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Protein 2A Is Not Required for Theiler's Virus Replication

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Nonpolar mutations were introduced into all 12 regions of the genome of Theiler's murine encephalomyelitis virus. In agreement with data previously reported for other picornaviruses, mutations in regions 2B, 2C, 3A, 3B, 3C, and 3D totally abrogated viral RNA replication. Viruses with deletions in each of the capsid proteins retained RNA replication proficiency, although they were unable to propagate from cell to cell. As reported previously, mutations in the leader protein did not impair RNA replication or virus production in BHK-21 cells. Surprisingly, region 2A also appeared to be dispensable for the replication process. Indeed, up to 77 of the 133 amino acids of 2A could be deleted without significantly affecting RNA replication. 2A mutant viruses had only a slow cytopathic effect for BHK-21 cells and were totally avirulent for mice. As was the case for mutants lacking the leader protein, viruses with deletions in 2A propagated in BHK-21 cells, but their propagation was highly restricted in L929 cells.

Theiler's murine encephalomyelitis virus (TMEV; also referred to as Theiler's virus) is a picornavirus responsible for a chronic demyelinating disease of the central nervous system of the mouse (8, 21, 35) (for reviews, see references 4 and 31). This virus is classified as a cardiovirus together with encephalomyocarditis virus (EMCV) and the closely related mengovirus. The genome of picornaviruses encodes a unique large polyprotein which undergoes autoproteolytic cleavage to yield all the mature proteins necessary for the viral cycle (for a review, see reference 38). Ten of these proteins are well conserved among picornaviruses: six proteins (namely, 2B, 2C, 3A, 3B, 3C, and 3D), which are either directly or indirectly involved in RNA replication, and four proteins (VP1, VP2, VP3, and VP4), 60 copies of which assemble to form the viral capsid. Two proteins encoded by Theiler's virus, the leader (L) protein and protein 2A, play unknown roles in the virus cycle.

Leader proteins are encoded by both cardioviruses and aphthoviruses. The leader protein of foot-and-mouth disease virus (FMDV; an aphthovirus), has protease activity (34) and participates in host cell shutoff through cleavage of the P220 component of the eIF4G translation factor (9). The leader proteins of cardioviruses (EMCV and Theiler's virus) show no similarity to that of FMDV. They contain a zinc-binding motif (6). Their role in replication and pathogenesis is unknown. Recently, it was shown that the deletion of the leader region in the genome of Theiler's virus had no effect on the replication and spread of the mutant virus in BHK-21 cells. In contrast, propagation of leader mutants was strongly inhibited in L929 cells and in vivo, resulting in complete attenuation of virulence (5, 17).

Protein 2A is encoded by all picornaviruses but differs strikingly among different subgroups of picornaviruses. In enteroviruses and rhinoviruses, protein 2A is a chemotrypsin-like serine protease that is responsible for primary cleavage of the polyprotein between VP1 and 2A (13, 37). It is also capable of cleaving the P220 component of the cap-binding protein complex, resulting in a strong shutoff of host protein synthesis (11, 18, 22, 37). Protein 2A has also been found to indirectly enhance the translation initiation rate from the internal ribosome entry site (15, 23, 40). Finally, several mutations in the 2A region of poliovirus have been shown to cause partial or complete inhibition of viral replication (26, 39).

In aphthoviruses, the 2A protein consists of no more than 16 or possibly 18 amino acids (10, 32). This short sequence has the ability to promote cotranslational cleavage at its C-terminal end in the sequence NPG/P, thereby dissociating the L-P1-2A and 2B-2C-P3 precursors during the synthesis of the nascent viral polyprotein.

In hepatitis A virus, protein 2A has no proteolytic activity. Protease 3C was shown to be responsible for both VP1-2A and 2A-AB cleavages (33). The role of protein 2A in hepatitis A virus remains unknown. 2A was found to be associated with protein VP1 as a precursor, PX (1). This precursor is detected in pentamers, but the 2A moiety of PX is eliminated in subsequent steps of capsid assembly. Surprisingly, a recombinant hepatitis A virus harboring a deletion in the 2A region conserved its infectious character in vitro and its virulence for marmosets (16).

The 2A protein of cardioviruses is about 140 amino acids long. It does not have any sequence homology with the 2A proteases of rhinoviruses and enteroviruses or with protein 2A of hepatitis A virus. Its C terminus is similar to the 16-amino-acid 2A protein of aphthoviruses and is involved in primary cleavage of the polyprotein, which occurs between proteins 2A and 2B (3, 10, 14, 28). The larger part of protein 2A does not resemble any identified protein sequence. Furthermore, the 2A protein largely diverges between Theiler's virus and EMCV, although it is highly conserved among various isolates of either virus. The role of protein 2A in cardioviruses is still a mystery.

