Orthopoxvirus Gene Expression in *Xenopus laevis* Oocytes: a Component of the Virion Is Needed for Late Gene Expression

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We have examined the feasibility of using *Xenopus laevis* oocytes microinjected with rabbit poxvirus as a system to study poxvirus gene expression. The injection of either intact virus or subviral cores resulted in accurate synthesis of viral proteins. This expression was dependent on the multiplicity of injected virus, with the optimal injected dose being equivalent to approximately 300 PFU per oocyte. Extensive viral gene expression including late viral protein synthesis was observed when intact virions were microinjected into the oocyte. However, the injection of subviral cores resulted in only early protein synthesis. When oocytes were injected with a mixture of subviral cores and the nonionic detergent-soluble fraction was removed from virus during the preparation of cores, both early and late viral proteins were synthesized. Therefore, the detergent-soluble fraction appears to contain a factor(s) required for the transition from early to late gene expression.

Rabbit poxvirus (RPV) is a member of the orthopoxvirus family and is characterized by its ability to replicate and develop within the cytoplasm of the infected cell (9). Gene expression is temporally regulated. Early or prereplicative gene expression begins rapidly after infection, is independent of de novo protein synthesis, and leads to the inhibition of host transcription and translation. Late gene expression depends on prior early gene expression and was classically thought to begin with the onset of viral DNA replication.

The cytoplasmic nature of this class of complex DNA viruses has sparked interest in the delineation of the enzymes and factors involved in the regulation of poxvirus gene expression. Enzymes which are known to be involved in this process include a virion-encoded, DNA-dependent RNA polymerase (14, 32), capping and methylating enzymes (11, 19-21, 24, 25, 35, 39, 41, 42), and a poly(A) polymerase (27-29). The development of in vitro systems to more precisely study viral transcription at the biochemical level has made possible the search for regulatory factors of transcription (5, 13, 36-38). One of the most surprising results of these studies has been the discovery that the capping enzyme, in addition to providing the 5'-guanylyl residue of the cap for mRNAs, also functions as a termination factor in the transcription of early genes (3).

While it is acknowledged that the poxviruses develop in the cytoplasm, recent evidence has suggested that certain specific components of the host cell nucleus are mobilized to the cytoplasm. These nuclear components include subunits of RNA polymerase II (Pol II) (23, 30, 43, 44). In addition, a cellular laminlike protein normally found in the perinuclear area redistributes in infected cells so that much more is found in the cytoplasm (1). Some of the published studies in which the mobilization of Pol II was examined suggested that the movement of the enzyme to the cytoplasm takes place in the absence of viral protein synthesis. This implies that a component of input virus is in part responsible for the process (23). An elucidation of which component(s) of the virion is involved in mobilization is technically difficult because of the problems inherent in selectively introducing

specific components, normally packaged as parts of mature virions, into cells.

The use of frog oocytes offers a system which potentially obviates these problems, provided the virus is capable of functioning normally in the oocyte environment. For example, microinjection of the oocyte would allow the introduction of intact virions, subviral cores, protein fractions, or even individual purified viral components. The oocyte system also allows for the unambiguous separation of the nuclear and cytoplasmic compartments, a problem which is not trivial in typical mammalian cells. The ability to reproducibly obtain pure cytoplasmic and nuclear fractions from oocytes (12) facilitates experiments designed to determine partitioning of either viral or host cell components between these two cellular compartments. Finally, the oocyte system has been characterized extensively and shown to faithfully translate injected heterologous mRNAs (6, 18, 40).

In this paper, we describe experiments to examine the feasibility of using frog oocytes as a system for the study of orthopoxvirus gene expression. We show that following injection of either intact virus or subviral cores, both extensive translation and presumably accurate viral gene transcription occur. In the case of intact virus, uncoating of the particle was efficient, rapid, and productive. We also show that while injection of whole virus leads to expression of both early and late viral genes, injected cores synthesize only early proteins. Finally, we present data demonstrating that a factor(s) within the material removed by nonionic detergent treatment of virus during the preparation of subviral cores is capable of restoring the ability of subviral cores to synthesize late viral proteins.

