Some Enzymatic Activities Associated with Purified Parapoxvirions

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Purified virions of milker's nodule virus, a parapoxvirus, were shown to contain an RNA polymerase, a nucleotide phosphohydrolase, and a protein kinase associated with or encapsulated within the DNA-containing core of the virus. In vitro, the activated viral RNA polymerase transcribed only 7 to 8% of the genome, in the form of 8S to 14S polyadenylated RNA molecules which were complementary to sequences present in milker's nodule virus DNA but not vaccinia virus DNA or DNA prepared from the host cells in which the virus was propagated. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis showed that in vitro, the activated viral protein kinase phosphorylated viral polypeptides of 95, 60, 33.5, 15, and 13.8 kilodaltons.

Milker's nodule virus (MNV) (2, 5, 21, 40) belongs to the genus *Parapoxvirus*, a subgroup of morphologically distinct viruses within the family Poxviridae (20, 28, 37). Like the orthopoxviruses, the primary site of parapoxvirus replication appears to be in the cytoplasm of infected cells (6, 15, 21). However, profound alterations in the structure of nuclei in parapoxvirus-infected cells have been noted, suggesting that there may be nuclear involvement in the replication cycle of parapoxviruses (65).

The unique morphology of parapoxvirions has been described in a series of electron microscopic studies (12, 47, 48, 62). Purified parapoxvirions have a complex polypeptide composition when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) which is distinct from that of orthopoxviruses analyzed under similar conditions (73). The genome of the parapoxvirions consists of a single, linear, double-stranded DNA molecule of about 85×10^6 to 90×10^6 daltons (39, 73) and a guanine-plus-cytosine content of 63% (73, 77). Like the DNA of the orthopoxviruses (7, 22), the DNA prepared from parapoxvirions is terminally cross-linked (39).

Purified orthopoxviruses, Yaba poxvirus, and insect poxviruses contain a complex of enzymes which are clearly involved in or can be postulated to be involved in the transcription of the viral genome (2-4, 16, 23, 31, 45, 57-59, 60, 63, 64, 67, 69, 72) or the subsequent modification (11, 18, 30, 34-36, 43, 44, 74, 75) and processing of

transcripts (55, 56). These enzymes are localized in the cores of the virions and are activated when the outer virion envelope is disrupted with 2-mercaptoethanol (β ME) and either a nonionic detergent or trypsin. Some of the enzymes have been purified to homogeneity and shown to be coded for by the viral genome (reviewed in references 41 and 42).

We report here that parapoxvirions contain a DNA-dependent RNA polymerase, a nucleotide phosphohydrolase, and a protein kinase. Conditions for assay of the RNA polymerase associated with purified virions are defined, and the transcripts are analyzed by sedimentation in sucrose gradients and by molecular hybridization experiments.

MATERIALS AND METHODS

Virus and cells. MNV (ATCC VR-634) was propagated in primary bovine embryonic kidney cells, primary bovine embryonic lung cells, and MDBK cells (ATCC CCL-22), an established cell line. The yields of virus and enzymes associated with the virus particles isolated from these various sources were found to be similar, and MNV was subsequently routinely grown in MDBK cells. Cells were propagated as monolayers in 32-oz. (ca. 960-ml) prescription bottles and fed with Eagle minimal essential medium plus 5% calf serum. After infection with virus, the cells were washed and refed with minimal essential medium containing 5% fetal calf serum. Infected cells were harvested 54 to 96 h postinfection, and the virus was purified as described previously (23), with the following modifications. Virus present in cell extracts was pelleted through a sucrose cushion consisting of 10 ml of 40% sucrose (wt/wt) overlaid with 5 ml of 20% sucrose (wt/wt) prepared in 1 mM phosphate buffer (pH 7.4). The pelleted virus was resuspended in 1 mM phosphate

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buffer (pH 7.4) with the aid of sonication and then banded by sedimentation in 20 to 40% sucrose gradients (25). Before the virus was used for the experiments to be described below, it was collected by centrifugation and rebanded in 20 to 40% linear sucrose gradients. In our hands, 4×10^{10} elementary bodies of purified MNV contained 100 μg of protein and yielded 4 to 5 μg of DNA (when the DNA was extracted as described below). On the basis of data obtained with virions in which the DNA was labeled with [^3H]thymidine, we estimate that this represents a yield of ~60% of the total DNA present in the virus particles. Vaccinia virus, strain WR, was propagated in HeLa cells and purified as described previously (6, 25, 27).

