Vaccinia Virus Late Transcription Is Activated in Vitro by Cellular Heterogeneous Nuclear Ribonucleoproteins*

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Vaccinia virus gene expression is temporally regulated, and three gene classes have been identified: early, intermediate, and late. Several virus-encoded proteins and an activity designated VLTF-X are required for maximum transcription in vitro of a template containing a late promoter. VLTF-X is present in both cytoplasmic and nuclear extracts prepared from uninfected mammalian cells and co-purifies with a late promoter DNA-binding activity. Here, extensive purification of VLTF-X has revealed that heterogeneous nuclear ribonucleoproteins A2/B1 and RBM3 co-purified with in vitro late transcription stimulation. Overexpression and purification of these proteins from Escherichia coli demonstrated that they both complemented for VLTF-X activity in vitro transcription reactions. These studies identify two host cell factors potentially contributing to poxvirus replication in vitro.

The heterogeneous nuclear ribonucleoproteins (hnRNPs) are a family of single-stranded nucleic acid-binding proteins involved in a variety of cellular functions including mRNA splicing, transport, and turnover (1). Approximately 20 major hnRNPs are known, and they are designated hnRNP A1 (the smallest at 34 kDa) to hnRNP U (the largest at 120 kDa). The most well studied member of this family, hnRNP A1, has been implicated in determining splice site selection of cellular mRNAs. The protein is predominantly nuclear but has been shown to shuttle between the nucleus and the cytoplasm, presumably as a chaperone for mRNA export from the nucleus (2). In addition to its role in cellular mRNA biogenesis, hnRNP A1 has been shown to bind to mouse hepatitis virus template RNA and has been implicated in the replication of this RNA virus (3), although its role in the mouse hepatitis virus life cycle has recently been challenged (4). hnRNP A2 is a closely related member of this family and, similar to hnRNP A1, is a modular protein containing two N-terminal RNA binding domains (RBDs) and a C-terminal glycine-rich domain implicated in protein-protein interactions (2XRBD-gly). hnRNP A1 and A2 are ~80% identical in the N-terminal 2XRBD domain (5), and the genes encoding these proteins presumably arose from a gene duplication event (6). The genes for both proteins encode RNAs that can be alternatively spliced; the alternative product to hnRNP A2 is designated hnRNP B1 and is identical to hnRNP A2 but with 12 additional amino acids at the extreme N terminus (5). RBM3 is a more recently described hnRNP closely related to hnRNP G but having only one RBD and a glycine-rich tail (7).

The poxviruses are DNA-containing viruses that replicate in the cytoplasm of eukaryotic cells and are pathogenic to many animal species. Gene expression in vaccinia virus, the prototypic member of the poxvirus family, is temporally regulated and can be divided into early, intermediate, and late phases. All three phases of gene expression rely on virally encoded factors and a viral multisubunit RNA polymerase with homology to eukaryotic RNA polymerase II. Transcription of the late genes requires a number of viral factors including the products of the A1L, A2L, and G8R genes (8–13). In addition, we have previously identified and partially purified a factor termed VLTF-X that is also required for late transcription (14, 15). Transcription complementation assays were used to demonstrate that VLTF-X activity is present in the cytoplasm and nucleus of uninfected HeLa cells, leading to the hypothesis that VLTF-X, unlike the other known late transcription factors, is a factor provided by the host cell. Also, a late promoter DNA binding activity co-purified with VLTF-X, suggesting that a biochemical role of this factor may be in late promoter recognition (15, 16).

In this study, we have further purified VLTF-X from uninfected HeLa cells and identified hnRNP A2 and RBM3 as two proteins that co-purified with VLTF-X activity. Both of these proteins were expressed and purified from Escherichia coli, and both were found to independently stimulate viral late transcription in vitro. The hnRNPA1 protein, on the other hand, did not have this activity. These results provide evidence that members of the hnRNP family may play a role in poxvirus transcription. Understanding this virus-host interaction is likely to give new insights into poxvirus replication and tropism as well as revealing novel functions for these cellular proteins.