MATERIALS AND METHODS

Virus and cells. Wild-type RPV Utrecht strain was obtained from the American Type Culture Collection. Viral stocks were prepared and purified as described by Moyer and Rothe (31). Rabbit kidney (RK-77) cells were obtained from J. DeMarchi, and human lung carcinoma (A549) cells were obtained from C. Tibbets of Vanderbilt University, Nashville, Tenn. The cell lines were propagated as monolayers in Eagle minimal essential medium (F-11; GIBCO Laboratories) supplemented with 10% fetal bovine serum, 2

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TABLE 1. RPV RNA polymerase transcription assay^a

Virus treatment	Trichloroacetic acid-insoluble counts (cpm)	
	Assay 1	Assay 2
Mock	32	14
NP-40	61	71
DTT	1,040	811
NP-40 + DTT	28,716	27,642

^a Treatments of virus and assays for transcriptions were performed in duplicate as described in Materials and Methods.

mM glutamine, 100 U of penicillin, 100 µg of streptomycin, and 0.1 mg of pyruvate per ml.

Preparation and labeling of infected tissue culture extracts. Confluent monolayers of RK-77 or A549 cells were infected with RPV at a multiplicity of 5 to 10 PFU per cell. The inhibitor cytosine arabinoside (40 µg/ml), rifampin (100 µg/ml), or hydroxyurea (50 mM), as indicated below, was present throughout the infection. Radiolabeled samples of early viral proteins were prepared from infected tissue culture cells labeled from 3 to 5 h postinfection in the presence of cytosine arabinoside or hydroxyurea. Radiolabeled late viral proteins were prepared from infected cells labeled 13 to 15 h postinfection either in the presence of rifampin or with no inhibitors present. Specifically, a 150-mm dish of infected cells was labeled with 250 µCi of [³H]leucine (ICN Pharmaceuticals) for 2 h in 10 ml of leucine-free medium. Immediately thereafter, the cells were scraped into the medium, chilled, pelleted by centrifugation at 800 × g for 5 min at 4°C, and suspended in 0.5 to 1.0 ml of RIPA buffer (0.15 mM NaCl, 1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 0.1% sodium deoxycholate, 10 mM Tris hydrochloride [pH 7.4], 100,000 U of aprotinin per ml) plus 1 mM phenylmethylsulfonyl fluoride. The cells were incubated at 4°C for 30 min and then sonicated briefly. Cellular debris was pelleted by centrifugation at 12,800 × g for 10 min at 4°C. The supernatant was removed and stored at -70°C prior to immunoprecipitation.

Preparation of subviral cores and soluble proteins from purified virus. The fractionation of virus into subviral cores and soluble proteins was performed essentially as previously described (34). Purified RPV (3 × 10⁵ PFU) was suspended in solubilization solution (0.05% Nonidet P-40 [NP-40], 10 mM dithiothreitol [DTT], 50 mM Tris hydrochloride [pH 8.5], 10 mM MgSO₄) and incubated at 4°C for 60 min. The subviral cores were then pelleted by centrifugation at 12,800 × g for 1 min at 4°C. The supernatant was removed and used as the soluble fraction. Complete removal of the viral membrane from the core pellet was assured by resuspending the core preparation in 10 times the original volume of solubilization solution followed by incubation and centrifugation as previously described. This twice-extracted core pellet was suspended in TE (50 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) and used for oocyte injections.

Viral RNA polymerase assay. Viral RNA polymerase activity was assayed essentially as described previously (14, 32, 34). Each reaction sample contained 4 mM ATP, CTP, and GTP; 0.8 mM UTP; 3 µCi of [4,5-³H]UTP (ICN), 50 mM Tris hydrochloride (pH 8.5), 10 mM MgSO₄, and 1.7 × 10⁶ PFU of purified RPV in a final volume of 250 µl. In addition, some samples also contained 0.5% NP-40, 10 mM DTT, or a mixture of both (Table 1). The samples were incubated at 37°C for 30 min and stopped by the addition of 2 ml of cold 20% trichloroacetic acid containing 50 mM sodium phosphate and 50 mM sodium pyrophosphate. Precipitation was

enhanced by the addition of 50 µl of 1% bovine serum albumin to each sample. Following a 15-min incubation at 4°C, the acid-insoluble nucleic acid was collected on a glass fiber filter, dried, and counted in a liquid scintillation counter.

Oocyte injection and methodology. *Xenopus laevis* oocytes were collected from mature female frogs anesthetized by hypothermia and were injected essentially as described by Colman (6). Individual oocytes were manually defolliculated, and unblemished stage 5 and 6 oocytes were selected for injection. The injections were directed into the vegetal pole of the oocyte. The intact virus or subviral fractions were suspended in TE immediately prior to injection. The total volume injected was approximately 40 nl per oocyte. The injections and subsequent incubations were carried out in either amphibian Ringer or modified Barth solution containing 100 U of penicillin and 100 µg of streptomycin per ml. The incubation temperature for all oocytes was between 25 and 27°C.

