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Nuclear Localization of Semliki Forest Virus-Specific Nonstructural Protein nsP2

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About 50% of Semliki Forest virus-specific nonstructural protein nsP2 is associated with the nuclear fraction in virus-infected BHK cells. Transport into the nucleus must be specific, since only trace amounts of nsP3 and nsP4 and about 13% of nsP1, all derived from the same polyprotein, were found in the nucleus. Subfractionation of [^{35}S]methionine-labeled Semliki Forest virus-infected cells showed that 80 to 90% of the nuclear nsP2 was associated with the nuclear matrix. Indirect immunofluorescence, with anti-nsP2 antiserum, showed the most intensive staining of structures which by Nomarski optics appeared to be nucleoli. In the presence of 1 to 5 μg of dactinomycin per ml the nuclei were stained evenly and no nucleoli could be found. Transport of nsP2 into the nucleus occurred early in infection and was fairly rapid. A cDNA encoding the complete nsP2 was isolated by the polymerase chain reaction technique and ligated into a simian virus 40 expression vector derivative. When BHK cells were transfected with this pSV-NS2 vector by the lipofection procedure, nsP2 was expressed in about 1 to 5% of the cells, as shown by indirect immunofluorescence. In positively transfected cells the immunofluorescence stain was most intensive in the nucleoli. Thus, Semliki Forest virus-specific nsP2 must have information which directs it into the nuclear matrix and, more specifically, into the nucleoli.

The alphaviruses code for four nonstructural proteins (nsP1 to nsP4), which all are translated from a large polyprotein, P1234, previously designated as ns250. The functions of these proteins in RNA replication have been studied intensively during the last 10 years (24, 69). Temperature-sensitive mutants of Sindbis virus have been instrumental in these studies (24, 69). One of the functions (proteins) represented by complementation group F (*ts*6) was deduced to be the actual polymerizing component (1, 27, 57). Another, *ts*11 (group B), was implicated in the synthesis of RNA minus strands (57). Group A mutants (*ts*15, *ts*17, *ts*21, *ts*24, and *ts*133) were defective in the synthesis of the subgenomic 26S RNA (27, 56). The same defect was reported for some representatives of complementation group G (27). A few group A mutants (*ts*24, *ts*17, and *ts*133) also displayed a defect in the shutoff on minus-strand RNA synthesis (56, 59, 60).

The thorough analysis by Strauss and collaborators has enabled researchers to establish the interrelation between the four Sindbis virus complementation groups (A, B, G, and F) and the four nonstructural proteins (nsP1 to nsP4) (19–21, 37, 51). Mutations in complementation group F mapped in the conserved regions of nsP4 (19), whereas the single representative of group B (*ts*11) had a single mutation in nsP1. Mutants *ts*17, *ts*21, and *ts*24 of group A had amino acid replacements in nsP2. Interestingly, mutants *ts*7 and *ts*18 of group G also had amino acid changes in nsP2, suggesting that the regulation of 26S RNA is carried out by nsP2 only (20). *ts*7 had, in addition to the amino acid change in nsP2, lethal replacement in nsP3. This is the only genetic indication of the involvement of nsP3 in RNA replication. These results for Sindbis virus must evidently be valid also for Semliki Forest virus (SFV), since their nonstructural proteins are highly homologous (24, 72).

Additional functions of the alphavirus nonstructural proteins are also emerging. The virus-specific, cytoplasmic, methyltransferase activity needed in the capping of viral RNAs (7, 8) has recently been suggested to be associated with nsP1 in Sindbis virus-infected cells (41). Recent results by Ding and Schlesinger (9) suggest that Sindbis virus-specific nsP2 has protease activity which cleaves the nonstructural polyprotein between nsP1 and nsP2 as well as between nsP2 and nsP3.

In the work described in this paper we have used monospecific antisera against the four nonstructural proteins (nsP1 to nsP4) of SFV (46, 72) to demonstrate, by indirect immunofluorescence and cell fractionation, that nsP2 is specifically transported into the nuclei of virus-infected and transfected BHK cells. In these cells, about 50% of nsP2 is associated with the nuclear matrix fraction. Light microscopy shows that nsP2 is enriched in the nucleoli.

MATERIALS AND METHODS

Cells and viruses. The cultivation of BHK cells and the propagation of the SFV prototype strain and *ts*1 mutant have been previously described (26, 55).

Radiolabeling and subcellular fractionation of cells. BHK cells grown on two 100-mm plastic dishes were infected with the wild-type virus or the *ts*1 mutant (50 PFU per cell). These cells were labeled with [^{35}S]methionine (100 Ci/mmol; Amersham Corp.) at 200 μCi per dish for various periods. After being labeled, the cells were washed twice with ice-cold phosphate-buffered saline, scraped from the dishes, and pelleted. All manipulations were done on ice unless otherwise mentioned. The pelleted cells were washed twice with isotonic buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 0.25 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 100 IU of Trasylol [Bayer AG, Leverkusen, Federal Republic of Germany] per ml) and resuspended in buffer A. The cells were disrupted by Dounce homogenization (25 strokes) in 1 ml of buffer A supplemented with 2 mM EDTA and 0.1 mM dithiothreitol. The nuclei were isolated by low-speed centrifugation (500 × *g* for 10 min), and the supernatant (cytoplas-

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mic fraction) was removed. This step was repeated, and the nuclei were then suspended in buffer B (10 mM HEPES [pH 7.4], 0.25 M sucrose, 2 mM $MgCl_2$, 0.1% Triton X-114, 0.1 mM PMSF, 100 IU of Trasylol per ml) and incubated for 15 min on ice. The nuclei were pelleted (500 × *g* for 10 min) and suspended in an appropriate buffer.

