Localization and phosphorylation of Semliki Forest virus non-structural protein nsP3 expressed in COS cells from a cloned cDNA

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The nsP3 protein of Semliki Forest virus is a phosphoprotein, which is processed from a large non-structural polyprotein. The nsP3 gene was isolated from the large coding region by the polymerase chain reaction technique and cloned into a eukaryotic expression vector. Using the constructed pSVNS3 expression vector it was shown that nsP3 could be phosphorylated in the absence of other virus-specific proteins. This suggests that the formation of a complex with the other non-structural proteins is not required for the phosphorylation of nsP3. About half of the synthesized nsP3, in pSVNS3-transfected COS cells, could be fractionated into the mitochondrial pellet fraction indicating that nsP3 is associated with large intracellular structures. Immunofluorescence microscopy of pSVNS3-transfected COS cells showed that nsP3 was found in the cytoplasm localized to vesicle-like structures. These results suggest that nsP3 contains information for specific interaction with large intracellular vesicular structures.

The genetic information of the Semliki Forest virus (SFV) genome, which is a positive single-stranded 42S RNA molecule of 11442 nucleotides, has been determined recently (Garoff et al., 1980a, b; Takkinen, 1986). Two-thirds of the 42S RNA contains an open reading frame encoding a large polyprotein, which is proteolytically processed to four non-structural proteins (nsP1 to nsP4) (Keränen & Ruohonen, 1983; Takkinen, 1986; Takkinen et al., 1990).

The synthesis of SFV RNA takes place in the cytoplasm. The genomic 42S RNA is transcribed into a complementary minus strand, which then functions as a template for the synthesis of new genomic 42S RNAs and of subgenomic 26S RNAs (Kääriäinen et al., 1987). By using a full-length cDNA clone of another alphavirus, Sindbis virus, for the production of infectious transcripts, Strauss and collaborators have been able to elucidate the relationship between the four complementation groups of Sindbis virus and the four non-structural proteins (Hahn et al., 1989a, b; Rice et al., 1987; Li & Rice, 1989). From these studies it seems likely that nsP1 is needed in the synthesis of the minus RNA strand, that nsP2 is necessary for subgenomic 26S RNA synthesis, and that nsP4 is the major elongation factor. The function of nsP3 remains obscure.

The nsP3 protein of SFV is a phosphoprotein, with phosphorylated serine and threonine residues (Peränén et al., 1988). The majority of the nsP3 protein in SFV-infected cells can be recovered from a mitochondrial pellet fraction (P15), where nsP3 seems to be more phosphorylated than in the supernatant fraction (S15) (Peränén et al., 1988). Recent studies have also shown that nsP3 can be found associated with large vesicular structures in infected cells (Froshauer et al., 1988; Peranen et al., 1988).

The fact that nsP3 is synthesized only in catalytic amounts and that it forms complexes with other non-structural proteins has made it difficult to characterize this protein further. To circumvent these difficulties the nsP3 gene of SFV was cloned as a separate unit, and expressed in eukaryotic cells. The results of these expression studies are presented in this paper.

The nsP3 protein is proteolytically processed from a polyprotein and the coding region lacks translation initiation and termination signals (Takkinen, 1986). To make the nsP3 gene functional, translation initiation and termination signals were linked to the coding region of nsP3. This was done by enzymic amplification using the polymerase chain reaction (PCR) procedure (Saiki et al., 1988). The presence of an ATG start codon in the 5' primer (5' ATCACCATGGCACCATCCTACAGAGTTAAG Y) allowed the incorporation of a translation initiation signal into the nsP3 gene, and a TAA stop codon in the Y primer (5' CTCAAGCT TATGCACCCGCGCGGCCTAGTCG 3') provided the gene with a translation termination signal. These primers also contained restriction sites overlapping the translation signals to facilitate cloning (see Fig. 1). A cDNA clone pT30, containing a DNA fragment corresponding to the region between nucleotide positions 1945
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Fig. 1. Schematic representation of the DNA constructs used for expressing the nsP3 gene of SFV in animal cells (for details see text). The nsP3 gene was amplified from plasmid pT30 by PCR and cloned into transcription vector pHOS1 creating plasmid pTSF3. The eukaryotic animal expression vector pSVNS3 was obtained by cloning the nsP3 gene from pTSF3 into pSVR6.

to 6638 on the SFV genome, was used as a template for the amplification of the gene.

