Proteolytic processing of a Murray Valley encephalitis virus non-structural polyprotein segment containing the viral proteinase: accumulation of a NS3-4A precursor which requires mature NS3 for efficient processing

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The proteolytic processing of a non-structural polyprotein segment from the cytoplasmic domain of NS2A to the C terminus of NS5 of Murray Valley encephalitis (MVE) virus was examined, when expressed from cDNA via a vaccinia virus recombinant, in transiently transfected COS cells, or synthesized by cell-free translation. Cleavages mediated by the virus-encoded proteinase domain in NS3 at the junctions of NS2A-2B, NS2B-3 and NS4B-5 were catalysed efficiently. However, the cleavage at the NS3-4A junction, also mediated by the NS3 proteinase, was greatly delayed. Little or no NS3 was found, but an 85K precursor molecule accumulated; this was identified as NS3-4A. Termination codons were introduced by site-directed mutagenesis at the junctions of the NS3-4A, NS4A-4B and NS4B-5 genes to generate C-terminal truncations of the MVE virus polyprotein segment. In expression studies of these constructs the predicted NS3-mediated proteolytic cleavages were catalysed, except for that at the NS3-4A junction. In co-infections and co-transfections with constructs encoding the MVE virus non-structural polyprotein region truncated at the C termini of NS3 or NS4A, efficient processing at the NS3-4A site was induced. Thus it appears that the MVE virus polyprotein is cleaved inefficiently in cis at the NS3-4A junction, whereas the site is processed efficiently in trans by mature NS3. The NS3-4A precursor is also seen in flavivirus-infected cells. Its function remains to be determined, but it could play a role in the replication of flavivirus, in view of the importance of polyprotein processing in the regulation of gene expression of positive-stranded RNA viruses, the modulation of processing at the NS3-4A site by NS3 or NS3-containing precursors described in the present study and the importance of NS3 as an integral part of the viral polymerase complex.

Introduction

During the replication of most positive-stranded RNA viruses the entire viral genomic RNA, or a 5′-terminal segment thereof, is translated into a polyprotein precursor. Viral polyproteins undergo co- and post-translational proteolytic cleavages mediated by cellular and viral proteinases (Bond & Butler, 1987; Hellen et al., 1989; Kräusslich & Wimmer, 1988; Wellink & van Kammen, 1988). Virus-encoded proteinases appear to be exclusively responsible for the cytoplasmic processing events (Bond & Butler, 1987; Hellen et al., 1989). Their action is highly specific for distinct cleavage sites, usually a pair of amino acids, with the flanking residues having some importance in substrate recognition (Blair & Semler, 1991; Hellen et al., 1989; Wellink & van Kammen, 1988). The processing of viral polyproteins often proceeds in a specific sequence, can be temporally regulated and frequently yields processing intermediates. These characteristics allow for a regulatory role of polyprotein processing in the replication of RNA viruses.

Here the proteolytic processing of a segment of the Murray Valley encephalitis (MVE) virus polyprotein encompassing the non-structural viral proteins with predominantly cytoplasmic intracellular location is described. MVE virus is a member of the Flaviviridae, a family of enveloped, positive-stranded RNA viruses. The flavivirus RNA is translated into a single polyprotein of approximately 350K (Chambers et al., 1990a; Rice et al., 1986; Westaway, 1987). Flaviviruses replicate in close association with the membranes of the endoplasmic reticulum (ER). The viral spike components (pr)M and E, as well as the non-structural proteins NS1 and probably NS4B are translocated across the membranes of the ER via a sequential set of signal and stop-transfer sequences (Nowak et al., 1989; Ruiz-Linares et al., 1989; Speight et al., 1988). The cleavages at the N termini of these proteins are probably mediated by the host enzyme signalase. The non-structural
proteins NS2B, NS3, NS4A and NS5 remain at the cytoplasmic side of the ER membranes. The cleavages at their N termini are predicted to be catalysed by a protease domain in NS3. Recently, the putative proteolytic activity of NS3 at the NS2A-2B, NS2B-3, NS3-4A and NS4B-5 junctions was confirmed. Consistent with molecular modelling predictions the protease activity resides within the N-terminal one-third of NS3, and is a serine protease which cleaves after dibasic amino acid pairs (Chambers et al., 1990c; Cahour et al., 1992; Falgout et al., 1991; Gorbalenya et al., 1989a; Preugschat et al., 1990; Preugschat & Strauss, 1991; Wengler et al., 1991). Deletion of NS2B abolishes the protease activity of NS3, implying that NS2B is an essential cofactor (Chambers et al., 1991; Falgout et al., 1991).

