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Polyomavirus Major Capsid Protein VP1 Is Capable of Packaging Cellular DNA When Expressed in the Baculovirus System†

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Using the p2Bac dual multiple cloning site transfer vector, the polyomavirus major capsid protein gene VP1 was cloned for expression in the baculovirus-insect cell expression system. The 5-day-infected cellular lysate from this recombinant preparation was purified by cesium chloride density gradient centrifugation. Capsid-like particles were observed in the resulting preparation. The purified particle preparation was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was shown to have accurately expressed the polyomavirus VP1 protein as cloned. It was found that the preparation revealed the presence of host histones in the stained gels, which is indicative of DNA packaging. To determine if cellular DNA was being packaged in the particles, Sf9 insect cells were prelabeled with [^{3}H]thymidine. The label was removed, and the cells were subsequently infected with a recombinant *Autographa californica* multiple nuclear polyhedrosis virus (Ac*M*NPV) carrying the polyomavirus VP1 gene. Upon purification through three cesium chloride gradients and DNase I treatment, capsid-like particles, containing [^{3}H]thymidine-labeled DNA, were isolated which were found to coincide with hemagglutination activity. Studies have indicated that the Ac*M*NPV appears to have the ability to fragment Sf9 cellular DNA. When infected with the recombinant Ac*M*NPV carrying the VP1 gene of polyomavirus, these host DNA fragments are being packaged by the VP1 major capsid protein; further, these DNA fragments have been shown to be approximately 5 kb in size, which corresponds to the size of the native polyomavirus genome. These studies demonstrate that the recombinant polyomavirus VP1 protein has the ability to package DNA in the absence of the minor structural proteins VP2 and VP3 and independently of the polyomavirus T antigens.

The murine polyomavirus is a member of the papovavirus group, which also includes the papillomaviruses as well as simian virus 40 (SV40). The genome of polyomavirus is composed of 5.3 kb of closed circular supercoiled DNA which encodes 6 proteins. Due to the fact that this relatively small genome can encode 6 proteins, polyomavirus has often been studied as a model for transcription, translation, and DNA replication (30). Three of the proteins produced in an active polyomavirus infection are the early T antigens. The T-antigen group includes small T, middle T, and large T, all of which appear to function in the induction and maintenance of the tumorigenic state as well as playing an essential role in the lytic cycle of polyomavirus infection in susceptible host cells. The genome of polyomavirus also encodes three late proteins, which function as the structural proteins of polyomavirus and are vital components of the capsid in native, wild-type polyomavirus virions. The three structural proteins are VP1, which is the major capsid protein and has a molecular mass of 45 kDa, and VP2 and VP3, which are the minor capsid proteins and have molecular masses of 35 and 25 kDa, respectively. In the native, wild-type polyomavirus virion, VP1 comprises approximately 80% of the protein found in the capsid of the virion, and VP2 and VP3 each make up about 10% of the capsid protein. The capsid of polyomavirus has been shown to be composed of 72 capsomere (pentamers) subunits which are arranged in an icosahedral lattice with a diameter of approximately 45 to 50 nm (3, 27, 30).

It has been previously demonstrated that bacterially expressed recombinant VP1 protein molecules will assemble into empty capsid-like structures in the presence of calcium, with a size and morphology similar to that found in the native polyomavirus capsids. When purified and resolved on stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, these structures show that only VP1 protein is present (15, 28). For many years our laboratory has been involved with investigations into the nature of the polyomavirus capsid structure and the roles played by each of the structural proteins in various aspects of capsid structure and function, such as expression, transport, and interactions with each other, as well as in assembly and maturation of the final progeny virions. In earlier studies, the bacterial expression systems have proven to be an invaluable resource for the production and purification of large quantities of all of the various structural proteins of polyomavirus (5–9, 15, 25). There is a feature of bacterially expressed proteins which must be considered when comparing their behaviors to those of native proteins, this feature being that they may lack many of the sophisticated posttranslational modifications associated with proteins produced in eukaryotic cells, many of which have been shown to occur in the native polyomavirus structural protein components (reference 4 and references therein).

Recently, the eukaryotic baculovirus-insect system has been utilized to express and produce important biological proteins. This system has been used to study structural proteins in a variety of other viral systems such as herpesvirus (reference 31 and references therein), hantavirus, poliovirus, rotavirus, mea-

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sles virus, rabbit hemorrhagic disease virus, human immunodeficiency virus, feline immunodeficiency virus, bovine immunodeficiency virus, Broadhaven virus, human papillomavirus (reference 2 and references therein), and SV-40 (19) as well as the plant viruses beet western yellows luteovirus (29) and potato leafroll luteovirus (20). The recombinant baculovirus system has also been successfully used to probe essential questions dealing with the polyomavirus life cycle, such as the expression of structural proteins and their cellular localization and transport to the nucleus (10, 11, 24), and to study protein-protein interactions involving the structural proteins (10, 11), the posttranslational modifications of the structural proteins, and the essential nature of calcium ions in VP1 capsid assembly (11, 21) as well as the packaging of DNA (26).

The eukaryotic baculovirus expression system also has features which are advantageous for our studies, namely the capacity to produce large quantities of polyomavirus capsid proteins for experimentation. While producing various polyomavirus capsid proteins, it was observed that the recombinant VP1 capsid-like particles, purified from 5-day-infected *Spodoptera frugiperda* (Sf9) cells, showed the presence of histones when visualized on SDS-PAGE gels. This observation was indicative of DNA packaging. Analysis of Sf9 insect cells that were prelabeled with [^{3}H]thymidine and then infected with the Ac*M*NPV-VP1 recombinant revealed packaging of host DNA in the VP1 capsid-like particles.

MATERIALS AND METHODS

Polyomavirus structural gene constructs. The VP1 gene was obtained by PCR as previously described by Chang et al. (6). The VP1 coding sequence was inserted into the p2Bac (Invitrogen, San Diego, Calif.) dual multiple cloning site transfer vector at the *Bgl*II restriction site. The transfer vector and linear *Autographa californica* multiple nuclear polyhedrosis virus Ac*M*NPV DNA were then used to cotransfect Sf9 cells. Recombinant Ac*M*NPVs were selected by plaque purification on Sf9 cells as described in the Invitrogen company protocols manual. The stock recombinant virus was then produced in Sf9 cells by using Grace's insect medium supplemented with lactalbumin hydrolysate, yeastolate, and 2% fetal calf serum (FCS) (Gibco, Grand Island, N.Y.) with amphotericin B and gentamicin sulfate.

