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The Vaccinia Virus H5R Gene Encodes Late Gene Transcription Factor 4: Purification, Cloning, and Overexpression

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The vaccinia virus late stage-specific transcription factor P3 was purified to homogeneity from HeLa cells that were infected in the presence of an inhibitor of viral DNA replication. The purified 36-kDa protein was digested with trypsin, and the peptides were analyzed by mass spectroscopy and amino-terminal sequencing. The purified factor was identified as the product of the vaccinia virus H5R open reading frame by both methods. A recombinant baculovirus was engineered to express the H5R open reading frame. The partially purified recombinant protein could replace the vaccinia virus P3 factor in transcription assays. On the basis of these findings, we assigned the H5R gene product the name viral late gene transcription factor 4 (VLTF-4). Unlike VLTF-1, -2, and -3, which are synthesized exclusively after viral DNA replication, VLTF-4 is synthesized before and after viral DNA synthesis. Indirect immunofluorescence of infected cells with anti-H5R protein antiserum demonstrated that VLTF-4 is diffusely distributed in the cytoplasm when DNA replication is blocked but is localized to discrete viral DNA-containing factories during a productive infection. Its expression pattern and subcellular distribution suggest that the H5R gene product may have multiple roles in the viral life cycle.

Poxviruses, of which vaccinia virus is the prototype, are unique among the DNA viruses in that they reproduce exclusively in the cytoplasm of host cells and encode enzymes and factors for the transcription and replication of their genomes. Gene expression is programmed and can be divided into three temporal phases: early, intermediate, and late. Regulation occurs primarily at the transcriptional level, and the genes composing each class have distinct *cis*- and *trans*-regulatory components. DNA replication plays a key role in the regulatory process and is required for expression of the intermediate and late stage genes (31).

The relative autonomy of vaccinia virus from the host cell makes it an excellent experimental system for biochemical and genetic studies of transcription. The components required for early gene transcription in vitro have been extracted from vaccinia virions (16, 40); they consist of the multisubunit RNA polymerase (4, 47), the RNA polymerase-associated early specificity factor RAP94 (1, 10), and the viral early transcription factor (7, 14). Additional proteins that have roles in transcription termination and mRNA modification include the capping (27, 30, 33, 46) and methylation (3, 44) enzymes and a poly(A) polymerase (13, 32). Infected cells, in which viral DNA replication was blocked, were used as a source for the isolation of viral intermediate transcription factors (VITFs), which include the viral RNA polymerase, capping enzyme (VITF-A) (19, 49), a free form of the RNA polymerase RPO30 subunit (VITF-1) (41), and a nuclear-associated host cell factor (VITF-2) (42). The products of three viral intermediate genes (A1L, A2L, and G8R) were identified by transfection assays as transactivators of late gene expression (22). Subsequently, all three were purified from virus-infected cells or isolated as recombinant proteins and were shown to be necessary for transcription in vitro (20, 23, 36, 51). The proteins encoded by G8R, A1L, and A2L are referred to as viral late transcription factor 1 (VLTF-1), VLTF-2, and VLTF-3, respectively. The transfection assay used to identify the three VLTFs could not provide information regarding the existence of late transcription factors that are synthesized prior to DNA replication. Evidence for the existence of one or two such factors, called P3 (25) and VLTF-X (50), was recently obtained. In the present study, we have purified the P3 factor to apparent homogeneity, and by mass spectroscopy and peptide sequencing we have identified it as the product of the vaccinia virus H5R gene. The new transcription factor, named VLTF-4, is distinguished from VLTF-1, -2, and -3 by its synthesis before as well as after DNA replication. VLTF-4 also associates with cytoplasmic DNA-containing viral factories.

MATERIALS AND METHODS

Purification of the vaccinia virus P3 factor. The P3 factor was purified from 290 liters of HeLa-S3 cells (Cell Culture Center; Minneapolis, Minn.) that were infected in 12-liter batches with the WR strain of vaccinia virus at 5 PFU per cell. The viral DNA replication inhibitor, cytosine β-D-arabinofuranoside (araC; Sigma), was added 1 h prior to infection and maintained at 44 μg/ml thereafter. Cytosolic (S100) and nuclear extracts were prepared at 18 h after infection essentially as described previously (11). Subsequently, both nuclear and cytoplasmic extracts were dialyzed against buffer A (50 mM Tris [pH 8.0], 0.1 mM EDTA, 0.01% Nonidet P-40, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol) plus 0.3 M NaCl. The pooled, dialyzed extracts were clarified by centrifugation at 40,000 × *g* for 1 h in a 60 Ti rotor at 4°C. The protein concentrations of the resulting cytosolic and nuclear extracts were approximately 4.0 and 2.8 mg/ml, respectively. The results of late gene transcription assays (described below) indicated that the bulk of P3 activity sedimented with the nuclei of infected cells.