In vitro assay of MNV-associated RNA polymerase. The standard assay mixture for in vitro assay of MNV-associated RNA polymerase contained 50 mM Tris-hydrochloride buffer (pH 8.0), 6 mM MgCl_2 , 10 mM βME , 0.05% Nonidet P-40 (NP-40), and 50 μg of Macaloid. This mixture is referred to as $1\times$ in vitro buffer. For RNA synthesis, in vitro buffer was supplemented with 5 mM ATP, 1 mM CTP, 1 mM GTP, and 50 μM [^3H]UTP (specific activity, 0.3 to 1.2 Ci/mmol). Virus (4×10^{10} elementary bodies or 100 μg of viral protein) was resuspended in $1\times$ in vitro buffer with the aid of sonication and added to the assay mixture. The total volume of the reaction mixture was 0.4 ml. Reaction mixtures were incubated at 35°C with continuous shaking. At appropriate intervals, samples were removed from the reaction mixtures into ice-cold 10% trichloroacetic acid (TCA) containing 0.1 M sodium pyrophosphate. After 30 min at 4°C , the precipitates which formed were collected by filtration through GF/C glass filters (Whatman, Inc., Clifton, N.Y.), and the precipitates were washed with 5% TCA containing 0.1 M sodium pyrophosphate, dried, and counted under a toluene-based scintillant. Conditions used in this laboratory for in vitro assay of the DNA-dependent RNA polymerase present in purified vaccinia virions have been described previously (13).

Sedimentation analysis of in vitro-synthesized RNA. In vitro RNA synthesis was terminated by the addition of Sarkosyl NL-97 (1.0%) and βME (2%) to the reaction mixtures. After mixing, the samples were incubated at 37°C for 10 min and layered onto 17-ml, 15 to 30% (wt/wt) linear sucrose gradients prepared in Tris-hydrochloride buffer (pH 7.9)–100 mM NaCl–10 mM EDTA–0.2% SDS. The gradients were centrifuged (Spinco SW27 rotor, 20 to 24 h, 24,000 rpm, 25°C) and fractionated, and the distribution of TCA-insoluble radioactivity in the fractions was determined as described previously (13). Cytoplasmic extracts of HeLa cells, analyzed in parallel gradients, provided rRNA and tRNA sedimentation markers of 28S, 18S, and 4S.

Purification of RNA. To the in vitro reaction mixtures (0.4 ml) we added the following: sodium acetate (pH 6; 0.01 M), NaCl (0.1 M), and EDTA (0.01 M) (ASE buffer). Sarkosyl NL-97 (1%) and βME (2%) were added, and the samples were incubated at 37°C for 10 min. The samples were deproteinized (3 times) with phenol-chloroform-isoamyl alcohol (25:24:1), we added 2 volumes of ethanol to the final aqueous phase, and the samples were then placed at -20°C for 15 to 18 h. The precipitated nucleic acids were collected by centrifugation, and the precipitates were dis-

solved in 200 μl of 10 mM Tris-hydrochloride buffer (pH 7.6) containing 10 mM MgCl_2 . Pancreatic DNase (RNase free; Worthington Diagnostics, Freehold, N.J.) (50 $\mu\text{g}/\text{ml}$) was added to the solutions, and the samples were incubated at 37°C for 30 min. The samples were then deproteinized and reprecipitated as described above and stored under ethanol at -20°C until needed.

Isolation of polyadenylated RNA. In vitro-synthesized RNA, purified as described above, was dissolved in binding buffer (0.12 M NaCl, 0.01 M Tris-hydrochloride buffer [pH 7.6], 0.1% SDS). Samples were applied to a column of polyuridylic acid-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) (0.6 by 2 cm) equilibrated with binding buffer, at room temperature. After the sample was applied, flow from the column was stopped for 5 min to allow binding of the polyadenylated RNA, and the column was washed with 10 ml of binding buffer. Polyadenylated RNA was displaced from the column with elution buffer (0.01 M Tris-hydrochloride buffer [pH 7.6], 0.01% SDS in 90% formamide). We added 50 μg of *Escherichia coli* tRNA per ml and 2.5 volumes of ethanol to the eluates, and the samples were placed at -20°C . For estimation of the amount of polyadenylated RNA present, that percentage of radiolabeled RNA recovered after two cycles of chromatography on polyuridylic acid-Sepharose 4B columns was calculated.

