Semliki Forest Virus-specific Non-structural Protein nsP3 Is a Phosphoprotein

By JOHAN PERÄNEN,* KRISTIINA TAKKINEN, NISSE KALKKINEN AND LEEVI KÄÄRIÄINEN

Recombinant DNA Laboratory, University of Helsinki, Valimotie 7, SF-00380 Helsinki, Finland

(Accepted 7 June 1988)

SUMMARY

Antisera were raised in rabbits against fusion proteins consisting of β-galactosidase and partial amino acid sequences of Semliki Forest virus (SFV)-specific non-structural proteins nsP1, nsP2, nsP3 and nsP4. The antisera were specific since each of them precipitated only one labelled protein of a size expected for nsP1, nsP2, nsP3 or nsP4 from lysates of [35S]methionine-labelled SFV-infected BHK-21 cells. The specific antisera also precipitated p220 (with sequences of nsP1, nsP2 and nsP3), p155 (nsP1 and nsP2) and p135 (nsP3 and nsP4) which have been previously shown to be cleavage products of the polyprotein precursor of the non-structural proteins. nsP1, nsP4 and most of nsP3, together with the virus-specific RNA polymerase activity, were in the mitochondrial pellet (P15) fraction of infected BHK-21 cells whereas nsP2 was evenly distributed between P15 and the supernatant fraction (S15). Only antisera directed against nsP3 sequences precipitated a labelled protein from cells incubated with [32P]orthophosphate during SFV infection. Treatment of the immunoprecipitate with calf alkaline intestinal phosphatase reduced the amount of labelled nsP3 considerably. Immunoprecipitated 32p-labelled nsP3, isolated by SDS-PAGE, was subjected to acid hydrolysis. Both phosphoserine and phosphothreonine but not phosphotyrosine could be identified in the hydrolysate. Approximately twice as much [32P]serine as [32P]threonine was detected in nsP3. P15 and S15 fractions were prepared from [35S]methionine- and 32P-labelled SFV-infected cells and the 35S/32P ratio of nsP3 was determined after immunoprecipitation and SDS-PAGE. The nsP3 in S15 was less heavily phosphorylated (about 50%) than P15-associated nsP3. Anti-nsP3 serum revealed large cytoplasmic vesicles in SFV-infected cells in indirect immunofluorescence microscopy.

INTRODUCTION

The structure and mode of expression of the positive strand RNA genomes of two alphaviruses, Semliki Forest virus (SFV) and Sindbis virus, have been extensively characterized (Garoff et al., 1980a, b; Strauss & Strauss, 1983, 1986; Strauss et al., 1984; Takkinen, 1986; Schlesinger & Schlesinger, 1986, Kääriäinen et al., 1987). Since the polymerase components are synthesized only in small amounts early in infection (Lachmi & Kääriäinen, 1977), the isolation and purification of sufficient amounts of the enzyme for analysis has been a very difficult task (Clewley & Kennedy, 1976; Ranki & Kääriäinen, 1979; Gomatos et al., 1980). The putative RNA polymerase components of SFV, non-structural proteins nsP1, nsP2, nsP3 and nsP4 (previously designated ns72), are synthesized as a large polyprotein (2431 amino acids) from the 5' region of the 42S genomic RNA (Lachmi & Kääriäinen, 1976; Glanville et al., 1976; Kalkkinen et al., 1981; Keränen & Ruohonen, 1983; Takkinen, 1986). The nsP1 is the major virus-specific protein which together with smaller amounts of nsP2 and nsP4, copurifies with template-associated RNA polymerase preparations (Ranki & Kääriäinen, 1979; Gomatos et al., 1980; Keränen & Ruohonen, 1983).
Recombinant DNA techniques have opened new possibilities for the study of the expression of structural proteins in animal cells (Kondor-Koch et al., 1983; Garoff, 1985; Melancon & Garoff, 1986, 1987; Rice et al., 1985) and in yeast (Wen & Schlesinger, 1986). Full-length cDNA constructs of Sindbis virus RNA have recently been reported, which can be used for transcription of infectious RNA (Rice et al., 1987). This has allowed new approaches to the study of the functions of different viral structural and ns proteins and their mutations. A recent paper by Hardy & Strauss (1988) describes the production of monospecific antibodies against amino acid sequences of the four Sindbis virus-specific ns proteins and their use in the study of the processing of the ns protein-containing polyprotein. Expression of defective interfering RNAs by recombinant DNA methods has been used to analyse which nucleotide sequences are essential for the replication of the alphavirus RNAs (Jalanko & Söderlund, 1985; Levis et al., 1986, 1987; Tsiang et al., 1985, 1988).

Here we have produced antibodies against fragments of the different ns proteins of SFV by using recombinant DNA techniques. The antibodies specifically precipitated all four of the ns proteins and their respective processing intermediates. nsP3 was the only ns protein that was labelled with \([^{32}\text{P}]\)orthophosphate during SFV infection. We have analysed the phosphoamino acids and intracellular location of nsP3 in SFV-infected cells.

