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Vaccinia Virus Morphogenesis Is Interrupted when Expression of the Gene Encoding an 11-Kilodalton Phosphorylated Protein Is Prevented by the *Escherichia coli lac* Repressor

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A conditional lethal vaccinia virus mutant, which constitutively expresses the *Escherichia coli lac* repressor and has the *lac* operator controlling the F18R gene (the 18th open reading frame of the *Hin*dIII F fragment of the vaccinia virus strain WR genome) encoding an 11-kDa protein, was previously shown to be dependent on the inducer isopropyl-β-D-thiogalactoside (IPTG) for replication (Y. Zhang and B. Moss, Proc. Natl. Acad. Sci. USA 88:1511–1515, 1991). Further studies indicated that the yield of infectious virus could be regulated by titration with IPTG and that virus production was arrested by IPTG removal at appropriate times. Under nonpermissive conditions, an 11-kDa protein reactive with antiserum raised to a previously described DNA-binding phosphoprotein (S. Y. Kao and W. R. Bauer, Virology 159:399–407, 1987) was not synthesized, indicating that the latter is the product of the F18R gene. In the absence of IPTG, replication of viral DNA and the subsequent resolution of concatemeric DNA molecules appeared normal. Omission of IPTG did not alter the kinetics of early and late viral protein synthesis, although the absence of the 11-kDa polypeptide was noted by labeling infected cells with [^{35}S]methionine or [^{32}P]phosphate. Pulse-chase experiments revealed that proteolytic processing of the major viral structural proteins, P4a and P4b, was inhibited under nonpermissive conditions, suggesting a block in virus maturation. Without addition of IPTG, the failure of virus particle formation was indicated by sucrose gradient centrifugation of infected cell lysates and by the absence of vaccinia virus-mediated pH-dependent cell fusion. Electron microscopic examination of infected cells revealed that immature virus particles, with aberrant internal structures, accumulated when synthesis of the 11-kDa DNA-binding protein was prevented.

Vaccinia virus, a member of the *Poxviridae*, has a large double-stranded DNA genome that encodes about 200 polypeptides and replicates in the cytoplasm (reviewed in reference 18). A variety of genetic and biochemical approaches are being used to investigate the life cycle of this complex virus. Recently, the *Escherichia coli lac* operator-repressor system was shown to be capable of regulating transcription of the vaccinia virus genome (5, 26). On the basis of this technology, it was possible to construct inducer-dependent conditional lethal (32) and nonlethal (27) mutants of vaccinia virus. The general strategy has been to (i) integrate the *E. coli lacI* gene under control of an early/late vaccinia virus promoter to achieve continuous synthesis of repressor, (ii) place the *lac* operator just downstream of the RNA start site of the target gene so that repressor binding will block transcription, and (iii) isolate recombinant viruses in the presence of the inducer isopropyl-β-D-thiogalactoside (IPTG) which binds repressor and permits transcription. Unlike temperature-sensitive mutants, which usually make a nonfunctional protein at elevated temperature, inducer-dependent conditional lethal mutants selectively inhibit synthesis of the target protein under nonpermissive conditions. In many circumstances, such as in studying virion assembly and morphogenesis, the interpretation of results may be simpler when a protein is absent rather than defective.

Using the above strategy, we constructed a recombinant vaccinia virus with the F18R gene (the 18th open reading frame of the *Hin*dIII F fragment of the vaccinia virus strain WR genome), which encodes a protein of 11 kDa (31), under *lac* repressor-operator regulation (32). Under nonpermissive conditions, transcription of the gene for the 11-kDa protein was prevented, plaques did not form, and the virus yield was decreased by 1,000-fold. Thus, expression of the F18R gene was essential for replication. Our continued investigations now demonstrate that in the absence of IPTG an 11-kDa phosphorylated protein that reacts with antibody to a previously described virion core-associated DNA-binding protein (11) was not made, proteolytic processing of virion core proteins did not occur, and virion assembly was blocked, resulting in the accumulation of immature particles with aberrant internal structures.

MATERIALS AND METHODS

Virus and cells. Vaccinia virus strain WR, referred to as wild-type virus (WT), was propagated in Hela cells and purified as previously described (16). Recombinant virus vRO11k (32) was replicated in the continuous presence of 5 mM IPTG. BS-C-1 cells were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum. Conditions for one-step virus growth were similar to those previously described (32), and a multiplicity of infection of 10 PFU was used.

Southern blot analysis of viral DNA. Cytoplasmic DNA from BS-C-1 infected cells was digested with *Bst*EII and separated by electrophoresis on an 0.8% agarose gel. The DNA was transferred to a GeneScreen Plus membrane (DuPont, NEN Research) and hybridized with a ^{32}P-labeled probe composed of the terminal segment of the vaccinia

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virus genome (17). For accumulation of vaccinia virus concatemeric junction fragments, cells were infected with the temperature-sensitive mutant *ts*21 (1) for 24 h at either 31 or 40°C before the DNA was extracted and digested with *Bst*EII.

Analysis of [^{35}S]methionine-labeled polypeptides by SDS-PAGE. Early and late [^{35}S]methionine-labeled infected cell proteins were analyzed as described previously (32) except that a 12% polyacrylamide gel was used for electrophoresis. For pulse-chase experiments, 2.5×10^6 BS-C-1 cells were infected for 7 h and then incubated in 1.5 ml of methionine-free MEM containing 50 μCi of [^{35}S]methionine for 30 min and then in MEM supplemented with a 100-fold excess of unlabeled methionine for another 12 h. For continuous labeling, 2.5×10^6 infected BS-C-1 cells were incubated in 3 ml of the above [^{35}S]methionine-containing medium supplemented with 0.3 ml of regular MEM for 12.5 h. Cells were lysed as above, and samples were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10% T + 3% C, where T is the total percentage concentration of both monomers (acrylamide and bisacrylamide) and C is the percentage concentration of the cross-linker relative to the total concentration, as previously described (30).

For electrophoresis of the samples from sucrose gradient fractions, proteins were concentrated by trichloroacetic acid precipitation before SDS-PAGE (10% T + 3% C). Precipitation was carried out by adding an equal volume of 20% cold trichloroacetic acid to 20- or 200-μl samples of each fraction of the gradient. After 1 h at 4°C, samples were collected by centrifugation in 1.5-ml conical tubes and the pellets were dissolved in 20 μl of 100 mM Tris-HCl (pH 6.8) by shaking them for 20 min at room temperature.

