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Characterization of Recombinant Polioviruses Expressing Regions of Rotavirus VP4, Hepatitis B Surface Antigen, and Herpes Simplex Virus Type 2 Glycoprotein D

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Recombinant polioviruses expressing antigens from rotavirus, herpes simplex virus type 2, and hepatitis B virus were generated. Fusion of the heterologous polypeptides to the amino terminus of the poliovirus polyprotein did not prevent myristylation of VP0, suggesting a novel mechanism of myristylation for these recombinant viruses. The effects of the parental genetic background, different foreign sequences, and different insert sizes on growth characteristics were compared. Both the size and the nature of the heterologous sequence appeared to be factors influencing the growth and stability of recombinant polioviruses. All of the recombinants showed a temperature-sensitive phenotype, regardless of the genetic background (attenuated or wild type) from which they were derived. Preliminary studies with transgenic mice carrying the poliovirus receptor gene are discussed.

Poliovirus is a human enteric pathogen that belongs to the *Enterovirus* genus of the *Picornaviridae* family and undergoes extensive multiplication in the human intestinal tract. It has a positive-sense single-stranded RNA genome of approximately 7.5 kb. The viral RNA encodes a single large polyprotein precursor that is cleaved by virus-encoded proteases to give rise to the mature structural and nonstructural proteins (6, 20, 21). The capsid protein precursor, P1, is cotranslationally myristylated and is cleaved into VP0, VP3, and VP1 (21, 30). The final processing step is the autocatalytic cleavage of VP0 into VP2 and VP4, which takes place after RNA encapsidation. A myristate group remains covalently linked to each VP4 molecule and plays an important role in the stability of the virus capsid (3, 4).

The use of polioviruses as vectors for the delivery of heterologous antigens to the intestinal mucosa has several advantages. Attenuated strains of poliovirus (Sabin strains) have proven to be very safe and efficacious. The use of these strains as vectors for heterologous antigens provides a system for making combined pediatric vaccines. Oral poliovirus vaccine results in generation of mucosal plus systemic immunity (19), which is advantageous for the generation of protection at the mucosal surface.

In previous work, we have characterized Sabin 3 recombinant viruses that carried fragments of the rotavirus outer capsid glycoprotein VP7 gene in two different locations of the poliovirus genome (15, 17). In the present study, we generated recombinant polioviruses carrying genes encoding several different antigens in a variety of poliovirus backgrounds. Characterization of vectors included comparison of the effects of attenuated and wild-type (wt) backgrounds and different poliovirus serotypes (type 3 and type 1) on the growth of recombinant viruses. The vectors constructed in wt backgrounds were generated with the aim of improving the replication of the recombinant polioviruses in transgenic mice carrying the poliovirus receptor gene (23).

The poliovirus vectors used in this work are described in Table 1. Type 3 vectors were constructed as previously described (17), using either the plasmid pLED3 (27, 29) or pT7Leon (25), containing a cDNA encoding the full-length genome of P3/Sabin or P3/Leon poliovirus, respectively. A multiple cloning site (MCS) encoding cleavage sites for the restriction enzymes *Not*I, *Hpa*I, and *Pac*I was incorporated to clone foreign sequences in frame with the poliovirus polyprotein (15, 17). The addition of short sequences encoding a 3C or a 2A protease cleavage site flanking the foreign insert resulted in the release of the heterologous polypeptide, leaving intact functional poliovirus proteins. The heterologous nucleotide sequence was carried in the viral genome, but the foreign polypeptide was not present in the mature virions. The vectors designated V1 and V2 (Table 1) were constructed in a P3/Sabin background. In all plasmids, the poliovirus sequences were cloned downstream from a T7 RNA polymerase transcription promoter, allowing production of infectious RNA (28).

A type 1 poliovirus vector constructed in a P1/Mahoney background (MoV-1.3), was kindly provided by Raul Andino (University of California, San Francisco) and has been described in a recent publication (1). This construct has an MCS at the 5′ end of the poliovirus polyprotein followed by a polyglycine sequence and an artificial $3C^{pro}$ cleavage site.

