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Conditional Lethal Expression of the Vaccinia Virus L1R Myristylated Protein Reveals a Role in Virion Assembly

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Within vaccinia virus-infected cells, the product of the L1R open reading frame is covalently modified by myristic acid at the penultimate NH_2-terminal glycine residue. Previously we have shown that while the L1R protein is a constituent of both intracellular mature virus particles and extracellular enveloped virions which are released from the infected cell, it is associated exclusively with the primary membranes surrounding the virion core. Given this rather specific localization, it was of interest to study the potential role of this essential gene in virus replication and morphogenesis. To this end, we have constructed a recombinant vaccinia virus in which expression of the L1R gene can be transcriptionally repressed. Without the inducer isopropylthiogalactopyranoside (IPTG), synthesis of the L1R protein was blocked, resulting in a total inhibition of plaque formation. Velocity sedimentation of viral particles labeled in the presence of [^{3}H]thymidine, grown in the absence of IPTG, revealed a substantial reduction in viral DNA incorporation into virions. Likewise, proteolysis of the major core proteins p4a, p4b, and p25K, believed to occur during the final stages of virion maturation, was severely impaired. In the absence of L1R expression, only immature virions could be detected by electron microscopy. Transient expression of a plasmid containing the full-length L1R gene driven by its own promoter was able to complement and rescue the defective phenotype. However, a plasmid bearing a mutation in the myristyl acceptor glycine residue was unable to biologically rescue the recombinant, and the protein was not detected in purified virions. *trans* complementation using a truncated, myristylated form of the L1R protein partially rescued the defective mutant. Collectively, these data suggest that myristic acid mediates essential interactions of the L1R protein with viral membranes and/or other virion components that lead to the productive assembly, maturation, and release of particles.

Poxviruses are among the largest and most complex eukaryotic viruses and are distinguished by replicating exclusively in the cytoplasm of the infected cell (10). The best-characterized member of this group, vaccinia virus (VV), regulates its gene expression in a temporal fashion during the viral replicative cycle that begins with entry of the virion into the host cell and terminates with the assembly of macromolecular structures to form an infectious particle.

This process of virion assembly has recently received notable attention. The traditional assumption that the immature spherical particle (IV) acquires its primary membrane by a unique de novo biogenesis mechanism (28, 29) has been challenged by studies using cryoelectron microscopy and the colocalization of virion proteins with cellular markers (26). The data derived from these later studies suggest that the IV acquires not one but two closely opposed membranes that are derived by budding through the intermediate compartment, lying between the rough endoplasmic reticulum and the Golgi stacks (25, 26). Maturation of the IV, involving molecular events that are still largely unknown, yields the characteristic brick-shaped particles that have been referred to as intracellular naked virion or intracellular mature virus (IMV). In any case, the IMV particle, which is enclosed by two distinct membranes, can lead to two other morphogenic forms: the four-membraned intracellular enveloped virion, by becoming enveloped by a cisterna derived from the *trans*-Golgi network (18, 25). The intracellular enveloped virion outermost membrane can then fuse with the host cell plasma membrane, giving rise to the three-membraned extracellular enveloped virus.

While electron microscopy (EM) data have provided a view of the virion at the ultrastructural level, other means must be used to investigate the function of known and unknown proteins at the molecular level in the life cycle of the virus. For example, the classical approach to study virus genetics involves the isolation and characterization of temperature-sensitive, deletion, drug-resistant, or drug-dependent virus mutants (reviewed in reference 3). Recently a novel method, which relies primarily on reverse genetic techniques, has been developed for the characterization of virion proteins (33). A recombinant conditional virus is constructed in which elements of the *Escherichia coli lac* operator-repressor system regulate and determine expression of the gene of interest. Using this technology, the virion encoded 11-kDa phosphoprotein, the product of the F18R open reading frame (ORF) has been shown to participate in morphogenesis (34). Similarly, the phenotype of the recombinant for the polypeptide encoded by the D13L ORF is observed to be arrested at the formation of immature viral envelopes, similar to the effect seen by treatment of infected cells with the antibiotic rifampin (15, 35, 27). Under the electron microscope, irregularly shaped structures were observed, which would appear more compacted under normal conditions. In addition, a recent report proposes a scaffolding role for the D13L protein in the formation of viral crescents (27).

In this report, we have extended the investigation of virion proteins that may be involved in the molecular events pertaining to morphogenesis, by constructing and characterizing a conditional, inducible VV recombinant for the L1R gene. This gene encodes a late protein that is covalently modified by the addition of the C_{14} fatty acid myristic acid to an amino-

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terminal glycine residue. The myristylated L1R protein is known to be localized to, and an integral component of, the membranes of the IMV (6, 19, 20). The L1R protein is of particular interest because of its membrane targeting and association signal that appears to reside both in the fatty acid moiety and in the first 12 amino acid residues synthesized (19). Previous work has indicated that this sequence, when fused to a normally soluble protein, is sufficient for localization of a protein to the primary membranes of the virus particle, although this fusion protein does not exhibit the same topology as the wild-type L1R protein (19).

Given that within the cell there exist a variety of different membrane destinations for myristylated proteins, including the plasma membrane, nucleus, endoplasmic reticulum Golgi complex, and mitochondria, it is remarkable that the L1R protein would specifically localize within the membranes of the assembling virion. Therefore, we sought to understand and determine whether this protein has a role during the morphogenic events that lead to the formation of mature particles. Our results indicate that both the L1R protein and myristic acid are involved in these events.

MATERIALS AND METHODS

Cells and virus. VV recombinant v*lacI* (a generous gift of B. Moss) was propagated on monolayers of BSC-40 (African green monkey kidney) cells maintained in modified Eagle's medium (MEM-E; Sigma cell culture reagents; Sigma, St. Louis, Mo.) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Whittaker M. A. Bioproducts, Inc., Walkersville, Md.), 2 mM L-glutamine, and 10 μg of gentamicin sulfate per ml at 37°C, 5% CO_2, and 95% humidity. Recombinant virus v*lacO*L1R was replicated in the continuous presence of 5 mM isopropylthiogalactopyranoside (IPTG). The v*lacO*L1R recombinant was grown and purified from infected BSC-40 cells by two successive cyles of sucrose gradient centrifugation and titrated by plaque assay as described previously (10).

Plasmid vector construction. An 1,143-bp DNA fragment containing the VV L1R promoter, the L1R ORF, and approximately 300-bp flanking regions was amplified from VV genomic DNA by using PCR (5) and oligonucleotide primers complementary to the 5′ (primer MR 20, 5′GATCGAGCT CAGAGTGTTCGAATGCCAATGTT3′) and 3′ (primer MR 21, 5′GTACCCTAGGATCTACAAATTTTTCATCCGC3′) ends of the DNA sequence. The PCR product was extracted twice with phenol-chloroform and concentrated by precipitation in ethanol. The major ethidium bromide-staining species was excised from a 1% agarose gel (Tris-borate-EDTA), phosphorylated by using T4 polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and ligated into the *Sma*I site of plasmid vector pUC118, resulting in pL1R. Using site-directed mutagenesis (12), a 22-bp synthetic *lac* operator sequence was inserted into pL1R, producing p*lacO*L1R (Fig. 1A). Plasmid p*lacO*L1R contains the VV L1R gene with the *lac* operator sequence located immediately upstream of the RNA start site. An *Xba*I-*Cla*I DNA fragment containing the bacterial *gpt* gene encoding xanthine-guanine phosphoribosyltransferase (17) regulated by the VV P7.5K promoter was excised from plasmid pTK7.5K*gpt*F1s, treated with the Klenow fragment of *E. coli* DNA polymerase I (Boehringer Mannheim), gel isolated, and ligated in the *Sal*I site of vector p*lacO*L1R (Fig. 1A). The resulting transfer vector p*lacO*L1R:7.5K*gpt* contains the L1R gene of VV with the *lac* operator inserted one nucleotide downstream of the transcription start site and with the *E. coli gpt* gene as a selectable marker for recombinant virus generation.