Assay of nucleotide phosphohydrolase. Assay mixtures for determination of nucleotide phosphohydrolase contained 100 mM Tris-hydrochloride buffer (pH 8.0), 3.3 mM MgCl_2 , 50 mM βME , 2 mM [$\gamma\text{-}^{32}\text{P}$]ATP (specific activity, 10 mCi/mmol), and 50 μg of viral protein (as purified MNV) in a final volume of 0.1 ml. Reaction mixtures were incubated at 37°C , and at appropriate intervals, 10- μl samples were removed and mixed with 2 μl of 0.25 M EDTA to terminate the enzyme reaction.

Samples were analyzed by thin-layer chromatography on plastic-backed, polyethyleneimine-cellulose thin-layer plates (Brinkmann Instruments, Inc., Westbury, N.Y.) developed in 0.85 M potassium phosphate buffer, pH 3.5 (14, 66). Nucleotides present in the experimental samples as well as in appropriate standards analyzed on the same plate were localized under a UV light source, the plate was dried, and autoradiograms were prepared (13) to define and locate the labeled species formed during the enzymatic reaction. Areas corresponding to the labeled species were cut out of the chromatogram and counted under a toluene-based scintillant.

Protein kinase assay. The reaction mixture for determination of protein kinase contained 50 mM Tris-hydrochloride buffer (pH 9.6), 3 mM MgCl_2 , 5 mM dithiothreitol, 0.02% NP-40, 1 mM [$\gamma\text{-}^{32}\text{P}$]ATP (specific activity, 120 mCi/mmol), and 100 μg of viral protein (as purified MNV) in a final volume of 0.5 ml. The final pH of the reaction mixture was 8.6. Reaction mixtures were incubated at 37°C , and at appropriate intervals, samples were removed and the incorporation of ^{32}P into hot and cold TCA-insoluble material was determined as described previously (58).

SDS-PAGE analysis of viral proteins. Protein kinase assay reactions were terminated by addition of 0.1 M EDTA, and proteins were precipitated by addition of an equal volume of ice-cold 10% TCA. The TCA precipitates were collected by centrifugation, washed

with cold acetone (2 times), and prepared for SDS-PAGE analysis as described previously (70).

Determination of protein concentration. The method of Lowry et al. (33), using crystalline bovine serum albumin as a standard, was followed for determining protein concentrations in experimental samples.

DNA isolation and purification. DNA from virions or cells was purified as follows. Virus (1 mg of viral protein as purified MNV) or the nuclear fraction prepared from 1×10^7 to 2×10^7 cells was suspended in lysis buffer (Tris-hydrochloride buffer [pH 8.0; 50 mM], EDTA [10 mM], Sarkosyl NL-97 [1.0%], β ME [50 mM] and proteinase K (500 μ g/ml). The mixture was incubated at 4°C for 30 min and then at 37°C for 4 to 6 h. NaCl (final concentration, 1 M) and sucrose (27% [wt/vol]) were added to the mixture, and the mixture was then deproteinized by extraction (2 times) with phenol (saturated with 0.1 M Tris-hydrochloride buffer, pH 8.0) followed by extraction (2 times) with chloroform-isoamyl alcohol (24:1). The final aqueous phase was dialyzed against sterile buffer (10 mM Tris-hydrochloride buffer [pH 7.6], 1 mM EDTA) and stored at 4°C. The 260:280 ratio of DNA solutions prepared in this manner was always >1.9 .

DNA-RNA hybridization. Viral or cellular DNA (about 30 μ g/ml) was denatured and fragmented by boiling for 15 min in 0.3 N NaOH (pH >12.5). After neutralization with HCl, the solution was diluted fivefold with $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), and appropriate amounts of the fragmented, denatured DNA were immobilized on nitrocellulose membranes (2.4 cm, type B-6; Schleicher & Schuell Co., Keene, N.H.). Filters containing various amounts of immobilized DNA were incubated at 65°C in solutions containing radiolabeled RNA dissolved in $6 \times$ SSC containing 0.1% SDS, and the filters were processed, incubated with pancreatic RNase (20 μ g/ml in $2 \times$ SSC), washed, dried, and assayed for bound radioactivity by liquid scintillation spectrometry as described previously (29, 49, 50).

