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Nuclear localization of Sindbis virus nonstructural protein nsP2

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ABSTRACT

In early infection, approximately 10% of nonstructural protein nsP2 of Sindbis virus was transported into the nuclei of virus-infected BHK-21 cells. Nuclear nsP2 was dominantly associated with nuclear matrix. During the course of infection, increasing amounts of nsP2 accumulated in the nuclear fraction. A prominent accumulation of nuclear nsP2 occurred early in infection, from 1 h to 3 h postinfection. Meanwhile, a weak NTPase activity was found to be associated with the immunocomplexed nsP2. Nuclear localization of nsP2 and its possible role were discussed in relation to the inhibition of host macromolecular synthesis.

Key words: *Sindbis virus, nonstructural protein nsP2, nuclear matrix.*

INTRODUCTION

Sindbis virus (abbreviated in SbV), the prototype virus of the Alphavirus genus in the family Togaviridae, is an enveloped, plus-stranded RNA virus [reviewed in 1]. The genomic 49S RNA, which is capped and polyadenylated, acts as a messenger for the production of 4 nonstructural proteins, nsP1 to nsP4. These 4 nsPs, in addition to nsP34 or some host cellular proteins are thought to form the transcriptase-replicase complex[2] that initiates viral replication by synthesizing a full-length minus strand complementary to the genomic plus-strand RNA.

The functions of these nonstructural proteins in RNA replication have been studied intensively[1], nsP1 functions in negative-strand RNA synthesis[3], and also shows a methyl-transferase activity needed for the capping of viral RNAs[4]. nsP4, which contains the conserved XGDD sequence common to many RNA-dependent RNA polymerases[5], is an essential component of the RNA polymerase and func-

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tions in elongation and possibly initiation[6-7], nsP3 and the polyprotein nsP34, for which no function has been identified, are found to be post-translationally phosphorylated[8], and act as the components of replication complexes[2]. Interestingly, nsP2 appears to be multifunctionah (i) It is required for the initiation of 26S subgenomic RNA synthesis[9]; (ii) It is involved in the shutoff of minus-strand synthesis[9]; (iii) It contains a thiol protease activity responsible for the proteolytic processing of nonstructural proteins in its C-terminal domain[10]; (iv) It has sequence motifs shared with NTP-binding proteins and helicases in the N-terminal domain[11]. Recently, nuclear localization of nsP2 in Semliki Forest virus-infected host cells[12], suggested an additional function of alphavirus nsP2, most probably related to its effects on host metabolism.

In this paper, we have demonstrated, by immunofluorescence, Western-blotting and immunoprecipitation, that early in infection, SbV nsP2 was transported into the nuclei and conjugated with the nuclear matrix.

MATERIALS AND METHODS

Cell, virus and infection.

All experiments were performed using monolayer cultures of BHK-21 cells. The SbV was originally obtained from Prof. M. J. Schlesinger, Washington University, U.S.A. Infection was performed at an input multiplicity of 50 PFU per cell for 1 h at 37℃. After virus absorption, the cells were incubated in Eagle's MEM supplemented with 2.5% calf serum at 37℃.

Subcellular fraction of cells

The nuclei were purified following the procedure described by J. PerSnen, et al[12]. Cell breakage was monitored by phase-contrast microscopy.

The nuclear matrix was obtained by sequential extraction described by Fey, et al[13] with some modification[16]. Briefly, the purified nuclei were suspended in CSK buffer (10 m*M* PIPES, pH 6.8, 100 m*M* KCl, 300 m*M* Sucrose, 3 m*M* $MgCl_2$, 1 m*M* EGTA, 0.1 m*M* PMSF) containing 0.5% (V/V) Triton X-100 for 10 min on ice. After centrifugation, the final nuclear pellet was resuspended in digestion buffer (10 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 10 m*M* $MgCl_2$) supplemented with DNase I (200 μg/ml) and incubated for 30 min at room temperature. The nuclease digestion was halted by adding ammonium sulfate to a final concentration of 0.25 M. After a 5-min incubation on ice, the nuclear matrix was pelleted by centrifugation.