In flavivirus-infected cells the viral polyprotein is processed rapidly, although some high Mr precursors can be detected (Chambers et al., 1990b; Cleaves, 1985; Smith & Wright, 1985). No clear picture has emerged from kinetic experiments as to whether a defined sequence of cleavages is followed. A polypeptide with an Mr of about 80K, and sometimes referred to as NV4½, has been observed in infected cells (Preugschat et al., 1990; Rice et al., 1986; Svitkin et al., 1981; Westaway, 1987). Peptide mapping of the tick-borne encephalitis virus NV4½ revealed that it is a precursor of NS3 (Svitkin et al., 1981).

In this paper a similar precursor-product relationship between an MVE virus-specific 85K polypeptide and NS3 is described. The former polypeptide is composed of NS3 and NS4A, and is the predominant or exclusive form of NS3 in vector-based and cell-free synthesis and processing of an MVE virus polyprotein fragment spanning from NS2A to the C terminus of NS5. The NS3-4A precursor is cleaved in the presence of mature NS3, and the processing intermediate might play a role in flavivirus replication.

Methods

Cells and virus. CV1 cells and 143B cells were grown in Eagle's MEM containing 5% foetal calf serum. COS-7 cells were grown in MEM containing 10% foetal calf serum. The prototype strain of MVE virus (MVE-1-51) was prepared as a 103 suspension of infected suckling mouse brain. Crude stocks of wild-type (wt) (strain WR) and (MVE-I-51) was prepared as a 103 suspension of infected suckling mouse brain. Crude stocks of wild-type (wt) (strain WR) and MVE virus cDNA. MVE virus cDNA was from a library used for the nucleotide sequence determination of the MVE virus genome (Dalgarno et al., 1986; Lee et al., 1990; Rice et al., 1988). Plasmid pMVE-NS3' was constructed by cloning a 1007 bp SphI-BglII fragment from p2/2/38 into p2/6/6 digested with Sphl and BglII. This plasmid encompassed a segment of the MVE virus genome from approximately nucleotide 5050 to nucleotide 10930 in the 3' untranslated region. Plasmid pMVE-NS5' was constructed by deleting 2049 bp from p2/1/22 after XhoI and Xhel digestion and religation. It encompassed a segment from nucleotide 3990 to approximately 5450, and overlapped with the MVE virus insert in pMVE-NS3'.

Vaccinia virus recombinant plasmids. The recombination vector pBcB-MVE-NS was constructed in two cloning steps. A 5663 bp HindIII fragment was subcloned from pMVE-NS3' into pBcB07 (Boyle et al., 1985) linearized with HindIII. The MVE virus cDNA containing pBcB07 vector was linearized by partial digestion with HindIII, and a 1318 bp HindIII fragment from pMVE-NS5' was inserted at nucleotide 5305. The newly formed recombinant vector pBcB-MVE-NS thus contained MVE virus cDNA corresponding to the region from nucleotides 3990 to 10930 on the MVE virus genome downstream from the vaccinia virus 7.5K promoter. The first initiation codon was at nucleotide 3997, corresponding to Met 1333, and was in a favourable context for the start of translation (Kozak, 1989).

