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Cooperation of Structural Proteins during Late Events in the Life Cycle of Polyomavirus

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The polyomavirus minor late capsid antigen, VP2, is myristylated on its N-terminal glycine, this modification being required for efficient infection of mouse cells. To study further the functions of this antigen, as well as those of the other minor late antigen, VP3, recombinant baculoviruses carrying genes for VP1, VP2, and VP3 have been constructed and the corresponding proteins have been synthesized in insect cells. A monoclonal antibody recognizing VP1, α-PyVP1-A, and two monoclonal antibodies against the common region of VP2 and VP3, α-PyVP2/3-A and α-PyVP2/3-B, have been generated. Reactions of antibodies with antigens were characterized by indirect immunofluorescence, immunoprecipitation, and immunoblot analysis. Immunofluorescent staining of mouse cells infected with polyomavirus showed all antigens to be localized in nuclei. When the late polyomavirus proteins were expressed separately in insect cells, however, only VP1 was efficiently transported into the nucleus; VP2 was localized discretely around the outside of the nucleus, and VP3 exhibited a diffused staining pattern in the cytoplasm. Coexpression of VP2, or VP3, with VP1 restored nuclear localization. Immunoprecipitation of infected mouse cells with either anti-VP1 or anti-VP2/3 antibodies precipitated complexes containing all three species, consistent with the notion that VP1 is necessary for efficient transport of VP2 and VP3 into the nucleus. Purified empty capsid-like particles, formed in nuclei of insect cells coinfected with all three baculoviruses, contained VP2 and VP3 proteins in amounts comparable to those found in empty capsids purified from mouse cells infected with wild-type polyomavirus. Two-dimensional gel analysis of VP1 species revealed that coexpression with VP2 affects posttranslational modification of VP1.

Three polyomavirus-encoded proteins, VP1 (45 kDa), VP2 (35 kDa), and VP3 (23 kDa), are synthesized in the late phase of productive infection. The corresponding viral genes are organized in an overlapping manner on the genome but are translated from three distinct mRNAs processed from one late transcript (34) to give 16S (VP1), 19S (VP2), and 18S (VP3) species. The minor late proteins, VP2 and VP3, are translated from the same open reading frame, with the amino acid sequence of VP3 corresponding to the carboxy-terminal two-thirds of VP2. The gene for the major late antigen, VP1, translated from a different open reading frame (16, 35), overlaps the 3′ end of VP2 and VP3 genes by 30 nucleotides.

The abundantly synthesized VP1 protein of polyomavirus has been reported to consist of six isoelectric species posttranslationally modified by phosphorylation and acetylation (3). VP1 has also been found to contain hydroxyproline (28). It has been proposed that differently modified VP1 species play a role in the attachment of virions to the cell surface and in the ability of viral particles to hemagglutinate erythrocytes (9). VP2 antigen has also been shown to be modified by covalent attachment of a myristyl moiety on the N-terminal glycine after its release by a processing procedure that removes the methionine residue (38). Data obtained with a nonmyristylated VP2 mutant virus, in which the target glycine of VP2 has been mutated to glutamic acid, demonstrate that the loss of the myristyl group does not completely abolish infectivity but the efficiency of viral propagation is substantially diminished (23).

After infection of cells with polyomavirus or simian virus 40 (SV40), all three late antigens, following translation, are rapidly transported into the nuclei. Sequences in the amino terminus of SV40 and polyomavirus VP1 proteins and in the carboxy terminus of the VP2 and VP3 proteins have been identified as being necessary for efficient transport to the nucleus, although the exact mechanism by which individual late antigens reach the nucleus is still unclear (6, 7, 15, 22, 30, 42, 43). In nuclei, all three late proteins, together with viral DNA and cellular histones, are assembled into new virions, composed of a nucleohistone core and a highly organized capsid shell consisting of 72 five- and six-coordinated capsomers, arranged in an icosahedral surface lattice (reviewed in reference 1; 32). In addition to the complete virions, empty capsids lacking the nucleohistone core can frequently be isolated. Although the minor antigens VP2 and VP3 are called capsid proteins, it has been shown that the major antigen, VP1, can itself assemble into empty capsid-like particles and related polymorphic aggregates and make three distinctly nonequivalent types of interpentamer contacts to produce 60 pentavalent and 12 hexavalent pentameric capsomers (25, 29). Moreover, capsid-like structures can form spontaneously even with VP1 expressed in *Escherichia coli*, in which it is not posttranslationally modified (33). These data indicate that neither VP2 nor VP3 is required for capsid-like structure assembly.

The question thus arises, what are the functions of the minor late antigens? With these viruses, it is known that mutants that cannot express VP3 are not viable (8) and that mutation of the amino terminus of VP2 considerably decreases infectivity of the virus (23). The mechanism of viral

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DNA-histone complex encapsidation is not known. Either the empty capsids are precursors of virions or capsid structures are formed around the nucleoprotein core from smaller morphological units (45). Recently, X-ray diffraction data of empty capsid and complete virion crystals were obtained for polyomavirus (17). An electron density map of the inside of the virion displayed 72 "prongs" of electron density extending from the core into the cavities of the VP1 pentamers. These prongs were proposed to be VP3, or the carboxy part of VP2 molecules, and it was suggested that they may function to guide the assembly of the highly ordered capsid on the nucleohistone core. Whether this is the case and by what mechanism it occurs, whether there are other functions for VP2 and VP3 in the viral life cycle, involving host cell interactions, and the actual role of the hydrophobic myristyl group on VP2 are all subjects for further studies. The purpose of this report is to address some of these questions. To this end, the late viral antigens have been expressed in an efficient eukaryotic system, and antigen-specific monoclonal antibodies have been generated to study properties of these proteins in vitro and in vivo, separately and in concert.

MATERIALS AND METHODS

Bacteria. *E. coli* DH5 was used for the propagation of transfer vectors and recombinant plasmids. When digestion with *Bcl*I was used, plasmids were propagated in *E. coli* JM110 (a *dam dcm* mutant derivative of JM101) (44).

Plasmids. The baculovirus transfer vectors pVL941, containing a *Bam*HI cloning site, and pVL1393, containing a multiple cloning site polylinker, obtained from M. Summers, were used for the construction of recombinant plasmids in which polyomavirus VP1, VP2, and VP3 genes, respectively, were placed under control of the polyhedrin promoter. pMJA2, containing the whole polyomavirus strain A2 genome inserted into the *Eco*RI site of plasmid pMJ (23), was used for isolation of the late genes.

