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The Vaccinia Virus I1 Protein Is Essential for the Assembly of Mature Virions

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The product of the vaccinia virus I1 gene was characterized biochemically and genetically. This 35-kDa protein is conserved in diverse members of the poxvirus family but shows no homology to nonviral proteins. We show that recombinant I1 binds to both single-stranded and double-stranded DNA in a sequence-nonspecific manner in an electrophoretic mobility shift assay. The protein is expressed at late times during infection, and approximately 700 copies are encapsidated within the virion core. To determine the role of the I1 protein during the viral life cycle, a inducible viral recombinant in which the I1 gene was placed under the regulation of the *Escherichia coli lac* operator/repressor was constructed. In the absence of isopropyl-β-D-thiogalactopyranoside, plaque formation was abolished and yields of infectious, intracellular virus were dramatically reduced. Although all phases of gene expression and DNA replication proceeded normally during nonpermissive infections, no mature virions were produced. Electron microscopic analysis confirmed the absence of mature virion assembly but revealed that apparently normal immature virions accumulated. Thus, I1 is an encapsidated DNA-binding protein required for the latest stages of vaccinia virion morphogenesis.

Vaccinia virus, the prototypical member of the *Poxviridae* family, is a complex DNA virus whose genome encodes approximately 200 proteins. Temporally regulated transcription and replication of the genome, as well as viral assembly, occur within discrete regions of the cytoplasm of the infected cell (29). Conventional electron microscopy has enabled a detailed description of viral morphogenesis. By categorizing wild-type (*wt*) infections and cataloging the intermediates which accumulate during nonpermissive infections with appropriate temperature-sensitive (*ts*) mutants, an ordered pathway of morphogenesis has been outlined (8, 29). This pathway has been placed within the context of eukaryotic cell biology with the help of various pharmacological reagents and cell biological markers. Vaccinia virus morphogenesis begins with the formation of spicule-coated membrane crescents which derive from the intermediate compartment between the endoplasmic reticulum and the Golgi apparatus (44). Subsequently, these viral membranes enclose granular material, forming spherical immature virion (IV) particles. IV particles then acquire electron-dense inclusions as nucleoid condensation occurs. Finally, the IV particles transform into the characteristic brick-shaped mature particles within which are seen a defined core. A small portion of these intracellular mature virions (IMV) become wrapped in a double layer of Golgi-derived membrane and are released from the cell by exocytosis (39). These cell-associated and extracellular enveloped particles (3) are responsible for distal viral spread.

A growing body of work on *ts*, drug-resistant, and inducer-dependent conditional lethal mutants has elucidated several of the gene products involved in directing viral assembly. A caveat to this genetic approach is that only the earliest step requiring a protein of interest can be defined. Mutants *ts* for the F10 protein kinase show no signs of immature membrane formation within the cleared areas of the cytoplasm; thus, the F10 protein is required for the onset of virion assembly (49, 51). The A17 protein, a component of the virion membrane, is also required at a very early stage of virion assembly, although morphogenesis proceeds further in the absence of A17 than in the *ts* F10 mutants (34, 35, 54). The cytoplasm of infected cells is still devoid of viral membranes, but electron-dense virosomes are visible. Moreover, numerous vesicles, presumably diverted from the intermediate compartment but blocked in the conversion to crescents, accumulate. The D13 protein (p65), the target of rifampin inhibition (2, 46), has been shown to be responsible for the characteristic rigidity of the virion membranes; in its absence, irregularly shaped, uncoated membrane structures are formed (60). The expression of the myristylated L1 protein, a component of the virion membrane, is required for progression beyond the stage of IV formation (32). In the absence of the viral early transcription factor, IV and masses of electron-dense material accumulate, but mature IMV are not seen (13). This surprising phenotype suggests that encapsidation of the early transcriptional machinery may be required for complete virion maturation. In the absence of the F18 gene product, an 11-kDa DNA-binding phosphoprotein (VP11) that is normally a major virion component, morphogenesis proceeds until IV formation, but nucleoid condensation is aberrant (58). The I7 protein, which shows homology to the type II DNA topoisomerase of *Saccharomyces cerevisiae*, also appears to be essential for nucleoid formation; IV particles enclosing aberrant "half-moon" structures accumulate during nonpermissive infections with a *ts* mutant carrying an I7 lesion (16).

On a fundamental level, the proteins required for the production of IV are involved in membrane reorganization whereas the proteins involved in the transition from IV to IMV include those which interact with DNA. Vaccinia virus contains two abundant DNA-binding proteins which are encapsidated in the virion core, VP11 (see above) and VP8, a 25-kDa protein encoded by the L4 gene (14, 17, 55, 56). VP8-deficient infections lead to the formation of IV which show a visible gap

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between the viroplasm and the surrounding membrane, suggesting that VP8 may mediate interactions between these compartments. These IV progress through morphogenesis, but the resultant IMV are noninfectious and defective in early transcription (52, 53). Together, VP11, VP8, and the I7 gene product are presumed to have roles in DNA condensation and/or genome organization which have yet to be well defined.

In this report, we identify a core protein of 35 kDa which is encoded by the I1 open reading frame (ORF). We demonstrate by S1 nuclease mapping, as well as by protein profile analysis, that I1 is a late protein. By constructing a vaccinia virus recombinant inducible for the I1 gene and characterizing its conditionally lethal phenotype, we demonstrate that this protein is required for the production of mature virions. In the absence of I1, arrest of morphogenesis occurs at a very late stage in the viral assembly pathway, as IV containing normal looking nucleoids accumulate but IMVs are absent. We also show that I1 is a DNA-binding protein and propose that it may work in conjunction with previously described DNA-binding proteins to achieve the final stage of morphogenesis.

MATERIALS AND METHODS

Materials. Restriction endonucleases, calf intestinal phosphatase, polynucleotide kinase, poly(dI-dC), and S1 nuclease were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) or New England Biolabs, Inc. (Beverly, Mass.). [γ-^{32}P]ATP and [^{35}S]methionine were purchased from Dupont NEN (Boston, Mass.).

Cells and virus. *wt* vaccinia virus (WR strain) stocks were prepared in suspension cultures of mouse L cells; v*lac*I and v*ind* I1 were amplified in monolayer cultures of monkey BSC40 cells. All cell lines were maintained in Dulbecco's minimal essential medium (DMEM) plus 5% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). Viral stocks were purified from cytoplasmic lysates by ultracentrifugation through 36% sucrose (40,000 $\times$ *g*, 80 min); banding of virions was accomplished by ultracentrifugation through 25 to 40% sucrose gradients (6,000 $\times$ *g*, 45 min). Titrations and plaque purifications were performed on BSC40 cells; neutral red was used to visualize plaques prior to picking, and crystal violet was used to stain analytical titrations.

Construction, expression, and purification of I1^{His}. The I1 ORF was amplified by PCR using vaccinia virus genomic DNA as a template. The upstream primer (5′TT*GGTACC***CATATG**GCGGAATTTGAAGA3′) introduced *Kpn*I (italic) and *Nde*I (boldface) sites upstream of and overlapping the initiating ATG codon (underlined); the downstream primer (5′TG*GGTACC***AGTACTTA**TTCAGCA TTACTTG3′) introduced an additional *Kpn*I site (italic) and a *Sca*I (boldface) just beyond the termination codon (underlined). The PCR product was digested with *Nde*I and *Sca*I and ligated to pET-14b DNA (Novagen, Madison, Wis.) previously linearized by digestion with *Nde*I and *Bam*HI (blunt). This recombinant plasmid was predicted to direct the synthesis of the I1 protein with a 20-amino-acid N′-terminal extension including a hexahistidine tag, yielding I1^{His}.

HMS174 transformants carrying pET14b-I1 were induced to express I1 by infection with bacteriophage λ CE6, which encodes the T7 RNA polymerase (45). After a 20-min adsorption, infected cultures were shaken vigorously, first at 37°C for 30 min and subsequently at room temperature for an additional 3 h. Cells were harvested and resuspended in 20 mM Tris (pH 7.9)–500 mM NaCl–5 mM imidazole as previously described (23). Cells were lysed by three cycles of freeze-thawing, followed by the addition of Triton X-100 (0.1%), and whole-cell extracts were clarified by centrifugation at 12,000 $\times$ *g* for 15 min at 4°C. These soluble lysates were then applied to nickel-Sepharose columns (Novagen). Columns were washed extensively and developed with buffer containing increasing concentrations of imidazole; the I1^{His} protein was eluted with 250 mM imidazole. The pooled fractions were dialyzed first against a buffer containing 500 mM NaCl, 20 mM Tris (pH 7.4), and 1 mM dithiothreitol (DTT) and finally against a buffer containing 300 mM NaCl, 20 mM Tris (pH 7.4), and 1 mM DTT. This concentration of NaCl was required to keep the concentrated I1 protein in solution. The protein concentration was determined using the Bio-Rad colorimetric assay with lysozyme as a standard. The samples was stored in 50% glycerol at −80°C.

Electrophoretic mobility shift assay (EMSA). Reaction mixtures (20 μl) contained 250 μg of bovine serum albumin per ml, 20 mM Tris (pH 7.4), 50 mM NaCl (except where indicated otherwise), 1 mM EDTA, 12.5% glycerol, various amounts of recombinant I1, as indicated, and 300 fmol of ^{32}P-labeled 24-mer oligonucleotide DNA. Both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) were used; probes were prepared as previously described (19, 20). Samples were incubated at 30°C for 15 min and resolved by electrophoresis through nondenaturing gels (6% acrylamide, 0.12% bisacrylamide) cast and run (20 V/cm, 4°C) in 0.5× TAE (20 mM Tris-acetate, 0.5 mM EDTA). Results were visualized by autoradiography.