Immunofluorescence microscopy

At 3 h p.i., SbV-infected BHK cells were fixed with 3% paraformaldehyde for 30 min on ice. After rinsing with PBS (pH 7.4), cells were permeabilized with 0.5% (V/V) Triton X-100 in PBS for 5 min on ice. Samples were then washed in several changes of PBS and blocked with 1% BSA (bovine serum albumin, fraction V) for 30 rain at 37℃. Cells were subsequently incubated with the primary antibody (rabbit monospecific antisera against nsP2, Hardy and Strauss[14], were kind gifts from Dr. C. M. Rice) at an appropriate dilution for 1 h at 37℃. After washing in PBS, samples were incubated with FITC-conjugated (sheep anti-rabbit) secondary antibody and then rinsed in PBS. Finally, the samples were mounted and scrutinized under an Opton fluorescence light microscope.

The Osborn's immunofluorescence procedure[15] was performed for the presence of nsP2 in the intermediate filament-lamina-nuclear matrix system as described previously[13, 16].

SDS-PACE and Western blotting

SDS-PAGE, unless otherwise stated, was performed according to Laemmli's method[17] in a 7.5% acrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membrane in transfer buffer (0.01% (W/V) SDS, 20% (V/V) methanol, 20 m*M* Tris-HC1, pH 8.3) according to Towbin's procedure [18]. Nitrocellulose membrane were rinsed in TBS (20 mM Tris-HCl, pH 7.5, 500 m*M* NaCl) and then blocked overnight with TBS containing 3% (W/V) BSA at 4℃. The membrane was probed at 37℃with the monospecific antisera against nsP2 as the primary antibody and a biotinylated-sheep-anti-rabbit IgG as the secondary antibody. The immunoblot was developed by using an avidin-alkaline phosphatase conjugate and BCIP and NBT as the substrate according to the manufacturer's instruction.

Radioimmunoprecipitation

Monolayer of BHK-21 cells was infected with SbV (50 PFU per cell). At 2.5 h p.i., 4 M NaCl was added to a final concentration of 0.25 *M*. After 40 min, the cell were washed and incubated with methionine-free MEM for 10 min followed by a 5-min label with 100 μCi of ^{35}S-methionine per ml (1000 μCi/m *M*, Amersham). Label was removed and unlabeled methionine (1 m*M*) was added. After 0, 10 and 30 min, the media were removed and cells were lysed with 1% SDS containing 2 mM PMSF.

For localization studies, a 30-min-label with 30 μCi of ^{35}S-methionine per ml followed by a 30-min-chase was used. For the nuclear sample, the purified nuclei resuspended in nuclear-lysis buffer (25 m*M* Tris-HCl, pH 8.0, 2.5 m*M* magnesium acetate, 2 m*M* $CaCl_2$, 0.05 m*M* EDTA, 0.01 m*M* DTT, 0.2 m*M* PMSF, and 12.5% glycerol), were incubated at 4℃ for 30 min in the presence of 100 μg/ml DNase I and RNase A and solubilized by the addition of an equal volume of 2% SDS containing 1 m*M* PMSF. Samples were heated at 95°C for 3 min, cooled, and centrifuged at 8,000 g for 5 min. The supernatant were subjected to immunoprecipitation. The immunoprecipitation was performed as described by Anderson and Blobel[19] with modifications according to Li, G. et al[8].