**EXPERIMENTAL PROCEDURES**

Extract Preparation and Protein Purification—To prepare VLTF-X, 35 liters of uninfected HeLa cells were harvested and processed for cytoplastic extracts as previously described (17). The nuclei were also processed into an extract as previously described (18). The cytoplastic extract (1.2 g of total protein) was adjusted to 0.25 M NaCl in buffer A (50 mM Tris (pH 8), 0.4 mM EDTA, 2 mM dithiothreitol, 0.01% IGEPAL (Sigma), and 10% glycerol) and passed over a 2.5 × 22-cm DEAE-cellulose column equilibrated in the same buffer. The flow-through from the column was collected (875 mg of total protein) and applied to a 12 × 1.5-cm single-stranded DNA cellulose (Sigma) column equilibrated in buffer A containing 0.25 M NaCl. The column was washed with 2 bed volumes of buffer and eluted with a 10-bed volume 0.25–1.0 M linear

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§ The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; RBD, RNA binding domain; PCR, polymerase chain reaction.
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NaCl gradient. Fractions were collected, and selected fractions were tested for transcription complementation after dialysis against buffer A containing 0.1 M NaCl. All fractions containing the peak of transcription activity were pooled (5.6 mg of total protein) and dialyzed against buffer A containing 0.05 M NaCl and applied to a 4-ml poly(U)-agarose (Sigma) column equilibrated with buffer A. The column was washed with 2.5 bed volumes of buffer A, 0.1 M NaCl and eluted with a 10-bed volume gradient of 0.1–1.0 M NaCl. Selected fractions were dialyzed in buffer A, 0.1 M NaCl and tested in transcription complementation reactions. Active fractions were pooled, dialyzed against buffer A, 0.1 M NaCl, and applied to a 2-ml heparin-agarose (Life Technologies, Inc.) column equilibrated with buffer A. The column was washed with 3 bed volumes of buffer and eluted with a 10-bed volume 0.1–0.3 M NaCl gradient. Selected fractions were dialyzed, concentrated by ultrafiltration, and tested in vitro transcription reactions.

The GSR and AIL proteins were purified from the extracts of recombinant baculovirus-infected Sf9 cells as previously described (14). Vaccinia virus RNA polymerase was purified from vaccinia virus-infected HeLa cells as previously described (14). The A2L protein was purified from baculovirus-infected Sf9 cells as previously described (8) or from *E. coli* strain BL21(DE3)pLysS that had been transformed with a pET3a (Novagen, Madison, WI) vector containing the A2L open reading frame in frame with a tract of 6 histidine residues present on the vector was identified by restriction and sequence analysis and was used to transform *E. coli* strain BL21(DE3)pLysS. Protein induction, purification, and identification from bacteria were performed as described above for the hnRNP A2 protein.

The hnRNP A1 protein was cloned by reverse transcription of HeLa cell RNA and amplification of the cDNA product with the primers 5′-GGG GGA TAC ATG TCT AAG TCA GAG-3′ and 5′-GGG GAA TTC TTA AAA TCT TCT GCC-3′ as described for RBM3 except that the annealing temperature for the PCR was 55 °C. The PCR product was digested with EcoRI and BamHI, and the ends were filled in as described above. This product was cloned into the pET3a vector (Novagen) that had been digested with NdeI and filled in. The recombinant vector was used to transform *E. coli* strain BL21(DE3)pLysS, and protein induction and lyase preparation were performed as for the A2L protein. The hnRNP A1 protein was purified by sequential chromatography over DEAE-cellulose, phosphocellulose, and single-stranded DNA cellulose column.

