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Expression of a Foreign Epitope by Porcine Reproductive and Respiratory Syndrome Virus

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The potential of porcine reproductive and respiratory syndrome virus (PRRSV) as a viral vector was explored by the insertion of a sequence encoding a foreign antigen into the infectious cDNA clone of the Lelystad virus isolate. An epitope of the hemagglutinin (HA) protein of human influenza A virus was introduced at the 5′ end and at the 3′ end of ORF7, in each case resulting in a fusion protein between the HA epitope and the nucleocapsid (N) protein. Furthermore, in the construct carrying the HA sequences at the 5′ end of ORF7, additional in-frame insertions encoding the autoprotease 2A of foot-and-mouth disease virus were made between the HA and ORF7 sequences to ensure the generation of a functional N protein from its hybrid precursor. When RNA transcripts from these full-length cDNA clones were transfected into BHK-21 cells, they were each found to replicate, to express the HA epitope, and to produce progeny virus. However, fusion of the HA epitope directly to the nucleocapsid protein either at the N terminus or at the C terminus adversely affected both the viability and the genetic stability of the recombinant PRRS viruses. Serial passage of the recombinant viruses on porcine alveolar macrophages demonstrated that these viruses had lost (part of) the HA epitope at passage four. In contrast, in the PRRS viruses expressing the HA epitope from a precursor cleavable by the autoprotease 2A peptide, the HA epitope was still intact after four passages, and no effect on the viability of these viruses was observed. Immunoprecipitation and pulse chase experiments revealed the efficient and presumably cotranslational cleavage of the HA epitope from the N protein by the 2A protease. Our results demonstrate the feasibility of using PRRSV as a viral vector that might be suitable for the delivery of antigens from other pathogens to the immune system of the pig. © 2000 Academic Press

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped positive-strand RNA virus, which belongs to the *Arteriviridae* family together with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus, and simian hemorrhagic fever virus (Meulenberg *et al.,* 1993b). The *Arteriviridae* family has recently been grouped within the order of *Nidovirales* together with the *Coronaviridae* (Cavanagh, 1997). The genome of PRRSV is a single-stranded polyadenylated RNA molecule of 15.1 kb, which contains at least nine partially overlapping open reading frames (ORFs). ORFs 1a and 1b encode the viral replicase genes, which are expressed from the genomic RNA. The other, smaller ORFs are located at the 3′ end of the genome and are expressed from subgenomic RNAs, which are produced as a 3′ nested set during replication (de Vries *et al.,* 1990; Meulenberg *et al.,* 1993a). They encode the structural proteins. ORF 7 codes for the nucleocapsid protein N, ORF 6 for the membrane protein M, ORF5 for the major envelope glycoprotein GP_5, and ORFs 2–4 for the minor envelope glycoproteins designated GP_2, GP_3, and GP_4, respectively (Meulenberg *et al.,* 1995). However, the structural nature of the GP_3 protein has recently been questioned (Mardassi *et al.,* 1998). Recently, a novel gene, ORF2a, was described for EAV (Snijder *et al.,* 1999). This gene encodes a new structural protein and is conserved in PRRSV and other arteriviruses.

PRRSV causes mainly respiratory problems in pigs and stillbirths in sows. The first PRRSV isolate was identified in 1991 in the Netherlands by (Wensvoort *et al.,* 1991) and was designated Lelystad virus (LV). Recently, an infectious cDNA clone was constructed from this isolate (Meulenberg *et al.,* 1998b). It provides a convenient tool for mutagenesis of PRRSV to introduce specific mutations, deletions, or substitutions into the viral genome. In this study, we used the infectious clone for the introduction of additional sequences to explore the potential of PRRSV as a viral vector. Since PRRSV grows specifically in porcine alveolar macrophages (PAMs), we expect a PRRSV vector to be able to efficiently deliver antigens to the immune system of the pig.

The construction of a viral vector raises basic questions as to the regions of the viral genome in which insertions are tolerated and about the maximum uptake capacity for foreign sequences. In this respect, two considerations are important. First, PRRSV, like other RNA

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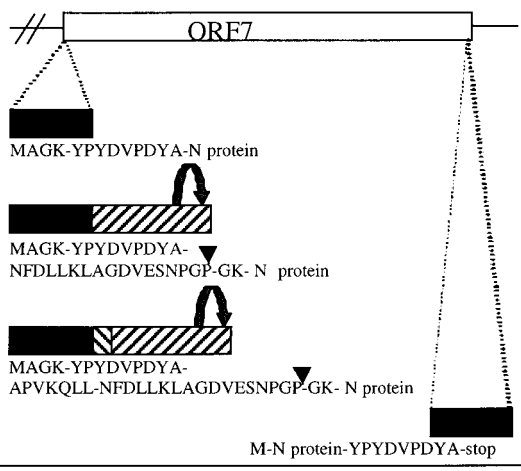

Mutation	Description	Construct
	*Pac*I-mutant of infectious clone LV	pABV437
	HA-N	pABV525
	HA-2A^{pro-17} -N	pABV523
	HA-2A^{pro-24}-N	pABV661
	N-HA	pABV526

FIG. 1. Design of the recombinant genome-length cDNA clones of PRRSV expressing the HA epitope. Mutations were introduced by PCR mutagenesis and cloned into the *Pac*I-mutant of the full-length cDNA clone, pABV437 (Meulenberg *et al.,* 1998b). The inserted sequences, the description of the mutant, and the clone numbers are indicated. The black box represents the HA epitope from influenza A (YPYDVPDYA), the large striped box represents 17 amino acids encoding for 2A^{pro} (NFDLLKLAGDVESNPGP), and the small striped box represents the extra 7 amino acids of the 3′ end of 1D of FMDV (APVKQLL). The region encoding the four N-terminal amino acids (MAGK) of N overlaps with the ORF6 region encoding the C-terminus of M. Hence, to keep the M protein intact, the region encoding the MAGK was retained upstream of the inserted HA/ HA-protease 2A sequence. The black triangle indicates the cleavage site of the protease 2A.