ATPase assay

BHK cells were infected with SbV of 50 PFU per cell and then labeled with ^{35}S-methionine (15 μCi/ml). At 3 h p.i., the cells were lysed in lysis buffer (0.1% SDS, 1% NP-40 (V/V), 100 mM NaCl, Tris-HCl, pH 8.0) for 15 min at 4°C. Then the lysates were cleared by centrifugation. And the supernatant was immunoprecipitated using antisera against nsP2 or nsP3 as described above. The ATPase activity of immunocomplex was measured as described by Schirmbeck and Deppert[20]. Immunocomplexes were washed 3 times with ATPase buffer (75 m*M* NaC1, 5 mM $MgCl_2$ and HEPES-NaOH, pH 7.4). Reaction was initiated by adding 10 μCi ATP γ-^{32}p (3,000 Ci/mmol) -25 μM unlabeled ATP to 100 μl of ATPase buffer. After 30 min incubation at 28°C, the immunocomplexes were pelleted and the supernatant was incubated with activated charcoal solution (7.5% in 50 mM HC1-5 mM H_3PO_4). Charcoal was pelleted and free ^{32}pi in the supernatant was measured by liquid scintillation counting. Background values were measured by analyzing immunoprecipitates from uninfected cells. After assaying, the immunocomplexes were washed and lysed from Protein A-Sepharose by the addition of SDS-PAGE loading buffer. Then the relative amount of nsP2 or nsP3 was estimated by the assay of ^{35}S in each lysate.

RESULTS

I. Processing and subcellular distribution of SbV nsPs

Pulse-chase experiment was carried out to visualize the kinetics of nsPs processing (Fig 1). It could be seen that early in infection, the nonstructural proteins were synthesized initially as polyproteins that were subsequently cleaved to produce

nsPs. After a 30-min chase, bands corresponding to each of the nonstructural proteins, nsP1 (60 Ka), nsP2 (89 Ka), and nsP3 (76 Ka) could readily be seen. But no nsP4 was detected in our study. In a deep-going experiment, infected BHK cells were pulse-labeled with ^{35}S-methionine for 30 min and were chased for 30 min in the presence of excess unlabeled methionine. Subsequently, the cells were fractionated into cytoplasmic and nuclear fractions, which were subjected to immunoprecipita-

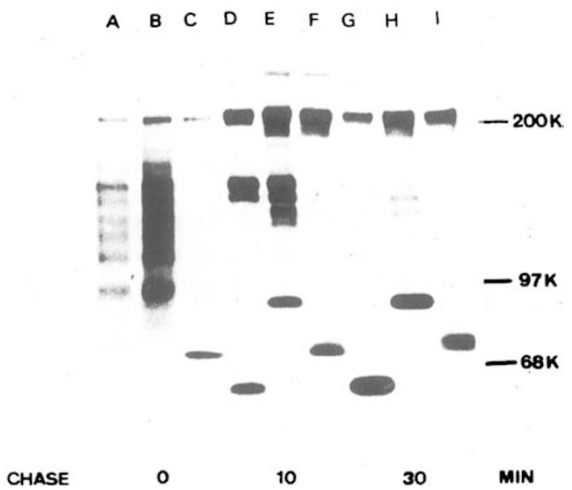

Fig 1. △ Synthesis and processing of SbV nonstructural proteins. At 2.5 h p.i., SbV-infected cells were subjected to 40-min hypertonic incubation and 10-rain methionine-free incubation. Cells were then pulse-labeled for 5 min in the medium containing 100 μCi of ^{35}S-methionine per ml, and chased for 0, 10, 30 min in the medium containing 1 m*M* unlabeled methionine. The cells were lysed, the lysates were immunoprecipitated, and the immunoprecipitates were analyzed by SDS-PAGE using a 10% acrylamide gel.
Lanes:A , B, C, 0 min chase; D, E, F, 10 min chase; G, H, I, 30 min chase.
Samples in lanes A, D, G were precipitated with anti-nsP1.
Samples in lanes B, E, H were precipitated with anti-nsP2.
Samples in lanes C, F, I were precipitated with anti-nsP3.