**METHODS**

*Plasmids.* The pEX1, -2 and -3 bacterial expression vectors and *Escherichia coli* strain NF1 were kindly provided by K. K. Stanley (EMBL, Heidelberg, F.R.G.). Plasmids pKTH310, pKTH330, pKTH337 and pKTH344, carrying cDNA fragments from the non-structural region of SFV, were the sources in all constructions (Takkinen, 1986). All DNA manipulations were done using standard protocols (Maniatis et al., 1982). DNA fragments were separated by electrophoresis in linear 2.5 to 10% polyacrylamide gradient gels and eluted by diffusion (Jeppesen, 1980; Maxam & Gilbert, 1980). Clones containing appropriate inserts were detected by SDS–PAGE of extracts from heat-induced *E. coli* cells. The positive clones were further characterized by restriction enzyme analysis.

pN1-10 was constructed by inserting a *SalI* fragment, corresponding to nucleotide positions 421 to 818 as defined according to the location on the genome of SFV, from pKTH344 into the *BamHI* site of pEX2. A *SalI–PstI* fragment (positions 1881 to 2799) containing the coding region for nsP2 was isolated from pKTH330. Plasmid pN2-4 was obtained by inserting this fragment into a *SalI–PstI*-deleted pEX1. Plasmid pKTH337 was used as source for the construction of pN2-16. This plasmid was obtained by cloning an *AluI* fragment (positions 3405 to 3808), encoding an nsP2 region, into the *Smal* site of pEX1. Plasmid pN4-8 contains an *AluI* fragment (positions 4158 to 4600) from pKTH337, a coding region for nsP3, inserted into the *Smal* site of pEX1. pN4-4 has an *AluI* fragment (positions 6330 to 6774) from pKTH310 cloned into the *Smal* site of pEX1. This fragment codes for a region of nsP4.

*Purification of fusion proteins.* Overnight cultures of *E. coli* strain NF1 containing plasmids encoding the β-galactosidase (β-gal) fusion gene were diluted 1:100 into 100 ml L broth containing ampicillin (50 μg/ml). Cells were grown at 30 °C until the optical density at 600 nm was 0.6. To induce synthesis of fusion proteins, cells were diluted with one volume of L broth at 54 °C, and then incubated for 90 to 120 min at 42 °C. The cells were cooled and harvested by centrifugation at 12000 g for 20 min. The pellet was resuspended in 1 ml buffer A (50 mm-Tris–HCl pH 8.0, 50 mm-NaCl, 1 mm-EDTA). Lysozyme was added to a final concentration of 1 mg/ml and the mixture was left for 40 min on ice. Then 3 ml 1% Triton X-100 in buffer A was added and incubated for 30 min on ice. Chromosomal DNA was sheared and the lysate was then centrifuged at 12000 g for 20 min. The pellet was resuspended in 1 ml of Laemmli sample buffer and boiled for 5 min. Remaining particulate material was removed by centrifugation in a microfuge for 5 min. The solution containing the fusion protein was subjected to preparative 7-5% SDS–PAGE (Laemmli, 1970). The position of the fusion protein could be directly visualized as follows. The lower glass plate was carefully cleaned and the upper plate was removed. Light scattering from the protein bands could be visualized by holding the gel at a suitably narrow angle against ordinary light. The lower limit of detection by this method was about 5 μg of protein per band. The protein band was excised and dried onto a dialysis membrane, and the protein was eluted in an ISCO model 1750 sample concentrator (Kalkkinen, 1986).

*Preparation of antisera.* Five groups of female rabbits were immunized with five different fusion proteins. About 100 μg of each fusion protein was mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously into the rabbit. Four weeks later booster injections were given biweekly with the same amount of fusion protein in incomplete Freund's adjuvant. Serum was collected 10 days after each injection.

*Cells and viruses.* The origin and cultivation of SFV prototype strain and BHK-21 cells have been described previously (Keränen & Kääriäinen, 1974; Saraste et al., 1977).
SFV-specific nsP3 is a phosphoprotein

Radiolabelling of cells. BHK-21 cells, grown on 60 or 100 mm plastic dishes, were infected with SFV (50 p.f.u./cell) and incubated at 37 °C. At indicated times post-infection (p.i.) the cells were exposed to methionine-free MEM for 30 min, followed by labelling with 200 μCi/dish of [35S]methionine (100 Ci/mmole; Amersham) for 15 to 30 min. They were then chased for 2 min (in pulse experiments) or 60 min (chase) in MEM containing a 20-fold excess of methionine. Specific labelling of SFV-infected cells under hypertonic conditions was done as previously described (Kääriäinen et al., 1978). Briefly, BHK-21 cells infected with wild-type SFV (50 p.f.u./cell) were treated at 2 h p.i. with MEM containing 335 mM-NaCl for 30 min. The hypertonic MEM was replaced by an isotonic medium (0.1 M-sucrose in MEM) and the cells were labelled for 30 or 60 min. In phosphate-labelling experiments SFV-infected BHK-21 cells received phosphate-free MEM at 2 h p.i. Thirty min later they were labelled with 500 μCi/dish of carrier-free [32P]orthophosphate (Amersham) for 2.5 h, in MEM lacking phosphate.