Construction of recombinant baculovirus transfer plasmids pVL-VP1, pVL-VP2, and pVL-VP3. The recombinant plasmid pVL-VP1 was constructed by ligation of the *Eco*RV-*Xba*I 1,584-bp fragment of plasmid pMJA2 (polyomavirus numberings 4106 [*Eco*RV] to 2522 [*Xba*I] are those of reference 35) with vector pVL1393, cleaved with *Sma*I and *Xba*I. Plasmid pMJA2 was cleaved with *Bcl*I and *Hin*dIII. A fragment 1,103 bp long containing the VP2 and VP3 genes (5021 [*Bcl*I] to 3918 [*Hin*dIII]) was isolated and ligated to pVL941, linearized with *Bam*HI, and partially cleaved with *Hin*dIII. Competent *E. coli* DH5 cells were transformed with the ligation mixture, and plasmid pVL-VP2 was selected. The recombinant plasmid pVL-VP3 was obtained by replacement of a pVL1393 fragment (cleaved with *Kpn*I in the polylinker and polyhedrin gene) with the *Kpn*I fragment cleaved from the pVL-VP2 construct containing the VP3 gene (C-terminal half of VP2 gene position 4693) and part of the baculovirus polyhedrin gene. Detailed construction maps are available on request.

Cells and viruses. *Spodoptera frugiperda* (Sf9) cells were grown as monolayer cultures at 27°C in standard TNM-FH medium containing 10% fetal calf serum (FCS) as described by Hink (19). *Autographa californica* nuclear polyhedrosis virus (AcNPV) was propagated as described by Summers and Smith (39).

Recombinant baculoviruses containing polyomavirus VP1, VP2, and VP3 genes, respectively, were prepared by in vivo allelic exchange between wild-type AcNPV DNA and the individual DNAs of recombinant transfer plasmids pVL-VP1, pVL-VP2, and pVL-VP3 as described previously (11). Sf9 cells were infected with 10 PFU of each recombinant baculovirus per cell for single and coinfections. A monolayer of cells was washed with TNM-FH medium, and the inoculum of virus was added. Cells were rocked at room temperature for 1 h. Complete medium with 10% FCS was then added, and cells were incubated at 27°C for various times. 3T6 Swiss albino mouse cells were grown at 37°C in a 10% CO_2–air humidified incubator, using Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine and 5% FCS. Infection of 3T6 cells with wild-type (strain A2) polyomavirus was performed as follows. Cells were plated onto 5-cm-diameter dishes (2×10^6 cells per dish) and washed after 24 h with DMEM, and virus (50 PFU per cell) in DMEM (0.5 ml) was added. Cells were incubated at 37°C for 90 min with rocking every 15 min, DMEM supplemented with 5% FCS was added, and cells were incubated under the same conditions for 36 h postinfection (p.i.) and then harvested.

Polyomavirus virions and empty capsids were purified from whole mouse embryo cells infected with strain A2 (0.05 PFU per cell) for 7 days by the procedure of Türler and Beard (40).

Preparation of antigens. Polyomavirus virions propagated on 3T6 mouse cells were used for purification of the VP1 antigen. VP2 antigen was isolated from lysates of insect cells infected with recombinant baculovirus. Both antigens were purified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Briefly, the gel was stained with a water solution of Page blue (Sigma) for product identification and destained with several changes of water. The VP1 or VP2 band was excised from the gel, and the corresponding protein was electroeluted. The resultant protein solution was dialyzed for 2 h against 0.2 M sodium bicarbonate–0.02% SDS and precipitated with 4 volumes of cold acetone. The sediment was resuspended in phosphate-buffered saline (PBS).

Hybridoma production. A female CBA/CF1 × BALB/c mouse was injected intraperitoneally with 20 to 50 μg of VP2 polypeptide in 100 μl of PBS homogenized with 100 μl of Freund's complete adjuvant. The mouse was boosted with antigen in incomplete adjuvant at 2 and 4 weeks. A final boost was administered without adjuvant; 3 days later, the mouse was sacrificed and the spleen was removed. Hybridomas were produced by standard methods (18), using the mouse SP2 myeloma cell line. A similar procedure was used to prepare a VP1 monoclonal antibody.

Screening of hybridomas. Hybridomas obtained from mice immunized with VP1 antigen were tested for secretion of anti-VP1 antibody directly, using lysates of polyomavirus-infected 3T6 cells. For production of anti-VP2 antibody, hybrid cells derived from the mouse immunized with VP2 antibodies were tested 7 days after fusion on nitrocellulose filters by dot blotting. Filters were soaked with lysates of recombinant baculovirus VL-VP2-infected and wild-type AcNPV-infected cells, respectively (3×10^7 cells in 15 ml), and then incubated for 30 min with 5% (wt/vol) milk powder in PBS, to block nonspecific protein binding sites. Hybridoma clones (5 μl) were spotted onto filters and incubated in a humid atmosphere at room temperature for 30 min, washed three times with PBS, then incubated with peroxidase conjugated anti-mouse antibody, washed, and visualized as described below for the protein blot immunoassay. Positive clones, giving a substantially stronger signal on filters soaked with lysates of cells infected with recombinant baculovirus than with the control wild-type virus, were further tested on

Western immunoblots from SDS-PAGE, using lysates of mouse 3T6 cells infected with polyomavirus. Selected antibody-producing cells were cloned three times.

Labeling of proteins with [^{35}S]methionine. Infected Sf9 cells were incubated for 1 h in methionine-free Grace's medium (GIBCO-BRL) to deplete the intracellular pool of methionine. Medium was then replaced with fresh methionine-free Grace's medium containing [^{35}S]methionine (>1,000 Ci/mmol; Amersham International plc); 100 μCi [^{35}S]methionine in 2 ml of medium was used per 10^6 cells. Cells were harvested after 3 h of incubation. Uninfected 3T6 cells or polyomavirus-infected 3T6 cells (36 h p.i.) were incubated for 1 h in methionine-free DMEM supplemented with 2 mM glutamine and 5% FCS (dialyzed overnight against PBS and filter sterilized). The medium was then removed and replaced with medium containing [^{35}S]methionine. Incubation was continued for 4 h. One millicurie of [^{35}S]methionine (>1,000 Ci/mmol) in 4 ml of medium was used per 10^7 cells on a 9-cm-diameter dish.