Mutagenized and sequenced cDNA (see below) with amber termination codons at the NS3-4A or NS4A-4B gene junctions was subcloned as 1061 bp XhoI-BstEII fragments into pBcB-NS3'-NS4A-T or pBcB-NS4A-T. Mutagenized cDNA with an ochre termination codon at the NS4B-5 gene junction was first subcloned as a 2570 NruI-Sphl fragment into plasmid p2/6/6 followed by subcloning as a 3744 base XhoI-Sphl fragment into pBcB-MVE-NS, in each case replacing the MVE virus wt fragment.

Eukaryotic expression plasmids. The eukaryotic expression vector, pcDNA1, was replicated in Escherichia coli strain MC1061/P3 as specified by the supplier (Invitrogen). To insert cDNA corresponding to the 3' terminal two-thirds of the MVE virus genome downstream from the cytomegalovirus and T7 promoters, a 5932 bp BamHI fragment was subcloned from pBcB-MVE-NS into pcDNA1 linearized with the same enzyme. This recombinant plasmid was digested with HindIII, and a 1318 bp HindIII fragment from pMVE-NS5' was inserted. The resultant plasmid, pcDNA-NS, contained a segment of the non-structural region of the MVE virus genome identical to that of plasmid pBcB-MVE-NS. Plasmids pcDNA-NS3'/T and pcDNA-NS4'/T, containing amber termination codons at the NS3-4A and NS4A-4B gene junctions, respectively, were constructed by subcloning XhoI-Xhel fragments from plasmids pBcB-NS3'/T or pBcB-NS4A/T into pcDNA-NS, replacing the wt sequence.

Oligonucleotide site-directed mutagenesis. In vitro mutagenesis was by the method of Kunkel et al. (1987). For termination mutants NS3/T and NS4A/T a 1980 bp HindIII–EcoRV fragment was subcloned from pBcB-MVE-NS into bacteriophage M13mp19. For termination mutant NS4B/T a 2666 bp EcoRV–Sphl fragment was subcloned from pBcB-MVE-NS into M13mp18. The mutating oligonucleotides were the following 20-mers: 5' CCGATGCCGTACCGTTCCC 3' for NS3/T, 5' CATATCTCTAGCGCACC 3' for NS4A/T and 5' CTGGCTTTTATCTTTGAAAC 3' for NS4B/T (mismatches are underlined). Competent E. coli DH5 cells were transfected with the mutagenesis mixture, and the resulting plagues were screened by dyeoxynucleotide sequencing.

Preparation of recombinant vaccinia virus. Homologous recombination into the vaccinia virus thymidine kinase gene using the temperature-sensitive mutant ts7 as the helper virus and subsequent bromodeoxyuridine selection were performed according to the protocol of Kieny et al. (1984). Recombinants were screened by SDS–PAGE of immunoprecipitates of infected, pulse-labelled, 143B cell lysates (see below), and were twice plaque-purified on 143B cells (Mackett et al., 1984).

Transient expression in COS cells. Transfection of eukaryotic expression vector DNA into subconfluent COS-7 cells grown in 50 mm plastic Petri dishes was by the DEAE–dextran method. DNA (5 to 10 μg) was suspended in 1.5 ml transfection medium (serum-free MEM
containing 250 µg/ml DEAE-dextran). Cell monolayers were washed twice with serum-free MEM, and the DNA-containing transfection medium was added. The monolayers were incubated for 4 h at 37 °C with occasional rocking in a CO2 incubator, the transfection medium was aspirated, and the cells were shocked by the addition of 2 ml Hanks' balanced salt solution containing 10% DMSO and 0.1% glucose at 22 °C for 1-5 min. This solution was removed, the monolayers were washed twice with serum-free MEM, and MEM containing 10% foetal calf serum was added. The monolayers were incubated for 2 to 3 days before pulse-chase experiments were performed.

