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Evasion of the Innate Immune Response: the Old World Alphavirus nsP2 Protein Induces Rapid Degradation of Rpb1, a Catalytic Subunit of RNA Polymerase II

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The Old World alphaviruses are emerging human pathogens with an ability to cause widespread epidemics. The latest epidemic of Chikungunya virus, from 2005 to 2007, affected over 40 countries in Africa, Asia, and Europe. The Old World alphaviruses are highly cytopathic and known to evade the cellular antiviral response by inducing global inhibition of transcription in vertebrate cells. This function was shown to be mediated by their nonstructural nsP2 protein; however, the detailed mechanism of this phenomenon has remained unknown. Here, we report that nsP2 proteins of Sindbis, Semliki Forest, and Chikungunya viruses inhibit cellular transcription by inducing rapid degradation of Rpb1, a catalytic subunit of the RNAPII complex. This degradation of Rpb1 is independent of the nsP2-associated protease activity, but, instead, it proceeds through nsP2-mediated Rpb1 ubiquitination. This function of nsP2 depends on the integrity of the helicase and *S*-adenosylmethionine (SAM)-dependent methyltransferase-like domains, and point mutations in either of these domains abolish Rpb1 degradation. We go on to show that complete degradation of Rpb1 in alphavirus-infected cells occurs within 6 h postinfection, before other previously described virus-induced changes in cell physiology, such as apoptosis, autophagy, and inhibition of STAT1 phosphorylation, are detected. Since Rpb1 is a subunit that catalyzes the polymerase reaction during RNA transcription, degradation of Rpb1 plays an indispensable role in blocking the activation of cellular genes and downregulating cellular antiviral response. This indicates that the nsP2-induced degradation of Rpb1 is a critical mechanism utilized by the Old World alphaviruses to subvert the cellular antiviral response.

Efficient replication of viruses in infected hosts relies not only on their replicative machinery and expression of virus-specific nonstructural and structural proteins but also on their ability to interfere with the antiviral response mounted in cells of both vertebrate and invertebrate origin. Different viruses have evolved numerous mechanisms to inhibit the cellular antiviral response. While the vast majority of them interfere with specific steps of cellular signaling pathways, the highly cytopathic alphaviruses induce a global inhibition of cellular transcription (15). The entire alphavirus life cycle proceeds in the cytoplasm and is completed within 24 to 48 h postinfection (p.i.) (33). Therefore, functional nuclei appear to play no role in alphavirus replication. This allows global inhibition of cellular transcription to serve as an efficient means of inhibiting the antiviral response without affecting virus replication and egress. The inability of infected cells to activate cytokine and chemokine expression makes them incapable of signaling and, importantly, of releasing type I interferon (IFN), which could induce an antiviral state in as-yet-uninfected cells.

The alphavirus genome is a single-stranded RNA of positive polarity with a cap at the 5′ terminus and poly(A) at the 3′ terminus (33). It encodes 4 nonstructural proteins that form a replicative enzyme complex and 3 structural proteins, which are translated from the subgenomic RNA and assemble viral particles. Both the New World (NW) and the Old World (OW) alphaviruses induce transcriptional shutoff within a few hours postinfection, suggesting that is an important characteristic of their replication. However, these geographically isolated alphaviruses employ different mechanisms for transcription inhibition (15). We have previously demonstrated that the capsid protein of NW alphaviruses, e.g., Venezuelan (VEEV) and eastern (EEEV) equine encephalitis viruses, encodes a unique peptide containing a classical nuclear localization signal and a supraphysiological nuclear export signal, which mediate the formation of a tetrameric complex with the nuclear export receptor CRM1 and importin-α/β (3). The accumulation of these complexes in virus-infected cells rapidly blocks the function of nuclear pores preventing nuclear import, which ultimately leads to the inhibition of cellular transcription. The capsid protein of the OW alphaviruses, such as Sindbis (SINV), Semliki Forest (SFV), and Chikungunya (CHIKV) viruses, does not exhibit this function and does not interfere with nucleocytoplasmic trafficking (15). In the OW alphavirus-infected cells, inhibition of transcription is achieved by different mechanisms mediated by the nonstructural protein nsP2 (1, 12, 15). However, to date the mechanism of nsP2-induced transcriptional shutoff has not been elucidated.

Alphavirus nsP2 protein is a multifunctional protein with three currently described enzymatic activities. It is synthesized as a part of the viral nonstructural polyproteins P123 and P1234, and its protease activity is required for proteolytic processing of the polyproteins into individual nonstructural proteins nsP1, nsP2, nsP3, and nsP4. This sequential proteolysis orchestrates the regulation of virus-specific RNA synthesis (33). Two other nsP2-specific activities (its ability to function as an RNA helicase and RNA 5′ triphosphatase) were initially proposed based on bioinformatic

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analysis and later confirmed biochemically (16, 18). Thus, nsP2 is required for viral RNA replication and transcription of the subgenomic RNA. However, only a small fraction of nsP2 is incorporated into viral replicative complexes (13). The main fraction of nsP2 is present both in the cytoplasm and cell nuclei, where it is not associated with other nsPs. The transcription inhibitory functions of the OW alphavirus nsP2 protein strictly depend on its accumulation in the nucleus (19). Indeed, some indirect data suggest that the nuclear form of nsP2 is conformationally different from that present in the cytoplasm (23).

In spite of the importance of the transcription inhibition phenomenon and the accumulation of a large amount of data about the negative effect of point mutations on nsP2 activity in transcription inhibition, the exact mechanism of nsP2 function in the induction of transcriptional shutoff remains obscure. Therefore, in this study, we further investigated changes in composition and function of cellular transcriptional machinery during OW alphavirus replication. Our data demonstrate that nsP2 proteins of OW alphaviruses induce rapid degradation of the catalytic subunit of polymerase II (RNAPII), Rpb1. This degradation of Rbp1 is independent of the nsP2-associated protease activity. Instead, nsP2 imitates activation of the transcription-coupled repair (TCR) mechanism, which leads to rapid, ubiquitin-dependent degradation of Rpb1 within 4 to 6 h postinfection. The nsP2-specific NTPase activity, associated with its helicase domain, is essential for the induction of transcriptional shutoff. Thus, the OW alphaviruses employ a previously unknown unique mechanism for robust inhibition of the cellular antiviral response.

MATERIALS AND METHODS

Cell culture. The BHK-21 cells were maintained at 37°C in alpha minimum essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS) and vitamins. The NIH 3T3 cells were obtained from ATCC and maintained in αMEM supplemented with 10% FBS and vitamins. Mosquito C7/10 cells were maintained in Dulbecco minimal essential medium (DMEM) supplemented with 10% heat-inactivated FBS and 10% tryptose phosphate broth (TPB).

Plasmid constructs. Plasmids encoding the viral genomes pSINV/green fluorescent protein (pSINV/GFP), pSINV/G/GFP, and pSINV/2V/GFP and VEEV replicons encoding the heterologous genes pVEErepL/nsP2/Pac and pVEErepL/GFP/Pac have been described elsewhere (12, 14, 19). The pVEErepL/nsP2h1-GFP/Pac and pVEErepL/nsP2h2-GFP/Pac plasmids were constructed based on pVEErepL/nsP2-GFP/Pac by introducing point mutations into the helicase domain of SINV nsP2 by PCR-mediated mutagenesis and standard cloning techniques. pVEErep/nsP2-GFP, pVEErep/nsP2L-GFP, pVEErep/nsP2G-GFP, and pVEErep/GFP plasmids were derived from pVEErep/nsP2-GFP/Pac, pVEErep/nsP2L-GFP/Pac, pVEErep/nsP2G-GFP/Pac, and pVEErep/GFP/Pac (14), respectively, by deleting the Pac-encoding subgenomic RNA sequence and the corresponding promoter. Plasmids encoding the VEErep/nsP2h1-GFP, VEErep/nsP2h2-GFP, VEErep/nsP2m-GFP, VEErep/nsP2sfv-GFP, and VEErep/nsP2chikv-GFP replicons were constructed by replacement of wild-type (wt) SINV nsP2 in pVEErep/nsP2-GFP with the corresponding SFV- and CHIKV-derived nsP2 sequences or mutant SINV nsP2. In all of the nsP2-containing constructs, the amino terminus of nsP2 was fused in frame with the ubiquitin (Ubi) sequence to achieve the intracellular synthesis of nsP2 proteins with a natural first amino acid. The schematic presentations of the constructs are shown in the figures. Sequences of the plasmids can be provided upon request.

Packaging of replicons and virus rescue. BHK-21 cells were coelectroporated with the *in vitro*-synthesized VEEV replicon RNAs and helper RNA Hvee/C+G1 as described elsewhere (37). Virus rescue from the plasmids was performed as described elsewhere (19). Viruses used for infection of C7/10 cells were produced in this mosquito-derived cell line.