Labeling of cells with [^{3}H]myristic acid. Sf9 cells infected with recombinant VL-VP2 baculovirus were labeled 24 h p.i. in complete TNM-FH medium containing 150 μCi of [^{3}H]myristic acid (47.5 Ci/μmol; Amersham) per 35-mm-diameter dish of cells. At 48 h p.i., cells were washed with ice-cold PBS and harvested. Similar procedures were followed for wild-type virus-infected 3T6 cells.

Harvesting of cells. Cells (Sf9 or 3T6) were washed in ice-cold PBS, scraped, and pelleted. Cell pellets were stored in liquid nitrogen.

Western blot analysis. 3T6 cells were lysed in lysis buffer (100 mM NaCl–100 mM Tris-HCl [pH 8.8]–0.5% Nonidet P-40 [NP-40] containing 0.2 trypsin inhibiting units of aprotinin per ml) (1 ml/10^7 cells) for 20 min on ice. Cell debris was pelleted, and the supernatant was resolved by SDS-PAGE (12% polyacrylamide gel) as described by Laemmli (24). The proteins were electrotransferred onto a nitrocellulose membrane filter in blot buffer (25 mM Tris base, 192 mM glycine [pH 8.3], 20% [vol/vol] methanol) in a cooled tank overnight (8 V/cm). The filter was washed in PBS containing 0.05% Tween 20 (PT) and then incubated for 30 min with PBS containing 0.05% Tween 20 and 5% fat-free milk powder (PTM) with gentle rocking. Incubation with specific antibody, diluted 1:5 for αPyVP2/3-A and αPyVP2/3-B or 1:10 for αPyVP1-A with PTM, was carried out for at least 1 h. The filter was then washed in PT three times for 20 min and incubated for 30 min with rabbit anti-mouse immunoglobulin (Ig)-peroxidase conjugate (DAKOPATTS) diluted 500 times in PTM. After being washed in PT as described above, the filter was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) [200 μl of DAB (20 mg/ml), 9.7 ml of PBS, 100 μl of 1% $CoCl_2$, 1% $Ni(NH_4)_2(SO_4)_2$, 10 μl of H_2O_2 (30%, wt/vol)] or with ELC reagent (Amersham) according to the manufacturer's instructions. Reactions were stopped by washing the filter in water.

Immunoprecipitation. Radioactively labeled polyomavirus-infected 3T6 cells were incubated in lysis buffer (100 μl/10^6 cells) for 20 min on ice and centrifuged. The supernatant was used as extract I. The residue was further extracted in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl [pH 7.4], 0.05% NP-40, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS) (100 μl) and clarified to yield extract II.

Cell extracts (30 μl) were incubated on ice for 1 h in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl [pH 7.4], 0.05% NP-40) containing 0.2% (wt/vol) gelatin (400 μl) with monoclonal antibody (30 μl of hybridoma culture fluid). For IgG1 isotype antibodies, 0.2 μl of rabbit anti-mouse antibody (DAKOPATTS) was added together with protein A-Sepharose. Washed protein A-Sepharose (50% suspension in NET) (30 μl) was added to antigen-antibody complexes and incubated with end-over-end mixing at 4°C for 30 min. Protein A-Sepharose immunoprecipitates were washed two times in NET buffer (1 ml) and two times in RIPA buffer (1 ml). Protein A-Sepharose sediment was then resuspended in Laemmli sample buffer, boiled for 5 min, and centrifuged. Supernatants were analyzed by SDS-PAGE and autoradiography.

Immunofluorescence of cells. Infected cells grown on glass coverslips were washed with PBS and fixed with acetone-methanol (1:1, vol/vol). Nonspecific antibody binding sites were blocked by incubation with PBS containing 0.25% gelatin and 0.25% bovine serum albumin for 30 min. The cells were incubated with monoclonal antibody (undiluted hybridoma medium) for 30 min, unbound antibody was removed by washing, and the cultures were then incubated in rhodamine isothiocyanate-conjugated rabbit anti-mouse antibody (50× diluted with PBS) for 30 min. After each incubation, the cells were extensively washed in PBS. After the final wash, coverslips were mounted in Aquamount (BDH Ltd., Poole, England), and cells expressing polyomavirus late antigens were visualized by fluorescence microscopy.

Isolation of polyomavirus capsid-like particles from insect cells. Insect cells were either infected with VL-VP1 baculovirus or coinfected with all three recombinant baculoviruses, VL-VP1, VL-VP2, and VL-VP3 (10 PFU of each per cell). Cells were harvested 72 h p.i., resuspended in buffer A (10 mM Tris-HCl [pH 7.4], 50 mM NaCl, 0.01 mM $CaCl_2$, 0.01% Triton X-100), and disrupted by sonication (10 amplitude microns [MSE Soniprep 150] for 3 s). Levels of unbroken cells and nuclei were monitored by microscopy. Cell debris was centrifuged (20 min, 10,000 × *g*). If unbroken nuclei were observed in the pellet, the pellet was resuspended in buffer A and sonicated again. After centrifugation, supernatants were combined and centrifuged for 3 h at 35,000 rpm in an SW41 rotor through a 20% sucrose cushion made up in buffer A. The pellet was resuspended in buffer A and loaded onto a 15 to 40% sucrose gradient. Fractions containing capsid-like particles were dialyzed against buffer A, concentrated on a CsCl gradient (40), and identified by electron microscopy.

Electron microscopy. Formvar (EMSCOPE)-coated grids were incubated for several seconds with 10-μl capsid samples, drained of surplus liquid, transferred to a drop of 3% phosphotungstic acid (pH 6.6), and left for 45 s. The grids were dried and stored at room temperature.

2D gel analysis. Proteins were analyzed by two-dimensional (2D) PAGE according to the method of O'Farrell (31), with minor modifications. ^{35}S-labeled uninfected or lytically infected 3T6 cells, and Sf9 cells either infected with VL-VP1 or coinfected with VL-VP1 and VL-VP2 or with VL-VP1 and VL-VP3, were harvested 36 h p.i., at a time when cellular enzymatic processes should still be functioning yet viral protein was abundant, by washing the cells in PBS and resuspending them in SB1 (0.3% SDS, 0.6 M β-mercaptoethanol, 28 mM Tris-HCl, 22 mM Tris base) (100 μl/10^6 cells). Cell lysates were incubated at 100°C for 5 min and then cooled on ice for 5 min. One-tenth volume of SB2 (24 mM Tris-base, 476 mM Tris-HCl, 50 mM $MgCl_2$, 1 mg of DNase I per ml, 0.25 mg of RNase A per ml) was added, and incubation on ice was continued for a further 9 min or until the lysates were no longer viscous. Proteins were precipi-