Radiolabeling and collection of oocytes. Groups of 3 to 10 oocytes were labeled with 250 µCi of [4,5-³H]leucine (ICN) in a total volume of 100 to 250 µl of incubation medium. Cells were labeled for 2 h at the normal incubation temperature and collected immediately thereafter. Labeled oocytes were washed two times with incubation medium, collected, and stored individually in 100 µl of RIPA buffer plus 1 mM phenylmethylsulfonyl fluoride. Storage was at -70°C prior to use.

Immunoprecipitations. All steps were at 4°C. Individual oocytes were thawed and briefly Dounce homogenized in 100 µl of RIPA storage buffer in order to rupture and homogenize the cell. NET-NP-40 (0.5% NP-40, 150 mM NaCl, 5 mM EDTA, 50 mM Tris hydrochloride [pH 7.4]) (100 µl) was then added to each sample followed by the addition of 25 to 50 µl of a 10% suspension of *Staphylococcus aureus* (Cowen strain) which had been heat killed, fixed, and stored in NET-NP-40 (17). The sample was mixed, incubated for 15 min, mixed again, and incubated for an additional 15 min. Samples were then centrifuged for 3 min at 10,000 × g to remove cellular debris as well as any proteins which adhered nonspecifically to the bacterial surface. The supernatant was removed and used for subsequent immunoprecipitations. Undiluted polyclonal rabbit anti-RPV serum (1 to 3 µl) or a mouse monoclonal antibody (MAb 94) (50 µl) culture supernatant was then added to each sample. The samples were mixed and incubated for 12 to 15 h. For the experiments in which the monoclonal antibody was used, 2 µl of a second antibody, anti-mouse immunoglobulin G (Sigma Chemical Co.), was then added (undiluted) and the sample was incubated an additional 2 h. All samples were then mixed with 25 to 50 µl of the 10% *S. aureus* suspension, incubated for 15 min, remixed, and incubated an additional 15 min. The bacterial pellets were collected by centrifugation at 10,000 × g for 1 min and 20 sec. The supernatant was discarded, and the pellet was suspended in 800 µl of NET-NP-40. The suspension was pelleted two additional times in order to minimize nonspecific adherence of protein. The final pellet was suspended in 50 µl of immunoprecipitation lysis buffer (2% SDS, 30 mM Tris hydrochloride [pH 6.8], 1.5% DTT, 20% glycerol, and 0.05% bromophenol blue) by water bath sonication. The samples were then heated to 100°C for 2 min and cooled to room temperature. Bacterial debris was pelleted by centrifugation at 10,000 × g for 3 min, and the resulting sample supernatants were analyzed by polyacrylamide gel electrophoresis. As controls, comparable amounts of radiolabeled extracts of uninfected and infected tissue

culture cells were immunoprecipitated in parallel with the oocyte samples by using the same procedures as those described for the oocytes.

Resolution of immunoprecipitated proteins. Samples of radiolabeled, immunoprecipitated proteins were separated by electrophoresis on 10% SDS-polyacrylamide (30:1 acrylamide/bis ratio) gels. Samples (50 μ l) containing the material collected from a single oocyte or comparable amounts of material derived from tissue culture samples were subjected to electrophoresis at 70 V for 16 h at room temperature in buffer containing 6.1 g of Tris base, 28.8 g of glycine, and 1.0 g of SDS per liter. Gels were processed for fluorography prior to autoradiography as described by Bonner and Laskey (2).

Monoclonal and polyclonal antibodies. The monoclonal antibody MAb 94, which recognizes both the 94-kilodalton (kDa) structural protein precursor of the RPV virion and its mature cleavage product (p62), was generated as previously described by Morrison et al. (22). Polyclonal antibodies were prepared from rabbits immunized with either acetone-fixed tissue culture cells infected with RPV or acetone-fixed purified RPV. Both immunization regimens involved the injection of the antigens diluted at a 1:1 ratio with complete Freund adjuvant. Each rabbit received a series of four such immunizations with a time interval of 7 days between each injection. At 2 weeks after injection 4, the animals were given a booster injection of antigen minus the adjuvant. Another booster injection was repeated 2 weeks following the first. The animals were bled 7 days after boost 2, and the antisera were stored at -70°C prior to use.