Metabolic labelling. Pulse labelling of infected CV1 cells or transfected COS-7 cells was as described by Lobigs & Garoff (1990). Labelling and chase periods are indicated in the legends to Fig. 1 to 5.

Immunoprecipitation, SDS–PAGE and fluorography. Immunoprecipitations using anti-MVE virus hyperimmune ascitic fluid, SDS–PAGE and fluorography were as described (Lobigs et al., 1990).

In vitro transcription and translation. RNA was transcribed from the T7 promoter on XbaI-linearized plasmids pcDNA-NS, pcDNA-NS3/T or pcDNA-NS4A/T in a reaction containing 40 mM-Tris–HCl pH 7.6, 6 mM-MgCl2, 2 mM-spermidine–HCl, 5 mM-DTT, 100 µg RNase/DNase-free BSA, 1 mM-cap analogue [mTG(5')ppp(5')G; Pharmacia], 1 mM-ATP, -CTP and -UTP, 0.5 mM-GTP, 50 units (U) RNasin and 15 U T7 RNA polymerase (Pharmacia), using approximately 1 µg DNA in a total volume of 50 µl. The reaction mixture was incubated at 37 °C for 1 h.

One microlitre from the in vitro transcription reaction was translated in a volume of 25 µl using a reticulocyte lysate translation kit (Boehringer Mannheim) as specified by the supplier. The potassium acetate and magnesium acetate concentrations were adjusted to 50 mM and 1 mM, respectively. Translations were carried out at 30 °C for 2 h. In some cases the radiolabelled translation products were chased by the addition of cycloheximide and methionine to 0.6 mg/ml and 1 mM, respectively, and further incubation for 2 h at 30 °C. Cell-free cleavage assays using unlabelled translation products were as described (Preugschat et al., 1990).

Results

Transient expression of an MVE virus cDNA segment encoding the non-structural proteins NS2, NS3, NS4 and NS5

Flavivirus-specific proteins are synthesized as a single polyprotein which is co- and post-translationally cleaved by host cell and virus-encoded proteinases. Here the proteolytic processing events on a large non-structural region of the MVE virus polyprotein were studied. Expression of the polyprotein segment was from cloned cDNA via a recombinant vaccinia virus, or transient COS cell transfection.

The vaccinia virus recombinant vv-NS encoded the C-terminal two-thirds of the MVE virus polyprotein. Initiation of translation from the 7.5K promoter was probably at the codon for Met 1333 in the C-terminal half of NS2A, and proceeded to the C terminus of NS5. Thus a translation product of 238K was expected. A protein profile of vv-NS-infected cell lysates, after immunoprecipitation with anti-MVE virus hyperimmune ascitic fluid is shown in Fig. 1 (lane 2). Several MVE virus-specific polypeptides were identified. These were a major band at 97K, and bands at 130K, 85K and 14K. The 97K and 14K products comigrated with the authentic MVE virus proteins NS5 and NS2B, respectively. Interestingly, NS3, which was routinely immunoprecipitated from MVE virus-infected cell lysates, was never apparent in vv-NS protein profiles. (A vaccinia viral polypeptide of 68K was sometimes precipitated, but could be distinguished from NS3 by its slightly faster electrophoretic mobility and diffuse appearance.) NS4A and NS4B with predicted Mts of 16K and 28K, respectively, were not seen in immunoprecipitates of MVE