viruses, has a concise genome. Since RNA viruses have evolved to optimal fitness, most of the genetic information is expected to be essential. Second, the ORFs that encode the structural proteins of the virus are partially overlapping. Insertions in overlapping regions would therefore result in the mutation of two structural proteins, which would almost inevitably lead to the production of a nonviable virus. In this study, we selected ORF7, which has only little overlap with the upstream ORF6, to explore the expression of foreign sequences. We introduced a nine-amino-acid epitope of the hemagglutinin (HA) protein of human influenza A virus (Kolodziej and Young, 1991) either at the N terminus or at the C terminus of the N protein. We additionally inserted sequences of the self-cleaving 2A protease (Robertson *et al.,* 1985; Ryan and Drew, 1994) of foot-and-mouth disease virus (FMDV) in frame between the HA encoding sequence and ORF7 at the 5′ end. Here we describe that the foreign HA epitope was expressed by PRRSV and that the sequence encoding this HA epitope was maintained in the viral genome, provided that the epitope was expressed as part of a cleavable N protein precursor from which it is liberated by the 2A protease.

RESULTS

Introduction of a HA epitope into the infectious clone of LV

The aim of this study was to explore the potential of PRRSV as a viral vector. This was performed by the introduction of a sequence, which encodes a foreign antigen, into the infectious clone of the LV isolate of PRRSV. We selected an epitope of nine amino acids of the hemagglutinin of human influenza A virus (HA epitope) (Kolodziej and Young, 1991). The HA epitope was chosen because of its limited size, which would reduce the chances of disturbing the replication of the virus or the expression or function of the protein to which it is fused. In addition, antibodies were available to detect the expression of the epitope. ORF7 was selected for insertion of the HA epitope for two reasons. First, subgenomic RNA7 is the most abundant mRNA produced in PRRSV-infected cells. Second, the HA epitope could be inserted here without mutating other ORFs, since ORF7 has only a very small overlap with ORF6 at its 5′ end while no other ORF occurs at its 3′ end. The HA epitope was introduced at the 5′ end of ORF7, without introducing mutations in the overlapping 3′ end of ORF6 (full-length clone pABV525; Fig. 1). In addition, the HA-tag was introduced directly upstream of the stop codon of ORF7, generating full-length clone pABV526 (Fig. 1). Since we could not predict the influence of the terminal extensions on the structure and function of the N protein, we additionally inserted sequences of the self-cleaving 2A protease of foot-and-mouth disease virus in frame between the HA encoding sequence and ORF7 at the 5′ end. Hence, the polyprotein would be proteolytically cleaved by protease 2A to release the HA epitope from the N protein. Sixteen amino acids of the protease 2A (Robertson *et al.,* 1985) plus a proline for cleavage at the

glycine-proline dipeptide motif junction were introduced, resulting in a full-length clone designated pABV523 (Fig. 1). Since the insertion of additional sequences from the 3′ end of the 1D capsid protein, flanking the protease 2A in FMDV, has been reported to increase the activity of $2A^{pro}$ (Donnelly *et al.,* 1997), we also produced a construct in which seven amino acids of this 1D capsid protein were inserted between the protease 2A and HA sequences, resulting in pABV661 (Fig. 1).

Analysis of PRRSV recombinants expressing the HA epitope

First, the expression of the structural proteins of PRRSV by the various transcripts of the recombinant full-length cDNA clones was tested to determine their ability to replicate. BHK-21 cells were transfected with RNA transcripts from pABV525, -523, -661, -526, and -437, the latter serving as a positive control. The BHK-21 cells transfected with the constructs stained positive in immunoperoxidase monolayer assay (IPMA) with MAbs directed against GP_3, GP_4, the M protein (not shown), and the N protein (Fig. 2A). BHK-21 cells transfected with transcripts from pABV525, -523, -661, and -526 also stained positive with MAb 12CA5 directed against the HA epitope. These results indicated that the HA encoding transcripts replicated in BHK-21 cells, that the subgenomic mRNAs were produced, and that the PRRSV proteins and the HA epitope were properly expressed by all four PRRSV mutants. In addition, it indicated that insertion of the heterologous sequences did not interfere with synthesis of subgenomic RNA7 and that the N protein, to which the HA epitope was fused, was expressed.

To examine whether the RNA transcripts from pABV525, -523, -661, and -526 were able to produce infectious virus, the culture supernatants of transfected BHK-21 cells were inoculated onto PAMs, as PRRSV does not infect BHK-21 cells. At 24 h after inoculation, PAMs stained positive in IPMA not only with MAbs directed against the PRRSV proteins GP_3, GP_4, M, and N, but also with the MAb directed against the HA epitope (Fig. 2B). However, staining with the HA-specific MAb was fainter than staining with the N-specific MAb for all constructs, which is most likely due to a lower affinity of MAb 12CA5. From these results we conclude that transcripts from the HA-encoding full-length cDNAs of PRRSV are able to produce infectious viruses that infect PAMs and express the foreign epitope.

Analysis of the stability of PRRSV mutants expressing the HA epitope

In order to investigate whether the recombinant viruses maintained the foreign sequences integrated in the viral genome, they were serially passaged in PAMs. After four passages, they were again tested by IPMA. When PAMs were infected with passage 4 of vABV525 and vABV526, only a few cells stained positive with the HA-specific MAb (Fig. 2C). However, many more cells stained positive with the MAb directed against the N protein. In contrast, the ratio of HA-positive to N-positive cells was much higher for PAMs infected with passage four of vABV523 and vABV661 (Fig. 2C) and was comparable with that of PAMs infected with passage 0 of these viruses. These results suggested that viruses encoding a HA epitope linked directly to the N protein lost the HA epitope, in contrast to viruses that contained the autoprotease 2A of FMDV.

