Inhibition of Transcription and Translation in Sindbis Virus-Infected Cells

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Alphaviruses are arthropod-borne viruses (arboviruses) that include a number of important human and animal pathogens. The natural transmission cycle of alphaviruses requires their presence at high concentrations in the blood of amplification hosts for efficient infection of mosquito vectors. The high-titer viremia development implies multiple rounds of infection that proceed in the background of the developing antiviral cell response aimed at blocking virus spread on an organismal level. Therefore, as for many viruses, if not most of them, alphaviruses have evolved mechanisms directed toward downregulating different components of the antiviral cell reaction and increasing viremia to a level sufficient for the next round of transmission. Using Sindbis virus (SIN) as a model, we demonstrated that (i) the replication of wild-type SIN strongly affects major cellular processes, e.g., transcription and translation of mRNAs; (ii) transcriptional and translational shutoffs are distinctly independent events, and their development can be differentially manipulated by creating different mutations in SIN nonstructural protein nsP2; and (iii) inhibition of transcription, but not translation, is a critical mechanism that SIN employs to suppress the expression of cellular viral stress-inducible genes in cells of vertebrate origin. Downregulation of transcription of all of the cellular mRNAs appears to be a very efficient means of reducing the development of an antiviral response. The ability to cause transcriptional shutoff may partially determine SIN host range and replication in particular tissues.

Alphaviruses are a group of widely distributed human and animal pathogens. Most of the nearly 30 members of the genus are transmitted by mosquitoes to vertebrates, which serve as amplifying hosts (18, 22, 49). In mosquitoes, alphaviruses cause a persistent, life-long infection that has little effect on the viability of their vectors (47). Infected vertebrates, in contrast, develop an acute disease, often characterized by high-titer viremia before either virus clearance by the immune system or host death (17, 19). Accordingly, alphaviruses usually establish a persistent or chronic infection in cultured mosquito cells and exhibit a highly cytopathic phenotype in cell cultures of mammalian and avian origin (49).

Sindbis virus (SIN) is one of the least pathogenic but most intensively studied alphaviruses. It can infect a wide variety of insect and vertebrate cell lines that are commonly used in experimental research. In the latter, SIN replication leads to the rapid development of a cytopathic effect and cell death within 24 to 48 h postinfection (9). The SIN genome is a single-stranded, almost 11.5-kb-long RNA of positive polarity (48). As with cellular mRNAs, it contains a 5' methylguanylate cap and a 3' polyadenylate tail, and after release from the nucleocapsids (54, 55), the genomic RNA is translated by cellular translational machinery to produce the viral nonstructural proteins nsP1 to nsP4. These proteins are encoded by the 5' two-thirds of the genome and, together with host factors, form the replicase/transcriptase or RNA-dependent RNA polymerase required for viral genome replication and transcription of

the subgenomic RNA from the replicative intermediate. The latter RNA is encoded by the 3' one-third of the genome and is translated into structural proteins that, along with the genomic RNA, comprise viral particles (35).

As in the case of other alphaviruses, SIN replication in vertebrate cells proceeds very rapidly and strongly affects fundamental processes in cell physiology (23). A few hours postinfection, cellular resources become redirected to the synthesis of viral structural proteins and viral genomes required for assembly of a large number of viral particles (9, 11, 15). The major changes in cellular macromolecular synthesis that we focused on in this study included the downregulation of (i) transcription and (ii) translation of cellular mRNAs. Inhibition of these critical processes has a strong effect on alpha/beta interferon (IFN- α/β) production by the infected cells (14, 41, 45) and is likely beneficial for SIN replication (12, 15) and partially determines viremia development.

The IFN- α/β activates the antiviral state in the uninfected cells and makes them resistant to infection with newly released virus particles. At the same time, the autocrine action of IFN- α/β can induce an antiviral response in already infected cells and suppress ongoing replication (12). Thus, the outcome of the infection on the organismal and cellular levels is determined not only by the availability of susceptible cells, but also by the balance between two competing processes: activation of cellular responses to viral infection and the ability of virus to suppress or overcome activation of the antiviral reaction.

Previously published data suggest that defined mutations in SIN nsP2 not only affect viral RNA replication, but also make mutants less cytopathic (7, 8) and incapable of suppressing activation of the antiviral response (12). nsP2 mutants induce significantly higher levels of IFN- α/β secretion, and this, in

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turn, attenuates virus infection both in vivo and in vitro (12). The secreted IFN not only protects the uninfected cells, but also stops virus replication in the cells already infected with the mutant viruses. These changes in replication likely result from the reduced ability of these viruses to inhibit host gene expression, leading to the efficient activation of more than 200 genes, the viral stress-inducible genes, among which at least a portion are likely involved in the development of an antiviral state (12).

In the present study, we further investigated changes in cellular macromolecular synthesis caused by SIN replication. We compared the effect of wild-type SIN and two mutants on the transcription and translation of cellular RNAs and activation of an antiviral response. Our results demonstrated that SIN-specific shutoffs of transcription and translation of cellular mRNAs are distinctly independent events; mutations in the nsP2 can independently affect downregulation of transcription and translation; and inhibition of transcription, but not translation, plays a critical role in downregulation of the antiviral response in SIN-infected cells.

MATERIALS AND METHODS

Cell cultures. BHK-21 cells were kindly provided by Paul Olivo (Washington University, St. Louis, MO). NIH 3T3 cells (mouse cells) were obtained from the American Type Culture Collection (Manassas, Va.). Both cell lines were maintained at 37°C in alpha minimal essential medium (α MEM) supplemented with 10% fetal bovine serum (FBS) and vitamins. L929 cells used for the biological assay of IFN- α/β were provided by Samuel Baron (University of Texas Medical Branch, Galveston, TX), and propagated in Dulbecco's modified Eagle's medium supplemented with 10% FBS.