Capsid-like particle purification. Sf9 cells were grown in 150-cm^2 flasks (Corning, Corning, N.Y.) containing Grace's medium with 5% FCS and antibiotics. Confluent cell cultures were then infected with recombinant Ac*M*NPV containing the polyomavirus structural gene VP1 (multiplicity of infection, approximately 10 PFU/cell). Virus adsorption was allowed for 1 h at room temperature, and then cultures were maintained in Grace's medium with 2% FCS at 27°C. Five days after infection the cells were harvested by low-speed centrifugation and were suspended in Buffer A (10 mM Tris, 50 mM NaCl, 0.01 mM $CaCl_2$, 0.01% Triton X-100); this was followed by three cycles of sonication at 45 s each. Protease inhibitors (aprotinin, leupeptin, phenylmethylsulfonyl fluoride, *N-p*-tosyl-L-lysine chloromethylketone, and L-1 tosylamide 2 phenylmethylchloromethylketone, all at 10 μg/ml [Sigma, St. Louis, Mo.]) and 200 U of receptor-destroying enzyme (BioWhittaker, Walkersville, Md.) were added to the lysate and incubated at 37°C for 30 min. The lysate was then centrifuged at 10,000 rpm for 30 min in a Sorvall HB-4 rotor, with the supernatant being saved. The pellet was reextracted in Buffer A and sonicated for three cycles of 30 s each. An equal volume of Freon was then added, and the mixture was shaken for 5 min; this was followed by centrifugation at 10,000 rpm for 30 min in the HB-4 rotor. The supernatant was saved and pooled with the original supernatant. The capsid-like particles were concentrated through 2 ml of a 20% sucrose shelf (in Buffer A) and centrifuged at 35,000 rpm for 105 min in a Beckman SW 41 rotor. The pellet containing the partially purified capsid-like particles was resuspended in a minimal amount of Buffer A and sonicated for 15 s to disrupt aggregates. Aliquots of this suspension were layered on top of a preset cesium chloride (CsCl) gradient (1.35, 1.32, 1.29, 1.26, and 1.23 g/ml) and centrifuged at 33,000 rpm for 15 h in a Beckman SW 50.1 rotor, and then fractions were collected. The isolated bottom band from the CsCl gradient was also treated with DNase I (Worthington Biochemical Corporation, Freehold, N.J.). The reaction mixture contained the capsid-like particles suspended in Buffer A, DNase I (final concentration per reaction mixture, 200 U/ml), and $MgCl_2$ (final concentration per reaction mixture, 1 mM). The reaction was allowed to proceed for 45 min at room temperature, and then the mixture was loaded on top of a preset CsCl gradient as described above. The activity of the DNase I enzyme was confirmed by allowing it to react with a target plasmid, pΔFlag, which is also approximately 5 kb in size, under the same conditions as those used to treat the recombinant capsid-like particles. Agarose gels of the resultant reaction mixture revealed that the target plasmid was completely degraded into fragments of less than 100 bp in size (data not shown).

To determine if Sf9 cellular DNA was being packaged by the recombinant VP1 protein, insect cells were prelabeled with [^{3}H]thymidine ([^{3}H]TdR). Sf9 cells were seeded in 150-cm^2 flasks containing Grace's complete medium with 2% FCS and 15 μCi of [^{3}H]TdR (American Radiolabeled Chemicals, St. Louis, Mo. [specific activity of 71.5 Ci/mmol]) per ml and maintained at 27°C. After 3 days, the labeled medium was removed and the unlabeled medium was replaced and incubated for an additional 3 h to reduce cellular pools of unincorporated [^{3}H]TdR. This medium was then discarded. Cells were then infected with the recombinant Ac*M*NPV carrying the polyomavirus VP1 gene; this was followed by the addition of fresh medium, and infection was allowed to proceed for 5 days. Capsid-like particles were then purified as described above.

Electrophoresis. (i) SDS-PAGE. The proteins of the CsCl-purified capsid-like particles were analyzed on a 12.5% acrylamide minigel (Serva, Atto, Japan) with a 0.33% *bis*-acrylamide cross-linker and visualized by Coomassie blue staining as previously described (16).

(ii) Immunoblotting. The proteins of the CsCl-purified capsid-like particles were resolved by using an SDS–12.5% PAGE gel, which was subsequently transferred to a nitrocellulose membrane (MSI, Westboro, Me.) by using a semi-dry system (PolyBlot from American Bionetics, Emeryville, Calif.) at a current setting of 2.5 mA/cm^2 of nitrocellulose. Membranes were then blocked in a solution of 3% dried milk powder in TBST (Tris-buffered saline [0.05 M Tris, 0.15 M NaCl], 0.05% Tween 20, pH 7.4) (Fisher, St. Louis, Mo.). Membranes were probed with an antibody cocktail consisting of rabbit antipolyomavirus polyclonal antibody (1:500) and rabbit anti-VP2/3 polyclonal antibody (1:500), which was raised against bacterially expressed recombinant polyomavirus VP2 (5). After subsequent washings in TBST, the membranes were probed with goat anti-rabbit antiserum conjugated to horseradish peroxidase (1:10,000) (Sigma Immunochemicals, St. Louis, Mo.). The bound secondary antibodies were visualized by developing the blot with an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, Ill.) in conjunction with exposure to BioMax X-ray film (Kodak, Rochester, N.Y.).

(iii) DNA analysis. The CsCl-purified recombinant capsid-like particles were analyzed to determine the size of the DNA within the capsid-like particles. The purified preparations were treated with proteinase K (final concentration, 200 μg), SDS (final concentration, 1%), and EDTA (final concentration, 0.025 M) and allowed to react at 37°C for 30 min. An equal volume of phenol-chloroform was then added and extracted for 5 min with gentle inversion. Phases were separated by centrifugation at 7,000 rpm for 5 min in a model 59A microcentrifuge (Fisher). DNA in the aqueous phase was precipitated by treatment with sodium acetate (0.3 M final) followed by the addition of three volumes of cold ethanol. Precipitation was allowed to proceed overnight at −20°C. DNA was pelleted by centrifugation at 10,000 rpm for 15 min at 4°C in a model 59A microcentrifuge. The DNA pellet was resuspended in a minimal amount of distilled water and resolved on a 0.8% agarose gel (in TBE [0.09 M Tris, 0.09 M boric acid, 0.05 M EDTA, pH 8.0]) as described previously (1).