The stability of the HA sequences integrated in the recombinant viruses was studied further by genetic analysis of the viral RNAs isolated from the culture supernatants after the different passages. The viral RNA was reverse transcribed and the region where the HA/protease sequences were inserted was amplified with flanking primers. For vABV525 and vABV526, the sizes of the fragments amplified after passages 0 and 1 were identical to that of the original cDNA clone from which they were derived. After further passages, however, fragments of smaller size appeared, the smallest of these comigrating in the gel with the fragment amplified from vABV437 (Fig. 3). In contrast, for both vABV523 and vABV661, the size of the amplified products remained identical to that of the original cDNA clone at all passages analyzed. Sequence analyses of the PCR fragments showed the loss of the sequence encoding the HA epitope from the viral genome of vABV525 and vABV526, starting at passage 2 (data not shown). For both viruses, the proportion of the PCR fragments lacking sequences encoding the HA epitope increased with increasing passage numbers. For vABV526, a new band of higher size was observed after several passages. However, the nature of this band could not be verified and is expected to be a nonspecific PCR product. These results indicated that the HA epitope was not maintained stably when it was fused directly to the N protein. However, for vABV523 and vABV661, the sequence data confirmed the complete stability of the region of the viral genomes containing the insertion during the four passages analyzed (data not shown).

Growth characteristics of PRRSV mutants expressing the HA epitope

In initial experiments we observed that vABV525 and vABV526 were produced in lower amounts than wild-type vABV437 and the recombinant viruses vABV523 and vABV661 after transfection of BHK-21 cells with RNA transcripts, as illustrated in Fig. 2B. This suggested that the fusion of the foreign epitope to the nucleocapsid protein adversely affected the viability of the virus. This was studied more carefully by analyzing the virus production from the RNA transcripts in BHK-21 cells in a

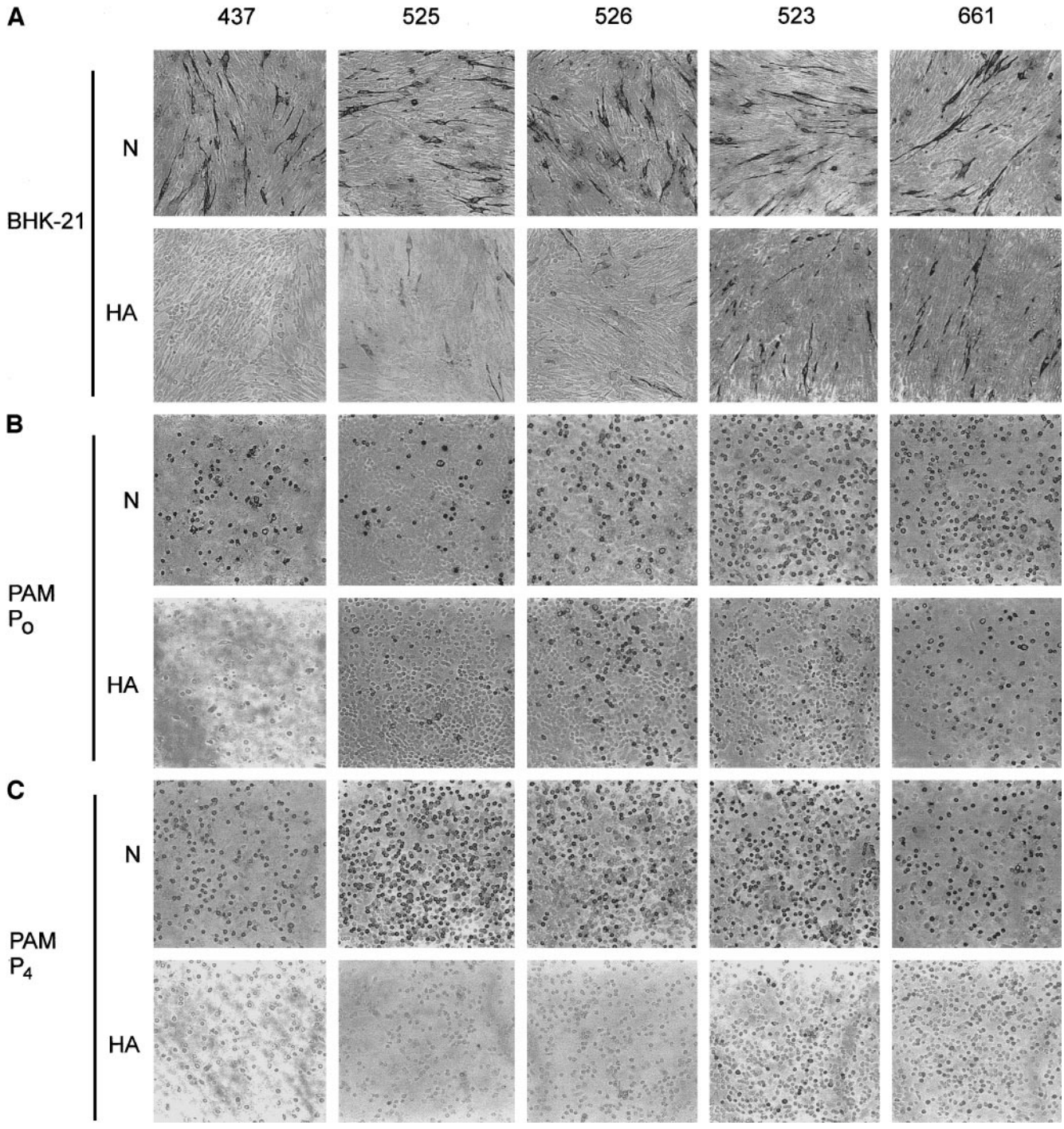

FIG. 2. Expression of the PRRSV proteins and of the HA epitope in BHK-21 cells transfected with RNA transcripts from pABV437, -525, -526, -523, and -661 (A), in PAMs infected with the supernatant (p0) of these transfected BHK-21 cells (B), and in PAMs infected with passage 4 (p4) of the PRRSV mutants expressing HA (C). Since p0 and p4 of vABV525 and 526 had far lower titers than those of vABV437, -523, and -661, a 20 times larger volume was used of the former to infect the PAMs for staining. Staining was performed with the N-specific MAb and with the HA-specific MAb at 15 h after transfection or infection in IPMA.

time course. Cells were transfected by electroporation with 10 μg of *in vitro* transcribed RNA from the full-length cDNA clones. Virus production was determined by end point dilution of the supernatant of transfected BHK-21 cells, harvested at various time intervals after transfection (Fig. 4).