Immunoblot analysis. Cells were infected with viruses at a multiplicity of infection (MOI) of 20 PFU/cell and with replicons at an MOI of 50 infectious units/cell. Cells were harvested at different times postinfection as specified in the figure legends and then pelleted and frozen. The cell pellets were suspended in equal volumes of protein gel loading buffer and analyzed using either standard 9% PAGE or 4 to 12% NuPAGE (Invitrogen). The following antibodies were used for Rpb1 detection: (i) the N terminus-specific rabbit polyclonal antibodies N20 or H224 (sc899 and sc9001; Santa Cruz), (ii) the S2-specific monoclonal antibody (MAb) H5 (MPY-127R; Covance), and (iii) the S5-specific MAb 4H8 (39097; Active Motif). Rabbit polyclonal and mouse monoclonal antibodies against nsP2 were custom produced in our lab. Additional antibodies used in these studies included α-Rpb6 (sc28711; Santa Cruz), α-Rpb8 (sc32122; Santa Cruz), α-Gft2b/TFIIB (4169; Cell Signaling), α-Ercc3/XPB (sc293; Santa Cruz), α-Ddx5 (ab21696; Abcam), α-Dhx9 (ab26271; Abcam), α-Ncl/C23 (sc13057; Santa Cruz), α-Ddx6 (A300-461A; Bethyl Laboratories), and α-hnRNP C1/C2 (2438; Epitomics). As a loading control, we used α-actin (ab14001 and ab6276; Abcam) or α-tubulin (Sigma). Immunoblots were quantified using Odyssey Imager, and protein signals were normalized to the loading controls, α-actin or α-tubulin.

Analysis of cytotoxicity of SINV nsP2 helicase mutants was performed as described elsewhere (14).

Immunofluorescence. BHK-21 or C7/10 cells were seeded in 8-well μ-slides (Ibidi) and infected with viruses at a concentration of 10^7 PFU/ml, sufficient to infect all of the cells in the monolayers. Then the cells were fixed at indicated times postinfection in phosphate-buffered saline (PBS) supplemented with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in the presence of 5% normal goat serum. Next, the cells were incubated with the below-indicated primary antibodies, followed by staining with appropriate fluorescent secondary antibodies. The following antibodies were used: α-Rpb1 (MAb F12, sc55492; Santa Cruz; or MAb 4H8, 39097; Active Motif), α-hnRNP A0 (5545; Cell Signaling), α-hnRNP A1 (8443; Cell Signaling), α-hnRNP K (4675; Cell Signaling), α-Dhx9 (ab26271; Abcam), and α-Ncl/C23 (sc13057; Santa Cruz). Nuclei were stained with Hoechst. Images were acquired on a Zeiss LSM700 confocal microscope with a 63 × 1.4 numerical aperture (NA) Plan-Apochromat oil objective.

Analysis of Rpb1 ubiquitination. BHK-21 cells were infected with SINV/GFP or SINV/G/GFP viruses at an MOI of 20 PFU/cell for 2 h. Cells were then lysed in a buffer, containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM MG132, and a proteinase inhibitor cocktail (Sigma) and sonicated, and the cellular debris was pelleted by centrifugation. Supernatants were incubated overnight with 40 μl of GSK-DSK2 beads (Millipore). After extensive washing, the bound proteins were eluted by boiling in protein gel loading buffer. The aliquots of total lysates (1/100) and GSK-DSK2 bead-bound proteins (1/4) were analyzed on 9% PAGE followed by Western blotting with MAb 4H8 to detect Rpb1.

RESULTS

SINV infection induces rapid degradation of the catalytic subunit of RNAPII, Rpb1, in vertebrate cells. Our previous studies demonstrated that within the first few hours postinfection, SINV efficiently inhibits the transcription of all cellular messenger RNAs and that this phenomenon depends on the integrity of nsP2 protein and its expression in a free, completely processed form (14). We hypothesized that such robust, nonspecific transcription inhibition points to nsP2 targeting directly to the cellular RNAPII complex. Thus, we analyzed changes in concentration of several RNAPII subunits during the course of wt SINV replication as well as replication of the previously described mutants, SINV/G and SINV/2V. SINV/G/GFP contains a P726G point mutation in the

carboxy-terminal domain of nsP2, which has deleterious effects on virus cytopathogenicity and makes it incapable of inhibiting cellular transcription and translation to the level detected in wt SINV-infected cells (1, 19). The SINV/2V/GFP contains a mutation in the cleavage site between nsP2 and nsP3 proteins, which prevents the release of free nsP2 and its translocation into the nucleus. As a result, SINV/2V/GFP did not inhibit cellular transcription but retained its ability to inhibit translation and thus caused cytopathic effect (CPE) (19). We have previously demonstrated that replication of both mutants induced a robust antiviral response and upregulated the expression of over 300 cellular genes, while the wt SINV inhibited cellular transcription, and this in turn prevented activation of the virus stress-inducible genes (19). All viruses also encoded green fluorescent protein (GFP) in their genomes under the control of a second subgenomic promoter (Fig. 1A). Its expression was required for monitoring the completeness of infection of all the cells, which was critical in these experiments.

BHK-21 cells were infected with wt SINV/GFP and indicated mutants, and we analyzed the fate of several RNAPII subunits (Rpb1, Rpb6, and Rpb8), proteins associated with the RNAPII complex (Ercc3/XPB and Gtf2b/TFIIB), and several nuclear proteins. By 7 h postinfection, all of the analyzed proteins were present at noticeably lower levels in the cells infected with SINV/GFP and its cleavage mutant SINV/2V/GFP (Fig. 1B). The SINV/G/GFP mutant had very little effect on the levels of the same proteins. Interestingly, we found that in the cells infected with SINV/GFP, but not with mutants, the largest subunit of RNAPII, Rpb1, became undetectable. This effect was confirmed by using three antibodies specific to different forms of Rpb1 (Fig. 2A and B). At the same time, the Rpb1 level was reduced only about 2-fold in SINV/G/GFP-infected cells and 5-fold in SINV/2V/GFP-infected cells. The stronger effect of the latter mutant virus on the level of Rpb1 was likely the result of the robust inhibition of translation caused by replication of this virus. However, Rpb1 remained readily detectable in SINV/2V/GFP-infected cells at 7 h postinfection, which implies that the complete disappearance of Rpb1 in SINV/GFP-infected cells cannot be simply explained by translation inhibition induced by virus. To confirm that Rpb1 loss in SINV/GFP-infected cells was not a result of translation inhibition, we estimated the half-life of Rpb1 in BHK-21 cells using puromycin (Pur), a known inhibitor of translation. The half-life of Rpb1 was approximately 6 h, which correlates well with that estimated for yeast Rpb1 (half-life of 5 h) (22).

To additionally confirm the loss of Rpb1 from the SINV/GFP-infected cells, we stained the SINV/GFP-, SINV/G/GFP-, or mock-infected cells at 7 h postinfection with antibodies specific to the Rbp1 N terminus, thus recognizing all of its forms. As expected, no Rpb1 could be detected in SINV/GFP-infected cells (Fig. 2C), while compared to mock-infected cells, the SINV/G/GFP infection led to only an ~2-fold reduction of the Rpb1-specific nuclear signal, which correlated with the data obtained by immunoblotting (Fig. 2B). The inability to detect Rpb1 in SINV/GFP-infected cells by both methods and with different antibodies was a strong indication that within a few hours postinfection, Rpb1 is completely degraded.

To rule out the possibility that the effect of Rbp1 degradation is specific only to BHK-21 cells, which are highly permissive for the replication of numerous viruses, including SINV, we analyzed the SINV-induced degradation of Rpb1 in murine fibroblasts, NIH 3T3 cells, which have no defects in type I IFN expression and signaling. By 7 h after infection with wt SINV/GFP, the level of total Rpb1 also decreased to 11%, and the levels of hyperphosphorylated forms were reduced to 19% (Rpb1 S5) and 13% (Rpb1 S2). The amount of total Rpb1 in the cells infected with mutant viruses did not significantly change and remained at 90% and 86% for SINVG/GFP and SINV/2V/GFP, respectively (Fig. 3).