Polyclonal antiserum preparation. The PCR-amplified I1 ORF described above was digested with *Xba*I and *Hin*dIII, and the resultant 735-bp fragment was ligated with pATH21 DNA which had been similarly digested. *Escherichia coli* HB101 transformants containing the pATH/I1 plasmid directed the synthesis of a 64-kDa protein containing the amino-terminal 336 residues (37 kDa) of TrpE and amino acids 20 to 265 of the I1 protein ($\cong$27 kDa) (21). This fusion protein was excised from sodium dodecyl sulfate (SDS)-polyacrylamide gels and used to inoculate rabbits. The specificity of the resultant polyclonal antiserum was confirmed by immunoblot analysis. Infected cell lysates were analyzed for [^{35}S]methionine I1 by immunoprecipitation, as described below.

Virion fractionation. Virions (10^{10} particles) were incubated in reaction mixtures containing 100 mM Tris-HCl (pH 7.9), 100 mM DTT and 0.1% Nonidet P-40 (NP-40) at 37°C for 30 min. The membrane fraction (supernatant) was separated from the cores (pellet) by centrifugation at 16,000 $\times$ *g* for 30 min at 4°C. The pellet was resuspended in 300 mM Tris-HCl (pH 8.5)–100 mM DTT–250 mM NaCl–0.1% deoxycholate and incubated at 4°C for 45 min. The solubilized fraction (supernatant) was separated from the insoluble core fraction (pellet) by centrifugation at 16,000 $\times$ *g* for 30 min at 4°C.

S1 nuclease analysis. (i) DNA probe preparation. The DNA probe, a 380-bp fragment containing the first 55 bp of the I1 gene, was generated from a suitable plasmid containing a portion of the *Hin*dIII I fragment of the vaccinia virus genome. After linearization with *Hin*dIII, the DNA was treated with calf intestine alkaline phosphatase to remove the terminal 5′-phosphates and radiolabeled with [γ-^{32}P]ATP in the presence of polynucleotide kinase. Finally, the 380-bp fragment was released by endonuclease digestion with *Bgl*II, resolved electrophoretically, and purified on glass beads (50).

(ii) RNA isolation. Cells were infected with *wt* vaccinia virus (multiplicity of infection [MOI] of 15). Early RNA was prepared from infected cells treated with cycloheximide (100 μg/ml) from 30 min prior to infection until the time of harvesting at 4 h postinfection (hpi). Late RNA was prepared from cells harvested at 8 hpi. Cells were lysed in 4 M guanidinium thiocyanate–25 mM sodium citrate (pH 7)–0.5% lauroylsarcosine–0.1 M β-mercaptoethanol, and total RNA was isolated following ultracentrifugation through cesium chloride as previously described (24).

(iii) Hybridization and S1 nuclease treatment. The hybridization reaction mixture (30 μl), which contained the DNA probe (40 ng; 10,000 cpm) and total RNA (20 ng of early or late RNA) in 80% formamide–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)–0.4 M NaCl–1 mM EDTA, was incubated for 10 min at 65°C to allow denaturation and subsequently incubated at 25°C for 24 h to allow the formation of DNA-RNA hybrids. S1 nuclease digestion was carried out by addition of a 300 μl of cocktail containing S1 nuclease at 400 U/ml, 20 mg of herring sperm DNA per ml, 280 mM NaCl, 30 mM sodium acetate (pH 4.5), 4.5 mM $ZnSO_4$, and 5% glycerol. The reaction mixtures were incubated at 25°C for 1 h, and the nucleic acids were recovered by organic extraction and ethanol precipitation. The resulting samples were resolved on a 9% urea-acrylamide gel and visualized by autoradiography.

Metabolic labeling. (i) Time course analysis. Confluent monolayers of BSC40 cells were infected with *wt* or recombinant vaccinia virus at an MOI of 15. Virus was adsorbed to cells for 30 min in DMEM, after which time the cells were fed with DMEM–5% fetal calf serum. At various times postinfection, cells were metabolically labeled in methionine-free DMEM (ICN Laboratories, Costa Mesa, Calif.) supplemented with [^{35}S]methionine at 100 μCi/ml for 30 min.

(ii) Pulse-chase. BSC40 cells were infected with *wt* virus or v*ind*I1 in the absence or presence of 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an MOI of 2. At 7 hpi, proteins were metabolically labeled with [^{35}S]methionine as described above. After 1 h of labeling, cells were either harvested immediately (pulse) or refed with DMEM containing unlabeled methionine and maintained at 37°C for an additional 4 h (chase). Cells were then harvested by centrifugation and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Immunoprecipitation and immunoblot analyses. For immunoblot analyses, samples were fractionated by SDS-PAGE and transferred electrophoretically to Immobilon-P membranes (Millipore Corp., Bedford, Mass.) in 10 mM CAPS (3-[cyclohexylamino]-1-propane-sulfonic acid) (pH 11) containing 10% methanol. After incubation with the appropriate primary and alkaline phosphatase-conjugated secondary antisera, blots were developed colorimetrically (Bio-Rad, Richmond, Calif.). For immunoprecipitation analyses (18, 24, 33), harvested cells were lysed in 1× phospholysis buffer (10 mM $NaPO_4$ [pH 7.4], 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) and clarified by centrifugation; after incubation with the appropriate antisera, antigen-antibody complexes were purified on protein A-Sepharose (Sigma, St. Louis, Mo.) and analyzed by SDS-PAGE and fluorography (4).

Preparation and isolation of viral recombinant v*ind* I1. (i) PCR. Recombinant PCR was performed as previously described (23, 58). The primers used to insert the *lac* operator downstream of the I1 endogenous promoter were primers 1 (5′ CTggatccAGATGATATGTATG 3′), 2 (5′ *AGAATTGTGAGCGCTCACAATT CATG*GCGGAATTTGAAGA 3′), 3 (5′ *TGAGCGCTCACAATTC***TATTT**AA GTTCACCAAAC 3′), and 4 (5′ CCggtaccTAATCATTGATAGAGAA 3′). The PCR products generated following incubation of primers 1-2 and 3-4 with vaccinia virus genomic DNA were used together as the template for a third PCR involving the outer primers 1 and 4, as previously described (23). This reaction yielded a DNA fragment (I2*op*I1) which spanned the I1 and I2 ORFs and within

which the 22-bp *lac* operator sequence (italic) had been placed between the endogenous I1 promoter (boldface) and the initiating ATG codon (underlined). This final PCR product was digested with *Hin*dIII (lowercase in primer 1) and *Kpn*I (lowercase in primer 4) and was ligated into pUC19 DNA which had been similarly digested. The DNA sequence of the I2*op*I1 insert was confirmed using a Sequenase version 2 kit (United States Biochemical, Cleveland, Ohio). The insert was recloned into a plasmid (pUCneo) (23) which contained the neomycin resistance (Neo^r) gene under the regulation of a constitutive vaccinia virus promoter (11). The final construct (pUCneoI2*op*I1) was used for transfection to generate recombinant v*ind* I1 virus.

(ii) Generation of v*ind* I1. v*ind* I1 was isolated by transient dominant selection as previously described (10, 23). Briefly, following transfection of the pUCneoI2*op*I1 construct into cells infected with v*lac*I (viral recombinant expressing the *lac* repressor), Neo^r virus was twice plaque purified in the presence of G418 (2 mg/ml) and IPTG (5 mM). A third round of plaque purification was performed in the absence of G418 to permit recombination between the two adjacent I1 genes contained within the genome of Neo^r recombinants. Resolution of these tandemly repeated genes by recombination could yield either *wt* virus or a virus containing a single inducible copy of the I1 gene (v*ind* I1). To distinguish between these two types of viruses, PCR was performed on isolated plaques, using the primers 5′ CTGTTCTAAGTGATGAG 3′ and 5′ CAATGC ACGGGCACTGAT 3′. *wt* plaques were predicted to produce a 196-bp product, whereas v*ind* I1 plaques containing the *lac* operator should produce a 218-bp product. Plaques identified as v*ind* I1 were subjected to a final round of plaque purification in the absence of G418 prior to expansion.

Electron microscopy. Cultures were infected at an MOI of 5 with either *wt* virus or v*ind* I1, the latter both in the absence and in the presence of IPTG. At 18 hpi, cells were fixed in situ with 2% gluteraldehyde and processed for electron microscopic analysis as previously described (49). Samples were examined on a JEOL 100CX-II microscope.

Southern blot analysis of viral DNA. BSC40 cells (1.3×10^6) were infected with *wt*, v*ind* I1 (both at an MOI of 2), or vROG8 (57) (MOI of 15) in the absence or presence of IPTG, as indicated. Cells were harvested at 18 hpi, and viral genomic DNA was prepared (25). For each analysis, 10% of the resulting DNA was digested with *Bst*EII and resolved on a 1% agarose gel cast and run in 1× TAE. Gels were alkaline transferred to Zeta-probe blotting membranes (Bio-Rad, Hercules, Calif.) and hybridized with a ^{32}P-labeled 1,200-bp *Pvu*II/*Eco*RI fragment derived from pSV9 (26, 27). This fragment contains the tandem repeats found at the termini of the vaccinia virus genome.