Both the time at which virus was first detected in the medium (10 h posttransfection) and the amount of virus produced in a time interval of 32 h (6.9 and 7.1 $TCID_{50}$/ml, respectively) were similar for BHK-21 cells transfected with transcripts from pABV437 and pABV523 (Fig. 4). Also, the amount of vABV661 produced within the 32-h

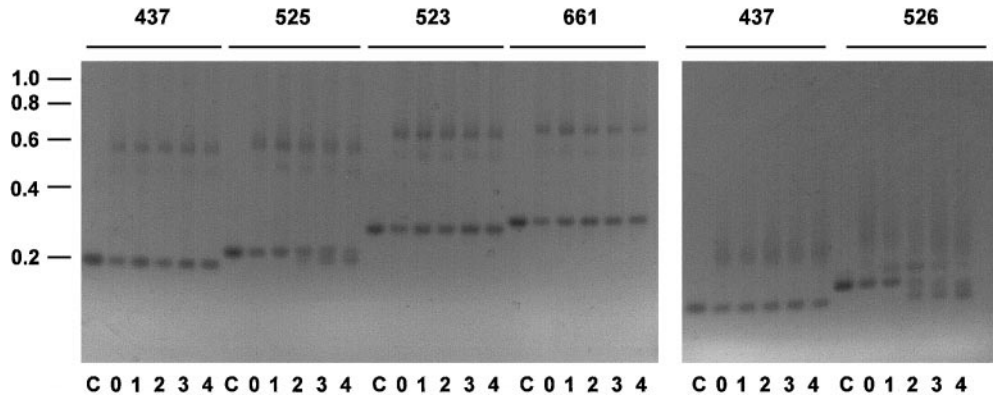

FIG. 3. RT-PCR analysis of viral RNA of vABV437, -525, -523, -661, and -526. Viral RNA was isolated from the supernatant of BHK-21 cells (p0) and from the supernatant of PAMs (p1–p4), as indicated beneath the lanes. The viral RNA was reverse transcribed and amplified by PCR, together with the original cDNA of the PRRSV mutants with primers that flank the insertions. Fragments were analyzed in a 2% agarose gel. The numbers on the left indicate the marker sizes in kilobases. Lane C: RT-PCR fragment derived from the original cDNA; lanes 0–4: RT-PCR fragment derived from RNA of passage numbers 0 to 4.

interval was comparable (6.8 $TCID_{50}$/ml) to that of vABV437, although the onset of virus production was delayed, infectious virus being detected first at 12 h after transfection. For vABV525 and vABV526 we confirmed our former observation that these viruses grew to significantly lower titers (5.3 and 5.4 $TCID_{50}$/ml, respectively) than did the viruses vABV437, vABV523, and vABV661 in the same time interval (Fig. 4). In addition, vABV525 and vABV526 became detectable later than wild-type vABV437, at 14 and at 12 h posttransfection, respectively. The results suggest that both C-terminal and N-terminal extension of the nucleocapsid protein with the HA epitope adversely affected the viability of PRRSV. However, cleavage of the N-terminal extension from the N protein restored the viability of PRRSV.

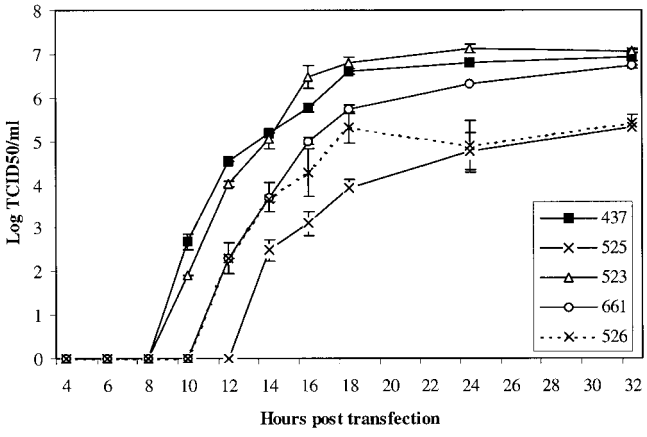

FIG. 4. Virus production of the wild-type recombinant virus vABV437 and of the HA expressing PRRSV mutants vABV525, -523, -661, and -526 in BHK-21 cells. BHK-21 cells were electroporated with approximately 10 μg of *in vitro* transcribed RNA and virus was harvested at the indicated time points. Virus titers were determined by end point dilution on PAMs (Wensvoort *et al.,* 1986). The mean titers of two independent experiments are shown for each time point.

