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The ORF, Regulated Synthesis, and Persistence-Specific Variation of Influenza C Viral NS1 Protein

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The open reading frame (ORF) and the regulated synthesis of the influenza C viral NS1 protein were analyzed in view of viruses possessing different biological activities. We provide evidence for a 246-amino-acid NS1-ORF, encoded by five viral strains and variants. Prokaryotic expression of the prototype NS1-ORF resulted in a product of 27 kDa, confirming the predicted molecular weight. Using an antiserum raised against recombinant NS1 protein, nonstructural proteins of wild-type virus were detected in infected cells for a limited course of time, whereas a persistent virus variant was characterized by a long-term nonstructural gene expression. As examined by infection experiments, the intracellular distribution of nonstructural protein was nuclear and cytoplasmic, whereas in NS1 gene-transfected cells, the cytoplasmic localization occurred in a fine-grained structure, suggesting an analogy to influenza A viral NS1 protein. Concerning persistent infection, NS1 protein species differing in sizes and posttranslational modifications were observed for a persistent virus variant, as particularly illustrated by a high degree of NS1 phosphorylation. Virus reassortant analyses proved the importance of the NS-coding genomic segment: the minimal viral properties required for the establishment of persistence were transferred with this segment to a monoreassortant virus. Thus the influenza C viral NS1 protein is a 246-amino-acid nuclear-cytoplasmic phosphoprotein that can be subject to specific variations being functionally linked to a persistent virus phenotype.

INTRODUCTION

The NS1 protein of influenza C virus is encoded by RNA segment 7, which is 935 nucleotides in length. An open reading frame (ORF) for NS1 had been proposed, coding for a polypeptide of 286 amino acids (Buonagurio *et al.,* 1986; Nakada *et al.,* 1985). In another report, however, an NS1-ORF of 246 amino acids in length was described after determining the complete NS gene sequence of C/Yamagata/1/88 and a partial sequence of C/Ann Arbor/1/50 virus (Hongo *et al.,* 1992). This controversial situation awaited further analysis. Functional aspects of the influenza C viral NS1 protein were less intensively studied than those of the corresponding protein of influenza A virus. Influenza A viral NS1 protein is known to be a nonstructural phosphorylated protein (Privalsky and Penhoet, 1981; Ward *et al.,* 1994) that is localized in the nucleus and nucleoli as well as at polysomes in the cytoplasm of infected cells. It is encoded by an unspliced mRNA from the NS1-ORF of segment 8 and is preferentially synthesized at early times in the virus cycle (reviewed by Lamb, 1989). The RNA binding activity of influenza A viral NS1 protein shows binding specificity for different targets, such as for poly(A)$^+$ sequences (Qiu and Krug, 1994), and leads to a regulated nuclear mRNA export (Alonso-Caplen *et al.,* 1992). Moreover, NS1 protein is associated with spliceosomes and inhibits pre-mRNA splicing (Fortes *et al.,* 1994; Lu *et al.,* 1994). Additional activities were postulated, such as NS1 protein, was shown to act as a positive regulator of translation (de la Luna *et al.,* 1995; Enami *et al.,* 1994; reviewed by Ortin, 1998).

In several studies on variant isolates of influenza A virus, the involvement of nonstructural proteins in the determination of different phenotypes was investigated (Lucas *et al.,* 1988; Ludwig *et al.,* 1995; Perdue, 1992; Urabe *et al.,* 1994; Ward *et al.,* 1995), particularly raising the question of NS-specific mechanisms leading to persistent infection. For influenza C virus, a persistence model was established in cultured canine kidney cells (Camilleri and Maassab, 1988). Viral persistence was transfered by secondary infection to a human lung cell line (Marschall *et al.,* 1996a), and persistent viral RNAs were detected in lung tissues of experimentally infected chickens (Helten *et al.,* 1996). Productive and nonproductive phases of persistence were observed (Marschall *et al.,* 1993; Zach *et al.,* in press), both associated with an extremely low degree of virus-induced cytopathology and apoptosis compared with lytic types of infection

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TABLE 1

Comparison of Partial Nucleotide Sequences Which Are Responsible for the Determination of Two Different NS1-ORFs

Influenza C virus	Nucleotide sequences 690–710[a]
C/AA-wt[b]	CCC TAT TGG GGA AAT GAA AAA
C/AA-pi[b]	CCC TAT TGG GGA AAT GAA ACA
C/JHB[c]	CCC TAT TGG GGA AAT GAA AAA
C/TAY[c]	CCC TAT TGG GGA AAT GAA ACA
C/ENG[c]	CCC TAG TGG GGN AAT GAA AAA
C/YAM[d]	CCC TAT TGG GGA AAT GAA ACA
C/AA[e]	CCC TAT TGG GAA ATG AAA CAC

[a] The presence of a distinct G residue at position 700 is indicated by underlining.

[b] Sequences determined from clones pGEX-NSwt and pGEX-NSpi.

[c] Sequences directly determined from RT-PCR products.

[d] Sequence published by Hongo *et al.* (1992) and partly reinvestigated here by the use of clone pCNS5-8.

[e] Sequence published by Buonagurio *et al.* (1986).

(Hechtfischer *et al.,* 1997; unpublished observations). The viral particles released from persistently infected cells were characterized by their typical properties due to a modified HEF surface glycoprotein, such as by altered receptor binding and acetylesterase activities (Marschall *et al.,* 1994). Distinct mutations in different genomic segments of the persistent variant were identified (e.g., for the viral polymerase gene PB2) (Lapatschek *et al.,* 1995).

In this study, we demonstrate that the NS1 protein of five influenza C viruses is encoded by an ORF of 246 amino acids that underlies variant-specific sequence alterations. Examination of NS1 protein synthesis revealed new aspects of protein kinetics, intracellular distribution, and posttranslational modification. With regard to persistent viruses, the importance of a mutant form of NS1 protein was illustrated by different approaches.