Immunoprecipitation. For immunoprecipitation the infected and labelled cells were washed with phosphate-buffered saline and lysed in 1% SDS. The DNA was sheared by passing the lyseate five times through a narrow gauge syringe needle and the lysate was immediately boiled for 2 min and diluted (1/10) in NET buffer (1% NP40, 50 mM-Tris–HCl pH 8.0, 400 mM-NaCl, 5 mM-EDTA, 0.02% NaN3 and 100 units/ml of Trasylol (Bayer, Leverkusen, F.R.G.)). Ten μl of each antiserum was used for 700 μl of the diluted lysate. After overnight incubation at 4 °C 250 μl of 2.7% (w/v) Protein A-Sepharose (Pharmacia) was added and incubated for 30 min at 37 °C. The proteins were analysed in 7.5% SDS–polyacylamide gels according to Laemmli (1970). Fluorography was carried out according to Bonner & Laskey (1974).

Cell fractionation. SFV-infected cells in 100 mm plastic dishes were used to isolate mitochondrial pellet (P15) and supernatant (S15) fractions in RS buffer (10 mM-Tris–HCl pH 8.0, 10 mM-NaCl) as described previously (Ranki & Kääriäinen, 1979). The P15 was resuspended in a volume of RS buffer equal to that of S15.

Phosphatase treatment. Calf intestinal alkaline phosphatase (CIAP; Boehringer Mannheim) was used for the treatment of immunoprecipitates obtained with anti-nsP3 serum. The immunoprecipitates bound to Protein A-Sepharose were washed with CIAP buffer (50 mM-Tris–HCl pH 8.0, 1 mM-MgCl2, 0.1 mM-ZnCl2, 1 mM-spermidine). To 1 ml of CIAP buffer 10 μl of CIAP was added followed by rotational incubation at 20 °C for 30 min. After incubation the Protein A-Sepharose beads were washed and the proteins eluted as in the standard immunoprecipitation procedure (Kuismanen et al., 1984).

Determination of phosphoamino acids. 32P-labelled nsP3 was precipitated from the cell lysate with anti-nsP3 serum as described above and separated by SDS–PAGE. The labelled protein band was eluted from the gel by shaking for 12 h at 37 °C in 0.05 M-NH4HCO3, 0.1% SDS, 5% 2-mercaptoethanol. Bovine serum albumin (10 μg) was added as a carrier and the protein was precipitated with 20% TCA. The pellet was washed with acetone (−20 °C) followed by drying. The dried precipitate was dissolved in 98% formic acid and lyophilized. Acid hydrolysis was carried out in 6 M-HCl for 120 min at 110 °C. After lyophilization the sample was put in the electrophoresis medium (acetic acid:formic acid:water, 78:25:897), pH 2, containing phosphopyrosine, phosphothreonine and phosphoserine (about 20 nmol of each; Sigma). Samples were electrophoresed for 120 min at 1 kV on cellulose thin layer plates (Merck). Chromatography was performed in isobutyric acid:ammonium hydroxide (0.5 M; 5:3). The phosphoamino acid markers were detected by staining with ninhydrin (200 mg/100 ml ethanol:acetic acid, 20:1, v/v). The 32P radioactivity on the thin layer plate was visualized by autoradiography.

For reversed phase (RP) HPLC analysis the hydrolysed nsP3 was dissolved in 20 μl 0.1 M-NaHCO3 pH 9-1 containing phosphoserine, phosphothreonine and phosphopyrosine (about 20 nmol of each) added as markers. Forty μl of recrystallized dimethylaminobenzene-4-sulphonyl chloride (DABS-Cl; Fluka) solution (1:3 mg/ml in acetonitrile) was added and the derivatization performed at 55 °C for 30 min. The mixture was dried in vacuo and the amino acid derivatives were dissolved in 50 μl of 70% ethanol. HPLC separation of the phosphoamino acid derivatives was performed on a Spherisorb S5 ODS2 column (0.46 × 25 cm) by using a 20 to 30% gradient of acetonitrile in 25 mM-sodium phosphate pH 6.5, 4% dimethylformamide (Chang, 1984) for 40 min at 1 ml/min. Detection was at 436 nm. Fractions corresponding to the positions of the added standards were collected and the radioactivity due to 32P was determined using a liquid scintillation counter.

Indirect immunofluorescence. BHK-21 cells infected with SFV were fixed at 4 h p.i. with 3% paraformaldehyde and permeabilized with 0.05% Triton X-100 (Kuismanen et al., 1982). Preimmune sera and immune sera were diluted 1:50. Swine anti-rabbit IgG conjugated to tetramethylrhodamine isothiocyanate (Dako, Copenhagen, Denmark) was used as second antibody.

