A Vaccinia Virus Late Transcription Factor with Biochemical and Molecular Identity to a Human Cellular Protein*

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A factor designated VLTF-X is required to support vaccinia virus late transcription in vitro. It has been found that a late promoter DNA binding activity cochromatographs and cosediments with VLTF-X activity. Current experiments show that VLTF-X activity is present in a variety of uninfected mammalian cell types and is indistinguishable from that recovered from infected cells based upon several criteria. VLTF-X activity from both sources displays the same purification profile over phosphocellulose and DNA affinity resins and has the same sedimentation coefficient. In addition, the factors purified from both infected and uninfected cells form protein-DNA complexes of identical electrophoretic mobility in the presence of vaccinia virus late promoter-containing DNA. The affinity of these factors for the late promoter probes is identical and late promoter-specific based on competition experiments. Moreover, VLTF-X purified from both sources bound to late promoter-containing DNA in the presence or absence of MgCl₂ and ATP and formed complexes resistant to heat inactivation. These experiments offer proof that vaccinia virus factor VLTF-X is a host cell protein that supports transcription of the viral late genes.

Poxviruses are large DNA viruses that replicate within the cytoplasm of host cells and encode most of the specific enzymes and factors required for their transcription and replication. Our knowledge of transcription regulation in poxviruses has come largely from studies of vaccinia virus, the prototypical member of this family. The vaccinia virus genome contains approximately 200 genes whose expression is tightly regulated based on transcription initiation. The life cycle of vaccinia virus is complex, with three temporally regulated classes of gene products. These genes can be divided into early, intermediate, and late classes based on their time of synthesis relative to viral DNA replication. The different gene classes are distinguished by their requirement for different transcription factors and by unique promoter sequences (1).

All of the components required for transcription of the early genes are packaged within the virion core, which is activated upon entry into the host cytoplasm. Fractionation of virion extracts has shown that two components are needed for early gene transcription initiation: the viral RNA polymerase and a protein designated VETF (for vaccinia early transcription factor) (2). Transcription of the intermediate class of viral genes requires the viral RNA polymerase, capping enzyme (3), the viral E4L gene product (4), and a factor apparently provided by the host cell (5).

Most of the factors necessary for late transcription are only present in the cell after the onset of viral DNA replication. Thus far, at least five factors, in addition to the viral RNA polymerase, have been identified as being necessary for maximal late transcription activity in vitro. These are the virally encoded products of the A1L, A2L, G8R, and H5R open reading frames (6–11) and a factor designated VLTF-X (6, 11). In addition, the presence of the viral G2R and A18L genes may play a role in transcript elongation or stability (12, 13).

Recent data have demonstrated that VLTF-X activity is found in uninfected HeLa cell cytoplasmic extracts and virion extracts, thus defining it as a unique late transcription factor (14). The presence of this factor in uninfected cells was surprising in that it potentially defined another host factor, besides that required for intermediate transcription, that participates in the viral transcriptional machinery. We have also shown that this factor copurifies from infected cells with a late promoter-specific DNA binding activity, which suggested that its biochemical role is in the recognition of vaccinia virus late promoters. In the present study, we demonstrate that VLTF-X affinity-purified from uninfected and infected cells shares the same size and biochemical and molecular properties. Based on these data, we conclude that VLTF-X purified from both preparations is most likely the same factor.

EXPERIMENTAL PROCEDURES

Purification of Factors

The A1L, A2L, and G8R proteins were expressed in a recombinant baculovirus system and purified as described previously (11). The A2L protein was also expressed as a glutathione S-transferase fusion protein and purified from Escherichia coli using glutathione-Sepharose. The vaccinia virus RNA polymerase was purified from infected HeLa cells to the glycerol gradient-pure stage as described previously (11).

Cell Fractionation

Uninfected HeLa cell extracts were prepared from the nucleus or cytoplasm as described by Abmayr and Workman (15). Whole cell extracts from a variety of cell lines (Fig. 1) were prepared as described previously (16) with the omission of the ammonium sulfate precipitation step.