Immunoprecipitation. BS-C-1 cells (2.5×10^7), infected with WT or vRO11k were pulse-labeled in 1.5 ml of methionine-free MEM containing 75 μCi of [^{35}S]methionine for 30 min between 7.5 and 8 h after infection. The cells were washed and suspended in 300 μl of 60 mM Tris-HCl (pH 6.8)–1% SDS–5% β-mercaptoethanol–10% glycerol lysis buffer. Twenty microliters of cell lysate, diluted with 200 μl of 50 mM Tris-HCl (pH 7.4)–150 mM NaCl–1% Nonidet P-40–0.1% SDS, was incubated with 10 μl of polyclonal antiserum raised against the purified 11-kDa DNA-binding protein (11) for 2 h at 4°C. The antigen-antibody complex was then incubated with 20 μl of protein A-Sepharose (Pharmacia) suspension (30%) for another 2 h at 4°C. The beads were collected by centrifugation and washed twice with 50 mM Tris-HCl (pH 7.4)–300 mM NaCl–0.1% sodium deoxycholate–0.1% SDS. Cell lysis buffer, 20 μl, was added to the beads which were heated at 95°C for 10 min, and the supernatant was applied to a 20% polyacrylamide gel.

Analysis of ^{32}P-labeled infected cell proteins. BS-C-1 cells (1×10^6) were labeled with 2 ml of phosphate-free medium containing 40 μCi of carrier-free [^{32}P]phosphoric acid (Dupont) from 3 to 24 h after virus infection. Cells were lysed with buffer containing 1% SDS, 5% mercaptoethanol, 60 mM Tris-HCl (pH 6.8), and 10% glycerol, and proteins were applied to a 20% polyacrylamide gel.

Cell fusion. At 12 h after infection, BS-C-1 cells were immersed in 37°C fusion medium [phosphate-buffered saline with 10 mM 2-(*N*-morpholino)ethanesulfonic acid and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)] at pH 5.6 or pH 7.4 for 2 min. The cells were then incubated with regular MEM. Polykaryon formation was examined by phase-contrast microscopy 2 h later.

Purification of [^{35}S]methionine-labeled virus particles. BS-C-1 cells (4×10^7) infected with vaccinia virus WR or vRO11k were labeled in 36 ml of methionine-free MEM plus 4 ml of regular MEM containing 300 μCi of [^{35}S]methionine (1,000 Ci/ml; Amersham) from 7 to 22 h after infection. The medium was then changed to regular medium free of isotope, and the cells were incubated for another 24 h. Cells were collected by scraping them into 0.5 ml of 10 mM Tris-HCl (pH 7.6)–10 mM NaCl–1.5 mM $MgCl_2$ and were subjected to Dounce homogenization. Nuclei were separated by centrifugation for 5 min at 900 rpm in an H6000A rotor in a Sorvall RC-3B centrifuge. After sonication, the supernatants were applied to linear sucrose gradients (25 to 40%) and centrifuged in a SW41 rotor at 13,000 rpm for 50 min at 4°C. Fractions of 0.7 ml were collected.

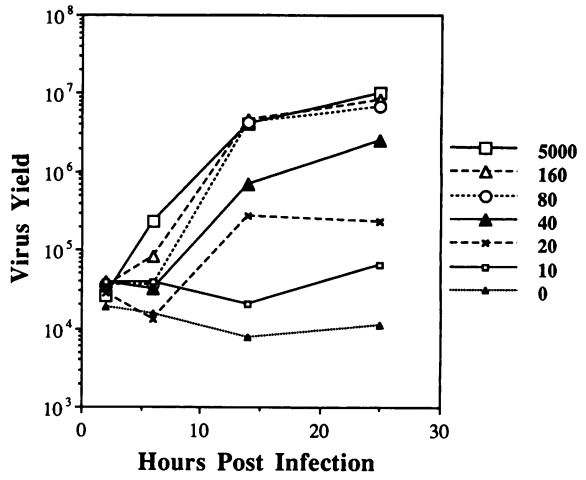

FIG. 1. Effect of IPTG concentration on one-step virus growth. BS-C-1 cells (5×10^5) infected with vRO11k at a multiplicity of infection of 10 PFU per cell were incubated with medium containing the indicated micromolar concentrations of IPTG. Virus yields from cells harvested at the indicated times after infection were determined by plaquing on BS-C-1 cells in the presence of 5 mM IPTG.

Electron microscopic analysis. At 12 h postinfection, the medium was removed and the cells were fixed by the addition of 2.5% glutaraldehyde in Millonig's buffer (8). After 1 h at 4°C, the cells were scraped but the pellet was stored in the same 2.5% glutaraldehyde buffer for another 0.5 h. The buffer was then replaced with Millonig's 0.13 M sodium phosphate buffer, pH 7.4. Transmission electron micrographs were prepared by Advanced Biotechnologies Inc.

RESULTS

Effects of IPTG concentration on virus replication. The inducer-dependent conditional lethal mutant vaccinia virus vRO11k contains the *E. coli lac* repressor gene under the control of a vaccinia virus early/late promoter and has the *lac* operator placed 3 nucleotides downstream of the RNA start site of the F18R gene (32). In our initial report, we showed that the formation of infectious vRO11k was dependent on the addition of inducer and that a nearly normal virus yield was achieved with 5 mM IPTG. For further studies, it was useful to determine the relationship between IPTG concentration and virus replication. No increase in virus yield was detected in the absence of inducer (Fig. 1). A small but significant increase in titer occurred with 10 μM IPTG, and the yield increased progressively with 20, 40, and 80 μM

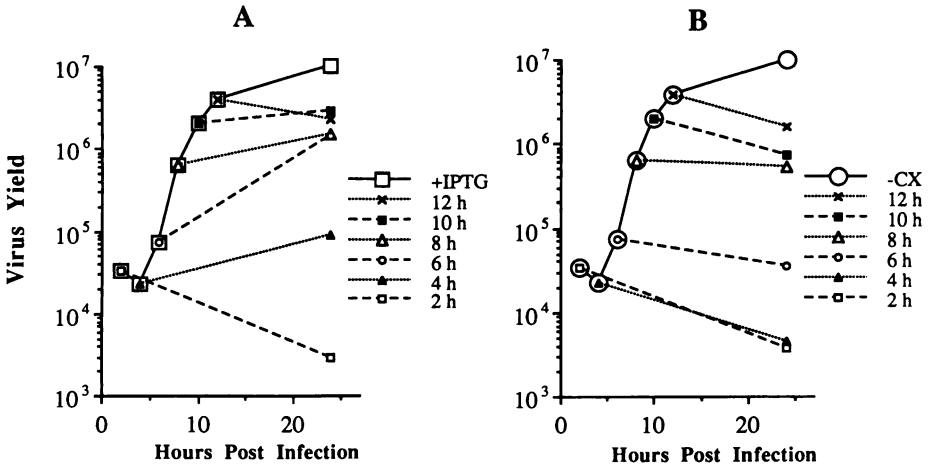

FIG. 2. Effect of IPTG withdrawal on virus replication. BS-C-1 cells in 160 μM IPTG were infected as described in the legend to Fig. 1. The cells were harvested at 24 h after infection, and the virus yields were determined in the presence of 5 mM IPTG. (A) The cell monolayers were washed with phosphate-buffered saline, and inducer-free medium was added at the indicated times after infection. (B) Cycloheximide (CX; 100 μg/ml) was added to the medium at the indicated times.