RESULTS

Production of SFV-specific ns-β-gal fusion proteins

To produce large quantities of SFV ns proteins for use as immunogens, we expressed portions of the coding region of each protein as a β-gal–ns fusion protein in E. coli. For this purpose we used the pEX1 and pEX2 bacterial expression vectors, which contain the bacteriophage λ Pr.
Proteins

nsP1 (537 aa)  nsP2 (798 aa)  nsP3 (482 aa)  nsP4 (614 aa)

Fig. 1. Regions of SFV-specific ns proteins expressed as β-gal fusion proteins in E. coli with pEX vectors (pN1-10, pN2-4, pN2-16, pN3-8 and pN4-4). The organization of the SFV genome region coding for the ns proteins is illustrated. The four ns proteins (nsP1 to nsP4) are translated as a polyprotein starting with the AUG codon at position 86 and ending with the termination codon at 7379. The nucleotide (nt) coordinates for each ns protein and fragments from them are indicated (upper part). Numbering of the amino acids (aa) inserts starts from the N terminus of each individual protein (lower part).

promoter and a cro–lacZ coding region, followed by a polylinker at the 3’ end (Stanley & Luzio, 1984). Appropriate cDNA fragments for each ns protein were ligated in phase via the polylinker to the coding region of the cro–β-gal sequence (Fig. 1). The resulting fusion proteins contained the cro–β-gal at their N terminus and the ns protein amino acid sequence at their C terminus.

In plasmid pN1-10 the cro–β-gal gene was joined to a DNA fragment encoding 131 amino acids of nsP1. Plasmids pN2-4 and pN2-16 represented 305 and 136 N- and C-terminal amino acids of nsP2, respectively. Plasmids pN3-8 and pN4-4 represented 146 N-terminal and 148 central amino acids from nsP3 and nsP4, respectively (Fig. 1).

Each of these plasmids was used to transform E. coli NF1, containing the clts857 repressor (Stanley & Luzio, 1984). Cells harbouring these plasmids were grown at 30 °C overnight and induced at 42 °C, the temperature at which the repressor is inactivated and the λ P_R promoter is functional. The cells were harvested after 2 h at 42 °C, and the fusion proteins were partially purified, as described in Methods. The denatured fusion proteins were analysed by SDS–PAGE (Fig. 2). The mobilities of the different fusion proteins were well correlated with their expected M_r values. All plasmids expressed the fusion proteins in quantities comparable to the control plasmid (Fig. 2, lane 1). For preparative purposes 100 ml cultures were treated as above and the fusion proteins were eluted from preparative SDS–PAGE gels. The 100 ml cultures usually yielded about 1 mg of purified fusion protein. The fusion proteins were used for immunization of rabbits as described in Methods.

Specificity of the antisera

The specificity of the antisera against the respective fusion proteins isolated from E. coli was tested by immunoprecipitation. BHK-21 cells in 60 mm dishes were infected with SFV at 37 °C and the cells were exposed to [35S]methionine (200 μCi/dish) for 15 min at 2-5 h p.i., the time when ns proteins are actively synthesized (Lachmi & Kääriäinen, 1976, 1977). The short pulse was chosen to label the previously characterized precursors of the ns proteins. After a 2 min chase, the cells were solubilized with 1% SDS and the lysate was reacted with the antisera raised against the different fusion proteins. The immune complexes were precipitated with Protein A–Sepharose. Analysis of the immunoprecipitates by 7.5% SDS–PAGE is shown in Fig. 3. Each of the antisera precipitated two to three major bands. Of these the smallest represented the mature ns proteins, nsP1, nsP2, nsP3 and nsP4 (Keränen & Ruohonen, 1983) (Fig. 3, lanes 1, 2, 3 and 4) and the larger ones represented precursors. The preimmune rabbit sera gave negative results (lanes 5, 6, 7 and 8). Anti-pN2-4 serum had the same specificity as anti-pN2-16 (Fig. 1) but a
Fig. 2. SDS (7.5%)-PAGE of β-gal–ns fusion proteins synthesized in E. coli. PAGE was by the method of Laemmli (1970) and gels were stained with Coomassie Brilliant Blue. Lane 1, pEX1 alone; lane 2, pN1-10; lane 3, pN2-4; lane 4, pN2-16; lane 5, pN3-8; lane 6, pN4-4.