A 100-ml phosphocellulose (Whatman P11) column (2.5 by 50 cm) was prepared according to the manufacturer's specifications. The column was sequentially washed with 1 liter of buffer A plus 0.3 M NaCl, 1 liter of buffer A plus 0.3 M NaCl containing 0.2 mg of bovine serum albumin (BSA; ICN) per ml, and 1 liter of buffer A plus 0.3 M NaCl at 2 ml/min. The nuclear extract (1.19 g in 388 ml) was divided into three aliquots, and each was applied to a phosphocellulose column at 1 ml/min. The column was washed with 500 ml of buffer A plus 0.3 M NaCl, and the P3 factor was eluted with 200 ml of buffer A plus 1.0 M NaCl.

A 40-ml Q-Sepharose (Pharmacia) column (2.5 by 30 cm) was prepared by washing with 500 ml of buffer A plus 50 mM NaCl. The P3-containing phospho-

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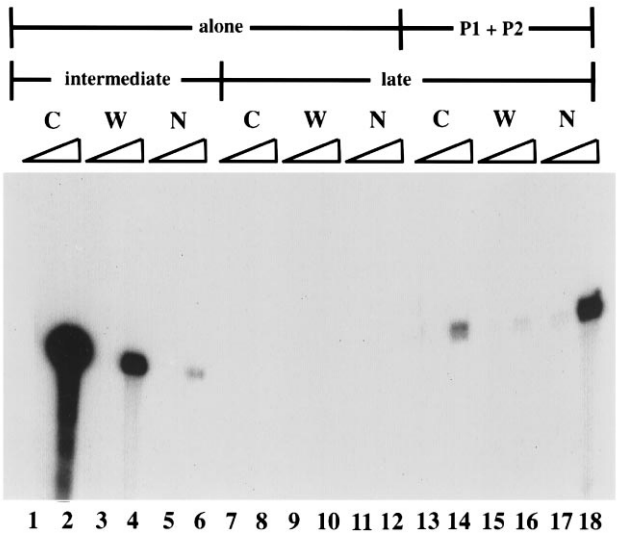

FIG. 1. The P3 factor sediments with infected-cell nuclei. Cytoplasmic and nuclear extracts were prepared from vaccinia virus-infected, araC-treated HeLa cells at 18 h postinfection. Individual extracts (1 or 10 μl) were analyzed by in vitro transcription assays with either an intermediate promoter (lanes 1 to 6) or a late promoter (lanes 7 to 11) template or were reconstituted with two fractions containing vaccinia virus RNA polymerase (P2) and VLTF-1, -2, and -3 (P1) and a late promoter template (lanes 13 to 18). RNA radiolabeled with [α-^{32}P]UTP was analyzed by electrophoresis through a 4% polyacrylamide–7.5 M urea gel and autoradiography. C, cytoplasmic fraction; W, 0.15 M NaCl–0.5% Nonidet P-40 wash of nuclear pellet; N, 0.45 M NaCl nuclear extract.

cellulose fractions (380 mg of protein in 700 ml) were pooled and diluted with buffer A to a NaCl concentration of 50 mM and applied to the Q-Sepharose column at 1 ml/min. The column was sequentially washed with three 250-ml portions of buffer A containing 0.05, 0.175, and 0.75 M NaCl at 2 ml/min. The bulk of the P3 factor was present in the 0.75 M NaCl eluate.

The P3 factor-containing fractions (228 mg of protein in 455 ml) were diluted with buffer A to a NaCl concentration of 0.175 M and loaded onto a second Q-Sepharose column (20 ml) equilibrated in the same buffer. The column was washed with 100 ml of buffer A plus 0.175 M NaCl, developed with a 300-ml continuous gradient of 0.175 to 0.5 M NaCl in buffer A, and then washed with 100 ml of buffer A plus 1.0 M NaCl at 1 ml/min. The peak of P3 factor activity eluted between 240 and 260 mM NaCl.

The fractions containing the peak of P3 factor activity (50 mg of protein in 45 ml) from the second Q-Sepharose column were pooled and diluted with buffer A to 0.1 M NaCl and were subsequently applied to a 10-ml double-stranded-DNA cellulose column (1 by 5 cm; Sigma) equilibrated with the same buffer. The column was washed with 50 ml of buffer A plus 0.1 M NaCl, developed with a 150-ml continuous gradient of 0.1 to 0.5 M NaCl in buffer A, and then washed with 50 ml of buffer A plus 1.0 M NaCl. The P3 factor eluted from this column between 220 and 260 mM NaCl.

The pooled fractions containing P3 factor activity (16 mg in 56 ml) were then dialyzed against buffer H (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.4], 0.1 mM EDTA, 2 mM dithiothreitol, 10% glycerol) plus 0.3 M NaCl and were applied to a Poros HS/M column (4.6-mm diameter, 100-mm length; PerSeptive Biosystems) equilibrated in the same buffer. The column was washed with 30 ml of buffer H plus 0.3 M NaCl, developed with a 30-ml linear gradient of 0.3 to 1.0 M NaCl in buffer H, and then washed with 15 ml of buffer H plus 1.0 M NaCl with the Waters 650E protein purification system. The P3 factor eluted between 480 and 520 mM NaCl from this column.

Protein concentrations were calculated by the Bradford assay (6) with BSA (Bio-Rad) as a protein standard. The NaCl concentrations of column fractions were calculated by plotting the measured conductivity against a linear titration of NaCl in the appropriate buffer.