Cleavage of viral and host cell DNA with restriction endonucleases, separation of the DNA fragments by

electrophoresis in agarose gels, transfer and immobilization of the separated fragments on cellulose acetate strips, and conditions for RNA hybridization as well as autoradiographic methods for detecting hybrids have been described previously (9, 13, 28, 71).

Sources of radioisotopes and reagents. Radioisotopes were purchased from New England Nuclear Corp., Boston, Mass., and Amersham Corp., Arlington Heights, Ill. Nucleoside triphosphates were purchased from Sigma Chemical Co., St. Louis, Mo., and Sarkosyl NL-97 was obtained from Chemical Additives Co., New York, N.Y. NP-40 was obtained from BDH Chemicals Ltd., Poole, England.

RESULTS

In vitro degradation of MNV. Incubation of purified milker's nodule (MN) virions with NP-40 and β ME under the conditions described by Easterbrook (17, 73) for the in vitro degradation of vaccinia virions resulted in the release of about 40 to 50% of the MN virion-associated proteins, generating "core-like" structures (73). This procedure also "unmasked" or activated a number of enzymatic activities which were apparently localized in, or associated with, the MNV cores. When MNV was incubated in the appropriate enzymatic reaction mixtures in the absence of nonionic detergents and β ME, no enzymatic activities (with the exception of the RNase activity described below) could be measured. In our hands, under the conditions described, the MNV cores (as revealed by electron microscopic analysis) were less stable than cores prepared from vaccinia virions. In most experiments, the MNV cores were generated directly in the reaction mixtures to avoid any manipulations which might further compromise their structure. In some MNV preparations, an RNase activity (apparently associated with the

FIG. 1. Kinetics of in vitro RNA synthesis with purified MNV. In vitro RNA polymerase assays were prepared with the standard in vitro reaction mixture described in the text. (A) Time course of in vitro synthesis with purified MNV. At the indicated times, samples of the reaction mixture were withdrawn and processed for measurement of incorporation of $[^3\text{H}]\text{UMP}$ into TCA-precipitable material as described in the text. (B) In vitro RNA synthesis reaction rate as a function of virus protein concentration. In vitro RNA polymerase assays were incubated for 20 min, terminated, and then processed as described in the text. (C) Comparison of in vitro RNA synthesis by enzymes present in equal amounts (as viral protein) of MNV and vaccinia virus. In vitro RNA synthesis assays were performed as described in the text. Symbols: $\circ$, MNV; $\bullet$, vaccinia virus.

surface of the virus particles) could be measured before the virus particles were disrupted. This RNase activity was not associated with all purified virus preparations, and we therefore assume that it represents a contaminant adsorbed to the surface of virus which was not removed during virus purification. A similar contamination of vaccinia virions has been reported (24). To remove the RNase from the surface of virus particles, MN virions were pretreated with NP-40 (0.5%, 30 min, 4°C), and the particles were collected by centrifugation and then added to the *in vitro* RNA synthesizing system (see Fig. 3).

***In vitro* RNA synthesis catalyzed by MNV-associated RNA polymerase.** Under the standard *in vitro* assay conditions described above, the rate of incorporation of labeled precursors into TCA-precipitable material was linear for at least 30 min (Fig. 1A) and was determined to be proportional to the concentration of protein added to the assay mixture (in the form of purified MNV) over the range from 35 to 200 µg of added protein (Fig. 1B). When equivalent amounts of virus (in terms of viral protein added) were compared under identical *in vitro* assay conditions, the level of RNA polymerase activity measured with MNV was always significantly lower than that measured with vaccinia virus (Fig. 1C).

All four nucleoside triphosphates were required for maximum RNA synthesis in the *in vitro* assay system (Table 1), although an appreciable amount of [³H]UMP was incorporated into acid-insoluble material when either CTP or GTP was omitted from the reaction mixtures. The radiolabeled, acid-precipitable reaction product synthesized *in vitro* by MNV-associated RNA polymerase was rendered acid soluble by digestion with pancreatic RNase or by alkaline hydrolysis, indicating that the product was in fact RNA (Table 1).

