JOURNAL OF VIROLOGY, Dec. 1993, p. 7264–7270
0022-538X/93/127264-07$02.00/0

Vol. 67, No. 12

Purification of the Late Transcription System of Vaccinia Virus: Identification of a Novel Transcription Factor

CYNTHIA F. WRIGHT* AND ASIMINA M. CORONEOS

Department of Cellular Pathology, Armed Forces Institute of Pathology, Washington, D.C. 20306-6000

Received 23 April 1993/Accepted 12 September 1993

We have resolved the in vitro late transcription system of vaccinia virus into four components consisting of RNA polymerase and three accessory factors. One of these additional factors is a 30-kDa protein which was previously shown to be required for late transcription in vitro and was indirectly shown to be the product of the G8R open reading frame. Another factor, of 17 kDa, was previously identified as a possible late transcription factor by an assay which demonstrated that the gene encoding it, A1L, was required for late gene expression in vivo. The G8R and A1L open reading frames have now been cloned into a baculovirus expression system, and the corresponding proteins have been purified. Both are necessary for late transcription in vitro, confirming that these intermediate genes encode late transcription factors. The third factor has a sedimentation coefficient consistent with a protein of 32 to 38 kDa. Experimental results suggest that this is a previously unidentified factor encoded by a vaccinia virus early gene. The RNA polymerase functioning in this system was purified from vaccinia virus-infected cells; however, it can be complemented by the RNA polymerase which is packaged in virions. The three smaller proteins and RNA polymerase are all necessary, and together are sufficient, for the synthesis of late viral mRNA in vitro.

Vaccinia virus provides an accessible system with which to study transcriptional regulation. Like other poxviruses, vaccinia virus replicates in the cytoplasm of infected cells and consequently must encode, in its 192,000-bp DNA genome, the enzymes needed for viral transcription and DNA replication. Vaccinia virus genes can be divided into three classes, early, intermediate, and late, on the basis of their expression relative to the synthesis of viral DNA. The machinery needed to synthesize early viral mRNA is encapsidated within the infectious viral particle so that transcription can begin immediately upon entry into a cell. Subsequent viral gene expression is controlled in a temporal manner: early gene products are required for DNA replication, whereas intermediate and late classes of genes are expressed only after DNA replication begins (for reviews, see references 7 and 8).

An extract made from HeLa cells infected with vaccinia virus and harvested late in infection can transcribe late genes (12, 18). This extract has previously been fractionated by chromatography on phosphocellulose into three crude components eluting at 0.1, 0.3, and 1.0 M NaCl (19). The 0.1 and 0.3 M components alone were capable of specific transcription, which could be stimulated somewhat by the addition of the 1.0 M fraction. Chromatography and glycerol gradient sedimentation of the phosphocellulose 0.1 M fraction resulted in the partial purification of an approximately 30-kDa factor, designated VLTF-1 (for vaccinia virus late transcription factor), that was necessary for late gene transcription in vitro. Subsequently, it was shown that three vaccinia virus intermediate genes, A1L, A2L, and G8R, encoding proteins of 17, 26, and 30 kDa, respectively, are necessary for late gene expression in vivo (6). These observations were partially reconciled in a series of experiments which indirectly demonstrated that VLTF-1 is the 30-kDa protein encoded by the G8R gene (17).

In this study, we further fractionated the phosphocellulose 0.3 M fraction and found that it is composed of two activities. One of these activities copurifies with RNA polymerase, and the other appears to be a novel transcription factor that we provisionally designate VLTF-2. We also show that the 17-kDa protein first identified by Keck et al. (6) is necessary for late transcription in vitro. These experiments lead to the description of a highly purified late gene-specific transcription system that depends on the presence of four components.

* Corresponding author.

MATERIALS AND METHODS

Assays. Protein concentrations were measured by the method of Bradford (2), with bovine serum albumin as the standard.

Nonspecific RNA polymerase assays were performed by using M13 DNA as the single-stranded template as described previously (19) except that reaction mixtures were incubated for 30 min.

Specific transcription assays were performed essentially as described previously (19) by incubating protein fractions with a plasmid containing a late promoter fused to 400 bp of DNA lacking G residues in the noncoding strand (11) (G-less cassette). With the exception of the experiments in Fig. 6, the template was supercoiled DNA isolated from *Escherichia coli* JM101 cells. In Fig. 6, plasmid DNA linearized with the restriction endonuclease *Sma*I was also used as the template. Reactions were carried out for 30 min at 30°C in a 50-μl total volume in a mixture of 40 to 50 mM NaCl, 2 mM dithiothreitol, 50 mM Tris-HCl (pH 8.0), 4 to 5% glycerol, 0.2 mM EDTA, 9% polyvinyl alcohol, 2 mM $MgCl_2$, 1 mM ATP, 0.1 mM CTP, 0.02 mM UTP, 5 μCi of [α-^{32}P]UTP, and 1 μg of DNA template.