Fragmentation of Sf9 cellular DNA. Sf9 cells were prelabeled for 3 days with [^{3}H]TdR in 150-cm^2 flasks as described above. These labeled cells were then distributed to 60-mm-diameter culture dishes and allowed to attach overnight in unlabeled Grace's medium with 2% FCS. The labeled Sf9 dishes were divided into three groups: one group remained uninfected, a second group was infected with wild-type Ac*M*NPV, and the third group was infected with the recombinant Ac*M*NPV carrying the polyomavirus VP1 gene. Viral adsorption was allowed to proceed for 1 h, and cells were then maintained in Grace's medium with 2% FCS. Appropriate cultures were analyzed at 24-h intervals over a 5-day period. Medium from the appropriate cultures was removed and subjected to low-speed centrifugation (2,000 rpm) to isolate the cells. The prelabeled cells from the appropriate cultures were then subjected to the Hirt extraction (17), which is used to isolate low-molecular-weight DNA. The previous low-speed-isolated medium was then centrifuged at 10,000 rpm to remove debris, and the supernatant was used to determine radioactivity in the medium. Radioactivity was determined by liquid scintillation counting.

Acid extraction of histones. Acid-soluble proteins were extracted from nuclei of Sf9 cells. Nuclei were isolated from uninfected Sf9 cells and extracted with 0.4 N sulfuric acid as previously described by Tweeten et al. (33). These isolated Sf9 cell histones were then used for comparison with commercial calf thymus histones (Mann Research Laboratories, New York, N.Y.), polyomavirus purified virions, and recombinant VP1 capsid-like particles by SDS-PAGE analysis.

Electron microscopy of recombinant capsid-like particles. CsCl-purified recombinant capsid-like particles were placed on a pioloform-coated grid, and the proteins were allowed to adsorb to the grid for 5 min. After a distilled water rinse, the sample was stained with a 1% aqueous uranyl acetate solution and examined with a Philips 201 electron microscope operating at 60 kV.

RESULTS

Using the p2Bac dual multiple cloning site transfer vector, the polyomavirus gene VP1 was cloned for expression in the baculovirus system. After 5 days of infection the cellular lysate from the recombinant preparation was purified by CsCl density

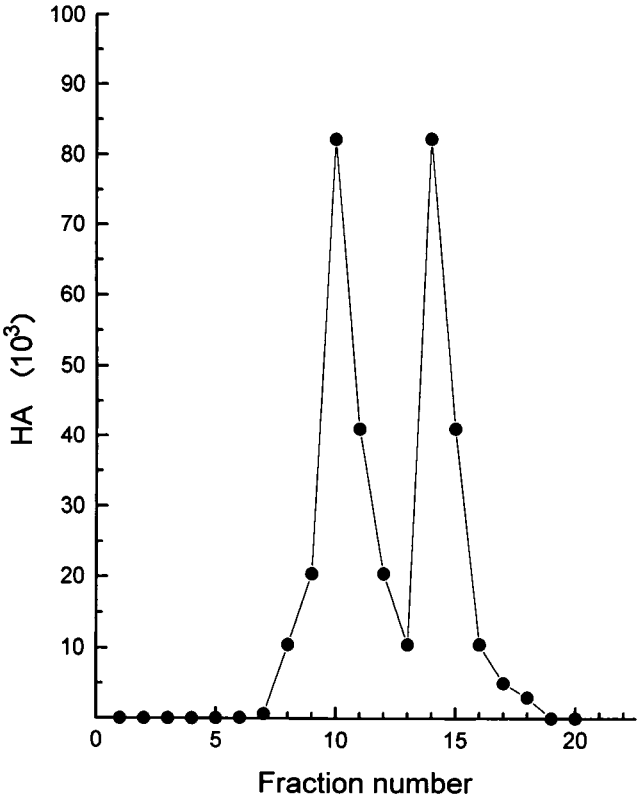

FIG. 1. CsCl purification of VP1 capsid-like particles from 5-day-infected cells. Sf9 cells were infected with Ac*M*NPV-VP1 and harvested 5 days after infection. Capsid-like particles were fractionated after CsCl density gradient centrifugation and assayed for hemagglutination activity.

gradient centrifugation. Fractionation of the gradient and analysis by hemagglutination assay revealed 2 peaks of activity (Fig. 1). Fifty percent of the hemagglutination activity was observed in the bottom band and approximately 50% was observed in the top band. Electron microscopy of the bottom and top bands revealed that the bottom band of particles appeared to be full virions and the top band appeared to consist of empty capsid-like particles (Fig. 2). The bottom band was recentrifuged in 2 additional CsCl gradients for further purification. Capsid-like particles were observed in electron micrographs of the preparation (Fig. 3B). These purified capsid-like preparations were found to have a buoyant density of 1.295 to 1.305 g/ml, as determined by refractometry. When the particles were measured they showed a diameter of 45 to 50 nm (Fig. 3B), which corresponds to that of native polyomavirus virions (Fig. 3A). Western blot analysis of this preparation revealed that the recombinant polyomavirus VP1 protein produced showed a molecular weight similar to that of the native polyomavirus VP1 (Fig. 4B, lane 3). SDS-PAGE analysis of these purified capsid-like particles revealed that the recombinant VP1 protein was produced as cloned (Fig. 4A, lane 3). It was also observed that histone proteins similar to the histones found in the native virions were present in the stained gel. The presence of histones was suggestive of possible DNA packaging in the particles. The most striking observation in this analysis was that histone-like proteins were present in the capsid-like particles composed only of recombinant VP1, in the absence of the minor capsid proteins VP2 and VP3, which are believed to play an essential role in the packaging of DNA in the native polyomavirus virions (14, 37). The histone-like proteins found in the recombinant capsid-like particles were then compared to a commercial preparation of calf thymus histones, histones isolated from uninfected Sf9 cells by acid extraction, and purified polyomavirus virions. These histone proteins were analyzed by SDS-PAGE (Fig. 5). It was found that the putative histone proteins in the recombinant capsid-like particles (lane 3) were similar to the Sf9 cell acid-extracted histones (lane 1), purified polyomavirus virion histones (lane 2), and commercially available calf thymus histones (lane 4).

