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The A2L Intermediate Gene Product Is Required for In Vitro Transcription from a Vaccinia Virus Late Promoter

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Previously, the in vitro late transcription system of vaccinia virus was resolved into four components: the 17- and 30-kDa products of the A1L and G8R intermediate genes, respectively, the viral DNA-dependent RNA polymerase, and an unmapped factor sedimenting at 32 to 38 kDa. Another protein, the 26-kDa product of the A2L open reading frame was predicted to be a late transcription factor on the basis of a transient-expression assay but was not recognized as being necessary for transcriptional activity in vitro. We now report that both the unmapped factor and the 26-kDa protein are required for transcription from a vaccinia virus late promoter in vitro. Since the 26-kDa protein has now been shown to be a *trans*-activator of late transcription and it is the product of a known gene, we suggest that it be designated VLTF-3.

The vaccinia virus genome encodes most of the proteins required for viral mRNA synthesis, including a multisubunit DNA-dependent RNA polymerase (2, 6, 10). Vaccinia virus gene expression is temporally regulated, and three discrete stages of expression are recognized: early, intermediate, and late (11). The viral polymerase is involved in the transcription of all gene classes, but distinct accessory proteins are required for each expression class. The progression of the replicative cycle depends upon the availability of these accessory proteins in concert with the accessibility of the genes themselves (for a review, see reference 10).

Early genes are transcribed immediately following infection, and all of the components required are present in the virion as a result of their synthesis and packaging during the late stage of the previous replication cycle (1, 4, 10). Intermediate transcription factors are synthesized during this early stage and are present in infected cells before DNA synthesis begins (7, 17). Interestingly, the transcription of intermediate genes requires DNA synthesis but does not require postreplicative protein synthesis, suggesting that intermediate genes are sequestered within the higher-order structures of the vaccinia virus genome (16, 17).

DNA replication inhibitors block intermediate and late gene expression in vaccinia virus-infected cells, leaving them poised to begin transcription from intermediate promoter sequences. The systematic transfection of viral DNA into such replication-compromised cells allowed the identification of the A1L, G8R, and A2L intermediate genes as necessary for late gene expression in vivo (7). These genes code for 17-, 30-, and 26-kDa proteins, respectively. A requirement for the 17- and 30-kDa proteins was confirmed by using a late promoter-dependent in vitro transcription system (8, 18, 20). An unidentified third factor was also shown to be required for transcription in this system, but evidence suggested that this factor was distinct from the 26-kDa protein (18). To date, no in vitro requirement for the 26-kDa protein has been demonstrated. We now report that the 26-kDa protein, in addition to an as yet unidentified factor, is necessary for in vitro transcription from a late promoter.

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MATERIALS AND METHODS

Reagents. Preimmune serum was taken from a New Zealand White rabbit prior to injection with antigen, while 30-kDa-protein-specific serum was prepared by standard methods. Antiserum directed against the 26-kDa protein was the generous gift of J. Keck. Other materials included cell culture reagents (Life Technologies Inc., Gaithersburg, Md.), Immobilon-P membrane (Millipore Corp., Bedford, Mass.), His-Bind resin (Novagen, Inc., Madison, Wis.), MAX-BAC baculovirus expression system (Invitrogen Corp., San Diego, Calif.), and protein A-Sepharose and general chemicals (Sigma Chemical Co., St. Louis, Mo.).

Cell growth, infection with virus, and extract preparation. Growth of HeLa S-3 cells, infection with the vaccinia virus WR strain, and cell extract preparation have been previously described (20, 21). The growth of *Spodoptera frugiperda* Sf-9 cells, their infection with *Autographa california* nuclear polyhedrosis virus, and transfection and screening procedures were performed essentially according to the manufacturer's instructions (5), with modifications as previously described (18). Sf-9 extracts were prepared by lysis in 10 times the packed cell pellet volume of 10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol (DTT), 1 μg of leupeptin per ml, 1 μg of aprotinin per ml, and 0.01% (vol/vol) Nonidet P-40.