Preparation of recombinant baculoviruses. The coding regions for the 30- and 17-kDa proteins were both cloned from purified vaccinia virus DNA by amplifying the appropriate areas of the genome through polymerase chain reaction. Sequences of the primers used to amplify the 30-kDa protein-coding region were 5′-TTTCATATGAGCATCCGTAT-3′ and 5′-CATTGGATCCATTAATCTAAAAACGCC-3′. Sequences of the primers used to amplify the 17-kDa protein-coding region were 5′-GTTCCGTAATCATATGGCTA

AGC-3′ and 5′-CTATTTAGGATCCAAAAAGC-3′. The polymerase chain reaction products were filled in with the Klenow fragment of *E. coli* DNA polymerase I and ligated to the baculovirus vector pBluBac2 (Invitrogen Corp., San Diego, Calif.), which had been digested with *Nhe*I and made blunt-ended by using the Klenow enzyme. Plasmids were screened by restriction enzyme and sequence analysis, and selected clones were transfected into *Spodoptera frugiperda* Sf9 cells along with linear *Autographa californica* nuclear polyhedrosis virus DNA. Screening of recombinant baculoviruses and amplification of viral stocks were done as described in the Invitrogen MaxBac manual (4).

Purification of the 17- and 30-kDa proteins. Sf9 cells were grown to a density of 2×10^6 cells per ml and infected with recombinant baculoviruses at a multiplicity of infection of 2. Infected cells were harvested after 48 h, washed with medium lacking serum, and resuspended in buffer A (0.01 M Tris-HCl [pH 8.0], 0.001 M EDTA, 0.005 M dithiothreitol, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml) at a ratio of 4 ml of buffer per ml of packed cell pellet. Cells were incubated on ice for 20 min and then broken open by several strokes of a Dounce homogenizer. All manipulations after this step were performed at 0 to 4°C. The lysate was centrifuged at 2,000 rpm for 5 min in 50-ml conical tubes in a Jouan refrigerated tabletop centrifuge. The supernatant was adjusted to 0.1 M NaCl–0.01% Nonidet P-40–10% glycerol and either stored at −70°C or used for chromatography. Prior to chromatography, extracts were centrifuged at 9,000 rpm for 10 min in a Sorvall SS34 rotor to pellet any particulate material. For the 17-kDa protein purification, the extract from 1 liter of Sf9 cells (47 ml at 8 mg of protein per ml) was applied to a 40-ml phosphocellulose column (Whatman P11; 1.5 by 23 cm) equilibrated in buffer B (0.1 M NaCl, 50 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, 2 mM dithiothreitol). The flowthrough protein was collected (50 ml at 6 mg of protein per ml) and applied to a 20-ml DEAE-cellulose column (Whatman DE-52; 1.5 by 11 cm) equilibrated in buffer B. The flowthrough protein was again collected (48 ml at 4 mg of protein per ml) and applied to a 12-ml reactive red 120-agarose column (1.5 by 7.5 cm; Sigma). The column was washed with 10 ml of buffer B and developed with a 100-ml 0.1 to 1.0 M NaCl gradient in buffer B. Fractions eluting between 0.15 and 0.36 M NaCl (10 ml at 1.8 mg of protein per ml) were pooled and dialyzed against buffer B. Aliquots of 200 μl were applied to 4.8-ml 15 to 35% glycerol gradients in buffer B containing 0.2 M NaCl. The gradients were centrifuged in an SW 55Ti rotor at 55,000 rpm for 16 h, and fractions were collected from the tube bottoms. Fractions containing the 17-kDa protein as assayed by specific transcription complementation and as visualized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were pooled.

For purification of the 30-kDa protein, the extract from 1 liter of Sf9 cells (30 ml at 6 mg of protein per ml) was applied to a 26-ml DEAE-cellulose column (1.5 by 15 cm) equilibrated in buffer B. The flowthrough protein was collected (47 ml at 1 mg of protein per ml) and applied to a 5-ml Affi-Gel Blue column (100/200 mesh; 1.2 by 4 cm; Bio-Rad) equilibrated in buffer B. The column was washed with 5 ml of buffer B and developed with a 50-ml 0.1 to 1.0 M NaCl gradient. Fractions eluting between 0.26 and 0.55 M NaCl (12 ml at 0.73 mg of protein per ml) were pooled (they were determined to contain the 30-kDa protein by Western immunoblot analysis) and dialyzed against buffer B for 2 h, and 11 ml was applied to a 2-ml hydroxylapatite column (Bio-Gel HT; 0.8 by 4 cm; Bio-Rad) equilibrated in buffer C (10% glycerol, 2 mM dithiothreitol, 0.01% Nonidet P-40, 10 mM sodium phosphate [pH 6.8]). The column was washed with 4 ml of buffer C and developed with a 30-ml 0.01 to 0.2 M phosphate gradient in buffer C. Proteins eluting between 0.05 and 0.065 M phosphate were pooled (5 ml at 0.14 mg of protein per ml) and dialyzed against buffer B for 2 h. Aliquots (200 μl) of the pooled hydroxylapatite material were run on 4.8-ml 15 to 30% glycerol gradients in buffer B containing 0.2 M NaCl. The gradients were centrifuged in a Beckman SW 55Ti rotor at 55,000 rpm for 19 h. Fractions containing the 30-kDa protein as assayed by specific transcription complementation and as visualized by SDS-PAGE were pooled.

