDa RNA polymerase gene template containing a single promoter directing only a runoff transcript gave similar results (data not shown).

**Purification of Transcription Factor**—Using the complementation assay, the transcription factor was detected in the DEAE-cellulose column II flow-through material. Upon chromatography of this fraction on a single-stranded DNA-cellulose column, the transcription stimulatory factor eluted at 0.18 M NaCl (Fig. 3). The factor eluted closely, but separately,

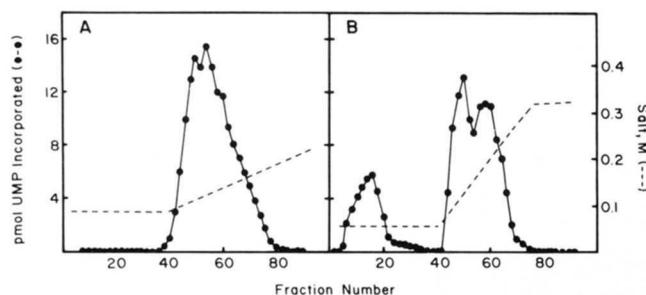


FIG. 1. Purification of vaccinia RNA polymerase. *A*, vaccinia virion extract that had been passed through DEAE-cellulose in 0.25 M KCl, was diluted to 0.1 M KCl with Buffer B and chromatographed on a second DEAE-cellulose column. Fractions 12–25 contained unbound protein. *B*, peak fractions from the DEAE-cellulose column were pooled, diluted to 0.05 M KCl with Buffer B, and loaded onto a single-stranded DNA-cellulose column. Unbound protein was found in fractions 4–22. The salt used for washing and elution was KCl in (*A*) and NaCl in (*B*). In both panels RNA polymerase activity was determined on a single-stranded DNA template.

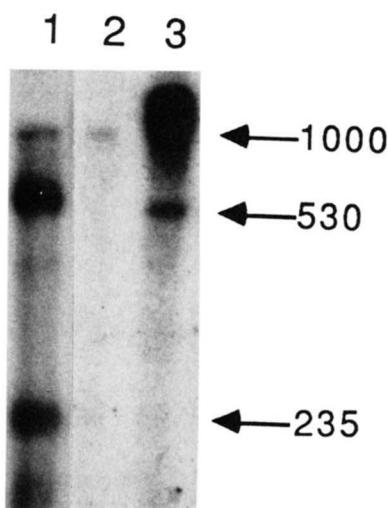
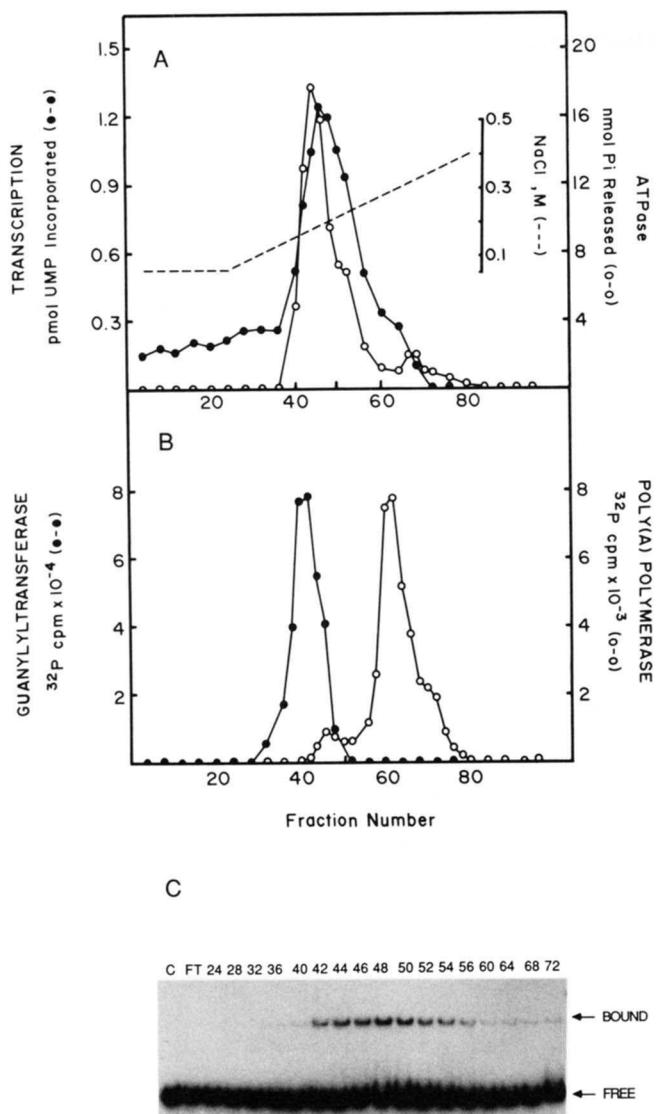


