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A Transcription Factor for Expression of Vaccinia Virus Late Genes Is Encoded by an Intermediate Gene

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A factor, designated VLTF-1, that is required in vitro for specific transcription of vaccinia virus late genes was previously isolated from vaccinia virus-infected cells. Subsequent genetic experiments identified three vaccinia virus genes, encoding proteins of 17, 26, and 30 kDa, that together *trans* activate late gene expression in vivo. The purpose of this study was to determine whether VLTF-1 corresponded to one of the three *trans* activators. Toward this end, VLTF-1 was further purified, the *trans*-activator genes were expressed in *Escherichia coli*, and antisera were made to the native and recombinant proteins. Antibody to the 30-kDa recombinant protein reacted on Western immunoblots with a protein of approximately M_r 30,000 that cochromatographed and cosedimented with VLTF-1 activity from virus-infected cells. Conversely, antibody to purified VLTF-1 bound to products produced by in vitro transcription and translation of the open reading frame encoding the 30-kDa *trans*-activator protein. Both antisera depleted VLTF-1 activity and blocked late gene transcription by partially purified extracts of vaccinia virus-infected cells. Taken together, these data demonstrate that the 30-kDa *trans* activator comprises part, if not all, of VLTF-1 activity.

Vaccinia virus, a poxvirus that replicates in the cytoplasm of eukaryotic cells, provides an accessible system with which to study transcriptional regulation. Vaccinia virus genes can be divided into three classes, early, intermediate, and late (for a review, see reference 7). Early genes are expressed upon viral infection and are required for DNA replication, whereas intermediate and late genes are expressed after the onset of DNA replication. Recent studies have shown that the DNA replication requirement for the expression of intermediate genes can be overcome if these genes are transfected into vaccinia virus-infected cells (11), whereas this is not true for late genes (3). Thus, the factors necessary for transcription of intermediate genes exist in the cell prior to DNA replication, but the templates become accessible only after DNA replication begins. These findings led to the suggestion that a subset of intermediate genes act as *trans* activators of late gene transcription (11).

Recently, two approaches have been taken to study the factors necessary for vaccinia virus late gene transcription. In the first, in vitro transcription systems that can utilize late genes as templates have been developed from extracts of vaccinia virus-infected cells (8, 12). Fractionation of one of these systems revealed that RNA polymerase and three or more additional factors were required for late gene specific transcription (13). One of these required factors, designated VLTF-1 (for vaccinia virus late transcription factor), was partially purified by column chromatography.

The second approach to identifying late transcription factors was the development of an in vivo complementation assay to identify fragments of vaccinia virus DNA necessary to *trans* activate a late promoter (5). In this assay, a late promoter-controlled reporter gene was transfected into vaccinia virus-infected cells in the presence of a DNA synthesis inhibitor. The block in late promoter activation, due to the presence of the inhibitor, was overcome by cotransfecting fragments of vaccinia virus DNA potentially coding for proteins of 17, 26, and 30 kDa. These *trans*-activator genes were classified as intermediate based on the criterion that transfected copies were transcribed in the absence of viral DNA replication. Thus, this assay provided direct evidence that at least a subset of intermediate genes are involved in regulating late gene expression.

In this report, we bring together the two approaches taken toward identifying late transcription factors and demonstrate that VLTF-1 activity is comprised partially, if not wholly, by the 30-kDa *trans*-activator protein. This finding validates both approaches and supports a cascade model for gene regulation by vaccinia virus.

MATERIALS AND METHODS

Extract preparation and specific transcription reactions. Growth and infection of HeLa S-3 cells, preparation of transcription extracts, and specific transcription assays were performed as previously described (13).