Plasmid constructs. Plasmids pwtSIN and pSIN/G, encoding the full-length genome of SIN with the additional subgenomic promoter driving the expression of green fluorescent protein (GFP), were described elsewhere (12). They encoded Toto1101-derived viral nonstructural genes and structural genes derived from the TE12 strain (30). pSIN/2V had essentially the same sequence as pwtSIN except for a previously described single point mutation leading to replacement of glycine by valine in the P2 position of the cleavage site between nsP2 and nsP3 (44). The nsP2 G₈₀₆→V mutation abolished nsP2/3 cleavage during P123 processing. A schematic representation of the recombinant genomes is shown in Fig. 1A.

RNA transcriptions. Plasmids were purified by centrifugation in CsCl gradients. Before the transcription reaction, plasmids were linearized using the XhoI restriction site located downstream of the poly(A) sequence. RNAs were synthesized by SP6 RNA polymerase in the presence of cap analog using previously described conditions (34). The yield and integrity of transcripts were analyzed by gel electrophoresis under nondenaturing conditions. Aliquots of transcription reactions were stored at -80°C and used for electroporation without additional purification.

RNA transfections. BHK-21 cells were electroporated using previously described conditions (29), and the infectious center assay was performed in parallel. Briefly, 1 μ g of in vitro-synthesized, full-length RNA was transfected into BHK-21 cells, and 10-fold dilutions of the transfected cells were seeded in six-well tissue culture plates containing subconfluent monolayers of naïve cells. After 1 h of incubation at 37°C, cells were overlaid with 2 ml of 0.5% Ultra-Pure agarose (Invitrogen), supplemented with MEM and 3% FBS. Plaques were stained with crystal violet after 48 h of incubation at 37°C. The residual electroporated cells were seeded into 100-mm dishes, and viruses were harvested after cytopathic effect was observed.

Viral replication analysis. NIH 3T3 cells were seeded at a concentration of 5×10^5 cells/35-mm dish. After 4 h incubation at 37°C, the subconfluent monolayers that formed were infected with different viruses at a multiplicity of infection (MOI) 20 PFU/cell for 1 h, washed three times with phosphate-buffered saline (PBS), and overlaid with 1 ml of complete medium. At indicated times postinfection, the medium was replaced, and virus titers in the harvested samples were determined by a plaque assay on BHK-21 cells as previously described (27).

RNA analysis. Cells were infected with viruses at the MOIs indicated in the figure legends. The intracellular RNAs were labeled with [³H]uridine, either in the presence of 1 μ g of dactinomycin (ActD)/ml or in the absence of this inhibitor of cellular RNA synthesis, using the conditions described in the figure legends. RNAs were isolated from the cells by RNA-Bee reagent, as recom-

mended by the manufacturer (TEL-TEST, Inc.), and then they were denatured with glyoxal in dimethyl sulfoxide and analyzed by agarose gel electrophoresis using the previously described conditions (5). In other experiments, RNAs were fractionated on oligo(dT) magnetic beads, as recommended by the manufacturer (Ambion), and this was followed by electrophoretic analysis. For quantitative analysis, the RNA bands were excised from the 2,5-diphenyloxazole (PPO)-impregnated gels, and the radioactivity was measured by liquid scintillation counting.

Analysis of protein synthesis. NIH 3T3 cells were seeded into six-well Costar dishes at a concentration of 5×10^5 cells/well. Four hours later, the cells were infected with different viruses at an MOI of 20 PFU/cell in 200 μ l of PBS supplemented with 1% FBS for 1 h. Then incubation continued at 37°C in α MEM with 10% FBS. At 2, 4, 8, and 16 h postinfection, the cells were washed three times with PBS and then incubated for 30 min at 37°C in 0.8 ml of Dulbecco's modified Eagle's medium lacking methionine, supplemented with 0.1% FBS and 20 μ Ci of [³⁵S]methionine/ml. Then cells were scraped from the dish into the incubation medium, pelleted by centrifugation, and dissolved in 100 μ l of standard loading buffer for protein electrophoresis. Protein concentration in the samples was measured using SYPRO Ruby protein dye (Molecular Probes) according to the manufacturer's instructions, and equal amounts of proteins were loaded onto sodium dodecyl sulfate-10% polyacrylamide gels (25). After electrophoresis, gels were dried, autoradiographed, and analyzed on a Storm 860 PhosphorImager (Molecular Dynamics).

The amount of radioactivity detected in the protein band corresponding to actin was used to evaluate the residual host cell protein synthesis. The results were normalized on the amount of radioactivity detected in the same fragments of the lane representing uninfected cells. Synthesis of viral structural proteins was evaluated by measuring the radioactivity in the protein band corresponding to SIN capsid.

Analysis of SIN nsP2 processing. We infected 5×10^5 NIH 3T3 cells in six-well Costar plates with wtSIN, SIN/G, or SIN/2V virus at an MOI of 20 PFU/cell. At 4 h postinfection, cells were washed with PBS and harvested, and equal amounts of proteins from each sample were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis. After transfer, the nitrocellulose membranes were stained with 0.5% Ponceau S (Fisher) in 1% acetic acid to control the quality of transfer, and processed by rabbit anti-nsP2 antibodies (diluted 1:1,000), kindly provided by Charles M. Rice (Rockefeller University). Horseradish peroxidase-conjugated secondary donkey anti-rabbit antibodies were purchased from Santa Cruz Biotechnology and used in a dilution of 1:5,000. The Western blotting Luminol reagent was used according to the manufacturer's recommendations (Santa Cruz Biotechnology).

IFN- α/β assay. The concentrations of IFN- α/β in the medium were measured by a previously described biological assay (51) with minor modifications. Briefly, L929 cells were seeded in 100 μ l of complete medium at a concentration of 5×10^4 cells/well in 96-well plates and incubated at 37°C for 6 h. Samples of medium harvested from infected NIH 3T3 cells were treated with UV light for 1 h and serially diluted in twofold steps directly in the wells with L929 cells. After incubation for 24 h at 37°C, an additional 100 μ l of medium with 2×10^5 PFU of vesicular stomatitis virus was added to the wells, and incubation continued for 36 to 40 h. Then cells were stained with crystal violet, and the endpoint was determined as the concentration of IFN- α/β required to protect 50% of the cells in monolayers from the vesicular stomatitis virus-specific cytopathic effect. The IFN- α/β standard for normalization of the results was received from the American Type Culture Collection.