The codon for the N-terminal myristic acid acceptor glycine residue of the VV L1R gene was altered to alanine by oligonucleotide site-directed mutagenesis. The synthetic oligonucleotide MR 26 (5′CTGTATACTTGCTGCGGCAGCC ATTTAAA3′) was used to generate plasmid vector pG1A from pL1R. Plasmid pΔC86 expresses a truncated form of the L1R protein in which the codons for the 86 C-terminal amino acids have been deleted. This vector was generated by digestion of plasmid pL1R with *Mun*I and *Bam*HI, treatment of the ends with Klenow enzyme, and ligation of the ends. Following ligation of the ends, the *Mun*I site is restored and a termination codon is introduced. All plasmids were subjected to dideoxynucleotide DNA-sequencing procedures (24) to confirm the authenticity of the inserts.

Isolation of recombinant virus. To generate the recombinant virus v*lacO*L1R, BSC-40 cells (1.4×10^6) were infected with 0.05 PFU of *vlacI* per cell. The infected cells were then transfected by using a liposome-mediated transfection protocol (22) with 5 μg of p*lacO*L1R:7.5K*gpt* and 1 μg of VV WR DNA. Selection for recombinant viruses containing the bacterial *gpt* gene as a result of a single-crossover event (Fig. 1B) was enriched at 24 h postinfection by addition of 25 μg mycophenolic acid (MPA) per ml (4). At 48 h, the infected cells were harvested and lysed by sequential freeze-thawing. The lysate was serially diluted and used to infect six-well plates of BSC-40 cells containing MEM-E supplemented with 2.5% fetal bovine serum and 25 μg of MPA per ml. The wells containing approximately 20 plaques were harvested, and the lysate was used to inoculate BSC-40 cells, which were then overlaid with agarose containing MPA. The gpt^+ virus obtained from separate plaques was used to inoculate BSC-40 cells without MPA, and DNA isolated from individual plaques was analyzed by PCR and agarose gel electrophoresis for the inserted sequence. PCR was performed with oligonucleotide primers MR 20, which is complementary to DNA sequences flanking the L1R gene at the 5′ end, and a *lac* operator primer (PlacO, 5′GAATTGTGAGCGCTCACAATTC3′) (Fig. 2A). The virus containing the *lac* operator sequence was plaque purified four additional times and then amplified to produce a viral stock.

Single-cycle growth curves. BSC-40 cells were infected with VV WR or with v*lacO*L1R in the presence or absence of IPTG, at a multiplicity of infection of 10 PFU per cell. Virus yields from cells harvested at the indicated times postinfection were determined by plaque assay on monolayers of BSC-40 cells in the presence of 5 mM IPTG.

Velocity sedimentation of thymidine-labeled virus particles. Confluent BSC-40 cells were infected with 5 PFU of v*lacO*L1R per cell in the presence or absence of 5 mM IPTG and incubated at 37°C in MEM-E with 5% fetal bovine serum. At 1 h postinfection, 1 μCi of [^{3}H]thymidine (80 Ci/mmol; New England Nuclear) per ml was added to the plates of infected cells. After a 24-h incubation period at 37°C in 5% CO_2, the infected cells were loosened from the plates with a rubber policeman and pelleted by centrifugation at $500 \times g$ for 5 min at 4°C. The infected cells were lysed by swelling in 10 mM Tris-Cl (pH 9.0) followed by Dounce homogenization, and the nuclei were pelleted by centrifugation. Samples were layered on 36% (wt/vol in 10 mM Tris-Cl [pH 9.0]) sucrose cushions, and virus particles were pelleted in an SW41 rotor at 18,000 rpm for 80 min at 4°C. Virus pellets were Duall homogenized, layered on 25 to 40% (wt/vol) sucrose gradients, and centrifuged at 13,500 rpm for 40 min at 4°C. Fractions (0.5 ml) were collected from the top of the gradient, using a density gradient