Computer analysis. Comparisons of the deduced amino acid sequence of I1 with entries in the available databases was performed by using the NCBI BLAST protocol (1). Alignment of the deduced amino acid sequences of the I1 proteins of vaccinia virus, variola virus, and molluscum contagiosum virus (MCV) was performed by using the Clustal algorithm and the Lasergene software package from DNASTAR (Madison, Wis.). The Kyte-Doolittle hydrophilicity plot of I1 was also prepared by using the Lasergene software package. For preparation of Fig. 1 to 5A, 6, 8, and 10, the original data were scanned on a SAPHIR scanner (Linotype-Hell Co., Hauppauge, N.Y.), using Adobe Photoshop software (Adobe Systems, Inc., San Jose, Calif.). Labeling of the figures was performed with Canvas software (Deneba Systems, Inc.), and final images were obtained by using a Kodak dye sublimation printer.

RESULTS

We have previously reported the overexpression and purification of the 90-kDa D5 protein, a DNA-independent nucleoside triphosphatase which plays an essential role in vaccinia virus DNA replication (9). When the overexpressed D5 protein was purified by conventional chromatography from vaccinia virus-infected cells, a prominent 35-kDa protein appeared to copurify. Specifically, both proteins eluted from DEAE-cellulose at 500 mM NaCl and subsequently from heparin agarose at 880 mM NaCl. The elution peaks on the final hydroxyapatite column were overlapping: D5 eluted in a broad peak from 180 to 300 mM $NaPO_4$, while the 35-kDa protein was recovered in fractions corresponding to 225 to 300 mM $NaPO_4$. Because we felt that copurification might indicate either a physical interaction between the two proteins or shared biochemical properties that might reflect shared functional properties, we determined the identity of the 35-kDa protein. The amino acid sequence obtained, EFEDQLVFN, indicated that we had purified the product of the vaccinia virus I1 gene. This gene is predicted to encode a protein of 35.8 kDa, in good agreement with our observations.

Subsequent analyses indicated that the purification of I1 with D5 was due to a fortuitous equivalence in their purification profiles rather than protein-protein interactions. I1 retained the same chromatographic properties when D5 was not overexpressed (not shown). In addition, repeated attempts to coimmunoprecipitate I1 by using a polyclonal anti-D5 serum were unsuccessful (not shown). Nevertheless, because little was known about the I1 protein (15, 38, 43) and because its purification profile suggested that it was likely to interact with DNA, we pursued our analysis of its biochemical and biological properties.

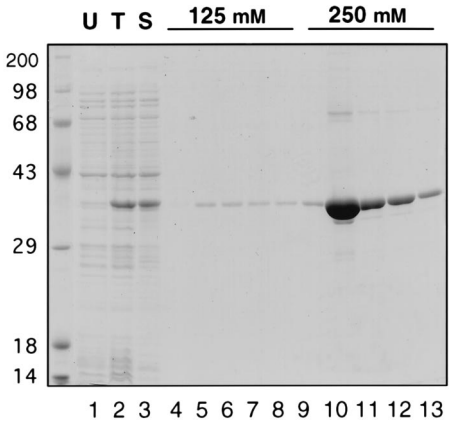

FIG. 1. Visualization of expression and purification of recombinant I1^{His}. Cell lysates from λ CE6-infected *E. coli* were prepared as described (Materials and Methods) and analyzed on an SDS–12% polyacrylamide gel. Proteins were visualized by staining with Coomassie brilliant blue. Lanes: 1, uninduced whole-cell extracts; 2, induced whole-cell extracts; 3, induced soluble lysates; 4 to 8, 125 mM imidizole elution; 9 to 13, 250 mM imidizole elution. Protein standards, with their molecular masses indicated in kilodaltons, are shown at the left.

Expression and purification of recombinant I1 protein. The I1 ORF was amplified and cloned into an appropriate pET vector (45) to allow overexpression within *E. coli* as an N′-terminally His-tagged protein. Upon induction, bacteria carrying the pET-I1 plasmid produced large amounts of soluble I1^{His} which could easily be purified to near homogeneity by chromatography on a nickel agarose resin (Fig. 1).

I1 binds to DNA in a sequence-nonspecific manner. As described above, the chromatographic properties of I1 are consistent with a protein which binds DNA. To test this possibility directly, an EMSA was performed with recombinant I1^{His}. The probe was a 24-mer oligonucleotide which was terminally radiolabeled and, in some cases, hybridized with its unlabeled complement as previously described (19, 20). Reactions containing ssDNA or dsDNA probe, purified I1, and an excess of bovine serum albumin were resolved by electrophoresis through nondenaturing gels as described in Materials and Methods. The mobility of both the ssDNA and dsDNA probes was shifted in the presence of I1 (Fig. 2, lanes 1 and 2 versus 3; lanes 4 and 5 versus 6), indicating that I1 does indeed interact with DNA. A given amount of I1 appeared to shift a similar amount of ssDNA or dsDNA probe, suggesting that the affinities of the protein for both types of DNA were roughly equivalent. Formation of the shifted complex was inhibited when an excess of a nonspecific nucleic acid competitor, poly(dI-dC), was included in the assay (not shown). We conclude that I1 binds to both ssDNA and dsDNA in a sequence-nonspecific manner.

Generation of a polyclonal anti-I1 serum. To facilitate our investigations of the I1 protein, we also prepared a polyclonal anti-I1 serum. Most of the I1 ORF (245 amino acids) was amplified by PCR and cloned into an appropriate pATH expression vector (21) to allow production of a TrpE-I1 fusion

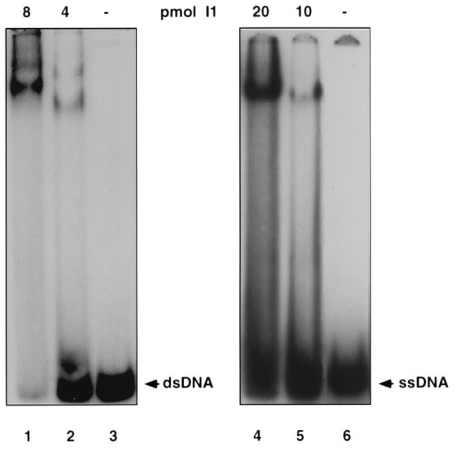

FIG. 2. Analysis of I1 binding to DNA in an EMSA. The EMSA was performed as described in Materials and Methods. The lanes contained purified $I1^{His}$ and DNA as follows: lanes 1 to 3, 300 fmol of ^{32}P-labeled ds oligonucleotide (dsDNA) with 8 pmol, 4 pmol, and no (−) I1 added; 4 to 6, 300 fmol of ^{32}P-labeled ss oligonucleotide (ssDNA) with 20 pmol, 10 pmol, and no I1 added. Gels were dried and analyzed by autoradiography.

protein which was used as the immunizing antigen. The serum specifically recognized the 35-kDa protein in both immunoblot and immunoprecipitation analyses (Fig. 3 and 4C, respectively).

I1 is encapsidated and localizes within the virion core. Because of the apparent abundance of I1 in vaccinia virus-infected cell extracts, and because it did interact with DNA, we considered that I1 might be encapsidated along with the viral genome. Indeed, we found that I1 was encapsidated by subjecting purified vaccinia virions to immunoblot analysis using our anti-I1 antiserum (Fig. 3, lane 1). When various numbers of vaccinia virions and various amounts of purified, recombinant I1 were analyzed in parallel, the signal obtained with 1.6×10^9 particles matched that seen with 62.5 ng of recombinant I1 (not shown). Thus, each virion contains approximately 670 molecules of I1.

To determine the subviral localization of I1, virions were treated with NP-40 (0.1%) plus DTT (100 mM) to release detergent-soluble membrane proteins from the cores, which were further partioned into soluble and insoluble fractions by treatment with deoxycholate (0.1%). Fractionated virions were then subjected to SDS-PAGE and immunoblot analysis with sera directed against D8, L4, and I1. The exclusive detection of

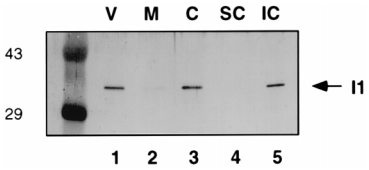

FIG. 3. I1 polypeptide is encapsidated within virion cores. *wt* vaccinia virions were fractionated into membrane and core components with NP-40 plus DTT (see Materials and Methods). Cores were further separated into sodium deoxycholate-soluble and -insoluble fractions. Samples were resolved on an SDS–12% polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride membrane for immunoblot analysis. The immunoblot was developed colorometrically after incubation with a polyclonal antiserum directed against a TrpE-I1 fusion protein. Each lane represents the yield from 2×10^9 virions. Lanes: 1, virions (V); 2, membrane (M) fraction; 3, cores (C); 4, soluble core (SC) fraction; 5, insoluble core (IC) fraction. The arrow indicates the 35-kDa I1 protein. Protein standards, with their molecular masses indicated in kilodaltons, are shown at the left.