Fig. 3. Analysis by SDS–PAGE of immunoprecipitated [35S]methionine-labelled proteins from SFV-infected BHK-21 cell lysate. Antisera against the following fusion proteins were used: pN1-10 (anti-nsP1), lane 1; pN2-16 (anti-nsP2), lane 2; pN3-8 (anti-nsP3), lane 3; pN4-4 (anti-nsP4), lane 4. The corresponding preimmune sera were used for the precipitations shown in lanes 5, 6, 7 and 8. Lane L represents the lysate before immunoprecipitation and lanes M show 14C-labelled marker proteins myosin (200K), phosphorylase b (92.5K), bovine serum albumin (67K), ovalbumin (46K) and carbonic anhydrase (30K) (from the top).

lower titre, and was thus not included in further experiments. The previously identified precursor of nsP1, nsP2 and nsP3, p220 (Lehtovaara et al., 1980), was immunoprecipitated with antisera against nsP1, nsP2 and nsP3 but not with anti-nsP4 (Fig. 3, lanes 1, 2 and 3). Anti-nsP1 and anti-nsP2 sera precipitated p155 (precursor of nsP1 and nsP2) (Fig. 3, lanes 1 and 2), whereas p135 was precipitated only with antisera against nsP3 and nsP4 (lanes 3 and 4). The entire ns polyprotein p250 was best visualized with antiserum against nsP4 (lane 4) but could also be seen in the immunoprecipitate of anti-nsP2 (lane 2). Upon longer exposure of the gel p250 could also be seen in the immunoprecipitates by antisera specific for nsP1 and nsP3 (not shown). The bands below p155 (lane 2) and below p135 (lane 4) most probably represent nascent polypeptides (K. Takkinen et al., unpublished data). When the pulse-labelled SFV-infected cultures were chased for 45 min in the presence of excess unlabelled methionine only nsP1, nsP2, nsP3 and nsP4 could be visualized (data not shown). We conclude that antisera raised against the selected fragments of different ns proteins were specific for the predicted final cleavage products of the polyprotein and also recognized the predicted processing intermediates of the ns polyprotein.
Fig. 4. Distribution of ns proteins in the P15 and S15 fractions of SFV-infected BHK-21 cells. The cells were harvested 4-5 h p.i., lysed in hypotonic RS buffer, and after removal of the nuclei, fractionated into P15 and S15. Immunoprecipitation was carried out with antisera against the four fusion proteins as in Fig. 3. Lanes 1 and 2 show immunoprecipitates with anti-nsP1, lanes 3 and 4 with anti-nsP2, lanes 5 and 6 with anti-nsP3 and lanes 7 and 8 with anti-nsP4 antiserum. The material shown in lanes 1, 3, 5 and 7 is from the S15 fraction and that shown in lanes 2, 4, 6 and 8 is from the P15 fraction. The labelled proteins found in S15 and P15 fractions before immunoprecipitation are shown in lanes S and P, respectively. The proteins were separated by SDS-PAGE in a 7.5% gel as in Fig. 3.

Subcellular distribution of nsP1, -P2, -P3 and -P4

We have previously shown that RNA polymerase activity in SFV-infected cells is found exclusively in the mitochondrial pellet fraction (P15) (Ranki & Kääriäinen, 1979). Upon further fractionation it remains strictly membrane-associated (Gomatos et al., 1980; Cross & Gomatos, 1981; Cross, 1983). SFV-infected BHK-21 cells in 100 mm dishes were labelled with 250 µCi/dish of [35S]methionine for 60 min at 2.5 h p.i. and chased for 60 min, during early infection, at the time when virus-specific components of the RNA polymerase are assembled (2.5 to 3.5 h p.i.). In this experiment the labelling was carried out under hypertonic conditions to achieve preferential labelling of virus-specific proteins (Ranki & Kääriäinen, 1979; Gomatos et al., 1980). After 60 min of labelling with [35S]methionine, the cells were allowed to recover for 60 min to ensure that the labelled proteins had time to assemble into the RNA polymerase-template complex. At 4.5 h p.i. the cells were lysed and the post-nuclear supernatant was used as a source of P15 and S15 fractions. Both fractions were exposed to the anti-fusion protein sera after solubilization with SDS and the labelled proteins were analysed by SDS–PAGE (Fig. 4).

All four ns proteins were found in the P15 fraction but nsP1 and nsP4 were exclusively found in P15 (Fig. 4, lanes 2 and 8). nsP2 was distributed almost equally between P15 and S15 (Fig. 4, lanes 3 and 4), whereas most of nsP3 was in P15 (Fig. 4, lane 6). Thus the RNA polymerase activity and all the ns proteins are confined to the membrane fraction as we have shown previously (Ranki & Kääriäinen, 1979), although in the previous study identification of the ns proteins was based mainly on their mobilities in SDS–PAGE.
SFV-specific nsP3 is a phosphoprotein

To discover whether one of the SFV ns proteins was a phosphoprotein, BHK-21 cells, infected with wild-type SFV in a 60 mm dish, were exposed to 500 μCi [32p]orthophosphate 2.5 to 5 h p.i. Nuclei-free cell lysates, treated with SDS, were subjected to immunoprecipitation with anti-fusion protein sera as before. Mock-infected cells and preimmune sera of rabbits immunized with the different fusion proteins were used as controls. Lanes 1 and 2 in Fig. 5 represent SDS–PAGE analysis of precipitates with preimmune and immune sera against nsP1 fusion protein, lanes 3 and 4 similar precipitations with anti-nsP2. Lane 5 shows that only antiserum against nsP3 precipitated a protein labelled with 32p. This protein had the mobility of nsP3, and could also be immunoprecipitated from SFV-infected chick embryo fibroblasts (CE cells). No labelled protein could be immunoprecipitated from mock-infected, 32p-labelled BHK-21 or CE cells (data not shown). Treatment of the immunoprecipitate with CIAP reduced the label of nsP3 considerably (Fig. 5, lanes 9 and 10).