In this work, we showed that protein 2A was dispensable for RNA replication of the viral genome. Furthermore, we observed that viruses with large deletions in the 2A region were infectious for BHK-21 cells, although they had restricted

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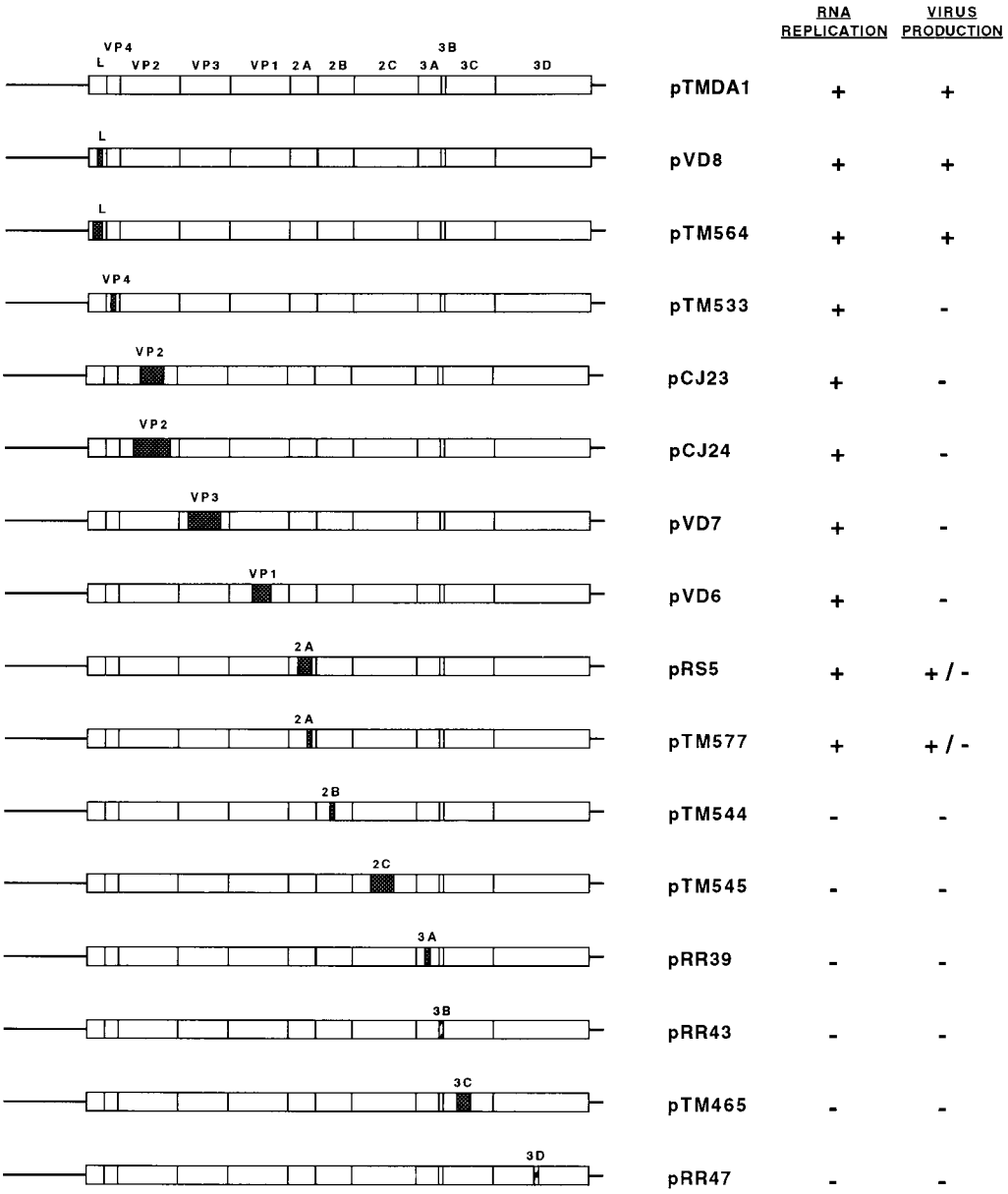

FIG. 1. Mutations introduced into the genome of Theiler's virus strain DA1. The genome of the virus is shown. Thin lines represent 5′ and 3′ noncoding regions, and boxes correspond to the regions encoding the different mature proteins. In-frame deletions are shown as stippled boxes, and amino acid replacements are symbolized by hatched boxes. The construction of mutants is detailed in Table 1. The replication abilities of mutants and infectious-particle formation are summarized on the right. +, positive; −, negative; ±, weakly positive.

spread efficiency. These viruses were totally avirulent for mice and were unable to spread in L929 cells.

MATERIALS AND METHODS

Cell lines and culture. BHK-21 cells were cultured in Glasgow-minimal essential medium supplemented with tryptose phosphate broth, penicillin-streptomycin, and 10% newborn calf serum. L929 cells were cultured in Dulbecco modified Eagle medium supplemented with penicillin-streptomycin and 10% fetal calf serum. Media and reagents for cell culture were purchased from Gibco.

Construction of viral mutants. pTMDA1 is a pBluescript derivative carrying the full-length cDNA of Theiler's virus strain DA (8). This plasmid is a clonal derivative of plasmid pTMDA (24), which turned out to be a mixture of at least two slightly different sequences. In vitro transcription of pTMDA1 from the T7 promoter to a unique *Cla*I site yields an RNA corresponding to the genome of TMEV which is infectious upon transfection in permissive cell lines.

Mutations were introduced in pTMDA1 into the 12 regions of the genome (Fig. 1 and Table 1). The mutations were either in-frame deletions or point mutations. Most mutants were obtained by deleting restriction fragments after eventually filling in restriction sites with T4 or Klenow polymerase. The deletion in 2C was obtained by treatment with nuclease *Bal* 31. Deletions in VP4 and 2B and point mutations in 3B and 3D were introduced by site-directed mutagenesis (19). As a replication-deficient control, we used pTM491, a pTMDA1 derivative carrying a *Stu*I out-of-frame deletion extending from region 2C to region 3C.