For subnuclear fractionation the nuclei were suspended in 500 μl of buffer C (10 mM HEPES [pH 7.4], 0.25 M sucrose, 100 mM NaCl, 0.5% Nonidet P-40 [NP-40], 0.1 mM PMSF, 100 IU of Trasolyl per ml) and kept for 30 min on ice. After the NP-40 treatment the nuclei were pelleted (500 × *g* for 10 min) and reextracted once with buffer C. The supernatants represent the nucleoplasmic fraction. The final nuclear pellet was suspended in 250 μl of buffer D (10 mM HEPES [pH 7.4], 50 mM KCl, 100 mM NaCl, 5 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM PMSF) supplemented with micrococcal nuclease (50 μg/ml) and incubated for 30 min at 37°C. The nuclease digestion was stopped by adding 250 μl of buffer E (10 mM HEPES [pH 7.4], 4 M NaCl, 1% NP-40, 10 mM EDTA, 0.1 mM PMSF). After a 10-min incubation on ice the sample was layered on a 100-μl 30% sucrose cushion containing 10 mM HEPES (pH 7.4) and centrifuged (12,000 × *g* for 15 min) to separate the chromatin (supernatant) from the nuclear matrix (pellet). The nuclear matrix was solubilized in 1% sodium dodecyl sulfate (SDS) by boiling.

Immunoprecipitation. Immunoprecipitation of SFV nonstructural proteins with monospecific antibodies has been described previously (46). Before immunoprecipitation the volume of each subnuclear fraction was made equivalent.

Immunofluorescence microscopy. BHK cells, infected with wild-type SFV or the *ts*1 mutant or transfected with plasmid, were processed for immunofluorescence microscopy as described by Kuismanen et al. (32). The antiserum directed against nsP2 was affinity purified by absorption to purified nsP2-β-galactosidase (46) immobilized onto nitrocellulose filter and eluted as described previously (36). Swine anti-rabbit immunoglobulin G conjugated to tetramethylrhodamine isothiocyanate (DAKO, Copenhagen, Denmark) was used as the second antibody.

Plasmid constructs. To construct the nsP2 gene, plasmid pPLH214 (P. Liljeström, unpublished data), containing the entire nonstructural cDNA region of SFV 42S RNA (base pairs 1 to 7390), was cleaved with *Xho*I. The linearized plasmid was used to amplify a 2,400-base-pair fragment encoding the nsP2 protein by the polymerase chain reaction (PCR) method of Saiki et al. (54), with minor modifications. For this purpose, two oligonucleotides were synthesized. The upstream primer was a 30-nucleotide oligonucleotide (5′-CTC ACC ATG GGG GTC GTG GAA ACA CCT CGC-3′) corresponding to nucleotides 1697 to 1722 (sense strand) with additional nucleotides, which contained a translation initiation codon and a *Nco*I site. The downstream primer was a 32-nucleotide oligonucleotide (5′-TC CAG ATC TTA ACA CCC GGC CGT GTG CAT GGC-3′) corresponding to nucleotides 4068 to 4090 (antisense strand) with additional nucleotides, which contained a translation termination codon and a *Bgl*II site. The PCR mix contained 50 ng of linearized pPLH214, 1 μg of each primer, 200 μM each deoxynucleoside triphosphate, 0.1% gelatin, and 1× PCR buffer (10 mM Tris hydrochloride [pH 8.35], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg of gelatin per ml) in a total volume of 0.1 ml. To denaturate the DNA, the reaction mixture was boiled for 5 min in a water bath and 2 U of *Taq* DNA polymerase (New England BioLabs, Inc., Beverly, Mass.) and 1 μl of 10% Triton X-100 were then added. Amplification was carried out for 15 cycles: denaturation for 75 s at 97°C, annealing for 2 min at 55°C, and extension for 20 min at 72°C. After the seventh cycle, additional *Taq* DNA polymerase (2 U) was added to the reaction mixture.