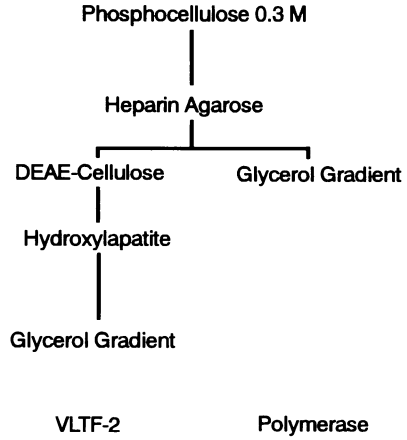

FIG. 1. Schematic representation of the separation of the phosphocellulose 0.3 M fraction into two components. See Materials and Methods for details.

Purification of the phosphocellulose 0.3 M fraction. Growth and infection of HeLa S-3 cells and preparation of transcription extracts were performed as previously described (19). Two separate preparations of infected cell extracts were made for the experiments reported here. The first was prepared from 11 liters of infected cells (prep 1), and the second was prepared from 22 liters of infected cells (prep 2). The chromatographic steps for both preparations were exactly the same, and the described enzymatic activities eluted reproducibly (Fig. 1). The crude extract from vaccinia virus-infected cells (27 ml at 10 mg of protein per ml [prep 1] or 70 ml at 10 mg of protein per ml [prep 2]) was applied to a phosphocellulose column (27 ml, 1.5 by 15 cm [prep 1] or 70 ml, 1.5 by 40 cm [prep 2]) in buffer B, and the column was washed with at least 3 bed volumes of buffer B. Proteins were then step eluted by washing the columns with 2 bed volumes of buffer B containing 0.3 M NaCl. The columns were washed a final time with approximately 1.5 bed volumes of buffer B containing 1.0 M NaCl. The proteins eluting at 0.3 M NaCl were dialyzed against buffer B for 2.5 h and applied to a heparin-agarose column (3 ml, 1.2 by 2.6 cm [prep 1], or 6 ml, 1.2 by 5.3 cm [prep 2]; GIBCO BRL). Columns were washed with at least 1 bed volume of buffer B and then developed with a 10-bed-volume 0.1 to 1.0 M NaCl gradient in buffer B. Fractions were collected and assayed for nonspecific RNA polymerase activity and also in specific transcription complementation assays. Proteins eluting from the heparin-agarose column between 0.17 and 0.22 M NaCl were pooled (4.5 ml at 2 mg of protein per ml [prep 1] or 5.5 ml at 1 mg of protein per ml [prep 2]), dialyzed against buffer B containing 0.05 M NaCl, and applied to a DEAE-cellulose column (1.2 ml, 0.8 by 2.4 cm [prep 1] or 1.5 ml, 0.8 by 3 cm [prep 2]) equilibrated in buffer B containing 0.05 M NaCl.

Selected fractions were assayed for specific transcription complementation, and active fractions (present in the flowthrough) were pooled. Aliquots of this material were concentrated three- to fivefold by ultrafiltration and layered over 4.8-ml 15 to 35% glycerol gradients in buffer B containing 0.2 M NaCl. Gradients were centrifuged in a SW 55Ti rotor at 55,000 rpm for 15 to 19 h. Cytochrome *c*, catalase, and bovine serum albumin sedimentation markers were centrifuged in parallel glycerol gradients. This material is referred to as glycerol gradient pure in figure legends. An additional aliquot of the DEAE flowthrough material was further purified over a hydroxylapatite column prior to glycerol gradient sedimentation as follows. Five milliliters of the DEAE flowthrough combined from preps 1 and 2 (at 0.32 mg of protein per ml) was applied to a 1.6-ml hydroxylapatite column (0.8 by 3.5 cm) equilibrated with buffer C. The column was washed with 3 ml of buffer C and developed with a 24-ml 0.01 to 0.2 M phosphate gradient in buffer C. Fractions were collected and assayed in specific transcription complementation assays. Proteins eluting between 0.13 and 0.16 M phosphate were pooled and dialyzed against buffer B for 2 h. Aliquots of this material were run on glycerol gradients as described above. These fractions were assayed for transcription complementation and run on SDS-polyacrylamide gels as shown in Fig. 4.

Proteins eluting from the heparin-agarose column between 0.4 and 0.56 M NaCl (the nonspecific RNA polymerase peak) were also pooled, dialyzed against buffer B, concentrated two- to threefold, and applied to 4.8-ml 15 to 35% glycerol gradients in buffer B containing 0.2 M NaCl. Gradients were centrifuged in an SW 55Ti rotor at 55,000 rpm for 5 or 8 h. Fractions were tested for nonspecific RNA polymerase activity, and active fractions were pooled as the source of RNA polymerase for reconstitution experiments.

Preparation of virion extracts. A soluble extract capable of early gene transcription was prepared from 18 mg of sucrose gradient-purified vaccinia virus as described by Rohrmann and Moss (10). Essentially, this method involves disrupting core particles with deoxycholate, NaCl, and dithiothreitol and passing the extract through a DEAE-cellulose column to remove nucleic acids. This extract was determined to be active for early transcription by supplying it with an early promoter/G-less cassette template (pSB24; a gift of S. Broyles, Purdue University) under the transcription conditions used for the late transcription assays.

Preparation of hydroxyurea-treated extracts. Vaccinia virus-infected HeLa cells blocked for DNA replication were prepared essentially as described by Vos et al. (15) except that cells were diluted 1 h postinfection with medium containing 20 mM hydroxyurea and harvested 6 h after infection. A soluble transcription extract was prepared from these cells as previously described (19).

