Analysis of mouse polyomavirus mutants with lesions in the minor capsid proteins

Petra Mannová,1 David Liebl,1 Nina Krauzewicz,2† Anna Fejtová,1 Jitka Štokrová,3 Zdena Palková,1 Beverly E. Griffin2‡ and Jitka Forstová1,2

1 Department of Genetics and Microbiology, Charles University in Prague, Viničná 5, 128 44 Prague 2, Czech Republic
2 Department of Virology, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 ONN, UK
3 Institute of Molecular Genetics, Czech Academy of Sciences, Flemingovo n. 2, 166 37 Prague 6, Czech Republic

Polyomavirus mutants E, Q and H, expressing non-myristylated VP2, were generated by replacing the N-terminal glycine residue with glutamic acid, glutamine or histidine, respectively. Viruses mutated in either VP2 or VP3 translation initiation codons were also prepared. All mutated genomes, when transfected into murine host cells, gave rise to viral particles. Infectivity of VP2N and VP3N viruses, as measured by the number of cells expressing viral antigens, was dramatically diminished, indicative of defects in the early stages of infection. In contrast, the absence of a myristyl moiety on VP2 did not substantially affect the early steps of virus infection. No differences in numbers of cells expressing early or late viral antigens were observed between wild-type (wt) and E or Q myrN viruses during the course of a life cycle. Furthermore, no delay in virus DNA replication was detected. However, when cells were left for longer in culture, the number of infected cells, measured by typical virus bursts, was much lower when mutant rather than wt genomes were used. In situ, cell fractionation studies revealed differences in the interaction of viral particles with host cell structures. The infectivity of mutants was affected not only by loss of the myristyl group on VP2, but also, and to a greater extent, by alterations of the N-terminal amino acid composition.

Introduction

Icosahedral polyomavirus (Py) capsids consist of 72 pentamers composed of three structural proteins, which are synthesized during the late stages of viral infection. The major capsid protein, VP1 (45 kDa), occupies 80% of the capsid protein mass. The two minor capsid proteins, VP2 (35 kDa) and VP3 (23 kDa), are encoded in an overlapping manner, with the sequence of VP3 being entirely contained within the C-terminal part of VP2. One molecule of either VP2 or VP3 is positioned in the centre of each pentamer (Barouch & Harrison, 1994). The minor capsid proteins are not essential for capsid formation and VP1 alone can self-assemble into capsid-like particles, the only requirement being the presence of Ca2+ ions (Leavitt et al., 1985; Montross et al., 1991). VP1 has been shown to consist of several isoelectric species (Bolen et al., 1981; Forstová et al., 1993). Forstová et al. (1993) observed that, in the baculovirus system at least, coexpression of VP2 is necessary for synthesis of all post-translationally modified VP1 species.

Although VP2 and VP3 possess their own nuclear localization signals, they are presumed to be transported to the nucleus (where virion assembly occurs) in complex with VP1 (Forstová et al., 1993; Barouch & Harrison, 1994). This is in agreement with observations that the coexpression of VP1 is needed for the stable nuclear location of VP2 and VP3 in heterologous expression systems (Stamatos et al., 1987;
Forstová et al., 1993). In contrast, VP2 and VP3 of the closely related simian virus 40 (SV40) can reach the nucleus independently of VP1 (Clever & Kasamatsu, 1991). SV40 minor proteins contain a C-terminal domain that includes a nuclear localization signal (Clever & Kasamatsu, 1991) and the DNA binding domain (Clever et al., 1993). Py VP2 and VP3, which do not bind DNA (Chang et al., 1993), have truncated C-terminal domains relative to their SV40 counterparts.

No specific functions in the viral life cycle have yet been assigned to VP2 and VP3. However, mutants of SV40 with deletions in the VP2 unique sequence have severely reduced growth (Cole et al., 1977). Sahli et al. (1993) selected revertants with wild-type (wt) properties during a study of Py mutants that failed to express either VP2 or VP3.