To determine if cellular DNA was being packaged in the particles consisting of VP1 alone, Sf9 cells were prelabeled with [^{3}H]TdR. Cells were then infected with recombinant Ac*M*NPV carrying the polyomavirus VP1 gene, and infection was allowed to proceed for 5 days. The VP1 capsid-like particles were purified by two consecutive CsCl density gradient centrifugations, with the capsid-like particle band being collected and treated with DNase I to eliminate any residual cellular DNA which might have been adhering to the exterior of the capsid-like particles. The nuclease-treated particles were then purified on a third CsCl gradient and fractions were collected. As seen in Fig. 6, a peak of radioactivity was found to coincide with a peak of hemagglutination activity. A small peak of radioactivity is visible in the upper portion of the CsCl gradient, which indicates that some DNA was being degraded by the nuclease activity, which may have removed extraneous DNA from the exterior of the particles or could have entered the particles which were slightly distorted by the harsh condi-

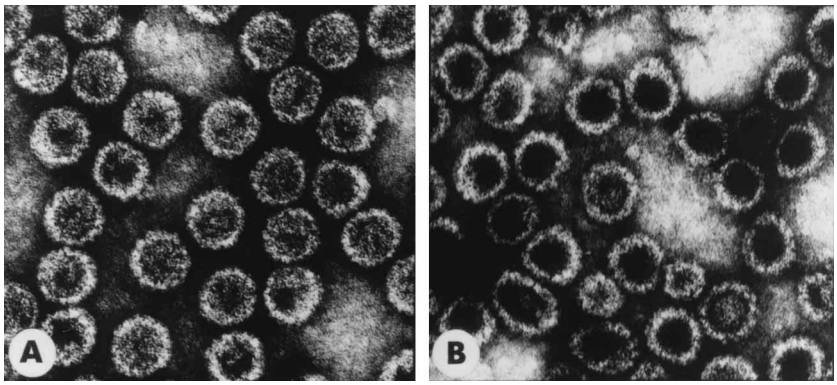

FIG. 2. Electron micrographs of VP1 capsid-like particles from the CsCl gradient purification shown in Fig. 1. (A) Fraction 10 from Fig. 1 showing full capsid-like particles. (B) Fraction 15 showing empty capsid-like particles.

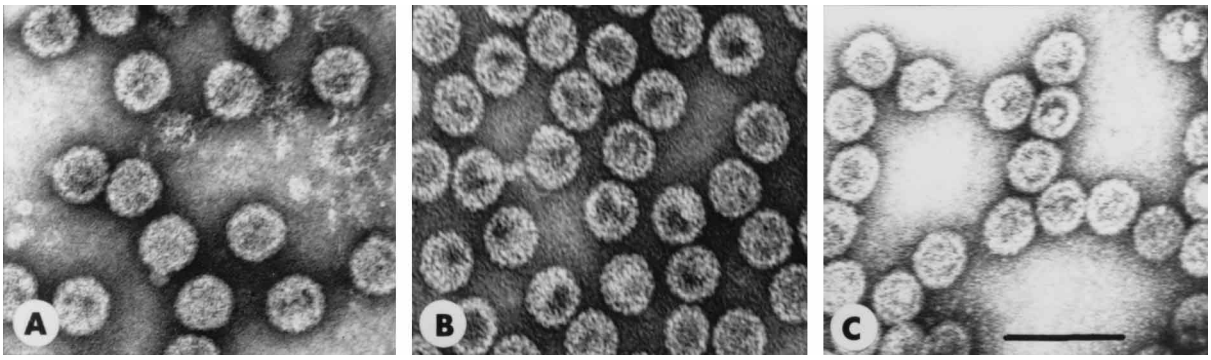

FIG. 3. Electron micrographs of native and recombinant capsid-like particles after three CsCl gradient centrifugations. (A) Native polyomavirus virions. (B) Recombinant VP1 capsid-like particles harvested 5 days p.i. without DNase I treatment. (C) Recombinant VP1 capsid-like particles 5 days p.i. treated with DNase I. Bar, 100 nm.

tions of numerous CsCl gradients. Upon comparing the structure of the nuclease-treated particles (Fig. 3C) with the nonnuclease-treated particles (Fig. 3B), it can be seen that the treated particles have a somewhat more irregular appearance.

The concomitant peaks of radioactivity and hemagglutination activity indicate that prelabeled Sf9 cellular DNA was being packaged by the assembled recombinant VP1 protein. The DNase-treated and nontreated VP1 capsid-like particles containing the [^{3}H]TdR-prelabeled cellular DNA isolated from the CsCl gradients were then analyzed by agarose gel electrophoresis to determine the size of the DNA found in these particles. As seen in Fig. 7 (lane 2 was not DNase I treated, lane 3 was DNase I treated), both the VP1 capsid-like particle preparations were found to contain DNA in the 5-kb range, which is similar to the polyomavirus DNA present in the native virus (see Fig. 10, lane 2).

Previous studies in the nuclear polyhedrosis baculovirus-insect cell system have shown that a specific endonuclease activity is detected in infected cells. It was reported that the enzyme degrades cellular DNA but not viral DNA (13, 17). With this observation in mind, experiments were designed to determine if the Ac*M*NPV infection was degrading the Sf9 cellular DNA and making these resulting fragments available for recombinant polyomavirus VP1 packaging. Sf9 cells were prelabeled with [^{3}H]TdR, as described above, to label cellular DNA. These cells were divided into three groups and used to compare the fragmentation of cellular DNA by wild-type Ac*M*NPV and by recombinant Ac*M*NPV carrying the polyomavirus VP1 gene (Ac*M*NPV-VP1). The third group was used as an uninfected control. The fragmentation of the Sf9 prelabeled DNA was monitored over a 5-day period by determining the cellular radioactivity released into the medium as well as by determining the amount of low-molecular-weight DNA inside the cells by the Hirt procedure (17). Examination of the radioactivity in the spent tissue culture medium revealed that the uninfected cells did not show any appreciable ^{3}H counts released into the medium; however, when the Ac*M* NPV-infected cells as well as the Ac*M*NPV-VP1-infected cells