IPTG. Further increases in IPTG concentration, however, had little effect on the final yield of vRO11k. These results are consistent with the effect of inducer concentration on plaque formation (32).

IPTG was withdrawn from vRO11k-infected cells at various times to determine the reversibility of induction. Presumably, as inducer is washed out, existing and newly synthezed repressor can bind to the operator and terminate further gene expression. A concentration of 160 μM IPTG, twice that needed for maximal virus induction, was used for this experiment. When IPTG was withdrawn at 2 h after infection, virus production was completely abolished, demonstrating that the inducer can be effectively washed out of the cells (Fig. 2A). Withdrawal of IPTG at 4 h after infection, which marks the onset of late viral protein synthesis, permitted a small increase in virus titer. When IPTG was removed at 6 h, virus yield increased over 10-fold, suggesting that significant quantities of the mRNA or protein product of the F18R gene had accumulated by the time IPTG was removed and repression was established. These results are consistent with the late regulation of the F18R gene (31). In a parallel experiment, the protein synthesis inhibitor cycloheximide was added to stop synthesis of all proteins (Fig. 2B). As expected, increases in virus yield were blocked at whatever time cycloheximide was added to the medium.

Evidence that the F18R gene product is a major phosphorylated DNA-binding protein. Vaccinia virion proteins of approximately 11 kDa have been described by many different investigators (see the Discussion). The best characterized of these is the DNA superhelix-binding phosphoprotein purified by Bauer and coworkers (11, 12). Monospecific polyclonal antibody to the latter protein, kindly supplied by W. R. Bauer, was found to immunoprecipitate a [^{35}S]methionine-labeled 11-kDa protein from cells infected with WT or with vRO11k in the presence of IPTG (Fig. 3A). With vRO11k in the absence of IPTG, however, the binding of a labeled protein to the antibody was not detected (Fig. 3A). Similar results were also obtained by Western immunoblotting (data not shown), providing evidence that the F18R gene product is the previously characterized DNA-binding protein.

Earlier reports suggested the possibility of several phosphorylated 11-kDa polypeptides in vaccinia virions (see the Discussion). In order to determine the state of phosphorylation of the product of the F18R gene, vRO11k-infected cells were labeled with [^{32}P]phosphate in the presence and absence of IPTG. The cell proteins were analyzed by SDS-PAGE, and autoradiography revealed a large number of phosphate-labeled bands (Fig. 3B). The only difference noted between the two samples was the absence of a labeled 11-kDa protein when IPTG was omitted. The simplest interpretation of these results is that there is one major phosphorylated 11-kDa protein which is the product of the F18R gene.

Viral DNA replication and processing under nonpermissive conditions. Since the F18R gene product appears to be a major DNA-binding protein, we were especially interested in determining whether the concatemeric forms of vaccinia virus DNA would still be processed into unit genomes with hairpin ends (21, 22) when synthesis of the 11-kDa protein was repressed. Initial dot blot analyses indicated that the quantity and kinetics of viral DNA synthesis were similar for WT and for mutant virus in the presence and absence of IPTG (data not shown). The restriction enzyme *Bst*EII digests the mature viral DNA molecule to generate a 1.3-kbp terminal fragment but produces a 2.6-kbp junction fragment from concatemeric DNA. When viral DNA from cells infected with vRO11k in the presence and absence of IPTG was digested with *Bst*EII, Southern blots revealed that the 1.3-kbp terminal band was predominant, indicating that resolution of concatemer junctions had proceeded efficiently (Fig. 4A). In addition, the amounts of terminal fragment were similar in the + and − IPTG lanes. As a control, we also analyzed the DNA formed by a temperature-sensitive mutant defective in late gene function; accumulation of the 2.6-kbp concatemer junction fragment was readily seen (Fig. 4B), as previously described (17).

Time course of protein synthesis under nonpermissive conditions. Since the proteins associated with the DNA template used for late gene expression are unknown, we wished to determine whether the absence of the 11-kDa DNA-binding protein has any effect on the pattern of viral polypeptides synthesized. vRO11k- and WT-infected cells were pulse-labeled at various times with [^{35}S]methionine, and the products were analyzed by SDS-PAGE. In this experiment, a

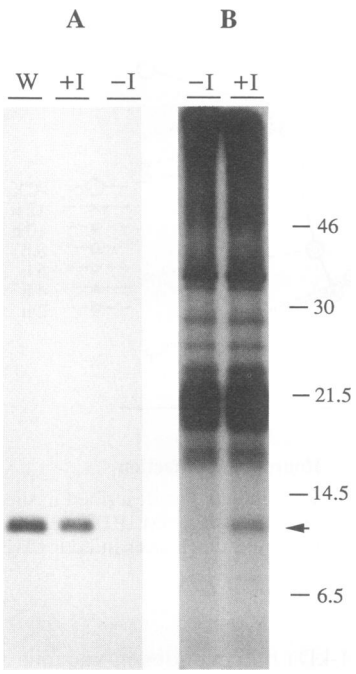

FIG. 3. Evidence that the F18R gene product is the major phosphorylated 11-kDa DNA-binding protein. (A) BS-C-1 cells were pulse-labeled with [^{35}S]methionine from 7.5 to 8 h after infection with WT (W) or vRO11k in the continuous presence (+I) or absence (−I) of IPTG. The cells were lysed, and the proteins that were immunoprecipitated with anti-VP11 polyclonal antibody (11) were treated with SDS and subjected to electrophoresis on a 20% polyacrylamide gel. (B) BS-C-1 cells infected with vRO11k in the presence (+I) and absence (−I) of IPTG were labeled from 3 to 24 h with [^{32}P]phosphoric acid. Infected cell lysates were treated with buffer containing SDS and resolved by electrophoresis on a 20% polyacrylamide gel. The positions of protein molecular markers (in kilodaltons) are indicated on the right. The position of the 11-kDa protein is marked by an arrow.