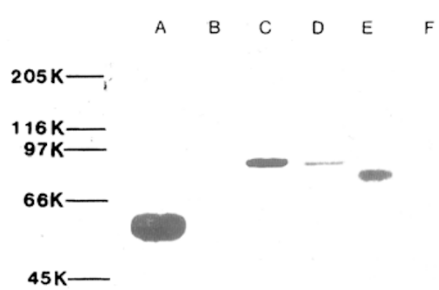

Fig 2. ◁ Distribution of SbV nonstructural proteins between cytoplasm and nucleus. Infected BHK cells were labeled at 2.5 h p.i. for 30 min with ^{35}S-methionine followed by a 30-min chase. Then the cells were harvested and fractionated into cytoplasmic (lanes: A, C, E) and nuclear (lanes: B, D, F) fractions. The fraction lysates were immunoprecipitated with antisera specific for nsP1 (lanes: A and B), nsP2 (lanes: C and D), and usP3 (lanes: E and F). The relative amounts of nsPs were evaluated by densitometric tracings of the exposed films.

tion. Fig 2 showed the distribution of nsPs in the cytoplasmic and nuclear fractions. No nsP1 and nsP3 were detected in the nuclear fraction, while at the same time about 10% of newly-synthesized nsP2 was transported into the nuclei. There was also no nsP4 detected in the nuclear fraction and only nsP34 could be seen in the cytoplasmic fraction (data not shown).

II. Immunofluorescence localization of nsP2

Indirect immunofluorescence was performed using a monospecific antisera against nsP2, followed by FITC -conjugated second antibodies. Most of virus-infected cells were intensely stained with fluorescence (Fig 3A), while mock-infected control cells showed negligible background fluorescence (Fig 3D). Diffusive and intensive fluorescence was localized in the cytoplasm of SbV-infected BHK cells (Fig 3B). Unlike predominent fluorescence localized in the nucleus of SFV-infected cell[12], only relatively weak nuclear fluorescence could be seen in our studies (Fig 3 B), suggesting

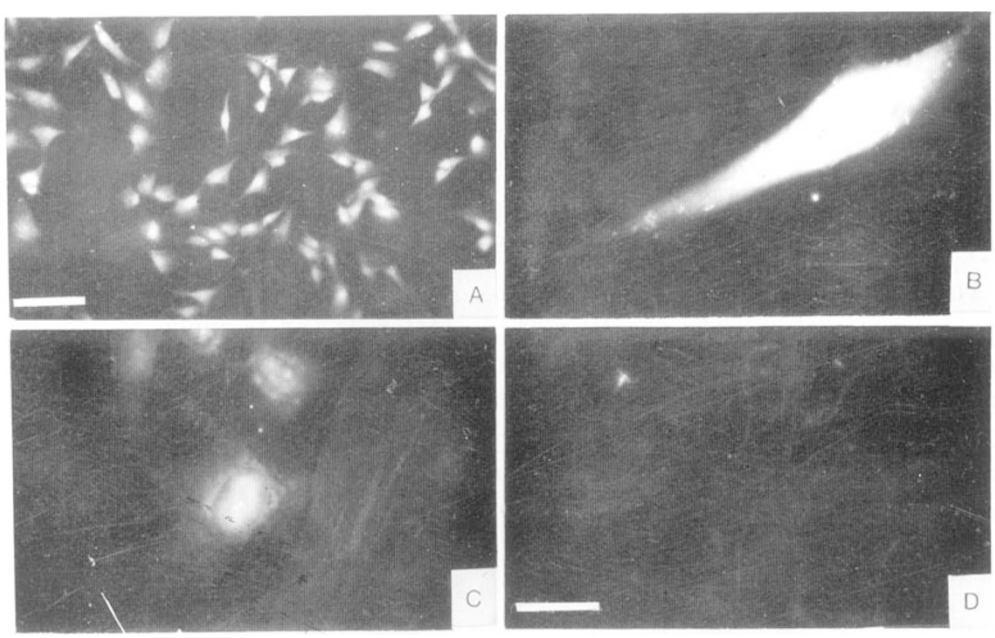

Fig 3. △ Immunofluorescence localization of nsP2 in SbV-infected BHK-21 cells.
A,B. Immunofluorescent staining of SbV-infected BHK-21 cells (3 h p.i.) with anti-nsP2 antibody.
C. Immunofluorescent staining of extracted SbV-infected BHK-21, cells (3 h p.i.) with anti-nsP2.
D. Mock-infected control.
Bars in A: 100 μm; B, C, and D: 15 μm. × 800

that only a small amount of nsP2 was transported into the nuclei. Analogous results were shown in Fig 2. When the cells were subjected to in situ sequential extraction for getting the intermediate filament-lamina-nuclear matrix system [13, 16], and stained with anti-nsP2, there was still apparent fluorescent staining in the nuclear compartment (Fig 3C), albeit very weak as compared to the cytoplasmic staining as shown in Fig 3B. In our opinion, the presence of speckled or lumped fluorescence in the nuclear compartment may imply that nsP2 was unevenly distributed throughout the residual protein skeleton of nucleus, the nuclear matrix.