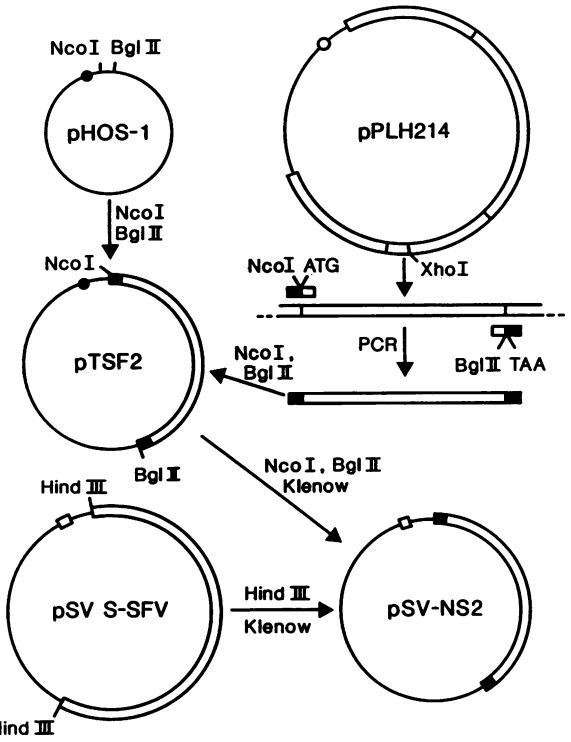

FIG. 1. Plasmid constructs. Plasmid pPLH214, which contains the complete coding region for SFV nonstructural proteins, was used as a template for PCR to obtain the complete coding region for nsP2 plus initiation and termination signals and the indicated restriction sites as described in Materials and Methods. The isolated *Nco*I-*Bgl*II fragment was inserted into the transcription-translation vector, pHOS-1, which is a derivative of pGEM3. For expression in mammalian cells the *Nco*I-*Bgl*II fragment containing nsP2 was ligated to pSV-S-SFV vector from which the *Hin*dIII fragment encoding SFV-specific proteins had been removed. This construction yielded the pSV-NS2 vector in which the nsP2 coding sequence was under the control of simian virus 40 early promoter.

DNA manipulations and cloning were done by standard methods (40). The amplified fragment was digested with *Bgl*II and *Nco*I, run in an agarose gel, and eluted with the GeneClean kit (Bio 101). The pTSF2 plasmid was constructed by inserting the fragment into a pGEM3 (Promega Biotec, Madison, Wis.)-derived transcription vector, pHOS-1 (J. Peränen, unpublished data), which had been cleaved with *Bgl*II and *Nco*I. For expression of nsP2 in animal cells, the vector pSV-NS2 was constructed by ligating the *Bgl*II-*Nco*I-cleaved and blunted fragment encoding nsP2 from pTSF2 into the pSV-S-SFV (a kind gift from Henrik Garoff, Department of Molecular Biology, The Karolinska Institute, Novum, Huddinge, Sweden) (31), which had been cleaved with *Hin*dIII and blunted and from which the SFV structural cDNA fragment had been deleted (Fig. 1).

For in vitro transcription the plasmid pTSF2 was cleaved with *Bgl*II and then transcribed as specified by the manufacturer (Transprobe T; Pharmacia, Uppsala, Sweden). The transcripts were used for in vitro translation, which was performed as recommended by the manufacturer (Promega Biotec).

Cell transfection. BHK cells were plated on cover slips at a density of 0.5 × 10^6 cells per 35-mm dish 1 day prior to

transfection. Cells were transfected with 1 μg of pSV-NS2 by the lipofection transfection procedure (14) with the Lipofectin kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The next day, the transfected cells were prepared for immunofluorescence microscopy (32).

RESULTS

Immunofluorescence localization of nsP2. We have recently reported the production of monospecific antisera against selected amino acid sequences of the four nonstructural proteins of SFV (46). Only anti-nsP3 gave a satisfactory staining in indirect immunofluorescence microscopy. When we affinity purified the anti-nsP2 antiserum and used the *ts*1 mutant of SFV, we could localize nsP2 by immunofluorescence in the infected BHK cells (Fig. 2C). The rationale for using *ts*1 was based on previous studies, which have shown that in *ts*1-infected cells the nonstructural proteins are produced in excess (33, 34, 58). Prominent fluorescence was observed in the nuclei of both wild-type and *ts*1 virus-infected BHK cells (Fig. 2A and C). In the nucleus an intensive fluorescent staining in most cells was localized to spherical or irregular structures, which by Nomarski optics could be identified as nucleoli (Fig. 2B and D). A weaker diffuse nuclear staining gave the background for the bright nucleolar fluorescence. In the cytoplasm we occasionally found stained vesicular structures on a background of a faint reticular staining (Fig. 2C and E). Only weak background fluorescence could be seen when uninfected cells were treated with the affinity-purified anti-nsP2 antiserum (Fig. 2G) or when virus-infected cells were treated with affinity-purified preimmune serum (Fig. 2H). The nuclear staining by anti-nsP2 antiserum could already be detected at 2 h postinfection (p.i.) (data not shown). When dactinomycin (1 or 5 μg/ml) was added at 1 h p.i., no distinct nucleolar structures could be seen at 6 h p.i. by Nomarski optics and the immunofluorescence was distributed all over the nucleus (Fig. 2E and F).

Isolation of nuclei and subnuclear compartments. We wanted to confirm the morphological findings by subcellular fractionation experiments. The conditions for isolation of intact nuclei and nuclear subcompartments were optimized. We found that isolation of nuclei under isotonic conditions gave the best and most reproducible results (see Materials and Methods). Inspection of isolated nuclei by light microscopy showed that they were intact and free of cytoplasmic tags. Analysis by SDS-polyacrylamide gel electrophoresis (PAGE) showed a typical protein pattern for nuclei (Fig. 3, lane 4; see especially the histones), whereas the cytoplasmic fraction was devoid of these proteins (Fig. 3, lane 3). Further purification of nuclei by mild Triton X-114 treatment removed cytoplasmic contaminants and probably proteins from the outer nuclear membrane, since the number of protein species was clearly reduced (Fig. 3, lanes 5 and 6). Subnuclear fractionation was performed by first extracting the nuclei twice with an NP-40-containing buffer to release the nucleoplasmic proteins (Fig. 3, lanes 7 and 8). NP-40-extracted nuclei were treated with micrococcal nuclease and high salt to obtain a soluble (chromatin) and an insoluble (nuclear matrix) fraction. The soluble fraction contained the histone proteins which are typical for chromatin (Fig. 3, lane 9), whereas the insoluble fraction contained the characteristic nuclear matrix proteins such as lamins (Fig. 3, lane 10).