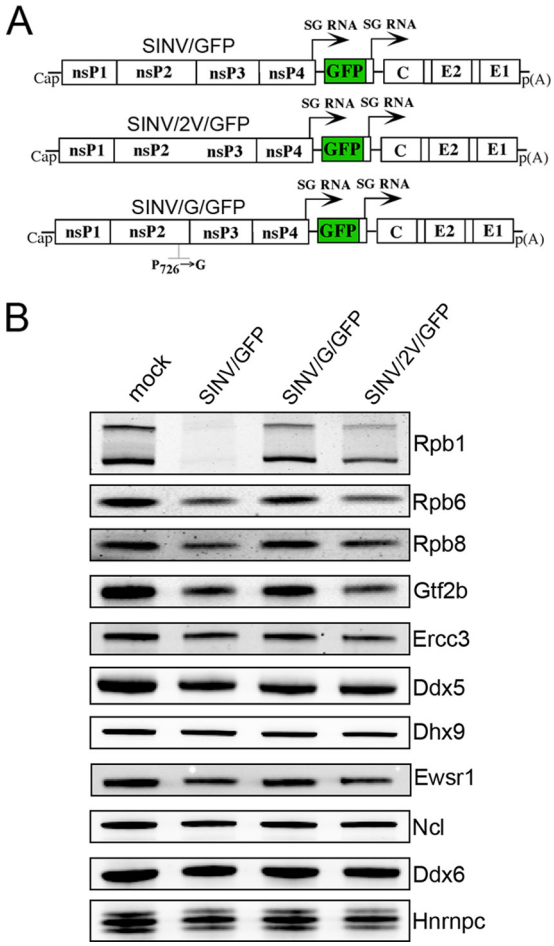

FIG 1 SINV encoding wt but not mutated nsP2 induces degradation of Rpb1. (A) Schematic representation of viral genomes. (B) BHK-21 cells were infected with SINV/GFP or SINV/G/GFP and SINV/2V/GFP mutants at an MOI of 20 PFU/cell. Cellular lysates were prepared at 7 h p.i. and analyzed by immunoblotting using antibodies against different nuclear proteins (see Materials and Methods for details). Abbreviations: Ddx5, DEAD (Asp-Glu-Ala-Asp) box helicase 5; Ddx6, DEAD (Asp-Glu-Ala-Asp) box helicase 6; Dhx9, DEAH (Asp-Glu-Ala-His) box polypeptide 9; Ercc3, excision repair cross-complementing rodent repair deficiency, complementation group 3 (or XPB, xeroderma pigmentosum group B); Ewsr1, Ewing sarcoma breakpoint region 1; Gtf2b, general transcription factor IIB; Nhrnpc, heterogeneous nuclear ribonucleoprotein C; Ncl, nucleolin; Rpb1, polymerase (RNA) II (DNA-directed) polypeptide A (POLR2A); Rpb6, polymerase (RNA) II (DNA-directed) polypeptide F (POLR2F); Rpb8, polymerase (RNA) II (DNA-directed) polypeptide H (POLR2H).

Rpb1 is present in cell nuclei in two forms: the hypophosphorylated (IIa) and hyperphosphorylated (IIo) forms (8). The carboxy-terminal domain (CTD) of Rpb1 contains multiple heptad repeats, $Y_1S_2P_3T_4S_5P_6S_7$, whose phosphorylation regulates different steps of transcription. Only the hypophosphorylated form can bind to the promoter, and a large fraction of this form is not

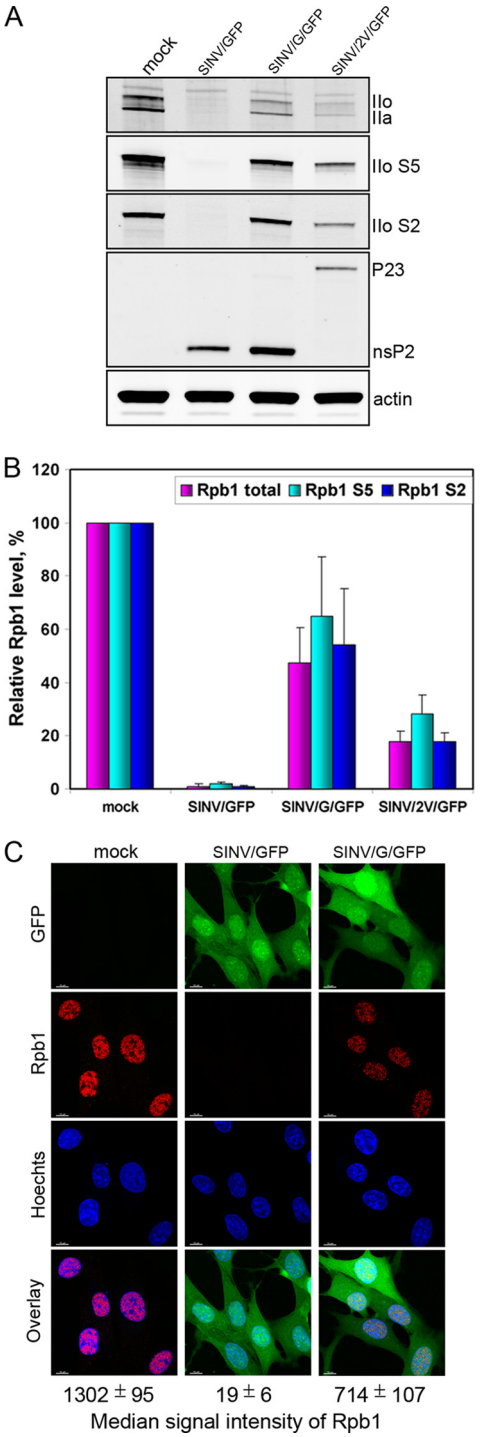

FIG 2 SINV/GFP induces degradation of all forms of Rpb1. (A) BHK-21 cells were infected as described in the legend to Fig. 1. Lysates were prepared at 7 h p.i. and analyzed by immunoblotting using antibodies recognizing different forms of Rpb1: total Rpb1 (IIa and IIo) and Rpb1 phosphorylated at CTD heptate residue serine 5 (IIo S5) or serine 2 (IIo S2). SINV nsP2 was detected by rabbit polyclonal antibodies. (B) Quantitative analysis of immunoblots presented in panel A. Mean values of three experiments and standard deviations (SDs) are presented. (C) Immunofluorescence analysis of total Rpb1 in BHK-21 cells at 7 h p.i. with different SINVs. GFP expression indicates virus replication. Images represent multiple-intensity projections of six optical sections. The median signal intensities of Rpb1-specific nuclear fluorescence were measured for at least 30 randomly selected cells. Bars, 10 μm.

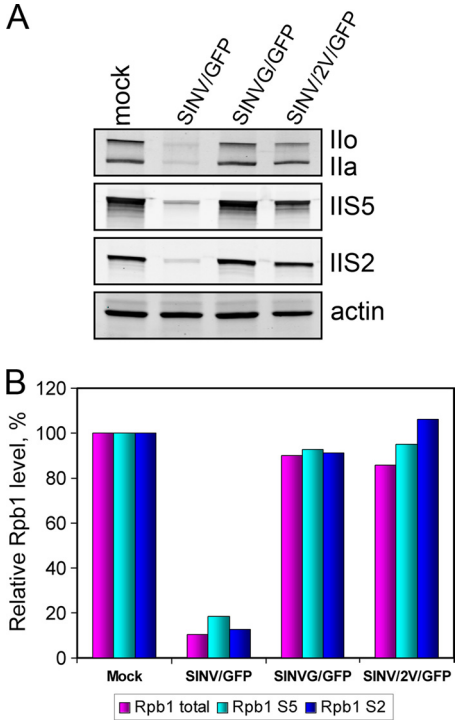

FIG 3 SINV/GFP, but not variants with mutated nsP2, induces Rpb1 degradation in NIH 3T3 cells. (A) NIH 3T3 cells were infected with SINV/GFP or attenuated mutants SINV/G/GFP and SINV/2V/GFP at an MOI of 20 PFU/cell. Lysates were prepared at 7 h p.i. and analyzed by immunoblotting using antibodies recognizing different forms of Rpb1 (see the legend to Fig. 2 for details). (B) Quantitative analysis of immunoblots presented in panel A.

engaged in RNA transcription. Phosphorylation of serine 5 (S5) of CTD heptads is a prerequisite of the initiation of transcription, while phosphorylation of serine 2 (S2), in turn, induces the formation of the elongating RNAPII complex. The inhibition of CTD phosphorylation can pause transcription, which causes Rpb1 degradation (24). Therefore, we next assessed the levels of total Rpb1 and its two hyperphosphorylated forms, S2 and S5, at different times postinfection. By 2 h postinfection, in BHK-21 cells infected with wt SINV/GFP, the total level of Rpb1 was already lower than 60%, and by 4 h, it was present at about 10% of its normal level (Fig. 4A). Importantly, at 2 h postinfection, wt nsP2 was present in the cells in less than 10% of its maximum level, which was normally achieved between 4 and 6 h postinfection. However, even at this low concentration, it was capable of causing a strong decrease in the Rpb1 level. The loss of Rbp1 hyperphosphorylated forms proceeded with similar kinetics, and both forms became completely undetectable by 8 h postinfection. Importantly, in cells infected with wt SINV/GFP, we did not detect preferential disappearance of a particular phosphorylated form and thus concluded that SINV did not inhibit any specific Rpb1 phosphorylation pathway. In SINV/G/GFP-infected cells, the level of total Rpb1 decreased at a drastically lower rate and was reduced to 37% of its initial concentration by 8 h postinfection, at which time no Rpb1 could be detected in SINV/GFP-infected cells (Fig. 4B). The reduction in the levels of hyperphosphorylated forms was even less evident, suggesting that either the larger fraction of Rpb1 was engaged in transcription or its phosphorylation level was higher. This correlates well with the observation that SINV/G/GFP infec-