Electron microscopy. To prepare samples for electron microscopy, oocytes were injected with or without virus into the distal region of the vegetal pole. Colloidal gold particles (15 nm) were included in the injection mix and used as a marker for the site of injection. Immediately or at 2 h postinjection, the oocytes were fixed overnight at 4°C with 3% glutaraldehyde, 2% paraformaldehyde, 2.5% dimethyl sulfoxide, 1% acrolein, and 0.14% CaCl_2 in 0.1 M cacodylate buffer. The oocytes were then further treated at room temperature for 2 h in 2% OsO_4 . The oocytes were flat embedded in Spurr embedding medium. The sections were poststained with uranyl acetate and lead citrate and examined at 60 kV with a JEOL 100-CX microscope.

RESULTS

Response of the oocyte to injection of RPV subviral cores. Amphibians are not natural hosts for RPV. Therefore, we first had to determine whether the *X. laevis* oocyte would respond to injected virus. Our first experiments, however, employed the injection not of intact virus but of subviral cores. The choice of subviral cores for these initial microinjection experiments was for the following reason. During a natural infection, after attachment of the virus to the cell, uncoating of the virion occurs via fusion of the viral membrane with the cell membrane or endocytic vesicle membrane during or immediately after viral penetration (4, 7, 8). The microinjection of the oocyte effectively eliminates contact of the virus with these membranes and therefore might prevent uncoating of the viral particle to yield active subviral cores. Any potential difficulties with the uncoating process are circumvented by instead injecting subviral cores directly into the cytoplasm. However, in order for subviral cores to be effective in the oocyte, core-associated transcription and subsequent translation still must take place. Furthermore, if the oocyte environment is to be considered analogous to that

FIG. 1. Response of *X. laevis* oocytes to injection of RPV subviral cores. Single oocytes maintained in either Ringer or modified Barth medium were injected with 40 nl of TE medium containing 300-PFU equivalents of RPV cores prepared as described in Materials and Methods. At the times after injection indicated, proteins were radiolabeled as described in Materials and Methods. Soluble radiolabeled proteins within the oocyte as well as samples of radiolabeled early and late viral proteins derived from infected rabbit kidney cells were immunoprecipitated as described in Materials and Methods. All samples were then analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography and autoradiography of the dried gel. (A) Lanes: 1, 2, and 3, samples derived from uninfected and early and late infected cells permissive for RPV; 4, immunoprecipitable proteins derived from mock-injected oocytes; 5, sample derived from oocytes injected with subviral cores, collected 24 h after injection. (B) Lanes: 1, 2, 3, and 4, samples derived from oocytes injected with subviral cores and collected 12, 24, 36, and 48 h postinjection, respectively. Molecular masses are indicated at the right. K, Kilodaltons.

of the mammalian cell, then the pattern of proteins initially expressed should be similar to the early pattern of viral protein synthesis observed during normal infections of mammalian cells.

The results observed when oocytes were injected with subviral cores are shown in Fig. 1. Also shown are controls for immunoprecipitated mock-injected oocytes (Fig. 1A, lane 4) and uninfected early or late viral immunoprecipitable proteins derived from infected rabbit kidney (RK-77) cells (Fig. 1A, lanes 1, 2, and 3, respectively). Comparison with infected cell cultures shows that within 24 h after the injection of cores, an early viral pattern of protein synthesis had been established. The early pattern of viral protein synthesis in oocytes was already apparent by 12 h following injection and persisted for periods up to 48 h (Fig. 1B). In

FIG. 2. Response of *X. laevis* oocytes to injection of intact RPV. Injections, preparation, and analysis of samples were as described in the legend to Fig. 1 except that injections were with intact virus. Lanes A, B, and C contain radiolabeled immunoprecipitable viral proteins synthesized 24, 36, and 48 h postinjection, respectively. Also shown, as controls, are samples of immunoprecipitable late and early viral proteins derived from infected permissive cells (lanes D and E, respectively) and from oocytes injected with subviral cores (lane F). The molecular masses of viral structural precursor proteins p4A (94 K) and p4B (65 K) are indicated. K, Kilodaltons.

experiments not shown, we have observed that this early pattern of viral gene expression persisted for up to 96 h or until oocyte death. The results of this experiment suggest that subviral cores can direct viral transcription within the oocyte environment and that those transcripts are accurately translated into early proteins. However, unlike what was observed in a normal productive infection, there was no progression to the expression of late genes.