III. Nuclear accumulation of nsP2 during the course of infection and subnuclear location of nsP2

To characterize the amount of nuclear nsP2 during the course of viral infection, the infected cells were collected at 1, 3 and 5 h postinfection. At each time, infected cells were fractionated into cytoplasmic and nuclear fractions. Nuclear sample, extracted from equal amount of cells at each time, was analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. Fig 4 showed that during the course of infection, increasing amounts of nsP2 accumulated in the nuclei. At 1 h postinfection, there was detectable amount of nsP2 in the nuclei. However, during the following 2 h, there was a significant increase in the accumulation of nsP2 in the nuclei. At 3 h postinfection the amount of nuclear nsP2 was about 5 times of that at 1 h postinfection. In a relatively late phase of infection, from 3 h to 5 h postinfection, only a slight increase in the amount of nuclear nsP2 could be seen. This result showed that nuclear accumulation of nsP2 occurred predominantly in the early phase of infection, from 1 h to 3 h postinfection.

The subnuclear location of nsP2 in Sb V-infected cells was investigated by fractionating the nuclear matrix as described in Materials and Methods. Each fraction was analyzed for the presence of nsP2 by immunoprecipitation and Western-blotting. Both results (Fig 5A and 5B) showed that nsP2 was dominantly associated with nuclear matrix, while almost no nsP2 could be identified in the nucleoplasmic fraction (including Triton X-100 release and DNase I digestion), nsP2 was also present in the isolated nucleolar fraction (data not shown).

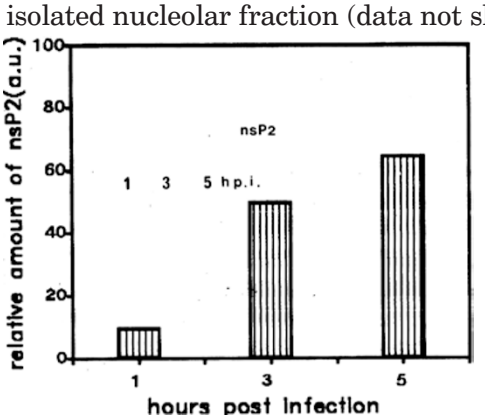

Fig 4. ◁ Nuclear accumulation of nsP2 during the course of infection. At various time periods postinfection, the nuclear fractions were isolated from SbV-infected BHK cells. And the nuclear samples from equal amount of cells were analyzed by 7.5% SDS-PAGE and Western blotting. Amounts of nsP2 were quantitatively evaluated by reflection densitometric tracing and given in arbitrary unit (a.u.).

IV. ATPase activity detected in nsP2 immunocomplex

To examine the proposed NTPase activity, we used the Schirmbeck and Deppert's procedure[20], nsP2 immunocomplex, extracted from infected cells at 3 h postinfection, was subjected to ATPase assay, nsP3 immunocomplex served as a control. As shown in Fig 6, immunocomplexed nsP2 showed a weak ATPase activity in comparison with that in mock-infected (as background value) or nsP3 immunocomplex. And this activity was slightly stimulated in the presence of polyA.