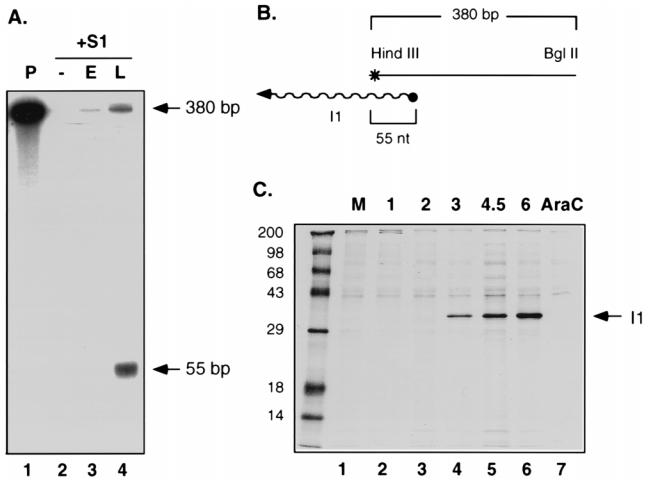

FIG. 4. Analysis of the pattern of I1 synthesis during *wt* infection. (A) Early and late mRNAs were isolated from vaccinia virus-infected cells as described in Materials and Methods and subjected to S1 nuclease protection analysis. Products of the S1 protection assay were fractionated on a 9% urea-acrylamide gel and visualized by autoradiography. Lanes: 1, 5′ radiolabeled probe (P); 2, probe incubated with S1 (−); 3, probe hybridized with early mRNA prior to incubation with S1 (E); 4, probe hybridized with late mRNA prior to incubation with S1 (L). +S1 indicates reactions in which S1 nuclease digestion was performed. (B) Schematic representation of the S1 nuclease protection analysis. The I1 transcript is represented by the wavy line with the arrowhead marking the 5′ terminus. The 380-nucleotide (nt) DNA was singly radiolabeled (*) at the free 5′ terminus of the template strand at a position 55 nucleotides downstream of the mRNA start site. (C) Temporal analysis of I1 protein synthesis during a *wt* infection (MOI of 15). Infected cells were metabolically labeled with [^{35}S]methionine for 30 min at the indicated times (1, 2, 3, 4.5, and 6 hpi). Protein lysates were prepared and subjected to immunoprecipitation with anti-I1 serum. Immunoprecipitates were resolved on an SDS–12% polyacrylamide gel and visualized by fluorography. The lanes correspond to immunoprecipitations of the following extracts: 1, mock infected (M); 2 to 6, harvested at 1, 2, 3, 4.5, and 6 hpi; (7) cytosine arabinoside (AraC) added at time of infection and cells harvested at 4.5 hpi. The arrow indicates the position of migration of I1. Protein standards, with their molecular masses indicated in kilodaltons, are shown at the left.

the vaccinia virus D8 protein in the membrane fraction (31) and L4 in the core fraction (23, 56) demonstrated the effectiveness of the fractionation (data not shown). The analysis performed with the anti-I1 serum revealed that the 35-kDa protein was retained in the core after NP-40–DTT fractionation and, furthermore, was recovered in the deoxycholate-insoluble fraction of the core (Fig. 3, lanes 2 to 5).

Temporal profile of I1 expression during *wt* vaccinia virus infections. We then proceeded to analyze the temporal profile of I1 expression *in vivo*. To analyze the expression pattern of I1 mRNA, an S1 nuclease protection analysis was performed. BSC40 cells were infected with *wt* vaccinia virus, and RNA was harvested at early (in the presence of cycloheximide) and late times postinfection as described in Materials and Methods. These RNA samples were hybridized with a terminally radiolabeled probe (380 bp) which spanned the first 55 bp of the I1 ORF (Fig. 4B); after hybridization, samples were treated with S1 nuclease and resolved on a denaturing acrylamide gel. A 55-nucleotide fragment was protected from nuclease digestion following hybridization with late but not early RNA (Fig. 4A; compare lane 3 with lane 4). I1 is therefore expressed during the late phase of the viral life cycle.

The kinetics of I1 protein synthesis were also monitored during a *wt* infection. Cells were infected with *wt* vaccinia virus (MOI of 15) and pulse-labeled with [^{35}S]Met for 30 min at various times postinfection. Lysates were prepared, and the I1 protein profile was analyzed by SDS-PAGE following immu-

noprecipitation with anti-I1 serum. The synthesis of I1 was first detectable at 3 hpi and continued at increased levels at both 4.5 and 6 hpi (Fig. 4C, lanes 1 to 6). No synthesis was seen when infections were performed in the presence of the DNA replication inhibitor cytosine arabinoside (Fig. 4C, lane 7), confirming that the expression of I1 is indeed part of the late phase of infection.

Construction of a recombinant, v*ind* I1, which allows controllable expression of the I1 gene. None of the available collection of *ts* mutants (5–7, 47) are predicted to have lesions within the I1 ORF. However, since I1 is expressed only at late times of infection, conditional repression of I1 can be achieved and used to probe the role of the protein in the viral life cycle (12, 23, 36, 37, 59). We therefore constructed an inducible recombinant, v*ind* I1, in which expression of the endogenous I1 gene is controlled by the *lac* operator/repressor system. Recombinant PCR technology was used to construct an I1 allele containing the *lac* operator inserted between the endogenous I1 promoter and the initial ATG; subsequently, a viral recombinant containing this inducible allele in place of the endogenous I1 allele was generated by homologous recombination and transient dominant selection as previously described (10, 23, 58). v*lac*I, a virus containing a constitutively expressed copy of the *lac* repressor gene within the nonessential thymidine kinase locus, was the recipient for the inducible allele (23). To identify plaques which had retained only the inducible I1 allele, PCR analysis was performed with primers flanking the I1 promoter. Whereas *wt* plaques yielded a PCR product of 197 bp, v*ind* I1 recombinants yielded a product of 222 bp (data not shown).

To confirm that I1 expression was regulated by inducer during infections with v*ind* I1, cells were infected (MOI of 2) in the presence or absence of IPTG. At 6 hpi, nascent proteins were metabolically labeled with [^{35}S]methionine for 1 h, cell extracts were prepared, and I1 was retrieved by immunoprecipitation. As the autoradiograph shown in Fig. 5A demonstrates, a 35-kDa protein was easily visible under conditions in which IPTG was added to the culture medium (lane 3). No I1 was detected in the absence of IPTG (lane 2). As expected, during *wt* infections I1, expression was equivalent in the presence and absence of IPTG (not shown). Thus, v*ind* I1 represents a recombinant in which synthesis of I1 can be tightly regulated by various experimental conditions: no I1 is detectable in the absence of IPTG, whereas in the presence of IPTG, I1 synthesis occurs at 50 to 100% of *wt* levels.

I1 plays an essential role during the viral life cycle. To determine if I1 repression influenced viral replication, we first compared plaque formation under induced and noninduced conditions. As illustrated in Fig. 5B, cells were infected with serial dilutions of v*ind* I1 in the absence and presence of the inducer, IPTG. After a standard viral adsorption for 30 min, the cells were fed appropriately and incubated at 37°C for 48 h. Plaques were observed only when IPTG was included in the medium. Thus, v*ind* I1 represents a conditional lethal mutant in which plaque formation is dependent on induction of I1 synthesis by IPTG.

Given the localization of I1 within the virion core and its known affinity for DNA, it seemed unlikely that the block to plaque formation during nonpermissive plaque assays with v*ind* I1 was due to a specific defect in the formation of cell-associated or extracellular enveloped virus. It seemed more likely that I1 was required for an earlier step during the viral life cycle. To confirm this and to determine the severity of the conditionally lethal phenotype of v*ind* I1, intracellular viral yields were analyzed. Cells were infected under both permissive and nonpermissive conditions at an MOI of 2 and harvested at 24 hpi. Subsequently, the cell-associated virus was titrated under permissive conditions (with IPTG). Omission of IPTG led to a decrease of 2 logs in infectious virus production (not shown).

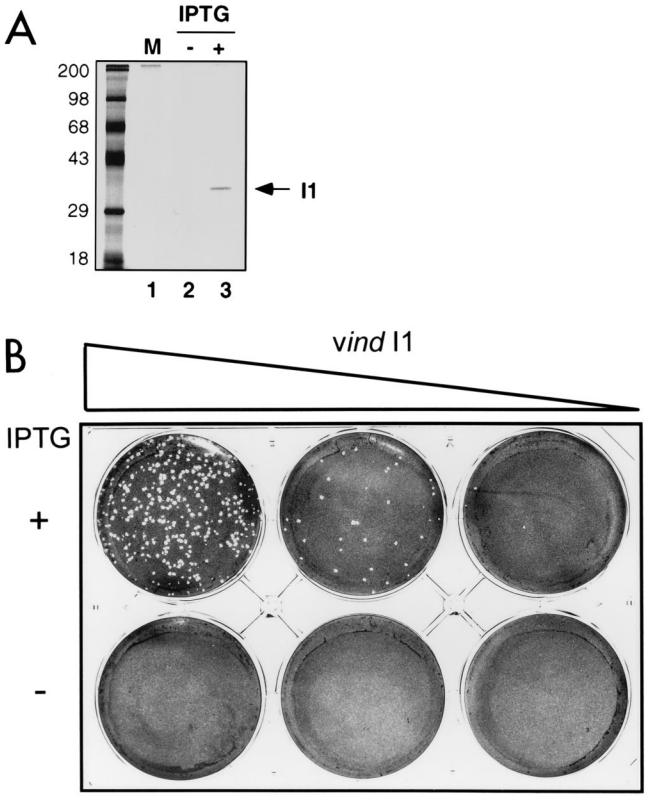

FIG. 5. Effect of IPTG on v*ind* I1 infections. (A) I1 protein synthesis in the presence and absence of IPTG. BSC40 cells were infected with v*ind* I1 (MOI of 2) in the presence (+) or absence (−) of IPTG and metabolically labeled with [^{35}S]methionine between 6.5 to 7.5 hpi. The cells were harvested, and lysates were subjected to immunoprecipitation with anti-I1 serum. The immunoprecipitates were analyzed by SDS-PAGE on a 12% polyacrylamide gel and visualized by autoradiography. (B) Plaque morphology in the presence and absence of IPTG. Confluent BSC40 monolayers were infected with serial dilutions of v*ind* I1, and the cultures were maintained in the presence (+) or absence (−) of 5 mM IPTG. At 48 hpi, plaques were visualized by staining with crystal violet.