Heterologous sequences incorporated into poliovirus vectors included fragments of rotavirus VP4 and VP7, herpes simplex virus type 2 (HSV-2) glycoprotein D (gD), and hepatitis B surface antigen (HBsAg) pre-S2. They are described in Table 2. Although the role of mucosal immunity in protection from HSV and hepatitis B virus infections has not been addressed, it is thought that since those viruses first encounter a mucosal surface during sexual transmission, stimulation of both the systemic and mucosal immune systems would be advantageous.

Rotavirus is the main etiologic agent of viral diarrhea in infants under 2 years of age. Rotavirus particles contain VP4

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TABLE 1. Poliovirus vectors[a]

Vector name (reference)	Parental virus	Insertion site	Insert description
pLED3V1	Sabin 3	5′ end	MCS + $3C^{Pro}$ site
pLED3V2 (17)	Sabin 3	P1-P2 junction	$2A^{Pro}$ site + MCS + $3C^{Pro}$ site
pLeon	wt PV3	5′ end	MCS + $3C^{Pro}$ site
pMoV-1.3 (1)	wt PV1	5′ end	MCS + poly(Gly) + $3C^{Pro}$ site

[a] Abbreviations: PV, poliovirus; Pro, protease.

spikes protruding from a smooth outer shell composed of VP7 (14, 22). Both outer capsid proteins are capable of inducing neutralizing antibodies. Rotavirus serotypes based on VP7 reactivity are called G types, and serotypes based on VP4 are called P types (14). Recombinant polioviruses carrying gene fragments of rotavirus VP7 have been described in a previous publication (17). The VP4 gene from the human rotavirus strain KU (serotype G1, P1A) was chosen for the present studies because it was reported to elicit antibodies that are able to neutralize the four most common G serotypes (G1 to G4) of viruses that cause disease in children (4a, 9). A cDNA of rotavirus KU VP4 was kindly provided by Mario Gorziglia (National Institutes of Health). Three different domains of VP4 were selected for expression in poliovirus vectors, as follows. (i) A 300-bp region of VP4 containing the protease cleavage site, designated C in this work, was used, since proteolytic cleavage of VP4 into VP8* and VP5* is required for rotavirus infectivity (10, 14). (ii) A region of rotavirus VP4 that includes the putative fusion domain (amino acids 384 to 404 [13]) was included, since this region, which has been localized to the ends of the virus spikes by electron microscopy (22), is thought to be involved in virus penetration into cells. This fragment (VP4F) also contains a linear neutralizing epitope (amino acids 296 to 313) that is well conserved in human rotavirus strains of serotypes G1, G3, and G4 (26). (iii) The entire amino-terminal VP8* subunit of KU VP4 (756 bp) was selected because the epitopes responsible for eliciting the neutralizing antibodies that cross-react with types G1 to G4 were mapped to this region (8, 9). It is worth mentioning that human rotaviruses with VP7 types G1, G3, and G4 share the same VP4 type (P1A).

gD is a virion envelope component of HSV-2 and is expressed on the surface of infected cells. When used as a purified immunogen, it elicits a neutralizing antibody response that protects mice against challenge with HSV-2 (7). A fragment of gD containing a neutralizing linear epitope bound by the monoclonal antibody DL6 (5) was selected for expression.

TABLE 2. Heterologous antigens inserted into poliovirus vectors[a]

Viral protein	Name	Fragment (amino acids)	Insert size[b] (nucleotides)
RV VP4	VP4C	178–279	300
	VP4F	278–450	513
	VP8	2–253	756
RV VP7	Δ150c13	216–295	237
	Δ101c9	225–295	210
HSV-2 gD	gD	260–335	225
HBsAg	PS2	120–175	165

[a] Abbreviations: C, cleavage; F, fusion; RV, rotavirus; PS2, pre-S2 region of HBsAg.

[b] The nucleotides encoding the MCS and the $3C^{pro}$ site are in addition to the sizes indicated.

Another heterologous antigen used in this work is a domain of HBsAg. The 165-bp (55-amino acid) region upstream of the gene sequence encoding the small HBsAg is called the pre-S2 region. Immunodominant epitopes recognized by human antibodies to hepatitis B virus have been mapped to the pre-S2 polypeptide (18). This region, which is also required for polyalbumin binding (12), was cloned into some of the available poliovirus vectors.