A single DNA fragment with an expected size of 1464 nucleotides could be detected when the amplified nsP3 gene was resolved by agarose gel electrophoresis (not shown). It was purified from the agarose gel using DNA sequencing (Sanger et al., 1977). The sequencing of the constructed nsP3 gene in pTSF3 revealed two amino acid substitutions compared to the sequence reported by Takkinen (1986). An arginine was replaced by a glutamine in a G to A nucleotide substitution at the nucleotide position 4776, and a glycine by an arginine in a G to A substitution at position 4817. These amino acid changes are in agreement with a revised version of the nucleotide sequence in the region of nsP3 (P. Liljestroem, personal communication), making it more like the nsP3 of Sindbis virus (Strauss et al., 1984).

In order to express the nsP3 gene in COS cells, the nsP3 gene was excised from pTSF3 and inserted directly into the eukaryotic expression vector pSVR6 creating plasmid pSVNS3 (see Fig. 1). Vector pSVR6 was obtained by cleaving pSV S-SFV (Kondor-Koch et al., 1983) with HindIII, and by substituting the structural cDNA region of SFV 26S RNA with a polylinker containing oligonucleotides C (5' AGCTGATATCG-GATCCAGATCTCATA 3') and D (5' AGCTTATGAG-ATCTGGATCGATCCATGGT 3').

The authenticity of the amplified and cloned nsP3 gene was checked by DNA sequencing (Sanger et al., 1977). The sequencing of the constructed nsP3 gene in pTSF3 revealed two amino acid substitutions compared to the sequence reported by Takkinen (1986). An arginine was replaced by a glutamine in a G to A nucleotide substitution at the nucleotide position 4776, and a glycine by an arginine in a G to A substitution at position 4817. These amino acid changes are in agreement with a revised version of the nucleotide sequence in the region of nsP3 (P. Liljestroem, personal communication), making it more like the nsP3 of Sindbis virus (Strauss et al., 1984).

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It is known from earlier studies that nsP3 of SFV is phosphorylated in SFV-infected BHK cells (Peranen et al., 1988). To find out whether nsP3 could also be phosphorylated in the absence of other SFV-specific proteins and in the absence of viral RNA synthesis, COS cells were transfected with pSVNS3 and 2 days later labelled with [32P]orthophosphate for 3 h. Nuclear-free SDS-treated lysates were subjected to immunoprecipitation with anti-nsP3 and the corresponding preimmune serum. As shown in Fig. 2 (lane 4) a phosphorylated band...
of 60K and also a smaller one were immunoprecipitated with anti-nsP3, while the preimmune serum did not precipitate any phosphoproteins (lane 6). The smaller of 60K and also a smaller one were immunoprecipitated proteolytic cleavage product because it was sometimes labelled with $^{32}$Porthophosphate when anti-nsP3 was not present in infected COS cells, probably represents a phosphoprotein detected in transfected COS cells, but with anti-nsP3, while the preimmune serum did not show any activity resides in P15 (Ranki & Käräriinen, 1979). The used for Western blot analysis (Towbin et al., 1979), and supernatant (S15) fractions were isolated from indicated cells. Lane 1, P15 from SFV-infected BHK cells; lane 2, S15 from SFV-infected BHK cells; lane 3, P15 from pBR322-transfected COS cells; lane 4, S15 from pBR322-transfected cells; lane 5, P15 from pSVNS3-transfected COS cells; lane 6, S15 from pSVNS3-transfected COS cells; lane 7, lysate from unfractionated tsl-infected BHK cells. Arrows indicate marker proteins: from the bottom 43K, 67K and 94K.

The nsP3 of SFV is localized to large vesicle-like structures in SFV-infected BHK cells (Peränen et al., 1988). The availability of plasmid pSVNS3 made it possible to investigate the intracellular localization of nsP3 in cells not expressing other SFV-specific proteins. COS cells were transfected with pSVNS3 using the lipofection procedure (Felgner et al., 1987), and 2 days later processed for indirect immunofluorescence microscopy. In transfected cells the affinity-purified anti-nsP3 antibody (J. Peränen, unpublished results) stained solid vesicle-like structures very similar to those seen in SFV-infected cells (Fig. 4). These structures seemed to be randomly distributed throughout the cytoplasm. Mock-infected cells showed no specific staining with the anti-nsP3 antibody.