RESULTS

Purification of the factors encoded by intermediate genes. Previous experiments had shown that the 30-kDa protein was essential for late transcription in vitro. Antibodies raised against the 17- and 26-kDa proteins demonstrated that they were also present predominantly in the phosphocellulose 0.1 M fraction and that they could be resolved from the 30-kDa protein by chromatography on Affi-Gel Blue and hydroxylapatite, respectively (5a). Adding back fractions enriched in the 17-kDa protein to the standard in vitro complementation assay (18) stimulated transcription; however, the stimulatory effect of adding back fractions enriched for the 26-kDa protein was much less pronounced (data not shown). Since both the 30- and 17-kDa proteins could be seen to stimulate transcription in vitro, and since the genes encoding these proteins were thought to be known, we expressed these gene products in a baculovirus system. Both proteins were soluble and active when expressed in this system, allowing them to be purified to near homogeneity (Fig. 2) and, importantly, providing preparations free from contaminating vaccinia virus proteins. Preparations of these proteins purified from baculovirus-infected cells added simultaneously to the in vitro transcription system could substitute for the phosphocellulose 0.1 M fraction purified from vaccinia virus-infected cells. Thus, these two proteins together appeared to constitute the active factors in this material. The ability of the proteins expressed in the baculovirus system to substitute for the factors purified from vaccinia virus-infected cells demonstrates directly that the A1L and G8R genes do indeed encode transcription factors and shows that these factors do not need to be modified by another vaccinia virus protein in order to be active.

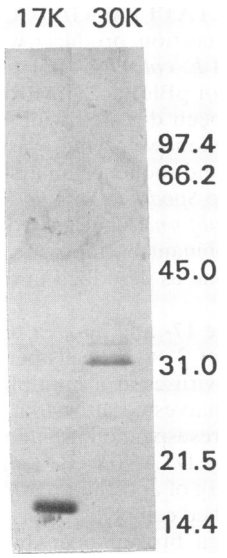

FIG. 2. Silver-stained SDS-polyacrylamide gel of the 17- and 30-kDa transcription factors purified from the baculovirus expression system. The factors loaded are designated above the lanes. Two microliters of the 17-kDa protein (glycerol gradient pure) and 10 μl of the 30-kDa protein (glycerol gradient pure) were loaded. The sizes of molecular weight markers (in kilodaltons) are indicated at the right.

Purification of the phosphocellulose 0.3 M fraction. It has previously been shown (19) that the phosphocellulose 0.3 M fraction is complex and contains the following activities: (i) nonspecific RNA polymerase activity as assayed with a single-stranded M13 DNA template and manganese as the divalent cation, (ii) early gene-specific transcriptase activity as assayed with a double-stranded DNA template containing an early gene promoter, and (iii) at least two factors needed for late gene transcriptase activity. One of these late factors cosedimented with the nonspecific RNA polymerase activity on a glycerol gradient, and the other cosedimented with a component that had an estimated molecular size of 32 kDa. Thus, these initial experiments using very crude components suggested that the phosphocellulose 0.3 M fraction contained, at a minimum, RNA polymerase, the early gene-specific transcription factor VETF, and one or more late gene-specific transcription factors.

We began purification of the phosphocellulose 0.3 M fraction by passing this material over a heparin-agarose column

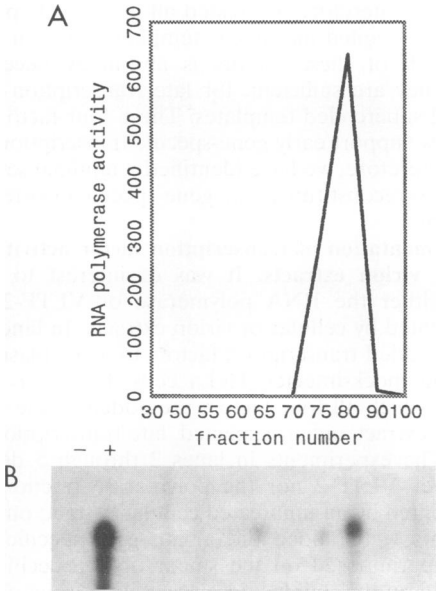

FIG. 3. Heparin-agarose chromatography of the phosphocellulose 0.3 M fraction. The phosphocellulose 0.3 M fraction was applied to a heparin-agarose column and eluted with a salt gradient. (A) Profile of fractions assayed for nonspecific RNA polymerase activity. Units are picomoles of UMP incorporated per 5 μl of column fraction. Fraction 80 contained a level of nonspecific RNA polymerase activity which was approximately 500-fold more than the activity of fraction 65. Because of the scale of the figure and this magnitude of difference between the peak and nonpeak fractions, the activity of the fractions surrounding the peak does not appear above the baseline of the figure. (B) Autoradiogram of the specific transcription reconstitution experiment. Column fractions (8 μl) were added to 5 μl of the 30-kDa protein (purified from vaccinia virus-infected HeLa cells over phosphocellulose, DEAE-cellulose, Affi-Gel Blue, hydroxylapaptite, and a glycerol gradient as described in reference 19), 5 μl of the 17-kDa protein (the flowthrough from the Affi-Gel Blue column used to purify the 30-kDa protein), and 2 μl of a phosphocellulose 1.0 M fraction in 50-μl transcription reactions. The phosphocellulose 1.0 M fraction was included, as it could stimulate transcription activity of these crude fractions severalfold. A negative control assay was performed without an added heparin-agarose column fraction (–), and a positive control assay was performed by adding 8 μl of phosphocellulose 0.3 M material (+). Fraction 65 contained 170 mM NaCl, and fraction 80 contained 400 mM NaCl.