Analysis of the N protein of PRRSV mutants expressing the HA epitope

The N protein expressed by the recombinant PRRSV RNA viruses was further analyzed by immunoprecipitation. The proteins in the lysates of BHK-21 cells transfected with RNA transcripts and of PAMs infected with the supernatants of these transfected BHK-21 cells were precipitated with either the N-specific MAb or the HA-specific MAb and analyzed by SDS–PAGE. As expected, only the N-specific MAb precipitated the N protein (15 kDa) from BHK-21 cells transfected with pABV437-derived RNA (Fig. 5A). Both antibodies precipitated a protein of 16 kDa from BHK-21 cells transfected with transcripts from pABV525 (Fig. 5A). The size of this protein corresponded to the calculated size of the HA–N fusion protein. However, a protein with an estimated size of 17 kDa was precipitated from BHK-21 cells transfected with RNA transcripts from pABV526 (Fig. 5A). The apparent size of this putative N–HA protein was somewhat larger than predicted. The protein was expected to have the same electrophoretic mobility as the HA–N fusion protein of pABV525. Both the 16-kDa and the 17-kDa protein were also precipitated with both the N-specific and the HA-specific MAbs from the lysates of PAMs infected with the culture supernatant of BHK-21 cells transfected with pABV525- and pABV526-derived RNAs (Fig. 5B).

When the lysates of BHK-21 cells transfected with RNA transcripts from pABV523 and pABV661 were analyzed, proteins with apparent molecular weights of 15 and 18 kDa or 15 and 19 kDa, respectively, were immunoprecipitated with the N-specific MAb (Fig. 5A). The 15-kDa protein was similar in size to the wild-type N protein. Polypeptides of 18 and 19 kDa are indeed predicted for the polyproteins of HA–$2A^{pro\text{-}17}$–N and HA–$2A^{pro\text{-}24}$–N, respectively. Immunoprecipitation with the HA-specific MAb confirmed that the 18- and 19-kDa proteins, but not

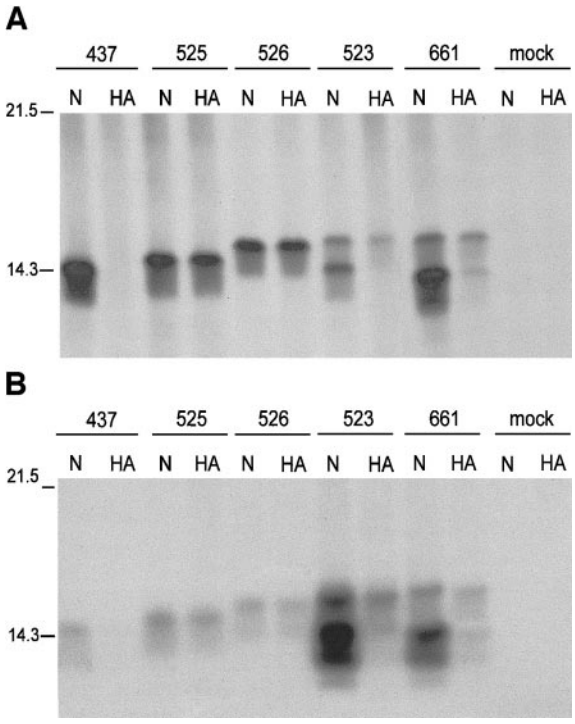

FIG. 5. Analysis of the N protein expressed by the PRRSV mutants expressing HA. Proteins were immunoprecipitated from lysates of BHK-21 cells transfected with RNA transcripts from pABV437, -525, -523, -661, and -526 (A) and from lysates of PAMs infected with the supernatant of transfected BHK-21 cells (B). Labeling was performed at 15 h after transfection (A) and infection (B), respectively, during 4 h. The immunoprecipitated proteins were analyzed by SDS–PAGE in a gel containing 14% acrylamide. The numbers on the left show the sizes of marker proteins in kilodaltons.

the 15-kDa protein, contained the HA epitope. The same protein bands were observed when immunoprecipitates from the lysates of PAMs infected with passage 0 of vABV523 and vABV661 were analyzed. The results indicated that in the context of the PRRSV genome FMDV $2A^{pro}$ was able to cleave the N protein from the HA–protease2A–N polyprotein both in BHK-21 cells and PAMs. However, cleavage of both polyproteins was not complete.

Analysis of the kinetics of cleavage by $2A^{pro}$ of FMDV

To further study the characteristics of cleavage by protease 2A in the context of the PRRSV genome, we performed pulse chase labeling experiments on BHK-21 cells transfected with transcripts from pABV523 and pABV661 (Fig. 6A) and on PAMs infected with the virus produced in these BHK-21 cells (Fig. 6B). The proteins were precipitated with the N-specific MAb. The wild-type N protein, as already observed in Fig. 5, was present after a pulse of 15 min both in BHK-21 cells and in PAMs for pABV523 and pABV661. In all cases, a band of approximately 12 kDa was precipitated, which has been observed before in immunoprecipitations of the N protein (Meulenberg *et al.,* 1995). It is considered a minor ORF7-derived product formed as a result of proteolytic degradation, premature translation stop, or internal initiation of translation. In addition, we observed that the intensities of both the wild-type N protein and the polyproteins of 18 and 19 kDa, precipitated with the N-specific MAb, remained quite constant during the chase. Hence, cleavage by FMDV $2A^{pro}$ is a rapid and probably cotranslational event, although we cannot exclude that cleavage occurs very rapidly posttranslationally. Densitometric analysis of the amount of radioactivity present in the 18-, 19-, and 15-kDa protein bands revealed that the polyprotein of vABV661 was cleaved more efficiently (approximately 85% after the pulse) than that of vABV523 (70%), both in BHK-21 cells and in PAMs. These ratios remained quite similar during the chases.