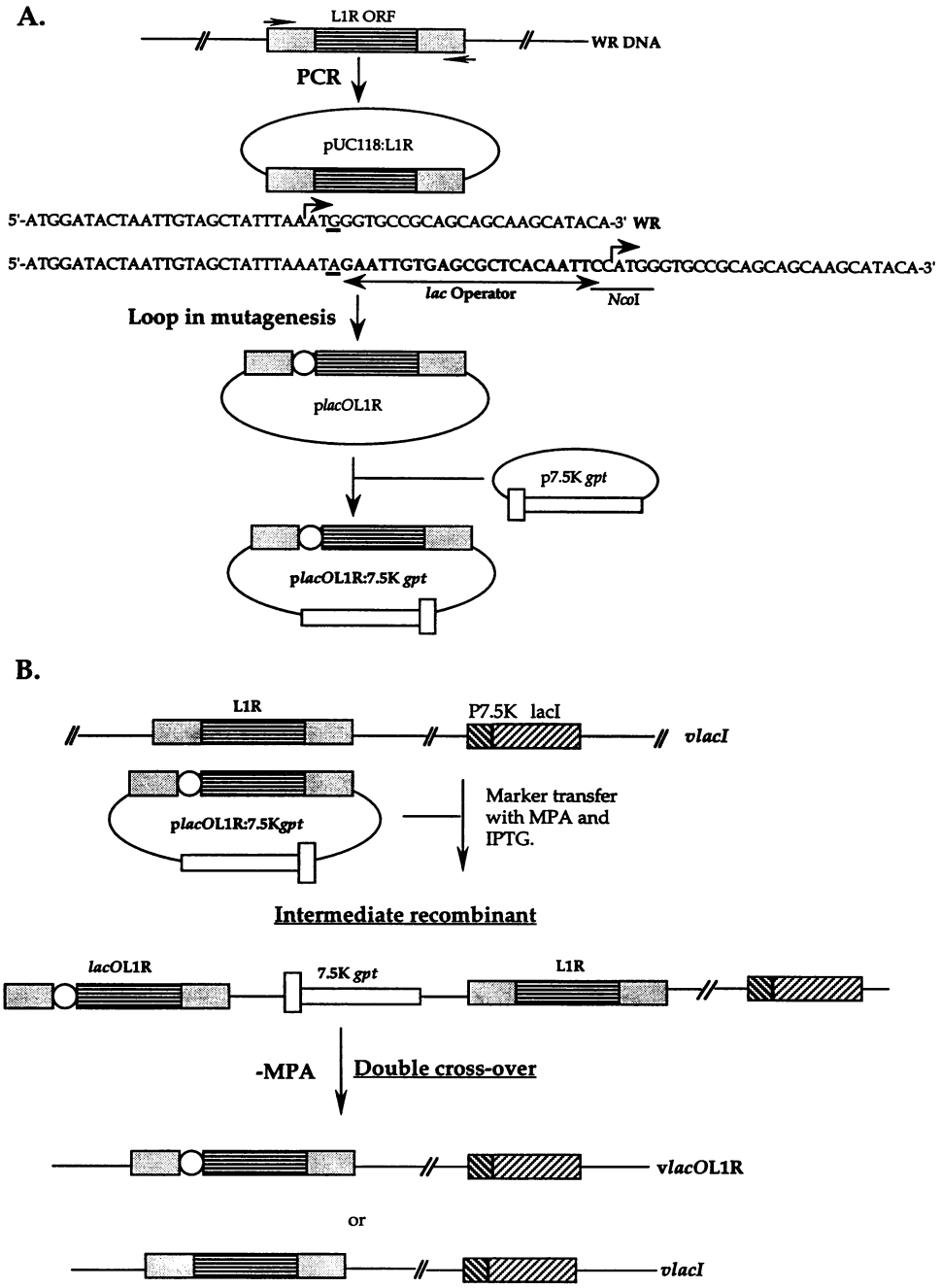

FIG. 1. Design and construction of the recombinant v*lacO*L1R. (A) An 1,143-bp DNA fragment containing the VV L1R late promoter and ORF was amplified by PCR and ligated into plasmid pUC118. The striped box represents the L1R ORF, while the gray boxes represent flanking sequences needed for recombination into the virion DNA. Oligonucleotide-directed site-specific mutagenesis was used to insert a 22-bp *lac* operator sequence and a new translation initiation codon immediately downstream of the TAAAT sequence which is utilized as the RNA start site. The third nucleotide of the wild-type translation initiation codon, G, was altered to A (underlined in bold for the WR strain of VV and for the insertion sequence), followed by insertion of the *lac* operator. An extra nucleotide was appended to this sequence to create a new *Nco*I site as an alternative means to screen for recombinant viruses. The *lac* operator sequence is schematically illustrated as a circle. The p7.5K*gpt* cassette was excised from plasmid pTK7.5K*gpt*F1s and ligated into p*lacO*L1R, resulting in p*lacO*L1R:7.5K*gpt*. (B) Plasmid p*lacO*L1R:7.5K*gpt* was used for marker transfer into the genome of *vlacI*, a recombinant virus which expresses the *lac* repressor protein, in the presence of IPTG. MPA was used to select for recombinant viruses which would contain the *gpt* gene between the wild-type copy of L1R and the mutated one as a result of a single-crossover event. Discontinuing selection with MPA would result in a second crossover event yielding genomes containing either the wild-type or the *lac* operator-altered L1R gene.

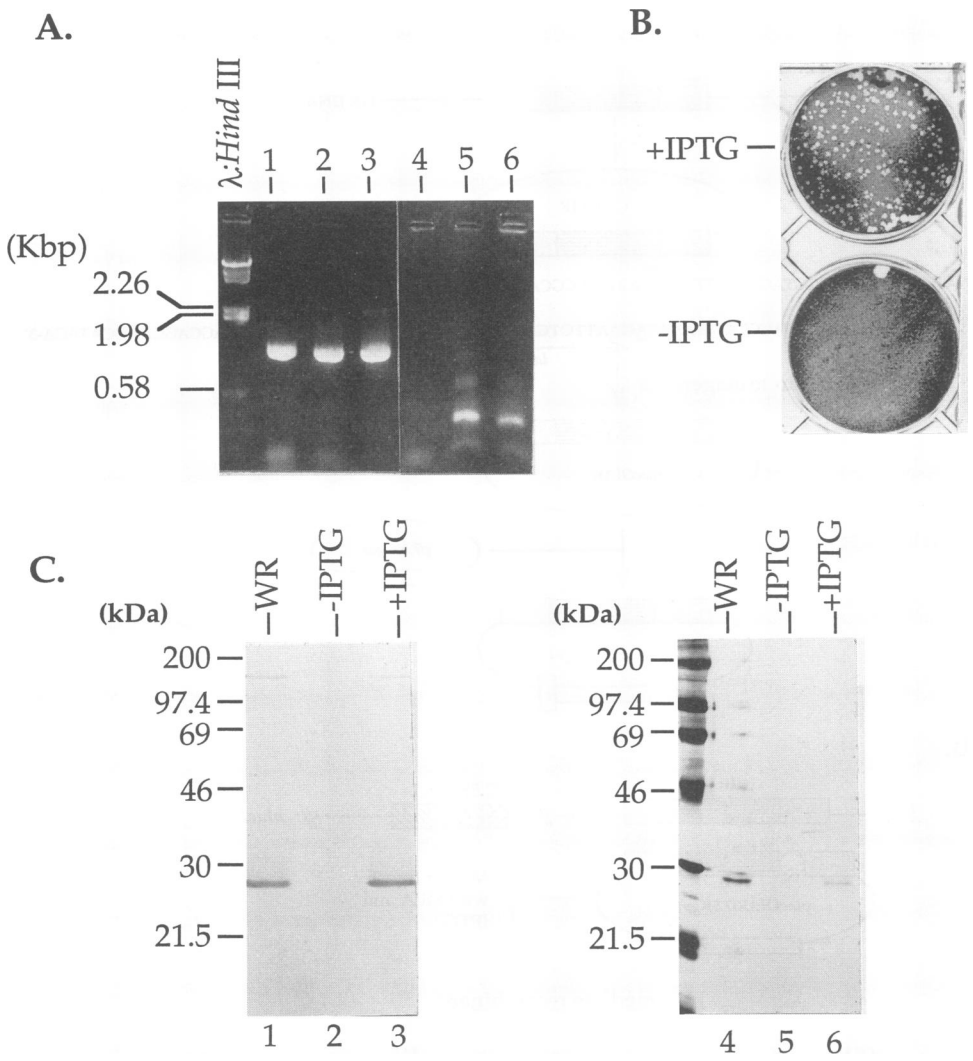

FIG. 2. Characterization of v*lacO*L1R. (A) Analysis of viral DNAs. Oligonucleotide primers complementary to the 5′- and 3′-flanking sequences of L1R (lanes 1 to 3) and to the *lac* operator sequence (lanes 4 to 6) were used to amplify a DNA fragment by PCR. Templates DNAs for PCR: lane 1, wild-type VV WR strain DNA; lane 2, v*lacO*L1R; lane 3, plasmid used for v*lacO*L1R construction, p*lacO*L1R; lane 4, WR DNA; lane 5, v*lacO*L1R; lane 6, p*lacO*L1R. (B) Plaque formation of v*lacO*L1R in the presence and absence of IPTG. Confluent monolayers of BSC-40 cells in a 12-well plate were infected with the recombinant v*lacO*L1R (~100 PFU per well) in the presence (+) or absence (−) of IPTG. At 30 h postinfection, cells were stained with crystal violet. (C) Western immunoblot and radioimmunoprecipitation analysis of viral proteins (lanes 1 to 3). BSC-40 cells infected with the VV WR strain or with v*lacO*L1R in the presence or absence of [^{3}H]myristic acid were harvested and lysed, and a portion of the extracts was subjected to separation on 15% polyacrylamide gels containing SDS or immunoprecipitated as described in Materials and Methods. For autoradiography (lanes 4 to 6), the gel was fluorographed and exposed to film. Extracts: lane 1, WR; lane 2, v*lacO*L1R (without) IPTG; lane 3, v*lacO*L1R with IPTG; lane 4, WR; lane 5, v*lacO*L1R (without) IPTG; lane 6, v*lacO*L1R (with) IPTG. Migration of protein molecular mass standards is indicated at the left.

fractionator (Isco model 185). Fifty-microliter aliquots of each fraction were analyzed for radioactivity by liquid scintillation counting. Peak fractions of the labeled virus were also analyzed by plaque formation.