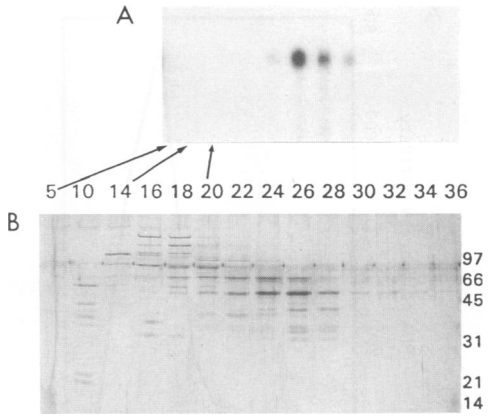

FIG. 4. Further purification of the factor eluting at 170 mM NaCl on heparin-agarose. The factor eluting at 170 mM NaCl on heparin-agarose was passed over DEAE-cellulose and hydroxylapatite columns and then applied to a glycerol gradient. (A) Autoradiogram of specific transcription reactions. Designated fractions (5 μl; labeled between the gels of panels A and B) from the glycerol gradient were added to 3 μl of glycerol gradient-pure RNA polymerase, 3 μl of 17-kDa protein purified from recombinant baculovirus-infected Sf9 cells (glycerol gradient fraction), and 5 μl of 30-kDa protein purified from recombinant baculovirus-infected Sf9 cells (hydroxylapatite fraction) in 50-μl transcription reactions. (B) Silver-stained SDS-polyacrylamide gel of fractions from the glycerol gradient. The sizes of molecular weight markers (in kilodalton) are indicated at the right.

and eluting it with a salt gradient (Fig. 1). Fractions from the column were tested both for RNA polymerase activity and for ability to complement late gene-specific transcription in vitro (Fig. 3). The RNA polymerase activity eluted from the column in a single peak at 400 mM NaCl. Specific transcription activity eluted in two peaks at 170 mM NaCl (fraction 65) and 400 mM NaCl (fraction 80). Neither of these peaks was as active as the original starting material; however, adding the two peak fractions together markedly stimulated transcription (data not shown), suggesting that both were needed for late transcription. The material eluting at 170 mM NaCl, which had a small but detectable level of nonspecific RNA polymerase activity, was then chromatographed further on a DEAE-cellulose column, with the result that the specific transcription activity eluted in the flowthrough of the column in 50 mM NaCl. Aliquots of the DEAE flowthrough material were either applied directly to glycerol gradients or chromatographed over hydroxylapatite prior to glycerol gradient sedimentation. After further purification, this material had no detectable nonspecific RNA polymerase activity and no longer worked without added RNA polymerase. Therefore, RNA polymerase, purified as described below, was also added to these reactions. Figure 4 shows transcription assays and a silver-stained protein gel of fractions from material applied successively to hydroxylapatite and a glycerol gradient. A protein of approximately 30 kDa copurifies most precisely with the transcription activity. There is a prominent protein of approximately 53 kDa, as well as other less prominent bands, also present in the active fractions; however, the presence of these proteins does not correlate as well with transcription activity. The sedimentation coefficient of this factor as determined from glycerol gradients is consistent with a protein with a molecular size in the range of 32 to 38 kDa. Thus, the 30-kDa protein seen on these gels is a strong candidate for the activity that we will provisionally designate VLTF-2.

The material eluting from the heparin-agarose column at 400 mM NaCl, which contained the peak of RNA polymerase activity, was also further purified by applying it to a glycerol gradient. Fractions were again tested for ability to complement late gene-specific transcription (in reactions that contained partially purified VLTF-2) and for nonspecific RNA polymerase activity. As can be seen in Fig. 5, the peak of RNA polymerase coincided precisely with the peak of specific transcription complementation, strongly suggesting that it is RNA polymerase which is providing the needed activity in this material. The multisubunit RNA polymerase is a major component of the active fractions, as judged by SDS-PAGE and subsequent silver staining (data not shown).

Reconstitution of late gene-specific transcription with highly purified factors. The experiment in Fig. 6 shows the activities of all of the highly purified factors tested together on both supercoiled and linear templates containing the same vaccinia virus late promoter. Recent evidence suggests that the topology of the DNA template can affect the spectrum of

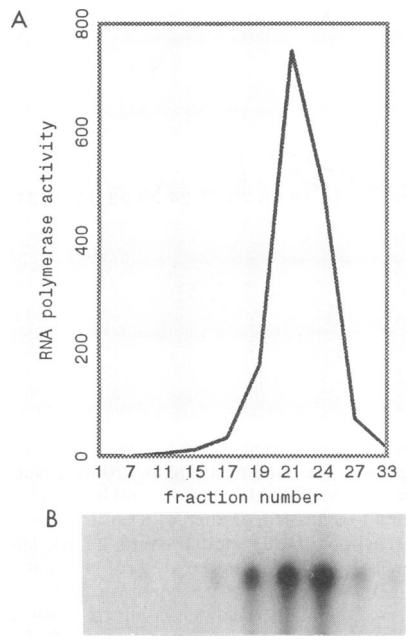

FIG. 5. Glycerol gradient sedimentation of RNA polymerase. The material eluting from the heparin-agarose column at 400 mM NaCl, which contained the peak of RNA polymerase activity, was further purified by applying it to a glycerol gradient. (A) Profile of fractions assayed for nonspecific RNA polymerase activity as in Fig. 2. (B) Autoradiogram of specific transcription reactions. Designated fractions (5 μl) were added to 5 μl of the 17- and 30-kDa proteins and 2 μl of the phosphocellulose 1.0 M fraction, all as in Fig. 3, and 5 μl of VLTF-2 (DEAE-cellulose fraction).

