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Myristylated Polyomavirus VP2: Role in the Life Cycle of the Virus

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The double-stranded genome of the small DNA tumor virus, polyomavirus, is enclosed in a capsid composed of a major protein, VP1, which associates as pentameric capsomeres into an icosahedral structure, and two minor proteins, VP2 and VP3, whose functions and positions within the structure are unknown. The N-terminal glycine of the VP2 coat protein has been shown to be cotranslationally acylated with myristic acid. To study the function of this modification and the role of VP2 in the life cycle of polyomavirus, the N-terminal glycine, critical to the myristylation consensus sequence, has been altered to a glutamic acid or a valine residue by site-directed oligonucleotide mutagenesis. The glycine→glutamic acid mutant DNA has been further studied. When transfected into cells permissive for the polyomavirus full lytic life cycle, this mutant DNA replicated at levels comparable to those of wild-type viral DNA, and small amounts of nonrevertant (mutant) virus could be harvested from the cultures. The virus particles viewed by electron microscopy appeared slightly distorted, but the ratio of full to empty particles was similar to that produced in a wild-type viral infection. Mutant virus was capable of reinfecting permissive cells but with a considerably reduced efficiency.

The acylation of eucaryotic functions, by posttranslational attachment of long-chain fatty acids to hydroxyl or sulfhydryl groups on amino acids, by cotranslational modification at the N termini of proteins, or by addition of glycophosphorylated groups to C termini of proteins, has been recently reviewed (27). Many cellular proteins are acylated, and in many cases the functions of the modifications relate to protein-membrane interactions. Whereas palmitylation (acylation with the 16-carbon saturated fatty acid) appears to be associated mainly with membrane-bound proteins, N-terminal myristylation (acylation with the 14-carbon saturated fatty acid) is found on a wider range of protein substrates. It is becoming increasingly apparent that the function of acylation, in particular myristylation, is not merely to anchor proteins to intracellular membranes. A number of viral proteins associated with virion assembly (including the *gag* proteins of retroviruses and the pre-S1 protein of hepatitis B virus), as well as with other late viral functions (including VP2 of papovaviruses, VP4 of picornaviruses, and M25 and M35 of vaccinia virus [10]), have been found to be N-terminally modified with myristic acid. The acylation of VP2 in polyomavirus should prove a good model for investigating roles of acylation in vivo and should shed light on the life cycle and infectivity of this virus and other members of the family *Papovaviridae* (including human polyomaviruses). The fact that it appears as a common modification among viral proteins suggests that defining its precise role could be useful in designing inhibitors of viral infectivity and would be particularly valuable were the myristylated coat proteins of various viruses to perform similar functions.

The life cycle of polyomavirus has been largely deduced from electron microscopic (EM) studies of cells at various time points postinfection (5, 17, 21). Detailed analyses involving isolation of pinocytotic vesicles (13, 14), assembly intermediates (32), and cell surface receptors (20; R. Garcea, personal communication) have also been carried out. However, few events in the life cycle are well characterized biochemically, and so far, none have identified conclusive roles for the minor coat proteins of polyomavirus, VP2 and VP3. In a recent paper describing myristylation of papovavirus proteins, we showed that the myristyl moiety was covalently attached to the N-terminal glycine of VP2 and that the modified protein was membrane associated (26). Here we have used site-specific mutagenesis to address questions regarding the biological function(s) of myristylation. Our findings support general observations that an N-terminal Met-Gly is essential for modification to occur (27). Viral DNA, in which the N-terminal glycine of VP2 has been mutated to glutamic acid, has been transfected into cells and assayed for DNA replication, viral gene expression, and virion assembly. Virions that contain the mutation have been isolated. We found that although the removal of the myristyl group from VP2 does not completely abolish the capacity of virions to infect host cells, the efficiency of viral replication is severely diminished.

MATERIALS AND METHODS

Construction of recombinant mutant and wild-type polyomavirus genomes. Mutagenesis of the GGA glycine codon at the N terminus of VP2 of polyomavirus (nucleotide 4999 of the A2 strain of polyomavirus [12]) was carried out by using an oligonucleotide primer, TL186 [5′-AGTGCGGCT(A,G,T) CCATTTTG-3′], which could theoretically generate three mutant sequences, Gly→Val (GGA→GTA), Gly→Ala (GGA→GCA), and Gly→Glu (GGA→GAA), in the single mutagenesis experiment. *Eco*RI-digested polyomavirus A2 viral DNA was cloned into the *Eco*RI site of pMJ1 to yield pMJA2 (pMJ1 is a 2,266-base-pair [bp] derivative of pAT153 [29] in which the DNA sequence between the *Hin*dIII and *Ava*I restriction enzyme sites has been deleted, with retention of the *Hin*dIII site). Subsequently, the small *Pst*I-*Bam*HI restriction enzyme fragment (1,144 bp, from coordinates 4632 to 484 in the polyomavirus A2 sequence) from pMJA2 was cloned into the M13mp9 single-stranded vector and digested with *Pst*I and *Bam*HI restriction enzymes to