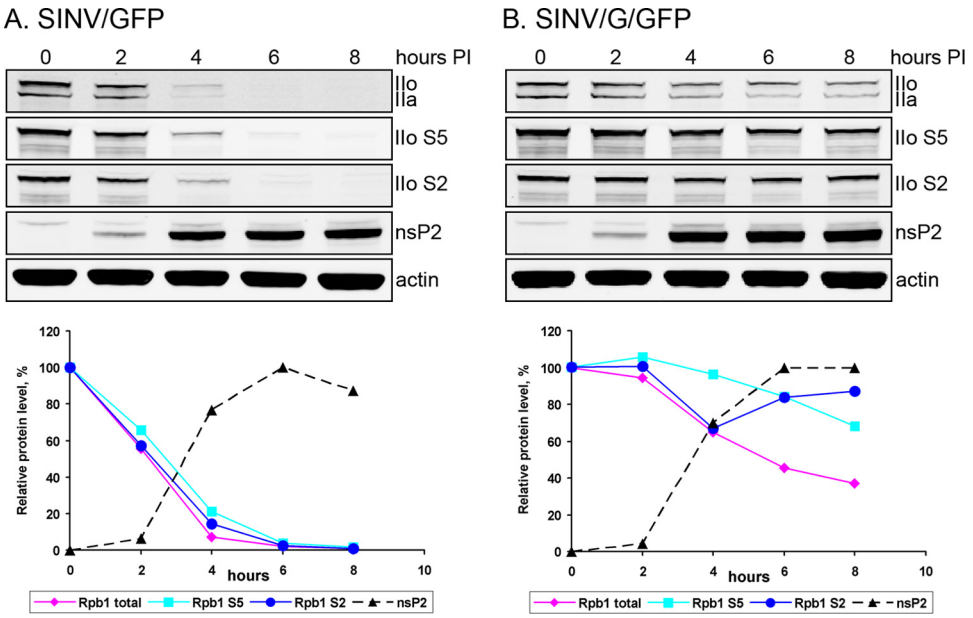

FIG 4 Rpb1 is completely degraded within the first 8 h postinfection with SINV encoding wt nsP2. BHK-21 cells were infected with SINV/GFP (A) or SINV/G/GFP (B) at an MOI of 20 PFU/cell. Cells were harvested at different times postinfection, and levels of Rpb1 and nsP2 were analyzed by immunoblotting with antibodies against different forms of Rpb1 and SINV nsP2. Immunoblots were quantitated as described in Materials and Methods.

tion induces a robust antiviral response that is accompanied by activation of the transcription of a large number of cellular genes (19).

SINV infection does not induce degradation of Rpb1 in mosquito cells. The characteristic feature of alphavirus infection is their acute infection of vertebrate hosts and their persistent replication in mosquito vectors (33). These two scenarios of virus replication are mirrored *in vitro*: in the cells of vertebrate origin, alphaviruses induce robust CPE within 24 to 48 h postinfection, but they persistently replicate in the cells of mosquito origin. The noncytopathic replication in mosquito cells is suggestive of the viruses' inability to interfere with the nuclear functions of insect cells. To test this hypothesis, we analyzed changes in Rpb1 levels in mosquito C7/10 cells after infection with SINV/GFP or SINV/G/GFP viruses. The antibodies directed to the N terminus of mammalian Rpb1 did not recognize the mosquito homolog. Thus, we applied only antibodies specific to the hyperphosphorylated form of Rpb1. Within 48 h postinfection, the amount of Rpb1 had decreased less than 2-fold in infected cells (Fig. 5A and B). Moreover, the small changes detected in the levels of Rbp1 were essentially

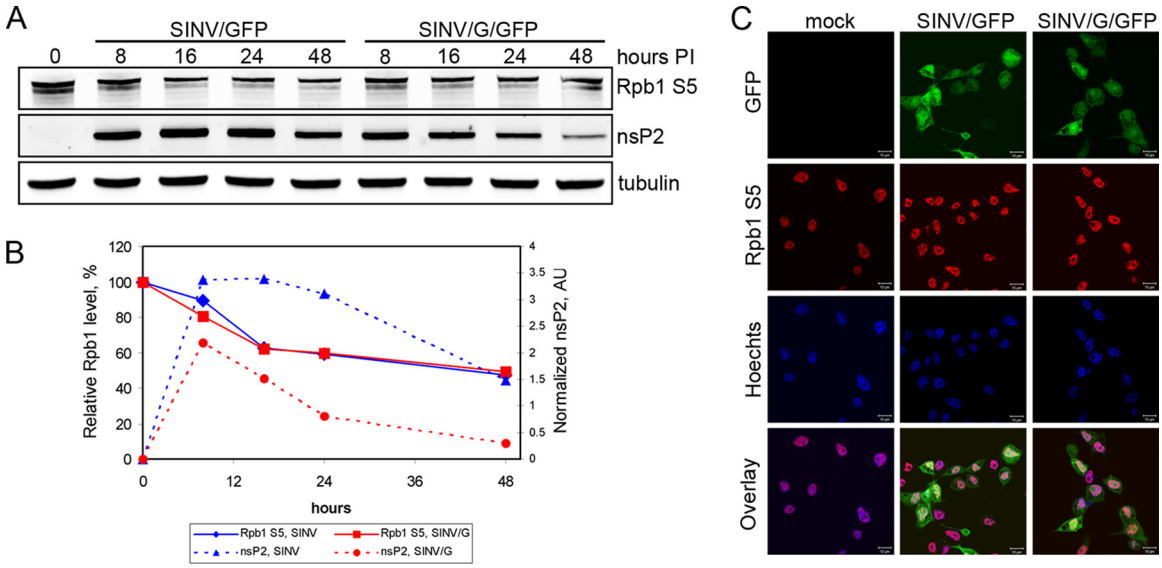

FIG 5 SINV infection does not induce Rpb1 degradation in mosquito cells. (A) C7/10 cells were infected with SINV/GFP or SINV/G/GFP. Cells were collected at different times postinfection and analyzed by immunoblotting using antibodies specific to hyperphosphorylated Rpb1 and SINV nsP2. (B) The results of quantitative analysis of immunoblots presented in panel A. (C) Immunofluorescence analysis of total Rpb1 in C7/10 cells at 24 h p.i. GFP expression indicates virus-infected cells. Images represent multiple-intensity projections of six optical sections. Bars, 10 μm.

the same in SINV/GFP- and SINV/G/GFP-infected cells. Similarly, Rpb1 was easily detected by indirect immunofluorescence in C7/10 cells infected with either wt SINV or mutant SINV/G virus (Fig. 5C). Thus, SINV replication does not induce Rpb1 degradation in mosquito cells.

nsP2 proteins of the OW alphaviruses are direct inducers of Rbp1 degradation in vertebrate cells. We next investigated whether SINV nsP2 is directly responsible for Rpb1 degradation and whether nsP2 proteins of other OW alphaviruses exhibit the same activity. We have previously demonstrated that wt SINV nsP2, transiently expressed from plasmid DNA or VEEV-based replicons, efficiently inhibits cellular transcription (14). In this study, we applied a similar approach to express nsP2 proteins of SINV and other, more pathogenic, OW alphaviruses. The nsP2-coding sequences of SINV, SFV, and CHIKV were cloned into VEEV replicons as GFP fusions (Fig. 6A). Previously, we had demonstrated that the addition of GFP to the carboxy terminus, but not to the amino terminus, of the SINV nsP2 protein had no negative effect on its transport to the nucleus and its ability to interfere with cellular transcription (14). In addition, nsP2 proteins were expressed as fusions with the ubiquitin gene (Ubi) and thus after processing, the released free form of nsP2 had its natural amino acids as a first amino acid. Its presence is critical for nsP2 function.