factors necessary to reconstitute specific transcription in vitro. Specifically, in vitro transcription of a supercoiled template containing the immunoglobulin heavy-chain promoter could be reconstituted by using fewer factors than was necessary for a linear template containing the same promoter (9). Thus far, we had used only supercoiled templates to purify the transcription factors. Therefore, we tested all four highly pure factors on both supercoiled and linear templates. As can be seen in Fig. 6, each of these factors is absolutely necessary, and together they are sufficient, for late transcription from both linear and supercoiled templates. These four factors together do not now support early gene-specific transcription (data not shown). Therefore, we have identified a minimal set of factors necessary to reconstitute a late gene-specific in vitro transcription system.

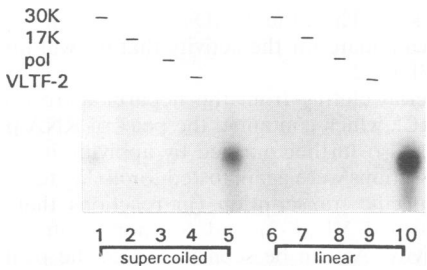

FIG. 6. Transcription reactions using highly pure transcription factors. In lanes 1 to 5, the template was supercoiled plasmid isolated from *E. coli* JM101 cells; in lanes 6 to 10, this template was digested with *Sma*I, which cleaves the plasmid in a unique site immediately after the G-less cassette sequence. In lanes 1 to 4 and 6 to 9, one of the transcription factors was omitted (designated with a dash on the same line as the factor designation); in lanes 5 and 10, all of the factors were added. Transcription reactions were performed with 7 μl of the 30-kDa protein purified from recombinant baculovirus-infected Sf9 cells (glycerol gradient fraction), 3 μl of the 17-kDa protein purified from recombinant baculovirus-infected Sf9 cells (glycerol gradient fraction), 5 μl of glycerol gradient-pure VLTF-2, and 3 μl of glycerol gradient-pure RNA polymerase.

Complementation of transcription factor activity with cellular and virion extracts. It was of interest to determine whether either the RNA polymerase or VLTF-2 could be complemented by cellular or virion extracts. In lanes 2 to 6 of Fig. 7, we added transcription factors to a cytoplasmic extract made from mock-infected HeLa cells. Lane 1 is a positive control in which all four factors are added in the absence of additional extract and, as expected, late transcription is reconstituted. The experiments in lanes 2 through 5 demonstrate that neither VLTF-2 nor the polymerase fraction could be complemented by an uninfected cellular extract; only when all four factors were added did a late gene-specific transcript appear superimposed on the smear of nonspecific products contributed by the uninfected extract alone (lane 6). Lanes 7 through 14 show the same type of experiment in which factors were added to an early gene-specific transcription extract made from purified vaccinia virions. The amount of the virion extract added to the transcription reactions had approximately the same amount of nonspecific RNA polymerase activity as did the added amount of the RNA polymerase purified from infected cells. The virion extract did not function alone to transcribe late genes (lane 7); however, in contrast to the experiments with the cellular extract, the system did function well when just the 17-kDa, 30-kDa, and VLTF-2 fractions were added to it (compare lanes 5 and 14). The three small factors do not function alone to transcribe late genes (Fig. 6, lanes 3 and 8). Therefore, the RNA polymerase packaged in the virion can substitute for the polymerase purified from infected cells. This experiment also demonstrates that VLTF-2 is not present in substantial amounts in the virion extract because all three factors must be added to the virion extract for an appreciable amount of transcription to occur. In lanes 15 to 20 of Fig. 7, we added transcription factors to an extract made from vaccinia virus-infected cells blocked for viral DNA replication by performing the infection in the presence of the drug hydroxyurea. This creates an extract which contains viral early proteins but not intermediate or late proteins. This extract also did not function alone to transcribe late genes (lane 15), nor did it function when any one of the late transcription factors was added to it (lanes 16 to 19). However, late transcription was reconstituted when just the 17- and 30-kDa factors were added to it, demonstrating that both the virion polymerase and VLTF-2 are present in this blocked extract. Taken together, these experiments suggest that VLTF-2 is the product of a viral early gene.