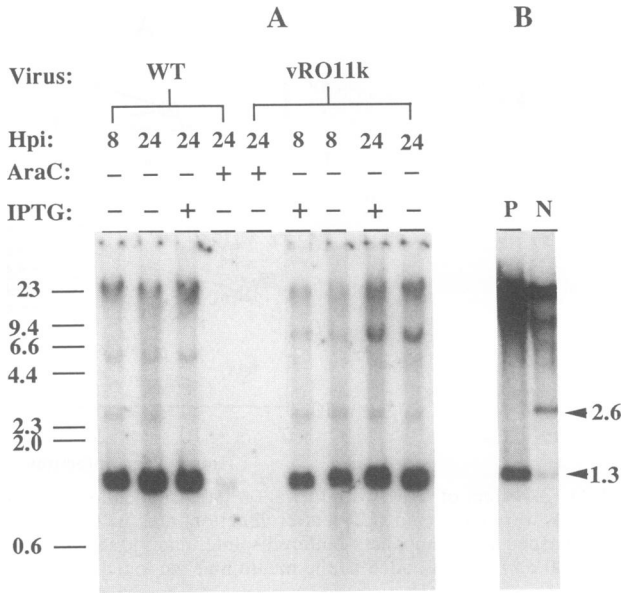

FIG. 4. Southern blot analysis of viral DNA. DNA from infected cells was digested with restriction endonuclease *Bst*EII, separated by electrophoresis on a 0.8% agarose gel, and transferred to a GeneScreen Plus membrane. Hybridization was with a ^{32}P-labeled DNA probe containing the short tandem repeats at the ends of the vaccinia virus genome. (A) DNA was obtained from cells at the indicated hours postinfection (Hpi) with WT or vRO11k mutant virus. Cytosine arabinoside (AraC) and IPTG were added as shown. The positions of DNA size markers (in kilobase pairs) are noted next to the autoradiograph. (B) DNA from cells infected with *ts*21 at the permissive 31°C (P) and nonpermissive 40°C (N) temperatures. The arrows point to the 1.3-kbp end fragment of mature DNA and to the 2.6-kbp concatemer junction fragment. Note that the probe also hybridized to the larger adjacent DNA fragment of the genome that also contains tandem repeats.

12% polyacrylamide gel was used to display a wide range of polypeptide sizes. As shown in Fig. 5, the typical shifts from host, to early, and to late viral protein synthesis occurred in vRO11k-infected cells, not only under permissive conditions but under nonpermissive conditions as well. The only apparent difference caused by omission of IPTG, absence of the 11-kDa band (Fig. 5), was previously demonstrated (32) by using a 20% polyacrylamide gel which provided high resolution of small polypeptides.

Examination of the autoradiographs (Fig. 5) also revealed a 40-kDa band among the proteins labeled at 5 h or later after infection with vRO11k in the presence or absence of IPTG but not after infection with WT. We presume that this band is the *E. coli lac* repressor, which has a molecular size of 38,600. This 40-kDa putative *lac* repressor band is also apparent in Fig. 6, which will be described in the next section.

Repression of 11-kDa protein synthesis blocks proteolytic processing of the major virion proteins. Several of the major proteins of the vaccinia virus core, including components 4a and 4b, are produced by proteolytic cleavage of higher-molecular-weight precursors (13, 19). Processing is inhibited by the drug rifampin (13), which blocks virus assembly at an early stage (7, 20, 23), leading to the suggestion that the biochemical and morphological events are coupled. Since the 11-kDa protein is packaged within the virion core, we wished to determine whether its absence would influence these maturational cleavage events.

Control experiments confirmed that the precursors P4a and P4b were labeled during a 30-min pulse with [^{35}S]methionine at 7 h after infection with wild-type vaccinia virus and that these precursors were mostly converted to 4a and 4b, respectively, during a 12-h chase (Fig. 6A). The identification of P4a and 4a was confirmed by immunoprecipitation with antibody against a shared sequence of P4a and 4a (data not shown). As expected, the conversion from precursor to mature polypeptide was blocked by rifampin. In cells infected with vRO11k, pulse-labeling of P4a and P4b occurred in the presence or absence of IPTG. With IPTG, processing of P4a and P4b appeared grossly normal although there was slightly more of the precursors remaining after the chase than had occurred with the wild-type vaccinia virus. In the absence of IPTG, however, only a trace of 4a and a small amount of 4b were formed. The slightly lower degree of inhibition of 4b formation, compared with that of 4a, was also noted with rifampin. Similar results were obtained with a 12-h continuous labeling protocol instead of a pulse-chase (Fig. 6A). Thus, synthesis of the 11-kDa DNA-binding protein is required for proteolytic processing of the major core proteins.

A related experiment was designed to determine whether the precursor proteins made in the absence of IPTG could be