For identification of phosphoamino acids, the SFV-infected BHK-21 cells were exposed to 32P between 2 and 5 h p.i., and nsP3 was purified by immunoprecipitation and SDS–PAGE. After elution from the gel, nsP3 was hydrolysed together with carrier protein in 6 M-HCl. Unlabelled phosphoserine, phosphothreonine and phosphotyrosine were added to the hydrolysate as markers and the amino acids were separated on thin layer plates (Fig. 6). The added phosphoamino acids (about 10 nmol each) were visualized by staining with ninhydrin and the 32P radioactivity on the plate was visualized by autoradiography. To quantify the 32P radioactivity in the individual spots the cellulose was scraped from the plate, the phosphoamino acids were dissolved and the radioactivity was determined by liquid scintillation counting.

Phosphoamino acid analysis was also carried out by using RP HPLC. For this purpose the acid hydrolysate containing the added unlabelled phosphoamino acids was treated with DABS-Cl and the resulting amino acid derivatives were separated on an RP column (Chang, 1984). The individual phosphoamino acid derivatives were collected by reference to the positions of the added standards (Fig. 7), and the radioactivity was determined by liquid scintillation counting.
Fig. 6. Identification of phosphorylated residues of nsP3 on cellulose thin layer plates by two-dimensional analysis. $^{32}$P-labelled nsP3 was isolated from SFV-infected cell lysate, immunoprecipitated with anti-nsP3 serum and purified by SDS–PAGE. The eluted protein was hydrolysed in 6 M-HCl and the phosphoamino acids were separated first by electrophoresis at pH 2 for 120 min at 1 kV toward the anode (from right to left), followed by chromatography in isobutyric acid:ammonium hydroxide (from bottom to top). The position of the origin (O) and the unlabelled markers phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) are indicated. The $^{32}$P label was visualized by autoradiography.

In the thin layer plates the phosphoserine and phosphothreonine spots, but not the phosphotyrosine spot, contained $^{32}$P radioactivity. Similarly, the phosphoserine and phosphothreonine derivatives but not the phosphotyrosine derivative contained $^{32}$P radioactivity in the collected RP HPLC fractions (Fig. 7). To investigate the possible destruction of the individual phosphoamino acids under the hydrolysis conditions used, a mixture of known amounts of phosphoserine, phosphothreonine and phosphotyrosine was analysed by the HPLC method before and after acid hydrolysis. The recovery of phosphoserine was 41%, that of phosphothreonine 53%, and phosphotyrosine 32%. These values were used to correct the quantities of $^{32}$P radioactivity obtained from the nsP3 hydrolysate. After correction for losses in the acid hydrolysis the ratios of $^{32}$P radioactivity recovered from phosphoserine and phosphothreonine were 2.3 and 1.8 in the thin layer and HPLC methods, respectively.

Since small amounts of nsP3 were found also in the S15 fraction (Fig. 4) we wanted to determine the relative phosphorylation ratios of the membrane-associated and free nsP3. BHK-21 cells infected with SFV were labelled either with $^{32}$P, or $[^{35}S]$methionine starting at 1.5 h and 22.5 h p.i., respectively. The isotopes were removed at 3 h p.i. from the medium and the cultures were chased with unlabelled phosphate and methionine for 1 h in the presence of cycloheximide (50 μg/ml). Protein synthesis was inhibited during the chase period to ensure that $^{32}$P would not incorporate into nsP3 molecules that were not labelled with $[^{35}S]$methionine, due to failure to chase the $^{32}$P.
SFV-specific nsP3 is a phosphoprotein

Fig. 7. Separation of DABS-phosphoserine (918 c.p.m.), DABS-phosphothreonine (660 c.p.m.) and DABS-phosphotyrosine (20 c.p.m.) by RP HPLC. Fractions corresponding to the individual phosphoamino acids were collected from the nsP3 hydrolysate and subjected to determination of $^{32}$P radioactivity in a liquid scintillation counter.