Preparation of recombinant baculoviruses coding for the A2L wild-type and mutant genes. The coding region of the A2L gene was amplified by PCR with purified vaccinia virus DNA as template. The primers GAG TGT TAA CAT GAA TCT ACG (A2L, 5′ end) and GCC ATT TAA TTA CGG AAC TAT (A2L, 3′ end) were used to amplify the native sequence, while the mutant form of the gene was generated with the primers CCG GTG GCC ATG AAT CTA CGA TTA AGT AGC GGT TG (A2L, 5′ end with a T-to-A base change from the wild-type sequence at the underlined position, which results in a cysteine-to-serine amino acid change at position 6 in the protein) and CCG GCT CGA GTA CTT CTA ATG AGC (A2L, 3′ end). The mutant PCR product was cut with *Msc*I and *Xho*I, and the *Xho*I end was filled in by the Klenow reaction. The wild-type and mutant genes were ligated separately into pBluBac2 (Invitrogen Corp.), previously cut with *Nhe*I and filled in by the Klenow reaction. The A2L-related DNAs of both the wild-type and mutated genes were sequenced by a cycle sequencing method (14) and found to match that of the WR strain except for the single point mutation as discussed above.

Protein purification. The 17- and 30-kDa late transcription factors were purified from recombinant baculovirus-infected SF-9 cells (18), while the vaccinia virus DNA-dependent RNA polymerase was purified from vaccinia virus-infected HeLa cells or virions as previously described (19).

The unmapped factor previously shown to be required for late transcription in vitro and tentatively designated VLTF-2 (18) will be referred to as VLTF-X in this work and until it is mapped to a specific gene. This is being done to avoid confusion with the 17-kDa protein which was also designated VLTF-2 (8) and to yield to the suggestion that the designations VLTF-3, etc., be reserved for factors mapped to specific genes and shown to have late-transcriptional stimulatory activity. A designation of the level of purity of a factor used in a given experiment will also be made for the sake of clarity (e.g., DEAE-pure VLTF-X).

VLTF-X was prepared by two different methods: that previously described, in which a vaccinia virus-infected HeLa cell extract prepared late in infection was purified over phosphocellulose, heparin-agarose, DEAE-cellulose, and hydroxylapatite columns and then sedimented on a glycerol gradient (18), and a novel method in which hydroxyurea-treated cell extract prepared on a small scale was purified over a nickel-chelate resin in batch mode ("nickel-pure"). In this

method, monolayers (four 100-mm^2 culture dishes) of BSC-1 cells were infected with vaccinia virus VTF7-3 at a multiplicity of infection of 20 and transfected with the plasmid pTM1 (12) essentially by the method of Fuerst et al. (3), except that a 2-h adsorption period was used and cells were maintained in the presence of 20 mM hydroxyurea. The extract was prepared by lysis in 500 μl of binding buffer (20 mM Tris [pH 7.9], 500 mM NaCl, 5 mM imidazole) followed by Dounce homogenization and centrifugation to remove membranes. Next, 400 μl of the recovered extract was mixed with 100 μl of His-Bind resin that was previously charged with nickel and equilibrated with binding buffer according to the manufacturer's instructions (13). Upon settling, the supernatant was removed and the resin was resuspended in 600 μl of binding buffer. The supernatant was again removed, and then the weakly interacting proteins (including VLTF-X) were eluted by suspending the resin in 600 μl of binding buffer containing 60 mM imidazole. The supernatants were dialyzed in buffer A (10 mM Tris [pH 8.0], 100 mM NaCl, 0.1 mM EDTA, 10% (vol/vol) glycerol, 0.01% Nonidet P-40), and then DTT (2 mM) was added and the supernatants were tested for the ability to support in vitro transcription in the presence of the 26-kDa protein. VLTF-X activity was found in the 60 mM imidazole fraction, while the vast majority of the protein was observed in the initial supernatant and wash steps (see Fig. 1, lanes 8 and 9). It is worth noting that this method was chosen as a result of gratuitous observations made while conducting other experiments; we do not advocate this as the best method for preparing VLTF-X.