The VP2s of both Py and SV40 are N-terminally modified with myristic acid (Streuli & Griffin, 1987). With many other viruses, it has been shown that fatty acid acylation of structural proteins plays an essential role in their life cycles. Myristylation of VP4 is important for the assembly and structural stability of poliovirus (Marc et al., 1990; Ansardi et al., 1992; Moscufo & Chow, 1992) and myristylation of the Gag polyprotein is necessary for lentivirus and retrovirus C type capsid formation and virion maturation (Rein et al., 1986; Bryant & Ratner, 1990). Myristylated capsid proteins can be involved in early stages of virus infection (interaction with receptors and entry) as shown, for example, for poliovirus and hepatitis B virus (Moscufo et al., 1993; Grippon et al., 1995). In general, fatty acid acylations of proteins serve as membrane targeting and anchoring signals. However, myristylation alone is not sufficient for the membrane location of modified proteins. An increasing number of recent studies points to a function of acylation (particularly myristylation) in the binding of modified proteins to specialized membrane domains. These domains could be sites where viral assembly occurs, as shown for poliovirus (Martin-Belmonte et al., 2000).

Myristic acid is co-translationally bound by an N-myristoyl transferase to an N-terminal glycine, the amino acid essential for this modification, after removal of the initiator methionine (reviewed by Johnson et al., 1994). Consensus sequences for myristylation have been found on the N terminus of the VP2 protein for all polyomaviruses. The function of this modification has been studied using myr− VP2 Py mutants with an altered N-terminal glycine. The absence of myristoyl moiety on VP2 (mutant E, with glutamic acid replacing glycine) did not abolish the ability of mutated virus to infect mouse cells (Krauzewicz et al., 1990). In another study, a lowered infectivity was observed with a mutant containing alanine substituted for glycine. This mutant apparently exhibited a delay in replication compared with wt (Sahli et al., 1993).

To elucidate the function(s) of the myristic acid modification of VP2 and possible effects of an altered N terminus of VP2 during the poliovirus life cycle, we created myr− VP2 mutants with different amino acids substituted for glycine. We also prepared mutants lacking either VP2 or VP3 in their virions. Mutants were prepared containing at least three nucleotide substitutions of wt sequences to limit the formation of revertants.

**Methods**

- **Bacterial strains, cells and plasmids.** *E. coli* DH5α or *E. coli* JM110 (dam− dcm−; Yanisch-Perron et al., 1985) were used for preparation of recombinant plasmids.

  Swiss albino mouse fibroblasts 3T6 (ATCC CCL 96) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4 mM glutamine and 10% foetal calf serum (FCS) at 37 °C in 10% CO₂. Primary cultures of whole mouse embryo (WME) cells were prepared as previously described (Hogan et al., 1994) and grown in DMEM with 10% FCS at 37 °C in 10% CO₂.

  The recombinant plasmid pMJG contains the complete sequence of wt polyomavirus strain A2, interrupted at the EcoRI site (position 1560, according to Griffin et al., 1981) and cloned into the EcoRI site of plasmid pMJ (Krauzewicz et al., 1990). The recombinant plasmid pMJE (Krauzewicz et al., 1990) contains the genome of polyomavirus strain A2, with a point mutation in the VP2 gene replacing the N-terminal GGA glycine codon with a GAA glutamic acid codon (mutated nucleotide 4998).

- **Construction of mutated polyomavirus genomes.** Plasmids pMJQ and pMJH were obtained from pMJG by replacing the BclI–EcoRV fragment, comprising the N terminus of VP2, with a BclI–EcoRV fragment prepared by PCR mutagenesis. Oligonucleotide primers 5’ AGCTGATC‐AAATGAAATTTTCAAATTCGAGGCCCGCA3’ introducing the substitution of the VP2 N-terminal glycine codon GGA by CAG (glutamine), and 5’ AGCTGATCAAGTAGATTTACCAAAATTCGAGGCCCGCA3’, with a histidine (CAT) codon, were used in the PCR reaction. In pMJH, a unique NsiI restriction site (ATGCAAT) was created by the mutagenesis.

  pMJM, with the ATG initiation codon for VP2 translation abolished, was prepared from pMJH by NsiI cleavage. Overlapping ends were blunt‐ended and religated to produce a frame‐shifted, three‐codon‐long open reading frame. pMJA was prepared by replacing the BclI–BamHI fragment of pMJG with a PCR fragment where the GCA codon for alanine had been substituted for the ATG VP3 translation start codon using the PCR primer 5’ GGCTGGATCCCGCCATGGTATCAACGCTGATCATTIC 3’.