P3 assays. The P3 factor activity in individual chromatographic fractions was determined as described previously (25) by a reconstituted late gene transcription assay which employs a plasmid (pCFW9 [52]) that contains (i) the viral 11K late promoter fused to a G-less cassette as the template and (ii) two phosphocellulose column fractions, P1 (containing VLTF-1, -2, and -3) and P2 (containing the virus-encoded DNA-dependent RNA polymerase).

To calculate the recovery of the P3 factor from each stage of the purification, we assayed several dilutions. One unit was defined as the number of femtomoles of UMP incorporated into RNA in 30 min at 30°C.

Mass spectroscopy and peptide sequencing. Purified P3 factor (approximately 200 pmol) was concentrated by vacuum microcentrifugation and loaded in two wells of a 0.1% sodium dodecyl sulfate (SDS)–14% polyacrylamide gel (26). One half of the gel was electroblotted onto a polyvinylidene difluoride (Immobilon P; Millipore) membrane, and the other half was stained with Coomassie blue. The membrane was stained with Ponceau S, and the protein was excised and subjected to trypsin digestion as described previously (9). One tenth of the 36-kDa digests and one half of the 30- and 25-kDa digests were subjected to matrix-assisted laser desorption ionization mass spectroscopy analysis with a Voyager RP mass spectrometer (PerSeptive Biosystems) (35). Masses of tryptic peptides obtained by mass spectroscopy were used by MOWSE (internet electronic mail server version 5.1; mowse@dl.ac.uk) to search a peptide mass database constructed from a theoretical trypsin digest of all proteins in the OWL database (34). Parameters used were a molecular weight filter of 40%, a 1-Da peptide mass tolerance, and a partial cleavage score factor of 0.4.

The Coomassie blue-stained protein band was excised and subjected to in situ tryptic endopeptidase digestion. Following high-performance liquid chromatography separation and subsequent mass spectroscopy, peptides were subjected to amino-terminal sequencing (W. M. Keck Foundation, Biotechnology Research Laboratory, New Haven, Conn.).

Expression of vaccinia virus H5R with a recombinant baculovirus. PCR was used to amplify a DNA segment containing a synthetic *Bam*HI restriction endonuclease site preceded by the entire open reading frame (ORF) of the vaccinia virus H5R gene and a synthetic *Hin*dIII restriction site. The amplified DNA was subsequently ligated into the vector pFastBac1 digested with *Bam*HI and *Hin*dIII, and the resulting plasmid (pGK335) was sequenced with the Applied Biosystems 373A fluorescent dideoxynucleotide system. Purified recombinant baculovirus (vBacH5R) was isolated, and then the titers of the virus were determined and the virus was amplified according to the specifications of the Bac-to-Bac expression vector system (Gibco BRL).

Recombinant H5R protein was prepared by infecting 3.0×10^7 *Trichoplusia ni* cells (High Five; Invitrogen) with 10 PFU of vBacH5R per cell. As a control, the same number of cells was infected with equivalent amounts of the wild-type baculovirus (E2 strain). At 48 h postinfection, cells were harvested by centrifugation and washed with ice-cold phosphate-buffered saline (PBS). Nuclear and cytoplasmic extracts were prepared as described above. The H5R protein was identified by Western blot (immunoblot) analysis with a rabbit polyclonal antiserum raised against a peptide containing amino acid residues 2 to 17 of the H5R protein. Subsequently, the nuclear extracts prepared from both vBacH5R and wild-type baculovirus-infected cells were applied to Poros HS/M columns essentially as described above. Individual fractions were dialyzed against buffer H plus 0.1 M NaCl and assayed for P3 factor activity as described above.

Intracellular localization of the H5R protein by indirect immunofluorescence. The H5R protein was localized in vaccinia virus-infected cells by indirect immunofluorescence. BSC-1 cells were grown on microscope slides to a confluency of 80% and were mock infected or infected at a multiplicity of infection of 5 in the presence or absence of araC (44 μg/ml). At the appropriate time after infection, cells were fixed in 3.0% paraformaldehyde in PBS for 30 min at room temperature. After permeabilization in 0.1% Nonidet P-40 in PBS for 4 min at room temperature, cells were blocked in PBS containing 0.25% BSA and 0.25% gelatin (PBSA). Subsequently, cells were RNase treated (Boehringer Mannheim; 10 mg/ml in PBS) for 1 h at 37°C. The primary antibody (anti-H5R at 1:500) in PBSA was incubated with cells for 1 h at room temperature. Secondary antibody (fluorescein isothiocyanate-conjugated donkey anti-rabbit immunoglobulin G; Jackson) was diluted in PBSA at 1:100 and incubated for 1 h at room temperature. DNA was stained with 10% propidium iodide (Boehringer Mannheim) in mounting medium for fluorescence (Vectashield; Vector). Cells were visualized with a Zeiss Axioplan microscope, and images were captured with a Bio-Rad MRC 1024 laser confocal imaging system.