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yield the template for the mutagenesis experiments. Oligonucleotide-directed mutagenesis was carried out essentially as described by Zoller and Smith (33). Briefly, the 5'-phosphorylated mutagenic oligonucleotide TL186 and the universal M13 sequencing primer (5'-GTAAAACGACGG CCAGT-3' [7]) were annealed to the single-stranded DNA template and extended with the DNA polymerase Klenow fragment in the presence of T4 DNA ligase. (A diagrammatic scheme for mutant construction is available upon request.) This mixture was transfected into *Escherichia coli* JM101, and mutant plaques were identified by plaque hybridization with end-labeled TL186 oligonucleotide under appropriate conditions (25). Positive plaques were detected by washing the filter lifts at increasing temperatures, up to 60°C, which was 4°C above the predicted melting temperature of the oligonucleotide (54 to 56°C). Apparently positive plaques were rescreened, and the sequence around the N terminus of the VP2 gene was determined, using the universal M13 sequencing primer and an oligonucleotide primer, TL187 (5'-GAGCTGTAATTTCGCCAT-3', corresponding to bp 4870 to 4887 in the polyomavirus A2 sequence).

Mutant M13 bacteriophage was used to infect *E. coli* JM110 (a *dam dcm* mutant derivative of JM101 [31]), replicative-form DNA was isolated (1), and the resulting DNA was cleaved with restriction enzymes *Bam*HI and *Bcl*I, which cleave polyomavirus A2 DNA at single sites (nucleotides 4632 and 5021, respectively). The smaller 390-bp fragment was isolated and ligated to the large *Bam*HI-*Bcl*I fragment of pMJA2, which had been similarly isolated from pMJA2 DNA grown in JM110, to generate a recombinant DNA containing the full-size Gly→Glu mutant polyomavirus genome. This recombinant, designated pMJA2E, was used in further studies. As a control for future experiments, the *Bam*HI-*Bcl*I small subfragment of the wild-type A2 virus isolated from the template that was utilized for the mutagenesis experiments was similarly religated to generate a wild-type recombinant DNA, designated pMJA2G. The presence of the mutations in full-length viral DNA in the correct orientation was confirmed by restriction enzyme analyses.

Preparation of viral DNA for transfection experiments. Full-length linear viral DNA was excised from the vector in the recombinants pMJA2, pMJA2E, and pMJA2G with *Eco*RI, and the gel-purified viral fragments (5,292 bp) were ligated at a high dilution (1 μg/100 μl). Circular mutant DNAs and linear concatemers generated in this manner were designated A2, E, and G DNAs, the latter in order to differentiate it from wild-type A2 DNA isolated from virions.

Assay of viral DNA replication. Swiss mouse 3T6 cells (ATCC CCL96) were plated onto 50-mm-diameter dishes at 10^6 per dish. After 24 h, the cells were either transfected or infected. Transfections were carried out with 50 or 500 ng of DNA by the dextran method. Briefly, cells were washed with Dulbecco modified Eagle medium (DMEM), and then 0.3 ml of DEAE dextran in DMEM (1 mg/ml) was added per 50-mm dish. After 10 min at room temperature, the dextran solution was removed, and 0.2 ml of DNA diluted in DMEM was added. After incubation at room temperature for 15 min, 5 ml of DMEM supplemented with 5% fetal calf serum (FCS) was added, and the cells were incubated at 37°C and 10% CO_2 until being harvested either 72 h or 7 days posttransfection. In infection experiments, cells were washed with DMEM and 0.5 ml of virus (0.1 PFU per cell) diluted in DMEM was added. Cells were incubated at 37°C and 10% CO_2 for 1.5 h with rocking every 15 min, and then DMEM supplemented with 5% FCS was added. Incubation at 37°C and 10% CO_2 was continued until harvesting of infected cells at 38 h postinfection.

Extracts of extrachromosomal DNA were prepared by the method of Hirt (15), and a portion of each sample was incubated for 2 h at 37°C with either *Hpa*II or *Mbo*I plus RNase A. Digested DNA was fractionated on a 1.4% agarose gel, and polyomavirus-specific DNA was identified with ^{32}P-labeled nick-translated whole viral DNA (18).