- **Preparation of wt and mutant viruses.** Wt and mutant genomes were excised from their respective plasmids with EcoRI and the resultant linear fragments diluted to a concentration of 3–4 μg/ml and ligated. Ligated mixtures were transfected into 3T6 cells. Cells were incubated at room temperature with DEAE–dextran (1 mg/ml in DMEM). After 10 min, dextran was replaced with DNA in DMEM (400 ng of circular relaxed genome per 8 × 10⁶ cells). DMEM with 10% FCS was added after a 15 min incubation at room temperature. Transfected cells were subcultured when they reached confluency. Cells were harvested after five passages, viruses isolated as described (Türler & Beard, 1985) and purified by centrifugation through a 20% sucrose cushion (25 000 r.p.m.; Beckman SW28 rotor, 3 h, 4 °C). Isolated mutants were designated A, M, H, E and Q according to the recombinant plasmids from which they originated. Particles were quantified by a haemagglutination test, as previously described (Türler & Beard, 1985).

- **SDS–PAGE and Western blot analysis.** Viral proteins were resolved on 12% SDS–PAGE (Laemmli, 1970) and electrotransferred on
to a nitrocellulose membrane in cooled blot buffer (0.3 % Tris base, 1.44 %
glycine, 20 % v/v methanol) at 2.5 mA/cm² for 3 h. For large T (LT)
antigen detection, blot buffer was supplemented with 0.1 % SDS and the
transfer carried out for 1 h. The filters were incubated in 5 % fat-free milk
in PBS and then with αVP1 (dilution 1:10) or αVP2/3-B (dilution 1:4)
mouse monoclonal antibodies (Forstová et al., 1993) or αLT 7 rat
monoclonal antibody (Dilworth & Griffin, 1982). Goat anti-mouse or
goose anti-rat immunoglobulins conjugated with horseradish peroxidase
(1:3000; Bio-Rad) were used as a secondary antibodies. Membranes were
developed with ECL Chemiluminescent Reagent (Amersham) and
exposed to X-ray film.

**Labelling of viruses.** 3T6 cells, infected with virus H, E, Q or wt,
were labelled 30 h post-infection (p.i.) with [³H]myristic acid (54 Ci/
mmol; Amersham) and products were analysed by SDS–PAGE. The gels were fixed, signal-enhanced with Amplify (Amersham) and autoradiographed for 7–9 weeks at −70 °C.

**Infection of cells.** 3T6 cells (at 70 % confluency) were washed with
DMEM and incubated with virus inoculum diluted in DMEM. Adsorption
of virus was carried out for 1 h at 37 °C. DMEW cells with 10 % FCS was
added and cells were further incubated.

**Immunofluorescence of cells.** 3T6 cells grown on glass coverslips
were fixed with methanol/acetic acid (1:1, v/v) for 3 min at room
temperature. Cells were incubated for 30 min with PBS containing 0.25 %
gelatin and 0.25 % BSA, and then for 1 h with αVP1, αVP2/3-A or αLT
antibody (diluted 1:10, 1:2 and non-diluted, respectively). Unbound
antibody was removed by washing with PBS. A secondary anti-mouse or
anti-rat FITC-conjugated antibody (Sevac), diluted 1:40, was added for
30 min and after washing with PBS, coverslips were mounted in 70 %
glycerol and observed with a fluorescence microscope.

**Plaque assay.** Plaque assays were performed on WME cells
according to Türlir & Beard (1985). Briefly, an equal number of
haemagglutination units (HAU) of mutant or wt virus was adsorbed on
WME cells by incubation for 90 min at 37 °C. After removal of
unadsorbed virus, cells were overlaid with 0.9 % Sea Plaque agarose
(FMC) in DMEM with 10 % FCS. Ten days p.i., living cells were stained
with 0.01 % neutral red and plaques were counted.

**Replication assay.** 3T6 cells were plated on 24-well dishes and
infected with mutant or wt virus (5 HAU/5 × 10⁶ cells). At indicated
times p.i., cells were washed three times with PBS and harvested by
lysis in 400 µl per well of lysis buffer (10 mM Tris–HCl, pH 7.5, 5 mM EDTA,
0.5 % SDS, 100 µg/ml RNase A). Samples were incubated with proteinase
K (100 µg/ml) for 1 h at 50 °C and the DNA-containing solutions
extracted with phenol–chloroform and ethanol-precipitated. DNA was
precipitated with ethanol, digested with EcoRI and resolved on 1 % agarose
gels. The DNA was transferred on to a nylon membrane (Zeta-Probe, Bio-Rad) by
capillary transfer in 0.4 M NaOH, 0.5 M NaCl and hybridized with [³²P]-labelled
Py genome probes in hybridization buffer (6 x SSC, 0.1 % SDS, 2 x
Denhardt’s solution (Maniatis et al., 1982), containing 100 µg/ml salmon
sperm ssDNA) at 68 °C for 20 h. Washing was performed with 0.1 x
SSC, 1 % SDS at 68 °C and membranes were exposed to X-ray film.