Recombinant polioviruses were generated after transfection of HeLa or Vero cells with RNA transcribed from the DNA constructs described above. In vitro transcription was carried out as described previously (17), and transfection was done by the DEAE-dextran method (27, 28). Recombinant viruses were identified by reverse transcription of the viral RNA followed by PCR amplification of the region of the genome containing the foreign insert with poliovirus primers (17), and titers were determined on Vero cell monolayers.

Fusion of a foreign polypeptide at the amino terminus of the viral polyprotein did not prevent myristylation of VP0. The amino-terminal glycine residue of poliovirus VP0 is covalently linked to a single molecule of myristic acid (*n*-tetradecanoic acid) and is required for virus infectivity (3, 4). The three-dimensional structure of poliovirus revealed that the myristate moieties in the mature virion are clustered near the fivefold vertices, suggesting that they may be involved in stabilizing interactions between the five protomer subunits that form a pentamer (4).

In nonrecombinant polioviruses, myristylation is a cotranslational modification: the initial methionine is cleaved by a cellular enzyme, and the exposed glycine is subsequently myristylated by the host enzyme *N*-myristoyltransferase. In recombinant polioviruses carrying a foreign insert at the amino terminus of the polyprotein, the glycine is exposed only after the cleavage of the QG junction by the virus-encoded protease 3C. This implies that at least two more steps are needed for the recombinant poliovirus polyprotein to become myristylated: the translation of the polyprotein for the protease to become available and at least one additional cleavage step. To test for proper myristylation, HeLa cell monolayers grown in 12-well plates were mock infected or infected with the poliovirus strains P3/Leon and P3/Sabin and with the recombinants Leon-VP4C, Leon-gD, and V1gD (Sabin background) at a multiplicity of infection of 10, as described previously (17). The proteins were labeled with 150 μCi of [^{3}H]myristic acid (NEN Research Products, Boston, Mass.) per ml at 3 h postinfection. After 3 h, the cells were lysed in radioimmunoprecipitation assay buffer and immunoprecipitated with a horse polyclonal antibody to type 3 poliovirus (16, 17).

Myristylation of VP0 and VP4 was found for all of the viruses tested (Fig. 1). Similar amounts of VP0 were labeled in all of the viruses, but the amount of labeled VP4 was significantly larger for P3/Leon and P3/Sabin, without foreign inserts (Fig. 1, lanes 2 and 3). Considering that the cleavage of VP0 into VP4 and VP2 takes place after the encapsidation of the viral RNA (21), these results are consistent with the data that the recombinants are slower in assembly than their parents without inserts (17).

The lysates from cells infected with recombinant polioviruses expressing gD or VP4C were also immunoprecipitated with the monoclonal antibody DL6 (anti-gD) or with a polyclonal antibody to the rotavirus VP4 protease cleavage peptide (C), respectively. The polypeptides gD and VP4C were not myristylated (data not shown).

Effect of temperature on the growth of wt and recombinant polioviruses carrying heterologous sequences. The attenuated Sabin strains of polioviruses (Sabin 1 to 3) have a temperature-

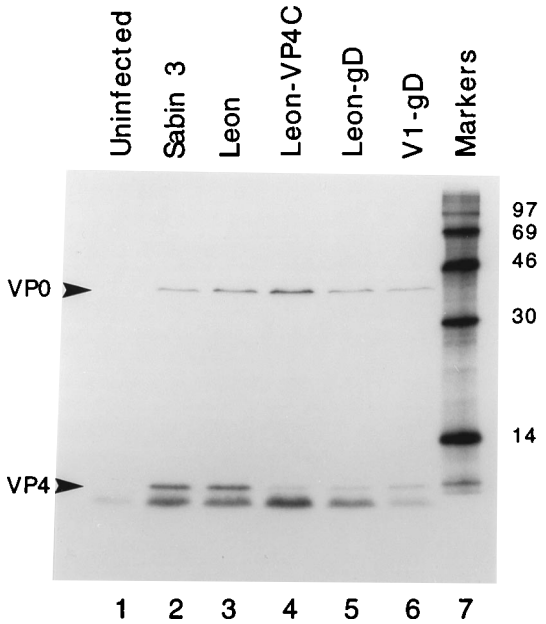

FIG. 1. Foreign sequences inserted at the amino terminus of the poliovirus polyprotein did not prevent myristylation of VP0. Proteins in infected cells were labeled with [^{3}H]myristic acid as described in the text and were immunoprecipitated with anti-type 3 poliovirus serum before separation in a 15% polyacrylamide gel. The recombinant polioviruses used and the positions of the myristylated proteins VP0 and VP4 are indicated. Numbers at the right are molecular weights in thousands.