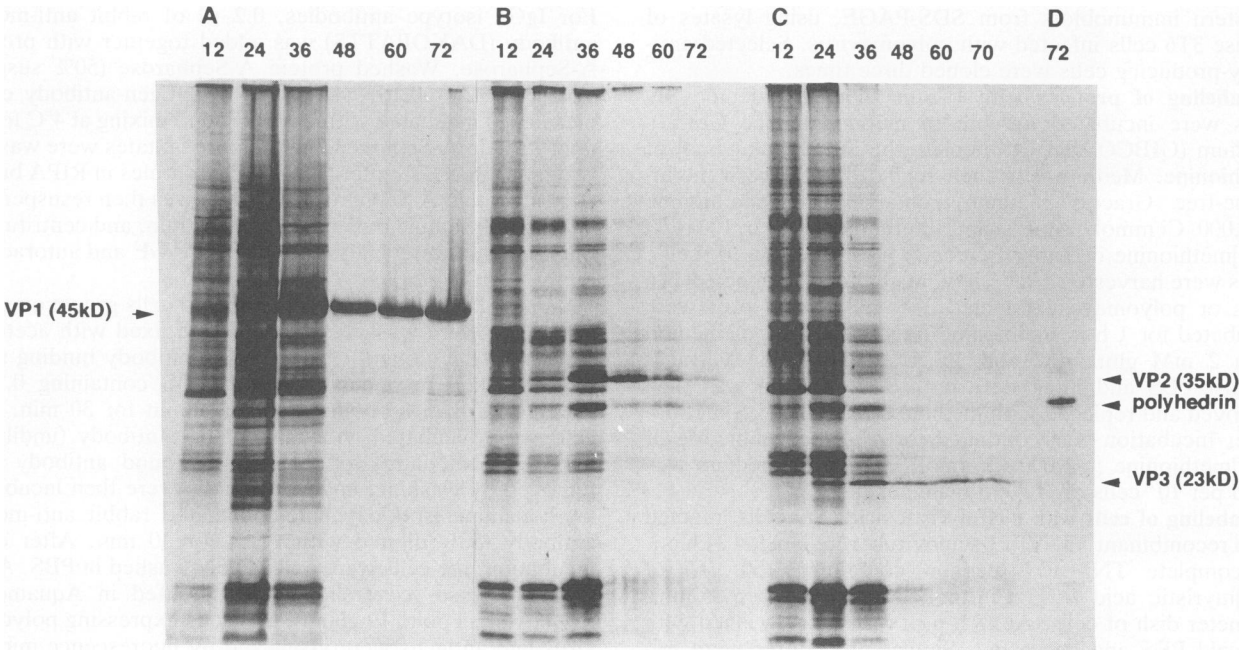

FIG. 1. Time course of production of polyomavirus late antigens in insect cells infected with recombinant baculoviruses. (A) VL-VP1; (B) VL-VP2; (C) VL-VP3; (D) wild-type AcNPV. Sf9 cells were labeled with [^{35}S]methionine for 3 h at the indicated times (hours) p.i. Products from total lysates of labeled cells were resolved on SDS–15% polyacrylamide gels as described in Materials and Methods.

tated by addition of 4 volumes of ice-cold acetone and incubation on ice for 30 min. Precipitates were harvested by centrifugation, air dried, and resuspended in a 1:4 ratio of SB1 to SB3 (9.9 M urea, 4% NP-40, 2.2% pH 3 to 10 ampholytes [Millipore], 100 mM dithiothreitol) (100 μl). Insoluble material was repelleted, and the supernatant (20 μl) was loaded on prefocused urea-acrylamide tube gels (18 cm by 2 mm, internal diameter) containing pH 3 to 10 ampholyte mix and focused for 17.5 h. Following brief equilibration (in 0.3 M Tris-base–0.075 M Tris-HCl–3% SDS–50 mM dithiothreitol–0.01% bromophenol blue), gels were loaded onto a second-dimension (24 by 24 cm) SDS–10% polyacrylamide gel. Gels were then fixed, dried, and autoradiographed for 6 h on Kodak XAR film. Unlabelled lysates of Sf9 cells, infected with VL-VP1 and VL-VP2, were fractionated as described above, Western immunoblotted, and probed with the anti-VP1 monoclonal antibody.

RESULTS

Production of polyomavirus late antigens by insect cells. The transfer plasmids pVL-VP1, pVL-VP2, and pVL-VP3 were constructed as described in Materials and Methods. Three recombinant baculoviruses containing polyomavirus VP1, VP2, and VP3 genes under the control of the polyhedrin promoter were prepared by cotransfection of transfer plasmids and purified DNA from cells infected with wild-type AcNPV baculovirus. Plaques containing the desired recombinant viruses were detected by visual screening, purified, and tested for the presence of polyomavirus late gene sequences by hybridization.

To monitor the expression of polyomavirus late antigens in insect cells, Sf9 cells were infected with either VL-VP1, VL-VP2, or VL-VP3 recombinant baculovirus and pulse-labeled with [^{35}S]methionine at various times p.i. Radiolabeled lysates were analyzed by SDS-PAGE (Fig. 1). Lysate from wild-type AcNPV-infected cells labeled 72 h p.i. was analyzed for comparison. From the time course study, it is apparent that at late times after infection, insect cells synthesized predominantly proteins with the sizes anticipated for VP1 (Fig. 1A), VP2 (Fig. 1B), VP3 (Fig. 1C), and polyhedrin (Fig. 1D), i.e., 45, 35, 23, and 30 kDa, respectively.

The level of synthesis reached a maximum at about 36 to 40 h p.i. but was maintained up to 60 h p.i., by which time recombinant VP1 and VP2 proteins became the predominant bands on Page blue-stained gels (data not shown). The highest expression was achieved with the VP1 gene, for which production of the protein reached the level of polyhedrin synthesis in wild-type baculovirus-infected cells. VP3 could be easily detected as a band on Page blue-stained gels, but the expression was substantially lower. The different levels of expression, highest for VP1 and lowest for VP3, were observed with five independent recombinant baculovirus isolates tested.

VP2 protein produced in insect cells could be resolved on SDS-gels into two bands with the same mobilities as the doublet of VP2 protein from polyomavirus-infected mouse cells (Fig. 2, lanes 1 and 3). The ratio of species in insect cells is about 1:1, while in lysates of polyomavirus-infected mouse cells harvested 36 h p.i., the slower-migrating VP2 band is present at a lower level. Labeling with [^{3}H]myristic acid confirmed that the recombinant VP2 protein is efficiently myristylated in insect cells (Fig. 2, lane 4).