The junctions of all deletions were sequenced to confirm that the phase was restored as expected. Furthermore, all the fragments subjected to site-directed mutagenesis that were used in subsequent constructs were entirely sequenced on one strand to ensure that mutagenesis did not introduce any unwanted alteration.

Analysis of RNA replication. To produce viral RNA, pTMDA1 derivatives were linearized with restriction enzyme *Cla*I and transcribed in vitro with T7 RNA polymerase (Boehringer Mannheim). In vitro-transcribed RNAs were run on nondenaturing 1% agarose gels in Tris-acetate buffer to estimate both the quantity and quality of RNA. Typically, 10 to 50 μg of RNA was electroporated

TABLE 1. pTMDA1 mutants constructed in this study

Plasmid	Protein	Junction[a]	Deletion or replacement[b]	Method used[c]
pVD8	L	CCT TCG---TCG AGC (^{50}Pro ^{51}Ser---^{53}Ser ^{54}Ser)	1-aa deletion	Deletion of *Bst*BI∗-*Xho*I∗
pTM564	L	CAT GTT---GTA CGC (^{5}His [Val]---^{68}Val ^{69}Arg)	61-aa deletion	Introduction of *Hpa*I by site-directed mutagenesis and deletion of *Hpa*I-*Bsi*WI∗
pTM533	VP4	GAA GGA---TCC AAT (^{17}Glu ^{18}Gly---^{26}Ser ^{27}Asn)	7-aa deletion	Site-directed mutagenesis with oligonucleotide TM39
pCJ23	VP2	CTT CGC---ATG GAC (^{100}Leu ^{101}Arg---^{153}Met ^{154}Asp)	51-aa deletion	Deletion of *Bst*BI∗-*Nco*I∗
pCJ24	VP2	GAC TTG---GCT AAT (^{66}Asp ^{67}Leu---^{221}Ala ^{222}Asn)	153-aa deletion	Deletion of *Msc*I-*Sph*I∗∗
pVD7	VP3	TCA GAT---CAA TTG ^{42}Ser ^{43}Asp---^{192}Gln ^{193}Leu)	148-aa deletion	Deletion of *Bgl*II∗-*Mun*I∗
pVD6	VP1	GGT ACT---ATG TGG ^{100}Gly ^{101}Thr---^{173}Met ^{174}Trp)	71-aa deletion	Deletion of *Asp*718∗-*Nde*I∗
pRS5	2A	CGG ATC---CGC GCA (^{34}Arg ^{35}Ile---^{113}Arg ^{114}Ala)	77-aa deletion	Deletion of *Bam*HI∗-*Bss*HII∗
pTM577	2A	ATT GAT---ATC CAT ^{81}Ile[Asp]---^{93}Ile ^{94}His	10-aa deletion	Site-directed mutagenesis with oligonucleotide TM51
pTM544	2B	CCT GGA---TCC GTG (^{64}Pro [Gly]---^{81}Ser ^{82}Val)	15-aa deletion	Site-directed mutagenesis with oligonucleotide TM40
pTM545	2C	GAA TGG---GGA CAA (^{17}Glu ^{18}Trp---^{124}Gly ^{125}Glu)	105-aa deletion	Deletion with *Bal* 31 nuclease
pRR39	3A	GCT CGC---TCT GAG (^{36}Ala ^{37}Arg---^{84}Ser ^{85}Glu)	46-aa deletion	Deletion of *Nru*I-*Sac*I∗∗
pRR43	3B	GCA (TAT→ACA) GCT (^{2}Ala ^{3}[Tyr→Thr] ^{4}Ala)	1-aa replacement	Replacement by site-directed mutagenesis with oligonucleotide TM31
pTM465	3C	AAT GTT---AAC GGC (^{104}Asn ^{105}Val---^{177}Asn ^{178}Gly)	71-aa deletion	Deletion of *Hpa*I
pRR47	3D	GGT (GAT→ACC) GAC (^{332}Gly ^{333}[Asp→Thr] ^{334}Asp)	1-aa replacement	Replacement by site-directed mutagenesis with oligonucleotide TM32

[a] Amino acids in parentheses and underlined nucleotides differ from those in the parental sequence.
[b] aa, amino acids.
[c] ∗, restriction site filled in with Klenow or T4 polymerase; ∗∗, restriction site blunt ended by the exonuclease activity of Klenow or T4 polymerase.

in 5×10^6 to 4×10^7 BHK-21 cells (20). For electroporation, cells were trypsinized, washed twice, and resuspended in ice-cold phosphate-buffered saline (PBS). Electroporation was performed in 0.4 cm-electrode-gap cuvettes with settings of 1,000 to 1,800 (usually 1,500) V, 25 μF, and 2,310 Ω with an Easyject + (Equibio) electroporator. After electroporation, cells were suspended in culture medium and distributed in several 6-cm-diameter petri dishes.

Typically at 4, 8, 12, 24, and 48 h posttransfection, total RNA was prepared from the cells of one petri dish by the method of Chomczynski and Sacchi (7). After 15 min of denaturation at 65°C in 5× SSC (0.75 M sodium chloride, 75 mM sodium citrate [pH 7])–8.75% formaldehyde, equal amounts of RNA preparations were spotted onto Nytran 12N (Schleicher and Schuell) nylon membranes. RNA was then fixed by 30 s of UV irradiation on a standard 312-nm UV transilluminator. Membranes were hybridized with a DNA probe corresponding to the entire genome of virus DA, labeled with [^{32}P]dCTP by the random priming method (ready-to-go kit; Pharmacia Biotec). Hybridization occurred overnight at 65°C in 0.5 M phosphate buffer (pH 7.4)–7% sodium dodecyl sulfate. Membranes were washed three or four times for 20 min each at 65°C in a solution containing 1% sodium dodecyl sulfate and 40 mM phosphate (pH 7.4), dried, and exposed.