GeneChip expression analysis. NIH 3T3 cells were seeded at a concentration of 3×10^6 cells per 100-mm dish. After 6 h incubation at 37°C, cells were infected with wtSIN, SIN/G, and SIN/2V viruses at an MOI of 20 PFU/cell in 1 ml of α MEM supplemented with 1% FBS. After 1 h of infection at 37°C, 9 ml of α MEM containing 10% FBS was added, and cells were incubated at 37°C for 4, 8, and 20 h. RNAs were isolated by using TRIzol reagent as recommended by the manufacturer (Invitrogen). RNAs were additionally purified using an RNeasy kit (QIAGEN). Considering transcriptional shutoff in the infected cells, equal amounts of so-called spike RNAs (the in vitro-synthesized polyadenylated RNAs, corresponding to the fragments of *Bacillus subtilis* Trp, Thr, Lys, and Phe genes) were added to equal amounts of sample RNAs (represented mainly by rRNAs). The corresponding oligonucleotide probes for these RNAs are present on the Affymetrix chips, and the corresponding values of the hybridization signals were used for normalization of the results of the hybridization data. cDNAs were synthesized using a T7(dT)₂₄ primer and the SuperScript Choice system (Invitrogen) using 20 μ g of sample RNAs. Biotin-labeled cRNA was synthesized on half of the cDNA using the RNA transcript-labeling kit (ENZO). cRNA was purified and fragmented according to the Affymetrix protocol. Equal amounts of cRNAs were hybridized to the murine genome U74A array (MG-U74A) in the Univer-

sity of Texas Medical Branch GeneChip Core Facility. The data were analyzed using GeneChip Suite software (Affymetrix).

RESULTS

Recombinant viruses. In the present study, we used three recombinant SIN viruses that differed in the sequence of one of the nonstructural proteins, nsP2 (Fig. 1A). The genomes of all of the recombinants encoded GFP, cloned under the control of an additional subgenomic promoter. The expression of this gene was used to monitor productive infection of all of the cells in the experiments. A complete and synchronous infection was critical for proper interpretation of the results aimed at measuring the residual protein and RNA synthesis in the infected cells. Another subgenomic promoter was used to drive the expression of SIN structural proteins.

To efficiently infect the NIH 3T3 cells that were used in most of the experiments, the structural genes were derived from the TE12 mouse-adapted strain of SIN (30). wtSIN contained unmodified nonstructural genes, and a SIN/G variant had a single substitution in nsP2 (P₇₂₆→G) that strongly reduced the cytopathogenicity of the virus, allowing it to persist in cells defective in IFN- α / β secretion or signaling. The effect of this mutation on SIN replication was described in detail in our previous publications (8, 12). Cells without defects in IFN- α / β signaling are able to inhibit and stop replication of SIN/G (12).

Another mutant, SIN/2V, also contained a single mutation that changed the glycine to valine in the P2 position of the cleavage site between nsP2 and nsP3. The effect of this mutation on viral RNA replication was previously described (44). The nsP2 G₈₀₆→V mutation prevented nsP2/3 cleavage, leaving these proteins in an unprocessed form, making them incapable of forming mature replicative complexes containing completely processed SIN nonstructural proteins. This mutation had a strong effect on virus replication by making it temperature sensitive (44). We were interested in further study of this mutant, because the mutation in the cleavage site was expected to change the intracellular distribution of nsP2 and its functioning in virus-host cell interactions.

All of the in vitro-synthesized RNAs were transfected into BHK-21 cells by electroporation, and viruses were harvested 24 h posttransfection. By that time, cells transfected with wtSIN and SIN/2V RNAs, but not with RNA of SIN/G, developed complete cytopathic effect. NIH 3T3 and BHK-21 cells were infected with the three viruses at the same MOI, and the processing of viral nonstructural proteins and intracellular distribution of nsP2 were analyzed by immunoblotting and staining of the infected cells with nsP2-specific antibodies (Fig. 1B and C, respectively). In SIN/2V-infected cells, we detected the presence of only the P123 and P23 precursors of the nonstructural proteins (Fig. 1B), indicating strong alterations in polyprotein processing. A very small, nonspecific protein band with the same molecular weight as nsP2 was always detected by nsP2-specific antibodies in the samples prepared from both SIN/2V- and mock-infected cells (Fig. 2B), making it impossible to completely rule out that very inefficient cleavage could occur. However, we did not detect an increase in intensity of this band when samples for Western blot analysis were harvested at 16 h postinfection (data not shown). The strong differences in the intracellular distribution of nsP2 (Fig. 1C)

and replication of the viruses, described in the succeeding sections, suggested that presumable residual cleavage could play a very small role (if any at all) in the SIN/2V phenotype.

wtSIN produced nsP2 that was uniformly distributed in the cell in a manner similar to that previously described for Semliki Forest virus (36). nsP2, likely in the form of P23 or P123, was found only in the cytoplasm of the SIN/2V-infected cells, and P₇₂₆→G mutation in SIN/G nsP2 surprisingly changed the distribution of this protein as well (Fig. 1C). The major fraction of this mutant nsP2 was detected in cell nuclei. Thus, the mutations introduced into the nsP2 coding sequence changed both the processing of the precursor and the intracellular distribution of nsP2.