Assay of virus production. 3T6 cells were plated at 10^6/50-mm dish. After 24 h, the dishes were either transfected (by the dextran method) with 50 or 500 ng of DNA or infected with 0.1 PFU of wild-type A2 virus per cell. Cells were sequentially harvested at 39 h, 72 h, 6 days, and 9 days by freeze-thawing three times. Resulting cell debris and media were sonicated for 5 s at 12 μm (amplitude) and assayed for hemagglutination (HA) titers by standard methods (28).

Isolation of virions and capsids by CsCl gradients. Cells were plated at 3×10^6/90-mm dish, transfected 24 h later with DNA (1 to 2 μg), and incubated for 7 days. Virus was purified, and virions and capsids were separated on CsCl, as described previously (28). Fractions were collected from the bottom of the gradient and assayed for viral particles by HA.

Preparation of virus for EM. Formvar (EMSCOPE)-coated grids were incubated on virion samples (10 μl) isolated from CsCl gradients for several seconds, drained of surplus solution, transferred to a drop of phosphotungstic acid (3%; pH 6.6), and left for 45 s. The grids were dried and stored at room temperature.

Labeling of viral proteins and viruses. 3T6 cells were plated at 2×10^5/35-mm-diameter dish. After 24 h, they were transfected by the dextran method with DNA or infected with the virus. Cells were then labeled at various times posttransfection or postinfection with either [^{35}S]methionine or [^3H]myristic acid. For ^{35}S labeling, cells were transferred to DMEM lacking methionine (DMEM-Met) and supplemented with 5% FCS (that had been dialyzed overnight against phosphate-buffered saline) for 1 h prior to labeling. The medium was then changed to DMEM-Met plus 5% dialyzed FCS (0.5 ml) containing 125 μCi of [^{35}S]methionine (>1,000 Ci/mmol) (Amersham International, plc) per 35-mm dish, and the cells were incubated at 37°C and 10% CO_2 for 4 h. For ^3H labeling, the medium was changed to DMEM supplemented with 10% tryptose phosphate broth (Flow Laboratories, Inc.), and 5% FCS and cells were incubated for 1 h at 37°C. The medium was then changed to DMEM plus 10% tryptose phosphate broth plus 5% FCS (0.5 ml) containing 150 μCi of [^3H]myristic acid (47.5 Ci/mmol) (Amersham) per 35-mm dish, and cultures were incubated at 37°C in 10% CO_2 for 4 h. To harvest, cells were washed twice in ice-cold phosphate-buffered saline, pelleted, suspended in lysis buffer (100 mM NaCl, 100 mM Tris hydrochloride [pH 8.8], 0.5% Nonidet P-40, 0.2 trypsin inhibitory units of aprotinin per ml) (100 μl), and incubated on ice for 20 min. Debris was removed by centrifugation in a Microfuge for 2 min.

To label viruses, 3 days before harvest the cell cultures were transferred to medium consisting of 50% DMEM-Met supplemented with 5% dialyzed FCS and 50% complete medium plus 5% FCS containing 1 mCi of [^{35}S]methionine per 90-mm-diameter dish. For ^3H-labeling, cells were incubated for 3 days prior to harvest in DMEM plus 10% tryptose phosphate broth–5% FCS containing 1 mCi of [^3H]myristic acid per 90-mm dish. Viral proteins were immunoprecipitated by standard methods with antibodies specific for early antigens or with antivirion serum, and the resulting immune