RESULTS

Purification of P3 factor from infected cells. To assay P3 factor activity, a late promoter template and two column fractions, one containing partially purified viral RNA polymerase and the other containing VLTF-1, -2, and -3 (25), were added to transcription reaction mixtures. We had previously reported that the P3 factor was expressed in vaccinia virus-infected cells that had been treated with araC to prevent viral DNA replication. However, insufficient protein was obtained for purification. In an attempt to improve our extraction procedure, the method of Dignam et al. (11) was used for separation of the cytoplasmic and nuclear fractions of cells. Since vaccinia virus replicates in the cytoplasm, we expected that the P3 factor would be found in this fraction. Unexpectedly, more than 90% of the P3 factor pelleted with the nuclei of infected cells treated with araC as determined by in vitro transcription assays (Fig. 1, compare lanes 14, 16, and 18). Subsequent Western

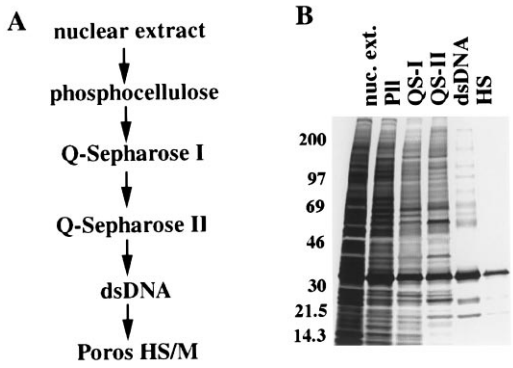

FIG. 2. Purification of the P3 factor. (A) Scheme of P3 factor purification. A nuclear extract from araC-treated vaccinia virus-infected HeLa cells was fractionated as indicated. (B) Analysis of P3 fractions. Samples (5 μl) containing the peak of P3 factor activity from the indicated columns were subjected to electrophoresis through a 0.1% SDS–4 to 20% polyacrylamide gel and subsequently silver stained. Sizes (in thousands) of the molecular weight standards are on the left. Nuc. ext., nuclear extract; P11, phosphocellulose; QS-1, Q-Sepharose I; QS-II, Q-Sepharose II; dsDNA, double-stranded DNA; HS, Poros HS/M.

blot analyses of cytoplasmic and nuclear extracts indicated that the bulk of P3 protein sediments with the nuclei (24). By contrast, more than 95% of the intermediate transcriptional activity was found in the cytoplasmic fraction (Fig. 1, compare lanes 2, 4, and 6). The absence of transcription products in lanes 7 through 12 (Fig. 1) indicated that synthesis of VLTF-1, -2, and -3 was blocked by araC, as anticipated because of their intermediate promoters (22). These data demonstrated that the P3 factor is physically separated from intermediate transcription factors and that it sediments with the nuclei of infected cells. We also infected cells in the absence of araC to determine how the P3 factor sediments in productively infected cells. Since VLTF-1, -2, and -3 are synthesized under these conditions, we partially purified the P3 factor by applying it to a phosphocellulose column at 0.3 M NaCl and eluting it at 1.0 M NaCl. In the absence of araC, we detected the P3 factor in the cytoplasmic and nuclear fractions (24).

Since few vaccinia virus proteins are expected to sediment with the nuclei of infected cells treated with araC, we considered that this property would aid in the purification of the P3 factor. We prepared nuclear extracts from approximately 290 liters of araC-treated HeLa cells infected with vaccinia virus and fractionated them according to the scheme in Fig. 2A. SDS-polyacrylamide gel electrophoresis (PAGE) analysis and subsequent silver staining of peak fractions throughout the purification indicated that a protein of 36 kDa had been purified to near homogeneity (Fig. 2B). The peak of P3 factor activity (Fig. 3B) coincided with a major polypeptide of 36 kDa and two minor proteins of 30 and 25 kDa (Fig. 3A). Depending on the amount assayed, the P3 factor stimulated late gene transcription from twofold to sixfold above basal levels (Fig. 4). Addition of excess P3 factor inhibited RNA synthesis, possibly because of transcriptional squelching (37). At this stage in the purification, approximately 70 μg, or 3.2 nmol (the theoretical molecular weight of the P3 factor is approximately 22,000; see below), of the P3 factor was purified over 800-fold (Table 1).

Identification of the P3 factor gene. To determine which gene(s) encodes the 36-, 30-, and 25-kDa proteins obtained from the final step of purification of the P3 factor, we used mass spectrometry and peptide sequencing. The electrophoretically separated proteins from fraction 49 (Poros HS/M column) were transferred to a membrane and digested in situ with trypsin, and then the eluted peptides were analyzed by mass spectroscopy to determine their molecular masses. The spectra obtained showed the presence of at least five peptides with identical molecular masses in each digest, suggesting that the three proteins are related (Table 2). It is likely that the 30- and 25-kDa polypeptides are either proteolytic cleavage products or processed forms of the 36-kDa polypeptide. To identify the gene encoding the P3 factor, the molecular masses of 12 tryptic peptides from a spectrum containing approximately 28 were entered into the MOWSE database searching program. This database matches the peptide fingerprint of an unknown polypeptide with the predicted fingerprints of all ORFs in the OWL database. Even though the entire database was searched, the only significant match was obtained with the peptide fingerprint of the vaccinia virus H5R gene product. Seven of the 12 peptide masses entered matched those of the predicted