DISCUSSION

Vaccinia virus gene expression is controlled in a highly regulated fashion once the virus infects a cell; early genes are made prior to viral DNA replication, intermediate genes are turned on once DNA replication begins but do not require concomitant protein synthesis (16), and late genes require both DNA replication and ongoing protein synthesis. These observations have suggested a cascade mechanism of gene regulation in vaccinia virus in which proteins required for entering

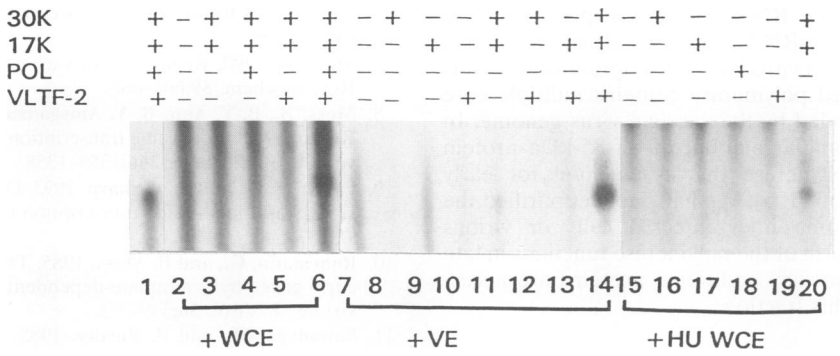

FIG. 7. Autoradiogram of transcription reactions performed to test complementation ability of cellular and virion extracts. The reaction in lane 1 contained 5 μl of the 30-kDa protein purified from recombinant baculovirus-infected Sf9 cells (hydroxylapatite fraction), 3 μl of the 17-kDa protein purified from recombinant baculovirus-infected Sf9 cells (glycerol gradient fraction), 3 μl of glycerol gradient-pure RNA polymerase (POL), and 5 μl of VLTF-2 (DEAE-cellulose fraction). The reactions of lanes 2 to 6 all contained 4 μl of mock-infected whole cytoplasmic extract (WCE) prepared from HeLa cells. In addition, these lanes contain the indicated transcription factors (+) in the amounts and purity as for lane 1. In lanes 7 to 14, 5 μl of an early gene-specific virion extract (VE) was added to every reaction. The indicated fractions also contained 2 μl of the 17-kDa protein and/or 8 μl of the 30-kDa protein, both purified from the baculovirus system (glycerol gradient fractions for both), and/or 5 μl of glycerol gradient-pure VLTF-2. In lanes 15 to 20, 5 μl of a cell extract prepared from cells infected with vaccinia virus in the presence of hydroxyurea (HU-WCE) was added to every reaction. The indicated reactions also contained 3 μl of the 17-kDa protein, 5 μl of the 30-kDa protein, or 3 μl of RNA polymerase, as in the reaction of lane 1, or 5 μl of VLTF-2 (hydroxylapatite fraction).

any one stage of the viral life cycle are synthesized in the previous stage.

Previous fractionation of a crude extract from vaccinia virus-infected cells showed that the phosphocellulose 0.1 and 0.3 M fractions were necessary for late transcription in vitro. The phosphocellulose 0.1 M fraction was further purified and shown to consist partially of an approximately 30-kDa protein. A subsequent assay, which could identify only genes of the intermediate class, identified three genes encoding proteins of 17, 30, and 26 kDa that are needed for late gene expression in vivo. Experiments using antibodies demonstrated indirectly that the 30-kDa protein identified by both assays was the same. The exact function of the 17- and 26-kDa proteins was not determined, and the other fractions necessary for late transcription were not further purified. Preliminary experiments done for this report demonstrated that infected cell fractions enriched in the 17-kDa protein could also stimulate late transcription in vitro (data not shown). Therefore, the genes encoding the 17- and 30-kDa proteins were cloned into a baculovirus expression system, and the corresponding proteins were purified. Both proteins purified in this manner were absolutely necessary for late transcription in vitro and added together could substitute for the phosphocellulose 0.1 M fraction purified from vaccinia virus-infected cells. This result demonstrates directly for the first time that these intermediate genes do indeed encode transcription factors necessary for the synthesis of late genes, thus further confirming a transcription factor cascade mechanism of gene regulation.

The in vivo experiments had the potential to identify intermediate gene products which are stage specific for late transcription; however, it was clear that other proteins, notably RNA polymerase and perhaps others, must also participate in synthesizing late gene products. Biochemical experiments had also shown that the phosphocellulose 0.3 M fraction was absolutely necessary for late transcription in vitro. Therefore, we further purified this fraction to determine exactly what activities are present. To this end, we applied the material to a heparin-agarose column and tested fractions for late gene-specific transcription complementation (by adding column fractions to the purified 17- and 30-kDa proteins) and for nonspecific RNA polymerase activity. Specific transcription activity eluted in two peaks, one of which corresponded to the nonspecific RNA polymerase peak. Both activities were further purified; one activity copurified with a novel activity that we designated VLTF-2, and the other copurified with nonspecific RNA polymerase. VLTF-2 activity was tentatively linked with a protein band of approximately 30 kDa on SDS-polyacrylamide gels. Therefore, the complete transcription system, as presented here, consists of the 17- and 30-kDa proteins, RNA polymerase, and VLTF-2. The phosphocellulose 1.0 M fraction is not added to this system because experiments have shown no, or only a very moderate, stimulation of the purified system with this material (data not shown).

Two lines of evidence suggest that VLTF-2 is not the 26-kDa protein (encoded by the A2L open reading frame) identified by the in vivo assay of Keck et al. (6). First, VLTF-2 is present in vaccinia virus-infected cells blocked for viral DNA replication by hydroxyurea. Previous studies have shown that no transcription is detectable from the A2L gene in the absence of DNA replication (6). Second, antibodies raised against the protein product of the A2L gene expressed in *E. coli* do not react at all on Western blots to which purified VLTF-2 has been bound (data not shown). Therefore, the function of the 26-kDa protein in late gene expression remains to be determined.