The optimal pH for *in vitro* MNV RNA polymerase activity was determined to be 8.0 (Fig. 2A). There was an absolute requirement for a divalent cation which could be satisfied by the addition of either Mg²⁺ or Mn²⁺ ions. When these ions were employed individually, the optimal concentrations of Mn²⁺ and Mg²⁺ were found to be about 2 and 6 mM, respectively (Fig. 2B). The optimal concentration of ATP was determined to be 5 mM (Fig. 2C). When ATP was omitted from the *in vitro* reaction mixtures, no RNA synthesis could be measured (Table 1); when ATP was used at the same concentration as CTP and GTP (1 mM), synthetic activity was minimal (Fig. 2C). Supraoptimal concentrations of ATP also resulted in a diminished rate of *in vitro* RNA synthesis (Fig. 2C). This may be attributed to the chelation of essential cations by excess ATP.

TABLE 1. Characteristics of MNV *in vitro* RNA polymerase activity

Treatment	Enzyme activity (pmol/liter per µg of viral protein)
Standard reaction	3.0
-ATP	0.125
-CTP	0.96
-GTP	0.69
+RNase ^a	0.306
+0.5 N NaOH ^b	0.096

^a After incubation under standard conditions, the reaction mixture was heated at 100°C for 2 min and then treated with 100 µg of pancreatic RNase per ml for 1 h at 37°C.

^b After incubation under standard conditions, the sample was treated with 0.5 N NaOH at 37°C for 2 h.

Partial characterization of transcripts synthesized *in vitro*. Sedimentation analyses of the RNA product synthesized *in vitro* by MNV-associated RNA polymerase showed that the RNA molecules were broadly distributed in the gradients from 6S to 18S with the bulk of the RNA sedimenting at 12S to 14S.

When some MNV preparations were used as the source of enzymes for *in vitro* RNA synthesis, the majority of the RNA species synthesized, when sized by centrifugation into sucrose gradients, sedimented at 4S to 6S rather than at 12S to 14S (Fig. 3A). These MNV preparations could be shown to contain an RNase activity which cleaved vaccinia mRNA (Fig. 3B). Macaloid and the RNase inhibitor prepared from human placentas (8) did not completely block this RNase activity (data not shown). It had been reported that preparations of purified vaccinia virions contain an RNase activity adsorbed to the surface of virus particles which is removed when the virions are converted to cores (24). We therefore treated MNV with NP-40, collected the detergent-treated particles by centrifugation, and then used them as the source of enzymes for *in vitro* RNA synthesis. Treatment of MNV with detergent alone, in this manner, did not activate the enzymes contained within the virus particles; it was necessary to include nonionic detergent and βME in the *in vitro* enzyme reaction mixtures to measure any enzymatic activities present in the virus. When detergent-treated virions were employed as the source of enzymes for *in vitro* RNA synthesis, the RNA molecules synthesized sedimented at 12S to 14S, and there was no evidence for cleavage of these transcripts to smaller products (Fig. 3C). The sedimentation behavior of heat-

FIG. 2. Optimal pH, divalent cation concentration, and ATP concentration in the in vitro RNA synthesis reactions. The standard in vitro reaction mixture containing MNV was used except for the indicated variations. After 20 min, in vitro reactions were terminated and processed as described in the text. (A) pH. (B) Mg^{2+} (●) and Mn^{2+} (○) concentrations. (C) ATP concentration.

denatured, in vitro-synthesized RNA was identical to that of the RNA molecules displayed in Fig. 3C. We concluded that the enzymes present in MNV catalyzed the synthesis of 12S to 14S RNA. The transcripts were similar in size to those reported to be synthesized by the enzymes present in vaccinia virions (42). The RNase activity associated with some MNV preparations that is responsible for the observed degradation of RNA synthesized in vitro was localized primarily on the surface of MNV particles. These experiments do not exclude the possibility that additional RNases may be present in the

MNV cores and are involved in the processing of primary transcripts, as has been described previously for vaccinia virus (51, 52, 55).