Immunodetection. (i) Immunocytochemistry. Cells were cultured on 24-well plates, washed with PBS, and fixed for 20 min in a 4% paraformaldehyde solution in PBS. All subsequent steps and washings occurred in PBS containing 1 mM $MgCl_2$. Cells were permeabilized by a 5-min treatment with 0.1% Triton X-100. Endogenous peroxidases were inactivated by a 5-min treatment with 0.3% H_2O_2. After being washed, cells were incubated with the primary antibody (anti-VP1 monoclonal antibody F12B3; kindly provided by Michel Brahic). The detection of positive cells was further performed with a biotinylated secondary antibody, horseradish peroxidase-conjugated streptavidin (LSAB-2 kit; DAKO), and by staining with diaminobenzidine.

(ii) Immunohistochemistry. The detection of viral antigen in paraffin-embedded sections was performed as previously described (2).

Quantification of noncytopathic viruses by RT-PCR. Culture supernatant (200 μl) containing the mutant virus to be titrated was mixed with 200 μl of several dilutions of a titrated cytopathic (wild-type) virus. Total RNA was extracted from each mixture and subjected to reverse transcription-PCR (RT-PCR). Primers were chosen to amplify the region in which the deletion occurred in the mutant. The proportion of wild-type and mutant viruses in each mixture was visualized after digestion with a discriminating restriction enzyme (pTM577 versus pTMDA1) or directly when the sizes of the amplified fragments differed significantly (pRS5 versus pTMDA1). The titer was calculated in PFU equivalents from the mixture that yielded equal amounts of mutant and wild-type sequences.

RESULTS

RNA replication and viral production of TMEV mutants in BHK-21 cells. We derived a set of mutants from pTMDA1, a full-length cDNA clone of the DA strain of TMEV. Nonpolar mutations, either in-frame deletions or point mutations, were introduced into all 12 coding regions of the genome as described in Materials and Methods (Fig. 1 and Table 1). Deletions were introduced into the central parts of proteins to minimize the risk of interference with the processing of the viral polyprotein by protease 3C.

For each mutant, we evaluated the ability to replicate viral RNA and to produce infectious viral particles. Therefore, viral RNAs were transcribed in vitro from the various cDNA clones and introduced into BHK-21 cells by electroporation. The amount of viral RNA in transfected cells was determined by dot blot hybridization (Fig. 2). Replication of the genome was monitored by quantifying viral RNA before completion of the first virus cycle (up to 12 h). Spread of the virus was further evaluated by monitoring the cytopathic effect in culture and the amount of viral RNA in transfected cells at later times.

As expected, pTMDA1 (the wild-type sequence) displayed efficient replication during the first round of replication and increased amounts of RNA at later times, confirming virus