VLTF-2 also does not appear to be any of the other previously identified vaccinia virus transcription factors. VETF, the vaccinia virus early transcription factor, is a heterodimer of 77- and 82-kDa polypeptides which is packaged in virions (3). VLTF-2 has a sedimentation coefficient inconsistent with that of VETF, and substantial amounts of active protein do not appear to be present in viral particles. This same reasoning excludes capping enzyme, which has been shown to be necessary for termination of early transcription (13) and initiation of intermediate transcription (14). VLTF-2 also has chromatographic properties different from those of VLTF-B, a recently described activity necessary for the transcription of intermediate genes in vitro (15). Thus, VLTF-2 appears to be the product of a viral early gene active uniquely in the transcription of late genes. We cannot rule out that vaccinia virus infection induces or modifies a cellular protein, but this scenario seems unlikely given the lack of precedence for such a mechanism.

We have found that the RNA polymerase present in the virion can complement the RNA polymerase fraction purified from infected cells in the in vitro late transcription system. It is known that the packaged polymerase contains multiple core subunits which are encoded by the vaccinia virus genome. In addition, there is a submolar amount of an 85-kDa protein associated with the polymerase that is essential for early gene-specific transcription (1, 5). We have not yet purified the polymerase fraction from either infected cells or virions enough to know which form of the polymerase functions in late transcription and whether any additional proteins are necessary for the activity of this fraction.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI31220 from the National Institute of Allergy and Infectious Diseases and by intramural funds from the AFIP.

We thank Jim Keck for generously providing the anti-26-kDa antiserum and for helpful discussions, James Sneeringer for help in cloning and expressing the A1L gene in baculovirus, and Ann Reid for critical reading of the manuscript.

REFERENCES

1. **Ahn, B.-Y., and B. Moss.** 1992. RNA polymerase-associated transcription specificity factor encoded by vaccinia virus. Proc. Natl. Acad. Sci. USA **89:**3536–3540.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Broyles, S. S., L. Yuen, S. Shuman, and B. Moss.** 1988. Purification of a factor required for transcription of vaccinia virus early genes. J. Biol. Chem. **263:**10754–10760.
4. **Invitrogen Corp.** MAXBAC manual version 1.5.5. Invitrogen Corp., San Diego, Calif.
5. **Kane, E. M., and S. Shuman.** 1992. Temperature-sensitive mutations in the vaccinia virus H4 gene encoding a component of the virion RNA polymerase. J. Virol. **66:**5752–5762.

5a. **Keck, J.** Personal communication.

6. **Keck, J. C., C. J. Baldick, Jr., and B. Moss.** 1990. Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late *trans*-activator genes. Cell **61:**801–809.
7. **Moss, B.** 1990. Regulation of vaccinia virus transcription. Annu. Rev. Biochem. **59:**661–688.
8. **Moss, B., B.-Y. Ahn, B. Y. Amegadzie, P. D. Gershon, and J. G. Keck.** 1991. Cytoplasmic transcription system encoded by vaccinia virus. J. Biol. Chem. **266:**1355–1358.
9. **Parvin, J. D., and P. A. Sharp.** 1993. DNA topology and a minimal set of basal factors for transcription by RNA polymerase II. Cell **73:**533–540.
10. **Rohrmann, G., and B. Moss.** 1985. Transcription of vaccinia virus early genes by a template-dependent soluble extract of purified virions. J. Virol. **56:**349–355.
11. **Sawadogo, M., and R. Roeder.** 1985. Factors involved in specific transcription by human RNA polymerase II: analysis by a rapid and quantitative *in vitro* assay. Proc. Natl. Acad. Sci. USA **82:**4394–4398.
12. **Schwer, B., and H. G. Stunnenberg.** 1988. Vaccinia virus late transcripts generated in vitro have a poly(A) head. EMBO J. **7:**1183–1190.
13. **Shuman, S., S. S. Broyles, and B. Moss.** 1987. Purification and characterization of a transcription termination factor from vaccinia virions. J. Biol. Chem. **262:**12372–12380.
14. **Vos, J. C., M. Sasker, and H. G. Stunnenberg.** 1991. Vaccinia virus capping enzyme is a transcription initiation factor. EMBO J. **10:**2553–2558.
15. **Vos, J. C., M. Sasker, and H. G. Stunnenberg.** 1991. Promoter melting by a stage-specific vaccinia virus transcription factor is independent of the presence of RNA polymerase. Cell **65:**105–113.
16. **Vos, J. C., and H. G. Stunnenberg.** 1988. Derepression of a novel class of vaccinia virus genes upon DNA replication. EMBO J. **7:**3487–3492.
17. **Wright, C. F., J. G. Keck, M. M. Tsai, and B. Moss.** 1991. A transcription factor for expression of vaccinia virus late genes is encoded by an intermediate gene. J. Virol. **65:**3715–3720.
18. **Wright, C. F., and B. Moss.** 1987. In vitro synthesis of vaccinia virus late mRNA containing a 5′ poly(A) leader sequence. Proc. Natl. Acad. Sci. USA **84:**8883–8887.
19. **Wright, C. F., and B. Moss.** 1989. Identification of factors specific for transcription of the late class of vaccinia virus genes. J. Virol. **63:**4224–4233.