Replication of SIN variants in NIH 3T3 cells. All three viruses were capable of efficient replication in NIH 3T3 cells (Fig. 2A). In agreement with previously published data, wtSIN grew more rapidly than did SIN/G and particularly SIN/2V. Nevertheless, titers of both mutants approached 10⁹ PFU/ml by 20 h postinfection. Further analysis of protein synthesis indicated that in spite of strong differences in infectious virion production, all three viruses produced structural proteins at different but comparable rates (Fig. 2B and C). The surprising phenomenon was that after 4 h of infection, the SIN/2V-infected cells reproducibly synthesized more capsid and glycoproteins (Fig. 2B and C) than did the cells infected with SIN/G but released 30- to 100-fold less infectious virus until at least 12 h postinfection. In repeated experiments, SIN/2V and wtSIN suppressed host cell protein synthesis equally efficiently (Fig. 2B and D), and by 8 h postinfection, cellular proteins were translated at 10% of the level found in uninfected cells. The SIN/G mutant was incapable of inhibiting translation at the same rate. At any time postinfection, SIN/G-infected cells sustained synthesis of cellular proteins above 40% of the level in uninfected cells. As we previously described (12), after 16 h, infected NIH 3T3 cells downregulated and stopped SIN/G replication, and cell metabolism returned to normal within the next 24 h (data not shown).

Based on the efficient translational shutoff found in wtSIN- and SIN/2V-infected cells, it was reasonable to expect that both viruses could suppress activation of a cell response to virus replication, particularly the release of IFN- α / β . However, this was not the case. As with the SIN/G mutant, SIN/2V induced a high level of IFN- α / β release (Fig. 3). In repeated experiments, the IFN concentration in the medium of cells infected by nsP2 mutants, but not by wild-type SIN, approached 600 to 800 IU/ml. These data indicated that the downregulation of translation observed in alphavirus-infected cells is probably insufficient to stop cytokine release, and an additional change(s) in the intracellular environment is required to suppress the antiviral response.

Inhibition of transcription in cells infected with different SIN variants. The possible explanation for the very different levels of IFN- α / β secretion from the cells infected with wtSIN and SIN/G or SIN/2V may lie in the inability of wtSIN to induce or of SIN/G and SIN/2V to suppress activation of IFN- α / β and other genes involved in the antiviral response. To test these hypotheses, we infected NIH 3T3 cells with the recombinant viruses, and RNA synthesis (in both the presence and absence of ActD in the medium) was analyzed by metabolic pulse-labeling of the RNAs with [³H]uridine at different times

FIG. 1. Schematic representation of the double subgenomic SIN genomes and analysis of nsP2 expression in virus-infected cells. (A) In all three genomes, the first subgenomic promoter drove transcription of GFP-encoding RNA, and the second one drove the transcription of the 26S subgenomic RNA encoding SIN structural genes, derived from the SIN TE12 strain. The SIN/G and SIN/2V variants differed from wtSIN by one amino acid change in nsP2, P₇₂₆→G and G₈₀₆→V, respectively. (B) Processing of nsP2 in virus-infected cells: 5×10^5 NIH 3T3 cells in six-well Costar plates were infected with wtSIN, SIN/G, or SIN/2V at an MOI of 20 PFU/cell. At 4 h postinfection, cells were harvested, and equal amounts of proteins were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis. After transfer, the nitrocellulose membranes were processed by rabbit anti-nsP2 antibodies and horseradish peroxidase-conjugated secondary donkey anti-rabbit antibodies. (C) NIH 3T3 cells were infected with wtSIN, SIN/G, or SIN/2V at an MOI of 20 PFU/cell. At 6 h postinfection, cells were fixed with 4% formaldehyde in PBS for 10 min, permeabilized with cold methanol for 1 min, and stained with rabbit anti-nsP2 antibodies and secondary goat anti-rabbit Texas Red-labeled antibodies. The intracellular distribution of nsP2 was analyzed on a Zeiss confocal microscope. The lower panel demonstrates profiles of fluorescence distributions in the sections indicated by arrows in the upper panel.

postinfection, followed by agarose gel electrophoresis (Fig. 4A, B, C, and D).

In the presence of ActD, we detected only the synthesis of viral genome RNA and two subgenomic RNAs in the infected cells (Fig. 4A). The SIN/G genome replicated four- to sevenfold less efficiently than did the genome of wtSIN (Fig. 4A and C), and the transcription of SIN/G subgenomic RNAs was proportionally lower as well. Replication of the SIN/2V genome 4 and 8 h postinfection was reproducibly higher than that found for wtSIN (Fig. 4A and C). The SIN/2V subgenomic RNAs were synthesized at a rate that was two- to threefold lower than that of wtSIN (Fig. 4A and data not shown), but this transcription was more efficient than that found for SIN/G subgenomic RNAs. These data correlated with higher levels of

SIN/2V structural proteins translation (compared to SIN/G) and contrasted with the lower rates of SIN/2V infectious virus release.

In the RNA samples isolated from infected cells metabolically labeled with [³H]uridine in the absence of ActD, we readily detected not only virus-specific RNAs, but also the presence of newly synthesized cellular messenger and pre-mRNAs and unprocessed and completely processed rRNAs (Fig. 4B). At different stages of processing, rRNAs formed distinct bands on the gel, while pre-mRNAs and mRNAs formed smears that were not present on the gels with the RNAs isolated from the ActD-treated cells (Fig. 4A). To confirm that heterogeneous smear-forming RNAs represented cellular pre-mRNAs and mRNAs, a few samples were fractionated

FIG. 2. Virus growth and protein synthesis in cells infected by SIN variants. NIH 3T3 cells were infected as described in Materials and Methods. (A) At the indicated times, medium was replaced and virus titers were determined by a standard plaque assay on BHK-21 cells. (B) Proteins were pulse-labeled with [³⁵S]methionine at the indicated times and analyzed on a sodium dodecyl sulfate-10% polyacrylamide gel. Gels were dried and autoradiographed. The positions of SIN structural proteins and uncleaved P23 are indicated. The position of the actin band is indicated by an arrow. (C) Viral protein synthesis in infected cells was evaluated by measuring the radioactivity in the band of the capsid protein (indicated by C on panel B), as described in Materials and Methods. (D) The synthesis of cellular proteins in the infected cells was analyzed as described in Materials and Methods, and the results were normalized on the amount of radioactivity detected in the uninfected cells.

on oligo(dT) columns (Fig. 5). All of the viral genome and sub-genomic RNAs and more than 50% of the heterogeneous RNA in both infected and uninfected cells bound to the oligo(dT) columns, indicating that the smear-forming RNAs indeed contained a significant fraction of poly(A)-positive cellular templates.