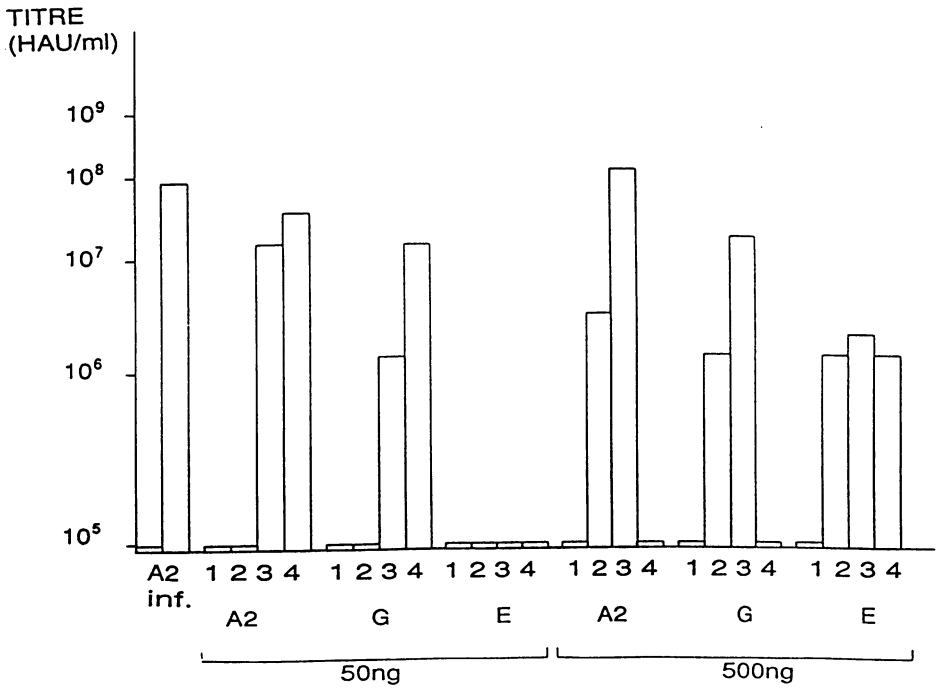

FIG. 1. Histogram showing comparison of virus titers from transfected cells harvested at different times. The HA titers (HA units per milliliter) of virus harvested from mouse cells infected with 0.1 PFU of wild-type virus per ml (A2 inf.) at 1.5 and 3 days is given, together with those from cells transfected with either 50 or 500 ng of wild-type (A2), reconstructed wild-type (G), or mutant (E) DNA at 1.5, 3, 6, and 9 days (time points 1 through 4, respectively). Data for A2 and G, using 500 ng of DNA, were not significant at time point 4, since all the cells were lysed.

complexes were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

RESULTS

A total of 40 positive clones were identified by hybridization with the oligonucleotide TL186 (see Materials and Methods). After rescreening, six positive plaques were chosen for sequence analysis. Three of these were found to contain the mutation for a Gly(G)→Glu(E) change, and one contained the mutation for Gly→Val(V); two of the clones were found to contain wild-type DNA. The Gly→Glu mutation, being a nonconservative amino acid replacement, was used in all further studies.

Infectivity of virus produced by mutant and wild-type DNAs. (i) HA assays. Cultures transfected with mutant (E) or wild-type (G) DNA were assayed for virus particles by HA, using guinea pig erythrocytes (28). The results of a typical experiment are shown as a histogram (Fig. 1). We observed that infection with virus produced progeny much more quickly (high to maximum titers within 3 days) than transfection with DNA; this is not surprising, since in viral infection the DNA would be delivered to the correct cellular compartment and in a state favoring replication and transcription, whereas in transfection this would not necessarily be the case. The transfections with small amounts (50 ng) of mutant E DNA failed to produce a titer at any time point, and even with larger amounts (500 ng), transfections produced only low levels of virus. Although virus was detected in the mutant E DNA transfections, the levels (as assessed by HA) did not increase in a time-dependent manner, as was the case with A2 and G DNA transfections. In cells transfected with 500 ng of DNA, the A2 and G DNAs produced a cytopathic effect which prevented meaningful results from being obtained for cells harvested at the last (9-day) time.

(ii) Plaque assays. In order to assess the proportion of infectious particles present in the mutant E virus preparation, plaque assays were performed on whole mouse embryo cells. Despite repeated attempts, no visible plaques were obtained for the mutant virus. At comparable titers (based on HA data) and comparable times, cell survival was observed with the mutant virus when wild-type virus had lysed all cells (data not shown).

(iii) Gene expression (assessed by protein analysis). The failure of the mutant E DNA viral titers to increase with time, coupled with plaque assay data, suggested that the virions produced were defective, being much less efficient at or incapable of reinfecting cells. To test this possibility, viruses from transfections with A2 or mutant E DNA, harvested at 7 days, were titered and equal numbers of HA units (corresponding to low titers, that is, 1 HA unit of virus per cell) were used to infect fresh 3T6 cells. After 7 days, the A2-infected cells showed cytopathic effects, whereas the morphology of the E virus-infected cells was that of a highly confluent but healthy culture. Cells were then labeled for 4 h with either [^{35}S]methionine or [^{3}H]myristic acid, and the radiolabeled protein lysates were immunoprecipitated either with monoclonal antibodies (C1 and C4 [6]) that recognize the common region of all three polyomavirus early antigens or with antivirion serum (Abtek Biologicals, Ltd.). Figure 2A shows the results obtained by immunoprecipitation and SDS-PAGE. Polyomavirus-specific proteins are clearly produced (Fig. 2A, lanes V and C) in cells infected with virus that was harvested from the initial mutant E DNA transfections, showing that the E virus produced is capable of