Analysis of polypeptides by immunoblotting. Infected cells were harvested, washed in phosphate-buffered saline (PBS; pH 7.0), and lysed by addition of 10 U of benzonase (EM Science, Gibbstown, N.J.). Immunoblot analysis was performed as described by Van Slyke et al. (31). Briefly, cell lysates were heated at 100°C for 3 min in a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (50 mM Tris [pH 6.8], 1% [wt/vol] SDS, 0.1% [vol/vol] 2-mercaptoethanol, 1% [vol/vol] glycerol). The proteins were separated on 15% polyacrylamide gels containing SDS and electrotransferred to nitrocellulose filters in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol) (30) for 40 min at 4°C and 25 V. The filters were washed once in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl [pH 7.5]) and blocked with 3% (wt/vol) gelatin (Bio-Rad, Richmond, Calif.) in TBS at room temperature for 2 h. The filters were washed three times with TTBS (0.05% Tween 20 in TBS) and then subjected to binding with an anti-*his*:L1R antibody (19) (dilution of 1:1,000) in antibody buffer (1% gelatin-containing TTBS) at room temperature overnight. After three washes in TTBS buffer followed by one in TBS, the filters were hybridized with an alkaline phosphatase-conjugated goat anti-mouse

antibody (dilution of 1:2,000; Bio-Rad) in antibody buffer for 2 h and washed with TTBS and TBS. The immunoblots were developed in *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (Bio-Rad) in carbonate buffer (0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$ [pH 9.8]).

Metabolic labeling and immunoprecipitation. BSC-40 cells (2.5×10^5) were infected at a multiplicity of infection of 5 infectious particles per cell with the WR strain of VV or with the recombinant virus v*lacO*L1R. At 6.5 h postinfection, the infected cells were pulse-labeled in 1 ml of methionine-free MEM-E containing 150 μCi of EXPRE^{35}S^{35}S protein labeling mix (11.04 mCi/ml [1,094.4 Ci/mmol]; Du Pont New England Nuclear, Wilmington, Del.) for 40 min. After 0 or 6 h of chase incubation in medium containing 100-fold-excess unlabeled methionine, the cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% deoxycholate, 1.0% Nonidet P-40, 0.1% SDS) at 65°C containing 10 U of benzonase. The concentration of SDS was adjusted to 0.4%, and the cell extract was diluted 10-fold in RIPA buffer. Five microliters of a polyclonal cocktail antiserum containing antibodies to the VV major structural proteins p4a/4a, p4b/4b, and p25K/25K (13, 31) was added to the RIPA buffer, and incubation was carried out overnight at 4°C with continuous rocking. One hundred microliters of a 20% (vol/vol) solution of protein A–Sepharose CL-4B beads (Sigma) was added to the antigen-antibody reaction, and incubation was resumed for 2 h at 4°C with continuous rocking. The immune complexes were precipitated by centrifugation in a microcentrifuge, and the beads were washed three times with RIPA buffer. The pelleted antigen-antibody-protein A complexes were released from the beads by heating at 100°C for 5 min in 25 μl of SDS-polyacrylamide gel electrophoresis sample buffer and resolved on SDS–11% polyacrylamide gels. Gels were processed by fluorography using 1 M salicylic acid, dried, and stored at −70°C while exposed to Kodak XAR-5 X-ray film.

For metabolic labeling with [9,10-^{3}H]myristic acid, the label (250 μCi/ml) was dried under nitrogen, dissolved by sonication in dimethyl sulfoxide, and diluted in MEM-E. After removal of the viral inoculum-liposome-DNA solution, the label was added to the cells, and incubation was resumed overnight at 37°C in 5% CO_2. Fluorography for ^{3}H-labeled proteins was performed by the method of Bonner and Laskey (1).

Transient expression. BSC-40 cells were infected with v*lacO*L1R at a multiplicity of infection of 10 PFU per cell by incubation for 60 min at 30°C in PBS containing 1 mM $MgCl_2$ and 0.01% bovine serum albumin. The conditions for transfections of plasmid DNA were described previously (19). Briefly, a liposome-mediated transfection protocol was used (22) in which 30 μl of liposomes was added to 1 ml of MEM-E followed by addition of 5 μg of plasmid DNA. After inversion of the tube and incubation at room temperature for 10 min, to allow for binding of the liposomes to the DNA, the viral inoculum was aspirated, replaced with the liposome-DNA solution, and incubated for 3 h at 37°C in a 5% CO_2 incubator.

EM. VV-infected cells were detached from the culture dish by lightly scraping with a rubber policeman. The cell pellet of a 500 × *g* centrifugation was fixed for 8 h in 2.5% glutaraldehyde in 200 mM cacodylate buffer (pH 7.4) and washed twice in cacodylate buffer. Embedding and sectioning of the samples was carried out by personnel at the OSU Electron Microscopy Laboratory. Samples were visualized on a Philips EM 300 electron microscope.

RESULTS

v*lacO*L1R is an inducible conditional-lethal VV recombinant. The goal of our study was to gain an understanding of the function of the L1R protein during the replication cycle of VV. We have previously shown that this gene product is an integral component of the primary membranes enveloping the virion (20) and is essential for virus replication in tissue culture cells (6). However, no temperature-sensitive mutants have been described for this locus. For these reasons, we constructed a recombinant virus in which synthesis of the L1R gene is under control of the *lac* operator-repressor system. A DNA fragment containing the entire L1R ORF with flanking sequences was amplified from VV WR DNA and ligated in plasmid vector pUC118 (Fig. 1A). By using insertion mutagenesis, the *lac* operator sequence was placed immediately downstream of the L1R gene transcription initiation site. Recombinant viruses were selected by using a transient dominant xanthine-guanine phosphoribosyltransferase selection scheme which takes advantage of the *E. coli gpt* gene (17). The *gpt* gene that is regulated by the VV early/late P7.5K promoter was ligated into the plasmid vector containing the L1R DNA fragment and the *lac* operator sequence, resulting in vector p*lacO*L1R:7.5k*gpt*. This plasmid vector was transfected into cells infected with a recombinant virus which expresses the *lac* repressor protein, *vlacI* (33), in the presence of 5 mM IPTG. By using MPA as a positive selection, recombination events between the plasmid vector and the viral genome were selected (Fig. 1B). These recombination events resulted in the *gpt* gene interposed between the wild-type copy of L1R and the mutated one. Because of the tandem arrangement of two L1R genes and the unstable nature of the *gpt* protein, discontinuing selection with MPA leads to a second recombination event in which the *gpt* gene is lost and either the wild-type virus is re-formed or a recombinant virus is formed (Fig. 1B). After selection with MPA, we isolated 17 plaques. Only one of these plaques contained the recombinant with the *lac* operator-modified L1R gene. The stable recombinant *gpt*-deficient virus was plaque isolated, and its DNA was screened by using PCR for insertion of the 22-bp region between the promoter and the L1R ORF. During PCR screening, primers which hybridized to the *lac* operator sequence or to a VV sequence approximately 300 bp upstream of the transcription start site were used (Fig. 2A). The recombinant virus was plaque purified an additional four times, and a virus stock culture was produced in the presence of 5 mM IPTG.