sensitive (*ts*) phenotype, while the wt strains do not (11). The effect of the foreign insert on the growth of wt and Sabin recombinant polioviruses in culture at different temperatures was studied. The viruses used in this study were wt recombinants carrying fragments from three different regions of rotavirus VP4 (VP4C, VP4F, and VP8) and Sabin 3 recombinants carrying a fragment of HSV gD or hepatitis B virus pre-S2. P3/Leon, P1/Mahoney, and P3/Sabin were included as controls. Virus titers were determined in plaque assays at 33.5, 37, and 39°C. The differences between the titers measured at different temperatures were calculated (Table 3).

As shown in Table 3, all of the recombinants with wt backgrounds behaved as *ts* viruses compared with their parents without inserts. The titers of the wt-derived recombinant viruses at 39°C were 2 to 3 log units lower than those at 37°C.

TABLE 3. Effect of temperature on growth of wt and recombinant polioviruses

Virus	Difference between titers (log PFU/ml) at the following temps (°C):	
	37 − 39	37 − 33.5
P3/Leon	0.13	0.07
P1/Mahoney	0.13	0.43
P3/Sabin	4.07	0.16
P3/Leon-VP4C	2.94	0.69
P3/Leon-VP4F	2.51	0.12
P3/Leon-VP8	2.49	0.46
P1/MoV-VP4C	2.84	0.02
P1/MoV-VP4F	2.83	0.10
P3/V1gD (Sabin)	6.06	0.14
P3/V2gD (Sabin)	5.60	0.12
P3/V1PS2 (Sabin)	5.09	0.55

Furthermore, the recombinants made in a Sabin 3 background were more *ts* than their Sabin 3 parent (titers at 39°C were 4.5 to 6 log units lower than titers at 37°C). Interestingly, V2gD, a virus derived from a Sabin 3 vector with a foreign insert located at the junction of poliovirus precursors P1 and P2, behaved in the same manner as the viruses that had the foreign insert at the 5′ end of the poliovirus polyprotein (Table 3). No significant differences between the *ts* phenotypes of recombinants made in a type 3 background and of recombinants made in a type 1 background were detected. No significant differences were found in the titers of all of the polioviruses at 33.5°C compared with 37°C.

We do not know which step in viral replication is affected by temperature for the recombinant polioviruses. The infectivities of recombinant polioviruses was found to be less stable than those of their parents after a 48-h incubation period at 37 and 39°C, although there was no significant difference in the stabilities of the infectivity after incubation at the different temperatures (unpublished data).

The insertion of foreign sequences may affect the stability of the secondary structure of the viral RNA. This could ultimately affect crucial steps at higher temperatures, such as encapsidation of the RNA, RNA transcription or translation, or the ability of the RNA to form a specific secondary structure capable of interacting with cellular and/or viral factors. Other features that are specific to the replication of the recombinant polioviruses, such as the addition of a novel cleavage step or the myristylation of VP0 after the cleavage of the foreign insert, may also contribute to the *ts* phenotype.

Comparison of type 1 and type 3 poliovirus vectors carrying the same rotavirus VP4 fragment. One-step growth curves were performed for comparative studies as described previously (17). Suspension cultures of HeLa cells were infected at a multiplicity of infection of 10. Aliquots were withdrawn at different times postinfection, and titers were determined by plaque assays.

Recombinant polioviruses in type 3 and type 1 backgrounds (Leon-VP4C and MoV-VP4C, respectively) showed similar growth kinetics (Fig. 2A). With both recombinant viruses, there is a 2-h delay in the assembly of infectious virions compared with that of the parent viruses without insert. The growth of both recombinant viruses is also slower, and the final titers are lower than those of their counterparts. However, P3/Leon-VP4C grew to lower titers than P1/MoV-VP4C. The recombinant P1/MoV-VP4C also showed larger plaques in plaque assays.