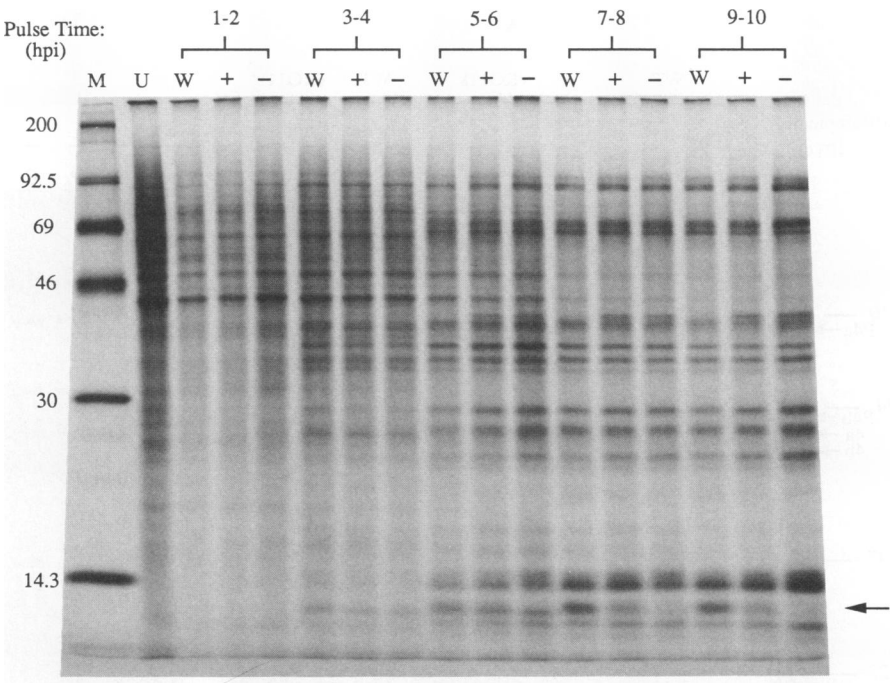

FIG. 5. Early and late polypeptide synthesis. BS-C-1 cells were infected with WT (W) or vRO11k in the presence (+) or absence (−) of 5 mM IPTG and pulse-labeled with [^{35}S]methionine between the indicated hours postinfection (hpi). The cell lysates were treated with SDS and analyzed on a 12% polyacrylamide gel. The position of the 11-kDa polypeptide is indicated by an arrow.

processed after the addition of inducer. Under the specified conditions, conversion of P4a to 4a still took place, although with some reduction in efficiency (Fig. 6B, lane −/+). This result indicates that concurrent synthesis of the 11-kDa protein with P4a and P4b is not required for the proteolytic processing event.

Virus-mediated pH-dependent cell fusion. Studies of Ichihashi and Dales (10), with a hemagglutinin-negative mutant of vaccinia virus, suggested that fusion of vaccinia virus-infected cells requires transport of mature progeny to the cell surface. Evidence for a similar requirement has been obtained (3a) for fusion mediated by acid pH (4, 15). Because repression of synthesis of the 11-kDa protein inhibited cleavage of the major structural proteins of vaccinia virus, which is a maturational event, we were interested to see whether fusion also was prevented. Control experiments (Fig. 7A and B) confirmed that the parental WR strain of vaccinia virus mediated cell fusion in a pH-dependent manner. Similarly, pH-dependent fusion occurred when cells were infected with vRO11k in the presence of IPTG (Fig. 7C and D). In the absence of IPTG, however, fusion was not observed (Fig. 7E and F) even after a prolonged (24 h) incubation (data not shown).

Inhibition of virus particle formation under nonpermissive conditions. Further experiments were designed to obtain more direct evidence for a block in virus assembly and to determine whether the noncleaved precursor proteins P4a and P4b were assembled into sedimentable particles under nonpermissive conditions. Homogenates of infected cells that had been labeled with [^{35}S]methionine were applied to a linear 25 to 40% sucrose gradient and centrifuged. Under these conditions, infectious virus particles sediment to the lower part of the gradient, whereas nonparticulate viral DNA and proteins remain near the top. Plaque assays indicated that gradient fractions 5 to 8 (Fig. 8) contained the most infectivity. SDS-PAGE of samples from the gradient of WR-infected cell material showed that certain proteins were present only in the top four fractions (note that only 20 μl of fractions 16 and 17 was analyzed, whereas 200 μl of all other fractions was used), indicating their nonparticulate nature whereas others, especially 4a and 4b, were most abundant in the fractions containing infectious virus. P4a and P4b, the precursors of the major core proteins, were most abundant near the top of the gradient, but some sedimented throughout, suggesting association with heterogeneous particulate matter. Essentially the same pattern was observed with lysates of cells infected with vRO11k in the presence of IPTG (Fig. 8). In the absence of IPTG, however, there was much less radioactive protein in the region of the gradient corresponding to virions. A small amount of 4a was present, coincident with low but detectable infectivity in those fractions. Significantly, the bulk of the P4a and P4b remained near the top of the gradient, suggesting that these proteins had not accumulated in immature particles that could be isolated by this procedure.

Inhibition of virus morphogenesis under nonpermissive conditions. Electron microscopy has provided the most information regarding the assembly of vaccinia virions (2). Under normal conditions, assembly is asynchronous and at late times there are mature virus particles as well as clusters of immature ones, appearing in cross-section as membrane arcs and circles with or without internal electron-dense nucleoid structures believed to contain DNA (Fig. 9A). Electron micrographs of cells infected with vRO11k in the presence of IPTG were indistinguishable from those of cells infected with WT, and both mature and immature particles were seen (Fig. 9B). By contrast, in the absence of inducer, almost all the particles were immature and many of these contained aberrant internal structures (Fig. 9C). Although the aberrant structures were less electron-dense than nucle-