**DNA content determination.** Aliquots of each virus (containing
the same amount of VP1, as estimated by SDS–PAGE) were diluted in TE
(10 mM Tris–HCl, pH 7.8, 1 mM EDTA) with 0.5 % SDS and 100 µg/ml
proteinase K. After incubation for 1 h at 50 °C, the DNA-containing
solution was extracted with phenol–chloroform and ethanol-precipitated.
DNA samples were resolved on 1 % agarose gels, transferred on to
membranes (Zeta-Probe, Bio-Rad) and viral DNA was visualized by
hybridization and autoradiography, as described above.

### Table 1. Mutated polyomaviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Sequence</th>
<th>Phenotype</th>
<th>p.f.u./ml</th>
<th>Specific infectivity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP2:</td>
<td>wt</td>
<td>Met Gly Ala…</td>
<td>myr VP2</td>
<td>2.0 × 10⁷</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*stopATG GGA GCC…</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>Met His Ala…</td>
<td>AtG CAT GCC…</td>
<td>No visible plaques</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Met Gln Ala…</td>
<td>ATG GAA GCC…</td>
<td>myr` VP2</td>
<td>5.0 × 10⁴</td>
</tr>
<tr>
<td>Q</td>
<td>Met Gln Ala…</td>
<td>ATG GAC GCC…</td>
<td></td>
<td>7.0 × 10⁵</td>
</tr>
<tr>
<td>M</td>
<td>Met Pro His Stop</td>
<td>ATG CGC CAC TGA</td>
<td>VP²⁻</td>
<td>No visible plaques</td>
</tr>
<tr>
<td>VP3:</td>
<td>wt</td>
<td>Met Ala Leu…</td>
<td>VP³⁺</td>
<td>2.0 × 10⁷</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*stopATG GGC TGG…</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Ala Ala Leu…</td>
<td>GGC CGC TGG…</td>
<td>VP³⁻</td>
<td>No visible plaques</td>
</tr>
</tbody>
</table>
Separation of virions and capsids by CsCl density gradients was performed according to Krauzewicz et al. (1990).

**Cell fractionation.** In situ fractionation of infected 3T6 cells was performed as previously described (Staufenbiel & Deppert, 1984). Briefly, cells were washed with KM buffer (10 mM MES, pH 6.2, 10 mM NaCl, 1.5 mM MgCl$_2$, 10 % glycerol, with 0.2 units/ml aprotinin) and sequentially extracted with buffer I (KM buffer plus 1 % NP-40, 0.1 mM EGTA, 1 mM DTT), buffer II (KM buffer plus 50 µg/ml DNase I), buffer III (KM buffer plus 2 M NaCl, 0.1 mM EGTA, 1 mM DTT) and buffer IV (KM buffer plus 50 µg/ml DNase I, 50 µg/ml RNase A). The residual material was solubilized by 1 % SDS in KM buffer. Aliquots of fractions were analysed by dot blot or SDS–PAGE and the presence of VP1 was detected with α-VP1 antibody.

**Electron microscopy.** Infected 3T6 cells grown on coverslips were washed with SB buffer (freshly prepared 0.1 M Na$_2$HPO$_4$, 0.1 M KH$_2$PO$_4$ at a ratio of 7:3, v/v, pH 7.2–7.4) and fixed with 3 % paraformaldehyde, 0.1 % glutaraldehyde in SB buffer at 4 °C for 20–50 min. Fixed cells were washed with SB buffer and dehydrated with increasing concentrations of ethanol on ice. Samples were flat-embedded into LR White resin (Polysciences Inc.). Ultrathin sections (60 nm) were mounted on grids, contrasted with uranyl acetate and Reynolds citrate and examined with a JEOL 1200 EX electron microscope.