By 8 h postinfection, wtSIN replication downregulated the synthesis of cellular mRNAs to an almost undetectable level, and the pattern of RNAs isolated at late times postinfection (8 and 16 h) from the cells not treated with ActD looked very similar to that found in the infected cells labeled with [³H]uridine in the presence of ActD (Fig. 4A and B), in that cell-specific RNA bands were barely detectable. At that time, the

SIN/G mutant-infected cells continued to synthesize mRNAs with 50% of the efficiency found in the uninfected cells (Fig. 4B and D). The mutation in the nsP2/3 cleavage site of SIN also affected the ability of the virus to inhibit cellular transcription. In SIN/2V-infected cells, RNA polymerase II-dependent RNA synthesis was downregulated more slowly than during wtSIN infection, and we detected cellular mRNA synthesis even at 16 h postinfection at the 40% level found in the uninfected cells. These data indicated that the mutations in nsP2 made SIN unable to inhibit RNA polymerase II-dependent cellular transcription.

Viral replication also strongly affected the synthesis and,

FIG. 3. Virus growth and IFN- α/β production by NIH 3T3 cells infected with wtSIN, SIN/G, and SIN/2V at an MOI of 20 PFU/cell. Virus titers and concentrations of IFN- α/β were determined as described in Materials and Methods in the same samples harvested at the indicated time points. These data represent one of a series of repeated experiments which generated very reproducible data. The error bars represent standard deviations.

most likely, the processing of the pre-rRNA. At late time points in wtSIN-infected cells, [3 H]uridine-labeled mature 18S and 28S rRNAs and the intermediate cleavage products of the precursor RNA were barely detectable (Fig. 4B and data not shown). In contrast, the rRNA intermediates were readily visible on the gels in the samples isolated at any time postinfection with SIN/G, and the replication of SIN/2V caused an intermediate-level downregulation of rRNA synthesis and processing. Unfortunately, the primary rRNA precursor transcript was the same size as the viral genomes, and it was thus difficult to precisely evaluate pre-rRNA synthesis.

To assess the effect of the inhibition of translation on the development of transcriptional shutoff, we performed a number of complementary experiments. NIH 3T3 cells were incubated in puromycin-containing medium, and RNA synthesis was evaluated after different times of drug treatment. Inhibition of translation by puromycin affected rRNA synthesis (Fig. 6A and B). Both the transcription and processing of pre-rRNA were strongly inhibited. The RNA polymerase II-dependent pre-mRNA synthesis was altered less, and even after 7, mRNAs were synthesized at 70% of the level found in the untreated cells. These data were confirmed by comparing the gene expression profiles in both untreated NIH 3T3 cells and cells treated with puromycin for 9 h. In contrast to wtSIN-infected cells, cells exposed to puromycin demonstrated a less than twofold decrease in the concentration of the mRNA pool even after 9 h of exposure to the drug (see below and data not shown). The later time points could not be analyzed because the cells started to die and detach after longer incubation with puromycin.

In additional experiments, NIH 3T3 cells were treated with ActD, and protein synthesis was analyzed after different treatment times. The results shown in Fig. 6C demonstrated that

inhibition of transcription led to inhibition of translation, but at a slower rate that strongly differed from the rapid downregulation of translation detected during wild-type SIN infection (Fig. 2B and D). This fact and the inability of SIN/2V to inhibit cellular transcription strongly argued against the possibility that translational shutoff normally detected during SIN infection could be the result of changes in transcription of cellular RNAs.

Taken together, the results indicated that replication of wtSIN, but not SIN/G and SIN/2V, strongly affected cellular mRNA synthesis. Moreover, the inhibition of cellular mRNA transcription and translation in wtSIN-infected cells appeared to be mostly independent events.

Microarray experiments. Our comparison of poly(A) RNA synthesis in cells infected with different viruses, described in the previous section, did not provide sufficient data about the entire pool of cellular mRNAs available for translation at different times postinfection with SIN variants. In particular, this method did not render information about induction of the viral stress-inducible genes. To compare in more detail the cells' reaction to infection with different SIN mutants, we performed a series of experiments using microarray-based technology. NIH 3T3 cells were infected with wtSIN, SIN/G, and SIN/2V, and their mRNA pools were analyzed by microarray gene profiling on Affymetrix mouse chips at 4, 8, and 20 h postinfection (see Materials and Methods for details).

Taking into consideration the downregulation of transcription and a possible decrease in the concentration of cellular mRNAs during infection, it was critical to establish a standard basis for normalization of the data. In the preliminary experiments, we found that in wtSIN-infected cells, rRNAs were more stable than mRNAs. In spite of the decrease detected in their synthesis, the concentration of rRNAs in the infected cells changed insignificantly, even at late times postinfection (data not shown). Thus, to normalize the hybridization data, we used the same amounts of total RNA (represented mainly by rRNAs) in all of the samples for cDNA synthesis and added the same amounts of reference RNAs (in vitro-synthesized *Bacillus subtilis* polyadenylated RNAs) to the samples prior to the reverse transcription reaction.

The wtSIN-infected cells demonstrated a detectable decrease in concentration of all of the mRNAs starting from 4 h postinfection, and a 20-fold lower concentration of the entire mRNA pool by 20 h (Fig. 7A, B, and C and Fig. 8). Out of 5,304 different mRNAs present in uninfected NIH 3T3 cells, a large fraction were already not present by 8 h postinfection with wtSIN, and only 377 genes were present in detectable concentrations by 20 h postinfection. We were unable to detect activation of any viral stress-inducible genes, including the standard genes involved in the IFN- α/β response.