Subcellular location of SFV nonstructural proteins. The distribution of nonstructural proteins in the cytoplasm and nuclei was examined by immunoprecipitation with monospecific antibodies against all four nonstructural proteins of SFV. BHK cells were infected with wild-type SFV or the *ts*1 mutant and pulse-labeled with [^{35}S]methionine early during infection (1.5 to 2.5 h), when the nonstructural proteins are maximally synthesized and the first replicase complexes are formed. After the pulse the cells were chased for 1 h in the presence of excess unlabeled methionine. The cells were fractionated into cytoplasmic and nuclear fractions, which were subjected to immunoprecipitations. The results for the wild-type-infected cells are shown in Fig. 4A. Most of nsP3 (Fig. 4A, lanes 5 and 6) and nsP4 (lanes 7 and 8) were in the cytoplasm. In contrast, nsP2 was distributed about equally between the cytoplasmic (Fig. 4A, lane 3) and nuclear (lane 4) fractions. Quantitation by densitometric tracings showed that about 13% of nsP1 and about 5% of nsP3 and nsP4 were found in the nuclear fraction (Table 1). The distribution of the four nonstructural proteins was almost the same in *ts*1-infected cells (Fig. 4B).

The subnuclear distribution of nsP2 in wild-type SFV-infected cells was investigated by fractionating the nuclei as described above for Fig. 3. When each fraction was analyzed for the presence of nsP2 by immunoprecipitation, it became clear that nsP2 was confined almost entirely to the nuclear matrix fraction (Fig. 5, lane 10). A small amount of nsP2 could also be detected in the nucleoplasm (Fig. 5, lanes 7 and 8), but virtually none was found in the chromatin fraction (lane 9). The relative amount of nsP2 in each fraction was estimated by densitometric tracings. The proportion of matrix-associated nsP2 varied between 70 and 90% in different experiments.

To be sure that the association of nsP2 with the nuclear matrix was not an artifact of the isolation procedure (12, 25), we mixed in vitro-translated nsP2 with unlabeled cells and fractionated them. After fractionation, virtually all the nsP2 was recovered in the cytoplasmic fraction (Fig. 6). This shows that the association of nsP2 with the nuclear matrix did not take place during the fractionation. It has recently been shown that the formation of intermolecular disulfide bonds between proteins during fractionation can render nuclear proteins nuclease and salt resistant (25). To examine this, we isolated nuclei and subnuclear compartments in the presence of 10 mM iodoacetamide. Iodoacetamide had no influence on the distribution of nsP2 in the nuclei: the same distribution was also seen in dactinomycin-treated cells (data not shown). We therefore conclude that the association of nsP2 to the nuclear matrix was not an artifact of the experimental conditions used.

Kinetics of nuclear transport of nsP2. To investigate the kinetics of nuclear transport of nsP2, SFV-infected cells were pulse-labeled for 1 min, chased for 3 and 7 min at 135 min p.i., and then fractionated into cytoplasm, nucleoplasm, chromatin, and nuclear matrix fractions. After 1 min, nsP2 and its precursors, P12 and P123, were in the cytoplasmic fraction. After the 3-min chase, nsP2 could already be clearly detected in the nucleus, mainly in the matrix fraction (Fig. 7, lanes 4). The precursor proteins remained in the cytoplasm (Fig. 7, 4- and 8-min panels, lanes 1). After the 7-min chase a clearly detectable amount of the nsP2 was found in the nuclear matrix fraction. Thus, transport to the nucleus and association to the nuclear matrix of nsP2 take place rapidly. Longer pulses with [^{35}S]methionine (5 to 15 min) followed by different chase periods allowed us to estimate that half of the nsP2 was transported to the nucleus within 15 to 20 min. The maximum proportion of nsP2 in the nucleus varied between 40 and 60% in different experiments.