To demonstrate that L1R gene expression in the recombinant virus was now dependent upon IPTG, cells infected with the WR strain of VV or with v*lacO*L1R were grown in the absence or presence of inducer. At 24 h postinfection, cells were harvested and the extracts were then subjected to Western blot (immunoblot) analysis using an antibody specific for the L1R protein (19). Similar levels of L1R protein were present for both VV WR and v*lacO*L1R grown in the presence of IPTG (Fig. 2C, lanes 1 and 3). However, the extract prepared from v*lacO*l1R-infected cells grown in the absence of IPTG showed no L1R protein band (Fig. 2C, lane 2), thus confirming the repression of synthesis of this polypeptide.

Since expression of the L1R gene from the recombinant was dependent on addition of 5 mM IPTG to the culture medium, a plaque assay was carried out with cells infected with v*lacO*L1R in the absence or presence of 5 mM IPTG. As shown in Fig. 2B, no plaques were visible when the inducer was omitted from the medium, thus confirming the essentiality of this gene product in the life cycle of the virion. To determine the state of myristylation of the L1R protein, v*lacO*L1R-

infected cells grown in the presence or absence of IPTG were labeled with [^{3}H]myristate. Protein extracts from these cells were immunoprecipitated with an anti-*his*:L1R antiserum and analyzed on SDS–15% polyacrylamide gels, and the gels were exposed to X-ray film. The autoradiography revealed that in extracts from the recombinant virus, a myristylated form of the L1R protein was not detected in the absence of IPTG (Fig. 2C, lane 5). A labeled L1R protein band was observed in extracts prepared from cells infected with the control virus, VV WR (lane 4), or with v*lacO*L1R grown in the presence of IPTG (lane 6).

Virus yield under nonpermissive conditions. To investigate the effect of the L1R protein on virus yield, a single-cycle growth experiment of the recombinant was carried out, and the results were determined by plaque titration assay (Fig. 3A). Cells infected with v*lacO*L1R when grown in the presence of IPTG produced virus titers comparable to those produced by the WR strain of VV. However, if v*lacO*L1R-infected cells were grown in the absence of IPTG, the virus yield was reduced by 3 log units. Experiments also demonstrated that the effects of IPTG appear to be reversible. We observed that when the inducer was removed from an infection at 4 h postinfection, the overall yield in virus dropped to levels comparable to those when cells were grown in the absence of IPTG. In contrast, if IPTG was added at 4 h postinfection, the virus yield would reach wild-type levels (data not shown).

Velocity sedimentation of viral particles. Since recombinant virus yield was dramatically reduced in the absence of inducer, it was of interest to determine whether viral particles were assembled. Cells infected with v*lacO*L1R and grown in the presence or absence of IPTG were incubated in the presence of [^{3}H]thymidine to label viral DNA. Virus particles were then purified and analyzed by velocity sedimentation (Fig. 3B). The peak fractions were analyzed for [^{3}H]DNA levels and also by plaque titration. A peak corresponding to labeled virus particles was present in the extracts from v*lacO*L1R-infected cells grown in the presence of inducer. However, few if any mature virus particles were present in the extracts from the infection in which IPTG was omitted from the medium. Virus titers in cells grown in the absence of IPTG were at least 2.5 log units lower than virus titers from cells grown in the presence of IPTG. These results indicate that the labeled DNA incorporated into virus particles that can be isolated by sucrose gradient centrifugation in the absence of IPTG is greatly reduced compared with the DNA isolated when the recombinant virus was grown in the presence of IPTG.

v*lacO*L1R can be genetically rescued by *trans* complementation. A transient expression assay was used in an attempt to complement the inhibition of L1R protein synthesis in cells infected with v*lacO*L1R in the absence of IPTG. Plasmid vector pL1R, which contains the authentic late L1R promoter and ORF, was transfected in BSC-40 cells which were infected with v*lacO*L1R-minus inducer. In this context, the VV L1R gene was expressed from the plasmid. Biological rescue of the recombinant mutant was measured by plaque formation assay of infected cell lysates (Fig. 4A). The results of these experiments indicate that the L1R protein synthesized from a plasmid can complement and rescue the defective phenotype of v*lacO*L1R when grown in the absence of IPTG.

Using this same approach, we analyzed the biological activity of two mutated forms of the L1R gene. In one mutant construct, the codon for the glycine residue, which is covalently linked to the myristic acid moiety, was altered to an alanine codon by site-directed mutagenesis. The resulting plasmid, pG1A, produced a mutated form of the L1R protein which was not myristylated (data not shown). In the *trans*-complementation experiments, this mutant protein did not efficiently rescue virus assembly. Another plasmid, pΔC86, was tested in the *trans*-complementation assay. This plasmid encodes for an L1R protein lacking the 86 carboxy-terminal amino acids. This carboxy-terminal portion of the L1R protein is predicted to posses a high degree of hydrophobicity and could potentially constitute a transmembrane domain. The virus titers recovered from the *trans*-complementation assay using this plasmid were approximately 1 log unit lower than the ones obtained in *trans*-complementation assays using pL1R (Fig. 4A). Three repetitions of this set of transient expression experiments yielded consistent results, suggesting the validity of the observed differences.

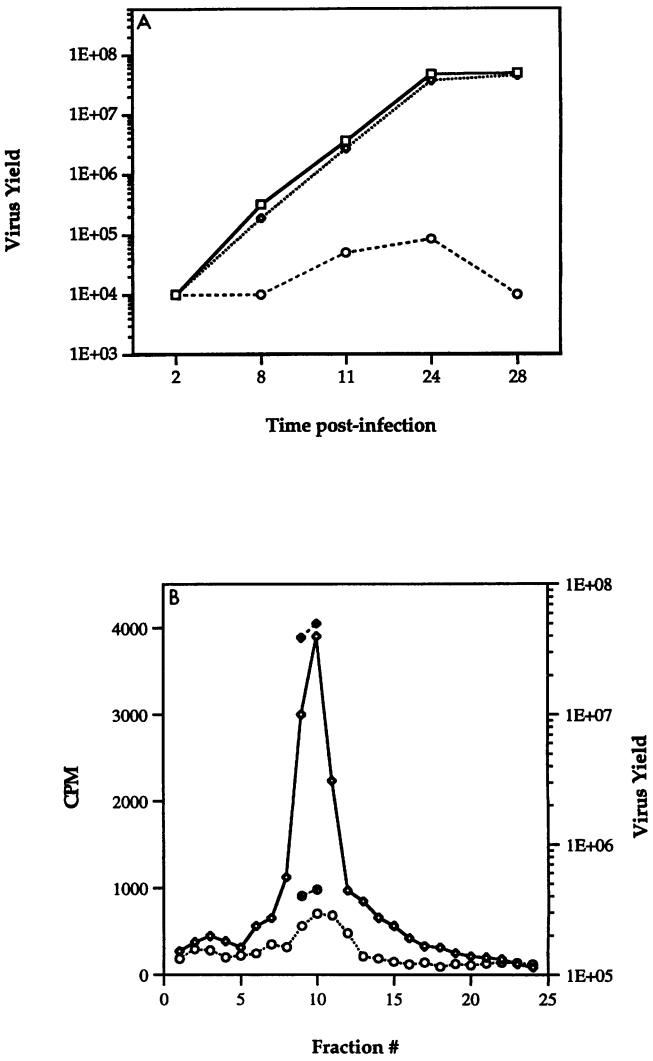

FIG. 3. (A) Effect of IPTG on v*lacO*L1R virus replication. BSC-40 cells infected with VV WR or with v*lacO*L1R in the presence or absence of IPTG were harvested at the indicated times, and the virus yield was determined by plaque assay of harvested virus in the presence of 5 mM IPTG. Symbols: □, VV WR; ◇, v*lacO*L1R plus IPTG; ○, v*lacO*L1R minus IPTG. (B) Velocity sedimentation of viral particles. BSC-40 cells were infected with v*lacO*L1R in the presence (◇) or absence (○) of 5 mM IPTG, and viral DNA was labeled for 24 h with [^{3}H]thymidine. Labeled viral particles were analyzed by velocity sedimentation, and titers of the peak fractions were determined by plaque titration (solid symbols).