Purification of VLTF-1. All steps were carried out at 4°C. Protein concentrations were measured by the method of Bradford (1), with bovine serum albumin as the standard. The dialyzed extract from 36 liters of HeLa cells (250 ml at 10 mg/ml) was applied to a 200-ml phosphocellulose column (Whatman P11; 40.5 by 2.5 cm) equilibrated in buffer A (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 (240 ml at 3.4 mg/ml) and applied to a 70-ml DEAE-cellulose column (2.5 by 15 cm; Whatman) equilibrated in buffer A. Again, the bulk of the unbound protein was pooled (240 ml at 1.6 mg/ml) and applied to a 32 ml Affi-Gel Blue column (100/200 mesh; 2.5 by 7 cm; Bio-Rad) equilibrated in buffer A. The column was washed with 50 ml of buffer A and then developed with a 500-ml 0.1 to 1.0 M NaCl gradient in buffer A. Fractions eluting between 0.22 and 0.32 M NaCl (50 ml at 0.28 mg/ml) were pooled, dialyzed against buffer A for 3 h, and applied to

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a 2-ml hydroxylapatite column (Bio-Rad Bio-Gel HT; 0.7 by 6 cm) equilibrated in buffer B (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 B and developed with a 40-ml 0.01 to 0.1 M phosphate gradient in buffer B. Fractions eluting between 0.085 and 0.1 M phosphate (11 ml at 0.32 mg/ml) were pooled and dialyzed against buffer A for 2 h. Portions of this hydroxylapatite pooled material were sedimented through glycerol gradients as follows: the material was concentrated two- to threefold by ultrafiltration, and then 0.2 ml was applied to a 3.8-ml 15 to 35% glycerol gradient in buffer A lacking Nonidet P-40. The gradients were centrifuged in a Beckman SW 60Ti rotor at 60,000 rpm for 17 h, and fractions were collected from the tube bottoms. Cytochrome *c*, bovine serum albumin, and catalase sedimentation markers were centrifuged in parallel glycerol gradients.

Expression of the GK1 ORF in *Escherichia coli*. The GK1 open reading frame (ORF) was subcloned into a modified pET-12a *E. coli* translation vector (9) by using the polymerase chain reaction to amplify the DNA and generate a 5′ *Sal*I site six nucleotides upstream of the initiation codon and a 3′ *Bam*HI site immediately after the termination codon. The polymerase chain reaction product was subcloned into the *Sal*I-*Bam*HI sites of the modified vector. This construct was used to transform *E. coli* BL21 (DE3) (10), and expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The protein product generated contained 22 amino acids of the *E. coli ompT* gene at the amino end.

Antibody preparation. To obtain antibodies against VLTF-1 purified from mammalian cells, the glycerol gradient-purified factor (equivalent to fraction 17 in Fig. 4) was emulsified in Ribi adjuvant (Ribi ImmunoChem Research, Inc.) and injected into a New Zealand White rabbit according to the manufacturer's instructions. The animal was boosted 16 days after the initial injection by the same protocol, bled 8 days later, boosted 2 weeks later, and then bled again after another 2 weeks. After clot formation, the serum was separated and stored in aliquots at −70°C. This serum did not react with VLTF-1 on Western immunoblots and thus was monitored for antibody production by immunoprecipitation and inhibition of transcription assays.

Antibodies to the GK1-OmpT fusion protein were obtained in the following manner. IPTG-induced recombinant *E. coli* was lysed in Laemmli electrophoresis sample buffer (6), and the soluble proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The protein bands were visualized by immersing the gels in ice-cold 0.25 M KCl, and the band containing the fusion protein was soaked in 50 mM Tris-HCl (pH 8.0)–0.1 mM EDTA–0.01% Nonidet P-40–1 mM dithiothreitol–10% glycerol. The eluted material was mixed with Ribi adjuvant and injected into New Zealand White rabbits. The rabbits were boosted after 2 weeks and bled after another 2 weeks. At the latter time, the rabbits received a second boost and were bled 2 weeks later. This serum was preadsorbed with both *E. coli* extracts and CV1 cell extracts; it reacted well with the GK1 protein product in Western blots but could only poorly immunoprecipitate the protein.

Western blot analysis. Proteins separated by electrophoresis on a 12.5% SDS-polyacrylamide gel were electrophoretically transferred to nitrocellulose. After transfer, the nitrocellulose filters were blocked in 5% nonfat milk solution in phosphate-buffered saline (PBS) (pH 7.6)–0.1% Tween 20. Antibodies were reacted with the filter in the same solution for 16 h, and the filter was then washed in PBS with 0.1% Tween 20. ^{125}I-labeled protein A was then added for 4 h, and the filter was washed as described above.