To study the kinetics of RNA synthesis, HeLa cells in suspension were infected at a multiplicity of infection of 10 in the presence of 5 μg of actinomycin D per ml and 150 μCi of [^{3}H]uridine per ml. Samples were withdrawn at different times postinfection and processed as described previously (17). For both poliovirus types, no significant difference in RNA synthesis was found when viruses carrying heterologous inserts at the 5′ end of the viral open reading frame were compared with their counterparts without inserts (Fig. 2B). Wild-type viruses P1/Mahoney and P3/Leon showed a dramatic decrease in RNA accumulation by 12 h postinfection, while the recombinant viruses still maintained a high level of RNA synthesis. This suggests that cell death is delayed in the latter virus, as would be expected from the lower growth rate.

Viruses generated in type 3 vectors containing a polyglycine sequence immediately upstream of the artificial cleavage site showed significantly lower titers (2 log units) and were more difficult to grow than type 3 viruses lacking this sequence. The same result was observed for both attenuated P3/Sabin and wt P3/Leon backgrounds (data not shown). In contrast, the pres-

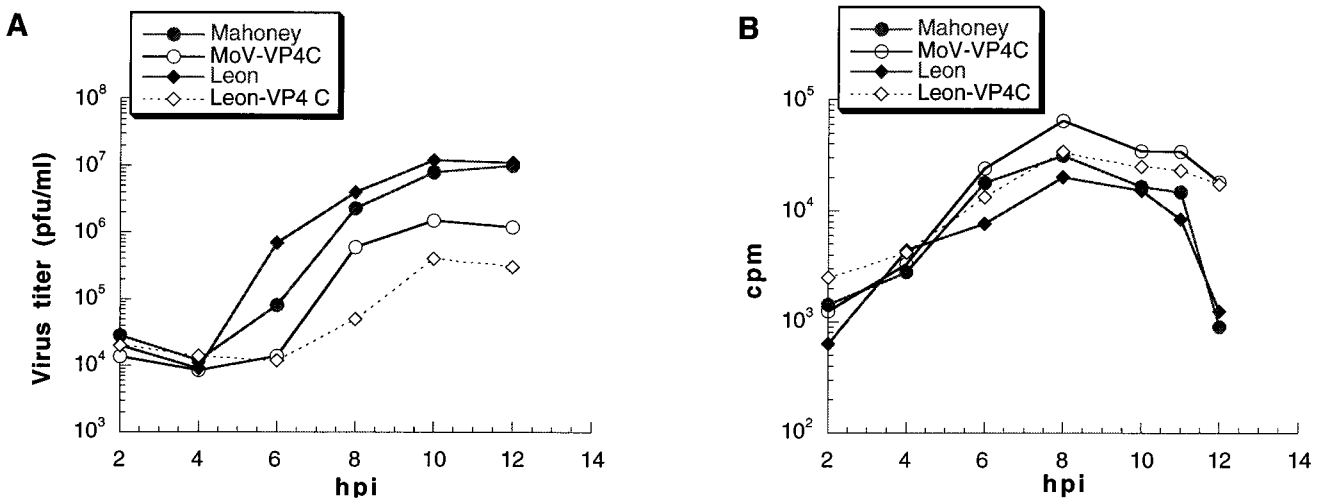

FIG. 2. Comparison of the growth of type 3 and type 1 poliovirus recombinants carrying 300-base insertions of rotavirus VP4 and of their respective parental viruses. One-step growth curves (A) and kinetics of RNA synthesis (B) are shown. Suspension cultures of HeLa cells were infected at a multiplicity of infection of 10 with P1/Mahoney, P3/Leon, MoV-VP4C, and Leon-VP4C. For single-step growth studies, samples were taken every 2 h, and the cells plus the supernatants were freeze-thawed three times before titration by plaque assay. For the RNA synthesis studies, total RNA was labeled with [^{3}H]uridine in the presence of 5 μg of actinomycin D per ml. Samples were taken every 2 h, and the radioactivity incorporated into trichloroacetic acid-precipitable material was measured by scintillation counting. The values for mock-infected cells have been subtracted from each of the corresponding values shown. hpi, hours postinfection.

ence of the polyglycine sequence seems to work very well in the wt type 1 vector, MoV-1.3 (1).