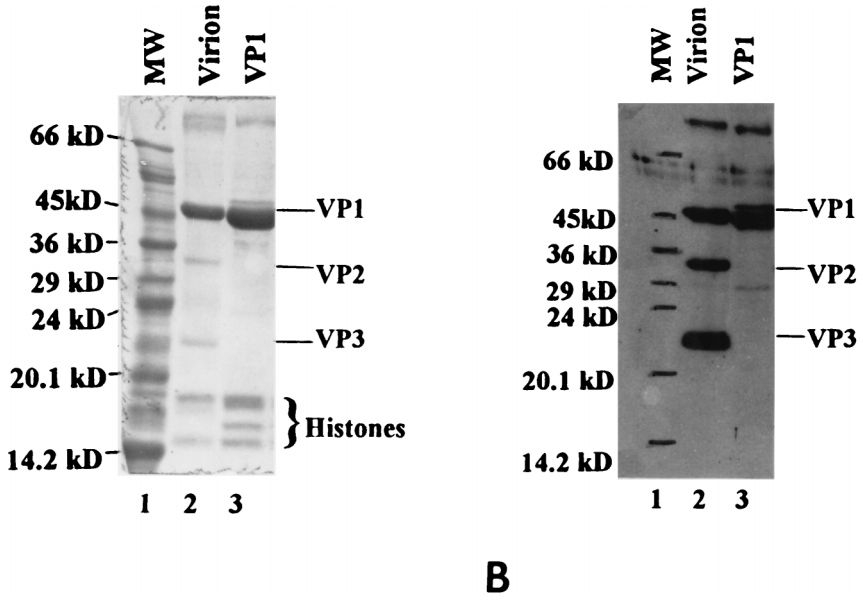

FIG. 4. SDS-PAGE and Western blot analysis of purified recombinant VP1 capsid-like particles. (A) Coomassie blue-stained SDS-PAGE gel of native polyomavirus virions and recombinant capsid-like particles, which were harvested 5 days p.i. and purified through three CsCl density gradient centrifugations. Lane 1, molecular mass (MW) marker; lane 2, native virions; lane 3, capsid-like particles (10 μg of protein). (B) Western blot of the same components from panel A. Lane 1, molecular mass marker; lane 2, native virion; lane 3, recombinant VP1 capsid-like particles (2 μg of protein).

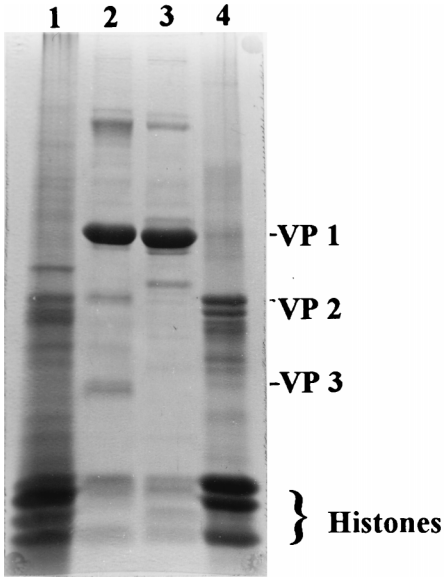

FIG. 5. SDS-PAGE analysis of histones. Lane 1, Sf9 cells which were acid extracted to isolate histone proteins; lane 2, purified native polyomavirus virions showing the VP1, VP2, VP3, and histone proteins; lane 3, the purified VP1 recombinant capsid-like particles; lane 4, commercial calf thymus histones.

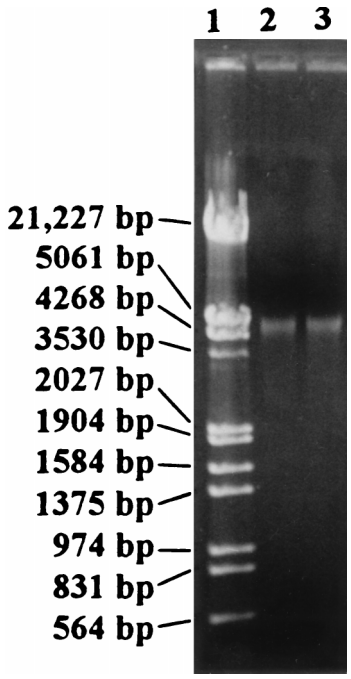

FIG. 7. Agarose gel analysis of DNA isolated from recombinant VP1 capsid-like particles. Capsid-like particles isolated from the purification described in the legend to Fig. 6 were analyzed on 0.8% agarose gels. Lane 1, DNA molecular weight marker (λ phage DNA cut with *Eco*RI and *Hin*dIII); lane 2, DNA isolated from particles not treated with DNase I; lane 3, DNA isolated from particles treated with DNase I.

were assayed for the release of radioactivity into the medium, it was found that there was a marked increase in radioactivity in the medium after 2 days of infection, indicating fragmentation of cellular DNA by the Ac*M*NPV infection (Fig. 8A). The cells from each of the above cultures were then extracted by the Hirt procedure (17), which is designed to isolate low-molecular-weight DNA from large genomic DNA. The uninfected cells did not show any marked increase in the formation of low-molecular-weight DNA, indicative of the lack of cellular DNA fragmentation. However, in both the infected cultures (wild-type Ac*M*NPV and Ac*M*NPV-VP1) the formation of low-molecular-weight DNA was found to increase by day 3 of infection and was found to increase dramatically thereafter, indicating that cellular genomic DNA was being fragmented by the Ac*M*NPV infection (Fig. 8B). To visualize the sizes of the DNA fragments that were present in the Hirt cellular extracts of the Ac*M*NPV-VP1-infected Sf9 cells (Fig. 8B), the extracts were subjected to agarose gel analysis (Fig. 9). After 1 day of infection (lane 2), small DNA fragments were not observed in the Hirt cellular extract. A 20-kb fragment was found 2 days after infection and thereafter (lanes 3 through 6), and by day 3, 5-kb fragments were found, reaching maximum levels after 4 days (Fig. 9, lane 5). These 5-kb fragments of cellular DNA are likely the material being packaged into the capsid-like particles by the recombinant VP1 protein.