In vitro transcription and translation of GK1 and immunoprecipitation using anti-VLTF-1. In vitro transcription and translation of DNA containing the GK1 ORF, using [^{35}S]methionine, was performed as described in the kit purchased from Promega. Labeled protein was immunoprecipitated by using protein-A Sepharose beads, washing them three times in Triton buffer (0.3 M NaCl, 0.05 M Tris-HCl [pH 7.4], 0.1% Triton X-100) and once in Triton buffer containing 0.1% SDS and 0.1% sodium deoxycholate.

Transcription inhibition. Transcription inhibition studies were performed by incubating 50 μl of preimmune or immune serum with 200 μl of protein A-Sepharose beads (a 20% slurry in PBS) with gentle mixing at 4°C for several hours to overnight. The beads were centrifuged from solution by a brief spin in a microcentrifuge and washed three times in Triton buffer and once with Triton buffer lacking NaCl. Then 30 μl of transcription extract (unfractionated) or 30 μl of the phosphocellulose 0.1 M fraction was added to the washed beads, and the beads were incubated with gentle mixing at 4°C for 3 h. The beads were centrifuged from solution, and the supernatant was assayed in transcription reactions.

RESULTS

Expression of the GK1 ORF. Previous results, obtained by using an in vivo complementation assay, demonstrated that three *trans*-activator genes predicted to encode polypeptides of 17, 26, and 30 kDa were necessary for late gene transcription (5). The 30-kDa coding region, designated GK1, was expressed in *E. coli*, and the protein product was used to immunize rabbits. The specificity of the anti-GK1 serum was first tested by Western blot analysis of extracts from *E. coli* carrying either the expression vector alone or the same vector into which the GK1 ORF had been cloned. The antibody recognized a band of 30 kDa only in extracts prepared from bacteria containing the GK1 ORF, and this band increased in intensity upon induction with IPTG (data not shown). The anti-GK1 serum was next used in Western blot analysis of extracts from vaccinia virus-infected cells and vaccinia virions. Figure 1 shows a time course of infection and demonstrates that the antibody recognized a band of approximately 30 kDa which first appeared in the infected cell extracts at about 4.5 h and accumulated mainly between 12 and 24 h after infection. The time course is in agreement with the observed late transcription of the GK1 ORF (5). Despite the late synthesis of the protein, it is not packaged in the virion (Fig. 1).

Purification of VLTF-1. In previous experiments designed to identify late transcription factors, extracts from vaccinia virus-infected HeLa cells were fractionated and tested for their ability to support late gene-specific in vitro transcription. These experiments led to the description of a late promoter-specific factor, designated VLTF-1, that is distinct from RNA polymerase and from previously identified early gene transcription factors. In this study, VLTF-1 was further purified by column chromatography and glycerol gradient sedimentation and assayed by using a late promoter (11K)/G-less cassette construct as a template (13). The first step of the purification scheme was chromatography over phosphocellulose. Previous experiments showed that all three fractions from the phosphocellulose column were needed for maximal late gene-specific transcription activity (13). The phosphocellulose 0.1 M fraction was then applied to columns

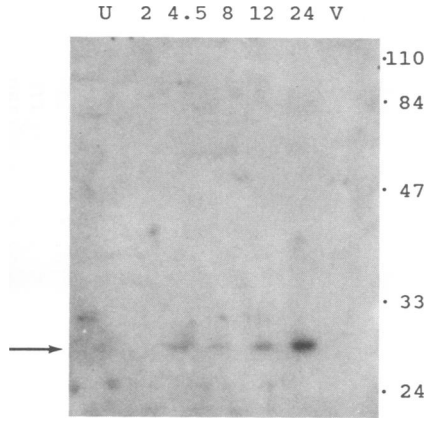

FIG. 1. Western blot analysis indicating the time course of appearance of the 30-kDa protein. Extracts of virus-infected cells (infected at a multiplicity of 10) or purified virions were prepared as described in Materials and Methods, resolved by SDS-PAGE, transferred to nitrocellulose, and incubated with anti-GK1 serum. Lanes: U, uninfected cells; 2 to 24, hours after infection; V, virion. The arrow indicates the position of the 30-kDa band; the apparent molecular masses, in kilodaltons, of mobility standards are shown at the right.