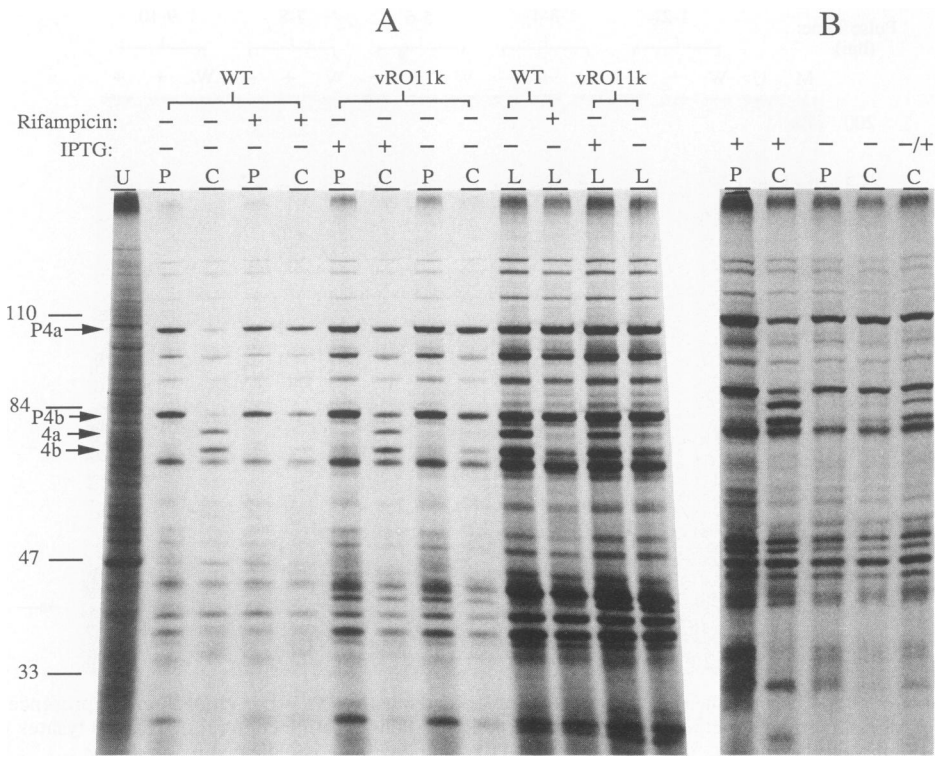

FIG. 6. Effect of IPTG deprivation on the cleavage of virion precursor proteins. (A) BS-C-1 cells in medium containing IPTG (5 mM), rifampin (100 μg/ml), or no additions were infected with the parental WR strain of vaccinia virus (WT) or with vRO11k for 7 h and labeled with [^{35}S]methionine for 30 min. Cells were harvested either after the pulse (P) or after a chase (C) with excess unlabeled methionine for another 12 h. For the long continuous labeling protocol (L), infected cells were incubated in [^{35}S]methionine-containing medium for 12.5 h. Cells were lysed, and the proteins were resolved by electrophoresis on a 10% polyacrylamide gel. The lane with uninfected cell extract is marked by a letter U. The molecular standards (in kilodaltons) are indicated by numbers, and the positions of P4a, P4b, 4a, and 4b are marked by arrows. (B) Cells were infected and labeled under the same conditions as described for panel A except that in the lane marked −/+, the infected cells were incubated and labeled in the absence of IPTG and the chase was carried out in 5 mM IPTG.

oids, their size, shape, and location suggested that they were related to them. Some sections of immature particles made in the absence of IPTG did contain structures with the electron density of nucleoids (Fig. 9C). Serial sectioning of infected cells would be useful to further investigate the internal structures of the immature particles.

DISCUSSION

The purpose of the present investigation was twofold. We wished to extend our initial study (32) regarding the general usefulness of inducer-dependent conditional lethal mutant viruses and also investigate the role of the 11-kDa protein product of the F18R gene in the replicative cycle of vaccinia virus. In particular, we were interested in learning whether the repression of synthesis of the 11-kDa protein would affect DNA processing, gene expression, or virion morphogenesis.

We found that under one-step growth conditions, the formation of vaccinia virus can be titrated by IPTG addition, with a maximum final yield achieved at 80 μM. The implied ability to regulate the level of gene expression provides a high degree of flexibility to the system. Previously (32), we demonstrated that addition of inducer at late times after infection still led to the rapid onset of virus production, indicating that IPTG could readily enter the cells and bind repressor. Here, we showed that IPTG added at the start of infection could be removed by medium changes at later times, leading to repressor-operator binding as shown by reduced virus yields. Removal had to be performed before accumulation of the 11-kDa protein, and the timing of the effect was consistent with the late expression of the F18R gene.

Examination of the translated sequence of the vaccinia virus genome reveals more than 20 open reading frames that can encode polypeptides of 9 to 13 kDa (6). For this reason, it is not clear whether the reports of various investigators refer to the same or different 11-kDa phosphoproteins. One or more core-associated phosphoproteins of approximately 11 kDa were first described by Sarov and Joklik (29), Rosemond and Moss (28), and Pogo et al. (25). The F18R gene, the object of the present study, was mapped by Wittek et al. (31), using antibody made to an 11-kDa protein isolated by PAGE of SDS-dissociated virions (9). Hiller and Weber (9) provided evidence that the polypeptide(s) that reacted with this antibody was basic, phosphorylated, exposed on the surface of virions, and interacted with actin-containing cytoskeletal elements. Bauer and coworkers (11, 12) purified by column chromatography a basic 11-kDa phosphorylated component of the vaccinia virion core that binds preferentially to superhelical DNA and which was suggested to have a role in genome condensation before or during packaging. Person-Fernandez and Beaud (24) purified and characterized a basic 11-kDa protein that could be phosphorylated by virion extracts in vitro and which binds to ribosomes and