Purification of VLTF-X

Previous Purifications—VLTF-X was purified from 30 liters of uninfected and vaccinia virus-infected HeLa cells as described previously (14) over sequential columns of phosphocellulose, heparin-agarose, DEAE-cellulose, hydroxylapatite, and phosphocellulose a second time. Preparation of Oligonucleotide Affinity Resin—A double-stranded late promoter-containing oligonucleotide affinity resin was prepared according to the protocol of Kerrigan and Kadonaga (17). Briefly, the
oligonucleotides 5′-GGATCCCTTTACCTTGGTCTTCTTCATGATA-TAGACTCG-3′ and 5′-GGATCCGGATCTTATTATGACGTTAAA-AAACAAAATAGAAA-3′ were annealed, ligated into multimeric chains (~7-mers) with T4 DNA ligase (Life Technologies, Inc.), and coupled directly to CNBr-activated Sepharose CL-B4 (Amersham Pharmacia Biotech). The affinity resin was equilibrated with buffer A (0.05 mM Tris-HCl (pH 8), 0.1 mM EDTA, 10% glycerol, 50 mM NaCl, 0.01% Nonidet P-40, and 2 mM dithiothreitol).

DNA Affinity Chromatography—Cytoplasmic extracts were prepared from uninfected and infected HeLa cells, passed individually over phosphocellulose columns, and eluted in step fractions of 0.1, 0.3, and 1.0 mM NaCl as described previously, except on a smaller scale (11). The 0.3 mM fractions (3 ng of protein) were dialyzed against buffer A and passed over a 1-ml column containing double-stranded calf thymus DNA-cellulose resin (Sigma) equilibrated in buffer A to deplete the extracts of nonspecific DNA-binding proteins. The flow-through fractions were collected and applied over a 1-ml column of the equilibrated affinity resin. The column was washed with 5 ml of buffer A, and proteins bound to the resin were collected by increasing NaCl concentrations (buffer A with 0.2, 0.5, and 1.0 mM NaCl). The presence of VLTF-X in the bound and flow-through fractions was monitored with mobility shift assays and transcription assays. All protein fractions from the 0.2, 0.5, and 1.0 mM eluates were dialyzed for 2 h against buffer A containing 0.01 mM salt prior to use in the above-mentioned assays. The 0.2 mM salt fractions, which contained VLTF-X activity, were pooled, reassembled over the affinity resin, and eluted as above to further purify the factor.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays contained varying amounts of poly(dI-dC)-poly(dI-dC) (Amersham Pharmacia Biotech) and 2–10 μl of protein samples. Mobility shift assays were conducted as described previously (14).

Target DNA Preparation

Radiolabeled DNA targets for the mobility shift experiments were prepared by using the plasmid pCFW7, which contains the wild-type promoter and flanking sequences of the vaccinia virus late gene expressing the 11-kDa protein product (p17R) cloned into pUC18 (16). Three additional radiolabeled DNA target oligonucleotides, 101/102 (5′-CTGATCTTAAAGGAACTCTTCAATTTT-3′, 5′-CTGATCTTAAAGGAACTCTTCAATTTT-3′), 60/61 (5′-GAAGCTTTCATATGTC-3′, 5′-GAAGCTTTCATATGTC-3′), and 80/81 (5′-CAATTTCATATGTC-3′, 5′-CAATTTCATATGTC-3′) were prepared for mobility shift experiments. To form the double-stranded DNA, 40-μl reaction mixtures containing a 25 μM concentration of each oligonucleotide, 250 mM NaCl, and 10 mM Tris-HCl (pH 8) were placed in a 1.5-ml tube in a beaker of boiling water, and the water was allowed to come to room temperature over several hours. To prepare the DNA probes, approximately 200 ng of DNA was used in a kinase reaction consisting of T4 kinase (20 units), 30 μl of [γ-32P]ATP (3000 Ci/mmol), and 0.2 mM of dATP, dGTP, dCTP, and dTTP; 50 mM Tris-HCl, 1 mM ATP, 0.1 mM CTP, 0.02 mM UTP, 5 μCi of [γ-32P]UTP (3000 Ci/mmol), 9% polyvinyl alcohol, 5% glycerol, and approximately 1 μg of DNA template.