FIG. 2. Transcription activity of purified vaccinia RNA polymerase. RNA polymerase prepared by DEAE-cellulose column chromatography (lane 1) and by subsequent DNA-cellulose column chromatography (lane 2) were assayed for transcription activity on the double-stranded VGF gene template. In each reaction 0.4 units of RNA polymerase were used. Lane 3 is DNA-cellulose-purified RNA polymerase plus 5  $\mu$ l of DEAE-cellulose column II flow-through fraction (0.2  $\mu$ g of protein). The RNAs of 235, 530, and 1000 nucleotides are the gene X runoff, the VGF gene terminated, and VGF nonterminated runoff transcripts, respectively.



**FIG. 3. DNA-Cellulose chromatography of vaccinia transcription factor.** DEAE-Cellulose column II flow-through material was diluted in half with Buffer B and chromatographed on a single-stranded DNA-cellulose column. *A*, Column fractions were assayed for stimulation of transcription by DNA-cellulose-purified RNA polymerase on the VGF gene template (●—●) and DNA-dependent ATPase (○—○). *B*, Guanylyltransferase (●—●) and poly(A) polymerase (○—○) activates. *C*, VGF promoter binding was assayed by gel electrophoretic mobility shift analysis. Two  $\mu$ g poly(dI-dC)·poly(dI-dC) was included in each binding reaction. Free and protein-bound VGF promoter DNA are indicated.

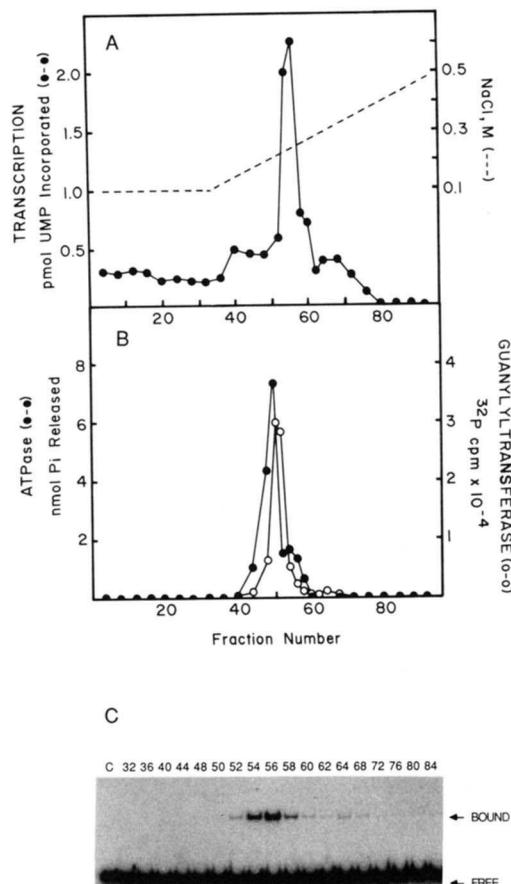
from the guanylyltransferase (mRNA-capping enzyme) and the major peak of DNA-dependent ATPase (36), which is nucleoside triphosphate phosphohydrolase I. A shoulder of ATPase, that probably contains nucleoside triphosphate phosphohydrolase II, overlaps the profile for transcription factor. The transcription stimulatory factor did not coelute with the mRNA 2'-*o*-methyltransferase, DNA topoisomerase, or the single strand-specific nuclease (data not shown).