DISCUSSION

In this study we have focused on the use of PRRSV as a viral vector for the delivery of a foreign epitope, the

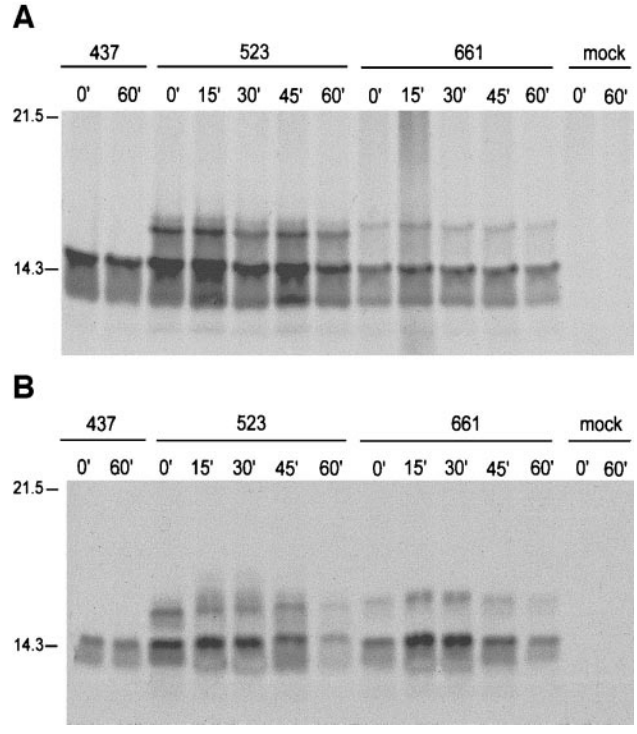

FIG. 6. Analysis of the kinetics of cleavage of the HA epitope from the N protein by $2A^{pro}$ in BHK-21 cells (A) and PAMs (B). Fifteen hours after transfection (A) and infection (B) the cells were pulsed for 15 min with L-[^{35}S]methionine. Chases were performed in medium containing 5 mM nonradioactive methionine for the periods indicated above the lanes. Lysates were prepared and immunoprecipitated with MAb 122.17 directed against the N protein. The immunoprecipitated proteins were analyzed by SDS–PAGE on a gel containing 14% acrylamide. The numbers on the left show the sizes of marker proteins in kilodaltons.

nine-amino-acid HA epitope from the hemagglutinin of human influenza A virus. We report that mutant cDNA clones in which HA sequences were inserted in frame with ORF7, either preceding or following it, yielded RNA that was able to replicate and to induce viral subgenomic RNAs, when transfected into BHK-21 cells. Both the PRRSV proteins and the HA epitope were produced from the subgenomic RNAs of the variously designed constructs. In addition, the transcripts all gave rise to the production of infectious virus. Yet those viruses that encoded the HA epitope as a direct fusion protein with the N protein lost their ability to express the epitope after serial passages on PAMs. This appeared to be due to the loss of sequences encoding the HA epitope from the PRRSV genome, as indicated by sequence analyses of the viral genomes. However, if the HA epitope was expressed as a cleavable extension of the N protein, the viruses grew normally and maintained the foreign sequences stably during serial passages while expressing the epitope.

The noncleavable extension of the N protein with the HA sequence either at the N or the C terminus was found not to be lethal to the virus. Yet this extension affected viral growth significantly and mutant viruses from which the foreign sequences had been deleted grew out selectively during serial passages. In contrast, the fitness of viruses expressing a cleavable extension of N appeared unaffected and the foreign sequences were stably maintained. Little is still known about the functioning of the N protein during different phases of the viral life cycle, but it is conceivable that its nonremovable extensions somehow interfered with processes during assembly or disassembly of virions. The N protein plays an important role during virus formation. During budding, it is involved in the packaging of viral RNA into a nucleocapsid as well as in the interaction of the nucleocapsid with the viral membrane proteins. Interference with either of these processes would be expected to lead to reduction in virus production, as we observed. The correct functioning of the N protein is also required in the initiation of a new infection. For instance, as an important step occurring shortly after viral entry, the N protein has to dissociate from the viral RNA to allow translation of the polymerase gene. Interference with the process of viral disassembly might thus result in a delayed onset of virus multiplication, which we actually observed. However, by electron microscopy we did not observe any differences in virus assembly and morphogenesis (data not shown). Still another way by which insertion of foreign sequences might reduce viral growth is by affecting the replication of the viral genome. This possibility is not quite unrealistic, since the inserts were located close to the 3′ untranslated region.

The 16 amino acid long FMDV $2A^{pro}$ peptide is known to exhibit its enzymatic cleavage activity also in the context of foreign sequences (Mattion *et al.,* 1996; Percy *et al.,* 1994; Ryan and Drew, 1994; Ryan *et al.,* 1991). We observed efficient cleavage of the polyprotein in which protease 2A had been inserted in frame between the HA epitope and the PRRSV nucleocapsid protein. The presence of additional neighboring FMDV sequences was not essential for cleavage. However, extension of the protease by seven amino acids, which are normally present directly upstream of $2A^{pro}$ in FMDV, increased the cleavage efficiency significantly. Since cleavage by protease 2A in the context of a foreign genome was found to be not as efficient as in the context of FMDV (Mattion *et al.,* 1996), in which no precursors spanning the cleavage site are detected during the native polyprotein processing, we assume that the conformation and sequence of the flanking regions influence cleavage efficiencies. The percentages of cleavage of the protease obtained in the context of PRRSV (70 and 85% for HA–$2A^{pro\text{-}17}$–N and 85HA–$2A^{pro\text{-}24}$–N, respectively) were in the same order of magnitude as in the context of other foreign sequences. For example, Ryan *et al.* (Ryan *et al.,* 1991) described the cleavage of a CAT–$2A^{pro}$–GUS polyprotein in which cleavage by a $2A^{pro}$ of 20 amino acids resulted in a cleavage efficiency of 80%. Other groups (Mattion *et al.,* 1996; Ryan *et al.,* 1991) showed that cleavage by protease 2A is a cotranslational event. Our results seem to confirm these findings, although we cannot rule out completely that cleavage occurs by very rapid posttranslational proteolysis. Since the 2A self-cleavage occurs between the amino acids glycine and proline, the released N protein predictably lacks the N-terminal methionine and possesses a proline instead of the alanine present at the N-terminus of the wild-type N protein. This change in amino acids is not likely to cause changes in the structure of the N protein since proline and alanine are both aliphatic residues and because a proline residue naturally occurs at this position in the N protein of the US PRRSV isolate VR2332 (Meulenberg *et al.,* 1998a). In our mutant virus, the resulting N protein indeed appeared to behave like wild-type N.