Characterization of recombinant poliovirus carrying inserts with different sizes in the same poliovirus vector. The effects of divergent heterologous sequences and insert sizes on the growth properties of recombinant polioviruses were studied. Results for recombinant polioviruses containing three different inserts in the P3/Leon background are shown in Fig. 3A. The sizes of the inserts are as follows: Leon-gD, 225 bases; Leon-VP4C, 300 bases; and Leon-VP4F, 513 bases. The growth rate appears to become slower as the insert size increases.

The effect of different inserts on the kinetics of growth of type 1 polioviruses is shown in Fig. 3B. For the recombinant polioviruses MoV-PS2 and MoV-VP4C, the insert lengths are 165 and 300 bases, respectively. The type 1 recombinants, like the type 3 recombinants, also showed a delay in the assembly of infectious viruses, but the final titers of both viruses were closer to those of their parents than was the case for the type 3 recombinant viruses. Another type 1 recombinant, MoV-VP4F, carrying a 513-bp VP4 fragment also grew to a similar final titer (data not shown).

Figure 3C shows a comparison between one-step growth curves of two Sabin 3 recombinant viruses carrying an insertion of rotavirus VP7 at the junction of P1 and P2 viral precursors (V2/Δ101c9 and V2/Δ150c13, carrying foreign insertions of 210 and 237 bases, respectively). The 210 bases of the VP7 inserts in these viruses were identical (13), but the latter insert had an extra 27 bases at the 5′ end. Despite the close similarity, the titers of the virus carrying the shorter insert were three to five times higher.

Considering that most of the heterologous inserts mentioned above had different sequences, it was difficult to differentiate between the influence of the different natures of the sequences (which may determine, for instance, misfolding at the protein or RNA level) and the influence of the different sizes on the growth of the recombinant poliovirus. However, the tendency was for polioviruses carrying smaller foreign inserts to grow better. Viable viruses were more readily obtained with insertions of less than 300 bases, and these inserts were more stably maintained in the poliovirus genome after serial passages in cell culture.

Recombinant polioviruses carrying the same insert in a wt or attenuated background were similarly attenuated for growth in tissue culture. The same insert, a 225-bp fragment of HSV gD, was cloned into P3/Sabin-derived or P3/Leon-derived vectors. Recombinant viruses were characterized and serially passaged, and their titers were determined. One-step growth curves showed the same kinetics of growth in tissue culture for both viruses, including the same 2-h delay in assembly of infectious viruses, possibly because of the presence of a new cleavage step during the replication cycle of the recombinants or because of delayed myristylation (Fig. 3D). It is not known how the presence of the foreign insert influences the neurovirulence phenotype of recombinant viruses derived from P3/Leon or P1/Mahoney strains, but these results suggested that the insert attenuated the growth of both recombinants in tissue culture to the same degree, regardless of whether the recombinant viruses had wt or attenuated backgrounds.

Expression of Leon-VP4C in transgenic mice generated antibodies that recognized poliovirus proteins and the VP4C fragment. Transgenic mice carrying the human poliovirus receptor (23, 24) were inoculated intraperitoneally with 10^6 PFU of the recombinant virus Leon-VP4C. All of the mice were tested for preexisting antibodies to rotaviruses by enzyme-linked immunosorbent assay and were found to be negative. The mice were boosted twice at 3-week intervals and bled 2 weeks after the last boost. The serum was used for immunoprecipitation of ^{35}S-labeled proteins from cells infected with MoV-VP4C recombinant poliovirus. As shown in Fig. 4, the VP4C polypeptide as well as poliovirus proteins were immunoprecipitated with serum from mice inoculated with Leon-VP4C (lane 1). Lanes 2 and 3 of Fig. 4 show the same labeled lysate immunoprecipitated with serum made against a peptide that maps to the VP4 cleavage region or with serum from transgenic mice inoculated with phosphate-buffered saline (PBS), respectively.