Analysis of Proteins

Protein concentrations were measured by the method of Bradford (19) using bovine serum albumin as a standard. Proteins were electrophoresed on discontinuous SDS-polyacrylamide gels and silver-stained.
performed in lane 8 GM 13258 (derivative of mouse A9 cells; human ovarian cancer; tumor cell line; lane 6). The reaction in lanes 5–9 additionally contained 3 μg of a cytoplasmic extract made from uninfected cells, and that of lane 4 additionally contained 3 μg of a nuclear extract from the same cells. The reactions of lanes 5–9 contained 3 μg of whole cell extracts made from Hi-5 insect cells (lane 5), STM 91-01 (a human malignant rhabdoid tumor cell line; lane 6), TTC 1240 (malignant rhabdoid tumor; lane 7), GM 13258 (derivative of mouse A9 cells; lane 8), and OVCAR3 cells (human ovarian cancer; lane 9). Standard transcription reactions were performed in 50 μl with approximately 1 μg of template.

and cytoplasmic compartments. Lanes 5–9 are reactions reconstituted with whole cell extracts made from various cell lines available in the laboratory. A factor complementing for VLTF-X is present in extracts from STM 91–01 and TTC 1240, both human malignant rhabdoid tumor cell lines, GM 13258 (lane 8, a derivative of mouse A9 cells), and OVCAR3 cells (lane 9, a human ovarian cancer cell line). In contrast, reactions reconstituted with extracts derived from the Trichoplusia ni (Hi-5) insect cell line only produced a smear of nonspecific products (lane 5). Thus, it appears as though a variety of mammalian cell lines contain this factor, as would be expected if this cellular protein is normally participating in vaccinia virus late transcription, since many different cell types are permissive for vaccinia virus infection.

**VLTF-X Purification from Uninfected and Infected Cells by Affinity Chromatography**

Our most recent studies have established that a factor that can provide VLTF-X activity is present in uninfected HeLa cells. As a first step toward distinguishing this factor from, or identifying it as, the complementing factor present in infected cells, we purified VLTF-X activity from both sources in order to compare their chromatographic profiles. In these experiments, cytoplasmic extracts from either infected or uninfected HeLa cells were partially purified by passage over a phosphocellulose column (11). Fractions containing VLTF-X activity were then passed over a double-stranded calf thymus DNA cellulose column to deplete the extracts of nonspecific DNA-binding proteins. Fractions containing VLTF-X activity were then applied to a vaccinia virus late promoter-coupled affinity resin. VLTF-X-containing fractions from this first affinity column were re-applied to the oligonucleotide affinity resin to obtain more concentrated factor with fewer impurities. In all cases, VLTF-X activity was measured both by mobility shift and transcription complementation reactions. Figs. 2, A and B, shows assays of fractions from the second oligonucleotide affinity column for both uninfected and infected cell extracts. In both cases, the mobility shift and corresponding transcription complementation activities were performed using 2 μl of flow-through fraction 1 (FT), 2 μl of 0.5 M fraction 1 (0.5M) or 2 μl of 0.2 M fraction 1 (0.2M). For the infected cell extract, transcription reactions in the last three lanes were performed using 2 μl of flow-through fraction 1, 2 μl of 0.2 M fraction 2, or 2 μl of 0.5 M fraction 1.