The RNA molecules synthesized in vitro by the DNA-dependent RNA polymerase associated with MNV cores hybridized with MNV DNA but not with DNA prepared from MDBK cells or from vaccinia virus, strain WR (Fig. 4). When RNA transcribed in vitro with a specific activity of $1.5 \times 10^5 \text{ cpm}/\mu\text{g}$ was hybridized to MNV DNA immobilized on cellulose acetate filters (0.26 μg of DNA per filter), saturation was reached when 1×10^3 to $1.2 \times 10^3 \text{ cpm}$ were

FIG. 3. Detection of an MNV-associated RNase activity by sedimentation analysis of RNA synthesized in vitro by virion-associated RNA polymerase. In vitro reaction mixtures, each containing 100 μg of virus protein, were incubated for 30 min, when the reaction was terminated and RNA was prepared for sucrose gradient analysis as described in the text. The labeled arrows indicate the positions of HeLa cell rRNA and tRNA markers analyzed in a parallel gradient. Sedimentation was from right to left. (A) Sedimentation behavior of in vitro-synthesized RNA when purified MNV with no NP-40 pretreatment was used as a source of enzymes. (B) ●, Sedimentation behavior of RNA synthesized in vitro with purified vaccinia virus as a source of enzymes; ○, labeled, in vitro-synthesized vaccinia RNA incubated with purified MNV which was not pretreated with NP-40. (C) Sedimentation behavior of in vitro-synthesized RNA when MNV pretreated with NP-40 was used as a source of enzymes.

annealed. We estimated that under the conditions described, 6.8 to 7.4% of the sequences present in MNV DNA were transcribed in vitro (assuming that asymmetrical transcription of the genome was occurring).

When the in vitro transcripts were purified and analyzed by chromatography on polyuridylic acid-Sepharose columns as described above, approximately 25 to 30% of the purified in vitro reaction products were bound to the column (two cycles of chromatography), indicating that the product was polyadenylated. The results suggest that MNV, like vaccinia virions (11, 43,

44), may also contain a polyribadenylate polymerase. The nature of this enzyme is presently under investigation.

Nucleotide phosphohydrolase activity in MNV. Since the in vitro transcription system with disrupted MNV was shown to have a requirement for a concentration of ATP which significantly exceeded the required concentration of the other three nucleotide precursors, we searched for additional virion-associated enzyme activities which would utilize ATP. Several such enzymatic activities have been identified and purified from vaccinia virions, including nucleotide phosphohydrolase(s) (23, 46, 57, 59, 60), protein kinase (16, 58), and polyribadenylate polymerase (11, 43, 44). Preliminary evidence for the presence of polyribadenylate polymerase activity in MNV has been presented above.

Purified MN virions were tested to determine if they contained an enzyme which was capable of hydrolyzing ATP as described above. The results of such experiments (Fig. 5) indicated that the MN virions contained nucleotide phosphohydrolase activity; maximum activity was measured by using reaction conditions similar to those described for assay of the enzyme present in vaccinia virions (57). When equivalent amounts (in terms of protein) of vaccinia virions or MN virions were utilized in the in vitro assay mixtures, the levels of nucleotide phosphohydrolase activity which could be measured were almost identical (Fig. 5). The ATP-hydrolyzing activity present in MNV was independent of in vitro RNA synthesis activity, as indicated by the active hydrolysis of ATP in the absence of other nucleoside triphosphates.

Protein kinase activity in MN virions was assayed by a modification of the method described by Paoletti and Moss (58). MN virions contained an enzyme which catalyzed the transfer of γ - ^{32}P from [γ - ^{32}P]ATP into hot, TCA-insoluble material (Fig. 6). The protein kinase activity measured in MN virions had the same requirements and was as active as the enzymatic activity measured in vitro with an equivalent amount of vaccinia virus (in terms of viral protein added) as a source of enzymes (Fig. 6). The ^{32}P -labeled products of the in vitro protein kinase assay were analyzed by SDS-PAGE as described above. Of the approximately 40 polypeptides which can be resolved when purified MNV is solubilized and the solubilized proteins are analyzed by electrophoresis in 10% SDS-polyacrylamide gels (70, 73), approximately 10 were determined to be significantly labeled with ^{32}P (Fig. 7). The major phosphorylated polypeptides (as determined by densitometric scans of autoradiograms) had masses of 95, 60, 33.5, 15, and 13.8 kilodaltons.