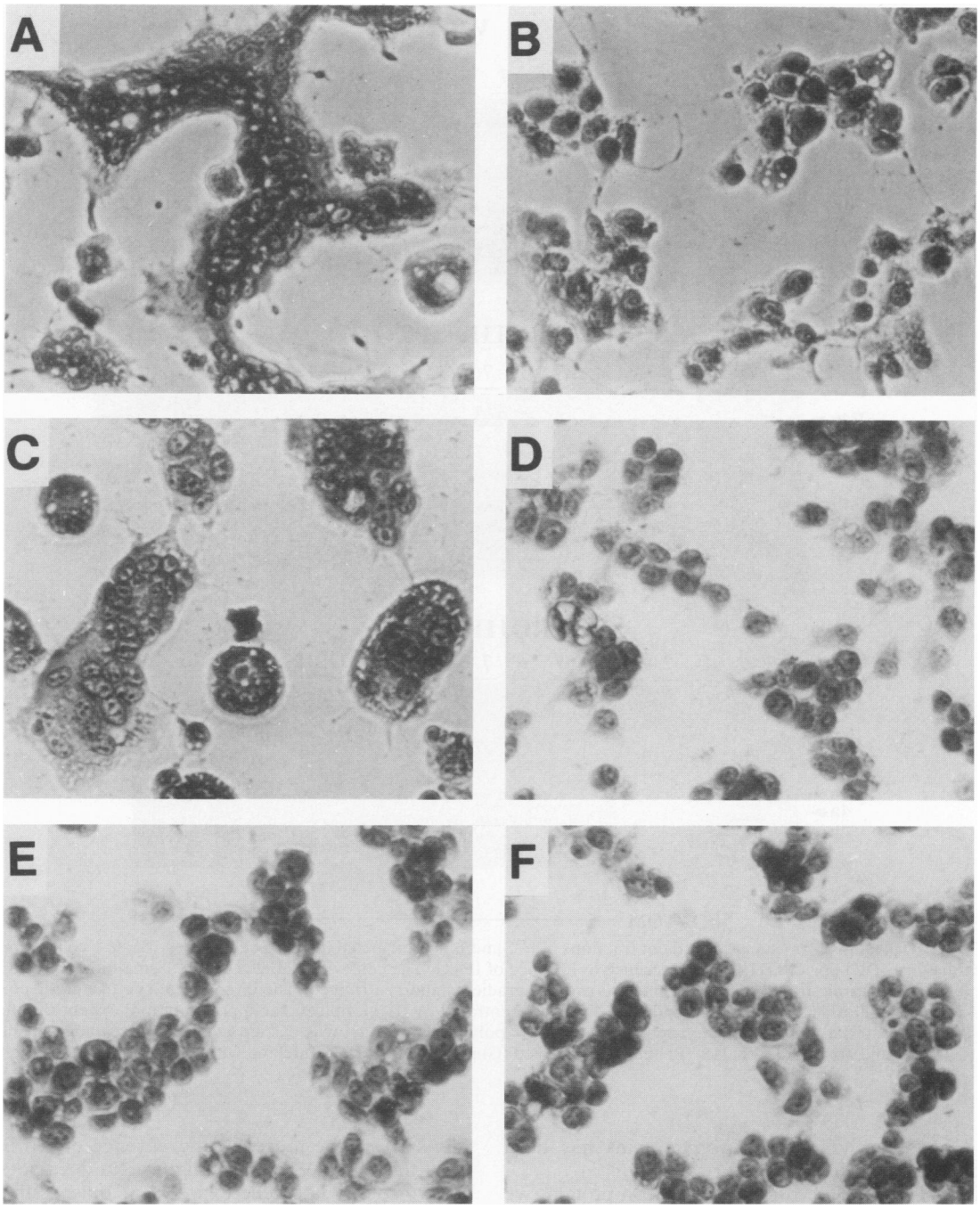

FIG. 7. Cell fusion. BS-C-1 cells infected with the wild-type parental virus (A, B) and with vRO11k in the presence (C, D) or absence (E, F) of IPTG. At 12 h, the medium was replaced with fusion medium at a pH of 5.6 (A, C, and E) or 7.4 (B, D, and F). After 2 min, the cells were returned to growth medium with or without IPTG as before. Polykaryon formation was examined by phase-contrast microscopy 2 h later.

inhibits protein synthesis. We found that antibody to the highly purified DNA-binding protein (11) reacted with an 11-kDa polypeptide made in cells infected with vRO11k in the presence but not the absence of IPTG, providing strong circumstantial evidence that the protein characterized by Bauer and coworkers is the product of the F18R gene. This interpretation, however, conflicts with N-terminal sequence data cited by these workers (11). In addition, our PAGE of ^{32}P-labeled infected cell proteins resolved only one 11-kDa polypeptide and this protein was absent when expression of the F18R gene was repressed, suggesting that there is only one major phosphorylated protein of this size. An alternate, but more complex and hence less likely, explanation is that the block in maturation prevents phosphorylation of additional 11-kDa proteins that comigrate with the product of the F18R gene. In this regard, a protein kinase which could be

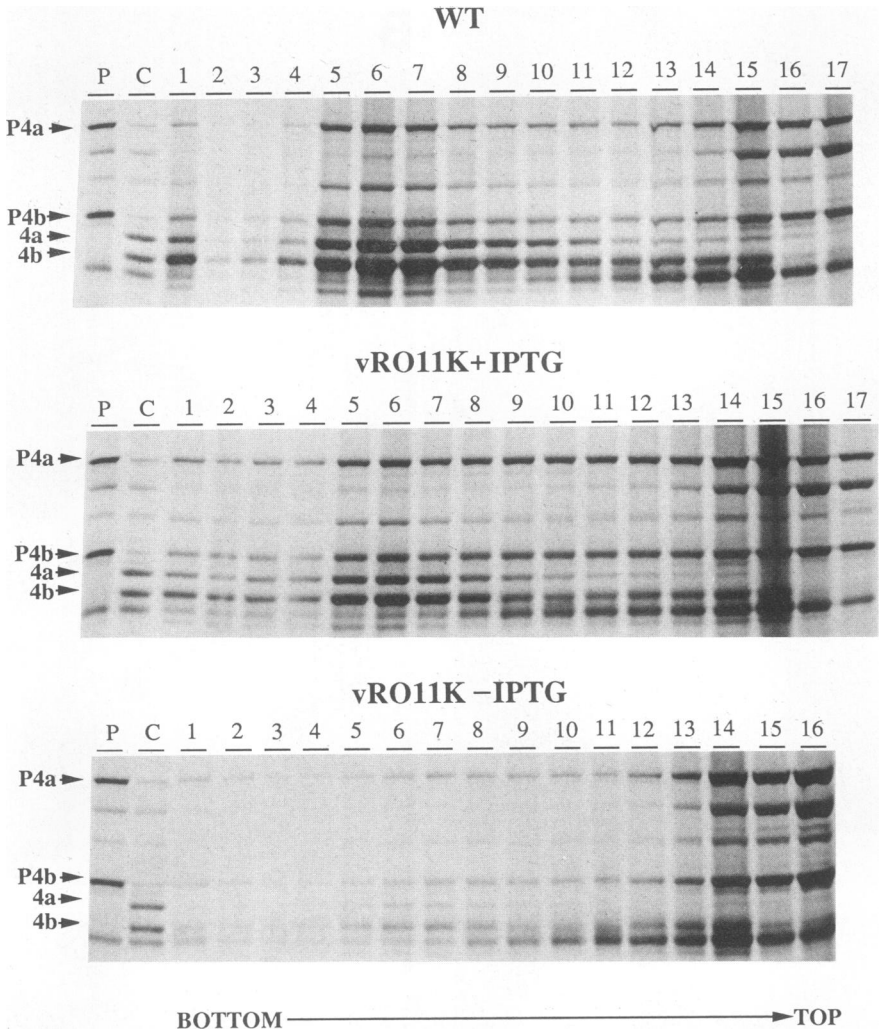

FIG. 8. PAGE of proteins from sucrose gradient fractions of [^{35}S]methionine-labeled infected cell lysates. BS-C-1 cells were infected with parental vaccinia virus (WT) or vRO11k in the presence or absence of IPTG. The cells were labeled with [^{35}S]methionine from 7 to 22 h after infection, and the cytoplasmic fractions were applied to sucrose gradients and centrifuged. The direction of sucrose gradient sedimentation is indicated. Samples (700 μl) were collected, and 200 μl was concentrated by trichloroacetic acid precipitation, except that for fractions 16 and 17 only 20 μl of material was used. Electrophoresis on a 10% polyacrylamide gel was carried out, and autoradiographs are shown. To provide P4a, P4b, 4a, and 4b markers, pulse- (P) and chase-labeled (C) proteins from cells infected with WT were added to the first two lanes of each gel.

involved in phosphorylation of virion proteins has been purified from vaccinia virus cores (14).