The 26-kDa gene product of the A2L gene was purified after expression in insect cells and preparation of extracts, performed as described previously for the 17- and 30-kDa proteins (18). The extracts were passed over a DEAE-cellulose column (bed volume, 1 ml; total protein, 10 mg) and equilibrated in buffer A containing 2 mM DTT, at 15 ml/h, and the 26-kDa protein was located in the flowthrough fractions by Western blotting (immunoblotting). The undialyzed 26-kDa protein fraction was passed over hydroxylapatite (bed volume, 1 ml; total protein, 10 mg), equilibrated in buffer B (20 mM sodium phosphate [pH 6.8], 10% (vol/vol) glycerol, 2 mM DTT, 0.01% Nonidet P-40), at 12 ml/h, and washed with 10 column volumes of buffer B. The retained proteins (including the 26-kDa protein) were eluted with buffer B containing 200 mM phosphate, then dialyzed in buffer A containing 2 mM DTT. The 26-kDa-protein-containing fractions were located by Western detection and specific-transcription assay and were then pooled, concentrated, and centrifuged on glycerol gradients as described for the 17- and 30-kDa proteins (18). The mutant 26-kDa protein was purified by an identical method except that no specific transcription was detected at any stage of the purification.

Specific transcription assay. Specific in vitro transcription assays were conducted under previously described conditions (18, 20, 21). Briefly, protein fractions were incubated with a supercoiled plasmid containing a late promoter fused to 400 bp of DNA lacking G residues in the noncoding strand (15). Reactions were conducted for 30 min at 30°C in a 50-μl total volume, in a mixture of 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM DTT, 0.2 mM EDTA, 2 mM $MgCl_2$, 1 mM ATP, 0.1 mM CTP, 0.02 mM UTP, 5 μCi of [α-^{32}P]UTP, 9% polyvinyl alcohol, 5% glycerol, and 1 μg of DNA template.

Immunodepletion. The immunoglobulin G-charged protein A-Sepharose resins were prepared by equilibration in buffer C (10 mM Tris [pH 7.6], 150 mM NaCl) and then incubation with antiserum for at least 15 min at 4°C with constant mixing followed by extensive washing with buffer C. The volume of serum used was approximately equal to the packed resin bed volume. The prepared resin was stored at 4°C for up to 2 weeks and immediately before use was equilibrated with buffer A containing 2 mM DTT and 250 U of RNasin per ml. Immunodepletion was conducted by incubating 1 volume of factors (individual amounts are described in the text) with 1/3 volume of the immunoglobulin G-charged resin for 1.5 h at 4°C on a rotation device and then allowing the resin to settle. The supernatant was then used in the transcription assays as described in the text.

RESULTS

Effect of adding the 26-kDa protein to the in vitro vaccinia virus late transcription system. Data obtained from transfection experiments suggested that the 26-kDa product of the A2L gene might play a role as a *trans*-activator of transcription from a late promoter (7); however, no such requirement had previously been observed with a late promoter-dependent in vitro transcription system (18). In order to confirm or rule out the participation of this protein in the in vitro system, a recombinant baculovirus containing the A2L gene was constructed and used to infect Sf-9 insect cells. Extracts from these cells appeared similar to those from uninfected Sf-9 cells by silver staining (Fig. 1, compare lanes 2 and 1, respectively) but contained an additional protein which migrated just ahead of the 30-kDa marker during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as detected by Western blotting (Fig. 2, compare lanes 2 and 1). The immunoreactive protein was partially purified by DEAE-cellulose and hydroxylapatite chromatography and was then tested in the in vitro transcription system. Numerical estimates of purity are not given since the band visualized by Western blotting could not unambiguously be associated with a stainable band. The addition of hydroxylapatite-pure 26-kDa protein to reaction mixtures containing the previously identified late factors (17- and 30-kDa proteins and VLTF-X) and the viral RNA polymerase gave inconsistent results from experiment to experiment. Essentially, a stimulatory effect on the order of two- to fivefold was sometimes, but not always, observed, and the variation appeared to depend upon which preparation of VLTF-X was used in the transcription reactions (data not shown). This suggested that if the observed variability was due to the presence of the 26-kDa protein as a contaminant of VLTF-X or another component of the system, then late transcription might require the 26-kDa protein.