The transgenic mice carrying the poliovirus receptor are being studied in several laboratories as a model to replace

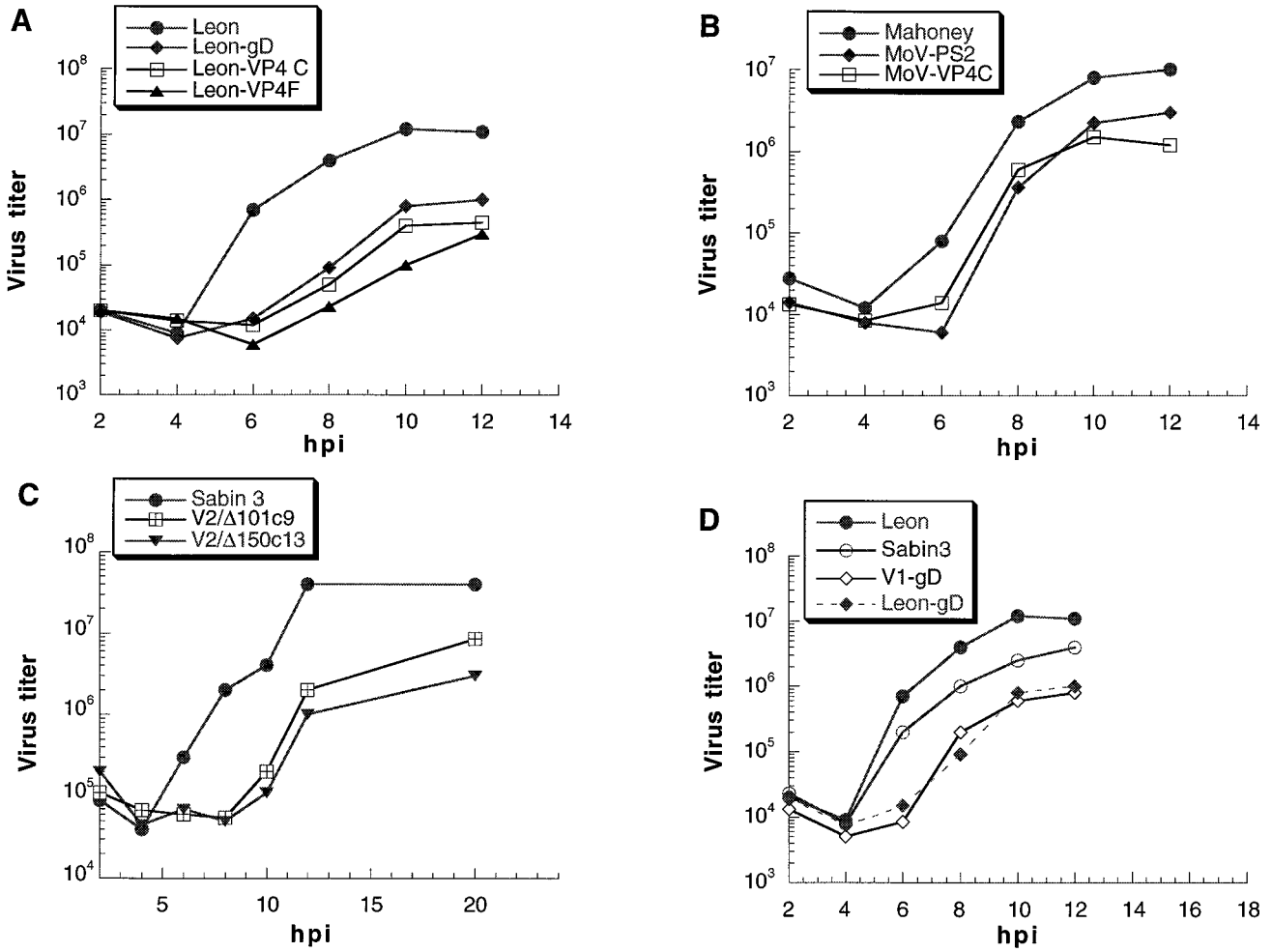

FIG. 3. Effect of different foreign gene insertions on the growth of type 3 or type 1 recombinant polioviruses. The experiments were performed as described in the legend to Fig. 2 for single-step growth curves. (A) One-step growth curves of P3/Leon recombinants carrying insertions of 250 bases (HSV gD), 300 bases (VP4C), and 513 bases (VP4F). (B) One-step growth curves of P1/Mahoney recombinants carrying insertions of 165 bases (hepatitis B virus PS2) and 300 bases (VP4C). (C) One-step growth curves of viruses carrying rotavirus VP7 insertions of 210 and 237 bases (V2/Δ101c9 and V2/Δ150c13, respectively) at the junction of P1 and P2 poliovirus precursors (for a further description of these two viruses, see reference 17). (D) One-step growth curves of poliovirus recombinants derived from P3/Leon (wt) or P3/Sabin (attenuated) strains carrying the same insert, a fragment of 225 bases from HSV gD. hpi, hours postinfection.