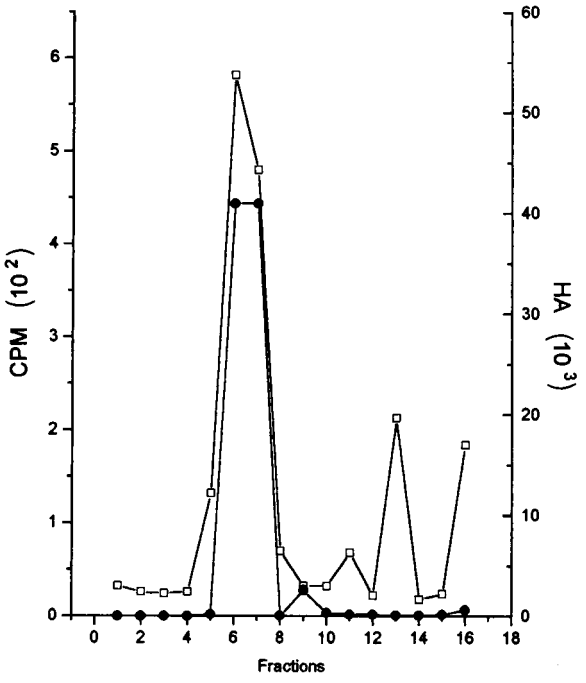

FIG. 6. CsCl purification of VP1 capsid-like particles from 5-day-infected cells. [^{3}H]TdR prelabeled cells were infected with Ac*M*NPV-VP1 and harvested 5 days after infection. Capsid-like particles were isolated after two CsCl density gradient centrifugations and were then treated with DNase I and subjected to a third CsCl gradient. Fractions were collected and assayed for hemagglutination activity (●) and radioactivity (□).

In an earlier study by Forstova et al. (11), the baculovirus recombinant system was used to express the three polyomavirus structural proteins. It was demonstrated that when all three polyomavirus structural proteins were coexpressed in Sf9 cells and harvested after 3 days of infection and purified by CsCl gradients, Western blot analysis revealed that capsid-like particles contained all three structural proteins. The proteins were present in the particles in ratios similar to that found in native polyomavirus virions (11). This study did not determine whether these particles packaged cellular DNA. Because this study was conducted at a relatively early time of infection (3 days postinfection [p.i.]), DNA packaging might not have been observable. Since our current findings show that the cellular

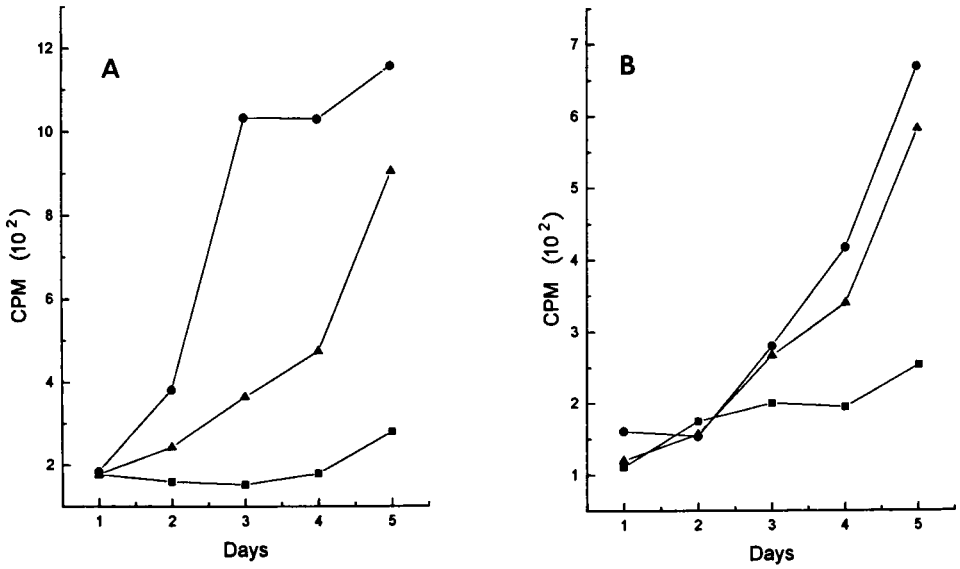

FIG. 8. Effects of Ac*M*NPV and Ac*M*NPV-VP1 infection on [^{3}H]TdR prelabeled Sf9 cells. (A) Extracellular radioactivity found in cell-free medium over a 5-day infection period. (B) Low-molecular-weight DNA extracted from [^{3}H]TdR prelabeled cells by the Hirt procedure (17) over a 5-day infection period. Symbols: ■, uninfected cells; ●, wild-type Ac*M*NPV-infected cells; ▲, recombinant Ac*M*NPV-VP1-infected cells.

DNA is starting to fragment after 3 days of infection (Fig. 8A and B), it was of interest to determine if 3-day-infected cultures were capable of expressing VP1 proteins that could package cellular DNA. Sf9 cells were prelabeled with [^{3}H]TdR and infected with Ac*M*NPV-VP1 as described above, and cells were harvested 3 days p.i. The VP1 capsid-like particles were purified as described above. These particles were analyzed by SDS-PAGE and were shown to package [^{3}H]TdR-labeled cellular DNA. It was found that the only bands that appeared in the Coomassie-stained gel were the polyomavirus recombinant VP1 protein and a small amount of the four histones contributed by the Sf9 cells. The amounts of DNA and histones found in these 3-day-infected particles were decidedly less than that found in particles isolated from 5-day-infected cultures (data not shown). To examine the size of the DNA being packaged by the recombinant capsid-like particles harvested from the Sf9 cells after 3 days of infection, the DNA was isolated and subjected to agarose gel electrophoresis (Fig. 10). Lane 2 represents native polyomavirus virion DNA, with the type I and type II forms of DNA. Lane 3 represents DNA harvested from capsid-like particles at 3 days postinfection (DNase I treated), and lane 4 represents DNA harvested from capsid-like particles at 5 days postinfection (DNase I treated). It is of interest to note that the DNA from the 3-day particles is approximately 3.5 kb and smaller, while that isolated from the 5-day particles is 5 kb, which is very similar to that of the native polyomavirus virions.