FIG. 4. Hybridization of MNV RNA synthesized in vitro to immobilized DNA restriction fragments. MNV, vaccinia virus, and MDBK cellular DNA were cleaved with *Hind*III and fractionated by agarose gel electrophoresis as described in the text. DNA fragments were transferred to nitrocellulose strips and hybridized to ^{32}P -labeled RNA (38). After washing and RNase treatment, the strips were autoradiographed. (I) Lane A, *Hind*III restriction fragments of vaccinia virus DNA; lane B, MNV DNA; and lane C, MDBK DNA; visualized by ethidium bromide staining. (II) Lane A', Autoradiograph of MNV ^{32}P -labeled RNA hybridized to vaccinia virus DNA; lane B', MNV DNA; and lane C', MDBK DNA. Numbers on left show the molecular weights.

FIG. 5. Nucleotide phosphohydrolase activity in MNV and vaccinia virus. In vitro nucleotide phosphohydrolase reaction mixtures, each with 50 μ g of virus as protein, were made as described in the text. At the indicated times, samples were removed and analyzed by polyethyleneimine-cellulose thin-layer chromatography as described in the text. Symbols: $\circ$, MNV; $\bullet$, vaccinia virus.

DISCUSSION

We have been able to demonstrate that purified MN virions contain a number of enzymatic activities. As has been shown for other members of the Poxviridae which have been examined in this regard, it was necessary to partially disrupt the MN virions to activate or unmask the enzymes encapsulated within the virus particles. This in vitro degradation may mimic the uncoating of virus particles which occurs after they infect an appropriate host cell. As has been proposed for the orthopoxviruses (41, 42), the enzymes present in parapoxviruses, as exemplified by MNV, could function to transcribe the parapoxvirus genome in an orderly fashion during the early stages of infection. Twelve or more enzymatic activities have now been detected in purified orthopoxvirus preparations (1, 4, 55; reviewed in reference 42). It is likely that a similar spectrum of enzymes are encapsulated in parapoxvirions. We plan to search for additional enzymatic activities in the context of studies in which the replication of parapoxviruses will be examined and compared with the replication of orthopoxviruses.

In an attempt to define conditions which would release cores from MNV in a manner analogous to that described for vaccinia virus (40), a variety of experimental conditions were

tested, and such variables as temperature, pH, and concentration of detergents (NP-40 and Triton X-100 were used), as well as various concentrations of β ME, were evaluated. Although our electron microscopic observations showed that core-like structures were released from MN virions under the conditions employed, we have also observed that these subviral particles were unstable (as compared with vaccinia virus cores), and further disruption beyond a core stage occurred (73). The activity of the MNV DNA-dependent RNA polymerase, which was less than the RNA polymerase activity measured with equivalent amounts of vaccinia virus in vitro, may have been limited by the relative instability of MNV cores in vitro. Additional experiments are needed to determine if there are conditions under which stable MNV cores can be prepared. It may be that the internal structure of parapoxvirions differs from that of the orthopoxvirions in a manner which precludes the preparation of subviral particles from MNV with the stability of orthopoxvirus cores.

The MNV-associated, DNA-dependent RNA polymerase activity described here shares several properties with the RNA polymerase activity found to be encapsulated into orthopoxviruses.

FIG. 6. Kinetics of protein kinase activity in MNV and vaccinia virus. In vitro protein kinase assays with 100 μ g of virus as protein were performed as described in the text. At the specified times, samples were removed and processed for analysis of TCA-precipitable radioactivity (58). Symbols: $\bullet$, MNV; Δ , vaccinia virus; $\circ$, no virus.

FIG. 7. SDS-PAGE analysis of in vitro-phosphorylated proteins of MNV. In vitro protein kinase reaction mixtures were incubated as described in the text. After incubation, samples from each mixture were precipitated with TCA, washed with cold acetone, and prepared for SDS-PAGE analysis (70). Lane A, Coomassie blue-stained MNV viral polypeptides recovered from the in vitro reaction mixtures; lane B, autoradiogram revealing ^{32}P -labeled, phosphorylated viral polypeptides. The molecular weights of selected MNV polypeptides ($\times 10^{-3}$) are indicated.