v*ind* I1 displays a "normal" phenotype under nonpermissive conditions. To determine if the absence of I1 had any effect on the progression of the temporally regulated cascade of viral protein synthesis, v*ind* I1-infected cells were pulse-labeled at various times with [^{35}S]methionine. The protein profiles were analyzed by SDS-PAGE. As illustrated in Fig. 6, repression of I1 does not affect protein synthesis, since the characteristic shift from host to early, and then to late, viral protein synthesis occurs under both permissive and nonpermissive conditions. We can deduce from the normal profile of late protein synthesis that DNA synthesis also proceeds normally. The protein profile observed is comparable to that seen during *wt* infections (not shown). This conditionally lethal phenotype, in which plaque formation is compromised but early, intermediate, and late protein syntheses occur in a timely manner, corresponds to the normal phenotype described for some of the *ts* mutants of vaccinia virus (5–7).

Mature virions are not produced in the absence of I1. Conditional lethal mutants of the normal class either can be deficient in virion morphogenesis or can produce mature virions which are noninfectious in the next round of infection. To

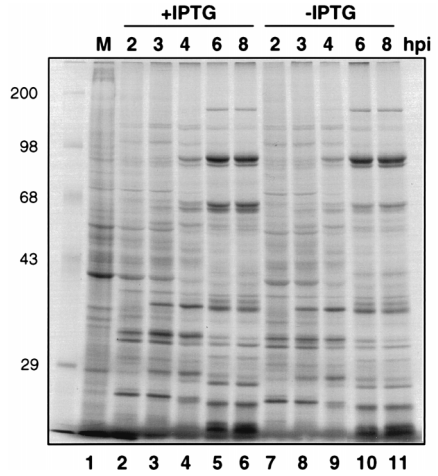

FIG. 6. Analysis of viral protein synthesis following infection with v*ind* I1. BSC40 cells were infected with v*ind* I1 (MOI of 15) in the presence (+IPTG) and absence (−IPTG) of IPTG. Infected cells were metabolically labeled with [^{35}S]methionine for 30 min prior to being harvested at the times indicated (2, 3, 4, 6, and 8 hpi). Cells were harvested and analyzed directly by SDS-PAGE (12% polyacrylamide gel). An uninfected cell lysate (lane M) was labeled in parallel with [^{35}S]methionine and served as a control. ^{14}C-labeled protein standards, with their molecular masses indicated in kilodaltons, are shown at the left.

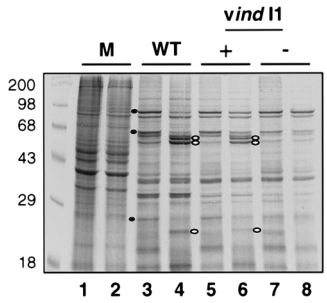

FIG. 8. Proteolytic processing of major structural proteins in v*ind* I1-infected cells. BSC40 cells were infected with *wt* virus (WT; lanes 3 and 4) or v*ind* I1 (lanes 5 to 8), the latter in the presence (+) or absence (−) of IPTG (MOI of 2). At 7 hpi, proteins were metabolically labeled with [^{35}S]methionine. After 1 h of labeling, cells were either harvested immediately (pulse; lanes 1, 3, 5, and 7) or refed with DMEM containing unlabeled methionine and incubated for an additional 4 h (chase; lanes 2, 4, 6, and 8). An uninfected control was treated similarly (M; lanes 1 and 2). The closed circles indicate the positions of migration of unprocessed precursors, and the open circles indicate the positions of migration of the mature products. Harvested cells were analyzed directly by SDS-PAGE (12% polyacrylamide gel) and subjected to autoradiography. ^{14}C-labeled protein standards, with their molecular masses indicated in kilodaltons, are shown at the left.

determine which of these possibilities holds true for v*ind* I1, BSC40 cells (1.6×10^8) were infected with v*ind* I1 (MOI of 2) under permissive and nonpermissive conditions. At 24 hpi, cells were harvested and virions were prepared from cytoplasmic extracts by ultracentrifugation through a 36% sucrose cushion and then by banding on a 25 to 40% sucrose gradient, as previously described (49).

A distinctive band of virions was recovered from permissively infected cells; however, no visible band was detected in samples prepared from nonpermissive infections (Fig. 7). The banded virions prepared from the permissively infected cells were extracted from the gradient by needle aspiration, and an equal volume was extracted from the corresponding position of the gradient representing the nonpermissive infection. Both samples were titrated in the presence of IPTG. The ratio of PFU recovered from the permissive versus nonpermissive infections was 115:1. These data show clearly that, under repressed conditions, v*ind* I1 infections arrest prior to the formation of mature IMV.

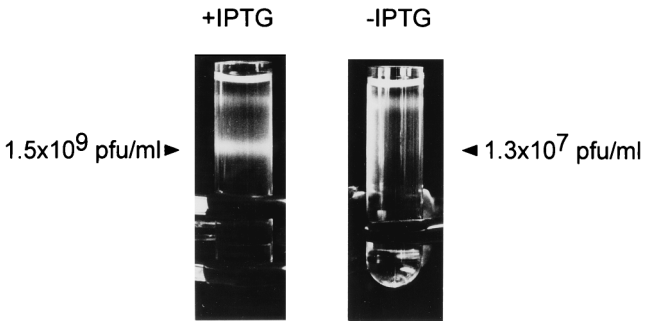

FIG. 7. Comparison of sucrose gradient-purified virions harvested from v*ind* I1 infections maintained in the presence or absence of IPTG. BSC40 cells were infected with v*ind* I1 at an MOI of 2, and cells harvested at 24 hpi. Intracellular virions purified by ultracentrifugation through 36% sucrose were subjected to a second ultracentrifugation on 25 to 40% sucrose gradients, as shown. Arrowheads indicate the positions of sedimentation of mature virions, which are clearly visible in the sample harvested from the permissive infection (+IPTG). No comparable band of virions is present in the sample harvested from the nonpermissive infection (−IPTG). The mature virion band was extracted from the +IPTG gradient by needle aspiration, and an equal volume was removed from the corresponding position of the −IPTG gradient. The virions were recovered by a final centrifugation, and the titers were determined as shown.

Proteolytic processing of late virion proteins is defective when I1 is repressed. The major structural core proteins of vaccinia virus, 4a, 4b, and VP8 (L4), are derived from precursor polypeptides (P4a, P4b, and pre-L4) by proteolytic processing. This processing has been shown to occur late during virion morphogenesis and is inhibited during nonpermissive infections in which virion assembly is blocked (30, 32, 34, 54, 58). Thus, the absence of proteolytic processing is a biochemical indicator of defective morphogenesis. Since nonpermissive infections with v*ind* I1 do not produce mature virions, we predicted that proteolytic processing would be deficient in the absence of I1 expression.

Infected cells were metabolically labeled for 60 min with [^{35}S]methionine at 7 hpi. Subsequently, cells were either harvested (pulse) or refed with medium containing unlabeled methionine and incubated for an additional 4 h (chase). Infections with *wt* virus or with v*ind* I1 in the presence or absence of IPTG were performed in parallel. The profiles seen with *wt* virus and with v*ind* I1 plus IPTG were comparable (Fig. 8, lanes 3 to 6). There is a conversion of the major structural protein precursors into their mature counterparts (lanes 3 versus 4 and 5 versus 6). However, the protein profile observed following infection with v*ind* I1 in the absence of IPTG indicates that little if any processing of P4a, P4b, or pre-L4 has occurred (lane 7 versus lane 8). Thus, synthesis of I1 is required for the proteolytic processing of the major core proteins, consistent with its requirement for IMV formation.

Electron microscopic analysis shows that repression of I1 synthesis allows production of immature but not mature virions. To determine at which stage viral morphogenesis arrests in the absence of I1, electron microscopy analysis was performed. Cells were infected with *wt* virus or with v*ind* I1 under permissive and nonpermissive conditions (MOI of 5); at 18 hpi, cultures were fixed in situ processed for electron microscopy as previously described (49).