Fig. 1. Proteolytic processing in a non-structural region, and C-terminal truncations thereof, of the MVE virus polyprotein expressed via vaccinia virus recombinants. A large C-terminal segment of the MVE virus polyprotein from NS2A to the C terminus of NS5 (vv-NS; lane 2), and C-terminal truncations thereof, precisely at the NS3-4A junction (vv-NS3/T; lane 3), NS4A-4B junction (vv-NS4A/T; lane 4) and NS4B-5 junction (vv-NS4B/T; lane 5) were expressed in CV1 cells via recombinant vaccinia virus. Wild-type vaccinia virus (vv-WR) is shown in lane 1. The cells were infected at multiplicities of 10 p.f.u./cell, pulse-labelled for 1 h at 5 h p.i., and the label was chased for 30 min. Cell lysates were subjected to immunoprecipitation with anti-MVE virus hyperimmune ascitic fluid, and analysed by 10 to 20% SDS–PAGE. The positions of the authentic MVE virus non-structural proteins NS5, NS3 and NS2B are given, and sizes of Mts, standards are indicated at the left.
virus- or vv-NS-infected cells. These results indicated that proteolytic processing in the non-structural region of the MVE virus polyprotein segment took place at three of the four predicted cleavage sites recognized by the NS3 proteinase, where the cleavage at the junction of NS3-4A was greatly delayed or defective in the vaccinia virus expression system. A polypeptide with an \( M_r \) of 85K, consistent with that of a NS3-4A precursor, was apparent in the vv-NS protein profile.

When the identical region of the MVE virus polyprotein, spanning from NS2A to the end of NS5, was expressed from cDNA in transiently transfected COS cells, a similar picture emerged (Fig. 4, lane 2). NS5 and NS2B could be clearly identified, and a polypeptide of 85K was immunoprecipitated, but NS3 was not seen.

**Cleavage site-specific termination mutants encoding polypeptide deletions on a MVE virus polyprotein segment: NS3-4A is a stable precursor**

The identity of the 85K polypeptide encoded by vv-NS as NS3-4A was confirmed when the protein profiles of vaccinia virus recombinants with truncations at the junctions of NS3-4A, NS4A-4B and NS4B-5 were compared. Termination codons were introduced by site-directed mutagenesis at the relevant positions on the MVE virus cDNA corresponding precisely to the C termini of NS3 (vv-NS3/T), NS4A (vv-NS4A/T) and NS4B (vv-NS4B/T). In the protein profiles of vv-NS4A/T and vv-NS4B/T, an 85K band identical to that in the vv-NS profile was seen (Fig. 1, lanes 4 and 5); however, no NS3 was apparent. Only from lysates of cells infected with vv-NS3/T, where translation is terminated at the C terminus of NS3, could a polypeptide with an electrophoretic mobility identical to that of the viral NS3 be immunoprecipitated (Fig. 1, lane 3); no 85K band was visible. The cleavages at the NS2A-2B and NS2B-3 junctions were catalysed in the vaccinia virus recombinants encoding truncated forms of the MVE virus polyprotein segment, since NS2B was synthesized. Additional precursor polypeptide bands appeared in the profiles of these recombinants. They were an 87K molecule in the vv-NS3/T profile (Fig. 1, lane 3), a 94K molecule in the vv-NS4A/T profile (Fig. 1, lane 4), and 95K, 98K and 102K molecules in the vv-NS4B/T profile (Fig. 1, lane 5).