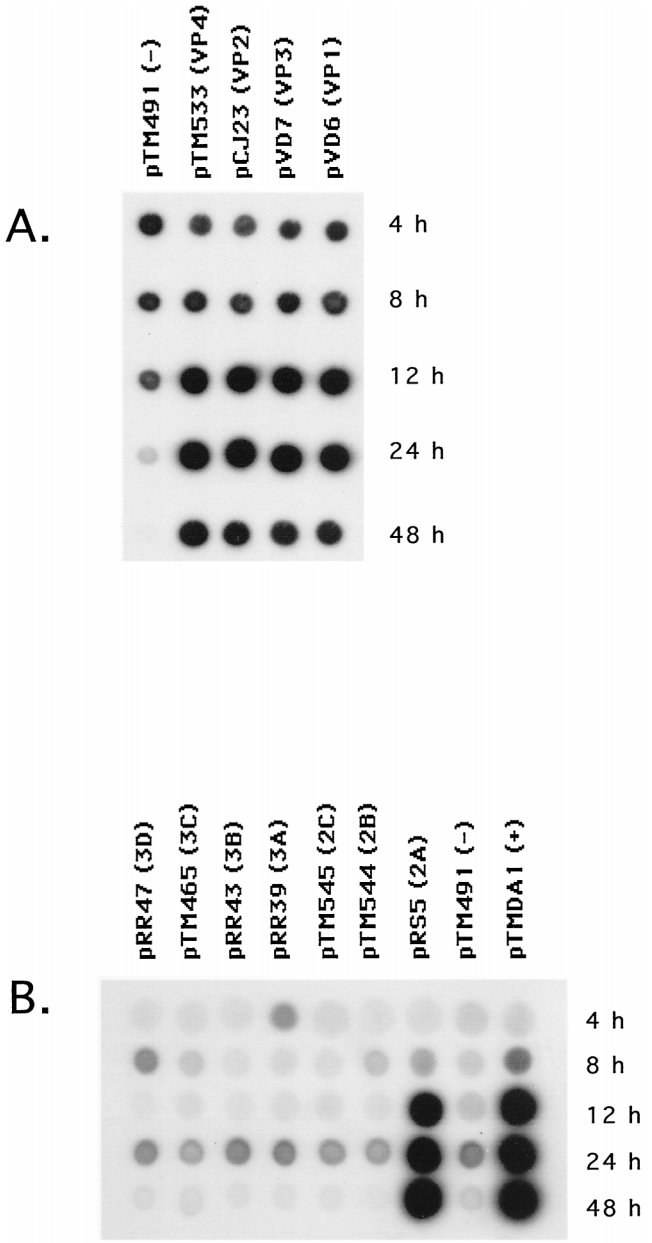

FIG. 2. Genomic RNA replication of DA1 mutants. In vitro-transcribed RNAs of the indicated mutants were transfected in BHK-21 cells. At several times posttransfection, total RNA was prepared from transfected cells and dot blot hybridized with a virus-specific probe to monitor viral RNA replication (early times) and virus propagation (late times). pTM491, used as a replication-negative control (−), is a pTMDA1 derivative carrying an out-of-frame deletion extending from the 2C region to the 3C region. (A) Viruses with deletions in capsid proteins; (B) viruses affected in 2A to 3D regions. +, wild type. Autoradiograms were scanned by using DeskScan II software (Hewlett-Packard). Light intensity was adjusted between blots with Adobe Photoshop LE.

spread in cell culture. RNA from pTM491, the negative control carrying an out-of-frame deletion in the replication functions, remained at background levels.

In agreement with data for other picornaviruses, TMEV genomes harboring mutations in regions 2B (pTM544), 2C (pTM545), 3A (pRR39), 3B (pRR43), 3C (pTM465), and 3D (pRR47) were unable to replicate at detectable levels. Indeed, viral RNA remained at backgrounds levels throughout experiments (Fig. 2).

Viral RNAs with deletions in all four regions, encoding the capsid proteins VP4 (pTM533), VP2 (pCJ23 and pCJ24), VP3 (pVD7), and VP1 (pVD6), were found to replicate as well as the wild-type virus did (data not shown). However, since they were unable to assemble into viable viral particles, the signal dropped after 24 h, confirming that these viruses were unable to spread to neighboring nontransfected cells (Fig. 2).

Genomes with a deletion in the leader protein (pVD8 and pTM564) not only had wild-type levels of RNA replication but also produced infectious viral particles upon transfection in BHK-21 cells. These viruses caused a complete cytopathic effect in BHK-21 cells about 60 to 72 h after transfection, only 2 to 12 h later than did the wild-type virus. The fact that the L protein is dispensable for virus production in BHK-21 cells is in agreement with previous data for strains DA (17) and GDVII (5).

To our surprise, the genome of mutant pRS5, containing a deletion encompassing more than one-half of the 2A region, appeared to replicate readily. Furthermore, this mutant was able to propagate in BHK-21 cell culture, as shown by progressive increases in the amount of viral RNA at 24 and 48 h and one or more passages after transfection. No clear cytopathic effect was detected in cells transfected with the 2A mutant before one or two passages of transfected cells. However, the formation of infectious particles occurred, as the culture supernatant of transfected cells was infectious for fresh BHK-21 cells. The virus derived from pRS5 could be passaged up to 20 times without any detectable alteration of the phenotype. The 2A region, amplified by RT-PCR, still contained the deletion after 20 passages, ruling out contamination or reversion to the wild type (Fig. 3). The 2A mutant virus failed to form visible plaques in BHK-21 cells.

The data presented above suggested that the 2A mutant replicated efficiently but was somewhat hindered in propagation from cell to cell in BHK-21 cell cultures. A likely explanation for this observation was that the large deletion in 2A affected the processing by protease 3C of the VP1-2A junction,

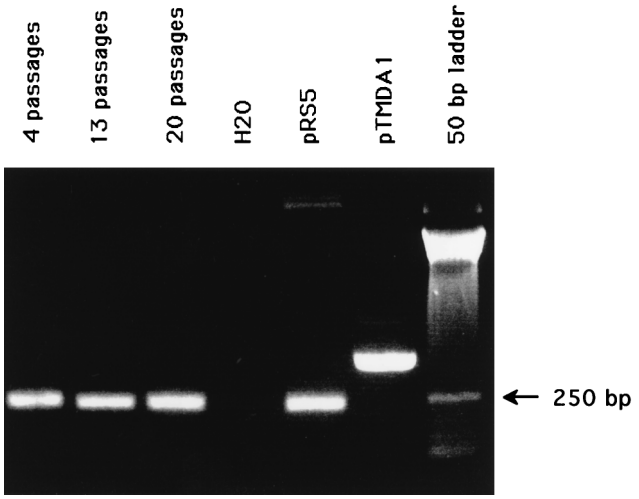

FIG. 3. Passage of the 2A mutant virus derived from pRS5. 2A sequences were amplified by RT-PCR from the supernatant of BHK-21 cells after 4, 13, and 20 cycles of infection with the 2A mutant derived from pRS5. 2A sequences were amplified in parallel from pRS5 (the parental mutant plasmid) and pTMDA1 (the plasmid carrying the wild-type sequence). H_2O, a negative PCR control. The arrow points to the 250-bp molecular size marker which is about the size of the PCR fragment expected for pRS5 (225 bp).