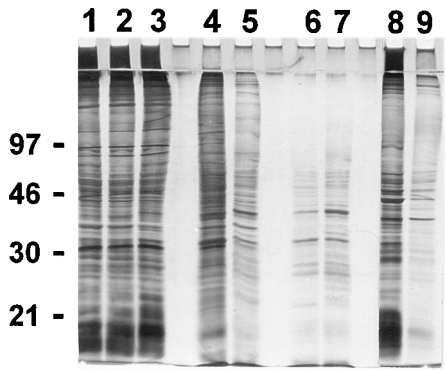

FIG. 1. Silver-stained polyacrylamide gel of partially purified late transcription factors. Protein preparations were separated by SDS-PAGE on a 12% gel and then silver stained. Lane 1, 1 μl of uninfected Sf-9 insect cell extract; lane 2, 1 μl of extract from insect cells expressing the A2L gene; lane 3, 1 μl of extract from insect cells expressing the mutated A2L gene; lane 4, 4 μl of hydroxylapatite-pure wild-type 26-kDa protein; lane 5, 7 μl of hydroxylapatite-pure mutant 26-kDa protein; lane 6, 9 μl of glycerol gradient-pure wild-type 26-kDa protein; lane 7, 3 μl of glycerol gradient-pure mutant 26-kDa protein; lane 8, 2.5 μl of extract from vaccinia virus-infected HeLa cells; lane 9, 5 μl of nickel-pure VLTF-X.

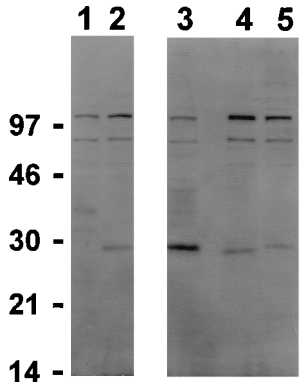

FIG. 2. Western visualization of the partially purified wild-type and mutant A2L proteins. After electrophoresis, proteins were electroblotted onto Immobilon-P membranes and then were probed with anti-26-kDa protein antiserum by standard methods and were visualized by chemiluminescence. Lane 1, 3 μl of uninfected Sf-9 insect cell extract; lane 2, 4 μl of extract from insect cells expressing the A2L gene; lane 3, 4 μl of extract from insect cells expressing the mutated A2L gene; lane 4, 3.5 μl of hydroxylapatite-pure wild-type 26-kDa protein; lane 5, 10.5 μl of hydroxylapatite-pure mutant 26-kDa protein.

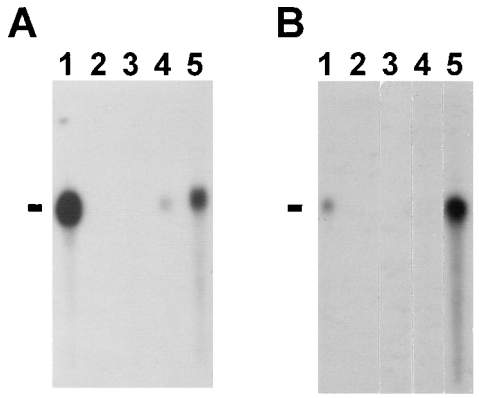

FIG. 3. Autoradiograms of gels after PAGE of transcription assays conducted with and without VLTF-X and the 26-kDa protein. (A) The transcription reaction mixtures loaded in lanes 1 through 5 contained 1.5 μl of 17-kDa protein, 2.0 μl of 30-kDa protein, and 2.0 μl of infected-cell purified RNA polymerase. Reaction mixtures also contained 5 μl of DEAE-pure VLTF-X (lane 1), no additional factors (lane 2), 5 μl of nickel-pure VLTF-X (lane 3), 3 μl of hydroxylapatite-pure 26-kDa protein (lane 4), or 5 μl of nickel-pure VLTF-X and 3 μl of hydroxylapatite-pure 26-kDa protein (lane 5). (B) The transcription reaction mixtures loaded in lanes 1 through 5 contained 3 μl of 17-kDa protein, 3 μl of 30-kDa protein, and 3 μl of RNA polymerase purified from virions. Reaction mixtures also contained 5 μl of hydroxylapatite-pure VLTF-X (lane 1), no additional factors (lane 2), 5 μl of nickel-pure VLTF-X (lane 3), 2.5 μl of glycerol gradient-pure 26-kDa protein (lane 4), or 5 μl of nickel-pure VLTF-X and 2.5 μl of glycerol gradient-pure 26-kDa protein (lane 5).