**Fig. 2.** Electrophoretic mobility shift assays, transcription assays, and SDS-polyacrylamide gel electrophoresis of fractions from the oligonucleotide affinity purification of VLTF-X from infected and uninfected HeLa cells. A and B, 3 μg of protein from the phosphocellulose 0.3 M fractions from either uninfected (A) or infected (B) cells was passed over a double-stranded DNA cellulose column, and flow-through fractions were collected and passed two times over a Sepharose resin to which an oligonucleotide containing a vaccinia virus late promoter had been coupled. Step elutions were performed with buffers containing 0.2, 0.5, and 1.0 M NaCl. Electrophoretic mobility shift assays were conducted in 20-μl volumes, with approximately 1 ng of 32P-labeled late promoter-containing DNA fragment as described under "Experimental Procedures." 3 μl of protein from the affinity column fractions was used along with 10 ng of poly(dl-dC)poly(dl-dC) as a nonspecific competitor. Fraction numbers are indicated above the lanes. Positions of the free (F) and bound (B) probes are indicated. The lane designated with a minus sign was a control in which protein was omitted. The specific transcription reactions were conducted as described for Fig. 1 and contained 1.7 μl of A1L protein, 2 μl of G8R protein, 2 μl of A2L protein, and 2.5 μl of RNA polymerase purified from infected cells. The positive/negative controls were as described for Fig. 1. For the infected extract, the transcription reactions in the last three lanes were performed using 2 μl of flow-through fraction 1 (FT), 2 μl of 0.2 M fraction 1 (0.2M) or 2 μl of 0.5 M fraction 1 (0.5M). For the infected cell extract, transcription reactions in the last three lanes were performed using 2 μl of flow-through fraction 1, 2 μl of 0.2 M fraction 2, or 2 μl of 0.5 M fraction 1. C, 30 μl of concentrated, affinity purified protein from uninfected (Uninf.) and infected cells (Infec.) were loaded onto a 12.5% SDS-polyacrylamide gel and subjected to electrophoresis. The gel was subsequently silver-stained using a Bio-Rad kit. The sizes of molecular weight markers are indicated at the left.
tion activity bound to the affinity column and eluted with 0.2 M NaCl. In no case could transcription activity be uncoupled from band shift activity. Thus, the factor present in infected and uninfected cell extracts has the same purification profile over phosphocellulose, double-stranded DNA cellulose, and oligonucleotide affinity columns. The most pure fractions from the oligonucleotide affinity columns were concentrated 10-fold, subjected to SDS-polyacrylamide gel electrophoresis, and silver-stained (Fig. 2C). This analysis revealed several faint bands of which common, approximately 35, 40, and 55-kDa bands were visible.

Biochemical Characterization of Affinity-purified VLTF-X from Infected and Uninfected Cell Extracts

Specificity of DNA Binding—The specificity of late promoter binding by affinity-pure VLTF-X from both cell sources was tested by performing assays in which early or late vaccinia virus promoter-containing DNA fragments were allowed to compete with the labeled late promoter probe (Fig. 3A and B). As previously observed with VLTF-X purified by conventional chromatography from infected cells (14), the early promoter-containing competitor did not affect the shifted complexes when present at 10 ng (a 58-fold molar excess over the target probe). The complexes were only partially inhibited at 20 ng of early oligonucleotide (a 116-fold molar excess). In contrast, the late promoter-containing DNA oligonucleotide almost completely inhibited complex formation at 10 ng, a 66-fold molar excess over the target probe.

Thermostability, Divalent Cation, and ATP Effects on Protein Binding—In a further effort to distinguish the purified infected and uninfected cell proteins from one another, the DNA-protein complexes from each preparation were subjected to a variety of biochemical tests. The influences of ATP, MgCl₂, and heat on the stability of complex formation were investigated. Fig. 4A and B, show the effects of different MgCl₂ and ATP concentrations on the binding of VLTF-X to promoter-containing DNA. VLTF-X bound to DNA in the presence of up to 20 mM MgCl₂ and 1 mM ATP. Heat treatment of affinity-purified VLTF-X-DNA complexes from both cell sources at 30, 37, 47, 65, and 100 °C for 10 min showed that the complexes were stable even at temperatures of 100 °C for 10 min (Fig. 4C).

Fig. 3. A and B, electrophoretic mobility shift assays with affinity-purified VLTF-X from uninfected and infected cells. Each reaction mixture contained 1 μl of VLTF-X purified from uninfected cells or 4 μl of VLTF-X purified from infected cells and the indicated amount of competitor (in nanograms) with the exception of lane 1, which contained only the target DNA and no competitor. Twenty nanograms of early competitor was approximately a 116-fold molar excess over the target DNA, and 20 ng of late oligonucleotide was approximately a 132-fold molar excess over the target DNA. The positions of free (F) and bound (B) probes are indicated. The lane designated with a minus sign is a control in which protein was omitted.