Antibody production. Two positive first-passage clones which immunoprecipitated the VP1 protein from lytically infected cells were detected in hybridomas derived from spleen cells of mice injected with VP1. One of them was cloned to produce a line secreting anti-VP1 antibody, αPyVP1-A, isotype IgG1. In the case of VP2, 20 clones from the first passage produced antibody which strongly reacted specifically with both recombinant baculovirus-infected in-

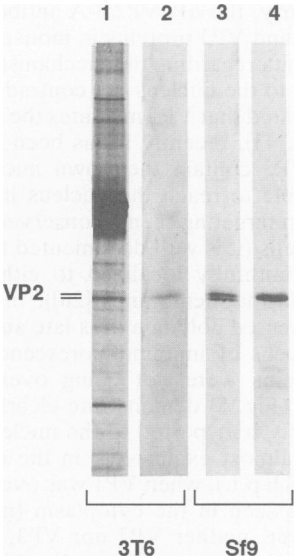

FIG. 2. Myristylation of VP2 expressed in insect cells. Immunoprecipitates of polyomavirus-infected 3T6 mouse cell lysates labeled with [^{35}S]methionine (lane 1) or [^{3}H]myristic acid (lane 2) were resolved by SDS-PAGE. For comparison, proteins from whole lysates of Sf9 cells resolved by SDS-PAGE, infected with VL-VP2 baculovirus and labeled with [^{35}S]methionine (lane 3) or [^{3}H]myristic acid (lane 4), are shown.

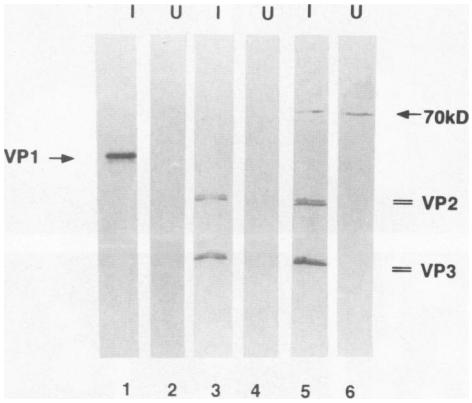

FIG. 3. Western blot analysis of polyomavirus-infected (I lanes) or uninfected (U lanes) 3T6 mouse cell lysates fractionated on SDS–15% polyacrylamide gels. The blots were incubated with mouse monoclonal antibodies αPyVP1-A (lanes 1 and 2), αPyVP2/3-A (lanes 3 and 4), and αPyVP2/3-B (lanes 5 and 6), incubated with rabbit anti-mouse antibody-peroxidase conjugate, and developed with DAB.

sect and polyomavirus-infected mouse cell proteins immobilized on nitrocellulose filters. All stable clones analyzed recognized epitopes from the carboxyl terminus of the VP2 molecule, common to both VP2 and VP3 proteins. To date, no stable clone for VP2 alone has been obtained, suggesting that the VP2 unique region, including the myristyl moiety, may be less immunogenic. (This is not necessarily the case with SV40, for which isolation of a hybridoma line secreting antibody reacting with the unique part of VP2 has been described [21].) Two hybridomas were further cloned to produce homogeneous lines expressing antibodies αPyVP2/3-A, isotype IgG1, and αPyVP2/3-B, isotype IgG2a.

Characterization of antibodies by immunoblotting. Antibody specificities were analyzed on Western blots. Figure 3 shows an immunoblot of proteins obtained from uninfected (lanes 2, 4, and 6) and polyomavirus-infected (lanes 1, 3, and 5) mouse cell lysates resolved by SDS-PAGE. Antibody αPyVP1-A reacted with a single band corresponding to VP1. Antibodies αPyVP2/3-A and -B reacted with VP2 and VP3 proteins, identifying a doublet for both VP2 and VP3 (lanes 3 and 5). We observed a doublet for VP3 in virions and capsids (see Fig. 4B), but the nature of the presumed modification which causes this separation into two components is not known. Antibody αPyVP2/3-B, in addition, cross-reacted with a cellular protein of ca. 70 kDa (lanes 5 and 6), indicating that αPyVP2/3-A and αPyVP2/3-B recognize different epitopes in the VP2/VP3 sequence.

Immunoprecipitation. The antibodies obtained could immunoprecipitate native proteins from lytically infected mouse cell lysates even though the antigens used in their preparation were initially purified from SDS-PAGE. All three polyomavirus late antigens were found in immunoprecipitates obtained by using either αPyVP1-A or αPyVP2/3-A and -B, as VP2 and VP3 form complexes with VP1 (Fig. 4A), indicating that none of the antibodies interfere with sequences involved in the interaction between the minor antigens and VP1. (In SV40 VP3, a determinant [single-letter amino acid code KARHKRRNRSSRS] for noncovalent interaction with VP1 has been found within the 13 carboxy-terminal amino acids of the antigen [14]. These sequences are not, however, present in polyomavirus VP3, and the domain on the polyomavirus VP2/3 molecule interacting with VP1 is as yet unknown, as are the location and nature of the reciprocal VP1 domain involved in VP2 and VP3 interaction for both polyomavirus and SV40.) In addition,

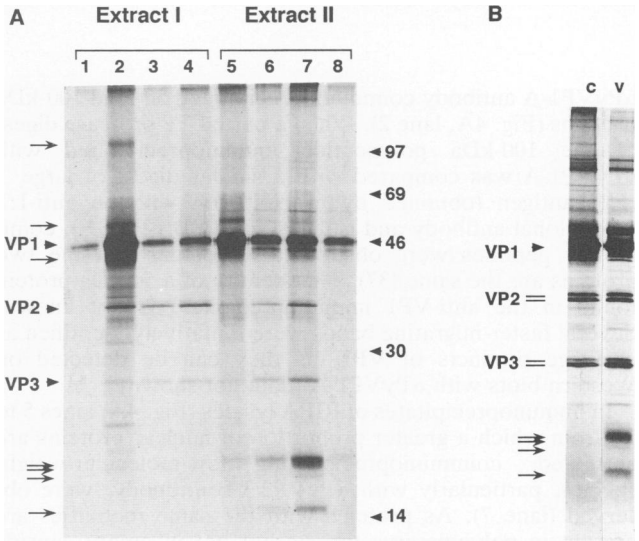

FIG. 4. (A) Immunoprecipitation of products of polyomavirus-infected 3T6 mouse cell lysates labeled with [^{35}S]methionine. Immunocomplexes were precipitated with αPyVP1-A (lanes 2 and 5), αPyVP2/3-A (lanes 3 and 6), and αPyVP2/3-B (lanes 4 and 7). Nonspecific antibody was added in lanes 1 and 8. Extract I, lysis buffer extract; extract II, clarified RIPA buffer extract of insoluble residue from extract I. Arrows indicate coimmunoprecipitating cellular proteins. Sizes are indicated in kilodaltons. (B) Profile of proteins contained in polyomavirus empty capsids and virions fractionated by SDS-PAGE. Lanes: C, empty capsids: V, virions. Arrows indicate locations of cellular histones.