Previously, it was shown that crude extracts of vaccinia virions contain a protein that has a high affinity for the VGF promoter region (35). The extract was incubated with end-labeled 90-nucleotide VGF promoter DNA fragment in the presence of a vast excess of nonspecific DNA competitor poly(dI-dC)·poly(dI-dC). The complex was detected as a DNA band with the electrophoretic mobility of a 300-nucleotide fragment. Analysis of DEAE-cellulose column II fractions

indicated that, while some promoter-binding material was bound to the column with the RNA polymerase, most of the factor flowed through the column (data not shown). Upon DNA-cellulose chromatography of the transcription factor, VGF promoter-binding protein eluted in fractions 42–56 as did the transcription factor (Fig. 3C). While it is not apparent in this experiment, we frequently detect materials eluting at other positions from the DNA-cellulose column that form minor protein-DNA complexes with different electrophoretic mobilities. We have not determined what, if any, relationship these proteins bear to the transcription factor.

Fractions with transcription activity from the single-stranded DNA-cellulose column were pooled and chromatographed on a phosphocellulose column. The transcription stimulatory factor eluted from phosphocellulose at 0.22 M NaCl in fractions 52–60 (Fig. 4A). Again the factor eluted closely to but separately from the capping enzyme and nucleoside triphosphate phosphohydrolase I (Fig. 4B). A trailing peak of ATPase, however, coincided with the transcription factor (Fig. 4B). The VGF promoter-binding protein eluted in the same fractions as the transcription factor (Fig. 4C).

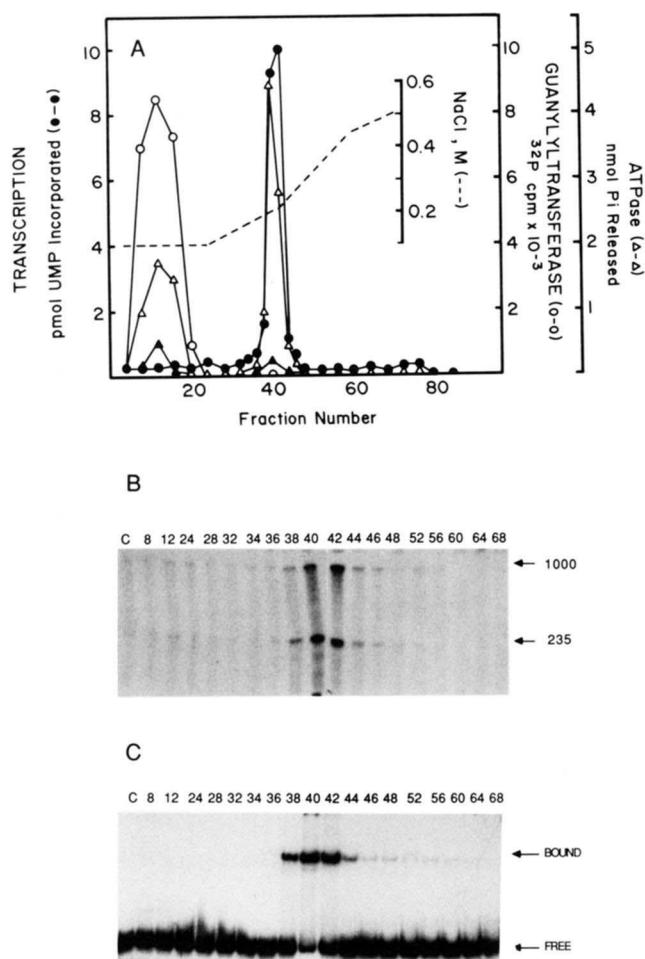
We considered that the transcription factor might be the promoter-binding protein and therefore sought to exploit this property for purification by linking a reiterated VGF promoter sequence to a Sepharose support (27). Chromatography of