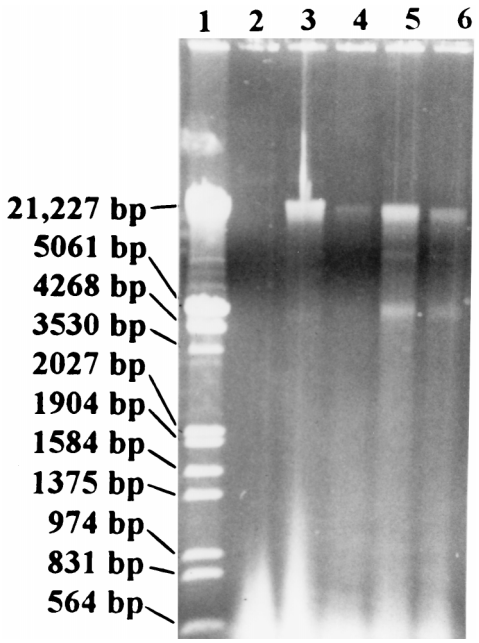

FIG. 9. Agarose gel analysis of DNA obtained from the Hirt cellular extracts of Ac*M*NPV-VP1-infected cells. Lane 1, DNA molecular weight marker (λ phage DNA cut with *Eco*RI and *Hin*dIII); lane 2, 1 day p.i.; lane 3, 2 days p.i.; lane 4, 3 days p.i.; lane 5, 4 days p.i.; lane 6, 5 days p.i.

DISCUSSION

The capsid of polyomavirus consists of three structural proteins. Of these structural proteins, VP1 is considered to be the major capsid protein since it comprises approximately 80% of the total protein in the capsid. Several laboratories have demonstrated that polyomavirus capsids can be produced from recombinant VP1 protein and which consist solely of the VP1 protein. It has been shown that bacterially expressed recombinant VP1 can self-assemble into capsid-like particles (15, 28). In subsequent studies, investigators utilizing a eukaryotic baculovirus expression system were able to demonstrate the assembly of polyomavirus capsid structures in the nuclei of insect cells (10, 11, 21, 24). Kosukegawa et al. showed the assembly of SV40 baculovirus-expressed recombinant VP1 protein into capsid-like structures in Sf6 insect cells (19). In these reports, with the baculovirus system, the structural proteins did not exhibit any packaging of DNA. However, in a recent report by Pawlita et al. it was demonstrated that the baculovirus-ex-

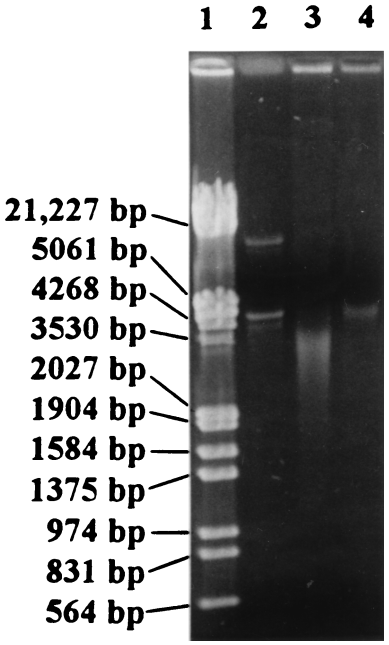

FIG. 10. Agarose gel analysis of DNA isolated from capsid-like particles isolated from 3- and 5-day-infected cells. Sf9 cells were prelabeled with [^{3}H]TdR and infected with Ac*M*NPV-VP1. Infected cells were harvested 3 or 5 days p.i. Capsid-like particles were isolated from both cultures by two CsCl density gradient centrifugations followed by DNase I treatment and a third CsCl gradient. DNA was extracted and analyzed on an agarose gel. Lane 1, DNA molecular weight marker (λ phage DNA cut with *Eco*RI and *Hin*dIII); lane 2, polyomavirus DNA; lane 3, DNA from 3-day p.i. capsid-like particles; lane 4, DNA from 5-day p.i. capsid-like particles.

pressed VP1 of B-lymphotropic papovavirus was capable of packaging DNA late in infection (26).

The other two structural proteins, VP2 and VP3, are the minor capsid proteins, each totalling approximately 10% of the total protein in the virus capsid. It has long been believed that VP2 and VP3 are required for the packaging of DNA inside the viral capsid since these proteins are found in the virion particle (14, 37). An electron density map of the polyomavirus virion obtained by X-ray crystallographic studies, with a resolution of 25 Å, indicates that VP2 and VP3 proteins are arranged inside the VP1 capsid construct as a structure with 72 prong-like extensions which invade the axial cavity of the VP1 pentamers. This arrangement seems to suggest that the minor capsid proteins VP2 and VP3 function to connect the major capsid protein VP1, arranged as the outer capsid shell, to the inner nucleohistone core of the virus (14). The core of the native polyomavirus virion contains the genome of the virus, which is arranged as a closed circular, double-stranded, supercoiled DNA molecule of approximately 5,300 bp. This DNA core of the virus is arranged around host-cell-contributed histones, which are believed to play an integral role in the packaging of the DNA into the capsid (4, 30). Forstova et al. have suggested that the minor capsid proteins form a bridge between the major capsid protein, VP1, either by direct interaction with the viral minichromosome or through interactions with the histones found associated with the minichromosome (11). These studies indicate that VP2 plays a role in the direction of modifications on VP1, which then modulates the efficiency of viral production in an infected cell. DNA packaging was not observed in the recombinant capsid-like particles produced (11).

In this investigation, we infected Sf9 insect cells with a recombinant baculovirus carrying the VP1 major capsid protein gene only, and the minor capsid proteins or the T antigens were not present. Upon harvesting and purifying the capsid-like particles we found the presence of host-derived histones in addition to cellular DNA inside these particles. The only possible source of histones in this system is the Sf9 cells, since the Ac*M*NPV does not possess histones but rather a small basic protein which fulfills this function in the native baculovirus (33, 34). While we agree that the minor capsid proteins VP2 and VP3 play a vital role in native polyomavirus capsid assembly, we suggest they do not seem to be required in the system used for our studies. This may be possible because some cellular factor, contributed by the Sf9 cells, or some Ac*M*NPV-encoded protein, may be replacing the functions of VP2 and VP3, since these minor capsid proteins are absent in our system. This possibility is also supported by the results of experiments conducted by Brady et al. in which intact polyomavirus virions were dissociated by treatment with ethyleneglycol-bis-*N*,*N*′-tetraacetic acid (EGTA) and dithiothreitol and the dissociation products were isolated by centrifugation through sucrose gradients (3). The resulting DNA-protein complex was shown to have a sedimentation value of 48S. Upon SDS-PAGE analysis and autoradiography it was shown that the proteins found in this 48S DNA-protein complex consisted only of VP1 and host-derived histones (3). These data also seem to indicate that the minor capsid proteins VP2 and VP3 may not be needed for the scaffolding of VP1 onto a viral minichromosome. Further, it has also been reported that VP1 and histone proteins of polyomavirus virions are the only DNA binding proteins, when analyzed by Southwestern blotting. It was also found that the DNA binding was nonspecific, since DNA other than that of polyomavirus could bind to these proteins (6, 25). Montross et al. (24) were able to demonstrate the assembly of recombinant VP1 capsid-like particles, without the aid of VP2 or VP3, in the nucleus of infected insect cells; however, packaging of DNA was not observed. It is possible that this was due to the relatively early time in the infection process that these particles were examined.