of DEAE-cellulose, Affi-Gel Blue, and hydroxylapatite and sedimented through a glycerol gradient (Fig. 2). In all cases, purification of VLTF-1 was followed by the ability of various fractions to complement the phosphocellulose 0.3 and 1.0 M fractions (Fig. 3 and 4). The sedimentation coefficient of the factor as estimated by the glycerol gradient was 2.4S, which corresponds to an apparent molecular weight of approximately 25,000.

Anti-GK1 antibodies recognize a 30-kDa protein that copurifies with VLTF-1. Since VLTF-1 appeared to be similar in size to the 26- and 30-kDa proteins predicted by the ORFs identified by the in vivo complementation assay, we desired to determine whether VLTF-1 corresponded to one of these two factors. Preliminary experiments showed that the anti-GK1 antibodies recognized a band of approximately 30 kDa in Western blots of SDS-polyacrylamide gels in which the phosphocellulose 0.1 M fraction was analyzed (data not shown). Therefore, we used the anti-GK1 serum in additional Western blot analyses on fractions from the Affi-Gel Blue and hydroxylapatite affinity columns used to purify VLTF-1. In Fig. 3 it is apparent that the anti-GK1 serum recognized an approximately 30-kDa protein which copurified with the specific transcription complementation activity through chromatography on the Affi-Gel Blue column. When this same analysis was performed on the hydroxylapatite fractions, the serum again recognized a 30-kDa protein; however, the peak of transcription did not quite correlate with the peak of 30-kDa protein as measured by Western blotting, perhaps because an active form of the protein elutes slightly faster than the bulk of protein on this column. For comparison, this figure also shows the silver-stained protein gels of these same fractions, demonstrating that they still contained many proteins.

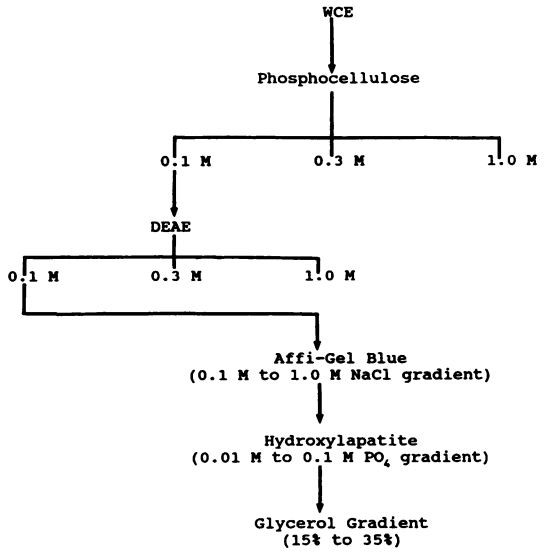

FIG. 2. Flow chart of the purification scheme for VLTF-1. The concentrations of NaCl used to step elute the phosphocellulose and DEAE-cellulose columns are indicated as 0.1 M, 0.3 M, and 1.0 M. WCE, whole cytoplasmic extract from vaccinia virus-infected HeLa cells.

The anti-GK1 serum was also used in a Western blot analysis of fractions derived from glycerol gradient sedimentation of VLTF-1. A silver-stained protein gel of these gradient fractions revealed a major protein band of approximately 30 kDa present only in the fractions containing transcription complementation activity (Fig. 4C). Once again, the anti-GK1 serum bound to a 30-kDa protein that cosedimented with the transcription activity across the gradient. These results strongly suggested that the GK1 ORF encodes a component of the transcription factor VLTF-1.