RESULTS

Nucleotide sequence analysis was performed to determine the ORF encoding the NS1 protein of influenza C viruses. For this, RT-PCR products of the genomic RNA segment 7 of five virus isolates were generated and used for cloning or direct sequence determination (Table 1). Complete sequences of the two virus variants of strain C/Ann Arbor/1/50 (C/AA-wt and C/AA-pi) and partial sequences of the strains C/JHB, C/TAY, and C/ENG were analyzed. The sequence around the NS1 start codon was determined separately by the use of RT-PCR products derived from an additional pair of PCR primers (see Materials and Methods). Data were compared with the putative NS1-ORFs described in previous studies. The NS1-ORF resulting from our analysis is composed of 738 coding nucleotides and is defined by an ATG start codon at position 27 and a TAA stop codon at position 765. All NS1-ORF sequences analyzed (including a reinvestigation of NS1 of strain C/YAM) contained an additional G residue at nucleotide position 700, which was missing in sequences published previously. The integrity of this nucleotide position was confirmed by several independent sequencing templates and by PCRs involving enzymatic proof reading activity. Thus the NS1-ORF described here differs from that reported in earlier studies (Buonagurio *et al.,* 1986; Nakada *et al.,* 1985) but is identical to that described recently (Hongo *et al.,* 1992).

Given this information, we deduced the complete NS1 amino acid sequences of viruses C/AA-wt and C/AA-pi (Table 2). A polypeptide of 246 amino acids in length was determined including nine coding mutations differing between the two viruses. Five of these changes (amino acid positions 39, 84, 96, 189, and 232) were characteristic for the variant C/AA-pi and did not occur in published sequences of other viral strains. The C-terminal part of C/AA-wt and C/AA-pi viral NS1 protein varieties (starting at amino acid 225) was distinct from the NS1-ORF of viral sequence C/AA, as explained by the additional G residue at position 700.

Expression of the NS1-ORFs of variants C/AA-wt and C/AA-pi (Fig. 1A) was performed in *Escherichia coli* and GST-NS1 fusion proteins were detected on Western blots (Fig. 1B). A small variation in protein sizes of the respective clones was visualized (in particular, by extended runs of electrophoretic separation), indicating that slightly varied gel mobilities might be due to the amino acid changes presented in Table 2. Cleavage of wild-type GST-NS1 protein by thrombin digestion resulted in a NS1 product of ~27 kDa (data not shown). This is consistent with the theoretical molecular mass of 27.8 kDa derived from the NS1-ORF described here (246 amino acids) and differs from that formerly calculated as 32.4 kDa (286 amino acids). GST-NS1 protein was used to raise rabbit antiserum Z-NS1 for the characterization of native viral NS1 protein in infected cells.

An infection kinetic of the virus variants C/AA-wt and C/AA-pi in MDCK cells was monitored at the time points 8 h and 1, 2, 3, 5, and 7 days postinfection (p.i.) by indirect immunofluorescence double-stainings of NS1/NP and NS1/HEF proteins (data not shown). Wild-type virus C/AA-wt was characterized by an early onset (8 h) of nonstructural protein synthesis, showing a maximum between days 1–3, and a steady reduction at later time points that ended in a complete loss of signals. Structural proteins NP and HEF produced by C/AA-wt virus paralleled this kinetic but showed a slight delay in their onset as well as in termination of synthesis. The persistent virus variant C/AA-pi, however, started at 1 day p.i. to produce continuously detectable amounts of viral nonstructural proteins and, in a delayed fashion, of structural proteins as well. No reduction in protein synthesis was noted within the period of 7 days investigated. In kinetic experiments described elsewhere, NS1 protein synthe-

TABLE 2

NS1 Nucleotide and Amino Acid Sequences Determined for Two Viral Variants[a]

[a] Small dots in the nucleotide sequence and bold dots in the amino acid sequence represent identity; the termination codon is indicated by hyphens. Noncoding sequences at the 5′ (26 nucleotides) and 3′ termini (168 nucleotides) in mRNA sense, as published for strain C/California/78 (Nakada *et al.,* 1985), were not determined. Variations of putative amino acid phosphorylation sites differing between the C/AA-wt and the C/AA-pi sequences are underlined. Amino acid changes that did not occur in published sequences of other viral strains are in bold. GenBank accession numbers: AF102026, AF102027.