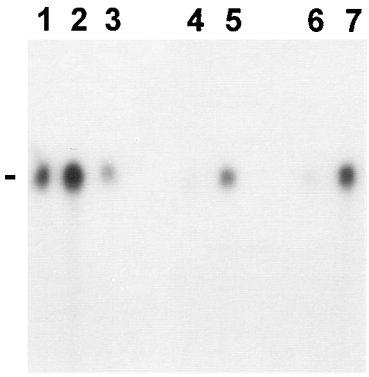

FIG. 4. Autoradiograms of gels after PAGE of transcription assays performed on factors treated with protein A-Sepharose beads charged with various antisera. Prior to any treatment, 19 μl of 17-kDa protein, 18 μl of 30-kDa protein, 23 μl of RNA polymerase from infected cells, 33 μl of DEAE-pure VLTF-X, and 26 U of RNasin were mixed together to give a single solution of factors. Aliquots of factors were incubated at 4°C, as described in Materials and Methods, with the following: lanes 1 and 2, no resin; lane 3, preimmune resin; lanes 4 and 5, 30-kDa-protein-specific resin; lanes 6 and 7, 26-kDa-protein-specific resin. Following the treatment at 4°C, the reactions were held on ice while the resin was allowed to settle and the supernatants were removed to new tubes, and then 11.6-μl aliquots of the treated factors were used in transcription assays in which no additional factors were added (lanes 1, 3, 4, and 6), 2 μl of 26-kDa protein was added (lanes 2 and 7), or 2 μl of 30-kDa protein was added (lane 5).

Dependence of in vitro late transcription on the A2L gene product as well as a factor purified from hydroxyurea-treated vaccinia virus-infected HeLa cells. As an alternate approach to its preparation, VLTF-X was purified from hydroxyurea-treated cells, since the A2L intermediate gene should not be expressed under these conditions. Additionally, the method of purification was altered in the hope of purifying VLTF-X away from any A2L gene product which may have been expressed despite the block in DNA synthesis. This strategy yielded VLTF-X preparations which did not stimulate transcription from a late promoter unless the reaction mixtures were also supplemented with the 26-kDa protein, as demonstrated in Fig. 3A. Assays were performed using a vaccinia virus late promoter driving the specific transcription of a G-less cassette sequence, as described in Materials and Methods. The transcription reaction mixtures contained appropriate amounts of the 17- and 30-kDa proteins and the infected-cell purified polymerase, but individual reaction mixtures contained either VLTF-X, the 26-kDa protein, or both. DEAE-pure VLTF-X, prepared from late extracts, was able to support specific transcription without addition of the 26-kDa protein (lane 1), but no transcription was observed when VLTF-X was not added to the reaction mixtures (lane 2). In contrast, nickel-pure VLTF-X prepared from hydroxyurea-treated infected cells did not support transcription (lane 3) unless the 26-kDa protein was also added to the reaction mixtures (lane 5). Importantly, when VLTF-X was not explicitly added, the 26-kDa protein alone did not support transcription on the same order of magnitude as did the control reactions (lane 4); however, extended exposure revealed some specific signal in this lane. This is most likely due to contaminating traces of VLTF-X in the polymerase preparation. To test this possibility, a similar experiment was performed in which the RNA polymerase purified from virions was used in place of the infected-cell purified polymerase (Fig. 3B). The basic result was the same, except that no transcription was detected in the absence of VLTF-X (lane 4). Thus, it appeared that our original preparations of VLTF-X contained the 26-kDa protein as a contaminant and that we had then prepared VLTF-X which was free of such contamination, resulting in the newly observed dependence of in vitro transcription upon the externally supplied 26-kDa protein.