**NS3-4A precursor in MVE virus-infected cells**

In lysates of flavivirus-infected cells high \( M_r \) precursor proteins are frequently seen, some of which have a \( M_r \) similar to that of NS3-4A (Cleaves, 1985; Preugschat et al., 1990; Smith & Wright, 1985; Svitkin et al., 1981; Westaway, 1987). Fig. 2 shows immunoprecipitates from vv-NS3/T- and vv-NS4A/T-infected cells which were co-electrophoresed with those from MVE virus-infected cells lysed at 16 and 24 h post-infection (p.i.). (The recombinant vv-NS4A/T was used for the further characterization of NS3-4A because expression levels higher than in cells infected with vv-NS were achieved.) A number of MVE virus-specific protein precursors larger than NS5 as well as two distinct polypeptides of 80K and 85K were precipitated (Fig 2). The 85K band had an electrophoretic mobility identical to that of NS3-4A from vv-NS4A/T-infected cells, suggesting that in vivo the cleavage at the NS3-4A junction occurs less efficiently than do other NS3-mediated cleavages. No significant difference in the relative amounts of NS3 and NS3-4A was seen at the end of the latent period after 16 or 24 h infection with MVE virus (Fig. 2, lanes 4 and 5). MVE virus-specific proteins could not be detected during the latent period at 8 h p.i. (not shown). Thus it is not clear whether the appearance of NS3-4A in MVE virus-infected cells is temporally regulated.
Expression of the MVE virus non-structural protein genes via vaccinia virus recombinants has shown, so far, that the proteolytic cleavage at the NS3-4A junction, thought to be catalysed in an intramolecular reaction by a proteinase domain in NS3, was greatly delayed. To test whether the mature NS3 proteinase would promote the processing at the NS3-4A site in a bimolecular reaction double infections with vv-NS3/T and vv-NS4A/T, at a ratio of 2:1, were performed (Fig. 3a). As a reference, pulse-chases of cells infected with only vv-NS3/T or vv-NS4A/T at the same multiplicities used in the double infections are shown (Fig. 3a, lanes 1, 2, 3 and 7, 8, 9).

The NS3-4A site was efficiently processed in double infected cells. The intensity of the NS3-4A band was greatly reduced after a 30 min labelling and 5 min chase interval, and the polypeptide was virtually absent after 60 and 120 min chases (Fig. 3a, lanes 4, 5, 6). Cleavage of the NS3-4A precursor was probably to the mature NS3 and NS4A products. However, this could not be verified, since NS4A could not be detected, and NS3 derived from NS3-4A processing would comigrate with NS3 encoded by vv-NS3/T. In the pulse-chase of cells infected with only vv-NS4A/T the intensity of the NS3-4A band diminished after 60 and 120 min chase intervals (Fig. 3, lanes 1, 2, 3). This was caused by the loss of cells from the monolayers due to the severe cytopathic effect of vaccinia virus infection, and not by proteolysis of NS3-4A. When pulse-chase experiments were performed in suspension cultures the intensity of the NS3-4A band did not diminish markedly (data not shown).

The predominance of NS3 relative to NS3-4A in double infected cells seen in Fig. 3(a) could have been a consequence of either the greater infectivity of vv-NS3/T in comparison to vv-NS4A/T, or the higher expression levels achieved from the former recombinant, although this was unlikely since the two vaccinia virus recombinants differed only in the position of a termination codon in the MVE virus insert, and had equal expression efficiencies of the MVE virus genes. As additional evidence that the processing at the NS3-4A site seen in Fig. 3(a) was due to the presence of mature NS3 provided by vv-NS3/T, double infections with vv-NS4A/T at 5 p.f.u./cell and increasing m.o.i. of 0.5, 5 and 10 p.f.u./cell with vv-NS3/T were performed (Fig. 3b).

At a low ratio of vv-NS3/T to vv-NS4A/T little cleavage at the NS3-4A site took place, whereas at ratios of 1:1 and 2:1 most of the NS3-4A precursor was processed (Fig. 3b, lanes 3, 4, 5). Thus the NS3-4A site appears to be a substrate for cleavage mediated in trans by the mature NS3 proteinase.