Fig. 4. Electrophoretic mobility shift assays to determine the effect of ATP, MgCl₂, and temperature on affinity-purified VLTF-X from uninfected and infected cells. A and B, each reaction mixture contained 1 μl of VLTF-X purified from uninfected cells, 2 μl of VLTF-X purified from infected cells, 10 ng of poly(dI-dC)·poly(dI-dC), and the indicated amount of ATP or MgCl₂. C, each reaction contained 3 μl of VLTF-X purified from uninfected cells, 2 μl of VLTF-X purified from infected cells, and 10 ng of poly(dI-dC)·poly(dI-dC). The mixtures were incubated at the indicated temperatures for 10 min prior to electrophoresis. Mobility shift assays were conducted as described previously for Fig. 2. Autoradiograms of the gels are shown.
UV Cross-linking Demonstrates That 35- and 55-kDa Proteins in both Uninfected and Infected Cell Extracts Bind to a Viral Late Promoter

The experiments performed above indicated that a protein with identical late promoter DNA binding specificity and biochemical properties is present in uninfected and infected cells. To confirm that the same protein(s) from infected and uninfected cells is binding to viral late promoters, a UV cross-linking experiment was performed. A uniformly $^{32}$P-labeled late promoter-containing DNA fragment was incubated with crude infected or uninfected 0.3 M phosphocellulose extracts under band shift conditions. Transcription reactions performed with these extracts confirmed that the infected cell preparation contained VLTF-X and viral RNA polymerase activity and that the uninfected cell preparation contained only VLTF-X activity (data not shown). The complexes were irradiated with UV light, digested with DNase I, and subjected to SDS-polyacrylamide gel electrophoresis. Fig. 5A demonstrates that an approximately 35-kDa protein was the most prominent protein cross-linked in both extracts with a less prominent species of approximately 55-kDa also visible (lanes 1 and 6). The specificity of this cross-linking was verified by performing a competition experiment using vaccinia virus early or late promoter-containing oligonucleotides as competitors as described previously (14). The addition of up to an approximately 6000-fold molar excess of early promoter-containing oligonucleotide had no effect on either cross-linked species (lanes 2, 3, 7, and 8). In contrast, the late promoter-containing DNA inhibited complex formation of both cross-linked proteins (lanes 4, 5, 9, and 10). To correlate the cross-linking results with our previous observations, 5-μl aliquots of the above reaction mixtures were also analyzed on a mobility shift gel (Fig. 5B). The DNA binding activities of both types of extracts correlated with the above observations showing the protein-DNA complexes being relatively unaffected by an approximately 6000-fold molar excess of early oligonucleotide but almost completely eliminated by a 3000-fold molar excess of late oligonucleotide.

Glycerol Gradient Sedimentation Analysis

Previous glycerol gradient sedimentation analysis of VLTF-X purified from infected cells showed that the sedimentation coefficient of this factor was consistent with a protein with a mass of approximately 32–38 kDa (11). To estimate the mass of VLTF-X affinity-purified from uninfected cells, the affinity-purified protein was sedimented through a 15–35% glycerol gradient. Gradient fractions were assayed for VLTF-X activity by mobility shift assays (Fig. 6). The sedimentation coefficient was calculated to be 3.0 S by comparison of the sedimentation position of marker proteins on parallel gradients as described under “Experimental Procedures.” Using the Martin and Ames equation (20), the approximate molecular mass of VLTF-X from uninfected cells was calculated to be between 35 and 40 kDa. Thus, the molecular mass determination of affinity-purified VLTF-X from uninfected cells is consistent with that of the infected cell VLTF-X (11).