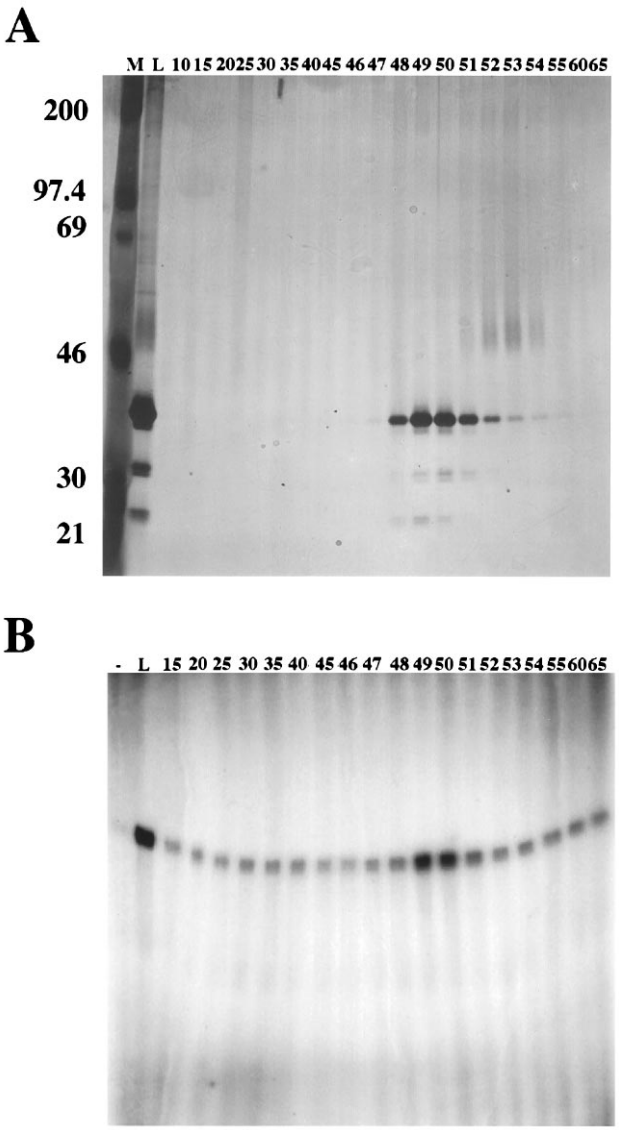

FIG. 3. Analysis of Poros HS/M fractions. (A) Silver-stain analysis of Poros HS/M fractions. Samples (10 μl) were electrophoresed through a 0.1% SDS–15% polyacrylamide gel and subsequently silver stained. M, molecular weight markers; L, material loaded onto column. Sizes (in thousands) of markers are on the left. (B) In vitro transcription assays of Poros HS/M fractions. The indicated fractions (10 μl) were analyzed by in vitro transcription assay as described for Fig. 1. −, no P3 factor added; M, molecular weight markers; L, loading material.

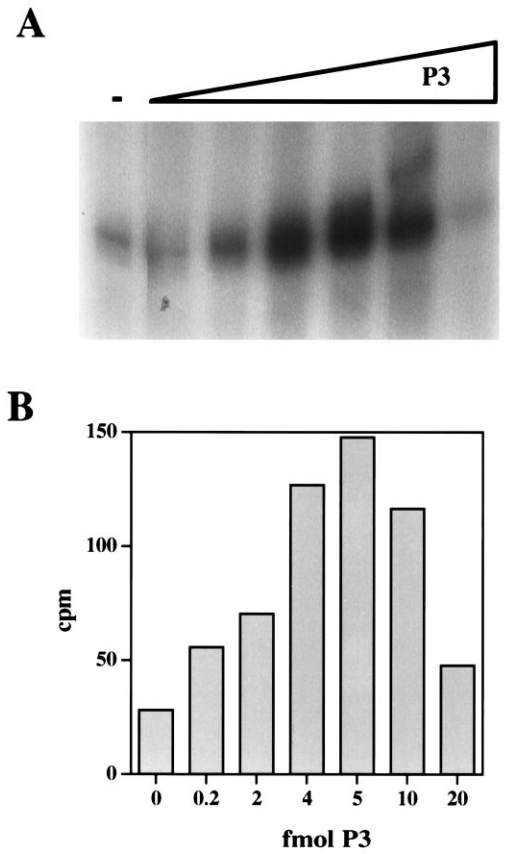

FIG. 4. Titration of P3 factor in a late gene transcription assay. (A) In vitro transcription assays were done in the absence (−) or presence of increasing amounts of Poros HS/M fraction 49 (Fig. 3). (B) Quantitation of in vitro transcription assays. A Betascope 630 (Betagen) was used to quantitate radioactively labeled RNA bands.

peptides of the H5R protein. Further analysis of the mass spectroscopy data confirmed the identity of an additional three H5R peptides in the spectra. To confirm the identity of the 36-kDa protein, two tryptic peptides of 1,818 and 862 Da (as determined by mass spectroscopy) were sequenced by the Edman degradation procedure. The sequence of the 1,818-Da peptide matched the predicted sequence of the H5R gene product from amino acids 153 to 170 (Table 2). No sequence data were obtained for the 862-Da peptide, whose molecular mass matched the predicted molecular mass of an acetylated form of the amino-terminal peptide of the H5R protein.