To obtain insight into the role of the 11-kDa protein, we investigated the synthesis of DNA, early and late proteins, and virus particles under nonpermissive conditions. Vaccinia virus DNA is made early in infection as long concatemeric molecules and is subsequently resolved into unit-length genomes (21, 22). Since the F18R gene is expressed at late times, we expected and found that the earlier event of viral DNA replication occurred with normal kinetics. Concatemer resolution, however, is dependent on late gene expression (3, 17), and we considered that the 11-kDa DNA superhelix-binding protein might be involved in this process. Nevertheless, concatemer resolution to form the mature ends of the DNA was nearly complete under both permissive and nonpermissive conditions.

Pulse-labeling with [^{35}S]methionine and SDS-PAGE indicated that early and late viral protein synthesis proceeded similarly in the presence and absence of IPTG, suggesting that the 11-kDa DNA-binding protein does not play a regulatory role in late gene expression. Indeed, the only difference noted was the absence of the 11-kDa polypeptide under nonpermissive conditions. Additionally, in the presence and absence of IPTG, the pattern of polypeptides that were labeled with inorganic phosphate appeared identical except for the absence of an 11-kDa band.

Pulse-chase experiments with radioactive amino acids provided a clue as to the stage of vRO11k replication that was blocked. We found that in the absence of IPTG, proteolytic cleavage of the major virion proteins P4a and P4b was inhibited. This effect was reminiscent of that observed with rifampin (13), a drug that blocks a very early step in virus morphogenesis (7, 20, 23). Electron microscopy confirmed that there was a block in virus assembly when synthesis of the 11-kDa protein was repressed. That block, however, occurred at an intermediate stage of morphogen-

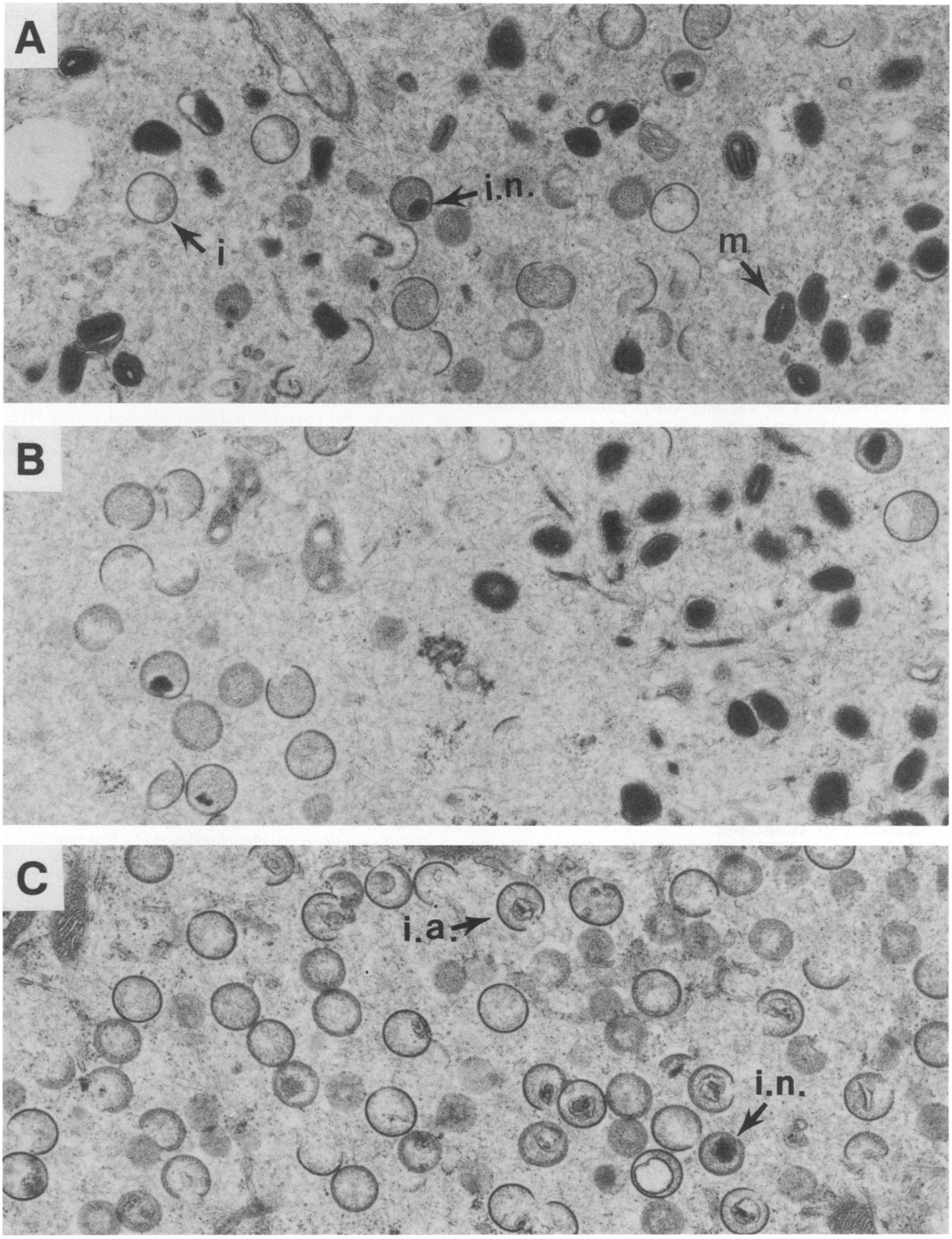

FIG. 9. Electron microscopy. BS-C-1 cells (10^7) were infected with 10 PFU per cell of wild-type vaccinia virus (A) or vRO11k in the presence (B) or absence (C) of 5 mM IPTG. Samples were prepared for transmission electron microscopy as indicated in Materials and Methods. m, mature virion; i, immature virion; i.n., immature virion with nucleoid; i.a., immature virion with aberrant internal structure.

esis and resulted in the accumulation of immature particles, many of which contained an aberrant internal structure. In size, shape, and location, the aberrant structures resembled DNA-containing nucleoids. The lower electron density of the aberrant structure, compared with that of a nucleoid, was of particular interest since the 11-kDa protein is thought to have a histonelike role in condensation of the genome (11, 12). The protein and DNA composition of these particles could not be determined, because a discrete peak was not detected by sucrose gradient centrifugation. It would be of additional interest to know whether P4a and P4b are present in the defective particles, since an association between the 11-kDa protein and P4a and P4b has been reported (9). Further information on this subject may be obtained by immunoelectron microscopy.

In conclusion, we have demonstrated that the *E. coli lac* operator-repressor system provides a powerful tool to study the roles of essential genes that are required for assembly and morphogenesis of vaccinia virus. Extension of this approach to additional vaccinia virus genes is in progress.

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