**FIG. 5.** SDS-polyacrylamide gel electrophoresis (A) of extracts from uninfected and infected cells UV-cross-linked to a late promoter-containing probe and electrophoretic mobility shift assay (B). A, a uniformly $^{32}$P-labeled late promoter-containing probe was incubated with 3 μg of phosphocellulose 0.3 M extracts from uninfected and infected cells in the presence of increasing amounts of vaccinia virus early or late promoter-containing DNA. The DNA competitor and the amounts used are indicated above the lanes. The reactions were conducted in 20-μl volumes under mobility shift conditions as described in Fig. 2 and contained 50 ng of poly(dI-dC)poly(dI-dC) as a nonspecific competitor. 10-μl aliquots of the mixtures were UV-cross-linked and treated with 2 units of DNase I, and the $^{32}$P-tagged proteins were fractionated on a 12.5% SDS-polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane and visualized by autoradiography. The positions of the protein molecular mass markers are indicated on the left. The positions of the 35- and 55-kDa cross-linked bands are indicated by arrows. B, 5-μl aliquots of the above reaction mixtures were subjected to mobility shift analysis prior to the UV cross-linking reaction as described for Fig. 2. The positions of the free (F) and bound (B) probes are shown. The lane designated with a minus sign is a control lacking protein. An autoradiogram of the gel is shown.

**FIG. 6.** Electrophoretic mobility shift assays with fractions from a glycerol gradient loaded with VLTF-X affinity-purified from uninfected cells. Reactions were conducted in 20-μl volumes and contained 10 μl of fractions from the glycerol gradient and 10 ng of poly(dI-dC)poly(dI-dC). Mobility shift assays were conducted as described previously for Fig. 2. The positions of free (F) and bound (B) probe are shown. The positions of the marker proteins in a parallel gradient are indicated by the arrows above the gel. The lane designated with a minus sign is a control lacking protein.
To further understand the biochemical role of VLTF-X in late promoter recognition, studies were conducted to determine the DNA residues that are critical for protein binding. Fig. 7A shows the sequence of a portion of the late promoter fragment (273 bp) currently being used as a probe as well as three additional late promoter-containing DNA fragments designated 60/61, 60/61 M, and 101/102. The 60/61 oligonucleotide is similar to the portion of the probe between residues -31 and +11 and has been shown in vivo to function as a strong late promoter (21). The 60/61 M oligonucleotide is essentially the same as oligonucleotide 60/61 except for a single mutation in the TAAAT sequence motif common to all vaccinia virus late promoters is highly conserved and essential for transcription of late genes in vivo (21, 22). Also, a promoter containing a TGAAT mutation was previously tested in the in vitro transcription system and shown to be nonfunctional (16). In vivo studies have also shown that an A/T-rich tract within approximately 30 bp upstream of the TAAAT sequence is also necessary for late promoter function (21–23). The 101/102 oligonucleotide contains a portion of the late promoter region excluding the upstream tract of T residues. Previous studies have shown that the TAAAT sequence motif common to all vaccinia virus late promoters is highly conserved and essential for transcription of late genes in vivo (21, 22). Also, a promoter containing a TGAAT mutation was previously tested in the in vitro transcription system and shown to be nonfunctional (16). In vivo studies have also shown that an A/T-rich tract within approximately 30 bp upstream of the TAAAT sequence is also necessary for late promoter function (21–23). The 101/102 oligonucleotide contains the late promoter region from −9 to +18; the main difference between this and the 60/61 oligonucleotide is that the latter contains the upstream tract of T residues. In preliminary studies (Fig. 7B), mobility shift assays conducted with phosphocellulose II-purified VLTF-X (14) and the indicated amount of nonspecific competitor (poly(dI-dC)·poly(dI-dC)). The target DNA used is indicated below the lanes of the gel. D, electrophoretic mobility shift reactions contained 2 μl of VLTF-X affinity-purified from uninfected cells, 10 ng of poly(dI-dC)·poly(dI-dC), and the indicated amount of single stranded DNA competitor. The target DNA was the full-length 273-bp probe. Twenty nanograms of single-stranded oligonucleotide was an approximately 162-fold molar excess over the target DNA. All mobility shift assays were conducted as described for Fig. 2.
To determine whether the TGAAT mutation affects the binding of VLTF-X to the DNA fragment, affinity-purified VLTF-X from uninfected cells was allowed to complex with 60/61 and 60/61 M oligonucleotides (Fig. 7C). VLTF-X bound to the wild-type and mutant probes with equal affinity and had an activity that was resistant to over 1000 ng of poly(dI-dC)poly(dI-dC).