Mature NS3 promotes NS3-4A processing in transiently transfected COS cells

The SDS-PAGE profile of immunoprecipitates of COS cells transfected with the eukaryotic expression vector pcDNA-NS has been described above (Fig. 4, lane 2). This construct was proteolytically processed at the NS2A-2B, NS2B-3 and NS4B-5 junctions, but failed to be cleaved at the NS3-4A junction. When COS cells were transfected with plasmids expressing truncated forms of the MVE virus polyprotein segment from cDNA containing termination codons precisely at the end of the NS3 or NS4A genes (pcDNA-NS3/T and pcDNA-NS4/T, respectively), mature NS3 (69K) or the NS3-4A precursor (85K) were synthesized (Fig. 4, lanes 3 and 4, respectively). The NS3-4A precursor was stable during the 2 h labelling and 1 h chase interval. Co-transfection with pcDNA-NS3/T and pcDNA-NS4A/T resulted in efficient cleavage at the NS3-4A junction (Fig. 4, lanes 5 and 6). In addition, NS2B (14K) was immunoprecipitated; this could be resolved by electrophoresis through 15% gels (data not shown). These data support...
Fig. 4. COS cell transfections showing NS3-4A cleavage mediated by mature NS3 in trans. COS cells were pulse-labelled for 2 h at 2 days post-transfection, and the label was chased for 1 h. MVE virus-specific polypeptides were immunoprecipitated and analysed by 10% SDS-PAGE. In transfections with a single plasmid (lane 2, pcDNA-NS; lane 3, pcDNA-NS3/T; lane 4, pcDNA-NS4A/T), 5 µg DNA was used. In lanes 5 and 6 co-transfections with plasmids pcDNA-NS3/T and pcDNA-NS4A/T are shown, where 2.5 µg DNA of each plasmid (lane 5), or 2.5 µg and 13 µg, respectively (lane 6), were used; lane 1, mock transfection. Sizes of Mr standards are shown on the left.

the earlier observation that mature NS3 promotes the cleavage of the NS3-4A site in trans.

**Cell-free synthesis and processing of MVE virus non-structural polyprotein segments**

Cell-free translation of RNA transcribed from the eukaryotic expression vector pcDNA-NS yielded two polypeptides with electrophoretic mobilities similar to those of the authentic MVE virus non-structural proteins NS5 (Fig. 5, lane 2) and NS2B (which was resolved through 15% SDS-polyacrylamide gels; data not shown). *In vitro* synthesized NS5 migrated as a doublet. NS3 was virtually absent from the protein profile, but instead the NS3-4A precursor was immunoprecipitated from the translation reactions. Translations carried out in the absence of microsomal membranes, which should have abolished the putative signalase-mediated cleavage at the NS4A-4B junction, yielded a polypeptide with an electrophoretic mobility slightly faster than that of NS3-4A, suggesting that the MVE virus polyprotein segment was cleaved between NS4A and NS4B at an alternative site, not mediated by signalase.

Cell-free translation of RNA transcribed from plasmids pcDNA-NS3/T and pcDNA-NS4A/T was performed with the aim of developing an *in vitro* cleavage assay. Transcription/translation of pcDNA-NS3/T or pcDNA-NS4A/T produced the mature NS3 and an additional 87K band (Fig. 5, lane 3), or the precursor NS3-4A and a 95K band (Fig. 5, lane 4), respectively, as well as NS2B (not shown). A minor translation product from pcDNA-NS4A/T RNA comigrated as a doublet with NS3. A 2 h chase following translation did not alter the profile for pcDNA-NS4A/T (Fig. 5, lane 6). When radiolabelled translation products from pcDNA-NS4A/T were incubated for 2 h with unlabelled NS3 from *in vitro* transcription/translation of pcDNA-NS3/T, no significant processing of the NS3-4 precursor could be detected (Fig. 5, lane 7). Thus NS3-4A is a stable precursor, and appears to be resistant to further proteolytic processing after release from the nascent polypeptide chain, even in the presence of mature NS3.