Baculovirus expression of H5R. To demonstrate that H5R encodes the P3 factor, we constructed a recombinant baculovirus (vBacH5R) that contains this ORF. Preliminary observations indicated that, as in araC-treated vaccinia virus-infected HeLa cells, most of the P3 factor was found in nuclear extracts of vBacH5R-infected insect cells (24). Crude nuclear extracts from vBacH5R- and wild-type baculovirus-infected cells were assayed for P3 factor activity by in vitro transcriptions. Initially, we observed minimal stimulation with the vBacH5R crude nuclear extract. However, addition of a wild-type baculovirus nuclear extract to a complete vaccinia virus late gene transcription system inhibited activity (24). Therefore, the extract containing the recombinant H5R protein was applied to a Poros HS/M column, and eluted fractions were assayed by Western blotting and in vitro transcription (Fig. 5). The level of transcription obtained with the partially purified H5R protein was significantly higher than that of the crude nuclear extract, and it was as high as that of the vaccinia virus-purified P3 factor. The bulk of H5R protein coeluted with the peak of P3 activity, demonstrating that recombinant H5R can replace the vaccinia virus-purified P3 factor. No P3 activity was observed in column fractions containing wild-type baculovirus proteins (24). These data demonstrate that the H5R gene product is the P3 factor. In this paper we refer to the P3 factor as VLTF-4.

Localization of VLTF-4 in infected cells. Our fractionation data suggested that VLTF-4 is associated with the nuclei of infected cells. Alternatively, the protein might simply sediment with nuclei after lysis. To resolve this question, we carried out indirect immunofluorescence by using an antibody raised against the amino terminus of the H5R protein. Cells were infected with vaccinia virus in the presence or absence of araC and analyzed at several times postinfection. In cells infected in the absence of araC, we observed VLTF-4 staining of large cytoplasmic bodies (Fig. 6). These bodies also stained with propidium iodide, indicating that VLTF-4 associates with large DNA complexes (virosomes or viral factories) in infected cells. In the presence of araC we observed diffuse staining of VLTF-4 throughout the cytoplasm. The anti-H5R antisera did not react with mock-infected cells, and as expected, only nuclei stained with propidium iodide. Indirect immunofluorescence of cells transfected with an H5R expression vector plasmid showed staining similar to that of araC-treated vaccinia virus-infected cells (24).

DISCUSSION

In this study we demonstrated that VLTF-4 is encoded by the vaccinia virus H5R gene. The H5R ORF (originally designated H6R) specifies a 203-amino-acid polypeptide with an M_r of 22,341 (43). In accordance with our results, it was previously reported that the H5R gene product migrates anomalously, between 35 and 40 kDa, in SDS gels (43). It was suggested that a proline-rich region and/or regions with several acidic or basic amino acids may contribute to its anomalous migration (17). H5R homologs have been identified in several poxviruses, including vaccinia virus Copenhagen strain (H5R, 99% identity [15]), variola virus (strain India-1967, I5R, 90% identity [28]), Shope fibroma virus (40% identity [48]), orf virus (F3R, 46% identity [12]), and *Molluscum contagiosum* virus (26% identity [45]). No significant homology was found with any other proteins.

Using antisera directed against solubilized virion proteins coupled with hybrid-arrest in vitro translation, Gordon et al.

TABLE 1. Purification of the vaccinia virus P3 factor

Fraction[a]	Amt of protein (mg)[b]	Vol (ml)	Amt of P3 (U)[c]	Sp act (U/mg)	Yield (%)	Purification factor (fold)[d]
NE	1,190	388	46,560	39.1	NA[e]	NA
P-11	380	203	21,924	57.7	47	1.5
QS-1	228	455	32,760	143.7	70	3.7
QS-2	50	45	9,790	195.8	21	5.0
dsDNA	16	56	3,752	234.5	8.1	6.0
HS	0.07	3.5	2,240	32,000	4.8	818

[a] NE, nuclear extract; P-11, phosphocellulose; QS, Q-Sepharose; dsDNA, double-stranded DNA; HS, Poros HS/M.
[b] Determined by the Bradford assay with BSA as a standard.
[c] Transcriptional activity is expressed as picomoles of UMP incorporated into RNA in 30 min at 30°C.
[d] Calculated as the ratio of the specific activity of each fraction to that of the crude nuclear extract.
[e] NA, not applicable.