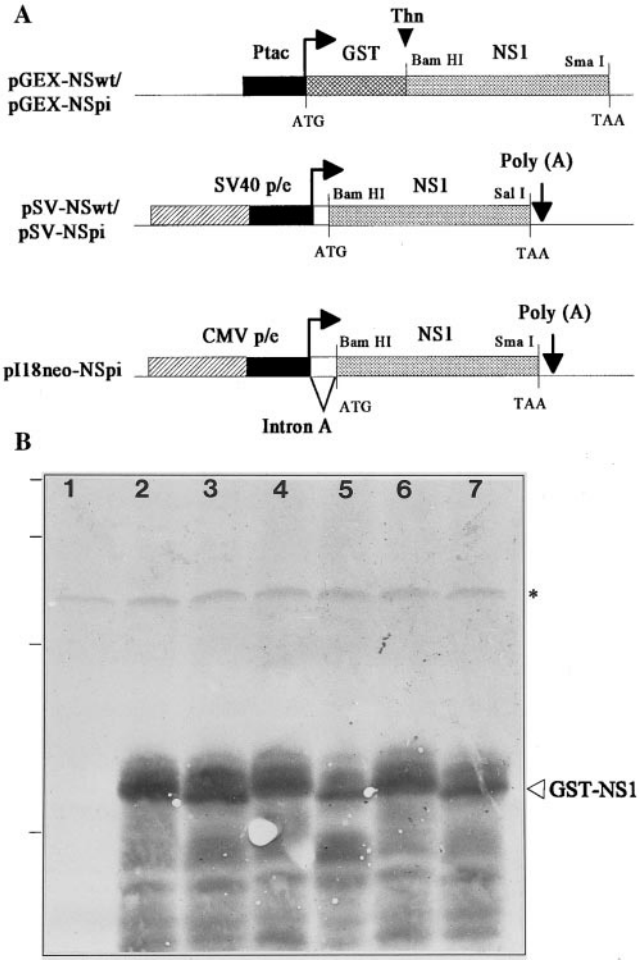

FIG. 1. Expression plasmids and prokaryotic production of GST-NS1. (A) For prokaryotic expression (top), NS1-ORF was placed downstream of the tac-promoter (P_{tac}) and the glutathione-*S*-transferase coding sequence (GST) to generate GST-NS1 fusion protein. Thn indicates thrombin cleavage site. For transient eukaryotic expression (middle), NS1 production was controlled by the SV40 promoter/enhancer (SV40 p/e) and the terminal polyadenylation consensus sequence [Poly(A)]. For stable eukaryotic expression (bottom), a *neo*-selectable construct was used, allowing that NS1 production was constitutively driven by the CMV promoter/enhancer (CMV p/e) linked to the splicing signals of CMV intron A. (B) Lysates of three pGEX-NSwt clones (lanes 2, 4, and 6), three pGEX-NSpi clones (lanes 3, 5, and 7), and vector clone pGEX-2T (lane 1) were separated by extended SDS–PAGE (to the point where GST was released into the flowthrough) and subjected to Western blotting. The specific staining of recombinant proteins (GST-NS1) was achieved by the use of anti-GST antibodies (Pharmacia). Note a slight variation in molecular weights between pGEX-NSwt and pGEX-NSpi clones (arrowhead). Asterisk indicates cross-reaction with a bacterial protein (Yu-Sherman and Goldberg, 1992); marker bands indicated on the left (High Molecular Weight Standard Mixture, Sigma), 116/97.4/66/45 kDa.

sis of the persistent virus C/AA-pi was monitored along a period of 55 days (Marschall *et al.,* 1998). Thus NS-specific expression of the persistent virus is not subject to a strict down-regulation on the protein or on the RNA level (Zach *et al.,* in press), as described for the late phase of wild-type viral replication. With regard to the long-term maintenance of viral persistence, however, protein synthesis may occur in changes between productive and nonproductive phases (Marschall *et al.,* 1993).

Concerning the subcellular localization of viral NS1 protein, cytoplasmic and nuclear staining signals (i.e., cells stained in either or both of the two compartments) were obtained. MDCK cells infected with C/AA-pi virus were double-stained for nonstructural and nucleoprotein (NP) synthesis 3 days p.i. (Figs. 2, A–C). Both viral proteins did not underly a strict subcellular compartmentation in infected cells but were frequently distributed over the whole cell bodies. In cells transfected with either NS1 or NP genes separately, however, distinct localizations of the synthesized proteins became detectable. Interestingly, the predominant cytoplasmic distribution of NS1 protein occurred in a fine-grained structure as particularly illustrated by confocal immunofluorescence (Fig. 2E). This microscopic pattern clearly differed from that of recombinantly expressed NP protein, which showed a massive nuclear accumulation (Fig. 2F). Thus NS1 and NP proteins of the persistent virus variant possess specific localization properties that are clearly detectable in transfected but less defined in infected cells.

The virus variant-specific synthesis of NS1 protein was further demonstrated in infected MDCK cells by Western blot analysis (Fig. 3A). C/AA-wt virus was shown to produce one major NS1 product at 20 h p.i. (lane 2), which was reduced to lower amounts at 50 h p.i. (lane 3) and disappeared at later time points as determined in different experiments. In contrast to this, C/AA-pi virus induced the synthesis of two forms of NS1 proteins, both detectable at variable time points of productive persistent infection (lane 4), whereas none of these NS1 products were present during nonproductive intervals (lane 5). As a control, viral matrix protein (M) was detected in parallel using the same cell samples. The differences in virus variant-specific gene expression were also reflected by the quantities of M protein synthesis, whereas no variation in M protein sizes was determined (Fig. 3B). Performing recombinant expression of NS1 in transfected MDCK-NSpi cells, the variant nature of persistent-type NS1 protein was confirmed. In these cells, the two forms of NS1 proteins were synthesized (Fig. 3C), which were indistinguishable from those seen in persistently infected cells. This indicates that the modified NS1 expression of the virus variant C/AA-pi is independent from the action of further viral proteins.

Detailed analysis of NS1 protein varieties was performed by RIPA using infected cell lysates. C/AA-wt virus-derived NS1 protein was determined with a molecular weight of 27 kDa, identical to that produced by other viral strains (Fig. 4a). An additional faint band of 28 kDa was detectable at times (data not shown). The C/AA-pi variant, however, produced NS1-specific proteins of 26.5 and 27.5 kDa, which were not found in samples of other