Response of the oocyte to injected intact RPV virions. Injection of subviral cores into oocytes resulted in the establishment of a persistent pattern of expression of early viral proteins (Fig. 1). However, unlike what occurs in a natural infection, there was no transition to late gene expression. We then examined the response of the oocyte to the injection of intact RPV virions. The results of this experiment (Fig. 2) surprisingly revealed a more complete pattern of viral gene expression than we observed following the injection of subviral cores. Unlike what was observed with subviral cores, by 24 h a pattern of viral proteins was seen that was both more extensive than that of cores and more similar to a late pattern of proteins. Additional viral proteins continued to appear at even later times, and each of these proteins corresponded to proteins seen at late times in a natural infection. Solely on the basis of molecular masses, it would appear that two of the protein bands correspond to the

FIG. 3. Effects of hydroxyurea on oocytes injected with intact RPV virions. Virions were injected into duplicate oocytes in the absence (lanes A and B) or presence (lanes C and D) of 50 mM hydroxyurea and radiolabeled as described in Materials and Methods. Samples were collected 42 h after injection, immunoprecipitated, and analyzed on polyacrylamide gels. As a control, a sample derived from tissue culture (A549) cells infected with virus in the absence of inhibitors (lane E) is included. The molecular masses of p4A (94 K) and p4B (65 K) are indicated. K, Kilodaltons.

precursor proteins of the major late core polypeptides P4A and P4B (94 and 65 kDa, respectively). By 48 h, the pattern of viral proteins was quite similar though not identical to that of late proteins observed for cells productively infected with RPV. Unlike subviral cores, which expressed only early proteins within oocytes, it would appear that intact virions can synthesize both early and at least some late viral proteins. Intact virions, therefore, can direct more complete expression of the viral genome than can subviral cores.

Analysis of oocytes injected with intact virus for the presence of late viral proteins. The results in Fig. 2 suggest that late viral gene products are synthesized in oocytes following injection with complete virus. The synthesis of late viral proteins should have been blocked by drugs such as hydroxyurea, which inhibit late viral protein synthesis by preventing viral DNA synthesis. The effect of the drug was to inhibit expression of many of the viral proteins, and the resulting pattern of expressed proteins which were drug resistant was similar to that of an early viral protein pattern (Fig. 3; compare lanes A and B with C and D). It should be noted that hydroxyurea was generally suppressive in terms of poxvirus gene expression. However, various exposures of the gels, while not shown, still support the notion that the pattern of proteins observed in the presence of the drug was early.

Further evidence of late viral protein synthesis in response

FIG. 4. Immunoprecipitation of viral proteins synthesized in oocytes with monoclonal antibody directed against a late structural protein. Proteins from infected A549 cell cultures or from oocytes injected with virus were immunoprecipitated (48 h after injection) with either polyclonal anti-RPV sera (lanes A to D) or with a monoclonal antibody (MAb 94) which recognized both the precursor (94-kDa) and processed form (62-kDa) of the viral structural protein p4A (lanes E to I). K, Kilodaltons. Cells were infected with wild-type virus in the absence of any inhibitor (lanes B and E), in the presence of CAR (lanes A and I), or in the presence of rifampin (lanes C and F). Infected cell samples were collected 5 h after infection in the presence of CAR or at 15 h postinfection otherwise. Oocytes were injected in the absence of inhibitors with either intact virus (lanes D and G) or with subviral cores (lane H).

to injected intact virus was provided by the experiment (Fig. 4) in which we used a monoclonal antibody (MAb 94) we prepared against p4A, one of the two major late structural proteins of the virion. This protein is synthesized late in infection, following the onset of DNA replication, as a 94-kDa precursor which is proteolytically cleaved during the maturation process to a 62-kDa form of the protein found within mature virions (15, 16, 26, 35). MAb94 recognizes both forms of the protein. As controls, we have included immunoprecipitations of cell cultures infected with RPV in the presence and absence of the inhibitors cytosine arabinoside (CAR) and rifampin. CAR is an inhibitor which prevents DNA replication and subsequent synthesis of late viral proteins; rifampin has no obvious effect on gene expression but instead blocks the proteolytic processing of late precursor proteins.

When infected tissue culture cells were examined, it could be seen that the addition of CAR blocked the appearance of both the 94- and 62-kDa forms of p4A (Fig. 4; compare lanes A and B). Addition of rifampin to infected cells (Fig. 4, lane C) drastically reduced levels of the mature 62-kDa cleavage

products of p4A but had relatively little effect on levels of the 94-kDa precursor. Oocytes injected with intact virus appeared to synthesize the 94-kDa structural precursor protein (Fig. 4, lane D).