The nsP2/GFP-encoding VEEV replicons were packaged into infectious viral particles, which were used to infect BHK-21 cells at the same multiplicity of infection (MOI) as described in Materials and Methods. At 8 h postinfection, the levels of the Rpb1 were analyzed by immunoblotting (Fig. 6B and C). Expression of nsP2 fusion proteins derived from different OW alphaviruses led to a decrease in total Rpb1 to a level below 10% of that found in cells infected with control VEEV replicon expressing GFP alone. The observed inability of expressed nsP2 proteins to mediate complete degradation of Rpb1 was due to their strong negative effect on the efficiency of replicon packaging, resulting in low titers of replicon-containing particles. As a result, based on GFP detection results, we were unable to infect more than 90% of cells in the monolayers. Nevertheless, the ultimate levels of Rpb1 degradation were found to be similar for all tested nsP2 proteins.

These results imply that a majority if not all of the OW alphaviruses are likely to use the same mechanism of transcriptional shutoff, which is mediated by degradation of the catalytic subunit of RNAPII, Rpb1. Importantly, the control VEErep/GFP replicon, expressing a VEEV-specific nsP2 in addition to GFP, did not induce similar degradation of Rpb1, confirming that VEEV and most likely other NW alphaviruses do not utilize the nsP2-dependent mechanism for transcription inhibition. We also performed a quantitative analysis of nsP2-GFP expression using a monoclonal antibody that recognizes nsP2 protein from all of the tested alphaviruses. The expression of nsP2-GFP proteins was found to be essentially the same as the expression of VEEV nsP2 encoded in the nonstructural polyprotein of the replicon (Fig. 6D). Thus, the difference in the effects of OW and NW alphavirus nsP2 proteins on the degradation of Rpb1 was not due to differences in the expression levels.

NTPase activity of SINV nsP2 is required for induction of Rpb1 degradation. The alphavirus nsP2 is a multifunctional protein. It exhibits several enzymatic activities and has at least four functional domains: the N domain helicase, protease, and C domain (Fig. 7A). The protease domain is responsible for processing

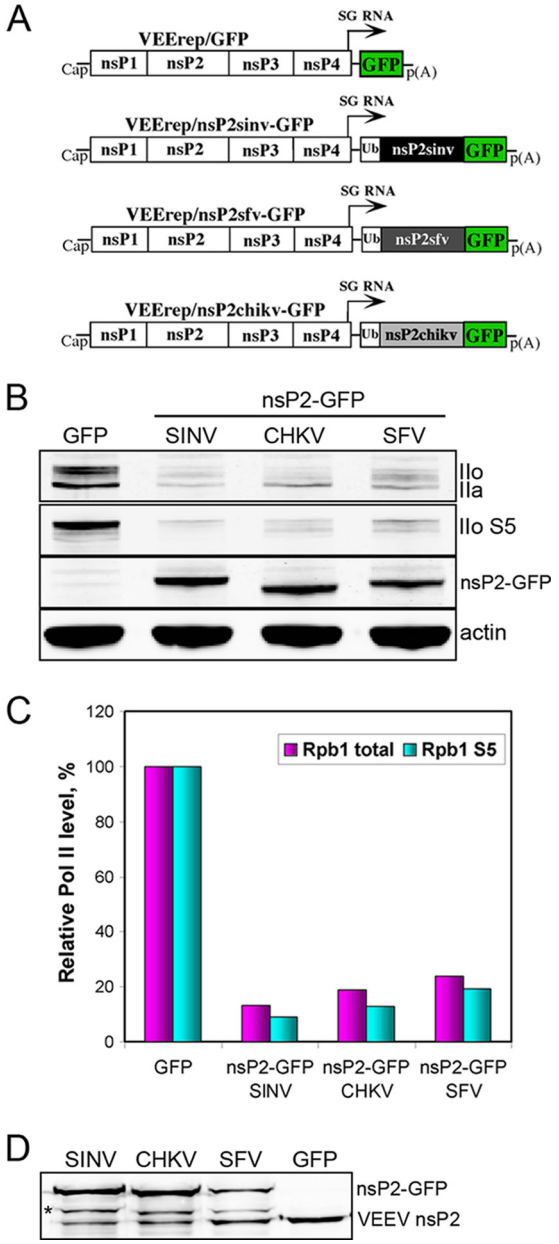

FIG 6 nsP2 proteins of different OW alphaviruses induce Rpb1 degradation with similar efficiencies. (A) Schematic representation of noncytopathic VEEV replicons encoding GFP fusions of nsP2 proteins derived from different OW alphaviruses. (B) Analysis of Rpb1 levels at 8 h p.i. with the indicated replicons. GFP-expressing replicon VEErep/GFP was used as a control. nsP2-GFP fusion proteins were detected with anti-GFP antibodies. (C) Results of quantitative analysis of immunoblots presented in panel B. (D) Comparative analysis of expression levels of different, heterologous nsP2 proteins and VEEV-specific nsP2 using mouse MAbs recognizing nsP2 proteins of several alphaviruses. The asterisk marks the product of nsP2-GFP degradation.

of the viral nonstructural polyproteins P123 and P1234 and was shown to function as a protease even in the absence of the N and helicase domains (35). It was logical to expect that nsP2 utilizes its protease activity to cleave Rpb1. However, we previously demonstrated that the inactivation of nsP2 protease by point mutations in the active site does not interfere with the protein's ability to inhibit cellular transcription (14). Moreover, using immunoblot-

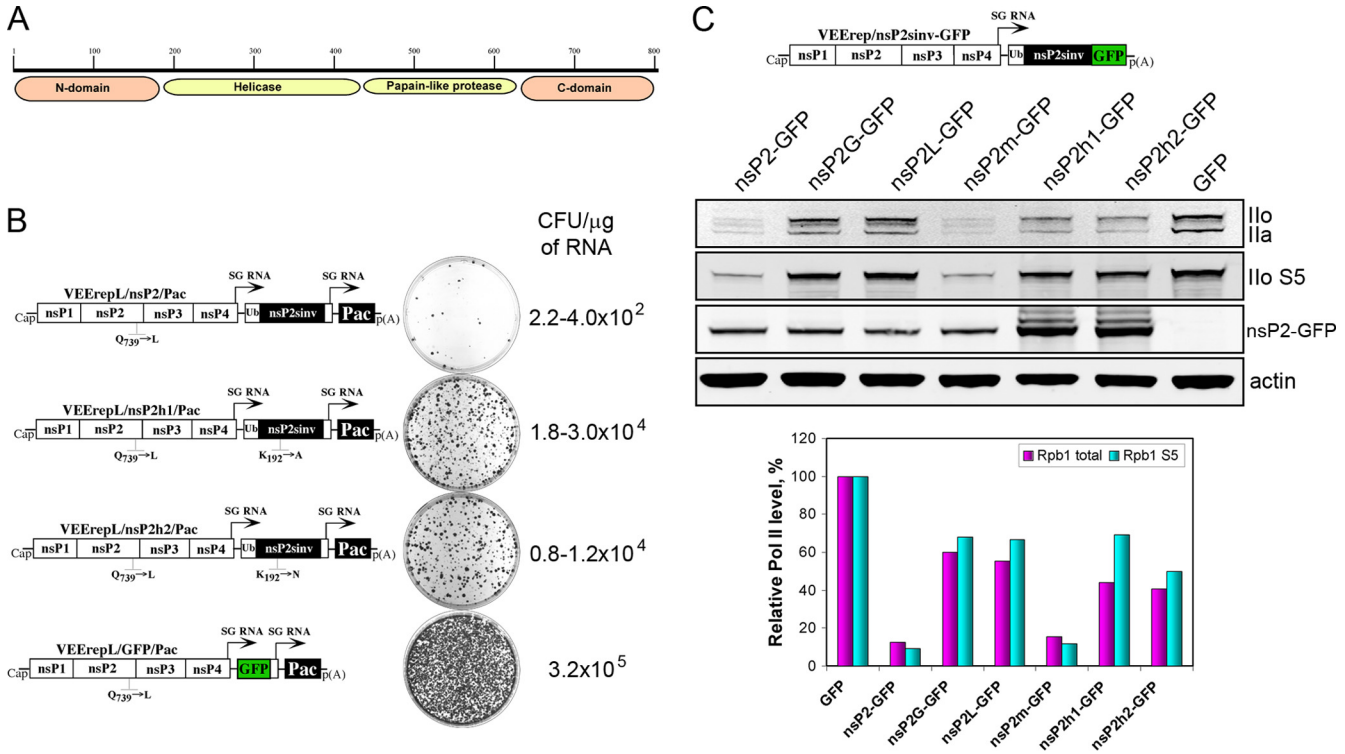

FIG 7 NTPase, but not protease activity of SINV nsP2, is essential for Rpb1 degradation. (A) Schematic representation of functional nsP2 domains. (B) Comparative analysis of the efficiency of Pur^r colony formation by VEEV replicons, expressing wt SINV nsP2, its two helicase mutants, and GFP. (C) Analysis of Rpb1 degradation in BHK-21 cells expressing GFP fusions of wt SINV nsP2 or its mutants. Schematic representation of the VEEV-based replicon is shown on the top. (Bottom) Results of quantitative analysis of the immunoblots.

ting, we did not detect any sequence-specific degradation products of Rpb1 that could be expected in the case of protease-specific cleavage. The C domain has an *S*-adenosylmethionine (SAM)-dependent methyltransferase-like fold, which was predicted to be enzymatically nonfunctional (31). The majority of mutations that abolish nsP2's ability to inhibit cellular transcription and CPE development were identified within this domain (1, 11). Additionally, in the current study, we demonstrated that the SINV/G/GFP mutant containing a point mutation in the C domain was incapable of inducing Rpb1 degradation. However, the isolated C domain or its combination with other domains did not inhibit cellular transcription, suggesting a complex interaction of all of the nsP2 domains (11). Interestingly, we have recently demonstrated that small insertions in the helicase domain can have a strong negative effect on SINV nsP2 cytotoxicity (11). Therefore, we investigated whether the helicase domain-associated enzymatic activity is directly involved in Rpb1 degradation and CPE development.