Although the predicted molecular weights of the noncleavable fusion proteins HA–N and N–HA were the same, they migrated differently upon SDS–PAGE. While the HA–N protein migrated according to its size, the N–HA polypeptide moved significantly slower. Anomalous behavior on SDS–PAGE has been documented earlier for various proteins (Waehneldt, 1975; Weber and Osborn, 1975). Protein mobility can be influenced by differences both in intrinsic charge (Armstrong and Roman, 1993; Graceffa *et al.,* 1992; Ohara and Teraoka, 1987) and in hydrophobicity (de Jong *et al.,* 1978) and can be affected just by simple point mutation, the effect depending sometimes on the location in the polypeptide chain. We have no explanation for the position-dependent effect of the extension of the N protein with the HA epitope. Intra- or intermolecular disulfide bonds are unlikely to be responsible for the higher mobility of the

N–HA fusion protein, since sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed under denaturing conditions in the presence of 2-mercaptoethanol. To exclude incomplete denaturation, we also analyzed the fusion proteins on SDS–PAGE in the presence of 3% urea, but the same differences in protein migration were still observed.

To our knowledge, this is the first report describing an arterivirus that stably expresses a foreign antigen and produces virus. The ability of arteriviruses to express a foreign gene has already been shown earlier for EAV (van Dinten *et al.,* 1997). However, no virus was produced from these constructs, since they lack the expression of one of the structural proteins. The application of the $2A^{pro}$ sequence for the construction of viral vectors that stably express a foreign epitope has been described before, such as for influenza virus (Percy *et al.,* 1994), foamy virus (Schmidt and Rethwilm, 1995), and poliovirus (Mattion *et al.,* 1996). Our results indicate that PRRSV has potential as a viral vector. Since PRRSV specifically infects alveolar macrophages, which are involved in antigen presentation to the immune system, it is conceivable that attenuated mutants of the virus can be used as vectors for the presentation of antigens of pathogens that cause respiratory diseases in pigs. As a first step, we are planning to study the ability of the HA-expressing PRRSV mutants to induce antibody responses against the foreign epitope *in vivo* by experimental infections in pigs.

MATERIALS AND METHODS

Cells and viruses

BHK-21 cells were grown in BHK-21 medium (Gibco BRL), completed with 5% FBS, 10% tryptose phosphate broth (Gibco BRL), 20 mM HEPES, pH 7.4 (Gibco BRL), 200 mM glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, 20 μg/ml kanamycin, 5 μg/ml polymixin B, and 0.2 μg/ml fungizone. Porcine alveolar lung macrophages were maintained in MCA–RPMI 1640 medium, containing 10% FBS, 100 μg/ml kanamycin, 50 U/ml penicillin, 50 μg/ml streptomycin, 25 μg/ml polymixin B, and 1 μg/ml fungizone. Serial passage of the recombinant PRRS viruses was started by inoculation of 500 μl of the culture supernatant of transfected BHK-21 cells onto 5×10^6 PAMs. The inoculum was removed after 1 h and 5 ml of fresh medium was added. The culture supernatant, containing the produced virus, was harvested approximately 48 h after infection. Serial passage was further performed by inoculation of 250 μl of the harvested culture supernatant onto 5×10^6 PAMs and, again, the culture supernatant was harvested after 48 h. Virus titers (expressed as 50% tissue culture infective doses [$TCID_{50}$] per milliliter) were determined on PAMs by end point dilution (Wensvoort *et al.,* 1986).

Construction of full-length genomic cDNA clones of LV encoding the HA epitope

PCR mutagenesis was used to introduce sequences into the *Pac*I-mutant of the genome-length cDNA clone of LV (pABV437) (Meulenberg *et al.,* 1998b). Four different full-length clones that contain the sequence of the HA epitope were made. The four PCR fragments, described below, were digested with *Hpa*I and *Pac*I and ligated into these sites of pABV437. Standard cloning procedures were performed essentially as described by Sambrook *et al.* (Sambrook *et al.,* 1989). Transformation conditions were maintained as described by Meulenberg *et al.* (Meulenberg *et al.,* 1998b) and sequence analysis was performed to confirm the introduced mutations. First, the sequence of nine amino acids encoding the HA epitope was introduced directly downstream of the start codon of ORF7 in pABV437. Two sequential PCRs were performed with primers LV192 and LV112 and with primers LV193 and LV112. The primers are listed in Table 1. This resulted in the generation of the full-length clone pABV525 (HA–N). Second, both the sequence encoding the HA epitope and the sequence encoding the 16 amino acids of protease 2A of FMDV plus a proline residue (Robertson *et al.,* 1985) were introduced directly downstream of the start codon of ORF7. Sequential PCR reactions were performed with primers LV139 and LV112 and with LV140 and LV112. This fragment was inserted in the full-length clone to generate pABV523 (HA–$2A^{pro-17}$–N). Third, the 9 amino acids of the HA epitope as well as 7 amino acids of the 3′ end of protein 1D of FMDV (Donnelly *et al.,* 1997) and the 17 amino acids of the protease 2A of FMDV were introduced directly downstream of the start codon of ORF7. We performed sequential PCR reactions with primers LV205 and LV112 and with LV206 and LV112 on pABV523. The insertion of the PCR product in pABV437 resulted in clone pABV661 (HA–$2A^{pro-24}$–N). Fourth, the sequence encoding the HA epitope was introduced at the 3′ end of ORF7 in a PCR with primers LV108 and LV194. This PCR fragment was inserted in pABV437, resulting in the full-length clone pABV526 (N–HA). Viruses derived from these recombinant full-length clones were designated vABV525, vABV523, vABV661, and vABV526, respectively (Fig. 1).