Identification of VLTF-X—To identify the proteins present in the transcriptionally active material, aliquots from fraction 29 from the heparin-agarose column were subjected to electrophoresis on a 10% SDS-polyacrylamide gel that was stained with Coomassie Blue. The protein bands present at ~20 and ~35 kDa were excised from the gel, trypsinized (in the gel matrix), and subjected to liquid chromatography/mass spectrometry on a Hewlett Packard 1100 HPLC and a Finningan quadrupole mass spectrometer. The resulting peptide fragmentation patterns were analyzed using the program SEQUEST (19) on an Aspens Systems BlackHawk 5 computer. This analysis identified two peptides from the 20-kDa protein, AMNGFQGDSR and GQGPDGT GGPGGGNGFQG GRE, that were identified for the 35-kDa band as belonging to heterogeneous nuclear ribonucleoproteins A2/B1 (GenBank′ accession number P22626).

Specific Transcription Reactions—Late promoter-specific in vitro transcription reactions were conducted in 50-μl final volumes as previously described (14), except that in some instances [α-32P]CTP was used in place of [α-32P]UTP with a concomitant decrease in UTP concentration from 0.1 to 0.02 mM and an increase in UTP from 0.02 to 0.1 mM in the final reaction.

Western Blot Analysis—Proteins were separated by electrophoresis on SDS-polyacrylamide gels and transferred to Immobilon-P (Millipore Corp., Bedford, MA) in a buffer containing 10% methanol, 0.025 M Tris, 0.19 M glycine, and 0.005% SDS. After transfer, filters were blocked with 5% nonfat dry milk in PBS for 1 h at room temperature. The filter was washed again and then incubated with the primary antibody for 1 h at room temperature. The filter was washed again and then incubated with peroxidase-conjugated anti-mouse antibody (Amersham Pharmacia Biotech catalog no. NA9310) diluted 1:4000 in PBS/milk for 45 min at room temperature. The filters were then washed and developed using the Amersham Enhanced Chemiluminescent Western blotting kit.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were performed as previously described (15) using ~0.4 ng/reaction of 32P-labeled target DNA, which was a double-stranded oligonucleotide made by annealing the oligonucleotides 5′-AAG GAT CCT TTT TGT TTT TTT CTA TGC TAT AAA TAA CCC CTG CCT TCC TCC CGG GAA CCC GC' and 5′-CCG AAT TCC CGG GAA GAG AA GAG GAT TTA TAG CAT AGA AAA AAA CAA AAA GGA TCC TCT TT-3′. This oligonucleotide contains the promoter region of the vaccinia virus late gene F17R and was also used as the late competitor oligonucleotide in the experiments of Fig. 7. The early promoter-containing oligonucleotide used in Fig. 7 contains the vaccinia virus VGF early gene promoter, and the sequence has been previously reported (15).

RESULTS

Purification of VLTF-X—Our previous results have established that a factor present in uninfected HeLa cells, provisionally named VLTF-X, can stimulate vaccinia virus late transcription in vitro (14–16). In order to identify and characterize an extract prepared from 35 liters of HeLa cells was purified using an empirically derived purification scheme consisting of sequential chromatography on DEAE-cellulose, single-stranded DNA cellulose, poly(U)-agarose, and heparin-agarose. In all cases, the presence of VLTF-X was monitored by assaying fractions for the ability to complement vaccinia virus late tran...
scription factors in *in vitro* transcription assays using a G-less cassette template (20) under the control of a vaccinia virus late promoter (Fig. 1). This scheme resulted in highly purified fractions in which only a few protein bands were visible by Coomassie Blue staining of SDS-polyacrylamide gels on which the heparin agarose fractions were run (Fig. 2). Two bands representing proteins of 20 and 35 kDa that co-purified with VLTF-X activity throughout the purification scheme were excised from a gel, trypsinized in the gel matrix, and subjected to liquid chromatography coupled with mass spectrometry. The peptides produced by this procedure were separated and sequenced by tandem mass spectrometry and compared with nucleotide data bases in order to identify the parental proteins. This endeavor identified the 20-kDa protein as RBM3 and the 35-kDa protein as heterogenous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1).