The full repertoire of virion intermediates was visible in cultures infected with *wt* virus (not shown) or with v*ind* I1 in

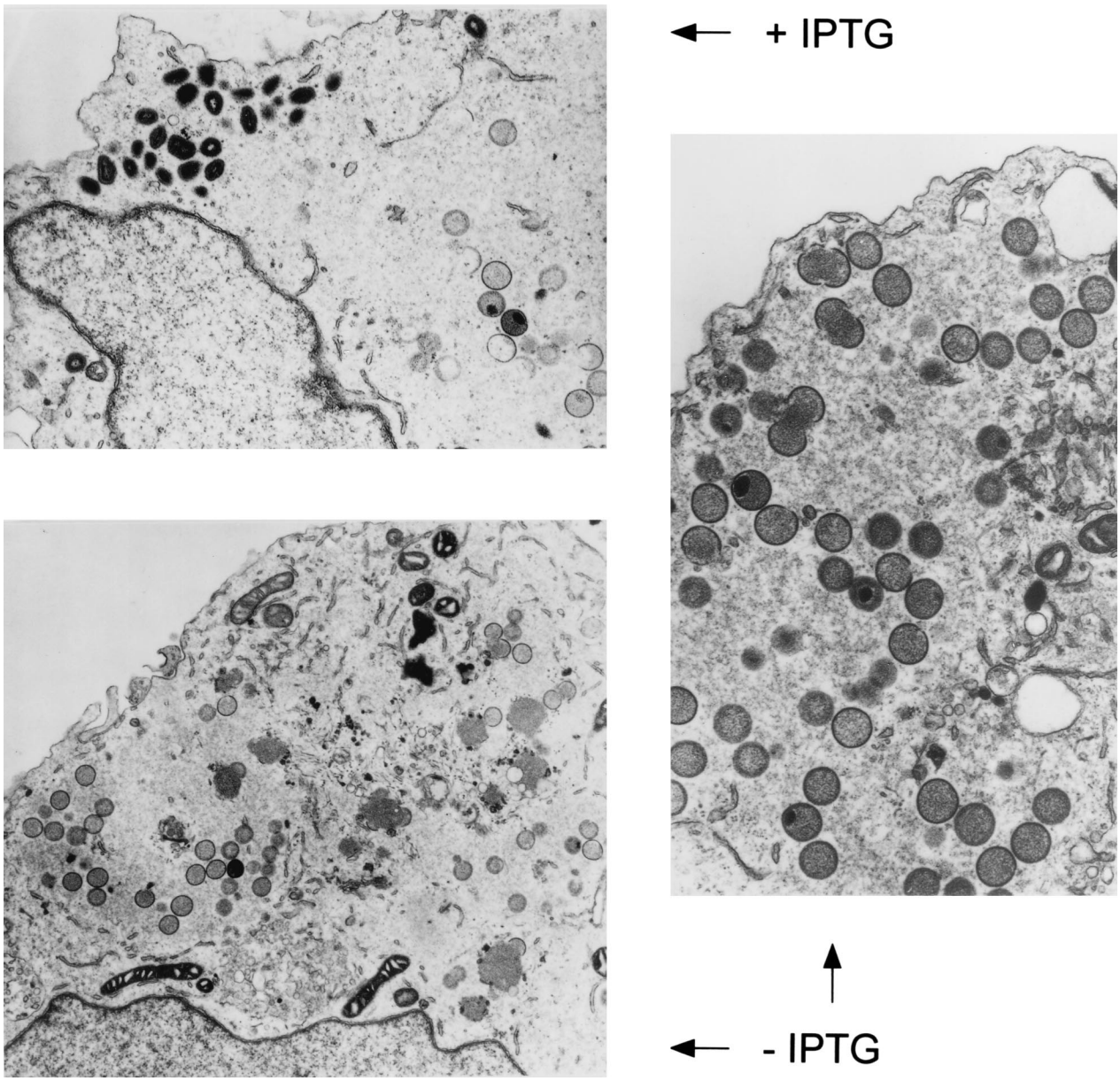

FIG. 9. Electron microscopic analysis of v*ind* I1-infected cells. Cells infected with wt (not shown) or v*ind* I1 (MOI of 5) under permissive (+IPTG) and nonpermissive (−IPTG) conditions were harvested at 18 hpi, fixed in situ, and processed for electron microscopy. Three representative micrographs are shown. Magnifications: top left, ×14,000; bottom left, ×10,500; right, ×9,600.

the presence of the inducer IPTG: crescents, IV, IV with nucleoids, and IMV (Fig. 9, +IPTG). In samples prepared from infections performed with v*ind* I1 in the absence of IPTG, crescents, IV and IV with typical nucleoids were abundant (Fig. 9, −IPTG). However, IMV were essentially absent, indicating that the expression of I1 is essential for the latest stages of morphogenesis.

I1 is not essential for the resolution of genomic DNA. The transition from IV to IMV is a morphologically complex one in which the virion changes from spherical to brick-shaped and a structured internal core appears. Although we presume that proteolytic processing occurs during this transition, we know little else of what biochemical events drive the reorganization. Even the time at which the genomic nucleoprotein complex becomes encapsidated is unclear. Because I1 is a DNA-binding component of the virion core, and because it is required for virion maturation, we felt that it might participate in the generation of mature, monomeric genomes for encapsidation. To test this possibility, cytosolic extracts were prepared from cultures infected for 18 h with v*ind* I1 (with and without IPTG), and cytoplasmic DNA was digested with *Bst*EII. This restriction enzyme releases a 1.3-kbp terminal fragment from mature genomes and a diagnostic 2.6-kbp fragment from concatemeric replication intermediates. Southern blot analysis using an appropriate probe revealed that the 1.3-kbp fragment was predominant, and the 2.6-kbp fragment essentially absent, in samples from both permissively and nonpermissively infected cultures (Fig. 10, lanes 1 and 2). The same result was seen with the parental virus that carries a *wt* I1 locus (v*lac*I) (Fig. 10, lanes 3 and 4). In contrast, in cultures infected with vROG8

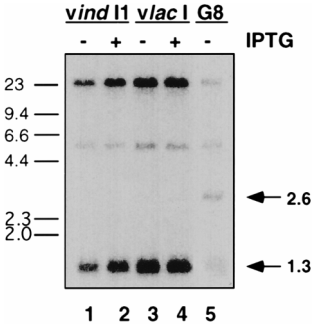

FIG. 10. Southern blot analysis of cytoplasmic viral DNA. BSC40 cells were infected with *wt*, *vind* I1 (both at an MOI of 2), or vROG8 (MOI of 15) in the absence (−) or presence (+) of IPTG, as indicated. Cells were harvested at 18 hpi, and viral genomic DNA was prepared and digested with *Bst*EII. Digested DNA was resolved on a 1% agarose gel, transferred to a Zeta-probe filter, and hybridized with a probe recognizing the termini of the viral genome as described in Materials and Methods. The arrows point to the 1.3-kbp fragment released from mature, monomeric genomes and the 2.6-kbp fragment released from unresolved concatemers. The probe also hybridizes to a larger, adjacent genomic fragment (common to monomeric and concatemeric molecules), as well as to undigested DNA. The sizes of DNA standards are indicated in kilobase pairs at the left.

(57), a viral recombinant in which expression of the late transcription factor G8 is IPTG dependent, the 2.6-kbp fragment diagnostic of impaired concatemer resolution could be observed (Fig. 10, lane 5). These results demonstrate that resolution of concatemeric intermediates is independent of I1 expression.

DISCUSSION

In this report, we have presented a biochemical and genetic characterization of the vaccinia virus I1 protein. The protein is predicted to contain 312 amino acids and to have a molecular weight of 35,844 and a pI of 8.6. As Fig. 11A illustrates, I1 is predicted to be extremely hydrophilic in character. A search of available sequence databases with the I1 amino acid sequence revealed no homology to any nonpoxviral proteins.

The chromatographic properties of I1 were consistent with a protein which might associate with DNA (retention on DEAE-cellulose, heparin agarose, hydroxyapatite, and ATP-agarose) (9, 18a), and this prediction was supported by the results obtained in EMSAs. We demonstrated that recombinant I1 binds to radiolabeled oligonucleotides in a sequence-nonspecific manner; comparable binding was obtained with both ssDNA and dsDNA. The I1-DNA complexes were retained during electrophoresis even in the absence of fixatives or cross-linking agents.

Although I1 had not been previously shown to be encapsidated (38), our data clearly indicated that approximately 700 copies of I1 are present within the virion core. When cores were further fractionated with sodium deoxycholate, I1 partitioned with the insoluble fraction. Whereas the components of