### Results

#### Creation of mutant polyomaviruses

The aim of this work was to try to elucidate the role(s) of the hydrophobic myristyl residue at the N terminus of VP2 in the life cycle of polyomavirus. Py wt genomic DNA, and DNAs carrying mutations in the N-terminal amino acid sequence of VP2 or VP3, as summarized in Table 1(A), were prepared and transfected into 3T6 cells. Transfection efficiency of mutated genomes and their translation into viral proteins initially proved comparable with that of the wt genome, as determined by indirect immunofluorescence of transfected cells using the α-VP1 antibody (data not shown). After prolonged incubation (up to 7 days), however, the titres of isolated mutant viruses were low in comparison with those of wt virus. Cells transfected with mutated genomes did not exhibit cytopathic effects (CPE), whereas CPE, a typical sign of Py growth in cultured cells, was visible by the fifth day post-transfection with the wt genome. Sufficient titres of mutant viruses could be obtained when transfected cells were subcultured several times.

For all studied mutants, we did not observe any revertants to the wt phenotype. The absence of VP2 in viral particles of mutant M and VP3 in viral particles of mutant A was verified by SDS–PAGE and immunoblotting (Fig. 1A). In agreement with earlier data (Krauzewicz et al., 1990), the myr− VP2 of mutant E migrated slightly more slowly on SDS–PAGE than myristylated wt VP2. VP2 of mutant Q migrated at an intermediate position between the VP2 of wt and mutant E (Fig. 1A). Isolated particles of mutant H contained only a very small quantity of VP2 (Fig. 1A), and very little VP2 was detected in infected cell lysates (data not shown). VP2 and VP3 of wt Py and all mutants were still resolved as double bands. In contrast to wt Py, no [H]myristic acid-labelled VP2 was found in lysates of cells infected with the H, E or Q mutants (Fig. 1B).

#### Infectivity

The ability of the isolated mutant viruses to infect cells and express viral proteins was followed by indirect immunofluorescent detection of LT and VP1 in infected cells at different times p.i. Multiplicities of infection were related to HAU of virus isolates. Haemagglutination activity is the property of VP1 and should not be affected by the absence of other capsid proteins. A correlation between HAU and the amount of VP1 in viral isolates was verified by SDS–PAGE and αVP1 immunoblotting (not shown). Ratios between virions and empty particles, not distinguishable by haemagglutination, were estimated by comparison of yields after the separation of both types of particles in CsCl density gradients. The ratios were approximately 1 for all viruses examined, similar to values previously obtained for mutant E virus and for wt (Krauzewicz et al., 1990). In support of these results, when DNA was isolated from the same number of HAU of viral particles, no difference in yield of viral DNA between mutants and wt was observed (not shown).

In initial experiments, the same number of HAU of mutants or wt were used to follow the time-course of infection during...
Fig. 2. Analysis of LT antigen expression. 3T6 cells were infected with H, E, Q or wt viruses (10 HAU/5 x 10^4 cells, i.e. approximately 6 p.f.u./cell, calculated for the wt virus). At the indicated hours p.i., cells were lysed, proteins resolved on SDS–PAGE and LT antigen detected by Western blotting using αLT antibody (A). In parallel, infected cells were fixed and indirect immunofluorescence was performed with αLT antibody (B).

The expression of LT antigen was detected by Western blot and by indirect fluorescence (Fig. 2A, B). No delay in LT antigen expression in E and Q mutant-infected cells relative to wt was observed. Nuclei staining positive for LT appeared at 10 h p.i. and the number of positively stained cells increased equally in mutant- and wt-infected cells (evaluated up to 23 h p.i.). The number of LT-positive cells (and the signal on Western blots) was substantially lower for the H mutant. The number of cells staining positive for VP1 was followed over 48 h. Levels were similar for mutants E and Q and wt, but substantially lower for mutant H (Fig. 3A). No significant change in location and intensity of staining of late antigens was observed when αVP1 or αVP2/3-A antibodies were used (not shown).