Antibodies raised against VLTF-1 immunoprecipitate a 30-kDa protein from reticulocyte lysates. In a further effort to confirm the relatedness of VLTF-1 and the protein encoded by the GK1 ORF, antibodies were raised against native VLTF-1 by injecting the glycerol gradient-purified protein into a rabbit. A [^{35}S]methionine-labeled lysate was made from vaccinia virus-infected BSC-1 cells, and immunoprecipitations were done with preimmune or immune serum. The immune serum precipitated a protein of 30 kDa from the cell lysate; this protein was not precipitated by the preimmune serum, which was the only discernible difference between the proteins precipitated by the preimmune versus the immune serum (data not shown). This anti-VLTF-1 serum was then used in immunoprecipitation reactions of [^{35}S]methionine-labeled extracts from reticulocyte lysates programmed with ORF GK1 RNA. The results (Fig. 5) demonstrate that the immune serum immunoprecipitated a 30-kDa protein from the lysate but the preimmune serum did not. Thus, the anti-VLTF-1 antibodies recognized the protein product from the GK1 ORF.

Inhibition of transcription with antibodies. We next tested whether the anti-VLTF-1 serum, as well as the anti-GK1 serum, could deplete biologically active VLTF-1 from infected cell extracts. Both sets of antisera, as well as preimmune sera, were bound to protein A-Sepharose beads. The beads were then incubated with the phosphocellulose 0.1 M extract (the material derived from the first step in the purification of VLTF-1). After centrifugation of the beads from the extracts, the supernatants were tested in in vitro transcription reactions containing the phosphocellulose 0.3 and 1.0 M fractions. In both cases, the beads incubated with the immune sera inactivated the extracts (Fig. 6, lanes 3 to 6); in the case of the anti-VLTF-1 serum, this inhibition appeared to be complete. Incubation of the 0.1 M extract