At 20 h postinfection, the SIN/G mutant activated 378 genes (Table 1), and the concentration of many other cellular mRNAs decreased, but all of them remained at detectable levels (Fig. 7D, E, and F). In contrast to the 20-fold drop found in wtSIN-infected cells, the total concentration of cellular mRNAs became only twofold lower than in uninfected cells (Fig. 8). The majority of the induced genes were previously characterized as viral stress-inducible genes (data not shown). The spectrum of genes activated by SIN/G infection was broader, and the levels of their activation were dramatically

FIG. 4. Synthesis of virus-specific and cellular RNAs in NIH 3T3 cells infected with different SIN variants. (A) We infected 5×10^5 NIH 3T3 cells in six-well Costar plates with wtSIN, SIN/G, or SIN/2V at an MOI of 20 PFU/cell. At the indicated time points, the medium in the wells was replaced by 0.8 ml of α MEM supplemented with 10% FBS, ActD (1 μ g/ml), and [3 H]uridine (20 μ Ci/ml). After 3 h incubation at 37°C, RNAs were isolated and analyzed by agarose gel electrophoresis as described in Materials and Methods. (B) Total RNA synthesis was analyzed using the conditions described in the legend to panel A, but the [3 H]uridine-containing medium was not supplemented with ActD. (C) The levels of viral genome replication were determined by excising the bands corresponding to 49S viral genome RNA from the gel shown in panel A, followed by measuring radioactivity by scintillation counting. (D) The levels of synthesis of cellular pre-mRNAs and mRNAs were determined by measuring radioactivity in the fragment of the gel in gel panel B (indicated by R). The data were normalized to radioactivity in the corresponding band in uninfected cells. G, SG1, and SG2 indicate the positions of viral genomic and subgenomic RNAs 1 and 2, respectively. Asterisks indicate the positions of rRNA precursors. 28S indicates the position of rRNA.

higher than those achieved in the NIH 3T3 cells treated with interferon (data not shown; manuscript in preparation). This was a strong indication that type IFN- α/β -dependent gene activation is only a component of viral stress-dependent response.

The mRNA pool was also not affected by SIN/2V infection as much as by wild-type SIN. By 20 h postinfection with SIN/

2V, we readily detected the activation of 267 genes, all of which were activated by SIN/G as well (Fig. 7G, H, and I and Table 1). Other mRNAs were present either at the same or less than fourfold lower concentrations, and similar to SIN/G-infected cells, the concentration of the entire pool of cellular mRNAs decreased only 3.4-fold (Fig. 8). Moreover, SIN/2V replication activated viral stress-inducible genes earlier than did the

FIG. 5. Analysis of poly(A) RNA in samples isolated from SIN-infected and uninfected cells. NIH 3T3 cells were infected with SIN/2V at an MOI of 20 PFU/cell. At 1 h postinfection, the medium was replaced by 0.8 ml of α MEM supplemented with 10% FBS, [3 H]uridine (20 μ Ci/ml), and ActD (1 μ g/ml) indicated as ActD+, or no drug (ActD-). After 3 h incubation at 37°C, RNAs were isolated and poly(A) RNAs were extracted with oligo(dT) MagBeads as described in Materials and Methods. The entire amounts of eluted and unbound RNA samples were analyzed by agarose gel electrophoresis. G, SG1, and SG2 indicate the positions of genomic RNA and subgenomic RNAs 1 and 2, respectively. Asterisks indicate the positions of rRNA precursors. 28S and 18S indicate the positions of rRNAs. Bound RNA indicates RNA eluted from the oligo(dT) MagBeads; flowthrough indicates RNAs that did not bind to the oligo(dT) beads.

SIN/G mutant. At 8 h postinfection, 312 and 217 genes were activated by SIN/2V and SIN/G, respectively (Table 1). Earlier and stronger activation of the spectrum of virus stress-inducible genes provided a plausible explanation for why SIN/2V induced IFN- α/β as efficiently as SIN/G (Fig. 3) despite causing a stronger translational shutoff. For example, by 8 h postinfection, the IFN- β gene was activated 9- and 104-fold in SIN/G- and SIN/2V-infected cells, respectively (data not shown).

The mRNA profiles were also examined in ActD- and puromycin-treated cells. As we expected, changes in the mRNA pool in puromycin-treated cells were less intense than those found during wtSIN infection (Fig. 7K). Few activated genes represented stress response genes (data not shown), and concentrations of other RNAs remained very similar to those in untreated cells. This fact was consistent with the finding that RNA polymerase II-dependent RNA synthesis continued to proceed in the presence of puromycin (Fig. 6A and B) and was an additional indication that translational shutoff cannot explain the very efficient inhibition of transcription in wtSIN-infected cells.

In ActD-treated cells, we detected a 2.2-fold decrease in the

concentration of the mRNA pool (Fig. 7J and data not shown) that generally correlated with slowly developing changes in cellular translation (Fig. 6C). Thus, inhibition of transcription might contribute, to some extent, to the profound translational shutoff detected in wtSIN-infected NIH 3T3 cells (Fig. 2B and D). However, efficient inhibition of translation, but not transcription, in the case of SIN/2V infection suggested that a mechanism(s) other than a simple decrease in the concentration of mRNAs is also involved in translational shutoff during SIN infection.

Taken together, the data implied that the designed SIN viruses expressed three different phenotypes: wtSIN was capable of causing both transcriptional and translational shutoff; SIN/2V efficiently downregulated translation but not transcription of cellular mRNAs; and SIN/G was inefficient in causing both downregulation events.

DISCUSSION

The most intensively studied alphaviruses, including Venezuelan and eastern equine encephalitis viruses and SIN, develop a biphasic disease in infected amplification hosts that is characterized by high-titer viremia before the appearance of virus in the brain and encephalitis development (13, 16, 17). Viremia is also a prerequisite of a natural transmission cycle of alphaviruses that is required for infection of mosquito vectors. Its development implies multiple rounds of infection that proceed in the background of the developing antiviral response, aimed at blocking the spread of infection (14, 24, 38, 41, 45).