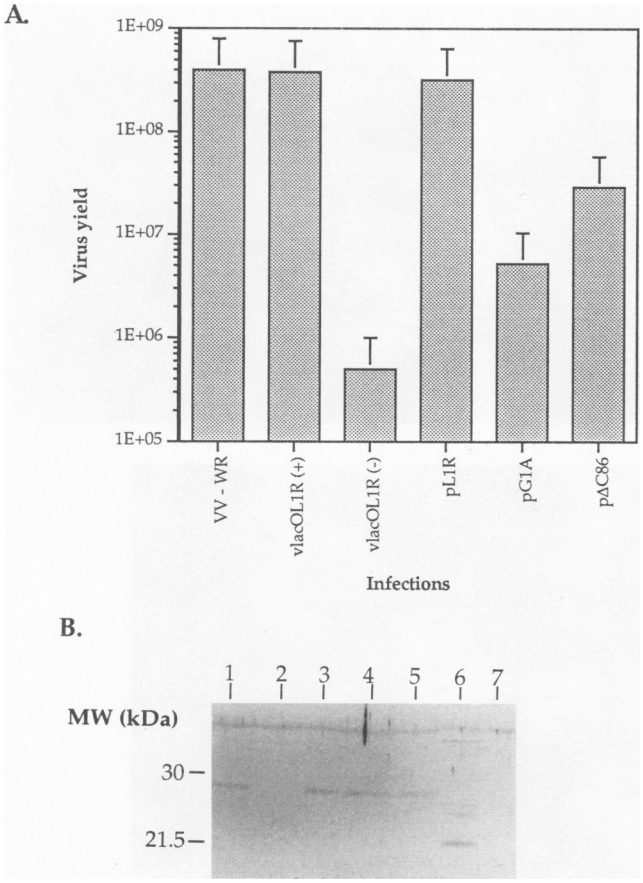

FIG. 4. Transient expression of pL1R, pG1A, and pΔC86 in v*lacO*L1R-infected BSC-40 cells in the absence of IPTG. BSC-40 cells infected with v*lacO*L1R were transfected with plasmid DNA by liposome-mediated transfection; after overnight incubation at 37°C, total cell extracts were prepared and analyzed as follows. (A) Cell extracts were titered by plaque formation. (B) Immunoblot analysis of extracts with anti-L1R antiserum. Infections and transfections: lanes 1 to 3, infection with VV WR, with v*lacO*L1R without IPTG, and with v*lacO*L1R with IPTG; lanes 4 to 6, DNA transfection with plasmids pL1R, pG1A, and pΔC86; lane 7, mock-infected cells. Positions and sizes of molecular mass standards are indicated at the left.

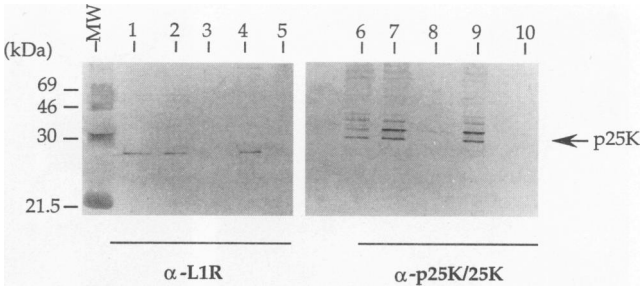

FIG. 5. Virion localization of the L1R and G1A proteins. BSC-40 cells infected with VV WR or with v*lacO*L1R (without) IPTG were transfected with pL1R or pG1A plasmid DNA, and intracellular virus particles were purified by velocity sedimentation on 36% sucrose cushions. Virus particles (approximately 10 μg) were examined on 15% polyacrylamide gels containing SDS and electroblotted onto nitrocellulose. Specific proteins were detected with either an L1R- or 25K-specific serum. Lanes: 1 and 6, VV WR; 2 and 7, v*lacO*L1R with IPTG; 3 and 8, v*lacO*L1R without IPTG; 4 and 9, transfections done with plasmid pL1R; 5 and 10, transfections done with plasmid pG1A. Molecular mass standards are indicated at the left.

In view of the fact that the G1A form of the L1R protein was not able to rescue the recombinant and lead to productive particle formation, we sought to characterize this protein in vivo. Immunoblot analysis of protein extracts prepared at 24 h postinfection revealed that plasmid pG1A encoded a protein of the correct size (Fig. 4B, lane 5). Using the same procedure, we were also able to detect a protein band for the truncated ΔC86 L1R protein (lane 6), which was approximately 9.5 kDa smaller than the wild-type L1R polypeptide (lane 4).

To further examine the role of myristate in particle formation, partially purified virus was prepared from v*lacO*L1R-infected cells transiently expressing the L1R or G1A protein. Approximately 10 μg of each virion preparation was subjected to electrophoresis on 15% polyacrylamide gels containing SDS. The separated proteins were then electrotransferred to nitrocellulose and analyzed by using standard immunoblotting techniques with an anti-*his*:L1R antiserum (19). The virus purified from the pL1R transfection contained a protein product (Fig. 5, lane 4) of the size of L1R, suggesting that the plasmid-encoded protein is present in mature virions. The G1A protein, however, was not detected by this analysis (lane 5). As a control, to confirm the presence of virions in these fractions, a polyclonal antibody specific for the virion core protein p25K/25K was used on identical samples (13). The 25K protein, which comprises approximately 7% of the virion mass, is synthesized late in infection as a larger-molecular-weight precursor (p25K). Proteolysis of p25K and the other major structural proteins of VV, such as p4a and p4b, is thought to be tightly regulated and coupled with morphogenic events. The antiserum used in this analysis recognizes both the precursor and the product of the 25K protein. This analysis revealed that the majority of p25K virion protein exists in its precursor form in the virions formed during transient expression from pG1A (lane 10). A fainter band representing the cleaved 25K product was barely visible in these blots. In contrast, the mature form of p25K (25K) was clearly observed in virus formed during transient expression of pL1R (lane 9). As a control, wild-type WR virus or v*lacO*L1R purified in the absence of IPTG was examined for the presence of L1R and 25K proteins (lanes 1 to 3 and 6 to 8). A faint band was visible for L1R protein in the preparation probed with L1R antibodies, and only a very faint precursor and product band was visible for the p25K and 25K polypeptides, indicating that although a small amount of L1R protein was synthesized (possibly as a result of low-level leak-through of the repression system used), virtually no mature particles were produced.

EM. The effect of the L1R protein on virus assembly was also analyzed by EM. This analysis revealed that virus particles from v*lacO*L1R-infected cells grown in the presence of IPTG were virtually identical to those from an infection with the wild-type VV WR (Fig. 6A and C). In contrast, electron micrographs from v*lacO*L1R-infected cells grown in medium from which IPTG was omitted showed most particles present to be arrested at the immature stage (Fig. 6B). Interestingly, in the micrographs prepared from v*lacO*L1R virus formed during transient expression of pL1R, mature virions were identified (Fig. 6D). However, EM analysis of v*lacO*L1R-derived virus grown in the presence of the nonmyristylated G1A form of L1R did not identify any mature virion particles (Fig. 6E). The only structures visible for this sample were virions in an arrested state of development.