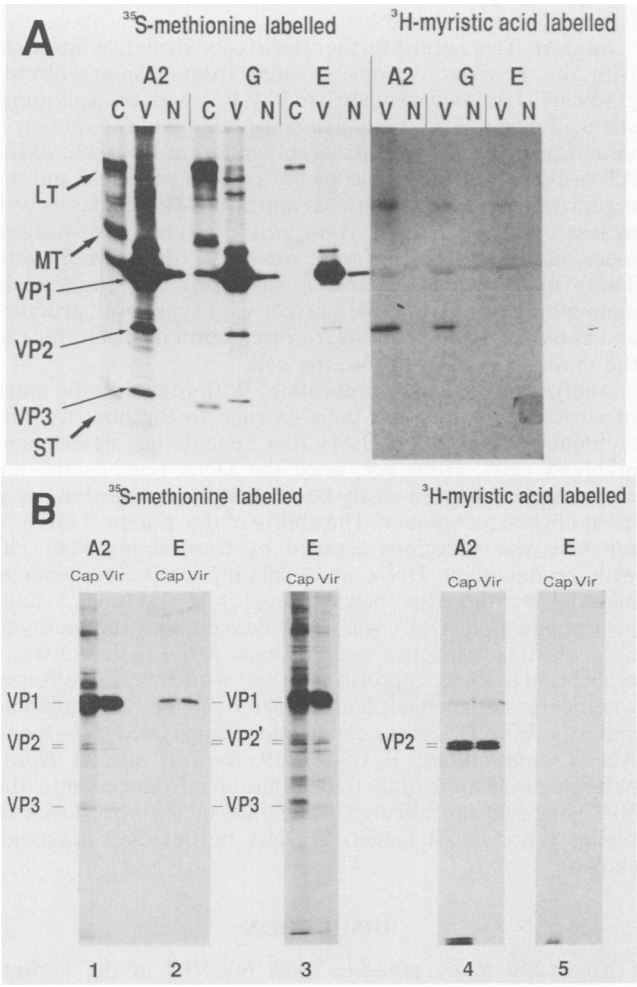

FIG. 2. (A) Protein patterns from cells infected with wild-type or mutant viruses. Protein lysates from 3T6 cells infected with 1 HA unit of wild-type (A2), reconstructed wild-type (G), or mutant (E) virus per cell, labeled after 7 days with either [^{35}S]methionine or [^{3}H]myristic acid, were immunoprecipitated with monoclonal antibodies specific for early viral antigens (lanes C), with an antivirion serum (lanes V), or with a nonimmune serum (lanes N). Products were separated by SDS-PAGE, and gels were fixed, enhanced with Amplify (Amersham), and autoradiographed on XAR-5 film (Eastman Kodak Co.) for 1 week (^{35}S-labeled proteins) or 7 weeks (^{3}H-labeled proteins). Positions of the early antigens, LT (100 kilodaltons [kDa]), MT (55 kDa), and ST (22 kDa), and the late antigens, VP1 (42 kDa), VP2 (35 kDa), and VP3 (23 kDa), are indicated. The nonmyristylated VP2 of the mutant E virus (VP2') migrates with a higher apparent molecular weight than that of wild-type VP2. (B) Protein patterns from isolated polyomavirus capsids and virions. Proteins were separated by SDS–12.5% PAGE from viruses isolated from cells transfected with either wild-type (A2) or mutant (E) DNA and labeled with [^{35}S]methionine or [^{3}H]myristic acid, as indicated. Lanes 1 and 2 show a 4-h exposure with only VP1 evident in the mutant (E) experiment; lane 3 is a 7-h exposure of the gel in track 2, in which minor capsid proteins are seen (Fuji film, used at room temperature). Lanes 4 and 5 were enhanced with Amplify and autoradiographed on preflashed XAR-5 film for 4 days at −70°C. On much longer exposure, no labeled bands were observed in either capsids (Cap) or virions (Vir) in lane 5. Positions of the late antigens (VP1, VP2, VP3) and the mutant E unmodified VP2 (VP2') are indicated.