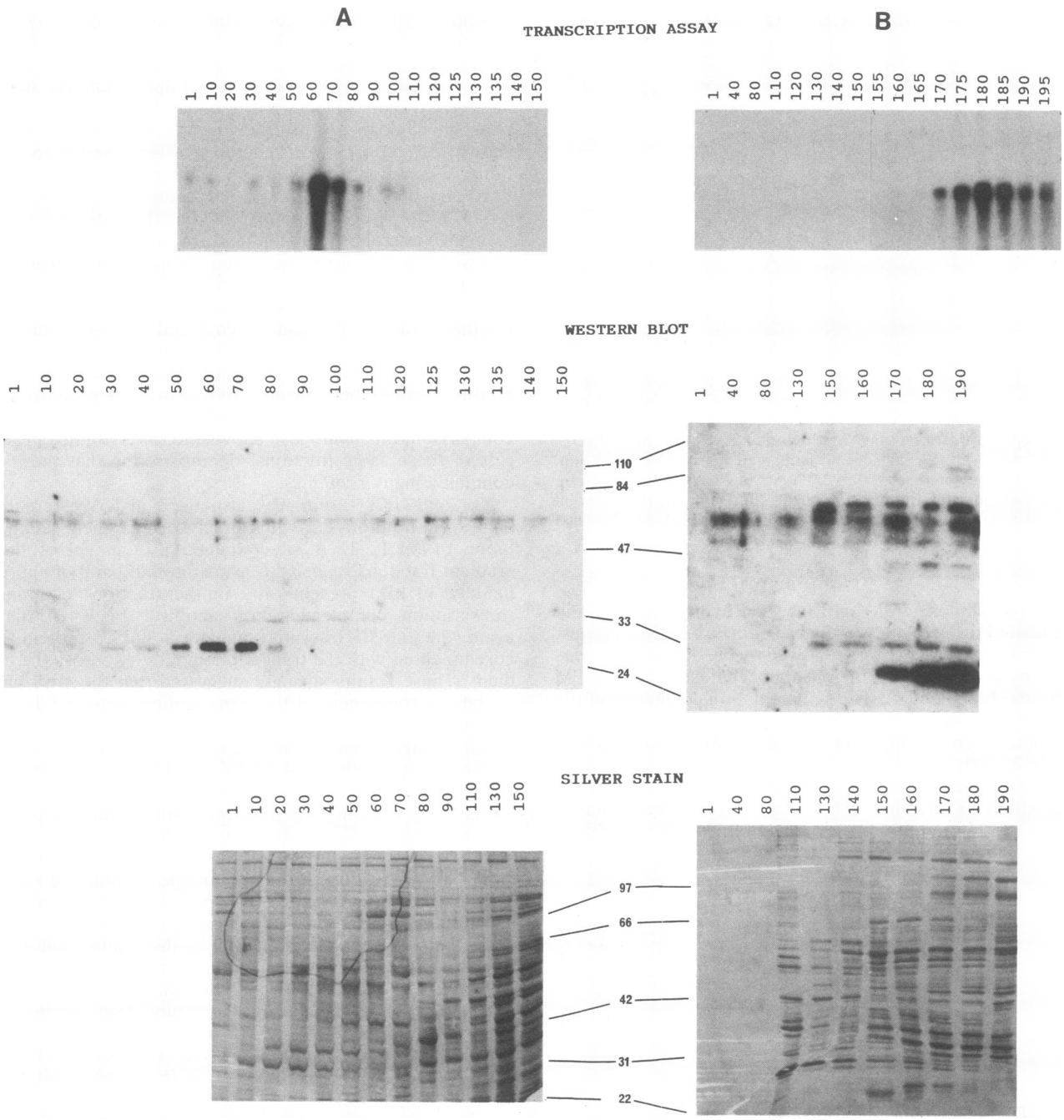

FIG. 3. Transcription assays, Western blot analysis, and silver-stained polyacrylamide gels of fractions derived from Affi-Gel Blue (A) and hydroxylapatite (B) chromatography of VLTF-1. For transcription assays, the various column fractions were dialyzed against buffer A, and then 10 μl was added to reaction mixtures containing the phosphocellulose 0.3 and 1.0 M fractions. The resulting RNA was applied to a 4% polyacrylamide gel. For Western blot analysis, 20 μl of the fractions were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated with the anti-GK1 serum. For silver staining, either 40 μl (A) or 20 μl (B) of the indicated fractions was again separated by SDS-PAGE and then silver stained. Fraction numbers are indicated above the lanes; the apparent molecular masses, in kilodaltons, of mobility standards are shown between the protein gels.

with the preimmune sera had no effect. In a further experiment, beads bound to either the anti-VLTF-1 serum or the preimmune serum were incubated with the unfractionated transcription extract and the supernatant was tested alone for transcription activity. The extract incubated with immune serum was inactive, while that incubated with preimmune serum was still competent for transcription (Fig. 6, lanes 8 and 9). Adding back hydroxylapatite-purified VLTF-1 to the extract depleted of VLTF-1 activity by the immune serum restored its transcriptional capability (Fig. 6; compare lanes 8 and 10). Similar experiments revealed that the anti-GK1 serum partially inactivated the unfractionated extract (data not shown). It is evident from this set of experiments that both sets of antibodies, when bound to