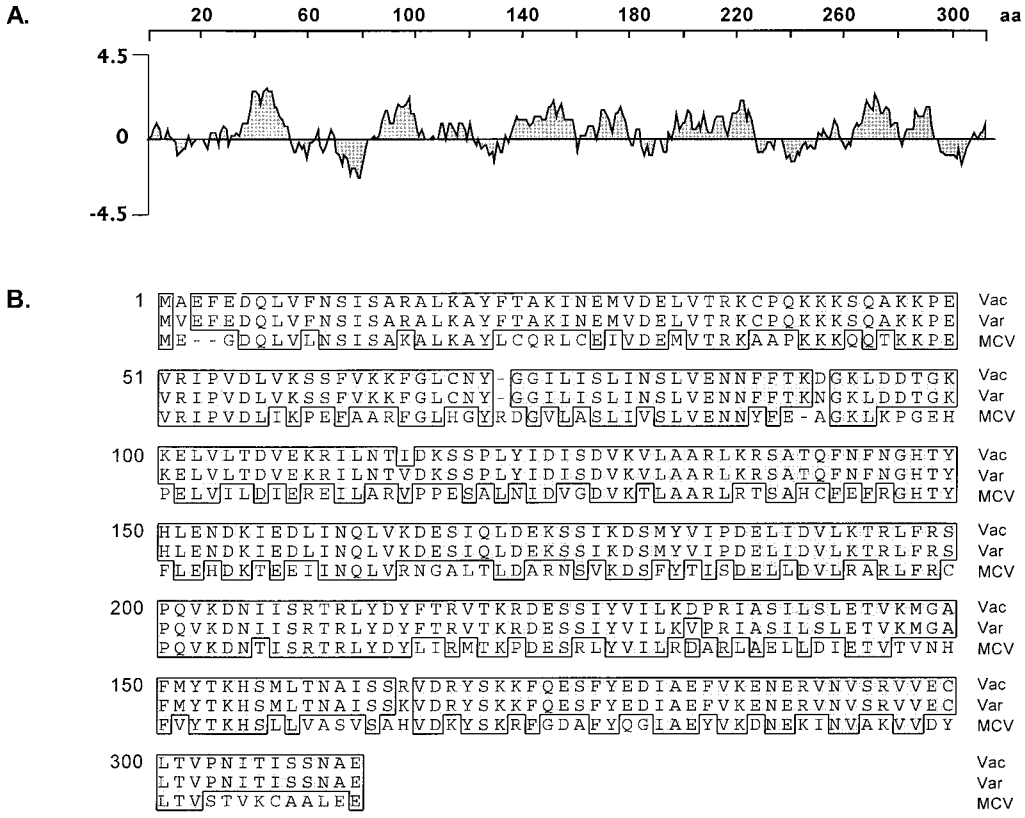

FIG. 11. Sequence analysis of the deduced I1 amino acid sequence. (A) Hydrophilicity of the vaccinia virus-encoded I1 protein. A Kyte-Doolittle hydrophilicity plot was generated for the deduced amino acid sequence (312 amino acids [aa]) of the I1 gene encoded by the Copenhagen strain of vaccinia virus (accession no. M35027), using Lasergene software. Hydrophilic regions are indicated as positive on the *y* axis, and hydrophobic regions are indicated as negative. (B) Comparison of poxvirus-encoded I1 homologs. The deduced amino acid sequence of the I1 gene encoded by the Copenhagen strain of vaccinia virus (Vac) and the homologs encoded by the Bangladesh strain of variola virus (Var) (accession no. L22579) and MCV type I (accession no. U60315) were aligned by using the Clustal algorithm and Lasergene software. The single-letter amino acid code is used; the amino acid positions of the vaccinia virus protein are shown at the left. Residues conserved in two or more proteins are shaded and boxed.

the viral transcriptional apparatus remain in the supernatant after this treatment, significant amounts of the viral DNA and associated proteins are recovered in the insoluble pellet (not shown), as well as the abundant structural proteins 4A and 4B. Our quantitation of the amount of I1 encapsidated revealed that it is far less abundant than the DNA-binding proteins such as F18 (27,000 copies per virion) or L4 (VP8) (7,000 copies per virion) and more comparable to enzymes such as the H1 phosphatase or F10 kinase (approximately 200 to 300 copies per virion) (22, 23, 48). Because several recent reports have underscored the importance of protein phosphorylation in regulating vaccinia virus morphogenesis and infectivity (23, 49, 51), the phosphorylation state of I1 was investigated. Although recombinant I1 was a good substrate for both the B1 and F10 kinases in vitro (not shown), repeated efforts to detect any phosphorylation of I1 in vivo were negative. No evidence of other posttranslational modifications, such as proteolytic processing, was obtained either.

Examination of the temporal profiles of I1 mRNA and protein synthesis indicated that the protein is expressed only at late times of infection, consistent with the presence of the TAAAT motif surrounding the translational (and transcriptional) start site and with previous observations (38, 40). Our determination that I1 was an encapsidated DNA-binding protein, and one whose essentiality had been previously suggested (43), stimulated further analysis of its role(s) in vivo. Because I1 is expressed only at late times postinfection, we were able to generate an inducible recombinant in which I1 expression was tightly controlled by the *E. coli lac* repressor/operator (v*ind* I1) (23, 36, 37, 59).

This recombinant exhibited a very tight conditional lethal phenotype: no plaques formed in the absence of inducer, and under these nonpermissive conditions the yield of infectious intracellular virus was reduced by 2 orders of magnitude. Nonpermissive v*ind* I1 infections exhibited a normal phenotype, in which biochemical events such as protein synthesis, DNA replication, and genome resolution were not perturbed. However, electron microscopy revealed that whereas IV were readily apparent, IMV were essentially absent. Moreover, the characteristic proteolytic processing of structural proteins that accompanies virion maturation also failed to occur.

In sum, these data indicate that I1 plays a key role in the transition from IV to IMV. This finding implicates I1 as playing a role at the latest step in morphogenesis which has been described. Conditionally lethal mutants with defective I7 protein form aberrant particles in which IV contain half-moon inclusions; recombinants in which F18 expression can be experimentally blocked are defective in nucleoid formation. Finally, particles assembled in the absence of viral early transcription factor have a phenotype similar to that described in this report, in which IV and IV with nucleoids accumulate but no IMV are seen (13).

IMV and IV are very distinct forms of the virus; IV are noninfectious, while IMV are infectious. Also, a major morphological transition occurs between the two forms of vaccinia virus, as IV have a spherical structure (within which a minority have a distinct nucleoid) and IMV have a distinct brick-shaped structure in which a dumbbell-shaped core is visible. The results obtained with a variety of inducible recombinants suggest that the transition from IV to IMV is likely to rely on a variety of DNA-binding proteins which may participate in multiple protein-protein and/or protein-DNA interactions. The data that we have obtained with v*ind* I1 support earlier observations that concatemer resolution occurs prior to this transition (28) but indicate that proteolytic processing of core proteins is likely to accompany or depend on this transition.

The IV that accumulate during nonpermissive v*ind* I1 infections may prove useful in determining when viral DNA becomes encapsidated and how IV differ from IMV at a biochemical level. The dramatic phenotype seen upon repression of I1 synthesis underscores the essentiality of I1 in the late stages of virion morphogenesis. This essentiality is also reflected in the high degree of conservation seen when the predicted sequences of the I1 proteins encoded by vaccinia virus, variola virus, and MCV are compared (Fig. 11B). The variola virus homolog is 98.4% identical to the vaccinia virus protein, and the MCV protein retains 54.8% identity. I1 is therefore among the proteins showing the highest similarity in vaccinia virus and the relatively distant MCV (41, 42). This conservation reflects I1's essentiality: in the absence of enzymatic function, such a high degree of conservation suggests that the sequence may be constrained by the need to conserve protein-protein interactions. The availability of v*ind* I1 and recombinant I1 protein should facilitate future studies of IMV morphogenesis.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Baldick, C. J., and B. Moss.** 1987. Resistance of vaccinia virus to rifampicin conferred by a single nucleotide substitution near the predicted NH2 terminus of a gene encoding an Mr62K polypeptide. Virology **156:**138–145.
3. **Blasco, R., and B. Moss.** 1992. Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. J. Virol. **66:**4170–4179.
4. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46:**83–88.
5. **Condit, R. C., and A. Motyczka.** 1981. Isolation and preliminary characterization of temperature sensitive mutants of vaccinia virus. Virology **113:**224–241.
6. **Condit, R. C., A. Motyczka, and G. Spizz.** 1983. Isolation, characterization and physical mapping of temperature sensitive mutants of vaccinia virus. Virology **128:**429–443.
7. **Condit, R. C., and E. G. Niles.** 1990. Orthopoxvirus genetics. Curr. Top. Microbiol. Immunol. **163:**1–39.
8. **Dales, S., V. Milanovanovitch, B. G. T. Pogo, S. B. Weintraub, T. Huima, S. Wilton, and G. McFadden.** 1978. Biogenesis of vaccinia: isolation of conditional lethal mutants and electron microscopic characterization of their phenotypically expressed defects. Virology **844:**403–428.
9. **Evans, E., N. Klemperer, R. Ghosh, and P. Traktman.** 1995. The essential replication protein encoded by the vaccinia virus D5 gene is a nucleic acid independent NTPase. J. Virol. **69:**5353–5361.
10. **Falkner, F. G., and B. Moss.** 1990. Transient dominant selection of recombinant vaccinia viruses. J. Virol. **64:**3108–3111.
11. **Franke, C. A., C. M. Rice, J. H. Strauss, and D. E. Hruby.** 1985. Neomycin resistance as a dominant selectable marker for selection and isolation of vaccinia virus recombinants. Mol. Cell. Biol. **5:**1918–1924.
12. **Fuerst, T. R., M. P. Fernandez, and B. Moss.** 1989. Transfer of the inducible lac repressor/operator system from Escherichia coli to a vaccinia virus expression vector. Proc. Natl. Acad. Sci. USA **86:**2549–2552.
13. **Hu, X., L. J. Carroll, E. J. Wolffe, and B. Moss.** 1996. De novo synthesis of the yearly transcription factor 70-kilodalton subunit is required for morphogenesis of vaccinia virions. J. Virol. **70:**7669–7677.
14. **Ichihashi, Y., M. Oie, and T. Tsuruhara.** 1984. Location of DNA-binding proteins and disulfide-linked proteins in vaccinia virus structural elements. J. Virol. **50:**929–938.