**Heterologous Expression of Proteins**—In order to determine if either RBM3 or hnRNP A2 could stimulate vaccinia virus late transcription *in vitro*, both proteins were expressed as histidine-tagged fusions and purified from *E. coli*. In addition, hnRNP A1 was similarly synthesized in *E. coli* but not histidine-tagged. All of the proteins were purified by binding to single-stranded DNA cellulose, and Fig. 3A shows a Coomassie Blue-stained gel of the purified proteins. For comparison, VLTF-X purified from HeLa cells is also shown on this gel. The RBM3 and hnRNP A1 proteins were expressed to high levels in the bacteria and purified to near homogeneity. The hnRNP A2 protein was not expressed as highly and eluted with contaminants, some of which can be seen in the RBM3 fraction as well (Fig. 3A). A Western blot analysis of the hnRNP A2 and RBM3 proteins using an anti-histidine antibody confirmed the presence of appropriately sized histidine tract-containing proteins in each sample (Fig. 3B).

**In Vitro Transcription with Recombinant Proteins**—The hnRNP A2, RBM3, and hnRNP A1 proteins were tested in *in vitro* transcription reactions to see whether any of them could complement for VLTF-X activity. The results of these reactions are shown in Fig. 4 and demonstrate that both hnRNP A2 (lanes 2 and 3) and RBM3 (lanes 4 and 5) stimulated late transcription *in vitro*. The hnRNP A1 protein, on the other hand, had only a very slightly detectable level of activity (lanes 6 and 7).

In Fig. 5 the activity of the recombinant hnRNP A2 and RBM3 proteins in *in vitro* transcription reactions is compared with fractions of VLTF-X purified from HeLa cells. On the *left side* of the figure (lanes 1–8), the template was an uncut plas-
mid containing a vaccinia virus late promoter, which was the template used in the standard assay in the purification of VLTF-X. Tested in this figure are the crude nuclear (NE) and cytoplasmic (CE) extracts prepared from HeLa cells, the pooled active fractions from the single-stranded DNA cellulose column (ss; see Fig. 1), and a fraction from the poly(U)-agarose column (pU). The control lane (lane 8) shows a reaction performed with an extract purified from E. coli that had been transformed with an empty pET15b vector and induced. This figure demonstrates again that the hnrNPA2 and RMB3 proteins, but not the control E. coli extract, stimulated late transcription and that the transcription products they produced were comparable in both size and amount to poly(U)-agarose-purified VLTF-X from HeLa cells. However, the figure also shows that the crude extract preparations from HeLa cells produced transcripts that appeared less discrete than those produced from the more highly purified proteins. In order to determine if this was perhaps read-through transcription continuing into the vector sequences from the G-less cassette, a template that had been linearized immediately 3' to the G-less cassette sequence was also tested with all of the fractions. With this template, the nuclear, cytoplasmic, and single-stranded DNA extracts produced more discrete transcripts that were the size of those produced by the purified proteins on the uncut template. Surprisingly, however, the poly(U) fraction and the hnrNPA2 and RMB3 proteins had minimal to no detectable activity on the linearized template. The possible reason for this result will be discussed below (see "Discussion").