In examining agarose gels of DNA isolated from the capsid-like particles composed only of recombinant VP1 it is evident that the size of this DNA is approximately 5 kb (Fig. 7, 9, and 10). It should be noted that when the capsid-like particles containing the prelabeled cellular DNA are isolated relatively early in the infection process of the Sf9 cells (3 days p.i.), a heterogeneous mixture of DNA molecules of various sizes is observed on agarose gels (Fig. 10). Even though a heterogeneous population of molecules exists, one does not find packaged cellular DNA exceeding the 5.3-kb size, even from particles isolated late in the infection (5 days) (Fig. 10). This is interesting when the size of the polyomavirus genome, which is 5.3 kb, is considered. Possibly the reason why DNA larger than 5 kb is not isolated is due to a physical size limitation imposed by the recombinant capsid-like structure. Perhaps the intrinsic nature of the polyomavirus capsid or recombinant capsid-like structure will not permit the packaging of DNA molecules larger than 5.3 kb.

The observation that the recombinant VP1 capsid-like particles were packaging cellular DNA made it essential that the source of the 5-kb DNA be identified. It has been reported by several investigators that the Ac*M*NPV has an intrinsic nuclease activity which degrades host cellular DNA (13, 18). We propose that this intrinsic nuclease activity imposed by the Ac*M*NPV infection, or the recombinant baculovirus Ac*M* NPV-VP1, makes 5-kb DNA molecules available for packaging (Fig. 8 and 9). We have demonstrated, through treatment of our recombinant capsid-like particles with DNase I, that this

DNA associated with the particles is not simply adhering to the exterior surface of these particles. This is supported by the fact the 5-kb DNA fragments are observed in both DNase I-treated and non-DNase-treated particles (Fig. 7), indicating that the capsid-like structure was protecting the DNA inside from the action of the DNase I. It is apparent that the majority of the cellular DNA is not simply adhering to the exterior of the capsid-like structures. If this were the case, DNA of various sizes would be visualized in the agarose gels, instead of the 5-kb fragments consistently isolated during this study. We suggest that since the cellular DNA fragments, produced by the nuclease activity of the recombinant baculovirus (Ac*M*NPV-VP1), still contain histones (Fig. 4 and 5), and that polyomavirus VP1 has inherent DNA and histone binding capability, the recombinant capsid-like particles are able to package DNA of the appropriate physical size, as dictated by the nature of the assembled capsid-like structure.

A possible assembly mechanism for the packaging of the cellular DNA-histone complex may be that the Sf9 chromosomal DNA, containing cellular histones, is fragmented by the endonuclease activity of the Ac*M*NPV-VP1 after 3 days of infection, as seen in Fig. 8B and 9. During this fragmentation process 5-kb fragments of DNA with histones are made available for packaging by the recombinant VP1 protein. The actual assembly of VP1 capsid-like particles containing DNA is not thoroughly understood at this time. However, earlier work by Yuen and Consigli (36, 37) and others (12) in the native polyomavirus-mouse infection system revealed that the assembly process is a step-wise addition of the structural proteins onto the minichromosome.

The importance of the minor capsid proteins, VP2 and VP3, in the assembly process of native polyomavirus virions is undeniable since they are found in the virion particle. However, in this system, one may envision that cellular factors or Ac*M* NPV gene products may also play a role in the packaging and assembly process. It is prudent to mention that the purified particles which were isolated from 5-day-infected cultures and analyzed by SDS-PAGE did not reveal the presence of other proteins in the stained gels, although their involvement in the assembly process merits further study.

The thought of polyomavirus capsids which contain sequences of DNA other than the polyomavirus minichromosome is not new. During the normal CsCl gradient centrifugation purification procedures for harvesting native polyomavirus from infected mouse cells in tissue culture, such particles are isolated as a natural by-product. These particles, which contain a mixture of mouse cellular DNA as well as polyomavirus minichromosome DNA have been shown to be pseudovirions (22, 23, 32, 35). Previous studies have shown that pseudovirions contain DNA which closely approximates the normal polyomavirus DNA in molecular weight. It has been demonstrated that these particles behave essentially like polyomavirus virions when subjected to such purification means as sucrose gradient centrifugation or CsCl density gradient centrifugation (32). Conditions in tissue culture which favor the production of pseudovirions include the infection of the culture with a high multiplicity of infection of input virus and the harvesting of virions late in the infection process (32). The capsid-like particles packaging the Sf9 cellular DNA may be analogous to the pseudovirion particles previously reported in the polyomavirus-mouse cell system.

Other investigators working with baculovirus-produced recombinant polyomavirus capsid-like particles have not observed the packaging of DNA in their systems. We suggest that this may be due to the relatively early times of harvesting (2 to 3 days p.i.) compared to the procedures used in our laboratory (10, 11, 21). Also, it is possible that the purification scheme for the recombinant capsid-like particles used in our laboratory may favor the recovery of particles which behave like DNA-containing polyomavirus virions in CsCl gradients. It is essential to note that the recombinant VP1 from the B-lymphotropic virus, a related papovavirus, was capable of packaging cellular (Sf9) DNA into capsid-like particles late in infection (26).

It is important to fully understand the phenomenon we report in this study, i.e., the process of packaging foreign DNA into recombinant VP1 capsid-like particles. The elucidation of the means by which this process is conducted would have a great impact on fully understanding the means of viral assembly. Future studies will entail characterization of the cellular DNA-protein complex packaged by the recombinant VP1 protein as well as that of possible cellular or Ac*M*NPV factors involved in the packaging process.

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