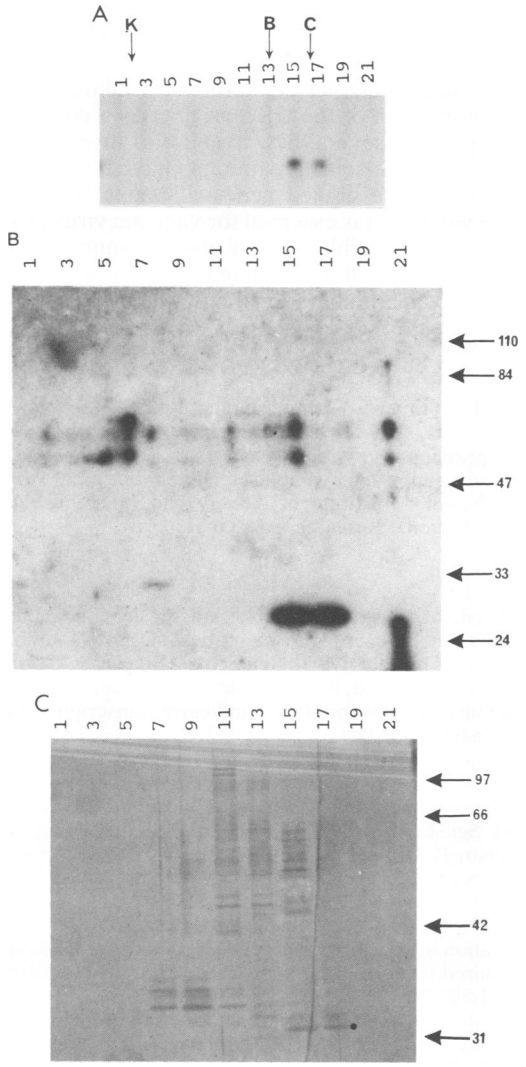

FIG. 4. Glycerol gradient sedimentation of VLTF-1. Shown are transcription assays (A), Western blot analysis (B), and a silver-stained gel (10 μl of each fraction applied to the gel) (C), performed as described in the legend to Fig. 3. Fraction numbers are indicated above the lanes; the apparent molecular masses, in kilodaltons, of protein mobility standards are shown at the right. Indicated above the autoradiogram in panel A are the positions of proteins used as molecular weight standards run in a parallel gradient: K, catalase; B, bovine serum albumin; C, cytochrome *c*. The prominent 30-kDa band present in fractions 15 and 17 of panel C is highlighted by a dot.

protein A-Sepharose beads, removed VLTF-1 activity from solution.

DISCUSSION

Biochemical fractionation of a transcription extract made from vaccinia virus-infected HeLa cells late in infection led to the description of a factor designated VLTF-1. This factor was shown to be necessary for transcription of vaccinia virus late genes in vitro; however, it was not highly purified, its importance as a transcription factor in vivo was not shown, and the gene which encodes it was unknown. In an unrelated series of experiments, using an in vivo complementation assay, three vaccinia virus genes encoding proteins of 17, 26,

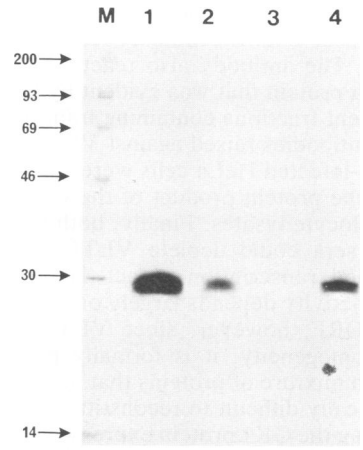

FIG. 5. Autoradiogram of proteins labeled in reticulocyte lysates. Lanes: 1 and 2, 5 and 1 μl, respectively, of a reticulocyte lysate programmed with GK1 RNA and incubated with [^{35}S]methionine; 3, 50 μl of labeled lysate immunoprecipitated with preimmune serum; 4, 50 μl of labeled lysate immunoprecipitated with anti-VLTF-1 serum; M, protein standards whose apparent molecular masses, in kilodaltons, are indicated at the left.

and 30 kDa were shown to be necessary for late gene expression; however, the actual function of these proteins was unknown. In this study, we have combined these two approaches and demonstrate that the 30-kDa ORF, designated GK1, encodes part, if not all, of VLTF-1 activity.

In the first set of experiments, antibodies raised against the gel-purified 30-kDa protein were found by Western blot analysis to cross-react with an M_r-30,000 protein that copu-