**Discussion**

Flavivirus protein biosynthesis involves the processing of a single polyprotein precursor into at least 10 polypeptides by cellular and viral proteinases. Here the proteolytic cleavages in a large segment of the MVE virus polyprotein extending from the cytoplasmic domain of NS2A to the C terminus of NS5 were studied using vaccinia virus and eukaryotic vector-based ex-
pression, or cell-free synthesis. Efficient proteolytic processing of the MVE virus polyprotein segment took place in the absence of viral replication. Proteins with an electrophoretic mobility similar to those of NS2B and NS5 were identified, indicating that the NS3 proteinase-mediated cleavages at the NS2A-2B, NS2B-3 and NS4B-5 sites took place. Interestingly, the cis cleavage at the C terminus of NS3 was greatly delayed or absent, and a stable 85K polypeptide was abundant. This polypeptide was identified as the NS3-4A precursor. The NS3-4A proteolytic site was subject to cleavage when mature NS3 was provided from a separate coding unit. This was demonstrated in two different expression systems; in vaccinia virus double infections and COS cell cotransfections with constructs encoding the NS3-4A precursor or the mature NS3 proteinase, the majority of NS3-4A was processed. Apparently the cleavage at the NS3-4A junction was catalysed efficiently only in trans by mature NS3, whereas the cleavages at the NS2A-2B, NS2B-3 and NS4B-5 junctions were mediated by the NS3 proteinase domain on the nascent polyprotein independent of processing at the NS3-4A site. The MVE virus structural proteins and NS1, when co-expressed in double infections with the recombinant vv-NS, did not promote the NS3-4A precursor cleavage (data not shown).

The proteolytic processing of polyprotein fragments of yellow fever virus and dengue virus has revealed some processing at the NS3-4A site. However, the processing at the NS3-4A junction was also much less efficient than that of other NS3-mediated cleavages, and NS3-4A precursors accumulated (Cahour et al., 1992; Chambers et al., 1991; Falgout et al., 1991).

The data presented here raise the question of whether a similar difference in cleavage site preference exists in the proteolytic processing of the MVE virus polyprotein in virus-infected cells. Polypeptides with Ms consistent with NS3-4A precursors have been reported, and an NS3-4A precursor was identified in MVE virus-infected cells. Attempts to test whether during the early phase of MVE virus infection the NS3-4A precursor was the more abundant molecule failed due to the low production of virus-specific proteins against a high host background during the long latent period in flavivirus replication. Late in virus infection the mature NS3 was predominant compared to its precursor.

The conflicting data of greatly delayed NS3-4A cleavage in vitro and relatively efficient cleavage in vivo could be reconciled by predicting a shift in the polyprotein processing pathway during MVE virus infection, from little cleavage early in infection to the gradual accumulation of mature NS3, and eventually efficient processing at the NS3-4A junction in a bimolecular reaction by mature NS3. Thus the processing intermediate NS3-4A and the processing end-product NS3 could be selectively generated, modulated by the inefficient cleavage in cis by NS3-containing precursors, and the efficient cleavage in trans by mature NS3. It remains uncertain whether the cleavage at the NS3-4A junction is catalysed solely on the nascent polyprotein chain, or can occur post-translationally on the NS3-4A precursor. Post-translational processing was not seen in in vitro cleavage assays, possibly because the NS3-4A junction is not accessible to cleavage after release of the precursor protein from the nascent polyprotein chain.

The transition from negative-strand RNA synthesis early in infection to predominantly positive-strand RNA synthesis at the end of the latent period is a common theme in the replication of positive-stranded RNA viruses, and has also been described for flaviviruses (Rice et al., 1986; Westaway, 1987). In the Sindbis virus model positive- and negative-strand RNA synthesis is regulated by the proteolytic processing of the non-structural region of the polyprotein, giving rise to intermediates and endproducts with different enzymatic activity (DeGroot et al., 1990). It is tempting to speculate that the NS3-4A precursor cleavage, which here is suggested to be temporally regulated, plays a role in flavivirus infections in the transition from negative-strand to positive-strand RNA synthesis. NS3 contains, apart from the viral proteinase, a nucleoside triphosphatase domain (Gorbalenya et al., 1989b; Wengler & Wengler, 1991) and is, in association with the polymerase NS5, an integral part of the replication complex (Chu & Westaway, 1987; Grun & Brinton, 1988).

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