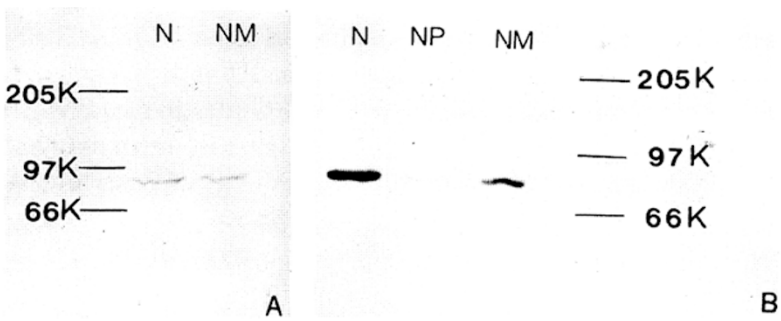

Fig 5. △ Subnuclear localization of SbV nsP2.
A. Western blotting of subnuclear fraction for nsP2.
At 3 h p.i., SbV-infected BHK cells were fractionated into nuclear and cytoplasmic fractions. The purified nuclei were further fractionated into nuclear matrix and nucleoplasmic fractions. Then the subnuclear fractions were analyzed by SDS-PAGE and Western blotting.
B. Immunoprecipitation of nsP2 in subnuclear fractions.
The subnuclear fraction lysates from radiolabeled SbV-infected cells were subjected to immunoprecipitation with antisera against nsP2.
Lanes: N. nuclei; NM, nuclear matrix; NP, nucleoplasmic fraction.

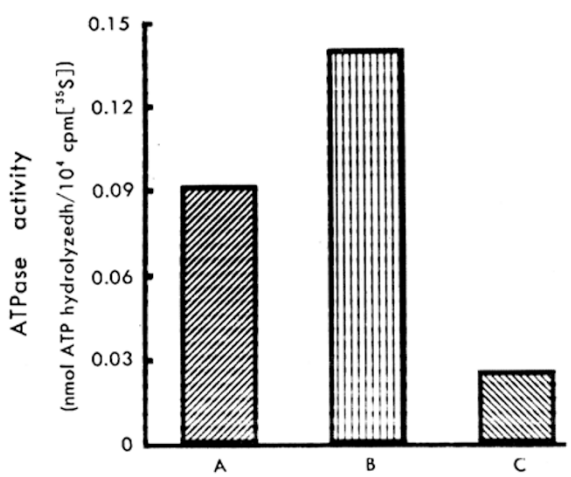

Fig 6. ◁ ATPase assay of immunocomplexed nsP2.
BHK cells infected with SbV were labeled with ^{35}S-methionine after infection. At 3 h p.i., the cells were lysed and immunoprecipitated for nsP2. The immunocomplexes were assayed for ATPase activity without (A) or with (B) 20 μM polyA. Immunoprecipitates from lysates of uninfected BHK cells served as the background values, and immunocomplexed nsP3 as the control (C). The counting values of ^{35}S in each immunoprecipitates represented the relative amount of nsP2 or nsP3

DISCUSSION

The results of our studies indicated that, early in infection, the nonstructural proteins of Sindbis virus were synthesized and processed. Approximately 10% of tlie newly-synthesized nonstructural protein nsP2 was transported into the nucleus of virus-infected BHK cell, while at the same time, no other nonstructural proteins, derived from the same polyprotein precursors, could be detected in the nuclear fraction. This implied that nuclear import of nsP2 must be specific. Furthermore, our results showed that the amount of nuclear nsP2 significantly increased during the early phase of infection; at relatively late phase of infection, only a slight increase was detectable. Thus, the nuclear accumulation of nsP2 most likely was not resulted from the cytopathic effects subsequent to virus infection. After entering the nucleus, most nsP2 displayed the ability to bind tightly to the nuclear matrix and exhibited an uneven pattern of distribution.

Nuclear import of virus-specific proteins encoded by RNA virus replicating exclusively in the cytoplasm has been previously reported. In the case of vesicular stomatis virus and Newcastle disease virus, some of the M protems were found to be associated with the nucleus in the nucleoplasmic fraction[21,22]. It has also been reported that a virus-specific 126-KDa protein of tobacco mosaic virus was bound to the chromatin of infected tobacco cells[23]. In addition, the core proteins of both Semliki Forest virus and dengue 4 virus were specifically localized in the nucleoli of infected cells[24,25]. Recent studies in Semliki Forest virus also demonstrated the nuclear localization of nsP2 in virus-infected host cells[12]. Considering the high sequence homology in alphavirus nsP2[1], nuclear localization of alphavirus nsP2 in vertebrate host cells seemed to be one of the characteristics of alphavirus.