Similar experiments using MAb94 are shown in Fig. 4, lanes E through I. The monoclonal antibody precipitated both forms of p4A from infected tissue culture cells (Fig. 4, lane E), only the 94-kDa precursor from cells infected in the presence of rifampin (Fig. 4, lane F), and neither form when cells were infected in the presence of CAR (Fig. 4, lane I). Examination of immunoprecipitated oocytes in the absence of inhibitors revealed the presence of the precursor 94-kDa protein but not of the processed form of the protein (Fig. 4, lane G). While we interpret these results to be a further indication of late viral protein synthesis, it is also interesting to note that we have never seen any indication that this protein is processed in oocytes. As expected, none of the protein was detected in oocytes injected with subviral cores (Fig. 4, lane H).

Analysis of the integrity of injected whole virions. It is clear that the injection of intact virions results in extensive transcription and translation of viral genes. Normally, intact virions are transcriptionally inactive because the virions must first be partially disrupted to form cores either during the adsorption and penetration of the cell in vivo or by disruption of the viral membrane in vitro by the addition of detergent and a reducing agent before the transcriptional machinery becomes functional. Therefore, in order for the virions to function within the oocyte environment, it is implicit that either the virus is activated or uncoated within the oocyte or the virus must be somehow activated prior to injection. We have examined the transcriptional state of the virus preparations used in these experiments. The results, in duplicate, are shown in Table 1. It is evident that without treatment with both detergent and reducing agent, the virions are transcriptionally inactive. It is also clear from this experiment that the virion preparation, when activated by the normal detergent and reducing-agent treatment, has the expected transcriptional potential. Therefore, since the virions are transcriptionally inactive prior to injections, they must be activated within the oocyte in the absence of the usual passage of the virus through the cellular membrane.

Electron microscopic analysis of virions after injection into oocytes. Since virions were activated when injected into oocytes, we attempted to visualize the processing of the virions following their introduction into the oocyte. Colloidal gold was coinjected with the virus to aid in locating the virus within the oocytes. It is clear that there were marked changes incurred following the injection of the virions and that these changes were induced rapidly (Fig. 5). Immediately following injection, virions which appeared normal and unaltered were readily found within the cytoplasm of the oocyte (Fig. 5A and B). However, within 2 h postinjection, the appearance of the virions had been markedly altered and visually appeared identical to cores as defined by Easterbrook (10) (Fig. 5C and D). These results would indicate that changes to the virus begin rapidly. Although we have examined many fields, there did not appear to be localization of the viral particles with any particular subcellular structure, membrane, or other site within the oocyte. After about 4 to 6 h, it became increasingly difficult to find any discrete viral structure.

Effect of input multiplicity of injected virus on gene expression. After repeated experiments of injecting oocytes with intact virus, it became apparent that viral expression was highly multiplicity dependent. For example, the injection of

FIG. 5. Electron micrographs of oocytes injected with RPV. Oocytes were injected and prepared for electron microscopy as described in Materials and Methods. Samples were taken for analysis either immediately (A and B) or 2 h (C and D) after injection with RPV.

concentrated stocks of virus ($>10^5$ PFU per oocyte) rapidly killed the oocytes. At very low concentrations of virus, we noted significant variations in both the amount of viral protein synthesis and the length of time needed for late viral protein synthesis to become apparent. Therefore, we sought to quantitate the effect of virus input multiplicity on the response seen in the oocyte. The results of this experiment (Fig. 6) demonstrate the pattern of viral protein synthesis observed 24 h after injection with various input levels of virus ranging from 3 to 12,000 PFU per oocyte. At input doses of 3 or 30 PFU per oocyte, little, if any, late viral protein synthesis was observed at 24 h postinjection (Fig.

6A, lanes A and B). Input levels of virus of 300 PFU per oocyte appeared to be ideal (Fig. 6A, lane C). The input optimum appeared fairly narrow, because expression was not nearly as extensive when higher levels (1,500 PFU per oocyte) were used (Fig. 6A, lane D). Larger doses (6,000 or 12,000 PFU per oocyte) actually appeared to be detrimental to the oocyte (Fig. 6A, panels E and F) and resulted in a strong diminution of viral expression. The inhibition of viral protein synthesis observed at higher concentrations of virus appeared to be nonspecific as overall oocyte protein synthesis, as measured by total acid-precipitable radiolabeled proteins, was also decreased. We routinely titrated each batch