The helicase and NTPase activities of alphavirus nsP2 were previously predicted and later biochemically confirmed (16). All of the known helicases contain highly conserved lysine in a so-called Walker A motif, and its replacement has deleterious effects on both NTPase and helicase activities (9, 17). Thus, to evaluate the role of the helicase domain in the nsP2-induced cell death, we replaced lysine 192 in SINV nsP2 with alanine (nsP2h1) or asparagine (nsP2h2) and expressed this protein from the noncytopathic VEEV replicon (VEErepL). This VEEV replicon is capable of establishing persistent replication in BHK-21 cells unless it expresses a cytotoxic protein from the subgenomic RNA (29). The replicons were designed as double subgenomic constructs: the first subgenomic promoter drives the expression of different forms of nsP2 or GFP. The second subgenomic RNA encodes puromycin acetyltransferase (Pac) (Fig. 7B). BHK-21 cells were electroporated with the *in vitro*-synthesized replicons' RNA and treated with puromycin to select the Pac-expressing cells containing replicons. In this assay, essentially all of the cells containing replicons encoding noncytotoxic proteins, such as GFP, establish Pur^r colonies within 6 days posttransfection, while cells with replicons expressing cytotoxic proteins die within 2 days posttransfection. In the repeated experiments, the control replicon expressing GFP (VEErepL/GFP/Pac) produced 3.2×10^5 colonies per μg of transfected RNA, while cells transfected with replicon expressing wt SINV nsP2 (VEErepL/nsP2/Pac) formed 3 orders of magnitude fewer colonies. These colonies contained VEErepL/nsP2/Pac replicons, in which SINV nsP2 contained either frameshift mutations, which led to expression of truncated protein, or point mutations in the C domain (reference 14 and data not shown). Most importantly, transfection of the cells with replicons expressing nsP2 with mutations in the Walker A motif of the helicase domain (VEErepL/nsP2h1/Pac or VEErepL/nsP2h2/Pac) produced Pur^r colonies 100-fold more efficiently than seen for those expressing wt nsP2, almost as efficiently as the GFP-expressing replicon. These data strongly suggested that the NTPase activity of nsP2 is required for cytopathogenicity.

The cytopathic effect of nsP2 could result from its ability to inhibit either cellular transcription or translation. To distinguish between these possibilities, we next analyzed whether helicase mutants were capable of inducing degradation of Rpb1. Both helicase

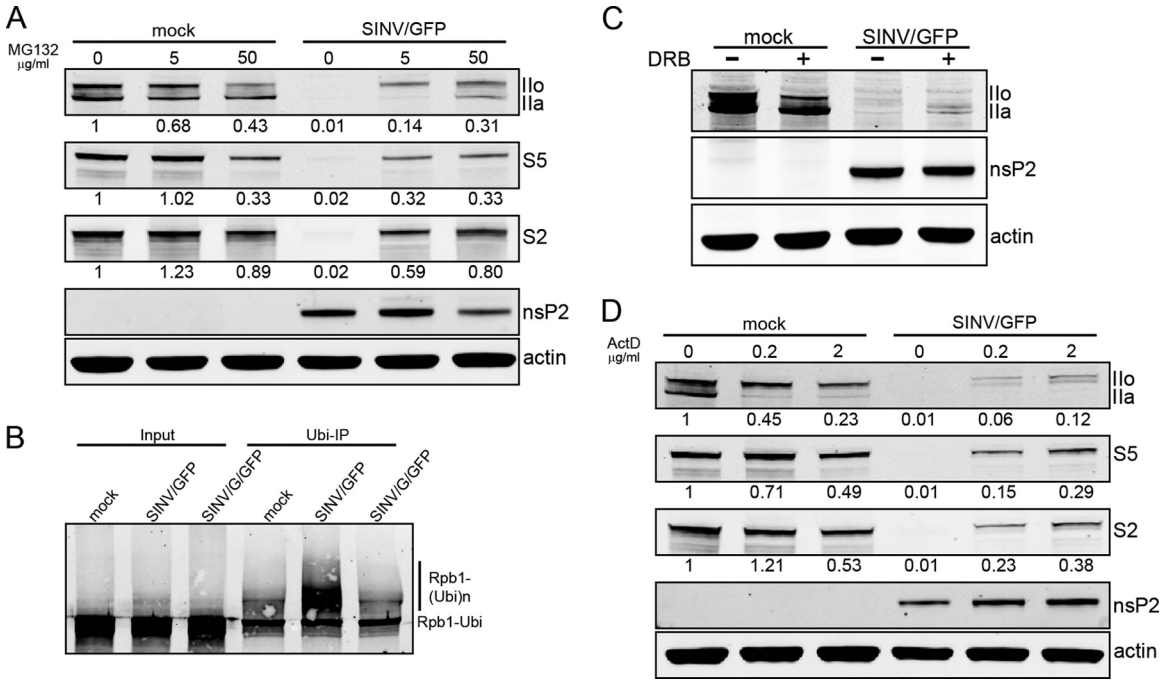

FIG 8 nsP2-induced Rpb1 degradation proceeds through its ubiquitination. (A) SINV/GFP- or mock-infected BHK-21 cells were treated with the indicated concentrations of the proteasome inhibitor MG132 for the entire duration of the infection (6 h). The presence of different forms of Rbp1 was analyzed by immunoblotting. (B) At 2 h p.i., ubiquitinated proteins from mock-, SINV/GFP-, or SINV/G/GFP-infected BHK-21 cells were precipitated with GSK-Dsk2 beads and probed for Rpb1 as described in Materials and Methods. (C and D) Mock- and SINV/GFP-infected BHK-21 cells were treated with 100 μg/ml of DRB (C) or the indicated concentrations of ActD (D) starting from 1 h or 0 h p.i., respectively. Lysates were prepared at 6 h p.i. and analyzed for the presence of Rpb1. Numbers indicate the normalized signal intensities relative to that in untreated, uninfected cells.

mutants were cloned into VEEV replicons as GFP fusions (Fig. 7C, top). These and three control replicons, expressing nsP2 with mutations in the protease (nsP2m-GFP, C481 to S) or C (nsP2G-GFP and nsP2L-GFP, P726 to G or L) domain, were packaged into infectious viral particles, which were used to infect BHK-21 cells at the same MOI. Cells were collected at 8 h postinfection, and Rpb1 levels were assessed by quantitative immunoblotting using two different Rpb1-specific antibodies (Fig. 7C). As expected, the replicons expressing SINV nsP2 with mutations in the C-terminal domain were very inefficient in the induction of Rpb1 degradation. The nsP2 containing an inactive protease domain, expressed by VEErep/nsP2m-GFP, induced degradation of Rpb1 as efficiently as the unmodified nsP2. This result confirmed that nsP2 protease activity is not required for the degradation of Rpb1 and inhibition of cellular transcription. In contrast, the point mutations in the Walker A motif of SINV nsP2 helicase domain (VEErep/nsP2h1-GFP and VEErep/nsP2h2-GFP) strongly reduced the nsP2-dependent degradation of Rpb1. This result demonstrated that a functional helicase domain is involved in the nsP2-mediated degradation of Rpb1.