Induction of a wide variety of cellular genes, e.g., viral stress-inducible genes, can strongly affect and/or completely stop viral replication (40). Their activation (i) depends on viral replication itself; (ii) depends on the presence of IFN- α/β in the medium or tissues; and (iii) can possibly be modulated by activation of other signaling pathways, like double-stranded RNA signaling mediated by the Toll-like receptor 3 (TLR3) (39, 40, 42). Therefore, many, if not most, of the viruses have developed a wide variety of mechanisms aimed at downregulating different components of the cell reaction, first of all the IFN- α/β response, and at increasing viremia to a level sufficient for virus transmission to susceptible organisms or arthropod vectors (2, 4, 21, 28, 32, 37, 43, 52). Alternatively, interference with cell reactions opens an opportunity for persistent infection (1, 20, 33). Intimate interactions between replicating viruses and a developing antiviral response represent a critical factor in viral pathogenesis. However, in the case of alphaviruses, our knowledge about this aspect of viral replication is far from complete.

Traditionally, the roles of different gene products and cell signaling pathways on virus replication are studied by using a variety of knockout mice incapable of activating some components of the antiviral reaction (26, 46, 58). Our study was based on a different approach. The previous results suggested that mutations in one of the viral nonstructural proteins, nsP2, had a profound effect on the ability of SIN to suppress activation of the antiviral response (12). Thus, we sought to further understand the mechanisms that SIN employs to suppress antiviral reactions in the infected cells, and IFN- α/β signaling in particular. Accordingly, we applied three different SIN variants to study the effect of changes in nsP2 on both transcription and

FIG. 6. Cellular RNA synthesis and cellular protein synthesis under conditions of translational and transcriptional shutoff, respectively. (A) NIH 3T3 cells were incubated in the presence of 5 µg/ml of puromycin or 1 µg/ml of ActD. During the indicated time intervals after treatment was started, incubation continued in the same medium supplemented with [³H]uridine (20 µCi/ml) and at the same concentrations of drugs. At the end of the labeling time RNAs were isolated and analyzed by agarose gel electrophoresis as described in Materials and Methods. Asterisks indicate the positions of 45S pre-rRNAs and intermediate cleavage products, 28S and 18S indicate the positions of rRNAs, and R indicates fragments of the gel used for evaluation of

translation in NIH 3T3 cells, processes that could critically affect the development of the antiviral reaction in these cells that have no defects in IFN-α/β production and signaling.

Replication of SIN with the wild-type sequence of nsP2 (wtSIN) led to a very efficient inhibition of translation of cellular proteins and inhibition of transcription of mRNA and ribosomal RNAs. Processing of pre-rRNAs was also strongly affected, but based on the results demonstrated in Fig. 6, this effect could be most likely explained by translational shutoff and depletion of the short-lived proteins involved in processing and RNA polymerase I-dependent rRNA synthesis. Interestingly, the decrease in the concentration of mRNAs in wtSIN-infected cells that was detected in microarray experiments differed from that found in the cells treated with ActD (see Fig. 7B, C, and J). This finding suggested that an additional component employing RNA degradation might be involved in the decrease of mRNA concentration during wild-type virus infection. However, this hypothesis needs additional experimental support.

Downregulation of cellular macromolecular synthesis resulted in the inability of wtSIN-infected NIH 3T3 cells to produce IFN-α/β, but because of the strong total inhibition of cellular transcription, we cannot rule out the possibility that the viral stress-inducible genes are additionally suppressed in a more specific manner. For example, recent data suggested that antiviral gene expression is more specifically suppressed during Ross River virus infection (31).

The point mutation at amino acid 726 of nsP2 (SIN/G mutant) made the virus incapable of causing a profound inhibition of translation and transcription of the cellular mRNAs that were observed in wtSIN-infected cells. As we recently described, at an early point postinfection, replication of this mutant activated a protein kinase R (PKR)-dependent component in the inhibition of translation. However, a PKR-independent component, which plays the most critical role during wtSIN infection, was activated inefficiently and also only at an early time postinfection (15).

Surprisingly, the mutated nsP2 accumulated mainly in the nuclei of the infected cells, but did not affect transcription of cellular genes. We speculate that either the mutation in position 726 makes nsP2 incapable of binding or cleaving the protein factors involved in transcription regulation, or a change in its compartmentalization might be the reason for a change in its functioning. (The functional mechanism of nsP2 is under investigation.) nsP2 transiently expressed from plasmids in the

pre-mRNA and mRNA synthesis. (B) Fragments of the gel from panel A, corresponding to pre-mRNA and mRNA, 45S pre-rRNA, and 28S rRNA were excised and used to measure radioactivity by liquid scintillation counting. The results were normalized to radioactivity in the cells that were not treated with the drug. One of several reproducible experiments is shown. (C) NIH 3T3 cells were incubated in the presence of 1 µg/ml of ActD. Cellular proteins were metabolically pulse-labeled for 30 min with [³⁵S]methionine at the indicated times and analyzed on a sodium dodecyl sulfate-10% polyacrylamide gel. Gels were dried and analyzed on a Storm 860 PhosphorImager as described in Materials and Methods. The levels of cellular protein synthesis were determined by measuring radioactivity in the protein band corresponding to actin and normalized to radioactivity in the actin band in the cells not treated with ActD. One of the very reproducible experiments is shown.

TABLE 1. Changes in gene expression at different times postinfection of NIH 3T3 cells with the SIN/G and SIN/2V variants

Change in genes expression	SIN/G		SIN/2V	
	8 h	20 h	8 h	20 h
Number of genes activated more than threefold	217	378	312	267
Number of genes downregulated more than threefold	20	184	66	916

form of the P123 precursor or as a single protein also preferentially accumulates in the nuclei (I.F., unpublished data), suggesting that the presence of this protein in the cytoplasm is determined at least partially by the presence of functioning replicative complexes. Thus, the lower levels of SIN/G replication and lower numbers of replicative complexes, compared to those of wtSIN, and higher production of nsP2 in the absence of translational shutoff might lead to an accumulation of this protein in the nuclei.