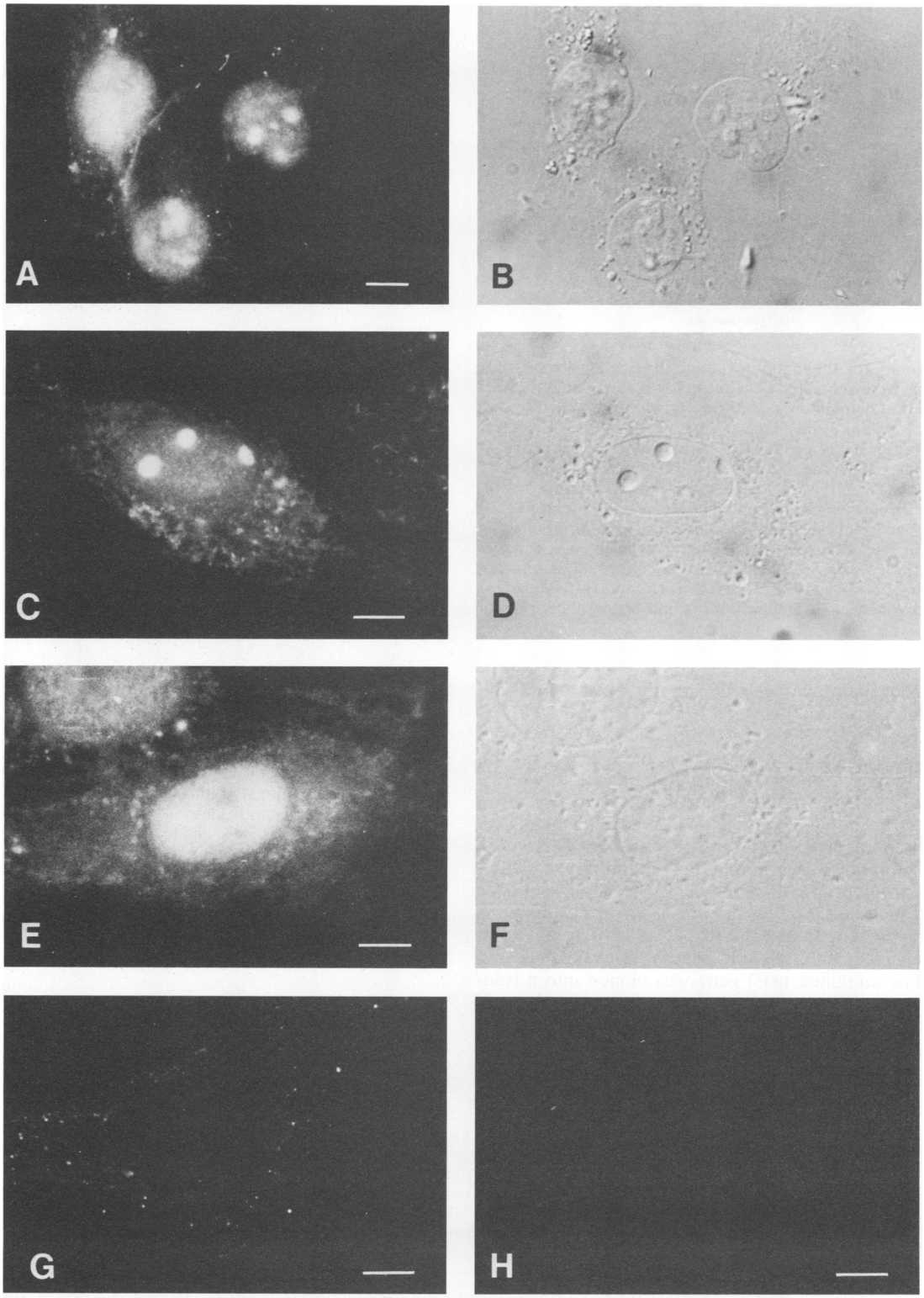

FIG. 2. Localization of nsP2 in SFV wild-type-infected (A and B), *ts*1 mutant-infected (C, D, E, F, and H), and mock-infected (G) BHK cells. The infected cells in panels E and F were treated with dactinomycin (5 μg/ml) from 0 to 6 h p.i. The cells were stained for indirect immunofluorescence at 6 h p.i. by using affinity-purified rabbit anti-nsP2 and preimmune serum (H) (46). Panels A, C, E, G, and H are immunofluorescence micrographs; panels A, E, G, and H were exposed identically. Panels B, D, and F are corresponding Nomarski images for panels A, C, and E, respectively. Bars, 10 μm.

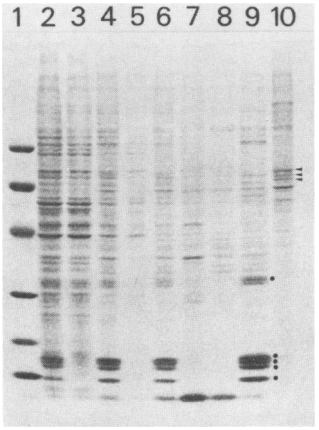

FIG. 3. Total proteins found in subnuclear fractions from BHK cells. Proteins were separated on a linear 7.5 to 15% gradient gel by the method of Laemmli (35) and stained with Coomassie blue. Lanes: 2, cell lysate; 3, postnuclear supernatant; 4, crude nuclear fraction; 5, Triton X-114 postnuclear supernatant; 6, Triton X-114-treated nuclear pellet; 7 and 8, nucleoplasmic fractions released by treatment with 1% NP-40; 9, supernatant released from NP-40-extracted nuclei after nuclease and high-salt treatment (chromatin); 10, nuclease- and high salt-resistant matrix fraction after pelleting and boiling in 1% SDS. Lane 1 contains molecular mass markers (from the top, 94, 67, 43, 30, 20.1, and 14.4 kilodaltons) (Pharmacia). The positions of histones (H1, H3, H2B, H2A, and H4) and lamins (A, B, C) have been indicated with dots and arrowheads, respectively.