Immunodepletion of the 26-kDa protein from mixed factors with reconstitution of transcription by addition of the 26-kDa protein. An alternate explanation for the results described above could be that the VLTF-X purified from hydroxyurea-treated infected-cell extracts and that purified from cell extracts prepared at later times of infection may represent different positions (26-kDa-dependent and 26-kDa-independent proteins). It was important to make this determination since the gene for VLTF-X has not yet been identified. Therefore, immunodepletion experiments were conducted to determine if the 26-kDa protein was required for in vitro transcription when traditional preparations of VLTF-X (obtained from late extracts) were employed (Fig. 4). The RNA polymerase, 17- and 30-kDa proteins, and DEAE-pure VLTF-X were mixed together as described in the legend to Fig. 4, and then aliquots of the mixed factors were incubated with preimmune, 30-kDa-protein-specific or 26-kDa-protein-specific immunoglobulin G-charged protein A-Sepharose. The 30-kDa protein depletion was used as an overall control for this procedure, since the conditions for successful depletion and reconstitution had previously been determined (20). The recovered supernatants were then tested for their ability to support late promoter-specific transcription.

As mentioned above, traditional VLTF-X preparations, the 17- and 30-kDa proteins, and the RNA polymerase were sufficient to support late transcription in this system (lane 1), and a stimulatory effect (1.75-fold) was seen when the 26-kDa protein was also included (lane 2). Treatment with the nonspecific resin lowered the overall level of transcription signal (lane 3), but the signal remained on the same order of magnitude as that of the untreated control (compare lanes 3 and 1). Exposure to the anti-30-kDa protein resin, on the other

hand, substantially reduced transcription by the treated factors (lane 4), but transcriptional activity was restored when purified 30-kDa protein was added back at the start of the transcription assay (lane 5). Similarly, when exposed to the anti-26-kDa protein resin, transcription was greatly reduced (lane 6), but complete restoration was achieved upon adding back hydroxylapatite-pure 26-kDa protein (lane 7). Treatment of the factors with the anti-26-kDa resin increased the relative stimulation of transcription by added 26-kDa protein more than fourfold compared with stimulation of untreated factors, confirming that our previous preparations of VLTF-X were contaminated with the 26-kDa protein and demonstrating a requirement for the 26-kDa protein in vitro.

Expression of a mutant A2L gene in baculovirus, partial purification, and comparison with the wild-type 26-kDa protein. Due to minor cross-reactivities in the antisera and also to the presence of insect proteins in the partially purified 26-kDa protein preparations, a second approach was taken to independently confirm that the 26-kDa protein was specifically required for late transcription. A point mutation of the A2L gene which results in a cysteine-to-serine amino acid replacement at position 6 of the protein had previously been shown to negate the ability of the A2L gene to stimulate late gene expression during in vivo transfection assays (7). Therefore, a construct with this point mutation was expressed in insect cells and the protein was purified in the same manner as the wild-type recombinant 26-kDa protein (Fig. 1 and 2). The mutant protein showed a purification profile similar to that of the wild type at each step in the purification (data not shown). Unlike for the wild-type protein, however, no transcriptional activity was detected for this protein at any stage of the purification. To confirm this observation, the relative amounts of the native and mutant proteins were determined by Western blotting and equivalent amounts of the relevant proteins were compared in the transcription assay. As can be seen by Western blot (Fig. 2), equivalent amounts of 26-kDa wild-type (lane 4) and mutant (lane 5) proteins at the hydroxylapatite-pure stage of purification could be prepared. This was also true of these proteins at the glycerol gradient stage of purity (data not shown). When identical amounts of these proteins were used in the transcription assay, however, only the native protein supported transcription (Fig. 5). Reaction mixtures which contained the 17- and 30-kDa proteins, nickel-pure VLTF-X, and the infected-cell purified RNA polymerase were prepared. When no 26-kDa protein was added (lane 1) no transcription was detected. Transcription was supported when either the wild-type hydroxylapatite-pure (lane 2) or glycerol gradient-pure (lane 4) 26-kDa protein was added to the reactions. In contrast, neither the hydroxylapatite-pure (lane 3) nor the glycerol gradient-pure (lane 5) mutant 26-kDa proteins supported any level of transcription.