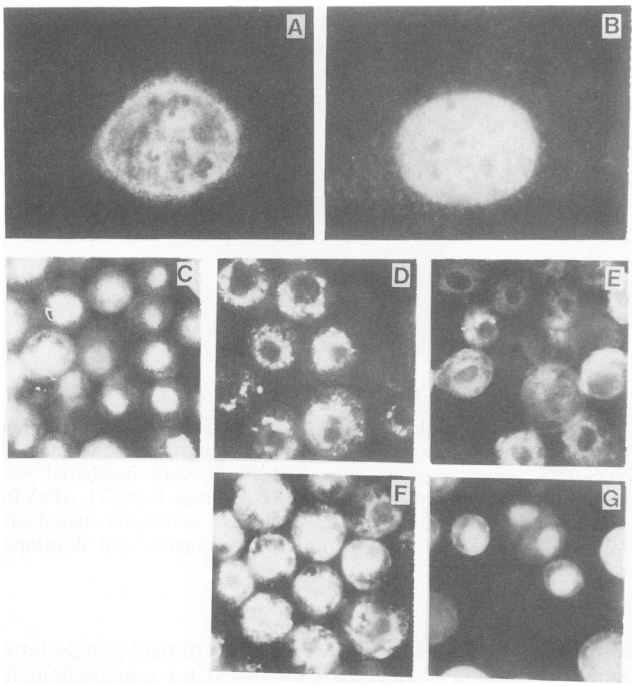

FIG. 5. Localization of late antigens in polyomavirus-infected 3T6 mouse cells and in recombinant baculovirus-infected insect cells examined by indirect immunofluorescence. Mouse cells infected with polyomavirus were fixed (36 h p.i.) and incubated with αPyVP1-A (A) and αPyVP2/3-A (B). Insect cells were infected with VL-VP1 baculovirus, fixed (24 h p.i.), and incubated with anti-VP1 antibody (C) or infected with VL-VP2 (D), infected with VL-VP3 (E), or coinfected with VL-VP1 and VL-VP2 (F) or VL-VP1 and VL-VP3 (G). Cells in panels D to G were fixed and incubated with anti-VP2/3 antibody. Magnification: A and B, ×1,320; C to G, ×330.

αPyVP1-A antibody coimmunoprecipitated 50- and 100-kDa proteins (Fig. 4A, lane 2). When a partial V8 protease digest of the 100-kDa polypeptide immunoprecipitated with αPyVP1-A was compared with a similar digest of large T (LT) antigen (obtained by precipitation with an anti-LT monoclonal antibody and isolated from SDS-PAGE), comigrating peptides were observed, suggesting that the two proteins are the same (37). The identity of a 50-kDa protein found in the anti-VP1 immunoprecipitate is not known. Several faster-migrating bands were tentatively identified as cleavage products of VP1, as they can be detected on Western blots with αPyVP1-A (data not shown).

In immunoprecipitates of RIPA lysates (Fig. 4A, lanes 5 to 7), from which a greater proportion of nuclear proteins are extracted, coimmunoprecipitating low-molecular-weight species, particularly with αPyVP2/3-B antibody, were observed (lane 7). As proteins with the same mobilities are present in polyomavirus virions but not in empty capsids (Fig. 4B), it was thought that those proteins might be histones. In subsequent experiments (data not shown), the 14-kDa species was shown to be recognized on Western blots by a monoclonal antibody against histone 2B (a gift from B. M. Turner) (41).

Indirect immunofluorescence analysis of subcellular localization of polyomavirus late antigens. Mouse 3T6 cells infected with polyomavirus 36 h p.i. and stained by immunofluorescence with the αPyVP1-A antibody showed the presence of VP1 antigen almost exclusively in the nucleus (Fig. 5A). Similarly, the αPyVP2/3-A antibody detected the majority of VP2 and VP3 proteins in mouse cell nuclei (Fig. 5B). Published data regarding the mechanism of transport of the late antigens to the nucleus are contradictory. Although it has been suggested that VP1 mediates the transport of VP2 and VP3 (22, 36, 43), recently it has been shown for SV40 that VP2 and VP3 contain their own nuclear localization signal and are able to reach the nucleus independently (7, 15). Since protein targeting seems conserved between insect and vertebrate cells (it is well documented that proteins can be secreted or faithfully localized to either the nucleus, cytoplasm, or plasma membranes [26]), the localization of individually expressed polyomavirus late antigens was monitored in insect cells by immunofluorescence at early times p.i., when proteins were not being overexpressed. The results obtained (Fig. 5) demonstrate clearly that VP1 protein was efficiently transported to the nucleus. At 24 h p.i., VP1 was found almost exclusively in the nuclei (Fig. 5C), and only later (48 h p.i.), when VP1 was overexpressed, was any fluorescence seen in the cytoplasm (not shown). Surprisingly, however, neither VP2 nor VP3, individually expressed, was found preferentially localized in nuclei (Fig. 5D and E). VP2 was mainly observed in discrete areas attached to or surrounding the outside of the nucleus, whereas VP3 had more diffuse localization in the cytoplasm. These observations indicate that the nuclear localization signal of both minor late antigens of polyomavirus not sufficient per se to direct their transport into the nucleus in insect cells. The data from immunoprecipitation experiments, demonstrating the ability of VP2 and VP3 protein to complex with VP1, suggested that both polyomavirus antigens might be transported into the nucleus in complex with VP1 molecules. Results of coinfection experiments were in agreement with this suggestion (Fig. 5F and G).