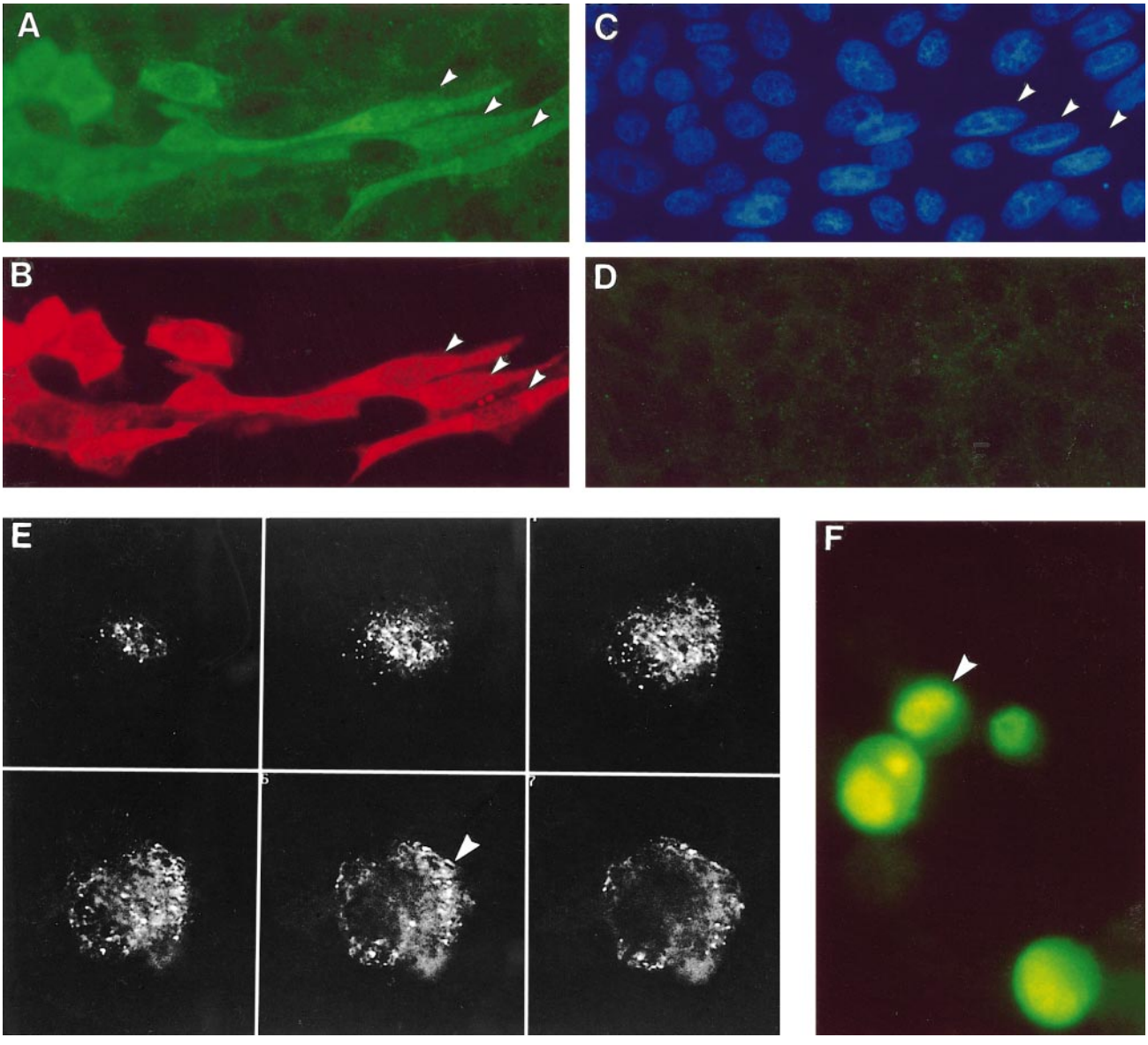

FIG. 2. Intracellular distribution of the viral proteins NS1 and NP during infection or recombinant expression. (A–D) MDCK cells were grown on Lab-Tek slides, infected with C/AA-pi virus or mock-infected, and incubated for 3 days. After fixation, the slides were assayed by immunofluorescence double-staining for the detection of viral NS1 (A, antiserum Z-NS1) and NP proteins (B, MAb-NP F17) in identical fields of infected cells (arrowheads); nuclei were counterstained by the use of DAPI reagent (C). Mock-infected cells served as a negative control (D, antiserum Z-NS1). (E) COS7 cells were transfected with pSV-NSpi and cultured on Lab-Tek slides. Three days after transfection, NS1 protein was detected by immunofluorescence analysis using antiserum Z-NS1. In confocal laserscanning microscopy, specific signals were monitored in serial layers of 0.5-μm distance from the top (upper left) to the medial cell body (lower right). Note the structured cytoplasmic staining pattern (arrowhead). (F) MDCK cells were transfected with pI18-NPpi, cultured for 1 day, and spotted onto glass slides for immunofluorescence analysis. Anti-C/JHB virus hyperimmune serum (H1/66) was used to visualize recombinant NP expression in a marked nuclear accumulation (arrowhead). Microscopic magnification, 400×.

viruses tested. This variation was considered to correlate with the distinct amino acid changes in the NS1 sequence, in particular to those concerning putative phosphorylation sites (Table 2). To address this item, we specified the RIPA analysis for phosphoproteins. As shown in Fig. 4b, we identified a NS1-specific phosphorylated polypeptide of 27.5 kDa for the C/AA-pi variant, which is identical with the 27.5-kDa protein detected in Fig. 4a, as indicated by the control lane (S). On overexposure, very small amounts of a 28-kDa NS1 phosphoprotein were also detectable for other viral strains (data not shown). These findings indicate that NS1 protein of influenza C virus is posttranslationally modified by phosphorylation and that the variant C/AA-pi produces a 27.5-kDa species of NS1 phosphoprotein in a constitutive manner during productive persistent phases.

The significance of variant NS1 expression for the determination of viral persistence was investigated by reassortant virus analyses. After a long-term selection procedure in infected MDCK cells, we succeeded in the

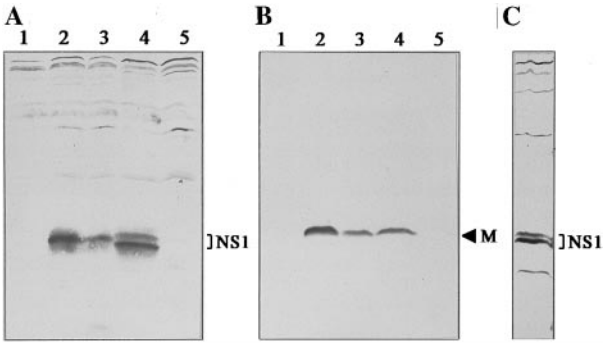

FIG. 3. Viral NS1 (A) and M proteins (B) produced in infected cells. MDCK cells were freshly infected with C/AA-wt virus and analyzed 20 and 50 h p.i. MDCK-pi cells were harvested at times with and without virus production (i.e., positive or negative hemagglutination signals from the culture medium). Lysates were separated by 17.5% SDS–PAGE, Western-blotted and exposed to the antiserum Z-NS1 or, as a control, to monoclonal antibodies against the matrix protein (MAb-M L2), respectively. Staining was performed by standard procedures using AP-conjugated antibodies. 1 indicates uninfected MDCK cells (MOCK); 2, MDCK cells infected with C/AA-wt virus for 20 h; 3, MDCK cells infected with C/AA-wt virus for 50 h; 4, MDCK-pi cells in a productive phase; and 5, MDCK-pi cells in a nonproductive phase. (C) Recombinant NS1 expression in MDCK-NSpi cells. MDCK cells were transfected with plasmid pI18neo-NSpi, selected for stably growing cell clones and analyzed by Western blotting as described in Fig. 3A.