The course of infection was then examined over several life cycles by staining infected cells over a 6-day period. As with wt, the number of cells infected with mutant E or Q increased until day 5, when a virus burst appeared with wt but not with
Fig. 3. Infectivity assay. (A) 3T6 cells on coverslips were infected with either mutant H, E, Q or wt viruses as indicated (black bars, mutant H; grey bars, mutant E; dark grey bars, mutant Q; white bars, wt) with an m.o.i. of 5 HAU/5 × 10^4 cells (3 p.f.u./cell calculated for the wt virus). At the indicated times p.i., cells were fixed and indirect immunofluorescence with αVP1 monoclonal antibody was carried out. The number of positively stained cells was scored per 10 microscopy fields for each virus. Mean values of two experiments are shown. (B) 3T6 cells infected with 1 HAU/5 × 10^4 cells (approximately 0-6 p.f.u./cell, calculated for the wt virus) of H, E, Q or wt viruses. Cells were fixed on the days p.i. (dpi) indicated, and stained and scored as in (A). (C) Immunofluorescent staining of 3T6 cells infected with 10^2 HAU of mutant A, 10^3 HAU of mutant M and 10^2 HAU of wt per 5 × 10^4 cells. Cells were fixed 48 h p.i. and indirect immunofluorescence with αVP1 monoclonal antibody was performed. Pictures were generated on an Olympus BX 60 fluorescent microscope equipped with a COHU High Performance CCD camera, and processed by Lucia 32G software. (D) 3T6 cells were infected (first infection) with mutant E or wt (10^2 HAU/5 × 10^4 cells). At 48 h p.i., infected cells were harvested and equivalence of infection was verified by VP1 immunofluorescent staining (on coverslips introduced into cultivation dishes). Positive cells per microscopic field were scored (10 fields for each virus). Subsequently, mutant and wt viruses were isolated from harvested cells and diluted to equal volumes. 3T6 cells infected with equal aliquots of virus isolates (second infection) were stained with αVP1 monoclonal antibody at 48 h p.i. and positive cells scored. Mean values of two experiments ± SD are given.
Polyomavirus VP2 and VP3 mutants

Fig. 4. Replication assay. 3T6 cells, infected with mutant or wt viruses (5 HAU/5 × 10⁴ cells, i.e. 3 p.f.u./cell, calculated for the wt virus), were lysed at the indicated hours p.i. (A) Total DNA was isolated, digested with EcoRI, resolved by electrophoresis and blotted as described in Methods. A band with a mobility corresponding to the size of linearized Py genome (5–3 kbp) was visualized by hybridization with an α-[32P]dATP-labelled probe against Py DNA. (B) Aliquots of lysates were resolved by SDS–PAGE and analysed by Western blot using α-VP1 monoclonal antibody.

not form visible plaques. The concentrations of viral particles needed to produce plaques in numbers comparable to wt were 400 times higher for the mutant E virus and approximately 28 times higher for the mutant Q virus (Table 1B). Mutant plaques were heterogeneous in size, only some of them reaching the size of wt plaques. When plaque assays were carried out on 3T6 fibroblasts, only wt plaques developed properly. No plaques were found with mutant H, and plaques from E and Q mutants were not sufficiently defined to be counted on 3T6 cells. The data were consistent with defects in the E and Q viruses being compensated for in epithelial cells in WME cultures, but not in fibroblasts.

Replication assay

To investigate further the different growth properties of the mutants, replication of viral DNA was studied during a 48 h life cycle. 3T6 cells were infected and DNA isolated as described in Methods. Fig. 4(A) demonstrates that the replication courses of wt and mutant genomes E and Q were comparable. Neither a time delay in the replication start nor significant quantitative differences were observed. Parallel detection of VP1 in lysates (Fig. 4B) showed that synthesis of VP1 followed DNA replication and no significant differences were observed between wt and mutants E and Q.

Only a small increase in DNA signal was detected with mutant H (Fig. 4A) and no DNA was detected with mutants A and M (not shown). The VP1 signal was below detection levels in lysates of cells infected with mutant H, A or M (not shown). These data are in agreement with previous experiments where substantially reduced infectivity of A, M and H mutants was observed in a 48 h cycle.

Electron microscopy studies

To find out whether there were differences between mutants and wt virus in their interactions with subcellular structures, electron microscopy (EM) studies were carried out. The data showed that cells successfully infected with mutant viruses exhibited no differences when compared with wt. New viral particles appeared in the nuclei in similar distinct patches under the nuclear membrane and in the proximity of nucleoli (Fig. 5A). Lysis of infected cells at 60 h p.i. and the release of virions could also be seen in both mutant- and wt-infected cells. At this time p.i., we could also see the adsorption of released viral particles and their internalization into monopino-cytic vesicles in surrounding non-infected cells (Fig. 5B). Thus, EM revealed no qualitative morphological differences between mutant and wt viruses.

Cell fractionation

Different methods of cell fractionation have been used previously to study SV40 and Py protein distribution within cells (Lin et al., 1984; Stamatos et al., 1987). In this study, cells were fractionated in situ according to the protocol of Staufenbiel & Deppert (1984), as described in Methods. Fig. 6 shows the distribution of VP1 in fractions of cells infected with either mutant E or wt virus. Newly synthesized VP1 accumulated predominantly in fraction 3, associated with chromatin and the cytoskeleton, and fraction 5, bound on the residual cytoskeleton and the nuclear matrix. Wt VP1 could also be found in the membrane fraction 1 (NP-40-soluble material), whereas only very small amounts of mutant E VP1 were reproducibly (in five independent experiments) detected in this fraction by dot blot (Fig. 6A). Surprisingly, when the samples were resolved by SDS–PAGE, VP1 of mutant E and wt in fraction 1 were observed at the same intensity (Fig. 6B).