Further competition experiments were conducted with the single-stranded oligonucleotides (60 or 61), used to form the 60/61 duplex to determine if affinity-purified VLTF-X binds to single-stranded DNA. In Fig. 7D, affinity-purified VLTF-X from uninfected cells was incubated with full-length target DNA and competitor single-stranded 60- or 61-oligonucleotides. Protein-DNA complexes were unaffected by a calculated 132-fold molar excess of either of the single strands of DNA.

**DISCUSSION**

We have previously described a factor present in crude, uninfected cell cytoplasmic extracts that could complement for the vaccinia virus late transcription factor designated VLTF-X. It was also found that a late promoter DNA binding activity cochromatographed and cosedimented with VLTF-X activity. These findings suggested that a cellular factor is needed to support vaccinia virus late transcription. In the present report, we have purified VLTF-X from uninfected and infected HeLa cell extracts in order to determine whether the factor present in both sources is indeed the same. The late transcription complementing factor from both infected and uninfected cells bound to a late promoter-containing DNA oligonucleotide affinity resin and eluted at the same salt concentration. These affinity-pure VLTF-X fractions were subjected to a variety of biochemical tests in an effort to determine if the proteins from these extracts were the same. Competition experiments demonstrated that an oligonucleotide containing a vaccinia virus late promoter sequence was much more effective in blocking the observed DNA-protein interaction of both factors than an oligonucleotide containing an early promoter. Steady-state levels of the complexes with both factors were unaffected by the presence of ATP or MgCl2, similar to the results seen with VETF (24, 25). We have also found that VLTF-X-DNA complexes from both cell sources were remarkably thermostable; DNA binding was only marginally inhibited when the complexes were subjected to boiling for 10 min. Finally, we have now confirmed that VLTF-X purified from uninfected cells has a similar mass as that purified from infected cells as estimated by glycerol gradient sedimentation.

To examine whether the same proteins from the uninfected and infected extracts were binding to the late promoter, a UV cross-linking experiment was performed. The preponderance of thymidine residues in the vaccinia virus late promoter region and the reactive nature of these residues in the presence of UV light (26) suggested that special reagents would not be necessary for adequate transfer of label from the DNA to the protein. Using this technique, approximately 35- and 55-kDa radioactivity were detected in uninfected and infected cell extracts. Both species bound specifically to the late promoter DNA fragment, since the presence of the labeled bands were competed with added unlabeled late promoter-containing oligonucleotide but not by comparable amounts of early promoter-containing oligonucleotide. Thus, while it is not possible at this time to tell which of these bands may be VLTF-X or what their relationship to each other may be, the results of this experiment are consistent with the premise that the factors from infected and uninfected cells are indistinguishable.

The indistinguishable DNA binding specificity, biochemical characteristics, and size of the proteins in infected and uninfected cells have led us to conclude that VLTF-X is a unique late transcription factor most likely encoded by a cellular gene. In this regard, we have found VLTF-X activity in a variety of mammalian cell lines but not in a cell line derived from insect cells. This result is as would be expected if a cellular protein is normally participating in viral late transcription, since vaccinia virus can replicate in a wide variety of cells.

We have also conducted a preliminary analysis to determine the sequences within the late promoter that are critical for DNA-protein interaction. Although mutations within the TAAAT sequence common to vaccinia virus late promoters dramatically reduce the level of transcription in vivo (21, 22) and in vitro (16), no effect on the interaction of VLTF-X with a late promoter containing a TGAAT mutation was observed. This result suggests that this sequence must have functions other than in the interaction with VLTF-X. We did, however, observe that a promoter lacking the upstream tract of T residues seems to be necessary for late promoter function in vivo did not bind VLTF-X, suggesting that these residues may play a role in factor binding or recruitment to the promoter.

In accordance with our results, recent studies by Zhu et al. (27) have identified a factor termed LPBP (for late promoter-binding protein) in uninfected HeLa cells that binds to vaccinia virus late promoters and stimulates late transcription in vitro. The binding of this factor to late promoters was also unaffected by a mutation in the TAAAT sequence. Considering the size and purification properties of this factor are similar to those of VLTF-X, we consider it likely that the two factors are identical.

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