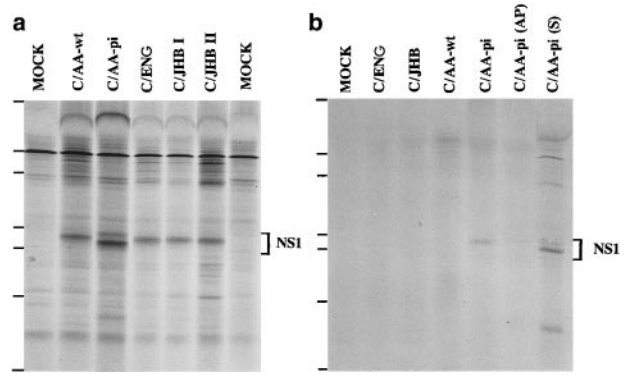

FIG. 4. NS1-specific proteins and NS1 phosphorylation identified for different influenza C viruses. (a) Immunoprecipitation of NS1-specific proteins was performed using lysates of cells infected with different influenza C viruses (C/JHB I and C/JHB II represent stock viruses from different sources). MDCK cells were infected for 36 h and labeled *in vivo* by an incubation in the presence of ^{35}S-methionine plus ^{35}S-cysteine (50 μCi ICN Tran^{35}S-Label/ml medium) for 24 h. Cell lysates were subjected to immunoprecipitation using antiserum Z-NS1, separated by SDS–PAGE and exposed to radiography films. (b) The demonstration of a posttranslational modification of NS1 polypeptides was achieved by immunoprecipitation of phosphoproteins. MDCK cells were infected with different influenza C viruses for 36 h and labelled *in vivo* by an incubation in the presence of inorganic ^{32}P-phosphate (330 μCi/ml medium, ICN) for 12 h. The immunoprecipitation of NS1-specific phosphoproteins was performed as described in Fig. 5a. Control reactions were carried out by dephosphorylation of the precipitate with alkaline phosphatase (AP) and by a precipitation control using a lysate labelled with ^{35}S-methionine/cysteine (S). Mock indicates uninfected cells.

isolation of a monoreassortant virus, C/R1, possessing characteristics of a persistent variant. The genomic structure of C/R1 virus consisted of a genetic background derived from the parental virus C/JHB, in a novel combination with the NS-encoding RNA segment originating from the persistent variant C/AA-pi (Table 3). In infection experiments, C/R1 virus behaved very similar to the variant C/AA-pi, concerning the long-term intracellular stabilization of viral RNAs, whereas RNA signals for C/JHB virus were not detectable >9 days p.i. (Fig. 5A). As depicted by Western blot analysis (Fig. 5B), the NS1-specific expression pattern was identical between the viruses C/R1 and C/AA-pi: two protein varieties of 26.5 and 27.5 kDa were stably produced during the time range analyzed. In contrast, C/JHB virus infection gave rise to the wild-type form of a 27-kDa NS1 protein, which was only transiently detectable at 1 day p.i. Determinations of individual biological characteristics of the reassortant virus C/R1 clearly illustrated its relatedness to C/AA-pi virus in establishing persistent infections in MDCK cells, whereas certain parameters also indicated a slightly different, intermediate phenotype with respect to both parental viruses (Table 4). As indicated by the predominance of continued nonproductive phases (i.e., the exclusive presence of viral RNA in the absence of virus production), it is suggestive that the C/R1 virus is equipped by the minimal genetic information required for persistent infection. Thus the influenza C viral NS-coding segment contributes as a major determinant to the establishment of *in vitro* persistence.

DISCUSSION

An influenza C viral NS1-ORF was identified that differs in its C-terminal part from an NS1-ORF reported earlier (Buonagurio *et al.,* 1986; Nakada *et al.,* 1985) and is identical to that described previously for strain C/Yamagata/1/88 (Hongo *et al.,* 1992). This finding has

TABLE 3

Genomic Structure of Reassortant Virus C/R1

Genomic segment	Restriction typing[a]	Sequencing[b]
PB2	C/JHB	C/JHB
PB1	C/JHB	C/JHB
P3	N.D.	C/JHB = C/AA-pi
HEF	C/JHB	C/JHB
NP	C/JHB	C/JHB
M	C/JHB	C/JHB
NS	C/AA-pi	C/AA-pi

[a] RT-PCR products of genomic segments were analyzed by a virus variant-specific restriction typing (Marschall *et al.,* 1998).

[b] Partial nucleotide sequences were determined for genomic sections of PB2 nt 1–309, PB1 nt 948–1543, P3 nt 784–1447, HEF nt 1013–1731, NP nt 1308–1756, M nt 383–720, and NS nt 126–843; the origin of segments was deduced from variant-specific positions (Marschall *et al.,* 1996; P3 sequences were identical).