Purification and analysis of capsid-like particles from insect cells. It has been shown that VP1 protein alone, expressed in insect cells, is able to assemble capsid-like particles in the nucleus but not in the cytoplasm (29). In our study, capsid-like particles, some of which appeared to contain DNA, were purified from insect cells coinfected with all three baculoviruses (expressing VP1, VP2, and VP3), and their protein content was analyzed by Western blotting (Fig. 6A). Such particles, purified on sucrose and CsCl gradients in several separate experiments, were found to be composed of VP1, VP2, and VP3 proteins, present in ratios similar to that found in CsCl-purified empty capsids from lytically infected mouse cells (Fig. 6B). Some empty capsid preparations have been reported to contain essentially only the VP1 protein (17). However, in this and other (38) studies using mouse 3T6 and whole mouse embryo cells infected with wild-type strain A2 virus, empty capsids have repeatedly been found to be composed of all three late antigens (Fig. 4B and 6B). Particles isolated in gradient fractions shown in Fig. 6A, viewed by electron microscopy, were found to be capsid-like in structure (Fig. 6C).

2D gel analysis of metabolically labeled VP1 species synthesized in insect cells in the presence and absence of VP2 or VP3. Fractionation of VP1 from lytically infected 3T6 cells by high-resolution, large-format 2D gel electrophoresis revealed that the 6 to 7 isoelectric species previously identified (2, 3) could be further resolved into at least 10 distinct species (Fig. 7b and c) that were absent in proteins from an uninfected 3T6 cell lysate run in parallel (Fig. 7a). When only the VP1 protein was expressed in insect cells, species 3, 8, 9, and 10 were underrepresented, whereas species 7 was overrepresented (Fig. 7d). A similar pattern was observed when

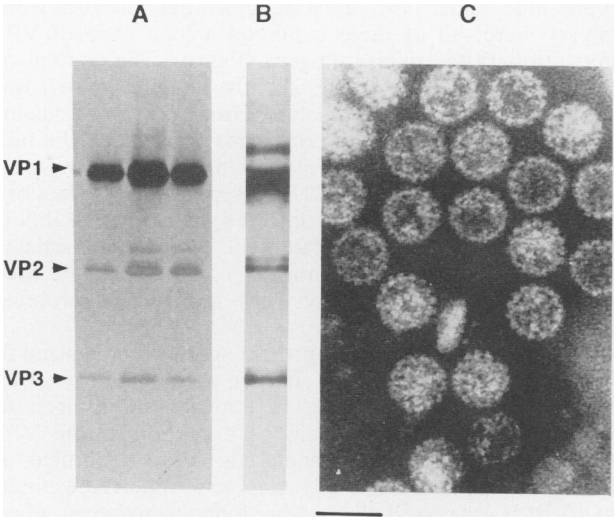

FIG. 6. Purified polyomavirus capsid-like particles from insect cells coinfected with baculoviruses expressing VP1, VP2, and VP3 proteins. (A) Western blot analysis of the three peak fractions of capsid-like particles from CsCl density gradient (buoyant densities from 1.28 to 1.30 g/cm^3) probed with a mixture of αPyVP1-A and αPyVP2/3-A. (B) Western blot analysis of empty capsids purified from wild-type polyomavirus-infected mouse cells, for comparison. (C) Electron microscope photograph of particles from the peak fractions analyzed in panel A. Bar = 50 nm.

VP1 was coexpressed with VP3 (Fig. 7f). However, lysates of insect cells in which VP1 protein was coexpressed with VP2 resulted in a pattern of VP1 subpopulations observed visually (Fig. 7e), and confirmed by densitometer tracings, which more faithfully reproduced that obtained with wild-type polyomavirus-infected mouse cells. These species were related to VP1, as confirmed by their reaction with the VP1-specific antibody on Western blots (Fig. 7g). These data suggest that VP2 mediates specific posttranslation modifications of a part of the VP1 molecule population.

DISCUSSION

Polyomavirus late antigens VP1, VP2, and VP3 have been expressed in insect cells, and specific antibodies have been raised in order to study the life cycle of polyomavirus and, in particular, the functions of the minor late structural proteins, VP2 and VP3. Proteins from recombinant baculoviruses were stable in insect cells and expressed to higher levels than in polyomavirus-infected mouse cells. Although all three proteins were transcribed from the same strong polyhedrin promoter, the levels of expression nonetheless differed. This difference was particularly apparent on Page blue-stained gels. In polyomavirus-infected mouse cells, the observed late antigen synthesis is thought to reflect the ratio of individual mRNAs; 16S RNA encoding the major protein, VP1, represents 80% of total viral mRNA (5, 20). The regulation of messages was presumed to occur during splicing and polyadenylation, as the late mRNAs are processed from one primary transcript. This cannot be the case in baculovirus-mediated expression, however, in which case only coding sequences of the individual antigens (without leader sequence) were introduced into baculovirus transfer vectors and the polyhedrin polyadenylation signal was utilized. Moreover, when Stomatos et al. (36) expressed polyomavirus late proteins in CV1 (African green monkey kidney) cells by using a vaccinia virus vector, Northern (RNA) blot analysis demonstrated approximately equal levels of mRNAs, but the ratio of expressed proteins, 20 (VP1):5 (VP2):1 (VP3), was similar to that observed in the insect cells. Therefore, the control of expression may lie at the level of translation.

The antibodies raised against baculovirus-expressed proteins immunoprecipitated all three late antigens from lysates of polyomavirus-infected mouse cells (Fig. 4) irrespective of whether the antibody was specific for VP1 or for VP2 and VP3. In addition, immunocomplexes of the late antigens, precipitated with αPyVP2/3-B antibody, contained abundant amounts of low-molecular-weight proteins, at least one of which was identified as a cellular histone. However, only traces of histones could be detected in precipitates with αPyVP1-A antibody even though much greater amounts of VP1 were present. This finding suggests that the minor