Table 1. Different phosphorylation levels of nsP3 in P15 and S15 fractions as evidenced by labelling with $^{32}$P and $[^{35}$S]Methionine

<table>
<thead>
<tr>
<th>Isotope labelling</th>
<th>Radioactivity of nsP3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P15</td>
</tr>
<tr>
<td>$[^{35}$S]Methionine</td>
<td>8288 (81%)</td>
</tr>
<tr>
<td>$^{32}$P</td>
<td>2076 (91%)</td>
</tr>
<tr>
<td>$[^{35}$S/$^{32}$P</td>
<td>40</td>
</tr>
</tbody>
</table>

Equal amounts of cells from $[^{35}$S]methionine- and $^{32}$P-labelled cultures were lysed and fractionated into P15 and S15, and the nsP3 was immunoprecipitated and analysed by SDS–PAGE. The radioactive band at the position corresponding to nsP3 was cut out and its radioactivity was determined after solubilization in NCS. The distribution of $^{32}$P and $[^{35}$S] labels differed; about 20% of $[^{35}$S]methionine was in the S15 fraction in accordance with the result shown in Fig. 4. The amount of $^{32}$P label in S15 was only about 10% (Table 1). From the $[^{35}$S/$^{32}$P ratios it can be estimated that the phosphorylation of nsP3 in S15 was about half of that seen in P15.

Subcellular localization of nsP3 by immunofluorescence

In all of the above experiments immunoprecipitations were carried out from lysates or fractions treated with SDS to denature the proteins. We tested the ability of the anti-nsP3 serum to react with less denatured proteins in paraformaldehyde-fixed infected cells as well. After fixation the cells were permeabilized with Triton X-100, and treated with the antiserum or preimmune sera followed by TRITC-conjugated swine anti-rabbit IgG. The majority of SFV-infected cells treated with anti-nsP3 antiserum showed cytoplasmic fluorescence accompanied
by staining of large vesicle-like structures (Fig. 8a). No staining of mock-infected cells or infected cells treated with preimmune serum could be seen (Fig. 8b). Antisera against nsP1, nsP2 and nsP4 failed to stain the infected cells in the indirect immunofluorescence test, indicating that the antibodies were not directed against natural epitopes in the respective proteins.

**DISCUSSION**

The tentative identification of SFV ns proteins associated with active RNA polymerase-template complex has been based on preferential virus-specific labelling in hypertonic media (Ranki & Kaiariainen, 1979; Gomatos et al., 1980). As the complete nucleotide sequence of SFV 42S RNA has been determined (Takkinen, 1986) other approaches have become available to study the function of the ns proteins. In this report we have expressed selected parts of each ns protein as a β-gal-ns fusion protein in *E. coli*. Antibodies raised against purified denatured fusion proteins were specific for the known ns proteins as evidenced by immunoprecipitation of lysates from [35S]methionine-labelled BHK-21 and CE cells which had been infected with wild-type SFV. When a 15 min pulse of [35S]methionine was given to the infected cells, the previously identified precursors p250 (containing nsP1, nsP2, nsP3 and nsP4), p220 (nsP1, nsP2 and nsP3), p155 (nsP1 and nsP2) and p135 (nsP3 and nsP4) could also be immunoprecipitated with the appropriate antisera (Fig. 3). Recently, monospecific antibodies have been produced against amino acid sequences of the four ns proteins of Sindbis virus (Hardy & Strauss, 1988). The virus-specific proteins were also expressed in *E. coli* as fusion proteins. The antibodies also recognized the precursor proteins, and were used to study the kinetics of the proteolytic processing of the ns polyprotein.

Fractionation of cells into P15 and S15 fractions revealed that nsP1 and nsP4 were exclusively associated with the P15 fraction, which has all the viral RNA polymerase activity of SFV-infected cells (Ranki & Kääriäinen, 1979; Gomatos et al., 1980; Cross & Gomatos, 1981). The methyltransferase activity responsible for the methylation of the SFV cap structure is confined to P15 as well (Cross, 1983). About 80% of nsP3 was found in P15, whereas nsP2 was distributed evenly between the P15 and S15 (Fig. 4 and 8). Thus nsP2 and nsP3 could play a regulatory role in some steps of SFV RNA synthesis. Interestingly, nsP2 has been previously shown to have specific affinity for ribosomal RNA to which it can be cross-linked by u.v. irradiation (Ranki et al., 1979).

The specific association to membranes of nsP3 was demonstrated by indirect immunofluorescence of fixed, permeabilized SFV-infected cells. The fluorescent staining was confined to large cytoplasmic vesicles (Fig. 8a) of similar size (about 1 μm) to the cytoplasmic vacuoles described...
SFV-specific nsP3 is a phosphoprotein

previously (Acheson & Tamm, 1967; Friedman & Berezesky, 1967; Grimley et al., 1968). These structures are typical of alphavirus-infected cells and have been suggested to be the site of the virus-specific RNA synthesis (Grimley et al., 1968, 1972).

When lysates of 32P-labelled SFV-infected cells were immunoprecipitated with the antisera produced against sequences of the four ns proteins the only labelled protein detected was nsP3. The following results suggested that nsP3 is a genuine phosphoprotein. (i) No labelled product could be precipitated from mock-infected 32P-labelled cells (not shown). (ii) Preimmune serum failed to precipitate any labelled proteins. (iii) Treatment of the immunoprecipitate, obtained by anti-nsP3 serum, with CIAP reduced the radioactivity in the nsP3 band significantly (Fig. 5, lanes 9 and 10).