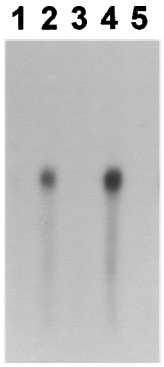

FIG. 5. Autoradiograms of gels after PAGE of transcription assays in which the wild-type and mutant 26-kDa protein were employed as transcription factors. Equivalent amounts of the wild-type or mutant 26-kDa proteins, at the hydroxylapatite or glycerol gradient stages of purification (as determined by Western analysis), were used in standard transcription reaction mixtures containing 1.5 μl of 17-kDa protein, 2.0 μl of 30-kDa protein, 5 μl of nickel-pure VLTF-X, 2.0 μl of infected cell-purified RNA polymerase and no added 26-kDa protein (lane 1), 1.67 μl of the hydroxylapatite-pure wild-type 26-kDa protein (lane 2), 5 μl of the hydroxylapatite-pure mutant 26-kDa protein (lane 3), 2 μl of the glycerol gradient-pure wild-type 26-kDa protein (lane 4), or 3.8 μl of the glycerol gradient-pure mutant 26-kDa protein (lane 5).

DISCUSSION

Prior genetic analyses with transient expression assays identified the vaccinia virus A1L, G8R, and A2L intermediate genes as necessary for transcription of a cotransfected reporter gene under late promoter control in replication-compromised cells (7). The encoded proteins with molecular masses of 17, 30, and 26 kDa, respectively, were hypothesized to be late *trans*-activators. This hypothesis was partially borne out when the 17- and 30-kDa proteins were shown to be necessary for in vitro transcription in a late promoter-dependent system (8, 18, 20). The 26-kDa protein, however, was not previously recognized as necessary for in vitro transcription. In addition, an unidentified factor (VLTF-X), which has not yet been mapped, was shown to be necessary for in vitro transcription and preliminary evidence suggested that this factor did not correspond to the 26-kDa protein (18). First, it did not cross-react with antibodies directed against the 26-kDa protein, and further, whole-cell extracts from hydroxyurea-treated infected cells could substitute for this activity. Nonetheless, direct evidence that this factor was not the 26-kDa protein was lacking. This report demonstrates that the factor referred to as VLTF-X can be purified from hydroxyurea-treated infected cells, that it is distinct from the 26-kDa protein, and further, that both VLTF-X and the 26-kDa protein are necessary for in vitro transcription from a late promoter.

The 26-kDa protein and VLTF-X were distinguished when the newly purified VLTF-X was used in the late transcription system (Fig. 3). Transcription was readily detected when both the hydroxylapatite-pure 26-kDa protein and nickel-pure VLTF-X were present in the reaction mixture, but little or no specific transcription was detected when only one of these factors was present. Furthermore, this requirement for both factors held true whether the infected-cell purified (Fig. 3A and 4) or the virion-purified (Fig. 3B) polymerase was used.

The purification of VLTF-X from hydroxyurea-treated infected cells extends previous observations that extracts from such cells can substitute for VLTF-X activity in the transcription assay (18) and supports the modifications to the cascade model of vaccinia virus gene regulation put forward by Kovacs et al. (9). Those researchers also found that a factor (designated P3) purified from cells blocked at the early stage of infection complemented factors purified from untreated infected cells to stimulate late transcription. Furthermore, the P3 factor is similar to VLTF-X in its elution from heparin agarose. However, it differs from VLTF-X in its elution from phosphocellulose (9, 18). Thus, it remains to be determined whether VLTF-X and the P3 factor represent the same or different proteins.

The immunodepletion of 26-kDa protein from traditional preparations of factors, in combination with the ability of 26-kDa protein purified from a heterologous source to restore transcriptional competence, demonstrates that one or more of our previous factor preparations was contaminated with the

26-kDa protein. The results of the other experiments in this report as well as personal observations suggest that it was our previous preparations of VLTF-X which contained this contaminating activity. Thus, the results presented here are in full agreement with all prior work reported by this laboratory.

Finally, antibody-independent confirmation that transcription from a late promoter in vitro specifically requires the 26-kDa protein was obtained by comparison of this wild-type protein with a similar protein containing a single amino acid change. Despite similar conditions of expression and purification, as well as the observed similarities in its immunoreactivity and purification profile, equivalent amounts of the mutant protein showed no activity in the in vitro system. Therefore, stimulation of transcription by a functionally homologous insect cell protein was effectively ruled out.