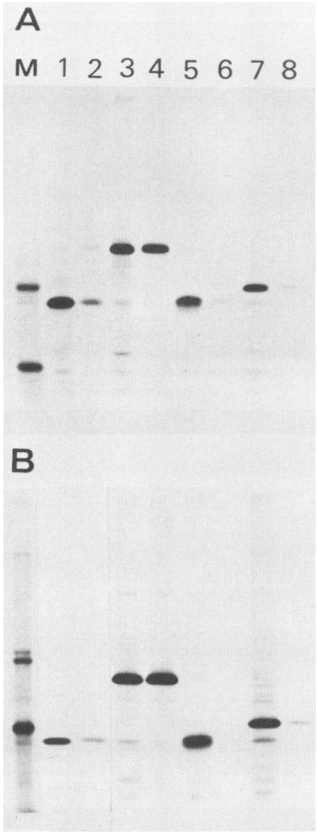

FIG. 4. Distribution of SFV nonstructural proteins between cytoplasm and nucleus. BHK cells infected with wild-type SFV (A) or the *ts*1 mutant (B) were labeled for 1 h at 1.5 h p.i. with [^{35}S] methionine followed by a 1-h chase. At 3.5 h p.i. the cells were harvested and fractionated into cytoplasm (including Triton X-114 postnuclear supernatant) (lanes 1, 3, 5, and 7), and Triton X-114-treated nuclei (lanes 2, 4, 6, and 8). The fractions were subjected to immunoprecipitation with immune sera against nsP1 (lanes 1 and 2), nsP2 (lanes 3 and 4), nsP3 (lanes 5 and 6), and nsP4 (lanes 7 and 8). Lane M contains ^{14}C-labeled molecular mass markers (from the top, 200, 92.5, 46, and 30 kDa [Amersham Corp., Arlington Heights, Ill.]). SDS-PAGE in a 7.5% acrylamide gel was performed by the method of Laemmli (35).

Inhibition of protein synthesis during the chase period did not affect the distribution of nsP2 (Table 1).

nsP2 in transfected cells. nsP2 is proteolytically processed from a large polyprotein. Thus, the nsP2 gene has no translation initiation or termination codons of its own. To construct a functional nsP2 gene we synthesized two primers, which corresponded to the 5′ and 3′ ends of the cDNA sequence encoding nsP2. The primers were supplied with a translation initiation and a termination codon, respectively. Using these primers, we amplified the nsP2 coding sequence by the PCR method (54). Plasmid pPLH214, which contains a cDNA fragment spanning the whole nonstructural region of 42S RNA (see Materials and Methods), was used as a template. The amplified nsP2 gene was cloned into a transcription vector. When transcripts made from this plasmid, pTSF2, were translated in vitro, a protein of the expected size was synthesized, confirming that the reading frame was open (Fig. 6). To express the nsP2 gene in animal cells, we inserted it into a eucaryotic expression vector, creating the plasmid pSV-NS2 (Fig. 1). BHK cells were transfected with pSV-NS2 by the lipofection method (14). After 16 h the transfected cells were processed for immunofluorescence microscopy by using the affinity purified nsP2 antibody. Positive immunofluorescence was detected in transfected cells, in which the nucleoli were often brightly stained (Fig. 8). In contrast to the SFV-infected BHK cells, the cytoplasm was stained very weakly.

DISCUSSION

In the present study we could demonstrate, by indirect immunofluorescence, that a substantial amount of nsP2 was transported to the nucleus in SFV-infected BHK cells. Morphological observations were supported by results from cell fractionation, which revealed that [^{35}S]methionine-labeled nsP2 was found in the nucleus. At the same time, only small amounts of nsP1, nsP3, and nsP4, derived from the same polyprotein precursor as nsP2, were transported into the nucleus. The specificity of the transport of nsP2 was supported by experiments in which exogenously synthesized nsP2, added to the cells before disruption and fractionation, was not found in the nuclear fraction (Fig. 6). Since the actual amounts of intracellular and in vitro-synthesized nsP2 are not known, the value for this control alone can be regarded as strongly suggestive but not absolute.

Labeled nsP2 was already associated with the nuclear matrix 4 min after synthesis and reached a plateau after about 30 to 40 min of chase. Similar transport rates have been reported for simian virus 40 large T antigen (53, 62) and nucleoplasmin (52). The nuclear matrix has an essential role in organizing DNA into dormant and active transcription units (6), as well as in DNA replication (17, 42, 43, 45, 73). The nuclear matrix also seems to be an assembly and replication site of many DNA viruses (3, 28, 47, 48, 63, 64, 68) and retrovirus oncoproteins (5, 11, 66). Why certain

TABLE 1. Distribution of SFV-specific nonstructural proteins between cytoplasm and nucleus

Virus	% of nonstructural proteins[a]							
	nsP1		nsP2		nsP3		nsP4	
	C	N	C	N	C	N	C	N
Expt 1[b]								
Wild type	87	13	56	44	96	4	95	5
*ts*1	87	13	49	51	98	2	94	6
Expt 2[b]								
Wild type			42	58				
*ts*1			40	60				
Expt 3[c]								
Wild type			46	54				
Wild type plus cycloheximide			45	55				

[a] C, Cytoplasmic fraction; N, nuclear fraction.
[b] Infected BHK cells were labeled for 1 h with [^{35}S]methionine at 1.5 h p.i., followed by a chase of 1 h.
[c] Infected cells were labeled for 15 min with [^{35}S]methionine at 225 min p.i., followed by a 90-min chase in the absence or presence of 100 μg of cycloheximide per ml. After the chase, the cells were scraped from the dish and cytoplasmic and nuclear fractions were isolated. The cytoplasmic fraction includes the Triton X-114 wash from the nuclear pellet. The fractions were immunoprecipitated with the respective antisera before SDS-PAGE analysis (7.5% acrylamide). The percentages are based on densitometric tracings of the exposed films.

proteins have affinity specifically for nuclear matrix, or how they interact with it, is so far unknown.