In addition to the requirement for the partial disruption of the virion for activating the enzyme, similar requirements for divalent cations and the four nucleoside triphosphates were defined. The excessive requirement for ATP (relative to the other nucleoside triphosphates) parallels the situation revealed when the RNA polymerase activity present in vaccinia and rabbit poxviruses was first analyzed (31, 45). Since these original observations, it has been demonstrated that a number of enzymes present in orthopoxvirions utilize ATP as a substrate (reviewed in references 42 and 56). We detected two and possibly three enzymes in purified MN virions in addition to RNA polymerase which

utilized ATP as a substrate. Experimental evidence was presented that a protein kinase and a nucleoside triphosphate phosphohydrolase are present in purified MNV. In addition, we could show that the transcripts synthesized in vitro by the MNV RNA polymerase were polyadenylated. By analogy to the results obtained with orthopoxviruses (11, 30, 44, 49), MNV may therefore contain a polynucleotide adenylyltransferase. Definitive experiments addressing this question must still be performed. A variety of functions for the protein kinase (42, 45) and nucleoside triphosphate phosphohydrolase (42, 53) during transcription or assembly of orthopoxviruses have been proposed, but experiments to test these hypothetical functions have not been performed. The role of the enzymes described here during MNV replication remains to be explored.

The average length of the transcripts synthesized by the MNV-associated RNA polymerase was determined by sedimentation in sucrose gradients to be approximately 1,000 to 1,500 nucleotides (8S to 12S). The in vitro transcripts hybridized to the restriction endonuclease fragments of MNV DNA immobilized on nitrocellulose strips, but no hybridization to MDBK cellular DNA or vaccinia virus DNA could be detected. Molecular hybridization experiments revealed that sequences corresponding to 7 to 8% of the MNV genome were transcribed in vitro. Kates and Beeson (29) and Nevins and Joklik (49) estimated that in vitro-synthesized vaccinia virus transcripts hybridize to 7 and 25% of vaccinia virus DNA immobilized on filters, respectively. Paoletti and Grady (54) and Boone and Moss (10), using DNA-RNA hybridization techniques in solution in which RNA was in excess, determined saturation values which ranged from 40 to 50% for in vitro-synthesized vaccinia RNA. The reasons for the disparity in the published saturation values have been discussed (10, 54), but the matter remains to be completely resolved. We were cognizant of these considerations when we undertook our hybridization studies. We therefore also hybridized ^{32}P -labeled, fragmented (8.6S in alkaline sucrose) MNV DNA (1.2×10^5 cpm/ μg of DNA) to excess cold, in vitro-synthesized MNV RNA and measured the percentage of labeled DNA that formed hybrids after digestion of the samples with *Aspergillus oryzae* nuclease S1 (510 U/ μg) (10). Using this approach, we were able to estimate that 8.2 to 9.6% of the DNA was transcribed in vitro; these values were in general agreement with those measured when RNA was hybridized to DNA immobilized on filters. As noted above, the reliability of these estimates may be dependent on the manner in which the enzyme encapsulated in MNV is activated and

on the relative stability of the transcription complexes as represented by MNV cores in vitro.

The presence of encapsulated enzymes in parapoxviruses is a feature they share with orthopoxviruses and may be common to all the viruses classified within the Poxviridae family. Similarly, parapoxviruses (39, 76), like orthopoxviruses (7, 22), avipoxviruses (32), and *Molluscum contagiosum* (61), contain DNA genomes with cross-linked termini. These shared characteristics could be applied in the classification of new virus isolates as poxviruses. However, African swine fever virus, an iridovirus, also shares these properties (37, 68). These features therefore are not unique to a particular family of viruses but may be common to all DNA viruses which replicate in the cytoplasm of their host cell. Morphology and shared antigenic determinants (19, 37, 78) remain the most important criteria for classification of DNA viruses as Poxviridae.

Hruby et al. (26) have reported that MDBK cells are nonpermissive for vaccinia virus growth. Vaccinia virus, strain WR, was able to infect MDBK cells, early viral proteins were synthesized, and viral DNA synthesis was completed, but no progeny were assembled (26). The MDBK cell line is permissive for MNV. Comparative studies utilizing MNV- and vaccinia virus-infected MDBK cells may provide information concerning the manner in which vaccinia virus replication is aborted and may provide insights into the manner in which the host-range specificity of poxviruses is maintained in nature.

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