Thus, the defect in nsP2 of the SIN/G mutant made this virus incapable of inhibiting transcription and translation of cellular mRNAs in the cells without the defects in IFN- α/β production or signaling. This, in turn, leads to efficient IFN release, followed by clearance of the virus from the infected cells and protection of the uninfected cells.

The SIN/2V variant, containing a mutation in the cleavage site between nsP2 and nsP3, demonstrated an intracellular distribution of the nsP2 that differed from those found during wtSIN and SIN/G infections. nsP2 was present in the form of P23 and P123 products, and the virus demonstrated a strong change in phenotype when its replication was compared to those of wtSIN and SIN/G. The SIN/2V genome replicated more efficiently than did the genomes of the other variants used, and the SIN/2V structural proteins were synthesized only at a level twofold lower than those of the proteins in the wtSIN-infected cells, but at a higher rate than that found during SIN/G infection. Nevertheless, the release of SIN/2V infectious particles did not correlate with RNA replication and structural protein synthesis, and it was 10- to 100-fold less efficient, at least until 10 to 12 h postinfection (Fig. 2A).

The replication rate of this mutant virus correlated with previously published data (44), but the present study suggests that a growth deficiency of SIN/2V might be explained not only by alteration of replication and transcription of virus-specific RNAs, but also by the effects of incomplete P123 cleavage on the packaging of the RNA genomes into infectious viral particles. We speculate that this defect could be a result of either less efficient presentation of the RNA or a difference in the localization of the RNA replication machinery relative to nucleocapsid assembly sites or in functioning of processed nsP2 and/or nsP3 in virion assembly, etc. Alphavirus cleavage mu-

FIG. 8. Decrease in concentration of cellular mRNA pool at different times postinfection with wtSIN, SIN/G, and SIN/2V. Concentrations of cellular mRNAs were evaluated by comparing the total normalized fluorescence of chips during GeneChip analysis. Total fluorescence was normalized based on the signals corresponding to the reference (in vitro-synthesized *B. subtilis* polyadenylated RNAs) that were added to the same amounts of RNA samples prior to the reverse transcription reaction (see Materials and Methods for details). The difference between different experiments was within 20% of measured values.

tants probably need additional investigation to elucidate the supplementary functions of the nonstructural proteins. However, our study focused on a particular feature of SIN/2V, its interaction with host macromolecular synthesis. The inability of SIN/2V to produce completely processed nsP2 and/or possibly its inability to produce nsP2 capable of translocating to the nucleus made this virus incapable of causing the transcriptional shutoff characteristic of wtSIN replication. On the contrary, more than 312 cellular genes, representing viral stress-inducible genes, were induced. They were induced earlier and to higher levels than those in SIN/G-infected cells, and the residual protein synthesis, particularly at the early stages of the infection, was sufficient for production of IFN- α/β that was easily detected in the medium. Thus, the alteration of SIN nonstructural polyprotein processing made SIN/2V incapable of blocking cell signaling.

Taken together, the results of this study suggest that, in contrast to many viral infections, SIN (and possibly other alphaviruses) employs total suppression of transcription and translation to inhibit cell reactions on virus replication (reviewed in reference 41). More specific mechanisms, aimed at downregulating expression of particular genes, are likely beneficial for the viruses that demonstrate slower replication rates, develop persistent infection, or require nuclei for viral repli-

FIG. 7. Results of GeneChip analysis of mRNA expression in cells infected with wtSIN, SIN/G, and SIN/2V or treated with ActD or puromycin. NIH 3T3 cells were infected at an MOI of 20 PFU/cell with wtSIN (A, B, and C), SIN/G (D, E, and F), and SIN/2V (G, H, and I) or treated with 2 μ g/ml of ActD (J) or 5 μ g/ml puromycin (K). At the indicated times postinfection or posttreatment with drugs, RNAs were isolated, and analysis was performed as described in Materials and Methods and Results. Relative hybridization signal intensities are plotted in a logarithmic scale. The y axis represents signal intensities in virus-infected or drug-treated cells. The x axis represents the signal intensities in control NIH 3T3 cells. The parallel lines indicate 2-, 3-, 10-, and 30-fold changes in signal intensities in samples from the treated cells.

cation. SIN replicates very efficiently both in mice and in cultured cells, and the high-level release of infectious virions can be detected within the first 3 to 4 h postinfection. SIN replication does not require nuclear functioning and proceeds efficiently in the presence of ActD. Thus, the transcriptional shutoff does not affect virus replication but prevents activation of viral stress-inducible genes and, first of all, IFN- α/β signaling. Inhibition of translation also does not appear to inhibit virus replication. In contrast, it might be beneficial for the rapid production of viral structural proteins. Alphaviruses developed a translation enhancer in the capsid coding sequence of the subgenomic RNA that is highly efficient under conditions in which translation is inhibited by replicating virus (10).

The phenomenon of inhibition of cellular transcription during virus infection is not unique. It has been previously described for poliovirus- and vesicular stomatitis virus-infected cells (53, 56, 57) or cells expressing the nonstructural protein of Rift Valley fever virus (3, 6). It appears to be a fast, highly efficient means of downregulating cell response. In the case of SIN, transcriptional shutoff may develop in highly permissive cells and at least partially determine SIN host range and replication in particular tissues. It should be noted that, similar to any other RNA virus infection, SIN replication *in vivo* (in more complex and likely less permissive conditions) leads to cytokine release, indicating that transcriptional and translational shutoffs in some tissues are likely incomplete (50, 51).

In conclusion, we demonstrated that (i) replication of wtSIN strongly affects major cellular processes and transcription and translation of mRNAs; (ii) the inhibition of transcription leads to a 20-fold decrease in the concentration of a cellular mRNA pool within 20 h postinfection; (iii) transcriptional and translational shutoffs are distinctly independent events, and their development can be differentially manipulated by creating different mutations in SIN nsP2; and (iv) inhibition of transcription, but not translation, is a critical phenomenon that SIN employs to suppress expression of cellular viral stress-inducible genes in cells of vertebrate origin.

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R.G. and E.F. contributed equally to this work.

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