The immunofluorescence staining localized a substantial amount of nsP2 in the nucleoli of infected or transfected cells. It is known that in the presence of dactinomycin, the nucleoli disappear as morphological entities and their protein components, such as S1 protein and RNA polymerase I, may have different distributions in the nucleus (2, 44). In dactinomycin-treated SFV-infected cells no nucleoli could be distinguished. Immunofluorescence staining and cell fractionation revealed that nsP2 was also transported into the nucleus in the drug-treated cells. Similar results have been reported recently for dengue 4 virus core protein in mammalian and mosquito cells (71) as well as in lepidopteran cells (39).

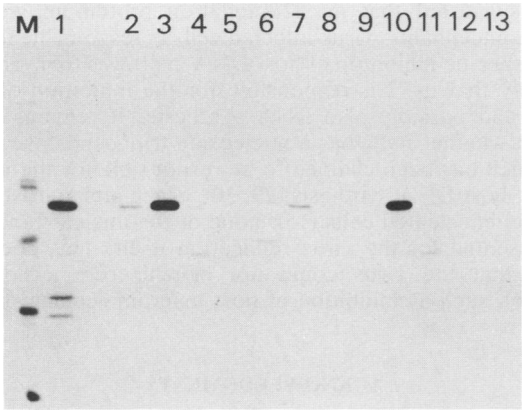

FIG. 5. Distribution of SFV nonstructural proteins in subnuclear fractions. BHK cells infected with wild-type SFV were labeled as in Fig. 4, and the harvested cells were fractionated into subnuclear fractions as performed for Fig. 3. Lanes: 1, postnuclear supernatant; 2, Triton X-114 wash of nuclei; 3 to 6, Triton X-114-treated nuclei; 7, nucleoplasmin first extraction with 1% NP-40; 8, second NP-40 extraction; 9, chromatin fraction; 10 to 13, nuclear matrix. Immunoprecipitations were carried out with antisera against nsP1 (lanes 4 and 11), nsP2 (lanes 1, 2, 3, 7, 8, 9, and 10), nsP3 (lanes 5 and 12), and nsP4 (lanes 6 and 13). SDS-PAGE was performed as in Fig. 4.

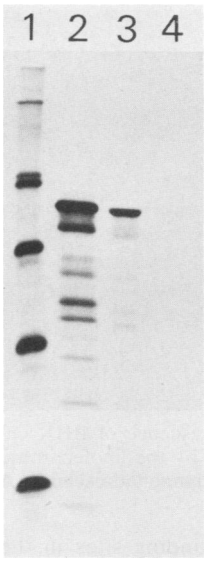

FIG. 6. Cell fractionation after addition of [^{35}S]methionine-labeled in vitro translational product of nsP2 gene to BHK cells before breakage as described for Fig. 3. Lanes: 1, molecular mass markers (from the top, 200, 94, 67, 43, 30, 20, and 14.4 kilodaltons); 2, in vitro translational product of pTSF2-transcribed mRNA coding for nsP2; 3, postnuclear supernatant; 4, nuclear pellet.

We have previously shown that nsP2 can be found associated with ribosomes in SFV-infected cells. nsP2 could be cross-linked to rRNA by UV irradiation, indicating that it is very close to the rRNA (50). Thus, it would be tempting to assume that nsP2 recognizes rRNA or ribosomal protein in the nucleolus during the biogenesis of ribosomes such as nucleolin, B23 (4), and ribocharin (22). Another possibility is that it binds to some nucleolus-specific proteins, which can be regarded as structural components of the nucleolus (RNA polymerase I, fibrillarin, etc.) (45, 67, 79). Since nsP2 is also transported to the nucleus in the presence of dactinomycin,

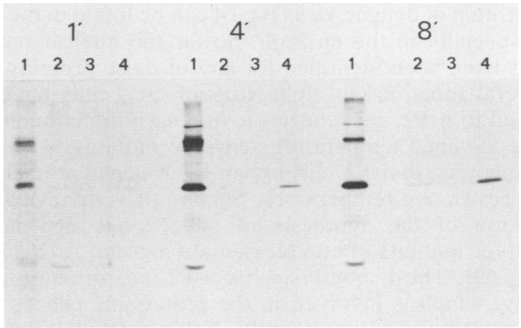

FIG. 7. Kinetics of transport of nsP2 into nuclei. BHK cells were infected with wild-type SFV and labeled for 1 min at 135 min p.i. followed by chases of 3 and 7 min. The harvested cells were fractionated as follows: lanes 1, cytoplasm (excluding postnuclear Triton X-114 wash); lanes 2, nucleoplasm (first extract with 1% NP-40); lanes 3, chromatin fraction; lanes 4, nuclear matrix. SDS-PAGE was performed as in Fig. 4.