**Western Blot Analysis for hnrNPA2**—The availability of a monoclonal antibody to hnrNPA2/B1 (21) allowed us to examine whether there was a correlation between the presence of these proteins and transcription activity in various fractions purified from cells. Fig. 6A shows a Western blot analysis of fractions from the purification of VLTF-X from HeLa cells. A doublet, most likely representing the presence of both hnrNPA2 and hnrNPA2/B1, is present in both the nuclear and cytoplasmic extracts of HeLa cells. A dilution series performed on these extracts demonstrated that these proteins were ~5-fold more abundant (per mg of total protein) in the nuclear extract (data not shown). The presence of hnrNPA2/B1 co-purified with transcription activity (see Fig. 1) across the single-stranded DNA cellulose and poly(U)-agarose columns. Similarly, the RMB3 protein, which could be tracked by Coomassie Blue staining of the protein fractions from the columns, also co-purified with transcription activity across these columns (data not shown). In the heparin-agarose fractions, it is apparent that both proteins are present in fractions past the transcription peak, suggesting that perhaps the proteins were becoming denatured during the purification process. Fig. 6B shows a Western blot analysis of whole cell extracts derived from a variety of cell lines that were previously tested for VLTF-X complementation activity (16). Briefly, we previously found that the mammalian cell lines STM91–01 (S7), T7T1240 (T7), GM13258 (GM), and OVCAR3 (OV) appeared to have VLTF-X, but the Hi-5 insect cell line did not. It is apparent from Fig. 6B
subjected to electrophoresis in parallel with the samples are designated as the primary antibody. The secondary antibody and blot visualization were performed as in Fig. 3B loaded onto 10% SDS-polyacrylamide gels, blotted, and incubated with a 1:800 dilution of an anti-hnRNP A2/B1 mouse monoclonal antibody (21). The recombinant hnRNP A2 and RBM3 proteins that had been highly purified by binding to both single-stranded DNA cellulose and a nickel affinity column were therefore tested in electrophoretic mobility shift assays. In Fig. 7, binding of both proteins to radiolabeled substrate DNA containing a late promoter sequence was examined. Both hnRNP A2 and RBM3 bound to the probe (lanes 1 and 9), resulting in multiple shifted species in both cases. Competition reactions performed with the late promoter-containing DNA (lanes 2–4 and 10–12) or with an oligonucleotide of similar length and base composition, but containing a vaccinia virus early promoter sequence (lanes 5–7 and 13–15), demonstrated that both oligonucleotides effectively competed with the probe DNA. Mixing the hnRNP A2 and RBM3 proteins did not result in the production of additional shifted species, nor did it appear to increase the specificity of the DNA-binding reactions (data not shown). Similarly, we have tested fractions from the single-stranded DNA cellulose, poly(U)-agarose, and heparin-agarose columns in mobility shift analyses using the late promoter (data not shown). While a variety of DNA binding activities were observed, thus far none of them have exhibited the markedly better competition with the late promoter-containing probe that we previously observed with cell extracts acquired via alternative purification schemes (15, 16). Whether this late promoter-specific binding activity can be reconstituted by a combination of factors once they have all been identified remains to be determined.

**DISCUSSION**

We have identified hnRNP A2 and RBM3 as potential cellular factors participating in vaccinia virus late transcription by three criteria: 1) both proteins have been identified by mass spectrometry in transcriptionally active highly purified fractions from HeLa cells; 2) both proteins stimulate late transcription in vitro when expressed separately and purified from bacterial cells, and 3) both proteins co-purified with late transcription complementation activity as demonstrated by Western blot analyses for hnRNP A2 and by Coomassie Blue staining of gels for RBM3.

The hnRNP A2 protein is an RNA and single-stranded DNA binding protein structurally similar to hnRNP A1. However, hnRNP A1 did not stimulate vaccinia virus late transcription in vitro to an appreciable degree. These two proteins are 80% identical in the N-terminal 2XRBD domain, but this identity drops to 30% in the glycine-rich C terminus may play a role in mediating protein-protein interactions specific for the viral transcription machinery. The exact cellular role of the hnRNP A2/B1 proteins is not clear. They are widely expressed in different tissues and are found predominantly in the nucleus in most normal tissues (21), although...
they become overexpressed and cytoplasmically distributed in some lung cancers such that they are being used as diagnostic markers of early disease (22–24). Inhibition of RNA polymerase II transcription with actinomycin D causes both proteins to accumulate in the cytoplasm suggesting that, like hnRNP A1, these proteins are also transcription-dependent shuttling hnRNP proteins (21). This has implications with regard to the potential role of these proteins in viral replication as vaccinia virus infection is known to inhibit RNA polymerase II transcription (25, 26), the net effect of which may be to cause these proteins to relocate to the cytoplasm, potentially enhancing viral transcription.