SINV nsP2 induces degradation of Rpb1 through a proteasome-dependent pathway. The above-described data demonstrated that nsP2 proteins of OW alphaviruses induce rapid degradation of Rpb1 independently of their protease activity. Thus, we argued that this protein could exploit already existing cellular mechanisms to degrade this critical RNAPII component. For example, cells utilize a specific mechanism for removal of stalled elongating RNAPII complex from damaged DNA during transcription-coupled repair (TCR) (34). When the elongating RNAPII complex meets the obstruction caused by bulky DNA lesions, Rpb1 is ubiquitinated and quickly degraded by proteasomes. Similarly, stalled Rpb1 poisoned by the specific inhibitor α-amanitin is degraded through the ubiquitin-dependent pathway (28). If nsP2 utilizes a similar mechanism, one would expect that Rpb1 degradation could be prevented by proteasome inhibitors. To test this hypothesis, we added proteasome inhibitor MG132 to the culture medium at the beginning of SINV/GFP infection, and the Rpb1 level was examined at 6 h postinfection. Partial protection of Rpb1 was evident even at low concentrations of MG132 (Fig. 8A). In the presence of 50 μM MG132, the level of Rpb1 was found to be similar in SINV/GFP- and mock-infected cells. Accordingly, the indirect immunofluorescence confirmed the presence of Rpb1 in the nuclei of SINV/GFP-infected cells treated with MG132 (data not shown). These results strongly indicate that SINV-induced Rpb1 degradation is proteasome dependent and can be prevented by proteasome inhibitors.

Next, we examined whether SINV replication causes an increase in the level of polyubiquitinated Rpb1. BHK-21 cells were collected at 2 h postinfection, and a pool of ubiquitinated proteins was precipitated using GSK-Dsk2 beads that specifically bind ubiquitin and ubiquitinated proteins (2). The amount of ubiquitinated Rpb1 was assessed by immunoblotting (Fig. 8B). Samples derived from SINV/GFP-infected cells at this early time postinfection demonstrated the presence of polyubiquitinated Rpb1 at levels higher (~3.5-fold) than that detected in the samples of mock- or SINV/G/GFP-infected cells. This was a strong indication that nsP2 directs Rbp1 into the ubiquitin-dependent degradation pathway.

During TCR, stalled Rpb1 is targeted to ubiquitin-dependent degradation only in the context of an elongating RNAPII complex,

when the CTD is hyperphosphorylated at serine 2 (21). To test if this requirement is true for nsP2-mediated Rpb1 degradation, we applied the nucleoside analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) to inhibit the formation of elongating RNAPII complexes during SINV infection. Addition of DRB to the cells prevents the hyperphosphorylation of Rpb1 and RNAPII transition from the initiating to the elongating form (7). BHK-21 cells infected with SINV/GFP or mock infected were treated with 100 μM DRB for the duration of infection, and the level of Rpb1 was evaluated at 6 h postinfection. In uninfected cells, the amount of the hyperphosphorylated form was dramatically reduced, demonstrating that BHK-21 cells are sensitive to DRB (Fig. 8C). However, no recovery of Rpb1 was observed in SINV/GFP-infected cells upon treatment with DRB. This result suggested that nsP2 does not specifically target the elongating form of Rpb1.

Next, we examined whether the inhibition of transcription by a different inhibitor, actinomycin D (ActD), would affect Rpb1 degradation in SINV-infected cells. Although ActD stalls the RNAPII complex, it acts as a reversible inhibitor and does not induce immediate degradation of Rpb1. Inhibition of cellular transcription with ActD in SINVG/GFP-infected cells reduced Rpb1 degradation, and this effect was dependent on ActD concentration (Fig. 8D). At 6 h postinfection, in infected cells treated with 2 μg/ml of ActD, Rpb1 was retained at 12% of its normal level, while only ~1% of Rpb1 was detected in the mock-treated, infected cells. This result suggested that ActD inhibited access of nsP2 to the RNAPII complex.

Altogether, these data demonstrate that nsP2 proteins of OW alphaviruses induce ubiquitination of Rpb1 followed by its rapid degradation by proteasomes.

Wild-type, not mutant, nsP2 causes a redistribution of nuclear RNA-binding proteins. It has been proposed that SINV replication induces the relocalization of several RNA-binding proteins, such as hnRNP A1 and hnRNP K, from the nucleus to the cytoplasm to facilitate virus replication (6, 20, 25). However, these studies did not consider the effect of global inhibition of cellular transcription on the nuclear structure. It has been shown that the inhibition of cellular transcription by several drugs leads to dramatic changes in nuclear morphology and redistribution of nuclear proteins, including a shift from mainly nuclear to cytoplasmic localization of a large number of shuttling RNA-binding proteins, whose nuclear import is mediated by transportin 1 or transportin SR (26, 27, 30, 32). Therefore, the redistribution of nuclear RNA-binding proteins during SINV infection might simply be a result of transcription inhibition and not directly regulated by a replicating virus.

To test this hypothesis, we evaluated the cellular distribution of several RNA-binding proteins in cells infected with SINV/GFP or SINV/G/GFP viruses. At 7 h after SINV/GFP infection, when transcription is already severely downregulated and no Rpb1 is present, we observed dramatic relocalization of hnRNP A0 and hnRNP A1 (Fig. 9A and B). The cytoplasmic accumulation of hnRNP K was less evident, which correlates with its ability to translocate to nuclei not only by the transportin 1-mediated pathway but also via the classical nuclear transport pathway (Fig. 9C) (26). No alteration in the intracellular distribution of hnRNP C1/C2 was detected (data not shown) as was previously reported in the case of ActD-mediated transcription inhibition (26). In contrast, SINV/G/GFP virus, which does not inhibit transcription, had no effect on the cellular distribution of any analyzed

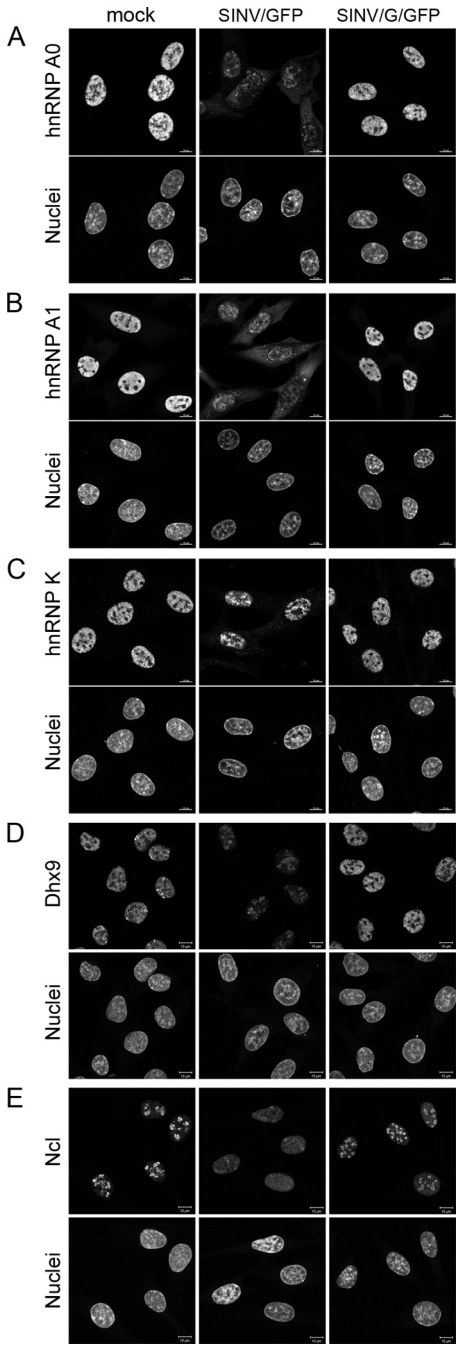

FIG 9 Relocalization of several RNA-binding proteins occurs only in the cells infected with the wt nsP2-encoding SINV. Mock-, SINV/GFP-, or SINV/G/GFP-infected BHK-21 cells were immunostained with hnRNP A0 (A)-, hnRNP A1 (B)-, hnRNP K (C)-, Dhx9 (D)- or Ncl (E)-specific Abs at 7 h p.i. Infection of all of the imaged cells was confirmed by detection of GFP, expressed by replicating virus (images are not shown).

hnRNPs. Considering that SINV/G/GFP replicates in BHK-21 cells almost as efficiently as SINV/GFP (10) and that its replication proceeds in the absence of relocalization of the nuclear RNA-binding proteins, it seems unlikely that their redistribution during SINV infection is essential for SINV replication. We have also found that the cellular distribution of several other nuclear proteins was strongly changed in the cells infected by SINV/GFP but

not SINV/G/GFP (Fig. 9D and E). Thus, we concluded that relocalization of nuclear proteins during wt SINV infection is not required for virus replication *per se* but most likely represents a consequence of cellular transcription inhibition.