reinfecting permissive cells. Immunoprecipitation of ^{35}S-labeled lysates with common region antibodies (Fig. 2A, lanes C) resulted in production of the three viral early antigens, the large, middle, and small T antigens (LT, MT, and ST, respectively), and antivirion serum precipitated the late antigens VP1, VP2, and VP3 (Fig. 2A, lanes V). However, the E mutant VP2 (VP2') migrated more slowly than the major band of wild-type VP2 in SDS-PAGE, consistent with an altered modification. With the A2 infections, [^{3}H] myristic acid labeling of the cells gave a polyomavirus-specific VP2 protein (Fig. 2A, lanes V) with a size indistinguishable from that of VP2 metabolically labeled with [^{35}S] methionine. However, no band was observed in immunoprecipitates of E mutant-infected cells, and longer exposures (up to 7 weeks) failed to reveal any proteins that would correspond to myristylated VP2 in these cells. Thus, the E mutant appears to produce a nonmyristylated VP2 gene product (consistent with the absolute requirement for an N-terminal glycine in the myristylation recognition sequence) which has a slower mobility in SDS-PAGE than does wild-type VP2. Densitometer tracings (not given) showed that the ratio of E mutant antigens, with respect to one another, resembles those found in A2-infected cells; all other antigens (except VP2) had migration rates in SDS-PAGE indistinguishable from those of their wild-type counterparts.

Similar infections with the wild-type and mutant viruses harvested at an earlier time (44 h postinfection), before significant reinfection could occur, resulted in the same protein profile (data not shown). Of particular note is the fact that viral protein levels were considerably lower than those resulting from wild-type infection. Since proteins observed will have resulted from the original infection with equal titers of each virus, this suggests that the major loss of efficiency of the mutant viral replication occurs at some stage prior to packaging or exit of virions from the cell.

Structure analysis of mutant viruses. (i) Fidelity of mutant. To confirm that the virus isolated from mutant-DNA-transfected cells had not undergone any significant reversion to wild type but still contained nonmyristylated VP2, virions and capsids were isolated from [^{35}S]methionine- and [^{3}H] myristic acid-labeled cells after 7 days. Total ^{35}S-labeled proteins were fractionated by SDS–12.5% PAGE and analyzed in both virions and capsids from E- and G-transfected cells. In the case of the mutant, fewer viral products were observed, and the VP2 doublet migrated more slowly than the corresponding wild-type VP2 proteins (even though the same number of cells were producing VP1 after 2 days in wild-type- and mutant-transfected cultures, as assayed by immunofluorescence) (Fig. 2B). Also, even on prolonged exposures, there was no ^{3}H labeling of VP2 in mutant virions or capsids. (The presence of viral proteins was confirmed in these tracks by Western blotting [immunoblotting] [data not shown].) Immunoprecipitation of ^{35}S-labeled virions and capsids with antivirion antisera gave data similar to those shown in Fig. 2B.

These results with purified virus particles confirm those obtained with the whole-cell lysate experiments. Since the viruses and viral products analyzed resulted from multiple rounds of infection, that is, over a period of 14 days in the case of the whole-cell lysate experiments (viruses harvested 7 days after transfection were used to infect cells, which were then processed after 7 days), if the mutant E virus reverts to a wild-type phenotype during replication, the reversion frequency is very low and cannot explain the infectivity data obtained. The fidelity of mutant DNA was

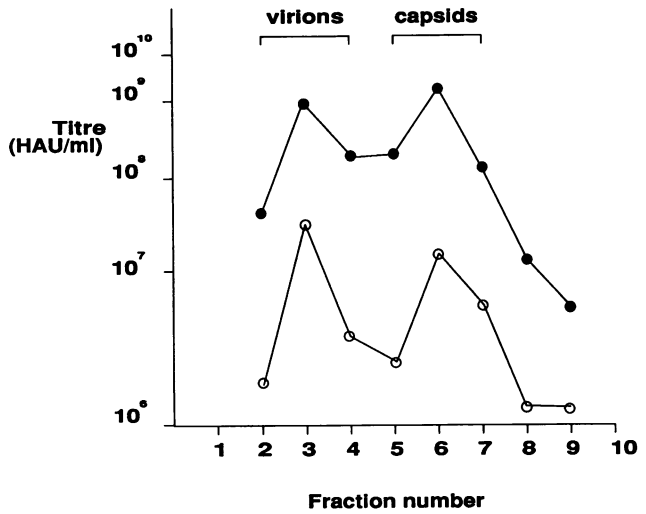

FIG. 3. Fractionation of viral products on CsCl density gradients. 3T6 cells were transfected with wild-type (●) or mutant (○) DNA and harvested after 6 days. Viral particles were concentrated through a sucrose cushion and fractionated on a CsCl density gradient into virions (density, 1.33 g/ml) and capsids (density, 1.29 g/ml), with their relative concentrations in each fraction ascertained by HA.

also confirmed by polymerase chain reaction analyses (done with R. P. F. Watkins), which suggested that if reversion occurred, it represented fewer than $1:10^4$ molecules (data not shown).