Discussion

In the present work, the functions of the Py minor capsid proteins, and particularly of the myristic acid modification of VP2, were studied. A consensus sequence for N-terminal myristylation is conserved in the VP2s of all known polyoma-viruses indicating the importance of this modification during their life cycle. Following receptor attachment, wt Py virions are internalized into monopino-cytic vesicles (Griffith &
Consigli, 1984) and, via as yet poorly characterized pathways, brought to cellular sites of uncoating and replication. Our results on the infectivity of isolated wt and myr⁻ viruses, and electron microscopy experiments, identified no role for myristic acid in these early events. This in part contradicts previously published work where a delayed onset of viral DNA and VP1 synthesis in cells infected with myr⁻ mutant as well as approximately 20 times lower specific infectivity in plaque assays compared with wt was observed. The virus used had the N-terminal glycine of VP2 changed by a point mutation to alanine (Sahli et al., 1993).

In our studies, three different myr⁻ mutants were studied. In addition to the previously described mutant E, where the N-terminal glycine of VP2 was replaced by glutamic acid (E), we also exchanged glycine with neutral glutamine (Q) and basic histidine (H). In plaque assays performed on WME cells, we...
Polyomavirus VP2 and VP3 mutants

observed a decreasing infectivity in the order wt → Q → E and no plaques were formed with the H mutant, suggesting that not only the absence of the myristyl modification but also the character of the N terminus of VP2 influenced its properties.

The specific infectivity (as measured by plaque assays) of mutant Q was in general agreement, although slightly reduced, with observations on the alanine mutant described by Sahli et al. (1993), but neither mutant Q nor E exhibited an early step defect described for the alanine mutant. Particles of myr−mutants were internalized efficiently in vesicles morphologically indistinguishable from those used by wt particles. Comparable courses of the first cycle of infection, as estimated by immunofluorescent staining of VP1, demonstrated the ability of myr−mutants to enter and infect the cell with the same efficiency as wt Py. We also observed no delay in the appearance of the early gene product, the large T antigen, nor any significant quantitative differences in the course of replication among the mutant E and Q and the wt genomes. In lysates of cells infected with mutant H and in isolated H virions, only very low quantities of VP2 were detected, possibly explaining why the H mutant had a phenotype more similar to that of the VP2−mutant M. A defect of the H mutant (as well as M and A mutants) consists apparently in the very low efficiency of the delivery of viral genomes into cell nuclei, i.e. in the early steps of viral infection. In a few cells, where genomes reached the cell nucleus, further development of viral progeny appeared not to be substantially affected as immunofluorescent staining of LT (Fig. 2B) or VP1 (not shown) and the appearance of virion progeny in the nucleus (Fig. 5A) were comparable with those of wt Py. At present, we cannot distinguish between the possibilities that mutation to H affects the stability of the VP2 gene transcript or its translation product, since basic amino acids on the N terminus can assign a protein for degradation via a ubiquitin pathway (Bachmair et al., 1986).