monkeys for poliovirus neurovirulence tests. In this case, the emphasis is on poliovirus replication in neural tissues. In order to test the immunogenicities of the foreign antigens expressed through poliovirus vectors, these viruses have to be able to replicate in other tissues. We found that following intramuscular inoculation of Sabin 3 recombinants into transgenic mice, the responses to our foreign antigens (which require viral replication) were low, although responses to poliovirus structural proteins (which do not require viral replication) were high. Relatively little work has been done on replication studies in transgenic mouse tissues other than neural tissue. P1/Mahoney poliovirus was found to replicate actively in transgenic mouse muscle after intramuscular injection (24). Similar studies in our laboratory found that P1/Mahoney and P3/Leon replicated in transgenic mouse muscle, but oral replication of any poliovirus (wt or Sabin) was not detected (results not shown). A limited replication of recombinant viruses made in a wt background after intraperitoneal inoculation (or even a single round of infection) might have been sufficient to elicit the immune response to the heterologous antigens found in this work. Similar results have been found by using defective "minireplicon" polioviruses (2). Studies are in progress to test the immunogenicities of recombinant polioviruses in cynomolgus macaques. The results shown in Fig. 4 indicate that studies with the transgenic mouse model are useful for preliminary testing of in vivo expression of heterologous antigens delivered by poliovirus vectors.

Final conclusions. Further studies are needed to understand the factors influencing the behavior of poliovirus carrying foreign antigens. The *ts* phenotype and the novel mechanism of myristylation are two features shared by all of the recombinants regardless of the parental background. There are other characteristics that seem to be dependent on the parental serotype or the nature of the sequences inserted. It appears that both the size of the inserted gene and the nature of the sequences are factors influencing the growth and stability of recombinant polioviruses. Studies to investigate the mucosal immune response to the heterologous antigens delivered by these recombinant viruses in an appropriate animal model are also crucial for further progress. A transgenic mouse able to support replication of polioviruses in the digestive tract will be an asset.

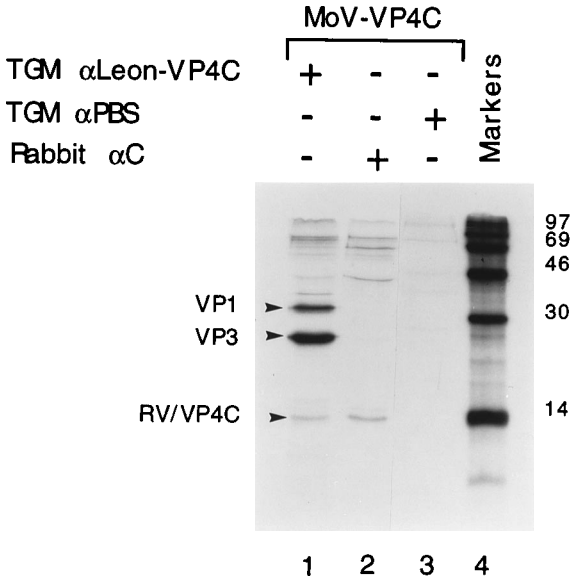

FIG. 4. Transgenic mice expressing the human poliovirus receptor and inoculated with the type 3 poliovirus-rotavirus recombinant Leon-VP4C generated antibodies to the foreign rotavirus fragment. Labeled proteins in cells infected with MoV-VP4C were immunoprecipitated with serum from transgenic mice (TGM) inoculated with Leon-VP4C (lane 1) or PBS (lane 3). Lane 2 shows labeled proteins immunoprecipitated with an antibody (α) made to a small peptide that maps to the VP4 cleavage site. The rotavirus VP4C fragment (RV/VP4C) and poliovirus proteins are indicated. Numbers at the right are molecular weights in thousands.

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