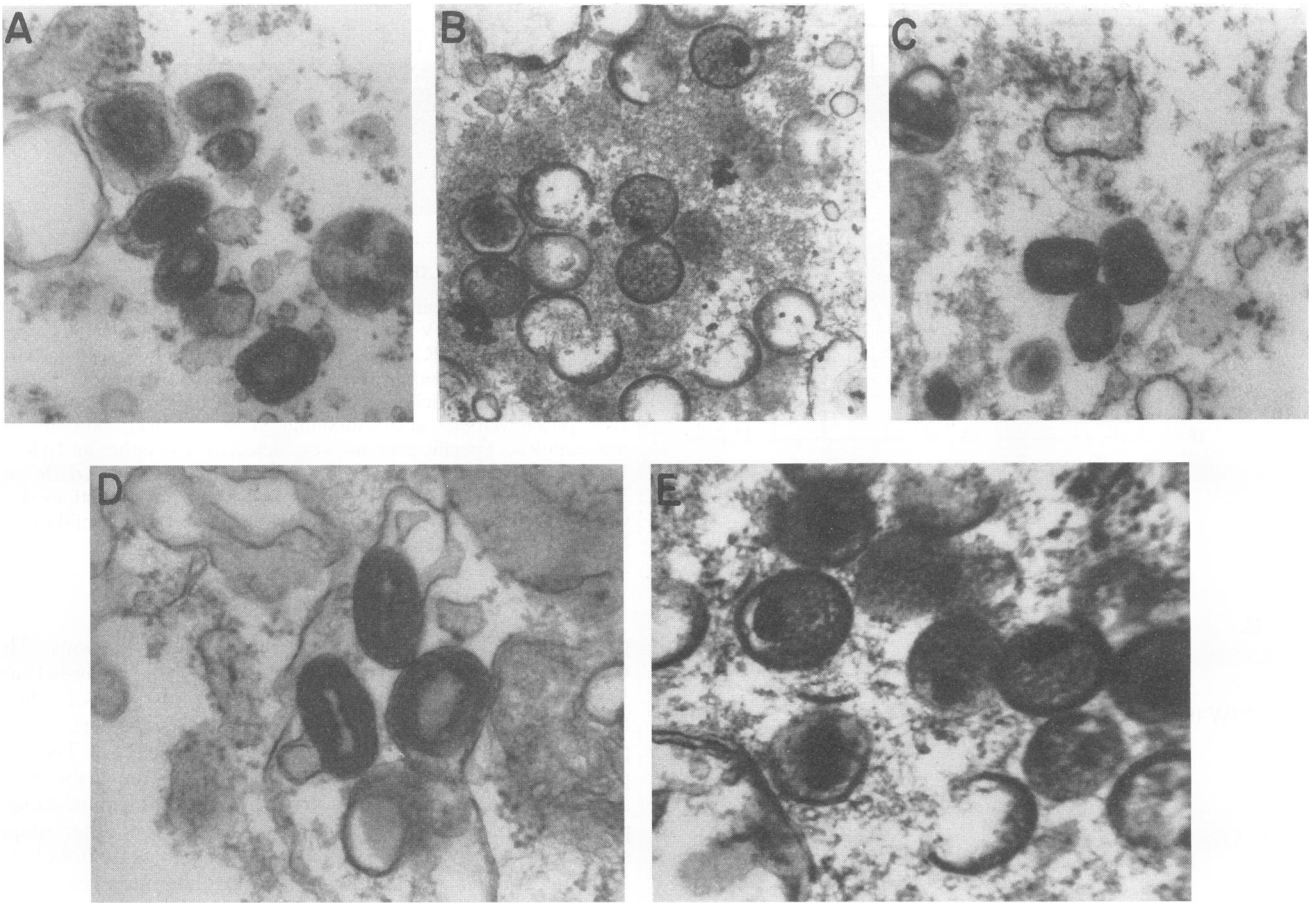

FIG. 6. EM analysis of v*lacO*L1R. VV-infected cells harvested and fixed for embedding were prepared for conventional transmission EM. (A) VV-WR; (B) v*lacO*L1R grown in the absence of IPTG; (C) v*lacO*L1R grown in the presence of IPTG; (D) particles recovered from a pL1R *trans*-complementation assay; (E) particles recovered from a pG1A *trans*-complementation assay. Magnifications: ×38,250 (A and C); ×29,750 (B), ×51,000 (D and E).

Effect of L1R protein on proteolytic processing of viral structural proteins. To achieve a better understanding of the block in virion assembly, a pulse-chase experiment was designed to determine whether the VV structural proteins 4a, 4b, and 25K remain uncleaved in the absence of inducer.

As a control, an infection with VV WR was pulse-labeled at 6.5 h postinfection for a 40-min period with [^{35}S]methionine (Fig. 7, lane 1) followed by a 6-h chase in excess unlabeled methionine. Protein extracts were then prepared, and the presence of the cleaved proteins 4a, 4b, and 25K was confirmed by immunoprecipitation of the labeled extracts with a cocktail of antibodies specific for these polypeptides (lane 2). The conversion of precursors to mature polypeptides was arrested in cells which had been infected with v*lacO*L1R in the absence of IPTG (lanes 3 [pulse] and 4 [chase]). Nevertheless, an identical infection with the recombinant virus performed in the presence of the inducer yielded a result similar to that for the wild-type virus (lanes 5 and 6).

DISCUSSION

The assembly of immature VV precursor particles and their transition to mature virion form is still poorly understood. An understanding of the mechanisms involved in this process could provide information not only relevant to virion assembly but also about determinants which may be involved in the release of the particles from the host cell and the ensuing infection of neighboring cells.

In this study, we provide evidence for the involvement of myristylation of the L1R protein in the events leading to the assembly of mature VV virions. By utilizing the *E. coli lac* operator-repressor system (33), a recombinant virus has been constructed in which the L1R gene can exist in a transcriptionally repressed state. Synthesis and expression of the L1R protein is entirely dependent on addition of the drug IPTG to the cell culture medium. Although previous reports have shown that the concentration of inducer can be titrated to levels of 80 μM without detecting significant reductions in virus yield (33), for this study we chose to use IPTG at a concentration of 5 mM. Physical evidence for the essentiality of this gene product in the VV life cycle was confirmed by the fact that no plaques were detected in v*lacO*L1R-infected cells grown in the absence of IPTG (Fig. 2B). Immunoblot analysis of protein extracts from v*lacO*L1R-infected cells (using an L1R-specific antibody) demonstrated that L1R accumulation was dependent upon IPTG (Fig. 2C).