Cloning and expression of the SFV nsP3 gene made it possible to study nsP3 in a situation where it is not participating in RNA synthesis. The results obtained show that nsP3 is phosphorylated in the absence of other virus-specific proteins and in the absence of SFV-specific RNA synthesis. Therefore, the phosphorylation of nsP3 cannot be mediated by any other virus-specific protein, meaning that nsP3 is either autophosphorylated or phosphorylated by a cellular kinase. However, the phosphorylation of nsP3 may be important for its interaction with other components of the replicase complex.

When the subcellular distribution of total nsP3 was monitored by Western blotting, it became clear that nsP3 was evenly distributed between P15 and S15, both in pSVNS3-transfected COS cells and SFV-infected BHK cells. It was reported recently that 80% of nsP3 was found in the P15 fraction when the subcellular distribution of nsP3 was studied in SFV-infected BHK cells pulse-labelled with $^{35}$Smethionine and chased (Peränen et al., 1988). This difference was also observed when the same fractionated lysates, which had been pulse-labelled with $^{35}$Smethionine and chased (pulsed 2 to 3 h p.i. and chased 3 to 4 h p.i.) and immunoprecipitated with anti-nsP3 antiserum, were analysed by Western blotting (4 h p.i.) (J. Peränen, unpublished results). The pulse–chase experiment detects the distribution of only a fraction of the nsP3 synthesized, whereas Western blotting shows the distribution of total nsP3. Although the reason for the difference between the two situations is unclear, it is possible that most of the newly synthesized nsP3 proteins attach initially to some structure in the P15 fraction, and then with time detach from the structure to enter the S15 fraction. The finding that the phosphorylated form of nsP3 is predominantly associated with P15 could mean that phosphorylation of nsP3 is necessary for the
Fig. 4. Localization of nsP3 in pSVNS3-transfected and SFV-infected COS cells by indirect immunofluorescence using anti-nsP3 antiserum. (a) SFV prototype strain-infected cell at 4 h p.i.; (b) mock-infected cells; (c) tsi-infected cells at 4 h p.i.; (d) pSVNS3-transfected cell. Bar markers represent 10 μm.

association of nsP3 with large structures in P15. The fact that nsP3 in transfected cells can also be recovered from P15 indicates that nsP3 in itself contains information for this association.

In SFV-infected BHK cells nsP3 is associated with large vacuole-like structures (Peränens et al., 1988) which are modified endosomes and lysosomes (Froshauer et al., 1988). An interesting observation was made when the intracellular localization of nsP3 in transfected COS cells was investigated in that similar vesicle-like structures were stained as in SFV-infected COS cells. This could mean that nsP3 has some intrinsic affinity for vesicle-like structures. It is most likely that these vesicles correspond to the large structures found in P15. The origin of these structures is unknown, but they could originate from endosomes or lysosomes as is the case for the cytoplasmic vacuoles in SFV-infected cells (Froshauer et al., 1988).

It has been shown that nsP3 is the least conserved of the alphavirus non-structural proteins, and that it is the only ns protein that contains no homology to non-structural proteins from RNA plant viruses (Strauss et al., 1988; Ahlquist et al., 1985). Although the function of nsP3 is unknown, it has been demonstrated that nsP3 is needed in RNA synthesis (Hahn et al., 1989b). The amino acid sequence of the non-structural open reading frame of rubella virus has very little similarity with the corresponding region of alphaviruses (Dominguez et al., 1990) but there is a region of similarity with the amino terminus of alphavirus nsP3 indicating that this region is of importance for alphavirus and rubella virus RNA synthesis (Dominguez et al., 1990). Preliminary results also suggest that nsP3 can be cross-linked to two of the three other ns proteins (J. Peränens, unpublished results) suggesting that nsP3 has at least two important domains, one for vesicular association and one for interaction with the other ns proteins.

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References


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