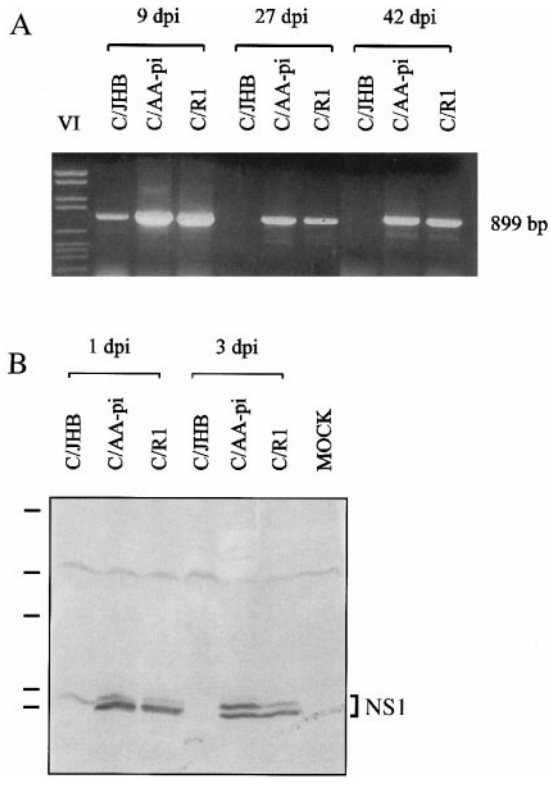

FIG. 5. Monoreassortant virus C/R1: long-term persistence of viral RNA and variant-specific pattern of NS1 protein synthesis. (A) Persistence of the viral NS coding segment was analyzed by RT-PCR in infected MDCK cells. An amplification product of 899 bp was detected for C/AA-pi or C/R1 viruses at 9, 27, and 42 days p.i. (dpi) for C/JHB virus at 9 days p.i. VI indicates DNA marker (2176, 1766, 1230, 1033, 653, 517, 453, 394, and 298 bp; Boehringer-Mannheim). (B) Infected MDCK cells were harvested 1 and 3 days p.i. Lysates were separated by 17.5% SDS–PAGE and subjected to Western blotting. The variant-specific pattern of viral NS1 protein was detected by the antiserum Z-NS1 and indirect alkaline phosphatase staining. Marker bands indicated on the left (Dalton Mark VII-L, Sigma; 66, 45, 36, 29, and 24 kDa); MOCK indicates uninfected cells.

implications on the primary structure of the NS1 protein (246 instead of 286 amino acids) and also of the NS2 protein (182 instead of 121 amino acids) because NS2 is known to be generated via a spliced mRNA derived from the same genomic segment (Nakada *et al.,* 1986). Protein data derived from recombinant NS1 expression and from infection experiments confirmed our sequence determination.

As illustrated by kinetic studies, viral NS1 protein is synthesized in the early period of infection. The subcellular localization of NS1 protein is nuclear and cytoplasmic, whereby the cytoplasmic localization may occur in typical fine-grained structures. This cytoplasmic staining pattern is reminiscent to the immune electron microscopic picture showing similar structures of influenza A viral NS1 protein recombinantly expressed in yeast cells and observations made in infected cells. Such electron-dense cytoplasmic inclusions were likely to correspond to a direct self-interaction of NS1 (Ward *et al.,* 1994). In fact, NS1 multimer formation was demonstrated using the yeast two-hybrid system (Nemeroff *et al.,* 1995).

In infection experiments, those influenza C viruses following a short-lived wild-type course of replication were characterized by the transiently limited synthesis of one major NS1 protein species, whereas a persistent variant continuously produced two protein species in comparable quantities along productive periods of persistence. This variant form of expression was attributed to the effects of NS1 gene mutation and posttranslational protein modifications: nine coding mutations were identified for the NS1 protein of the persistent variant with respect to wild-type virus. The occurrence of a mutant gene resulting in the production of a variant form of the NS1 protein was also reported for a persistent influenza A virus. Hereby, the NS1 variation was suggested to be involved in the development of the persistent virus phenotype (Lucas *et al.,* 1988). NS1 phosphorylation on threonine residues was demonstrated for influenza A/NWS/33 virus (Privalsky and Penhoet, 1981). The occurance of two NS1 proteins, differing in molecular weights and quantities from their wild-type counterparts, was found for influenza C/AA-pi virus and was explained by a distinct way of protein phosphorylation. This finding points out the significance of four potential threonine phosphorylation sites varying between the NS1 proteins of C/AA-pi and C/AA-wt viruses. In case of the persistent variant, the loss of two threonine residues on the one side and the gain of two different threonine residues on the other side might alter the effectiveness and stability of NS1 phosphorylation.

The production of two NS1 protein modifications was independent from other viral proteins as indicated by the recombinant expression in MDCK-NSpi cells. Hence, the identified persistent-type NS1 protein modifications are

TABLE 4

Biological Characteristics of Parental and Reassortant Viruses

Parameter	Viruses		
	C/JHB	C/AA-pi	C/R1
Allantoic growth rates in embryonated eggs[a]	High	Low	High
Persistent infections in MDCK cells[b]	No	Yes	Yes
Viral replication phases during persistence[c]		Mainly productive	Mainly nonproductive
NS1 protein variation[b]	No	Yes	Yes

[a] Viral progeny titers were determined by hemagglutination (HA) tests. High, ≤512 (C/JHB), ≤128 HAU/ml (C/R1); low, ≤4 HAU/ml (C/AA-pi, first egg passage). Titrations were performed in triplicate at least.

[b] Data shown in Fig. 5.

[c] Viral persistence was categorized by the predominance of positive (productive) or negative (nonproductive) HA test results obtained from long-term infected MDCK cells.

solely generated by cellular or NS1-derived processes. The two NS1 proteins might represent an unphosphorylated precursor and a phosphorylated target protein possessing different activities and being coexpressed during productive phases of viral persistence. Interestingly, Vero cells infected with the variant C/AA-pi do not support a long-term persistence and do not produce equal quantities of two coexisting NS1 protein species. In contrast, they show almost exclusively the production of the faster migrating, unphosphorylated 26.5-kDa NS1 protein (unpublished observation).

Furthermore, in studies on persistent virus RNA synthesis and transport, an aberrant manner of NS-specific transcription was described for the persistent variant C/AA-pi. The synthesis of segment 7 positive-strand RNA, continuing along the productive course of persistent infection, did not follow a specific down-regulation as it occurred in the late phase of wild-type virus infection (Zach *et al.,* in press). The lack of this down-regulatory event might explain in part the specialized kinetics of NS1 protein synthesis and consequences thereof.