FIG. 6. Viral gene expression in oocytes as a function of multiplicity of injection. Oocytes were injected at various input multiplicities of virus or cores as described in Materials and Methods. Proteins were radiolabeled 22 h after injection for 2 h, collected thereafter, and immunoprecipitated and analyzed on polyacrylamide gels as previously described. (A) Responses of oocytes to intact virus. The multiplicities of injection were 3 (lane A), 30 (lane B), 300 (lane C), 1,500 (lane D), 6,000 (lane E), and 12,000 (lane F) PFU per oocyte. Duplicate samples representing two individual oocytes are presented for each multiplicity. (B) Responses of oocytes to injection of cores at input multiplicities of 300 (lane A), 1,200 (lane B), and 12,000 (lane C) PFU per oocyte. The molecular masses of p4A (94 K) and p4B (65K) are indicated. K, Kilodaltons.

of virus prior to use to maximize both the response and reproducibility of the oocytes. The response of oocytes to subviral cores was not as fastidious, as a similar pattern of protein expression was observed over quite a wide range of input cores (30 to 12,000 cores per oocyte) (Fig. 6B, lanes A to C).

Addition of solubilized components back to subviral cores allowed late viral gene expression. Injection of oocytes with subviral cores resulted in only early viral protein synthesis, whereas injection of intact virions allowed additional expression of late viral gene products. We would predict, on the basis of these observations, that some material is removed during the preparation of subviral cores which facilitates the expression of late viral genes. This prediction has been tested by measuring the effect on protein synthesis when the solubilized material was added back to subviral cores prior to injection into oocytes (Fig. 7). The soluble fraction itself, when injected alone into oocytes, elicited only minimal levels of activity (Fig. 7, lanes A and B). Injection of the purified cores suspended in TE or in solubilizing solution alone (see Materials and Methods) yielded the expected limited early pattern of protein synthesis as previously shown (Fig. 1, 4, and 6B) (data not shown). High levels of solubilizing solution (NP-40, DTT, and MgSO_4) were eventually toxic and killed the oocytes. However, when the soluble fraction was added to the cores, we observed an extensive pattern of protein synthesis (Fig. 7, lanes E and F) virtually identical to that from oocytes injected with purified virus (Fig. 7, lanes C and D). It would appear, therefore, that expression of viral genes by purified cores is limited to early proteins because some component(s) essential for the transition to late gene expression is removed when viruses are

extracted to produce cores. Furthermore, the component is stable and can be added back to cores to reconstitute late gene expression.

DISCUSSION

The initial reason for exploring the *X. laevis* oocyte system was to examine whether the oocyte was a suitable environment to study the communication that occurs between the nucleus and cytoplasm in RPV-infected cells. Our work here focuses on the ability of the oocyte to allow poxvirus-directed gene expression following microinjections of either intact RPV or subviral cores. The expression of viral genes was assayed by gel electrophoresis of radiolabeled, immunoprecipitated proteins. The immunoprecipitations with a polyclonal anti-RPV antiserum served to minimize the background of host oocyte protein synthesis. From these experiments, we were able to make several conclusions. First, RPV genes were capable of being transcribed and accurately translated in the oocyte. Second, there was a remarkable difference between the patterns of viral proteins observed which depended on whether subviral cores or intact virions were injected. The injection of subviral cores resulted in the expression of only early genes, whereas the injection of intact virions resulted in the expression of both early and late genes. Furthermore, the early and late viral proteins observed in response to injected intact virions were expressed in a time-dependent, sequential order, as is seen in a natural infection.

In a natural infection, the expression of late genes is generally believed to be dependent on prior viral DNA replication. We have attempted to measure viral DNA

FIG. 7. Effects of virion soluble extract on protein expression in oocytes by RPV cores. Purified virions were fractionated into subviral cores and a soluble fraction after treatment with detergent and DTT. Oocytes were injected with soluble fraction alone (lanes A and B), purified intact virions (lanes C and D), or subviral cores which were supplemented with an amount of soluble extract equivalent to the amount stripped from the injected cores (lanes E and F). Newly synthesized proteins were radiolabeled for 2 h at 46 h after injection, oocytes were harvested, and proteins were immunoprecipitated and analyzed on polyacrylamide gels as described in Materials and Methods. The molecular masses of p4A (94 K) and p4B (65 K) are indicated. K, Kilodaltons.

synthesis within virus-injected oocytes by several procedures (data not shown). We have failed to detect any viral DNA synthesis in the oocyte under any conditions. After input virus was uncoated, we were unable to detect any immature or mature virus within oocytes up to 48 h postinfection (unpublished results), even though extensive late protein synthesis had occurred.