The major loss of infectivity and the absence of a significant CPE during long-term infection of 3T6 cells by myr−mutants suggested a defect at a late stage of the viral life cycle, during virion assembly or successful reinfection of the surrounding cells by viral progeny. However, the results presented on Fig. 3(D) showed that a slightly (half) lower yield of viral particles per infected cell was achieved with myr−mutants as compared with wt virus. On the other hand, the ratio of capsids and virions obtained proved to be similar. Electron microscopic studies showed that 3T6 cells that were successfully infected with myr−mutants finally lysed, releasing viral progeny. A poor reinfection by mutated viruses could more probably be explained by their altered interactions with host cell structures. In situ fractionation of wt Py-infected cells demonstrated that newly assembled virions were concentrated at the nuclear matrix and subsequently also appeared in chromatin and NP-40-soluble fractions. The absence of VP1 signals from myr−mutants in NP-40-soluble material on dot blots with native structures versus its presence on Western blots after SDS treatment suggests that the antibody epitopes of mutated viruses (in the reactive DE loop of VP1) and possibly other virion surface substructures might be masked due to altered interactions with host cell structures. In situ fractionation of wt Py-infected cells demonstrated that newly assembled virions were concentrated at the nuclear matrix and subsequently also appeared in chromatin and NP-40-soluble fractions. The absence of VP1 signals from myr−mutants in NP-40-soluble material on dot blots with native structures versus its presence on Western blots after SDS treatment suggests that the antibody epitopes of mutated viruses (in the reactive DE loop of VP1) and possibly other virion surface substructures might be masked due to altered interactions with host cell structures. No such effects were observed with wt-infected cells. In agreement with this, Krauzewicz et al. (1990) observed slightly changed mutant E virion shapes by electron microscopy. Together with the finding that cells infected with myr−mutant E produce slightly lower amounts of infectious viral particles than cells infected with wt virus, fewer virions may thus be available for reinfection. Moreover, inappropriate complexes of newly created virions with cell structures might affect the specificity of cell-surface interactions during reinfection events and direct entering particles to a non-productive route. Productive and non-productive routes of viral particle movement in cells have been recently suggested (Krauzewicz et al., 2000).

In contrast to myr−mutants, mutants lacking either VP2 (mutant M and mutant H also exhibiting VP2 deficiency) or VP3 (mutant A) exhibited defects even during the early stages of viral infections. The adsorption and uptake of mutated

Fig. 6. 3T6 cells infected with mutant E or wt and fractionated in situ 48 h p.i. Aliquots of fractions were dot blotted (A) or analysed by SDS–PAGE and Western blot (B). VP1 was visualized by immunodetection with αVP1 monoclonal antibody (A) or with a mixture of αVP1 and αVP2/3-B antibodies (B). Fractions (fr): 1, NP-40-soluble material; 2, DNase-treated; 3, high salt extraction (chromatin, cytoskeleton); 4, DNase/RNase; 5, SDS solubilization (nuclear matrix, residual cytoskeleton).
virions through monopinocytic vesicles proceeded efficiently, as observed by electron microscopy of cell sections (not shown). Although receptor-interacting epitopes lie within the VP1 molecule (Stehele et al., 1994) and even recombinant capsid-like particles, composed of VP1 only, are able to enter 3T6 cells (Forstová et al., 1995; Richterová et al., 2001), the specificity of entry and further fate of mutant particles could be affected by the absence of the minor capsid proteins. An et al. (1999) found that recombinant particles composed of both VP1 and VP2 were the most effective competitors of wt infection. This is in agreement with our finding that viruses lacking VP2 were substantially less infectious than those lacking VP3.

Coexpression of VP2 is needed for the proper phosphorylation of VP1, at least in a heterologous system (Forstová et al., 1993; Li et al., 1995). The unique N-terminal part of VP2 (and not the myristic acid moiety) was found to be involved in mediating phosphorylation of a threonine residue occurring in the BC loop of VP1. Others have suggested a role for this modification in virus attachment and entry (Li et al., 1995).

The lesion in mutants lacking minor capsid proteins could also affect later sorting and uncoating processes. Incoming SV40, as well as Py and polyoma VP1 capsid-like particles, accumulate in the endoplasmic reticulum (Pelkmans et al., 2001). The unique N-terminal part of VP2 (and not the myristic acid moiety) was found to be involved in mediating phosphorylation of a threonine residue occurring in the BC loop of VP1. Others have suggested a role for this modification in virus attachment and entry (Li et al., 1995).

When considered altogether, myristylation of Py VP2 did not appear obligatory for any particular infection step. With mny− mutants, no step was totally abolished. The myristyl moiety appeared to have a role in the fine-tuning of VP2 functions, possibly by maintaining a biologically active virion surface conformation. Its absence resulted in slightly changed interactions with cell structures and a concomitant decrease in virus progeny production. These effects accumulated during repeated reinfections. On the other hand, the expression of both minor capsid proteins and their presence in viral particles were essential for viral infectivity. The total absence of either VP2 or VP3 in virions was manifested even in early stages of infection, suggesting additional distinct functions for polyomavirus minor capsid proteins.

This work was generously supported in part by the HHMI USA (grant no. 75195-540501), the Grant Agency of the Czech Republic (the grant no. 204/00/0271), Ministry of Education of the Czech Republic (MSM 113100003), the Medical Research Council, UK, and by a grant from The Wellcome Trust, UK, including a fellowship to J.F.

References


Received 21 February 2002; Accepted 5 April 2002