**FIG. 4. Phosphocellulose column chromatography of vaccinia transcription factor.** Active fractions from the DNA-cellulose column were pooled, diluted to 0.1 M NaCl, and chromatographed on phosphocellulose. Fractions were assayed for stimulation of transcription by DNA-cellulose-purified RNA polymerase on the VGF gene template in *panel A* and DNA-dependent ATPase activity (●—●) and guanylyltransferase (○—○) in *panel B*. VGF promoter DNA-binding was assayed by gel mobility shift analysis in *panel C*. Two  $\mu$ g poly(dI-dC)·poly(dI-dC) was present in each reaction.

active phosphocellulose fractions on the DNA affinity column demonstrated that the factor was retained on the column in 0.1 M NaCl (Fig. 5A). All of the residual capping enzyme and much of the ATPase was found in the flow-through fraction. The VGF promoter-binding protein again coeluted from the column along with the transcription factor (Fig. 5C). Repeated copurification of the promoter-binding protein with the transcription factor further suggests that the two are identical. Interestingly, a DNA-dependent ATPase also cochromatographed with the transcription factor (Fig. 4A). Little ATPase activity was detected in these fractions when the DNA cofactor was omitted. The ATPase activity was stimulated some 20-fold by the presence of single-stranded DNA.

Analysis of the transcription products made by the addition of the DNA affinity column fractions to single-stranded DNA-cellulose-purified RNA polymerase showed that the initiation of transcription was specific for the early promoters on the VGF template. Fractions containing the vaccinia transcription factor stimulated transcription of the 1000 nucleotide



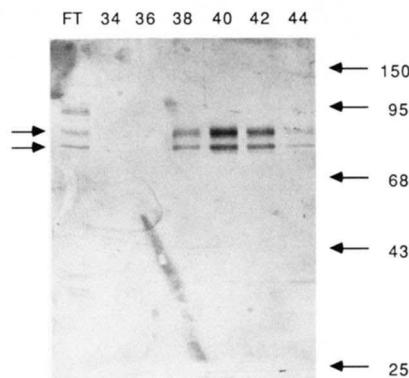
**FIG. 5. DNA sequence affinity chromatography of vaccinia transcription factor.** Active fractions from the phosphocellulose column were pooled, diluted to 0.1 M NaCl, and chromatographed on VGF gene promoter DNA linked to Sepharose. Fractions were assayed for stimulation of transcription on the VGF gene template by DNA-cellulose-purified RNA polymerase (●—●), guanylyltransferase (○—○), and ATPase in the presence (△—△) or absence (▲—▲) of single-stranded DNA cofactor (panel A). For ATPase assays ATP was present at 0.1 mM. Fractions 4–20 are flow-through material. Transcription products were also analyzed by polyacrylamide gel electrophoresis (panel B). The 235- and 1000-nucleotide RNAs correspond to the gene X and VGF runoff transcripts, respectively. VGF promoter-binding activity was assayed by gel mobility shift analysis (panel C).

runoff VGF RNA and the 235 nucleotide gene X RNA. Little or no 530-nucleotide transcript, indicative of transcription termination on the VGF gene, was seen. This observation is consistent with removal of the capping enzyme at this step of the purification and our previous demonstration that termination factor copurifies with that enzyme (34).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the DNA affinity column fractions revealed that most of the polypeptides did not bind to the column and appeared in flow-through fractions (Fig. 6). Fractions containing the transcription factor activity contain prominent polypeptides of  $M_r$  82,000 and 77,000. The elution profile of each polypeptide paralleled that of the transcription factor, indicating that one or both of these polypeptides are responsible for the stimulation of transcription. Densitometric scans of silver-stained gels indicated that these two polypeptides constituted 95% of the total protein in peak fractions. Minor polypeptides of  $M_r$  75,000 and 35,000 were also detected.

For quantification purposes 1 unit of transcription factor was defined as that which induces a 10-fold (about half-maximal) increase in the transcription of the VGF template under standard conditions. Because of the presence of RNA polymerase in fractions prior to the DEAE-cellulose column II step, measurement of the initial amount of transcription factor was not possible. From the second DEAE-cellulose column through the DNA affinity column, however, there was an 80-fold increase in activity (Table I).