Since the L1R protein is covalently modified by the addition of the myristic acid moiety to an NH_2-terminal glycine residue (6), it was of interest to ascertain whether the v*lacO*L1R recombinant would be labeled in the same way as the wild-type

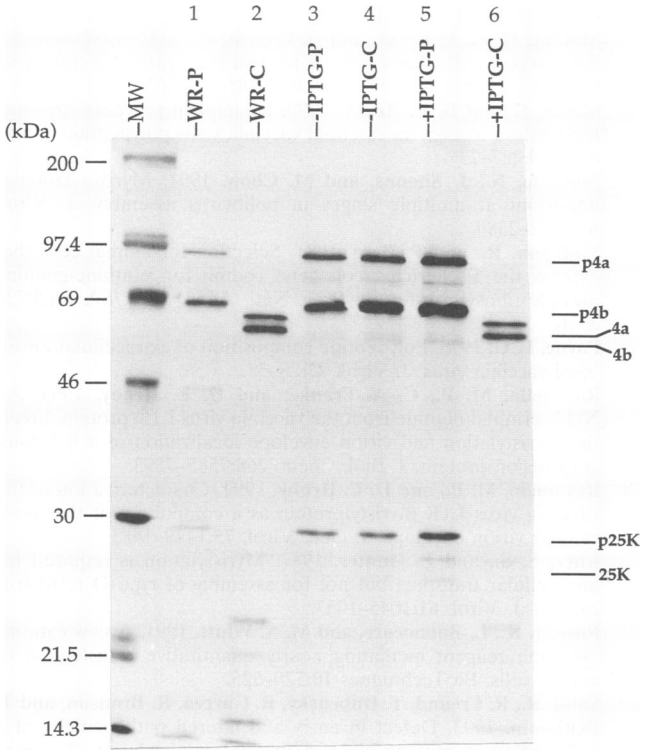

FIG. 7. Pulse-chase analysis of major core proteins p4a, p4b, and p25K in vivo. Monolayers of BSC-40 cells were infected with VV WR or with v*lacO*L1R in the presence or absence of IPTG. At 6.5 h postinfection, cells were incubated with a ^{35}S-labeled mixture of methionine and cysteine. Lysates were prepared either immediately (pulse [P]) or after and additional incubation for 6 h in the presence of 100-fold-excess unlabeled methionine and cysteine (chase [C]). Core proteins p4a, p4b, and p25K and their cleavage products 4a, 4b, and 25K were immunoprecipitated with a cocktail of monospecific antisera raised against these polypeptides and analyzed by polyacrylamide gel electrophoresis and fluorography. Lanes: 1, WR, pulse; 2, WR, chase; 3, v*lacO*L1R without IPTG, pulse; 4, v*lacO*L1R without IPTG, chase; lane 5, v*lacO*L1R with IPTG, pulse; 6, v*lacO*L1R with IPTG, chase.

virion in the presence of radioactive myristic acid. The complete lack of a labeled L1R protein band for cells infected with the recombinant in the absence of inducer (Fig. 2C, lane 5) was observed.

A *trans*-complementation assay was designed in an attempt to rescue the mutant phenotype observed in v*lacO*L1R grown in the absence of IPTG. Using a plasmid bearing a copy of the L1R gene driven by its native promoter, L1R protein could be produced in *trans* in v*lacO*L1R-infected cells. Under these conditions, we were able to observe biological rescue of v*lacO*L1R. The *trans*-expressed L1R protein could also be detected in infected cell extracts and in partially purified virions (Fig. 4B and 5). Samples from L1R *trans*-complementation assay were also examined by EM analysis. These experiments detected assembled VV IMV particles (Fig. 6D). This result indicated that successful assembly and maturation of virus particles was occurring when pL1R was transiently expressed from a plasmid. Taken together, these results suggest that the plasmid-encoded L1R protein can functionally replace the genomic L1R gene and biologically rescue the mutant phenotype of v*lacO*L1R.

To assess the influence of the myristate moiety on the function of L1R, plasmid pG1A was constructed. pG1A encodes for a mutant form of L1R protein in which the myristic acid acceptor amino acid, glycine, has been mutated to an alanine residue. *trans* complementation with pG1A allowed very limited virus assembly, as assayed by virus titer and EM analysis (Fig. 4A and 6). Under EM examination, the majority of the virus particles observed were arrested at the formation of viral crescent stage/immature particles. In addition, the mutant (G1A) protein did not seem to be present in the limited number of virions recovered in this assay (Fig. 5). When these virion preparations were probed with an antiserum for the VV structural protein p25K/25K, the unprocessed precursor p25K protein was the predominant form detected. A most likely hypothesis for the observed absence of the G1A protein from purified virions is that it may not be associated with viral membranes due to the lack of the myristic acid moiety. This hypothesis is supported by previous studies in which a nonmyristylated L1R-chloramphenicol acetyltransferase fusion protein was found predominantly in the cytoplasmic fraction (19). These results indicated a membrane targeting and localizing function for myristic acid in the L1R protein. In addition, the wild-type L1R protein can be extracted from VV virions only by treatment under denaturing conditions. This finding suggests that L1R is an integral component of the primary membrane(s) encircling the IMV particles (19, 20). An alternative hypothesis, that the G1A protein is simply very unstable in VV-infected cells, seems unlikely, since we could detect G1A protein at 24 h postinfection in v*lacO*L1R-infected whole cell lysates (Fig. 4B). Therefore, the reduction in virus titer observed in the presence of nonmyristylated L1R protein suggests that myristylated L1R is involved in the assembly and maturation of VV.

Computer analysis of L1R reveals that the carboxy terminus of this protein is hydrophobic (8). This observation suggests that the COOH-terminal region of L1R may also be involved in localizing (or anchoring) L1R to the virion membrane. Therefore, it was of interest to determine whether this portion of the molecule had any biological relevance in the *trans*-complementation assay. We observed that a truncated form of the L1R protein in which the codons for the C-terminal 86 amino acids have been deleted was able to biologically rescue the recombinant virus, albeit not to the same levels observed for pL1R (Fig. 4A). In the *trans*-complementation assay using the COOH-terminally truncated L1R protein, the virus yield was reduced by at least 1 log unit. Since both the full-length and truncated forms of L1R were modified by myristic acid, the difference in virus titer in this assay was not attributable to a lack of myristylation of the truncated protein (data not shown).

Direct proof for the involvement of myristic acid in the biological activity of eukaryotic viruses has been observed for many groups. In retroviruses, site-directed mutagenesis to either create or obliterate a penultimate glycine acceptor residue in Mason-Pfizer monkey virus (21), Rous sarcoma virus (32), or human immunodeficiency virus (7) has demonstrated that successful myristylation appears to be required for viral assembly and extracellular transport of virions. In type D retroviruses, capsid assembly can occur in the absence of myristylation, but the assembled capsids are not transported to the cell surface (21). On the other hand, mutations that destroy the myristylation in human immunodeficiency virus type 1 and type C retroviruses prevent virion assembly at the plasma membrane (9). In contrast, in duck hepatitis virus, myristylation plays a role in initiation of infection, i.e., in virus entry rather than assembly (14). A distinction between the roles of entry and assembly is often difficult to make. For example, in the case of poliovirus (2, 16) and polyomavirus (11), the characteristics of myristylation-defective mutants have suggested defects in either one or possibly both steps in the viral

replicative cycle. Most recently, myristylation of VP2 of polyomavirus has been shown to play a role early in infection, prior to the uncoating event, and appears to be essential for propagation in mice (23).

The technology of marker transfer has facilitated the study of the function of individual genes in VV. The use of the *lac* operator-repressor system to construct recombinant conditional viruses has provided information on the phenotype of the virion in the absence of a specific gene product. To date we have not pinpointed the exact stage at which the L1R protein appears to be involved in morphogenesis events. EM observations reveal that lack of the L1R protein or of myristic acid does not prevent or alter the characteristic formation of crescent-shaped structures during virion maturation. However, it appears that these do not develop further to form mature virion particles. One possibility is that myristic acid mediates important protein-protein interactions between the L1R protein and other virion polypeptides during morphogenesis. These interactions may help to hold virion structural proteins in a conformationally stable form until further maturation can take place.

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