In natural host cells, all poxvirus late proteins by definition are thought to depend on viral DNA synthesis for expression. Based on this definition, the protein precursors of the major core polypeptides referred to as p4A and p4B have been considered as late proteins. Considering the lack of detectable DNA replication in the oocyte, we wanted further proof that a protein of 94 kDa synthesized in response to injected whole virus was indeed p4A. Therefore, we used the monoclonal antibody MAb 94 to immunoprecipitate samples of infected oocytes injected with virus. This monoclonal antibody recognized both the p4A protein (94 kDa) and the 62-kDa cleavage product which was generated from the 94-kDa protein during morphogenesis of the virus. These experiments (Fig. 4) revealed not only that authentic

p4A was produced in the oocyte but also that the protein was not cleaved to the mature 62-kDa form. The failure to process p4A was seen during a natural infection in the presence of rifampin, an inhibitor of viral morphogenesis. This lack of the proper cleavage of one of the major core polypeptides might even be expected in the oocyte because there is no viral DNA synthesis. Under these conditions, no new DNA would be available for the initiation of viral morphogenesis.

To provide further evidence for the synthesis of late viral proteins in oocytes, we used an inhibitor of DNA replication, hydroxyurea, that blocks the expression of late genes in a natural infection. Oocytes injected with virus and incubated in the presence of hydroxyurea showed a diminished overall pattern of viral protein synthesis but nevertheless appeared to maintain an early viral protein pattern. Both observations are consistent with what is seen in a natural infection in the presence of hydroxyurea and further support the contention that many of the proteins seen without any inhibitors in the oocyte in response to intact virus are indeed late viral proteins, despite the fact we cannot demonstrate any viral DNA synthesis.

It has been well documented that intact virions are transcriptionally inactive and that gene expression commences only when the virion is uncoated. Since viral genes were expressed in the oocyte following injection of the virus, the virions must be uncoated within the oocyte, assuming the virus was intact and transcriptionally inactive prior to injection. Our results demonstrate that the virus we injected into the oocyte was intact, i.e., inactive unless somehow processed. We used an *in vitro* assay of RNA polymerase activity to prove that the virus was active only after treatment which permeabilized and removed the viral membrane. The untreated virions lacked any ability to transcribe (Table 1).

In a natural infection, the initial stage of uncoating of the virus is believed to occur via fusion of the viral membrane with either the cell membrane or an endocytic vesicle. Assuming fusion of the viral membrane with a cellular membrane to be a prerequisite for uncoating, we employed electron microscopy to look for possible compartmentalization of the virus or fusion of the virus with an oocyte cytoplasmic membrane early after injection of the oocyte (Fig. 5). Our results did not reveal any particular site of localization or cytoplasmic membrane involvement in the uncoating process. However, electron microscopy did indicate that within 2 h postinjection, nearly all of the virions were altered. Therefore, we conclude that productive uncoating of the virus within the oocyte is relatively rapid and quite efficient. However, the actual mechanism by which the virus is uncoated remains unclear.

We have also demonstrated that viral gene expression in the oocyte is clearly dependent on the multiplicity of the injected virus. We have shown that the optimal multiplicity for oocyte injections is 300 PFU per oocyte and that the expression of viral gene products decreases if either more or less virus is injected. However, it should be noted that each purified virus preparation can vary in its optimal oocyte multiplicity because of variations in the purity of the stock. Perhaps this reflects differences in the particle/PFU ratio. Therefore, each stock must be tested to determine its own optimal multiplicity. The injection of large numbers of virions into an oocyte appears to be lethal. Oocytes injected at high multiplicities (Fig. 6) reveal not only little or no viral gene expression but also a shutoff of endogenous oocyte expression and morphological changes consistent with severe and rapid oocyte deterioration (unpublished results).

Perhaps our most interesting observation relates to the transition from early to late gene expression. As previously stated, the injection of subviral cores resulted in the expression of only early genes, whereas the injection of intact virions resulted in the expression of both early and late gene products. During the preparation of subviral cores, the fraction of the virus that was solubilized by a nonionic detergent and a reducing agent was removed. This fraction has previously been shown to contain a number of viral proteins (33). We have shown that the injection of this soluble fraction together with subviral cores is capable of reconstituting the expression of late viral gene products. We conclude that this soluble fraction contains a factor(s) which is required for the transition from early to late gene expression.

These studies potentially reveal new aspects of poxvirus gene regulation. In this regard, the oocyte system may offer a rather unique experimental system in which to study these viruses. On the other hand, the oocyte is not a natural host cell for the orthopoxviruses. Therefore, additional studies are needed to determine whether our observations are truly reflections of natural regulatory events.

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