RBM3 is a more recently identified protein whose gene is located on the X chromosome and is proposed to be a member of the hnRNP family based on a BLASTX analysis of its sequence. This analysis revealed a close similarity to two human RNA-binding proteins, YRRM (also designated RBMY) and hnRNP G (7). RBMY is implicated as a factor important for spermatogenesis and is located on the Y chromosome (27). hnRNP G is encoded by the gene RRMX, which is the X chromosome homolog of RBMY (28). A Northern blot analysis revealed that RBM3 was expressed in a wide variety of human fetal tissues (7) and, in this regard, is similar to the hnRNP A2/B1 proteins. Our results have functionally demonstrated for the first time that the RBM3 protein is indeed capable of binding to both RNA and DNA.

The results of this study beg the question as to how cellular proteins mostly known for roles in mRNA biogenesis participate in the transcription of a DNA virus. It is known that several members of the hnRNP family have dual roles and also can directly bind to DNA regulatory elements to act as transcription factors. For example, hnRNP K is a transcription factor for the c-myc gene (29), and hnRNP A1 has been shown to bind to the human thymidine kinase promoter and negatively regulates expression of this gene (30). In the case of hnRNP K, it has been shown that the protein binds to a poly-pyrimidine tract (CT element) in the c-myc promoter (31). This region reacts with single-strand-specific chemical and enzymatic probes in vivo, and hnRNP K will not bind to this element unless it is present on negatively supercoiled DNA (32). This suggests that this DNA element may adopt a single-stranded or extruded conformation in vivo, as has been found for many promoters rich in polyurine or polypyrimidine tracts (33). Interestingly, it has been shown that one element of a vaccinia virus late promoter is a poly(T) tract and that abolishing this element dramatically reduces transcription (34–36). Extrapolating from the hnRNP K analogy, it is possible that hnRNP A2 and/or RBM3 do bind to this poly-T tract. However, these proteins may not be able to stimulate transcription from a linear template. Perhaps this region must be present on supercoiled DNA to provide the energy for melting that allows these proteins to bind and transcribe DNA efficiently. The DNA binding and competition studies of Fig. 7 demonstrate that both recombinant proteins do bind to double-stranded DNA containing a vaccinia virus late promoter, but this activity did not have the specificity seen previously with the more crude extracts purified from HeLa cells. Therefore, the relevance of the in vitro binding of these proteins to linear probes is yet to be determined, since they do not stimulate in vitro transcription from linear templates. One interpretation of these experiments is that yet another cellular factor may be required to stimulate vaccinia virus late transcription. Fig. 5 demonstrates that the ability of the reconstituted system to support late transcription from linear templates is apparently lost during chromatography of cellular extracts on poly(U)-agarose. Therefore, another factor, eluting elsewhere on this column, may be needed to aid in the sequence specificity of DNA binding or in melting of the DNA template. This activity is apparently not required for supercoiled templates in vitro but may be necessary to activate transcription from late promoters as they occur in vivo.

It should also be considered that hnRNP A2 and/or RBM3 may have a role in transcription apart from a DNA binding activity. It is possible that they bind to nascent RNA and stimulate transcription elongation by contacting the rest of the transcription machinery from this RNA-bound location, as has been demonstrated for the HIV Tat protein (37). Alternatively, they may function by binding to DNA to initiate transcription and then binding to nascent RNA to affect the turnover or translation of the subsequently produced mRNAs. The coupling of transcription of cellular mRNAs and the translational fate of these messages is a level of regulation being appreciated with increasing frequency in eukaryotic cells (38, 39).

In summary, in this paper we have demonstrated that two cellular proteins, hnRNP A2 and RBM3, can stimulate vaccinia virus late transcription in vitro. The hnRNP A2 protein, in particular, is consistent with previous observations regarding VLTF-X. Our previous glycerol gradient sedimentation results suggested that VLTF-X from uninfected cells was between 35 and 40 kDa and that it was located in both the nucleus and cytoplasm (16). However, given the resolution limitation of the sedimentation analysis, the molecular mass of RBM3 would not be entirely inconsistent with these results. Immunoprecipitation experiments can be performed when antibodies to both proteins are available, and the kinetics of viral replication can be analyzed when cell lines are developed that alter the levels of the proteins. Then we can determine if either, or both, of these proteins actually serves the physiologic role of aiding vaccinia virus transcription in vitro.

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