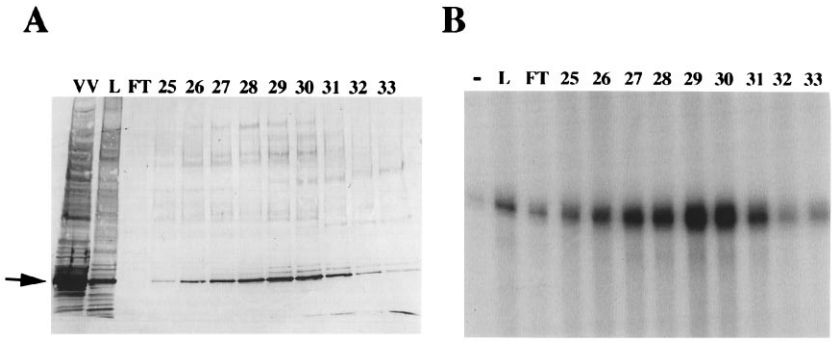

FIG. 5. Baculovirus-expressed H5R replaces vaccinia virus P3 factor. (A) Nuclear extracts were prepared from vBacH5R-infected *T. ni* cells and subsequently applied to a Poros HS/M column. Individual fractions (5 μl) from the Poros HS/M column were analyzed by Western blotting with a rabbit polyclonal antiserum (1:500) raised against an H5R synthetic peptide. Blots were incubated with a goat anti-rabbit immunoglobulin G antibody conjugated to alkaline phosphatase and developed with Western blue substrate (Promega). Arrow indicates the H5R polypeptide. VV, crude araC-treated vaccinia virus-infected HeLa cell nuclear extract; L, H5R-infected *T. ni* crude nuclear extract; FT, Poros HS/M flowthrough. (B) In vitro transcriptions, as described for Fig. 1, were done with individual fractions (5 μl) from the Poros HS/M column used for panel A. −, no added P3.

(17) concluded that the H5R gene encodes p35, a membrane protein involved in the biogenesis of the viral envelope. Antibodies presumably directed against this protein have been found to have neutralizing activity (18). However, direct sequencing of purified p35 protein from virions unambiguously demonstrated that it is encoded not by the H5R ORF but rather by the H3L gene (8). Immunoblot analyses with antisera raised against recombinant H5R or H3L proteins demonstrated that both proteins migrate as 35-kDa proteins by SDS-PAGE (53). However, the H3L protein is present in virions, and the H5R protein is found only in lysates of infected cells (53). Considering these findings and our inability to detect proteins in either virion membranes or cores that react with antibodies directed against H5R (24), we contend that VLTF-4 and p35 are not the same protein.

Unlike VLTF-1, -2, and -3, VLTF-4 is expressed prior to viral DNA replication. By high-resolution S1 nuclease analysis, it was determined that the H5R gene is transcribed from tandem promoters early and late in infection (43). Inspection of the H5R late promoter component does not show similarity to

TABLE 2. Summary of mass spectroscopy data

Protein (kDa)	MALDI mass (Da)	*D* (Da)[a]	H5R peptide sequence consistent with mass[b]	H5R amino acids
36	2,189.5	−2.70	(K)NEDIFPEDVIIPSTKPKTK(R)	34–52
	545.84	1.24	(R)ATTPR(K)	54–58
	615.91	1.21	(R)KPAATK(R)	59–64
	684.07	1.37	(K)VNHSAR(S)	124–129
	777.62	0.82	(R)SDLSDLK(V)	130–136
	859.74	0.57	(K)VATDNIVK(D)	137–144
	502.84	1.24	(K)IITR(I)	149–152
	1,817.30[c]	1.30	(R)ISAVSTVLEDVQAAGISR(Q)	153–170
	842.27	0.27	(R)QFTSMTK(A)	171–177
	1,348.74	1.24	(K)AITTLSDLVTEGK(S)	178–190
	524.56, 568.46, 598.78, 622.17, 636.08, 702.40, 1,871.30, 2,240.91[d]			
30	616.18	1.48	(R)KPAATK(R)	59–64
	684.98	2.28	(K)VNHSAR(S)	124–129
	503.10	1.50	(K)IITR(I)	149–152
	1,817.46	1.46	(R)ISAVSTVLEDVQAAGISR(Q)	153–170
	1,349.15	1.65	(K)AITTLSDLVTEGK(S)	178–190
	568.99, 598.57, 644.38, 660.23, 2,164.55, 2,193.97, 2,291.03[d]			
25	616.23	1.53	(R)KPAATK(R)	59–64
	684.08	1.38	(K)VNHSAR(S)	124–129
	502.94	1.34	(K)IITR(I)	149–152
	1,817.07	1.07	(R)ISAVSTVLEDVQAAGISR(Q)	153–170
	1,349.31	1.81	(K)AITTLSDLVTEGK(S)	178–190
	598.57, 660.07, 2,165.22, 2,194.68, 2,222.29, 2,275.33[d]			

[a] Difference between measured mass and calculated mass of H5R peptide.
[b] Residues before and after peptide are in parentheses.
[c] Peptide was microsequenced.
[d] Masses not attributed.