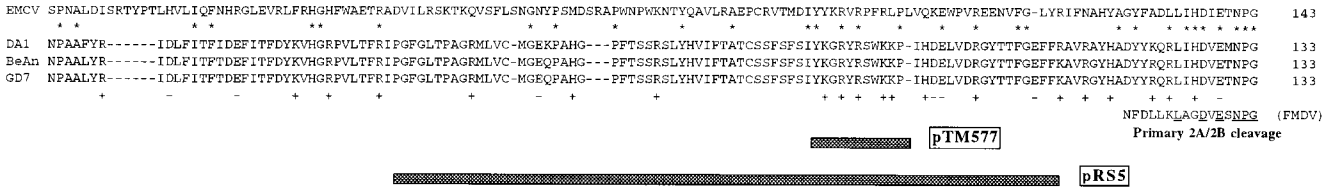

FIG. 4. 2A protein and deletions within 2A. The sequences of 2A proteins from EMCV (27), DA1 (24a), BeAn (30), and GDVII (GD7) (29) were aligned by using Clustal W (36). The sequences of 2A proteins from Theiler's virus isolates representative of the three phylogenic groups (25) have about 90% identity. In contrast, very few residues are conserved between the 2A proteins of Theiler's virus and EMCV (identity is indicated by an asterisk). Only the C terminus, which is responsible for primary cleavage of the polyprotein, shows significant identities with the sequences of EMCV and FMDV (10, 12). The extents of the deletions in 2A in pTM577 and pRS5 are indicated by shaded boxes. The 10-amino-acid deletion in pTM577 removes a predicted turn that is rich in positively charged (+) amino acids. −, negatively charged amino acid. Protein 2A is rather basic, with a predicted isoelectric point of 9.8. Dashes in the sequences indicate gaps introduced by the alignment procedure. Underlined residues in the FMDV sequence are conserved in both EMCV and TMEV.

leaving 2A fragments associated with VP1 and thus preventing the proper assembly of capsids. Hence, we introduced a smaller deletion in the 2A region. We deleted 11 amino acids from a sequence particularly rich in positively charged amino acids (Fig. 4). In this mutant, pTM577, the deletion left the first 108 amino acids of the protein and was thus unlikely to inhibit processing at the VP1-2A junction. As was the case for pRS5, RNA derived from pTM577 replicated well in BHK-21 cells. Although propagation of the virus derived from pTM577 generally appeared to be more efficient than that of the virus derived from pRS5, a cytopathic effect was hardly detectable 3 to 6 days after transfection (data not shown).

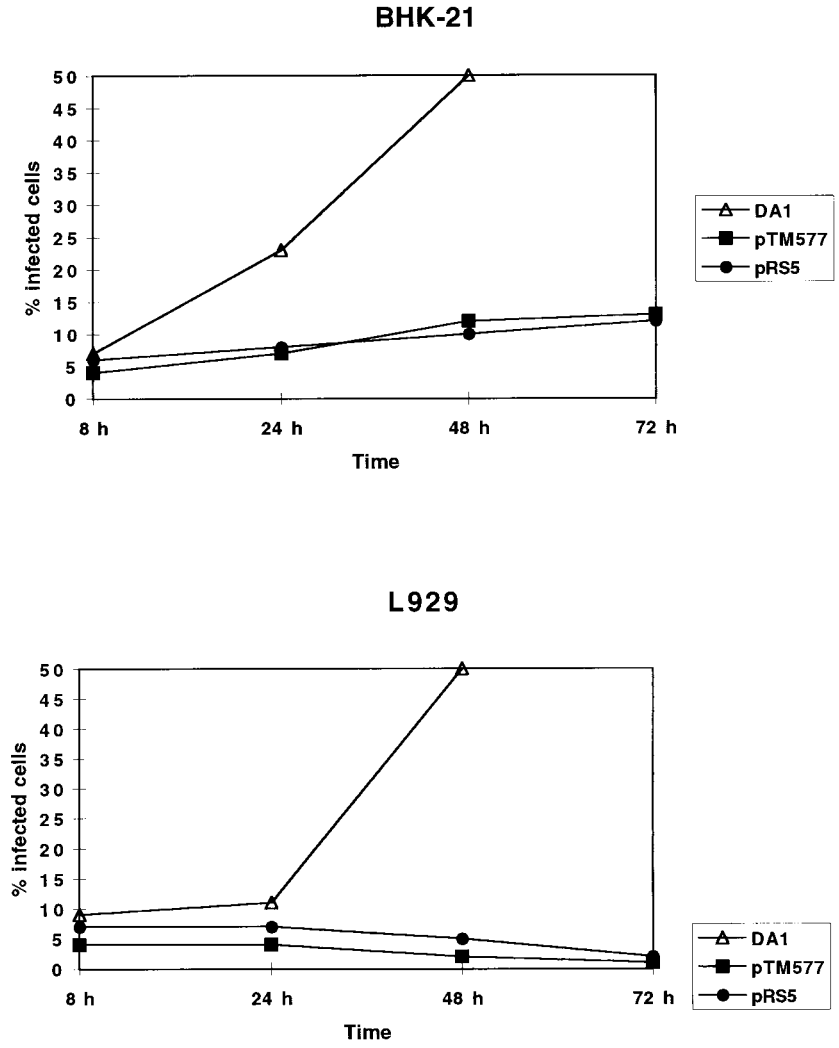

FIG. 5. Propagation of 2A mutant viruses in BHK-21 and L929 cells. In vitro-transcribed RNAs from 2A mutants (pRS5 and PTM577) and the wild-type virus (DA1) were transfected in BHK-21 and in L929 cells. After 8, 24, 48, and 72 h, the percentage of infected cells was estimated by the proportion of cells positive for viral antigen in immunocytochemistry. Each graph shows the proportion of infected BHK-21 or L929 cells as a function of time.