**Stimulation of Transcription of Other Early Genes**—The effect of the transcription factor on the VGF promoter (and the gene X promoter) suggests that the factor may be of general importance in the transcription of early genes. The effect of the factor on transcription of three other early genes was assessed. Transcription on templates containing the VGF gene, 7.5-kDa protein gene, 22-kDa RNA polymerase gene, and the 147-kDa RNA polymerase gene promoters all were stimulated by the presence of the factor (Fig. 7). Densitometry

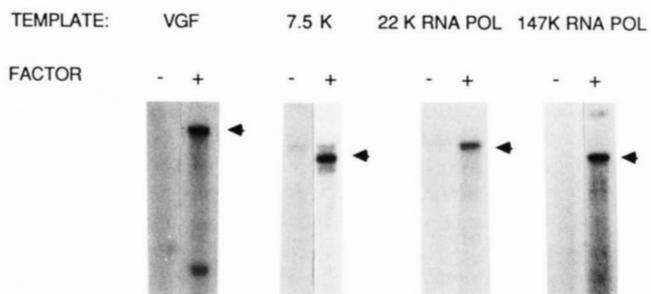


**FIG. 6. Polypeptide composition of the DNA affinity column fractions.** Thirty-μl samples of the indicated fractions from the DNA affinity column in Fig. 5 were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel (31). Polypeptides were detected by silver staining. Mobilities of marker polypeptides are indicated in kDa at right.

**TABLE I**  
Purification of vaccinia transcription factor

Fraction	Units	Protein mg	Specific activity units/mg
DEAE I	NA <sup>a</sup>	55.35	NA <sup>a</sup>
DEAE II	39,000	27.3	1,429
DNA-Cellulose	26,664	3.3	8,080
Phosphocellulose	4,785	0.344	13,910
DNA affinity	3,997	0.035	114,200

<sup>a</sup> Not applicable (see text).



**FIG. 7. Requirement for vaccinia transcription factor for *in vitro* transcription of early genes.** DNA templates containing the promoters of VGF (as well as gene X), 7.5-kDa protein, 22-kDa RNA polymerase, and 147-kDa RNA polymerase genes were transcribed by DNA-cellulose-purified vaccinia RNA polymerase in the absence (−) or presence (+) of the transcription factor. Transcript lengths are: 1000 (and 235), 700, 870, and 520 nucleotides, respectively.

of the autoradiographs indicated that the level of synthesis of the correct length run-off transcripts increased 35-, 20-, 18-, and 24-fold for the VGF, 7.5-kDa protein and 22-kDa RNA polymerase, and 147-kDa RNA polymerase gene promoters, respectively. The factor forms a stable complex as judged by electrophoretic mobility shift analysis, with each of these promoters (data not shown). The fact that the factor stimulated transcription from four different promoters suggests that the factor may function to promote transcription from all vaccinia early promoters.

It is noteworthy that the 7.5-kDa gene template encodes a late promoter immediately upstream of the early one (16). Synthesis of the predicted 780-nucleotide transcript originating from the late promoter was not observed, either in the absence or the presence of VETF. This result suggests that the vaccinia virion-derived transcription components are specific for early promoters, in agreement with previous studies on transcription using crude virion extracts (14).