Alphavirus replication in vertebrates is not dependent upon constant expression of the host genome. This is supported by the observation that neither enucleation nor 90 min of incubation in actinomycin D before infection affects virus production in vertebrate cells[26,27]. Our results, along with the recent findings in SFV[12], showed that alphavirus nsP2 is efficiently transported into the nucleus early in virus infection before the apparent cytopathic effects appear. Since none of the nuclear functions seemed to be essential for the virus replication itself, the nuclear accumulation of nsP2 implied an additional function of-nsP2, unrelated to viral RNA synthesis and autoprotease activity.

In this study, we also demonstrated that nsP2 was associated with nuclear matrix. It has been shown that the nuclear matrix is an important site of virus-cell interaction[28]. Viral DNA and virus-specific proteins have been found enriched in nuclear matrix preparation[20,29-31]. Meanwhile, it has become increasingly clear that nuclear matrix is implicated in chromatin looping, DNA replication, gene expression, and RNA processing and transport[32]. At the early phase of infection, nsP2 is transported into the nucleus and bound to nuclear matrix soon after its synthesis and processing. Therefore, it is quite possible that nsP2 exerts certain

regulatory function(s) by interacting with the component of nuclear matrix-binding replication or transcription complex.

Infection of vertebrate hosts with alphavirus is characterized by subsequent inhibition of host macromolecular synthesis[33]. Sindbis virus inhibits host protein synthesis by 55% and DNA synthesis by 88% in infected compared to those of mock-infected cells[34]. Recent results in our laboratory demonstrated that SbV infection was capable of making a rapid shutoff of cellular mRNA synthesis (in preparation). Infection of BHK cells with Western equine encephalitis virus also resulted in rapid inhibition of cellular DNA synthesis[33]. And it has been suggested that an 82-KDa nonstructural protein of WEE virus, which probably contained a NTPase activity, was responsible for the shutoff of cellular DNA synthesis[35]. Gorbalenya et al suggested that alphavirus nsP2 contained a conserved domain found in NTP-binding and helicase proteins[11]. In further support of this, a weak ATPase activity has been found to be associated with nsP2 immunocomplex in our studies. The ATPase activity of immunocomplexed nsP2 is much lower than that detected in WEE virus-infected cells. We reasoned that it may presumably be due to the blocking of anti-nsP2 antibody. Further studies are necessary to confirm the possible NTPase activity of nsP2. On the other hand, Atkins has shown that viral RNA synthesis was necessary for the inhibition of host DNA synthesis[34]. This suggested that one or more virus-specific proteins responsible for viral RNA replication should be involved in the inhibition of host DNA synthesis. Therefore, it is most likly that nuclear matrix-associated nsP2 is responsible for the inhibition of host DNA synthesis.

Recent findings in our laboratory showed that infection of BHK cells with SbV resulted in rapid inhibition of cellular mRNA synthesis(in preparation). In the early phase of infection, from 1 h to 3 h postinfection, the synthesis of cellular mRNA was drastically reduced; at 3 h after infection, it was only about 25% of that in mock-infected control. A ;further shutdown could be seen at 5 h after infection. In this paper, we showed that, a prominent accumulation of nsP2 in the nuclei occurred from 1 h to 3 h after infection. Afterwards, the amount of nuclear nsP2 was still increasing but at a slower rate. One can easily comprehend that the shutdown of host RNA synthesis subsequent to virus infection occurred concomitantly with the accumulation of nsP2 in the nuclei. Assuming that the virus-coded protein was involved in the shutoff of host macromolecular synthesis, we suggested that nsP2 is probably involved in the shutoff of host RNA transcription.

In conclusion, the nuclear localization of nsP2, along with the possible NTPase activity, suggested an additional function of alphavirus nsP2, most possibly unrelated to alphavirus replication, and involved in the shutoff of host cellular macromolecular synthesis.

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