The importance of NS1 protein for persistent infection was further illustrated by reassortment studies. In these experiments, the monoreassortant virus C/R1 showed a phenotype of infection that was similar to parental virus C/AA-pi, particularly concerning the long-term persistence of intracellular viral RNA. Although the possibility of different impacts of NS1 and NS2 proteins could not be distinguished by this approach, our results clearly indicate that the NS-coding genomic segment is a major determinant of viral persistence. Of note, not all the viral characteristics linked to a persistent phenotype were transferred with the mutant NS-coding segment. This situation suggests that further mutant viral gene products, minor determinants of persistence like PB2 (Lapatschek *et al.,* 1995), HEF (Marschall *et al.,* 1994), and M and NP (Hechtfischer *et al.,* submitted for publication), might act in a cumulative manner and might influence the stability and frequency of persistent infections. Analogous studies on the *in vitro* persistence of poliovirus mutants are in accordance with this scenario because they suggest a comparable hierarchy of viral determinants: distinct point mutations in viral capsid genes were identified as the minimal genetic markers of persistence, accompanied by mutations possessing importance for the frequency at which this viral phenotype was established (Duncan *et al.,* 1998; Pelletier *et al.,* 1998). In case of persistent Sindbis virus variants, a single coding mutation in the nonstructural gene nsP2 was described as the essential determinant for noncytopathic infection and viral persistence *in vitro.* Mutations identified in other genes were considered to play an additional role by decreasing the level of persistent viral RNA synthesis (Dryga *et al.,* 1997).

Concludingly, these results provide new insights in the general structure, the regulated synthesis and the modification of influenza C viral NS1 protein. Functional properties of NS1 need to be described in detail by future investigations. As an important point of this work, the identified variations in the mode of nonstructural gene expression are suggestive to play an essential role in the determination of persistent infection.

MATERIALS AND METHODS

Cells and viruses

MDCK, MDCK-pi, and MDCK-NSpi cells were cultivated in Dulbecco's modified Eagle's medium containing 10% (v/v) of fetal calf serum by refreshing the culture medium twice a week. The persistently infected MDCK-pi line was grown at virus-permissive (33°C) or nonpermissive temperatures (37°C) to induce productive or nonproductive phases (Marschall *et al.,* 1993, 1997). Stocks of wild-type influenza C/AA-wt and C/AA-pi, as well as the control viruses C/Johannesburg/1/66 (C/JHB), C/England/84 (C/ENG), and C/Taylor/1233/47 (C/TAY), were grown in MDCK cell cultures or in embryonated chicken eggs for 3–5 days at 33°C. Virus titers, in allantoic fluids or in cell culture supernatants, were determined by the hemagglutination microtitration method using 1% chicken erythrocytes in PBS. Infection of MDCK cells was performed in 12-well plates or in 12-cm^2 culture vessels. Monolayers were rinsed twice with PBS and overlaid with 500–1000 μl of virus inoculum diluted 1:2 in PBS. After an incubation for 1 h at 33°C under occasional shaking, the inoculum was removed by rinsing twice with PBS, and cells were cultivated in medium containing 2% (v/v) of fetal calf serum at 33°C.

Generation of reassortant viruses

The parental viruses C/JHB and C/AA-pi were coinoculated and grown in the allantoic cavity of embryonated hen's eggs. Progeny virus was harvested and passaged to MDCK cells by infection and long-term cultivation of the cultures. The reoccurrance of viral HA titers (after the common initial peak of virus replication and a characteristic HA-negative nonproductive phase) indicated the establishment of viral persistence. Virus was reisolated and subjected to several rounds of purification, that is, to viral growth on limited dilution inoculation in embryonated eggs or in MDCK cells (24-well plate assay; Marschall *et al.,* 1994) and to plaque purification in primary chicken kidney cells (Petri *et al.,* 1979). Putative reassortant viruses were screened by restriction typing of RT-PCR products before viral stocks were prepared by growth in embryonated eggs and stored at −70°C.

RT-PCR and sequence analysis

RT-PCR was performed by standard procedures as described before (Marschall *et al.,* 1995). In brief, virion

RNA was isolated and reverse transcribed by the use of the universal primer Uni1 positioned at the conserved 3′-ends of all vRNA segments (5′-AGCAAAAGCAGG-3′). cDNA was subjected to a 30-cycle PCR with temperature levels of 95°C for denaturation (1 min), 50°C for annealing (1 min), and 72°C for polymerization (3 min). Viral segments 7 (NS) and 5 (NP) were amplified by *Taq* or *Pwo* DNA polymerases or by an enzyme mix composed of both polymerases (Expand High Fidelity PCR System, Boehringer-Mannheim) using the following primer pairs (cloning linker sequences are given in brackets): C/7-27/1 5′-[TTGAGCTCTAG]ATGTCCGACAAAACAGTCAAATCAAC-3′ or C/7-27b/1 5′-[TTGAATTC]ATGTCCGACAAAACAGTCAAATCAAC-3′ or C/7-27c/1 5′-[TTGGATCC]ATGTCCGACAAAACAGTC-3′ and C/7-888/25′-[TTGTCGACCCGGGTACCTTTATTTAT]TTATATAAGTGAATTACACAAAG-3′ (RT-PCR products used in Fig. 5A, as inserts for pSV-NSwt, pSV-NSpi, pGEX-NSwt, and pGEX-NSpi or as direct sequencing templates); C/7-5/1 5′-GAAGCAGGGGTACTTTTCC-3′ and C/7-636/2 5′-CGGTAGCCATAACGAATCCGC-3′ (additional RT-PCR products spanning the 5′-NS1 gene section) C/5-1/1 5′-AGCAGAAGCAG-[AAGCTTTGGATCC]GAGATTTGGTTTTCAAAA-3′ and C/5-1756/2 5′-CAACAGTTGATCATAAAATA[GTCGACCCGGG]TGTGATGAATTTAATCTGAC-3′ (RT-PCR product as insert for pI18-NPpi).