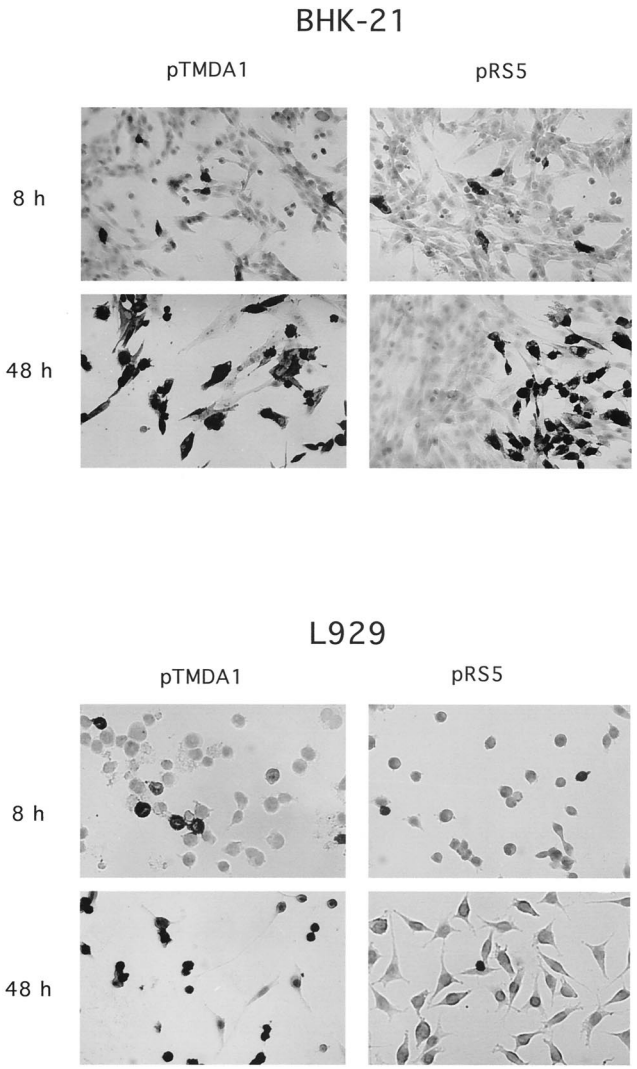

FIG. 6. Detection of infected cells by immunocytochemistry. Typical fields from the experiment presented in Fig. 5 are shown. Infected BHK-21 and L929 cells were detected by immunocytochemistry (stained dark) at 8 and 48 h posttransfection. The proportions of transfected cells were about the same (8 h posttransfection). Virus propagation was slower and more focal for the 2A mutant than it was for the wild-type virus in BHK-21 cells. Propagation of the 2A mutant in L929 cells was restricted. Pictures were digitalized by using a ScanJet 4C scanner and DeskScan II software (Hewlett-Packard). Light intensity was adjusted between pictures with Adobe Photoshop LE.

Propagation in BHK-21 and L929 cells. In order to confirm that 2A mutants replicated readily but were hindered in cell-to-cell propagation, we determined the numbers of infected cells after transfection of the genomes of 2A mutants and the parental strain. We used immunocytochemistry to discriminate between infected and noninfected cells. The proportion of infected cells was evaluated at 8 (before the detection of secondary infections), 24, 48, and 72 h after transfection for BHK-21 and L929 cells. As shown in Fig. 5 and 6, the proportions of infected cells detected 8 h after transfection were of the same order for wild-type virus and 2A mutants, showing that the transfection efficiencies were about the same and that the genomic RNAs of 2A mutants replicated and expressed viral capsid proteins. In BHK-21 cells, the proportion of cells infected by 2A mutants increased over time, showing that the virus was able to propagate from cell to cell. However, the increase was much slower for 2A mutants than for the wild-type virus, confirming their poor propagation in the cell population. In L929 cells, although the 2A mutants replicated readily, the viruses were unable to spread in culture. This observation was confirmed by determining the viral RNA content of cells by dot blot hybridization (data not shown).

Production of 2A mutant viruses. Since 2A mutant viruses did not form plaques on BHK-21 cells, we quantified the amount of virus in the culture supernatant by measuring the amount of viral RNA by comparative RT-PCR (see Materials and Methods). The production of 2A mutants by infected or transfected BHK-21 cells was generally about 10 times lower than the production of wild-type virus (10^6 PFU eq/ml and 10^7 PFU/ml, respectively).

Infection of mice with 2A mutant viruses. Six 3-week-old female FVB mice were inoculated in the right hemisphere with 10^4 PFU eq of pTM577 virus or parental virus DA1. Three mice from each group were sacrificed after 1 week, and the other three were sacrificed after 3 weeks. They were examined for infection of the central nervous system by immunohistochemistry. The wild-type strain produced the typical biphasic infection (21). Viral antigen associated with inflammation was mostly detected in the gray matter of the brain at 7 days postinfection, whereas it was more prominent in the white matter of the spinal cord by day 21. In contrast, for the 2A mutant virus derived from pTM577, very few infected cells were detected in mice at the site of injection 7 and 21 days after infection. No virus-positive cell was detected at any distance from the inoculation site. Accordingly, no inflammation was found in the spinal cord or brain except at the point of virus inoculation.

Inoculations of BALB/c nude mice confirmed the inability of the 2A mutant to spread in the central nervous system, even in the absence of a specific T-cell response. In these mice, viral spread was no more proficient than it was in immunocompetent mice.