In order to confirm this conclusion we subjected the 32P-labelled nsP3 to acid hydrolysis and phosphoamino acid analysis. The 32P radioactivity could be detected only in positions corresponding to phosphoserine and phosphothreonine. For the analysis we used both two-dimensional separation on thin layer plates and RP HPLC analysis. For the latter the amino acids were derivatized with DABS-C1 (Chang, 1984). This technique was also used to estimate the recovery of phosphotyrosine, phosphoserine and phosphothreonine under our conditions of acid hydrolysis. No consistent amounts of 32P radioactivity could be detected in the phosphotyrosine fraction even when taking into account the poor recovery (32%) of phosphotyrosine after acid hydrolysis. This strongly suggests that tyrosine is not phosphorylated in nsP3.

Many viruses specify phosphorylated proteins, some of which are constituents of the virions. Influenza A virus NP protein (Almond & Felsenreich, 1982; Privalsky & Penhoet, 1981), M1 protein (Gregoriades et al., 1984) and non-virion protein NS1 (Privalsky & Penhoet, 1981) are phosphorylated. Paramyxovirus P protein is heavily phosphorylated (Lamb & Choppin, 1977) as is the respective vesicular stomatitis virus (VSV)-specific NS protein (Sokol & Clark, 1973; Bell & Prevec, 1985). The avian and murine retrovirus gag proteins p19 and p12 are phosphoproteins (Lai, 1976; Erikson et al., 1977; Pal & Roy-Burman, 1975; Pal et al., 1975) as is also p30, which is less phosphorylated (Ikuta & Luftig, 1988). Phosphorylation has also been reported for the Sindbis virus capsid protein, with 0·03 to 0·1 mol phosphate per mol of protein (Waite et al., 1974).

Perhaps the best studied example of virion-coded phosphoproteins is the intracellular product of the v-src gene, pp60Src, since it plays a vital role in the malignant transformation of virus-infected cells (Hunter & Sefton, 1980) (for reviews, see Bishop, 1983, 1985). The simian virus 40 (SV40) large T antigen is a heavily phosphorylated protein (Tegtmeyer et al., 1977) with a number of well characterized functions in SV40 replication and cell transformation (Green, 1985).

In some cases the function(s) of the phosphorylation has been studied in more detail. The phosphorylation of serine residues 236 and 242 of VSV NS protein, presumably by the L protein-associated kinase, activates the RNA synthesis of isolated VSV nucleocapsids in vitro (Gill et al., 1986; Chattopadhyay & Banerjee, 1987; Banerjee, 1987). Phosphorylation is important for the binding of the pp12 nucleocapsid protein of avian retroviruses to ssRNA (Leis & Jentoft, 1983; Leis et al., 1984). Recent in vitro experiments suggest that the phosphorylation of SV40 T antigen plays an important role in the replication of the virus DNA (Mohr et al., 1987).

Reversible phosphorylation of proteins is a widely used mechanism in the control of metabolic and other cellular processes (for reviews, see Krebs & Beavo, 1979; Chock et al., 1980; Cohen, 1982). It would be tempting to assume that the phosphorylation of nsP3 would also serve a regulatory role in some steps of SFV RNA synthesis. The finding that nsP3 isolated from the P15 fraction, which has practically all the RNA polymerase activity of the cell lysate, is phosphorylated more heavily than the same protein in S15 (Table 1) might reflect the possible role of phosphorylation in the control of SFV RNA synthesis.

Analysis of temperature-sensitive mutants of SFV and Sindbis virus has revealed mutants with specific defects in the synthesis of complementary RNA (minus strands) (Sawicki et al., 1981a), in the shut-off of the minus strand synthesis (Sawicki et al., 1981b), in the polymerization of both minus and plus strands (Keränen & Kääriäinen, 1979; Sawicki et al.,
1981 b), as well as in the regulation of the initiation of the subgenomic 26S mRNA (Saraste et al., 1977; Sawicki et al., 1978; Kääriäinen et al., 1987). Studies of the stage at which nsP3 is phosphorylated in the above mentioned steps of RNA synthesis should reveal whether the phosphorylation plays a regulatory role in the RNA synthesis of SFV and other alphaviruses.

We would like to thank Ms Annikki Kallio and Ms Anna-Liisa Nyfors for skilful technical assistance. This work was supported by the Sigrid Juselius Foundation.

REFERENCES


KALKKINEN, N. (1986). Radio-sequence analysis; an ultra-sensitive method to align proteins and nucleotide
MELANCON, P. & GAP, OFF, H. (1986). Reinitiation of translocation in the Semliki Forest virus structural polyprotein:
MOHR, I. J., STILLMAN, B. & GLUZMAN, Y. (1987). Regulation of SV40 DNA replication by phosphorylation of T-
MOHR, I. J., STILLMAN, B. & GLUZMAN, Y. (1987). Regulation of SV40 DNA replication by phosphorylation of T-
2178 J. PERÄNEN AND OTHERS


(Received 12 April 1988)