15. **Johnson, G. P., S. J. Goebel, and E. Paoletti.** 1993. An update on the vaccinia virus genome. Virology **196:**381–401.
16. **Kane, E. M., and S. Shuman.** 1993. Vaccinia virus morphogenesis is blocked by a temperature-sensitive mutation in the I7 gene that encodes a virion component. J. Virol. **67:**2689–2698.
17. **Kao, S., E. Ressner, J. Kates, and W. R. Bauer.** 1981. Purification and characterization of a superhelix binding protein from vaccinia virus. Virology **111:**500–508.
18. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody absorbent. J. Immunol. **115:**1617–1624.
18a. **Klemperer, N., and E. Evans.** Unpublished data.
19. **Klemperer, N., D. J. Lyttle, D. Tauzin, P. Traktman, and A. J. Robinson.** 1994. Identification and characterization of the orf virus type I topoisomerase. Virology **206:**203–215.
20. **Klemperer, N., and P. Traktman.** 1993. Biochemical analysis of mutant alleles of the vaccinia virus topoisomerase I carrying targeted substitutions in a highly conserved domain. J. Biol. Chem. **268:**15887–15899.
21. **Koerner, T. J., J. E. Hill, A. M. Myers, and A. Tzagaloff.** 1991. High-expression vectors with multiple cloning sites for construction of trpE fusion genes: pATH vectors. Methods Enzymol. **194:**477–490.
22. **Lin, S., and S. S. Broyles.** 1994. Vaccinia protein kinase 2: a second essential serine/threonine kinase encoded by vaccinia virus. Proc. Natl. Acad. Sci. USA **91:**7653–7657.
23. **Liu, K., B. Lemon, and P. Traktman.** 1995. The dual specificity phosphatase encoded by vaccinia virus, VH1, is essential for viral transcription in vivo and in vitro. J. Virol. **69:**7823–7834.
24. **McDonald, W. F., V. Crozel-Goudot, and P. Traktman.** 1992. Transient expression of the vaccinia virus DNA polymerase is an intrinsic feature of the early phase of infection and is unlinked to DNA replication and late gene expression. J. Virol. **66:**534–547.
25. **Merchlinsky, M.** 1989. Intramolecular homologous recombination in cells infected with temperature-sensitive mutants of vaccinia virus. J. Virol. **63:** 2030–2035.
26. **Merchlinsky, M., C. F. Garon, and B. Moss.** 1988. Molecular cloning and sequence of the concatemer junction from vaccinia virus replicative DNA: viral nuclease cleavage sites in cruciform structures. J. Mol. Biol. **199:**399–413.
27. **Merchlinsky, M., and B. Moss.** 1986. Resolution of linear minichromosomes with hairpin ends from circular plasmids containing vaccinia virus concatemer junctions. Cell **45:**879–884.
28. **Merchlinsky, M., and B. Moss.** 1989. Resolution of vaccinia virus DNA concatemer junctions requires late gene expression. J. Virol. **63:**1595–1603.
29. **Moss, B.** 1996. Poxviridae: the viruses and their replication, p. 2637–2671. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology. Lippincott-Raven, Philadelphia, Pa.
30. **Moss, B., and E. N. Rosenblum.** 1973. Protein cleavage and poxvirus morphogenesis: tryptic peptide analysis of core precursors accumulated by blocking assembly with rifampicin. J. Mol. Biol. **81:**267–269.
31. **Niles, E. G., and J. Seto.** 1988. Vaccinia virus gene D8 encodes a virion transmembrane protein. J. Virol. **62:**3772–3778.
32. **Ravanello, M. P., and D. E. Hruby.** 1994. Conditional lethal expression of the vaccinia virus L1R myristylated protein reveals a role in virion assembly. J. Virol. **68:**6401–6410.
33. **Rempel, R., and P. Traktman.** 1992. Vaccinia virus B1 kinase: phenotypic analysis of temperature-sensitive mutants and enzymatic characterization of recombinant proteins. J. Virol. **66:**4413–4426.
34. **Rodriguez, D., M. Esteban, and J. R. Rodriguez.** 1995. Vaccinia virus A17L gene product is essential for an early step in virion morphogenesis. J. Virol. **69:**4640–4648.
35. **Rodriguez, D., C. Risco, J. R. Rodriguez, J. L. Carrascosa, and M. Esteban.** 1996. Inducible expression of the vaccinia virus A17L gene provides a synchronized system to monitor sorting of viral proteins during morphogenesis. J. Virol. **70:**7641–7653.
36. **Rodriguez, J. F., and G. L. Smith.** 1990. IPTG-dependent vaccinia virus: identification of a virus protein enabling virion envelopment by Golgi membrane and egress. Nucleic Acids Res. **18:**5347–5351.
37. **Rodriguez, J. F., and G. L. Smith.** 1990. Inducible gene expression from vaccinia virus vectors. Virology **177:**239–250.
38. **Ryazankina, O. I., A. I. Muravlev, V. V. Gutorov, N. N. Mikrjukov, I. O. Cheshenko, and S. N. Shchelkunov.** 1993. Comparative analysis of the conserved region of the orthopoxvirus genome encoding the 36K and 12K proteins. Virus Res. **29:**281–303.
39. **Schmelz, M., B. Sodeik, M. Ericsson, E. J. Wolffe, H. Shida, G. Hiller, and G. Griffiths.** 1994. Assembly of vaccinia virus: the second wrapping is derived from the trans Golgi network. J. Virol. **68:**130–147.
40. **Schmitt, J. F. C., and H. G. Stunnenberg.** 1988. Sequence and transcriptional analysis of the vaccinia virus *Hin*dIII I fragment. J. Virol. **62:**1889–1897.
41. **Senkevich, T. G., J. J. Bugert, J. R. Sisler, E. V. Koonin, G. Darai, and B. Moss.** 1996. Genome sequence of a human tumorigenic poxvirus: prediction of specific host response-evasion genes. Science **274:**813–816.
42. **Senkevich, T. G., E. V. Koonin, J. J. Bugert, G. Darai, and B. Moss.** 1997. The genome of molluscum contagiosum virus: analysis and comparison with other poxviruses. Virology **233:**19–42.
43. **Shchelkunov, S. N., O. I. Ryazankina, and P. V. Gashnikov.** 1993. The gene encoding the late nonstructural 36K protein of vaccinia virus is essential for virus reproduction. Virus Res. **28:**273–283.
44. **Sodeik, B., R. W. Doms, M. Ericsson, G. Hiller, C. E. Machamer, W. van't Hof, G. van Meer, B. Moss, and G. Griffiths.** 1993. Assembly of vaccinia virus: role of the intermediate compartment between the endoplasmic reticulum and the Golgi stacks. J. Cell Biol. **121:**521–541.
45. **Studier, F. W., A. L. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
46. **Tartaglia, J., A. Piccini, and A. Paoletti.** 1986. Vaccinia virus rifampicin-resistance locus specifies a late 63,000 Da gene product. Virology **150:**45–54.
47. **Thompson, C. L., and R. C. Condit.** 1986. Marker rescue mapping of vaccinia virus temperature sensitive mutants using overlapping cosmid clones representing the entire virus genome. Virology **150:**10–20.
48. **Traktman, P.** 1996. Poxvirus DNA replication, p. 775–798. *In* M. DePamphilis (ed.), DNA replication in eukaryotic cells. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
49. **Traktman, P., A. Caligiuri, S. A. Jesty, K. Liu, and U. Sankar.** 1995. Temperature-sensitive mutants with lesions in the vaccinia virus F10 kinase undergo arrest at the earliest stage of virion morphogenesis. J. Virol. **69:** 6581–6587.
50. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA **76:**615–619.
51. **Wang, S., and S. Shuman.** 1995. Vaccinia virus morphogenesis is blocked by temperature-sensitive mutations in the F10 gene, which encodes protein kinase 2. J. Virol. **69:**6376–6388.
52. **Wilcock, D., and G. Smith.** 1996. Vaccinia virions lacking core protein VP8 are deficient in early transcription. J. Virol. **67:**934–943.
53. **Wilcock, D., and G. L. Smith.** 1994. Vaccinia virus core protein VP8 is required for virus infectivity, but not for core protein processing or for INV and EEV formation. Virology **202:**294–304.
54. **Wolffe, E. J., D. M. Moore, E. J. Peters, and B. Moss.** 1996. Vaccinia virus A17L open reading frame encodes an essential component of nascent viral membranes that is required to initiate morphogenesis. J. Virol. **70:**2797–2808.
55. **Yang, W.-P., and W. R. Bauer.** 1988. Biosynthesis and post-translational cleavage of vaccinia virus structural protein VP8. Virology **167:**585–590.
56. **Yang, W.-P., and W. R. Bauer.** 1988. Purification and characterization of vaccinia virus structural protein VP8. Virology **167:**578–584.
57. **Zhang, Y., J. G. Keck, and B. Moss.** 1992. Transcription of viral late genes is dependent on expression of the viral intermediate gene G8R in cells infected with an inducible conditional-lethal mutant vaccinia virus. J. Virol. **66:**6470–6479.
58. **Zhang, Y., and B. Moss.** 1991. Vaccinia virus morphogenesis is interrupted when expression of a gene encoding an 11-kilodalton phosphorylated protein is prevented by the *Escherichia coli lac* repressor. J. Virol. **65:**6101–6110.
59. **Zhang, Y., and B. Moss.** 1991. Inducer-dependent conditional-lethal mutant animal viruses. Proc. Natl. Acad. Sci. USA **88:**1511–1515.
60. **Zhang, Y., and B. Moss.** 1992. Immature viral envelope formation is interrupted at the same stage by lac operator mediated repression of the vaccinia virus D13L gene and by the drug rifampicin. Virology **187:**643–653.