Cloning and transfer of expression plasmids

RT-PCR products containing the complete NS1-ORF were inserted into plasmid vectors pGEX-2T (Pharmacia), pSV1/2 (Marschall *et al.,* 1991), and pI18neo (see below) as indicated in Fig. 1A. Appropriate restriction sites of the inserts were generated either directly via primer linker sequences or indirectly via subcloning in additional transfer vectors. pI18neo was created by insertion of the PCR-derived neomycin resistance gene (pBK-RSV nucleotides 2689-35; Stratagene) into the *Hin*dIII site of eukaryotic expression vector pI18, which is driven by the human cytomegalovirus (CMV) immediate early promoter-enhancer linked to the CMV intron A. The NS1-coding *Bam*HI–*Sma*I fragment of pGEX-NSpi was inserted into pI18neo to generate the selectable expression plasmid pI18neo-NSpi. For constructing the control plasmid pI18-NPpi, an RT-PCR product stretching the NP-ORF of influenza C/AA-pi virus was generated and inserted into pI18 using restriction sites *Bam*HI–*Sma*I. Ligation was performed using the Rapid DNA Ligation Kit (Boehringer-Mannheim). Transformation of *E. coli* and screening for positive clones was carried out by standard procedures (Sambrook *et al.,* 1989). GST-NS1 proteins were produced in *E. coli* and purified by the use of RediPack GST Purification Module according to the protocol of the manufacturer (Pharmacia). The specific cleavage of GST from NS1 polypeptide fragments was performed by the incubation of thrombin (10 U/mg protein) for ≥2 h at room temperature. Purified GST-NS1 protein was used for subcutaneous immunizations of a rabbit to raise antiserum Z-NS1. This antiserum recognized recombinant and virus-produced NS1 proteins and possibly also the N-terminal part shared by NS1 and NS2 proteins (62 amino acids). However, NS2-specific polypeptides of the expected molecular weights were not detected in the different Western blot and RIPA experiments performed. Transfection of plasmids into eukaryotic cells was carried out by the electroporation method using a Gene Pulser (BioRad). Selection of stably expressing clones was achieved by the incubation of 1 mg/ml of geneticin (ICN). On transfection and selection using plasmid pI18neo-NSpi, MDCK-NSpi cell clones were obtained, which synthesized NS1 protein of the expected size, whereas NS2 coexpression (from a spliced NS-specific RNA) was not observed.

Indirect immunofluorescence test and confocal microscopy

Cells were either grown on Lab-Tek carriers (Nunc) or harvested from culture vessels, spotted onto glass slides, and fixed by a 15-min treatment with 3% of formaldehyde in PBS followed by permeabilization for 15 min in 0.1% of Triton X-100 in PBS at room temperature. Blocking was achieved by preincubation of the slides with nonspecific horse serum for 30 min at 37°C. The primary antibody (GST-NS1-specific antiserum Z-NS1, C/JHB virus-specific antiserum H1/66, MAb-NP F17, or MAb-HEF FCDD4·1/DA2·6·4) was incubated for 90 min and the secondary antibody (fluorescein-isothiocyanate- or rhodamine-coupled anti-rabbit or anti-mouse antibodies, respectively) was incubated for 45 min at 37°C before analysis by fluorescence microscopy. Double-stainings were performed as described elsewhere (Hechtfischer *et al.,* submitted for publication; Marschall *et al.,* 1998). Nuclear counterstaining was carried out using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

For confocal laser-scanning microscopy, Gelvatol coverslip mounting medium in glycerol solution was used in combination with DABCO antibleaching agent (both Sigma) to achieve rapid hardening of the labeled material and to prevent the fading of signals during microscopic analysis (Rodriguez and Deinhardt, 1960). Microscopy was performed with a LSM Rfl 1 microscope (Zeiss) by scanning serial layers of 0.5-μm distance at a magnification of 160×. Data were processed using the AVS-system computer program.

Western blot

Protein samples were denaturated under reducing conditions in boiling mix buffer (3% saccharose, 2% SDS, 20 mM Tris, pH 7.5, 5% β-mercaptoethanol, 0.03% bromophenol blue) and heated at 95°C for 10 min. Electro-

phoresis was performed by a 12.5–17.5% standard SDS–PAGE (Sambrook *et al.,* 1989) in electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). The transfer to nitrocellulose membranes was achieved in blotting buffer (192 mM glycine, 25 mM Tris, 20% methanol) using a semidry Trans-Blot apparatus (BioRad) at room temperature. Blots were blocked in a 5% milk powder solution and incubated with specific antibodies (dilutions, 1:100 for antisera and 1:2000 for monoclonal antibodies) for 4–15 h at room temperature. After washing, alkaline phosphatase (AP)-conjugated antibodies were incubated for 2 h at room temperature and subjected to color development using NBT/BCIP (Boehringer-Mannheim) as AP substrates.

Radioimmunoprecipitation assay (RIPA)

Viral proteins were labeled *in vivo* by the incubation of ^{35}S-methionine/cysteine or ^{32}P-inorganic phosphate in the supernatants of infected cell cultures. Cells were trypsinized and pelleted before protein extracts were generated by sonication in RIPA buffer (0.5% Nonidet P-40, 20 mM Tris, pH 9.0, 300 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2 mM EDTA, 2 mM PMSF, 10% glycerin). Then, 100 μl of each cell extract were used for precipitation with 100 μl of a preadsorbed antiserum (Z-NS1 dilution 1:20) for 4 h at room temperature. Specific precipitates were complexed with 100 μl of a solution of 3 mg of Protein A–Sepharose beads (Pharmacia) in RIPA buffer for 1 h at room temperature, washed repeatedly, and separated by SDS–PAGE. Gels were dried and exposed to Hyperfilm-βMax autoradiography films (Amersham) for 6–7 days.

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