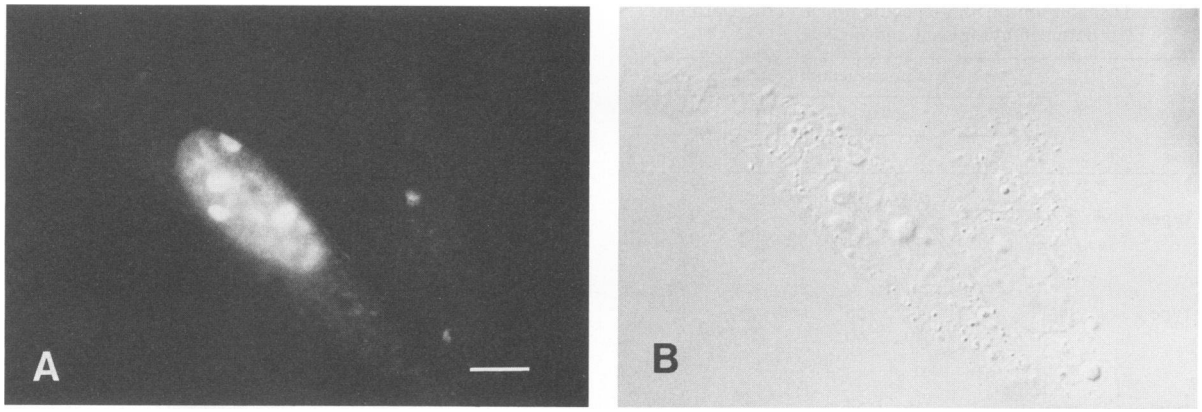

FIG. 8. Immunofluorescence of BHK cells transfected with the plasmid pSV-NS2 containing cDNA encoding full-length nsP2. Cells exposed to the plasmid by the lipofectin method (14) were prepared for indirect immunofluorescence 24 h later by using affinity-purified anti-nsP2 antiserum. Immunofluorescence (A) and Nomarski image (B) from the same field. Bar, 10 μm.

there must also be binding sites in the absence of visible nucleoli (Fig. 2E and F).

The fact that nsP2, expressed alone in transfected cells, also migrated into the nuclei, implies that the protein itself must have information for its nuclear targeting. Only in few cases has a specific signal or signals for the nuclear location (NLS) been identified by using deletion mapping and/or site-directed mutagenesis (10, 17). The common feature for the identified NLS seems to be a short stretch of basic amino acids, usually arginines and lysins. None of the identified viral or cellular nuclear targeting sequences can be found in the SFV nsP2.

There are some previous reports on transport into nuclei of proteins encoded by RNA viruses replicating exclusively in the cytoplasm. Lyles et al. (38) have recently shown evidence that some of the M protein of vesicular stomatitis virus was associated with the nucleus in the nucleoplasmic fraction. Similar observations have been made with Newcastle disease virus (13). Tobacco mosaic virus-specific 126-kilodalton protein, which is involved in viral RNA replication, has been reported to be bound to the chromatin of infected tobacco cells (75–78). The coat protein of alfalfa mosaic virus has also been found in the nucleus late in infection (74). Rift Valley fever virus-encoded nonstructural protein NS1 has been shown to accumulate in the nuclei of infected monkey kidney cells (70). Most recent reports by Tadano et al. (71) and Makino et al. (39) describe that the core protein of dengue virus type 4 can be found in the nuclei and especially in the nucleoli. So far, no nuclear targeting signals have been identified for any of these proteins.

Several functions in alphavirus-infected cells have been assigned to nsP2. First, it has a vital early function in RNA synthesis, since temperature-sensitive mutants, with mutations mapping in nsP2, display an RNA-negative phenotype at the restrictive temperature. Second, it is involved in the regulation of the synthesis of subgenomic 26S mRNA, typical for mutants of complementation group A (19, 20, 24, 27, 61, 69). Third, Sindbis virus nsP2 has an autoprotease activity, which is involved in the proteolytic processing of the nonstructural polyprotein (9). If this function is impaired, the processing of the polyprotein P1234 would be deficient, and this may explain the RNA-negative phenotype of nsP2 mutants. nsP2 can also bind to ribosomes, at least in SFV-infected cells (50). How this phenomenon is related to the observed functions of nsP2 remains open. In this study we have shown that nsP2 is efficiently transported into nucleoli relatively early in virus infection. This must be an additional function, most probably unrelated to the viral RNA synthesis and protease activities. Crude cell fractionation has shown that about half of the cytoplasmic nsP2 is located in the mitochondrial pellet (P15) (46), which contains all the virus-specific RNA polymerase activity (18, 49). Practically all of nsP4 and nsP1 and about 80% of nsP3 are located in P15, presumably as an RNA polymerase complex. They are evidently associated with the cytoplasmic vacuoles, which have been shown to be the site of virus-specific RNA synthesis (16, 46).

It can be estimated that about 25% of synthesized nsP2 is associated with the membranous structures in P15. Another 25% would be free in the cytoplasm. This would be compatible with the immunofluorescence results of cytoplasmic staining, which reveals vesicular structures (cytoplasmic vacuoles) on a weakly stained cytoplasmic background (Fig. 2). The rest of nsP2, about 50%, is transported to the nuclei, where it associates with the nuclear/nucleolar matrix. What is the function, if any, of nsP2 in the nucleus or nucleolus? So far, we cannot answer this question. Ishida et al. (23) have suggested that an 82-kilodalton protein in western equine encephalitis virus-infected cells is responsible for the virus-specific inhibition of host DNA synthesis (65). Thus, it is likely that nsP2 is responsible for the inhibition of host DNA and possibly also RNA synthesis. It remains to be shown whether nsP2 has a nucleoside triphosphatase activity which has been claimed to be responsible for the inhibition of host DNA synthesis (29, 30). Since alphaviruses can grow in enucleated cells (15), none of the nuclear functions are essential for the virus replication itself. This does not mean that the virus could not benefit from a nonvital function such as inhibition of host macromolecular syntheses.

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