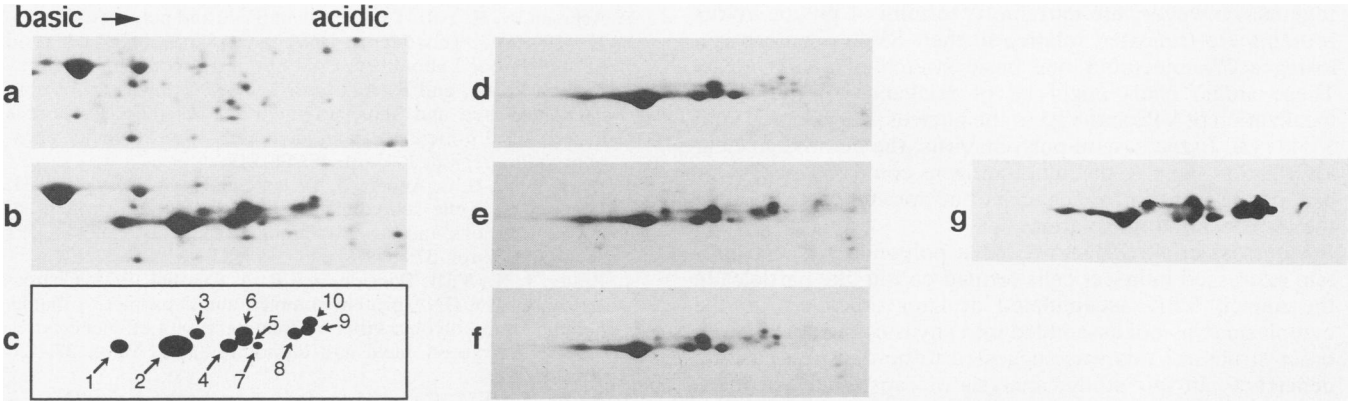

FIG. 7. Resolution of VP1 species on 2D gels. Proteins are fractionated in the first dimension by isoelectric point and in the second dimension by molecular weight. Shown is the portion of the gel containing [^{35}S]methionine-labeled VP1 species from uninfected 3T6 mouse cells (a), polyomavirus-infected 3T6 mouse cells (b), VL-VP1-infected insect cells (d), VL-VP1- and VL-VP2-coinfected insect cells (e), and VL-VP1- and VL-VP3-coinfected insect cells (f). (c) Graphic representation and numbering of wild-type polyomavirus VP1 species; (g) a Western blot of VL-VP1- and VL-VP2-coinfected insect cells, probed with anti-VP1 monoclonal antibody and developed for 2 min with ECL reagent (Amersham). The gel is shown in the orientation from basic to acidic pH in accord with earlier work on VP1 modification (3).

antigens may play a role in histone interaction, either directly or via DNA binding. Our preliminary results from immunoprecipitation of individually expressed late antigens from insect cell lysates suggest that the protein responsible for this interaction is probably VP2. Although it has yet to be demonstrated that VP1, the minor antigens, and histones are all present in the same complex, our observations are consistent with the conjecture, allowed by virion dissociation and X-ray diffraction studies (4, 17), that VP2 and VP3 form a link between the nucleoprotein core and the VP1 capsid shell. The early antigen, LT, has also been identified in complexes precipitated with αPyVP1-A antibody (37). A similar interaction between VP1 and LT proteins has also been observed with use of monoclonal anti-LT antibodies (10). Treatment of the anti-LT antibody immunoprecipitates with DNase I did not appear to disrupt this association, suggesting a direct interaction between these two proteins, the functional relevance of which remains to be determined.

Indirect immunofluorescence analysis of polyomavirus-infected mouse cells, performed 36 h p.i., detected all late antigens in the nuclei. Analysis of localization of individually expressed proteins in insect cells (Fig. 5) showed VP1 to be efficiently transported into nuclei, as expected, since both polyomavirus (6, 30) and SV40 (42) antigens have functional targeting sequences located at their amino termini. However, neither VP2 nor VP3 protein, when separately expressed, could be detected in the nucleus in insect cells. Whereas VP3 was dispersed throughout the cytoplasm, VP2 displayed a defined localization on structures surrounding the nuclear membrane. The myristyl group on the NH_2-terminal glycine of VP2 may account for the difference in location of VP2 and VP3. Similar observations were reported by Stomatos et al. (36) when polyomavirus late antigens were expressed separately by using vaccinia virus vectors. We conclude from these data that the transport of VP2 and VP3 into the nucleus is mediated by complexing with VP1, and results of cotransfection experiments were in agreement with this conclusion. In this regard, polyomavirus may differ from SV40; it has recently been reported that in microinjection experiments, VP2 and VP3 reach the nucleus in the absence of VP1 (7), although such data do not preclude a role for VP1 in transport in vivo (43). There is considerable homology between the minor capsid proteins of polyomavirus and SV40, and both contain a putative nuclear localization signal, P(Q/D)KKKR(R/K), in their carboxyl-terminal regions. However, the extreme C termini of polyomavirus proteins are truncated relative to their SV40 counterparts, losing a 27-amino-acid-long basic stretch of amino acids. These amino acids might be of relevance to the stable localization of VP2 and VP3 to the nucleus, as observed with SV40 (43). In the case of polyomavirus, the putative nuclear localization signal in the minor antigens could be a necessary part of the transport mechanism but not sufficient to effect the translocation in isolation.

Montross et al. (29) showed that polyomavirus VP1 protein expressed in insect cells formed capsid-like particles in the nuclei. VP1, accumulated at later times p.i. in the cytoplasm, was not assembled into any recognizable higher-order structure; this was suggested to be due to a calcium deficiency. In this study, analysis of capsid-like particles, which were assembled in cells coinfected with three separate baculoviruses expressing VP1, VP2, and VP3, was performed. The appearance of the minor antigens in capsid-like particles purified from sucrose and CsCl gradients, as analyzed on Western blots, suggested that VP2 and VP3 antigens, in complex with VP1, reached the insect cell nuclei. Several independent isolations of capsid-like particles were analyzed here; all of them exhibited a high ratio of VP1 compared with the minor antigens (Fig. 4). The content of VP2 and VP3 was, however, slightly lower than that observed in empty particles isolated from polyomavirus-infected mouse cells. This difference may be due to the fact that although the titer of each recombinant baculovirus used was high enough to ensure 90 to 100% efficiency of infection, not every cell was necessarily infected with the three different viruses. Thus, the population of capsid-like particles assembled from the three antigens could be mixed with particles formed with one or both minor viral proteins missing.

The 2D gel analysis of VP1 protein species synthesized in insect cells in the presence or absence of the minor proteins has revealed a possible function for the VP2 antigen in mediating specific modifications of a portion of the VP1 population; three different isoelectric forms identified in lytically infected mouse cell lysates, and underrepresented when VP1 is expressed alone, were found to reappear when VP1 was coexpressed with VP2 (Fig. 7). Acidic VP1 species were also absent in lysates of cells infected with virions obtained from a defective nonmyristylated VP2 mutant polyomavirus (23; our unpublished results), demonstrating that modifications directed by VP2 may be relevant to efficient virus production. Aberrant VP1 patterns have been previously noted in cells infected with mutant NG59 (12, 13). In this case, that several acidic species were missing was thought to be a consequence of the mutation in the middle T antigen gene, which resulted in a virus that grows poorly. Thus, as a working hypothesis, we propose that VP2, in addition to middle T antigen, can modify VP1 or interact with cellular structures and juxtapose VP1 molecules and cellular modifying enzymes during the transport of VP1-VP2 complexes to the nucleus.

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