#### DISCUSSION

The development of a soluble transcription system from vaccinia virions has opened the way for identification and characterization of the component enzymes and factors. We previously reported that glycerol gradient-purified RNA polymerase is deficient in terminating transcription and that capping enzyme itself or a factor copurifying with it, restored termination activity (34). In this report we describe the isolation of a form of RNA polymerase that transcribed vaccinia virus early gene templates at a greatly reduced rate. Fractions from a DEAE-cellulose column were found to restore activity and increase transcription 20–30-fold forming the basis for a reconstitution assay. The transcription factor eluted as a single peak from columns of single-stranded DNA-cellulose and phosphocellulose. The final step in the purification, chromatography on an affinity column containing tandemly linked synthetic oligonucleotides that have the sequence of an early gene promoter, separated the factor from previously identified vaccinia enzymes. Only two major polypeptides, with  $M_r$  77,000 and 82,000, coeluted with the transcription factor. The sedimentation rate of the native protein, as well as the continued association of both polypeptides with transcription activity (37), suggests that the factor is a heterodimer. The presence of a DNA-dependent ATPase activity associated with the purified transcription factor is an important finding that will be discussed further in the accompanying paper (37). Several lines of evidence indicate that this ATPase is distinct from previously described vaccinia nucleoside triphosphate phosphohydrolases.

Gel retardation studies had previously indicated the presence of a protein, in crude extracts of vaccinia virions, that bound specifically to early gene promoters. DNase I protection experiments with two early promoters demonstrated that the protein was associated with sequences between 14 and 30 nucleotides preceding the start site of transcription (35). We found that this promoter-binding protein copurified with the transcription factor. Furthermore, the same sequences were protected from DNase I digestion by the purified factor.<sup>2</sup> Analysis of several early genes indicated that only 30 nucleotides upstream of the start site of transcription are required for full promoter activity (16, 17) and that mutations within the factor binding region have drastic effects on transcription.<sup>3</sup> The correlation of functional promoter sequences and the factor-binding site argues that the interaction of the transcription factor with early promoters is an important event in the initiation of vaccinia transcription. Since the transcription factor can bind to promoter sequences in the absence of RNA polymerase, it might directly or indirectly facilitate attachment of the latter. An alternative possibility is that the transcription factor forms a complex with RNA polymerase prior to promoter binding. While we have no direct evidence for this, interactions of transcription and termination factors with RNA polymerase could explain the various forms obtained by glycerol gradient centrifugation (18, 34) and DEAE- and DNA-cellulose chromatography.

The factor stimulated transcription of four early gene templates tested, suggesting that the protein may be required for expression of all or most early genes. Furthermore, inspection of the sequences upstream of other known early transcription initiation sites reveals similarities to the factor-binding sequences within the VGF and 7.5-kDa promoters.<sup>4</sup> In accordance with the demonstrated role of the factor in transcription of early vaccinia virus genes, we have given it the acronym VETF.

At this time we do not know if vaccinia virus encodes the 77- and 82-kDa polypeptides associated with the transcription factor. However, the viral genome has been shown to encode the vaccinia RNA polymerase subunits (9), the DNA-dependent ATPase nucleoside triphosphate phosphohydrolase (39, 40), at least 1 subunit of the mRNA-capping enzyme (41), and the DNA topoisomerase (42), all of which are enzymes found within viral cores. Therefore, we anticipate that vaccinia virus also encodes the genes for the transcription factor.

Among the known eukaryotic transcription factors which interact with DNA sequences near the site of initiation of mRNA synthesis, three interact with a plethora of RNA polymerase II promoters: the TATA-binding factor (also known as factor TFIID, 2, 3), the CCAAT-binding factor (4), and the factor SP1 (38). Our current understanding of VETF suggests a similarity to the TATA-binding factor, which recognizes a sequence element with the consensus TATAAA or ATAAA centered at position −25 to −30 nucleotides preceding the RNA start site (2). Detailed comparisons of the proteins must, however, await further purification of the TATA-binding factor. The TATA sequence element regulates both the level and site of initiation of mRNA synthesis, presumably through interaction with the TATA-binding factor. The vaccinia transcription factor-binding sequence resembles the TATA sequence element in both its distance upstream of the start site and the A-T-rich nucleotide composition of its binding domain. The consistent location of the binding sequences of the two examined early promoters sug-

<sup>2</sup> S. S. Broyles, unpublished observations.

<sup>3</sup> A. Davison, unpublished observations.

<sup>4</sup> A. Davison, personal communication.

gests that the vaccinia factor will prove to be important in the determination of initiation site selection as well as influencing the amount of RNA produced.

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