(ii) Density gradient analysis of viral particles. Since the HA assay measures viral particles rather than infectious units, it was possible that the data above reflected a high proportion of noninfectious particles in the mutant E viral population. Since a role for VP2 in virion packaging has been suggested (26), the ratio of virions to empty capsids was measured for the A2 and mutant E viruses (Fig. 3). Viruses isolated from transfected cells were separated on a cesium chloride density gradient, and fractions were assayed by HA for virions and capsids. While overall lower titers were obtained for the mutant E virus, the ratio of full virions to empty capsids was the same as for G virus, suggesting that the failure to package DNA does not explain the data in earlier experiments. The results also indicate that removal of the myristyl group from VP2 has little effect on the packaging of DNA into capsids.

(iii) EM. To examine further the loss of virulence observed with the E virus, virions isolated from cesium chloride gradients (Fig. 3) were visualized by EM with phosphotungstic acid negative staining. The morphology of the mutant E virus was found to be considerably different from that of the A2 virus, in that the E particles were less spherical and the organization of the capsomeres appeared less regular as well as less compact (Fig. 4). Also, in the E virus preparation, more disrupted particles were observed (data not shown). These findings may suggest a structural role for VP2 in maintaining or creating the correct wild-type viral structure and could have implications for orientation of the surface of the virus prior to entry into the cell.

Analysis of viral DNA replication. With regard to the entry of viruses into cells and their passage to the nucleus, few techniques (other than EM) that reveal the mechanisms involved exist. However, once the DNA reaches the nucleus, replication can easily be studied by standard molecular biological techniques. The ability of the mutant E DNA to replicate was therefore assayed by transfecting 3T6 cells with recombinant DNA and isolating extrachromosomal material by the Hirt procedure (15) at 1.5 and 3 days posttransfection. DNA was then cleaved with the methylation-sensitive restriction endonuclease *Mbo*I to demonstrate replication in the eucaryotic cells and with *Hpa*II to observe whether gross changes had occurred in the viral genome patterns. Viral DNA was cleaved in all cases with *Mbo*I, and A2, G, and mutant E DNAs all showed normal *Hpa*II patterns, indicating that the mutation introduced into the VP2 gene had not affected the ability of the viral DNA to replicate nor could defective DNA be detected (data not shown).

DISCUSSION

There are many possible roles for VP2 in the various stages of the proposed life cycle of the papovaviruses (4), and clearly, since the myristyl modification is highly conserved among these viruses, it must confer a significant advantage to the virus. Given the chemical nature of the myristyl moiety and the fact that it is bound covalently to the protein, it is logical to assume that hydrophobic interactions

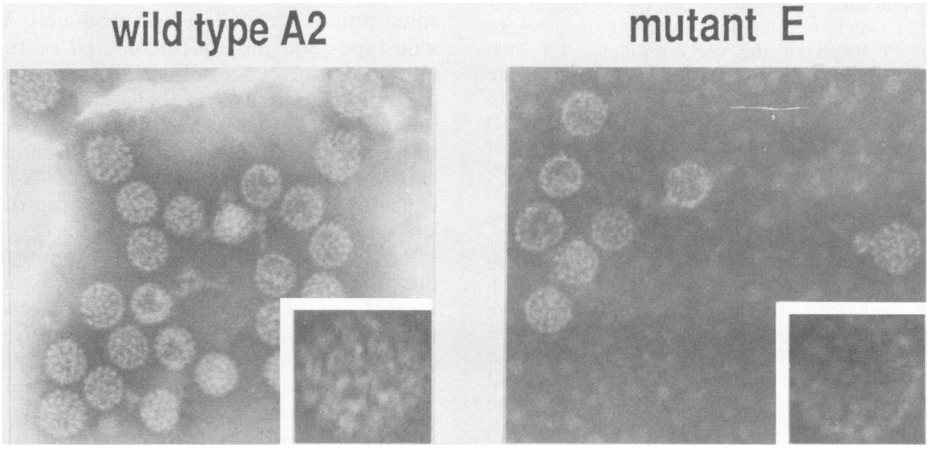

FIG. 4. EM of wild-type and mutant viruses. Viruses from 3T6 cells transfected with either wild-type A2 or mutant E DNA were purified on a CsCl gradient and visualized by EM. Magnification, ×120,000; inset magnification, ×345,000.

are involved. These could include interactions with membranes (e.g., plasma, pinocytotic vesicle, or nuclear interactions) or other hydrophobic proteins (e.g., receptors or cytoskeletal or nuclear matrix). Myristylation might also affect the tertiary structure of VP2.