DISCUSSION

We introduced in-frame deletions into all 12 coding sequences of the Theiler's virus genome. As expected from sequence similarities with other picornaviruses, mutations in regions 2B to 3D totally inhibited RNA replication. As observed for poliovirus, in-frame deletions introduced into any capsid protein did not affect RNA replication but prevented virus assembly and thus propagation of infection.

As described previously for strains DA (17) and GDVII (5), viruses with deletions in the leader (L) region retained full RNA replication proficiency. As originally described by Kong et al. (17), L mutant viruses were readily produced and caused a cytopathic effect and normal-sized plaques in BHK-21 cells, although their cell-to-cell propagation was inhibited in L929 cells compared with propagation of the wild-type virus. The leader proteins of Theiler's virus and mengovirus were inferred to interfere with the production of interferon by infected L929 cells and would thus be dispensable in BHK-21 cells since these cells do not produce interferon (17, 41).

We observed that 2A mutants also propagated to a certain extent in BHK-21 cells but not in L929 cells, suggesting that the effect seen for mutants in the leader protein might not be very specific.

The role of protein 2A remains totally unknown. The C-terminal 16 amino acids of the 2A protein of Theiler's virus have extensive sequence identities with those of EMCV and mengovirus and with the 16-amino-acid protein 2A of FMDV (Fig. 4). This sequence is known to promote cotranslational

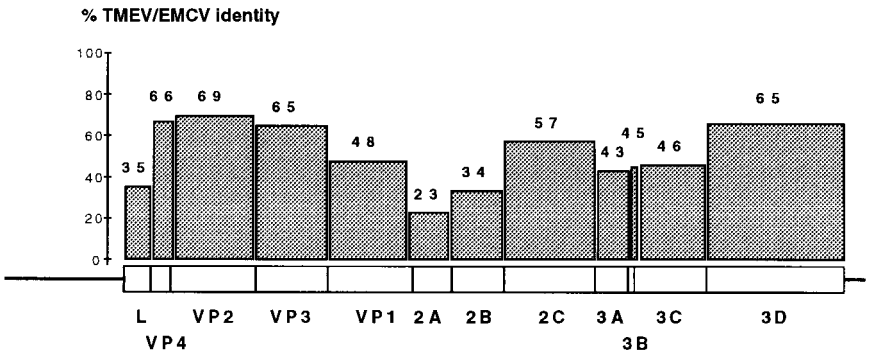

FIG. 7. Identity between proteins of Theiler's virus and EMCV. The genome of cardioviruses is depicted under columns showing the percentages of identity between TMEV and EMCV at the amino acid level for the indicated viral proteins (27). Noncoding regions are represented by horizontal lines. Coding regions are symbolized by boxes. The percentage of identity is indicated above each column.

primary cleavage of the polyprotein between the 2A and 2B regions in the NPG/P consensus sequence (14, 28, 32).

Apart from this short sequence, the 2A protein of Theiler's virus does not have any obvious identity with the 2A proteases of poliovirus and rhinovirus, with protein 2A of hepatitis A virus, or even with the 2A proteins of other cardioviruses. The 2A proteins from Theiler's virus and EMCV have 23% identity; the identity is even less (19%) if one does not take the conserved C terminus into account. This contrasts with the capsid proteins (VP4 to VP1) and the replication proteins (2B to 3D), which have 60 and 55% identity, respectively (Fig. 7).

We observed that a large deletion in protein 2A did not detectably impair RNA replication. This contrasts with the data reported for poliovirus, in which 2A clearly participates in the RNA replication process (26, 39). Although RNA replication was efficient, the overall virus cycle was affected in 2A mutants, suggesting a role for 2A in viral entry, assembly, or release. Indeed, although this virus propagated in BHK-21 cells, the spread of infection was much slower than that of the wild-type virus; therefore, a cytopathic effect was not always detectable in infected cultures and the virus did not form visible plaques in BHK-21 cells. We cannot definitely rule out any mild, undetected effect of the 2A deletion on replication that affects the global speed of infection, but this seems rather unlikely. Our data suggest that the 2A protein of Theiler's virus is essential for a function which is unrelated to genome replication. One hypothesis is that 2A has no role per se, but the sequence acts as a spacer between VP1 and 2B to ensure the proper folding kinetics required for efficient and time-controlled processing of the polyprotein. This fits with the unexpectedly high divergence found between the 2A proteins of Theiler's virus and EMCV, but it would be somewhat surprising in view of the high conservation of the 2A sequence among TMEV isolates. The fact that RNAs from 2A mutants replicate indicates that the deletion does not interfere significantly with primary cleavage of the polyprotein between 2A and 2B. Accordingly, both 2A mutants retained the C terminus of the protein, which is responsible for this cleavage. However, subsequent processing of the P1 region by protease 3C may be affected, thus hindering virus assembly.

Alternatively, 2A may play a specific role in either virus assembly or cell death. 2A may modulate virion formation by being transiently incorporated in a capsid precursor as protein PX of hepatitis A virus (1).

2A mutants of Theiler's virus were totally avirulent for both immunocompetent and nude mice. The virus was found to replicate at the site of inoculation but was unable to spread at some distance. This observation contrasts with the case of hepatitis A virus, where 2A mutant viruses retained their virulence for marmosets (16). Experiments are under way to clarify the role of this intriguing protein.

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