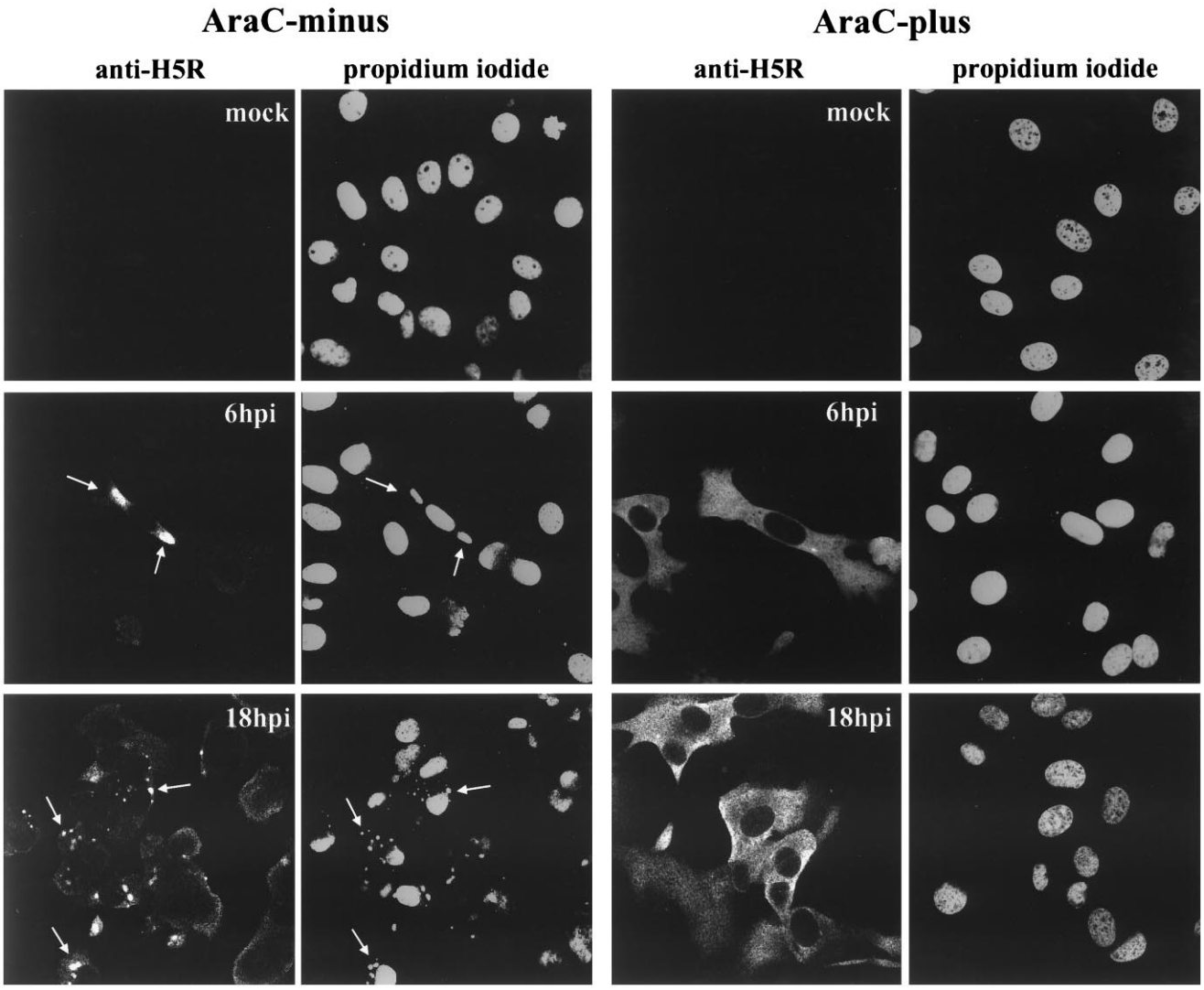

FIG. 6. VLTF-4 localizes to virosomes in infected cells. Cells were mock infected or infected in the presence (AraC-plus) or absence (AraC-minus) of araC. At the indicated times postinfection (pi), VLTF-4 was visualized by indirect immunofluorescence by staining with anti-H5R antibody. DNA was visualized directly by staining with propidium iodide. Arrows indicate virosomes.

the sequences of three intermediate promoters (2), suggesting that the H5R promoter is not of the intermediate type. Its synthesis prior to DNA replication raises an interesting question about its function: is it required for other viral processes, such as intermediate gene transcription and/or viral DNA replication? Assays developed with purified components will aid in elucidating the role of VLTF-4 in these processes.

Our finding that VLTF-4 is associated with virosomes is consistent with its role as a transcription factor, since late gene transcription (24) and viral DNA replication (21) occur within these cytoplasmic compartments. Interestingly, Mohandas et al. found that recombinant H5R was targeted to intranuclear viral replication compartments (virogenic stroma) when expressed with a baculovirus vector in insect cells (29). These data demonstrate that VLTF-4 is targeted to analogous compartments in vaccinia virus-infected mammalian cells and baculovirus-infected insect cells. Lastly, we find that although VLTF-4 is cytoplasmic in araC-treated infected cells, it sediments with the nuclei during fractionation. It is known that virosomes sediment with nuclei during fractionation; however, virosomes presumably do not form in the presence of araC. Although we do not know why VLTF-4 sediments with nuclei, its doing so was fortuitous and aided in its purification.

It was recently (5) demonstrated that the H5R gene product is phosphorylated in vitro by the vaccinia virus B1R protein kinase 1. However, it has not been possible to link VLTF-4 phosphorylation with transcriptional activation in vivo since cells infected with temperature-sensitive mutants with mutations in B1R fail to replicate viral DNA, thus precluding the transcription of late genes at the nonpermissive temperature (38, 39). The presence of kinase activity in our in vitro transcription assays has thus far prevented us from determining whether VLTF-4 is regulated by phosphorylation. Work is currently under way to determine if additional factors or modifying enzymes are required for late gene transcription.

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