We have observed that failure to myristylate polyomavirus VP2 does not result in a total loss of infectivity. However, while the mutant E virus was infectious, it had only 1/10 (or less) the efficiency of wild-type virus, as estimated by the reduced levels of mutant versus wild-type virus titers, after subsequent rounds of infection (Fig. 1). There are several explanations which could account for these observations. Since HA was used to determine viral titers, one explanation is that the actual infective titer of mutant virus was lower than that measured because fewer or defective viral genomes were being packaged. This does not seem to be the case, as analysis of the mutant E and wild-type virions (Fig. 2B) showed that the ratio of full to empty capsids was the same for both viruses, and with regard to potentially defective genomes, the *Hpa*II digests of mutant DNA gave the normal viral restriction enzyme pattern. (Defective viral DNA, on a scale required to produce the loss of infectivity seen in the E mutant, would be clearly apparent as anomalies in the restriction enzyme profile [11].) There is more reason to suspect that the altered morphology of the mutant virus (Fig. 4) is responsible for the observed reduction in efficiency, either by impairing entry into the cell by disrupting normal receptor interactions or by altering the stability of the virions. By analogy, in rhinoviruses, a small conformational change in the receptor-binding site results in the blocking of binding (8, 23). In the present study, such a conformational change may be induced by distortion in the viral particle as a whole. Alternatively, although the mutant virus appears to be as stable as the wild type at 4°C in culture media (unpublished results), in EM preparations (under standard pH conditions) a higher proportion of disrupted particles was observed in mutant- than wild-type-infected cells, raising the possibility that the distorted virus could be more sensitive to disruption under the low-pH conditions present in endocytotic vesicles (30). This could result in a proportion of particles being destroyed before the vesicles fuse with the nuclear membrane.

It is conceivable that VP2 is involved in transport across the nuclear membranes, although this is difficult to test. However, once the viral genome has been uncoated and released for replication in the nucleus, it would appear that the single base change that abolishes myristylation acts as a silent mutation; replication of mutant E DNA, as assessed by examination of Hirt extracts, appears similar to wild-type DNA. This, perhaps, is not surprising, since the region of the DNA altered in the mutant is not known to be involved in any binding sites of regulators of replication or transcription and does not appear to code for any known proteins involved in these processes. Once the viral late transcripts have been translated, the products are then transported back into the nucleus for assembly. Some cooperation between the viral coat proteins in binding to the cytoskeleton and passage to the nucleus (simian virus 40) (H. Kasamatsu, personal communication) and arrangement on the nuclear framework (for polyomavirus) (24) has been observed. However, our data do not suggest that myristyl VP2 is involved in any of these processes, although this cannot be rigorously excluded as yet.

Little is known about the mode of exit of the progeny virus from the cell, but it is generally assumed that cell death and passive disruption serve to release the virus into the surrounding medium (4). Recent data (2) suggest that release of simian virus 40 from epithelial cells is not a passive event. However, our results indicate that in the case of polyomavirus, the reduced production of mutant virus is due primarily to processes independent of virus packaging and release, at least in fibroblast cells in vitro. For example, on infection with an equal titer of mutant E or A2 virus, fewer translation products were produced in the first round of infection with the mutant E virus. In addition, the results from the plaque assay support the notion of inefficiency of infection events, since plaque formation is not just a measure of the lytic step but depends as well upon reinfection. (It is perhaps worth noting that mutants of simian virus 40 with an internally altered VP2 gene grow poorly, producing tiny plaques only after 15 to 20 days, compared with the large plaques produced in 8 to 10 days by wild-type viruses [3].) These data, taken together with the altered morphology of the mutant virus, observed by EM, suggest that myristyl VP2 is critical in those stages in the viral life cycle that include entry into the cell, passage to the nucleus, and uncoating.

Several lines of evidence from studies with other viruses suggest a role for myristylated coat proteins in viral entry. For example, VP4 of poliovirus, which is structurally similar to papovaviruses, is thought to interact with the cellular membrane during the initial stages of infection, since viral particles which are adsorbed to the cell surface and then eluted are found to be lacking this protein (9). However, in the case of poliovirus, lack of myristylation in the precursor and VP4 capsid proteins results in total loss of infectivity (19), contrary to our observations. Recently, Franke et al. (10) have reported the myristylation of two late proteins in the intracellular form of vaccinia virus, which appears to enter the cell via an endocytotic mechanism (unlike the extracellular form of vaccinia virus, which is thought to enter via fusion of the viral envelope with the cell membrane [22]). While interactions during endocytosis could be important in the case of polyomavirus, the data presented here suggest that myristylation is most relevant in one or more of the various stages involving viral attachment to cells, entry, and uncoating. Our data further support the notion that although myristylation of VP2 is not totally necessary for infection, it is a requirement for efficient viral infection.

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