In summary, transcription from a vaccinia virus late promoter in vitro requires the 26-kDa protein product of the A2L gene in addition to the previously reported factors VLTF-X, the 17- and 30-kDa proteins, and the viral DNA-dependent RNA polymerase. Since the 26-kDa protein has now been shown to be a *trans*-activator of late transcription and it is the product of a known gene, we suggest that it be designated VLTF-3.

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REFERENCES

1. **Broyles, S. S., and B. S. Fesler.** 1990. Vaccinia virus gene encoding a component of the viral early transcription factor. J. Virol. **64:**1523–1529.
2. **Broyles, S. S., and B. Moss.** 1986. Homology between RNA polymerases of poxviruses, prokaryotes, and eukaryotes: nucleotide sequence and transcriptional analysis of Mr 147,000 and 22,000 subunit gene of vaccinia virus. Proc. Natl. Acad. Sci. USA **83:**3141–3145.
3. **Fuerst, T. R., E. G. Niles, W. F. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
4. **Gershon, P. D., and B. Moss.** 1990. Vaccinia virus early transcription factor subunits encoded by late genes. Proc. Natl. Acad. Sci. USA **87:**4401–4405.
5. **Invitrogen Corp.** 1994. MAXBAC manual, version 1.5.5. Invitrogen Corp., San Diego, Calif.
6. **Jones, E. V., C. Puckett, and B. Moss.** 1987. DNA-dependent RNA polymerase subunits encoded within the vaccinia virus genome. J. Virol. **61:**1765–1771.
7. **Keck, J. G., 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.
8. **Keck, J. G., G. R. Kovacs, and B. Moss.** 1993. Overexpression, purification, and late transcription factor activity of the 17-kilodalton protein encoded by the vaccinia virus A1L gene. J. Virol. **67:**5740–5748.
9. **Kovacs, G. R., R. Rosales, J. G. Keck, and B. Moss.** 1994. Modification of the cascade model for regulation of vaccinia virus gene expression: purification of a prereplicative, late-stage-specific transcription factor. J. Virol. **68:**3443–3447.
10. **Moss, B.** 1990. Regulation of vaccinia virus transcription. Annu. Rev. Biochem. **59:**661–668.
11. **Moss, B., B. Ahn, B. Amegadzie, P. D. Gershon, and J. Keck.** 1991. Cytoplasmic transcription system encoded by vaccinia virus. J. Biol. Chem. **266:** 1355–1358.
12. **Moss, B., O. Elroy-Stein, T. Mizukami, W. A. Alexander, and T. R. Fuerst.** 1990. New mammalian expression vectors. Nature (London) **348:**91–92.
13. **Novagen, Inc.** 1994. pET system manual, 4th ed., p. 22–23. Novagen, Inc., Madison, Wis.
14. **Reid, A. H., M. Tsai, D. J. Venzon, C. F. Wright, E. E. Lack, and T. J. O'Leary.** MDM2 amplification, p53 mutation, and accumulation of the p53 gene product in malignant fibrous histiocytoma. Diagn. Mol. Pathol., in press.
15. **Sawadogo, M., and R. Roeder.** 1985. Factors involved in specific transcription initiation by the human RNA polymerase II system. J. Biol. Chem. **259:**5321–5326.
16. **Vos, J. C., M. Sasker, and H. G. Stunnenberg.** 1991. Vaccinia virus capping enzyme is a transcription initiation factor. EMBO J. **10:**2553–2558.
17. **Vos, J. C., and H. G. Stunnenberg.** 1988. Derepression of a novel class of vaccinia virus genes upon DNA replication. EMBO J. **7:**1183–1190.
18. **Wright, C. F., and A. M. Coroneos.** 1993. Purification of the late transcription system of vaccinia virus: identification of a novel transcription factor. J. Virol. **67:**7264–7270.
19. **Wright, C. F., and A. M. Coroneos.** 1995. The H4 subunit of vaccinia virus RNA polymerase is not required for transcription initiation at a viral late promoter. J. Virol. **69:**2602–2604.
20. **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.
21. **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.