Sequence analysis

The regions of the full-length cDNA clones originating from the PCR products were analyzed by nucleotide sequencing. Sequences were determined with the PRISM Ready Dye Deoxy Terminator cycle sequencing kit and the ABI PRISM 310 Genetic Analyzer (Perkin–Elmer).

In vitro transcription and transfection of BHK-21 cells

The full-length genomic cDNA clones were *in vitro* transcribed and BHK-21 cells were transfected with the

TABLE 1

Sequences of the Primers Used to Introduce the Foreign Sequences by PCR and of the Primers Used to Sequence the Introduced Mutations

Primer (nt. position)	Sequence of the primer[a]	Orientation	Purpose
119R64R (14932)	5′ TCGCGTGACTTCTACATCC 3′	+	Sequencing
LV19 (14571)	5′ GGTTAACCTCGTCGACTATG 3′	−	Sequencing
LV20 (15066)	5′ CCTGATTAAAAGCTTGACCCC 3′	+	Sequencing
LV73 (14577)	5′ ATACTCCCGGGTCATCCTAAGCAGCCAGGGGAA 3′	+	Sequencing
LV76 (15088)	5′ TCTAGGAATTCTAGACGATC(T)40 3′	−	RT-PCR
LV108 (14566)	5′ GGAGTGGTTAACCTCGTCAAGTATGGCCGGTAAA AACCAGAGCC 3′	+	ORF7-HA
LV112 (14958)	5′ CCATTCACCTGACTGTTTAATTAACTTGCACCCTGA 3′	−	*Pac*I-site
LV139 (14609)	5′ AACTTTGACCTTCTCAAGTTGGCCGGCGACGTCGAG TCCAACCCAGGGCCCGGTAAAAACCAGAGCCAGAAG 3′	+	HA–2A^{pro-17}–ORF7
LV140 (14609)	5′ GAGTGGTTAACCTCGTCAAGTATGGCCGGTAAATACC CATACGATGTTCCAGATTACGCTAACTTTGACCTTCTC 3′	+	HA–2A^{pro-17}–ORF7
LV192 (14609)	5′ AAATACCCATACGATGTTCCAGATTACGCTAACCAGA GCCA 3′	+	HA–ORF7
LV193 (14609)	5′ AGTGGTTAACCTCGTCAAGTATGGCCGGTAAATACCC ATACG 3′	+	HA–ORF7
LV194 (14971)	5′ ACTGTTTAATTAAGCGTAATCTGGAACATCGTATGGGTA ACTTGCACCCTG 3′	−	ORF7–HA
LV205 (14609)	5′ GATGTTCCAGATTACGCTGCACCGGTCAAACAGCTGTT GAACTTTGACCTT 3′	+	HA–2A^{pro-24}–ORF7
LV206 (14609)	5′ GAGTGGTTAACCTCGTCAAGTATGGCCGGTAAATACCC ATACGATGTTCCAGAT 3′	+	HA–2A^{pro-24}–ORF7

[a] The restriction sites are underlined. The outlined boxes represent the HA epitope. The boxes with the dotted outlines represent sequences of FMDV.

resulting RNA either using Lipofectin (Gibco BRL) or by electroporation, as described before by Meulenberg *et al.* (Meulenberg *et al.,* 1998b).

Infection of PAMs

To rescue infectious virus, the culture supernatant of BHK-21 cells was harvested 24 h after transfection and used to inoculate PAMs. After 1 h the inoculum was removed and fresh culture medium was added. Approximately 15 h after infection the culture supernatant was harvested and PAMs were washed with PBS, dried, and stored at −20°C until the immunoperoxidase monolayer assay was performed.

Immunoperoxidase monolayer assay

Immunostaining of BHK-21 cells and PAMs was performed by the methods described by Wensvoort *et al.* (Wensvoort *et al.,* 1986). Monoclonal antibodies (MAbs) against GP_3 (122.14), GP_4 (122.1), the M protein (126.3), and the N protein (122.17) (van Nieuwstadt *et al.,* 1996) were used to detect the expression of PRRSV proteins. MAb 12CA5 (Boehringer Mannheim) was used to detect the expression of the HA epitope.

Genetic analysis of genomic RNA of recombinant viruses

To analyze the viral RNA in the culture supernatant of PAMs, 500 μl of culture supernatant was diluted with an equal volume of proteinase K buffer (100 mM Tris–HCl [pH 7.2], 25 mM EDTA, 300 mM NaCl, 2% [wt/vol] sodium dodecyl sulfate), and 0.2 mg Proteinase K was added. After incubation for 30 min at 37°C, the RNA was extracted with phenol–chloroform and precipitated with ethanol. The RNA was reverse transcribed with primer LV76, and PCR was performed using primers LV19 and LV73 flanking the foreign sequences of vABV525, vABV523, and vABV661 and with primers LV20 and 119R64R flanking the foreign sequence of vABV526. The amplified fragments were analyzed in 2% agarose gels and the PCR fragments were excised from the gel and purified with SpinX columns (Costar). Sequence analysis of the fragments was performed using the antisense primer of the PCR.

Radioimmunoprecipitation

Metabolic labeling and immunoprecipitation of proteins expressed in BHK-21 cells and PAMs was performed essentially as described previously by Meulenberg and Petersen den Besten (Meulenberg and Petersen den Besten, 1996). MAbs 122.17 and 12CA5 were used to immunoprecipitate the N protein and the HA epitope, respectively. BHK-21 cells and PAMs were labeled at 15 h posttransfection and postinfection, respectively. In the pulse chase experiments, the cells were labeled for 15 min with L-[^{35}S]methionine (Amersham). The cells were then washed and chased with cold me-

dium containing 5 mM methionine for 15, 30, 45, and 60 min. Samples were analyzed by SDS–PAGE using a 14% acrylamide gel.

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