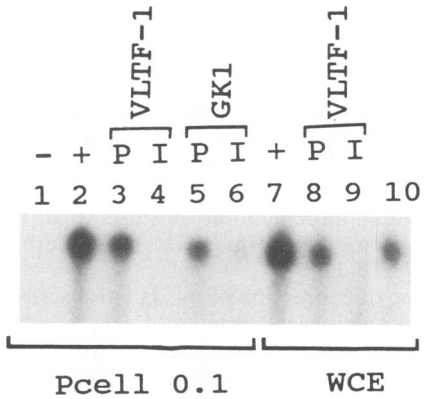

FIG. 6. Autoradiogram of transcription reactions. Lanes 1 to 6 were performed with the reconstituted system (containing phosphocellulose 0.3 and 1.0 M fractions); lanes 7 to 10 were performed with the unfractionated whole cytoplasmic extract. Lanes: 1, negative control without added phosphocellulose 0.1 M (Pcell 0.1) fraction; 2, positive control with 5 μl of Pcell 0.1 added; 3 to 6, 10 μl of supernatant from Pcell 0.1 fraction incubated with protein A-Sepharose beads bound to preimmune serum from a rabbit receiving VLTF-1 (lane 3), anti-VLTF-1 serum (lane 4), preimmune serum from rabbit receiving GK1 protein (lane 5), and anti-GK1 serum (lane 6); 7, 5 μl of unfractionated extract (WCE); 8 and 9, 10 μl of supernatant from the unfractionated extract incubated with protein A-Sepharose beads bound to preimmune serum from a rabbit receiving VLTF-1 (lane 8) or anti-VLTF-1 serum (lane 9); 10, same as lane 9 except that the reaction mixture also contained 5 μl hydroxylapatite-purified VLTF-1 extract.

rified with VLTF-1 transcription activity through columns of phosphocellulose, DEAE-cellulose, Affi-Gel Blue, and hydroxylapatite. The antibody also reacted with an approximately 30-kDa protein that was evident by silver staining in glycerol gradient fractions containing transcription activity. In addition, antibodies raised against VLTF-1 purified from vaccinia virus-infected HeLa cells were capable of immunoprecipitating the protein product of the GK1 ORF made in vitro in reticulocyte lysates. Finally, both the anti-GK1 and anti-VLTF-1 sera could deplete VLTF-1 activity from a partially purified transcription extract. Thus, we have shown that VLTF-1 activity depends largely on the protein product of the GK1 ORF; however, since VLTF-1 has not been purified to homogeneity, it is formally possible that it is composed of a mixture of proteins that includes GK1. It has proven technically difficult to reconstitute an active in vitro system by using the GK1 protein expressed either in bacteria or in reticulocyte lysates (data not shown). However, the anti-GK1 serum, directed against only one vaccinia protein, can almost completely inactivate transcription extracts. Also, the silver-stained protein gel of the glycerol gradient fractions containing VLTF-1 activity shows only one band at 30 kDa copurifying with transcription activity. For these reasons, we consider it unlikely that VLTF-1 is more than one protein, but proof will await the reconstitution of an active late transcription system from purified components.

Aside from a short leucine zipper motif, the primary sequence of GK1 showed no significant relationship to any known viral or cellular proteins (5). On the basis of the fact that transfected copies of the gene could be expressed in the absence of DNA replication, the gene was classified as intermediate. Western blot analysis using the anti-GK1 serum is consistent with this intermediate classification, since we show that it is present in infected cells starting at about 4.5 h postinfection and is stable until at least 24 h postinfection.

Whether VLTF-1 functions in transcription initiation or elongation is currently unknown. Attempts to demonstrate DNA binding by using partially purified preparations of the factor have been unsuccessful (unpublished results); this suggests that its role in transcription may be mediated through RNA polymerase or other regulatory factors. The fact that VLTF-1 activity can be restored to an unfractionated transcription extract previously depleted of VLTF-1 through immunoprecipitation suggests that if complexes with other factors do form, these factors are not entirely bound to VLTF-1.

The timing of expression of this late transcription factor is consistent with a cascade model of gene regulation. In this model, the factors necessary for the expression of any one gene class are synthesized in the previous phase of the infection (2, 4, 5, 11). Thus, the fact that GK1 is an intermediate gene allows its product to be available in the cell in time to support transcription of late genes. Previous reports have shown that the genes which encode the subunits of the vaccinia virus early gene transcription factor, VETF, are late genes (2, 4), which allows their protein products to be packaged in the virion for the next round of infection. We show here that the GK1 protein is still present in the cell late in infection and yet is not packaged in the virion, consistent with the absence of late transcription by virus cores. The mechanism by which early transcription factors are selectively packaged remains unknown.

The identification of the GK1 product as a component of VLTF-1 opens up several new avenues of investigation. As yet, no temperature-sensitive mutants have been mapped to the GK1 ORF; the cloned gene will make it possible to determine whether it is essential for vaccinia virus growth. It will now also be possible to more easily acquire the purified protein for additional biochemical studies as well as to perform a systematic mutational analysis of the gene.

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