DISCUSSION

The hallmark of alphavirus replication in vertebrate cells is the rapid development of CPE, which occurs within 24 to 48 h postinfection and results in cell death. We and others have previously demonstrated that for the large group of alphaviruses, which are distributed mostly in the Old World, CPE development depends on the expression of the wt form of the viral nonstructural protein nsP2 (5, 10, 14). This protein exhibits nuclear localization and causes cell death by inducing rapid and global inhibition of cellular transcription. The ability of a single viral protein to turn off the entire cellular transcriptional machinery is very intriguing but also a very difficult phenomenon to study, since the same nsP2 protein has numerous functions in virus replication. The previous conventional approaches, which were based on extensive mutagenesis, did not dissect the mechanism of nsP2-induced transcription inhibition but suggested that multiple domains of nsP2 function cooperatively, and mutations in at least three domains affect SINV nsP2's inhibitory activity (11).

In this study, we have discovered that in vertebrate cells nsP2 proteins of the OW alphaviruses induce rapid degradation of a large, catalytic subunit of the RNAPII complex, Rpb1. This new nsP2 function was detected in both virus-infected cells and those expressing nsP2 protein alone. We demonstrated that Rpb1 degradation can be induced by nsP2 proteins derived from several OW alphaviruses and thus concluded that most likely all of the OW alphaviruses inhibit cellular transcription by inducing Rpb1 degradation.

Interestingly, nsP2-induced degradation of Rpb1 does not depend on its protease activity. Instead, by inducing Rpb1 ubiquitination, nsP2 utilizes an existing cellular protein degradation pathway. In that, nsP2-mediated Rpb1 degradation is similar to the transcription-coupled repair (TCR) pathway. The key step of the TCR pathway is a ubiquitination of the catalytic subunit of RNAPII, Rpb1, followed by its degradation by the RNAPII-associated proteasomes, which allows rapid repair of the transcribing DNA strand (21). The characteristic feature of TCR is that it recognizes stalled RNAPII complexes only in the elongating form. This preferential targeting of the elongating complex is probably due to the requirement of CTD-specific serine 2 phosphorylation for the recognition of the stalled polymerase, though the exact mechanism of the stalled polymerase detection remains poorly understood. Similarity between nsP2-mediated Rpb1 degradation and TCR suggests that nsP2 might also induce degradation by stalling the RNAPII complex. Requirement of the nsP2 helicase domain for Rpb1 degradation, which could mediate binding of nsP2 to DNA or modification of the DNA, further supports this possibility. However, we found that inhibition of the elongating RNAPII complex formation by DRB does not abrogate Rpb1 degradation. In addition, in this scenario, the RNAPII should also be stalled and should induce Rpb1 degradation in insect cells, but this is not the case. Thus, the mechanism utilized by nsP2 for Rpb1 ubiquitination appears to be more complex and cannot be explained by merely stalling RNAPII.

Our previous data suggested the involvement of several nsP2 domains in transcriptional inhibition. The results of this study confirmed that at least two nsP2 domains are required for efficient degradation of Rpb1. The mutations in a Walker A motif of the helicase domain severely impaired SINV nsP2's ability to induce Rpb1 degradation. Helicase activity of nsP2 is poorly studied; however, biochemical analysis suggests that similar to well-characterized helicases of other RNA viruses, alphavirus nsP2 binds to double-stranded DNA (dsDNA) (unpublished data). Nevertheless, the finding of the requirement of NTPase activity for Rbp1 degradation was very surprising, since it implied that helicase domain-associated activities might play an important role(s) in nsP2-mediated Rpb1 degradation. A possible explanation for this phenomenon might be that, as known for some of the nucleic acid-binding proteins, nsP2 requires active NTPase for tight binding to nucleic acids or locking them in specific conformations. This hypothesis needs further experimental support. However, regardless of the mechanism, we hypothesize that nsP2-DNA binding is critical for the nuclear function of this protein. This conclusion is additionally supported by our analysis of Rbp1 degradation in the infected cells, which were additionally treated with ActD. The latter treatment had no effect on virus replication, but it partially prevented Rpb1 degradation. The protective effect of ActD can likely be explained by its competition with nsP2 for DNA binding and/or altering the conformation of the RNAPII complex, thus preventing nsP2 interaction with Rpb1. Hence, this result implies that nsP2 targets Rpb1 in the context of an RNAPII complex associated with DNA. Thus, we propose that that protein-DNA binding is a prerequisite for nsP2-mediated proteolysis of this RNAPII subunit. However, further biochemical analysis of nsP2 is needed to support this hypothesis.

The role of the C domain of SINV nsP2 in virus-induced transcriptional inhibition has been well demonstrated (10, 14). Here, we found that the previously described attenuating mutations in the C domain also made nsP2 protein incapable of inducing Rbp1 degradation. All of the mutations in the C domain, which abolished its transcription inhibitory activity, but not translocation into the nuclei, were confined to the same area on the protein surface. Although we cannot completely rule out the possibility that the C domain can potentially contribute to the nsP2-DNA binding, we favor the idea that it mediates a protein-protein interaction with the RNAPII complex.

Based on the presented data, we propose that at least two nsP2 domains are essential for Rpb1 ubiquitination and that each domain works cooperatively, mediating different interactions to induce Rpb1 ubiquitination. First, nsP2 binds to cellular DNA through its helicase domain (Fig. 10). Next, (i) nsP2 binds to the RNAPII complex through its C domain and modifies the RNAPII complex in such a way that it can be recognized by ubiquitin ligases leading to Rpb1 ubiquitination or, alternatively, (ii) nsP2 might directly facilitate binding of the ubiquitin ligases to the RNAPII complex. Both mechanisms imply the interaction of nsP2 with some proteins of the RNAPII complex. However, so far, we have been unable to detect direct interaction of nsP2 with proteins of the RNAPII complex in coimmunoprecipitation (co-IP) experiments (data not shown). This could be a result of the rapid dissociation of the complex upon the degradation of ubiquitinated Rpb1. In addition, nsP2 appears to interact with many cellular proteins, which makes it difficult to identify a specific interaction involved in the Rpb1 ubiquitination process.

The outcome of the virus infection strongly depends on the ability of the virus to escape the cellular antiviral response. Ac-

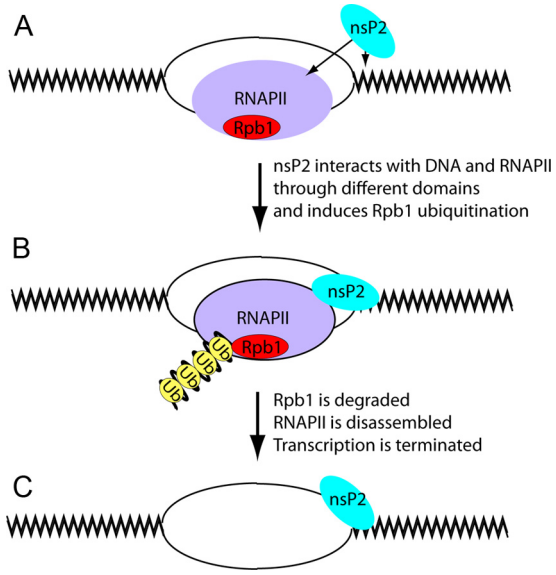

FIG 10 Proposed model of nsP2-mediated Rpb1 degradation. (A) nsP2 protein produced by the Old World alphaviruses translocates to the nucleus and binds to DNA. This interaction is mediated by its helicase domain and is most likely not sequence specific. When translocation of RNAPII is stalled at the nsP2-DNA complex, nsP2 interacts with RNAPII through its C domain. (B) This interaction induces polyubiquitination of Rpb1 followed by its degradation and disassembly of the RNAPII complex (C).

cordingly, viruses have developed numerous approaches to inhibit the cellular response. The alphaviruses are no exception and they evade the antiviral response by rapid inhibition of cellular transcription, which completely prevents the activation of antiviral genes. This approach appears to be very efficient, as the alphaviruses have independently evolved two different mechanisms to achieve it. The NW alphaviruses utilize their capsid protein for inhibition of nucleocytoplasmic traffic, which, in turn, leads to the inhibition of transcription (3), and here we demonstrate that the OW alphaviruses utilize their nonstructural protein nsP2 to rapidly degrade a catalytic subunit of the RNAPII complex. Interestingly, neither approach functions in insect cells, in which alphaviruses establish noncytopathic, persistent infection. The ability of these viruses to persist in insects is critical for their natural circulation. Thus, alphaviruses appear to exploit the difference between vertebrate and insect cells for efficient inactivation of antiviral response in the vertebrate host without affecting insect vectors.

Alphaviruses are not the only group of RNA viruses that directly target cellular transcription in order to prevent activation of the antiviral response. Another example is the *Bunyaviridae* family. Different members of this family inhibit functions of different components of the RNAPII complex by utilizing the nonstructural protein NSs. The NSs proteins have no or low homology between different members and appear to be dispensable for viral RNA replication. Rift Valley virus NSs induces degradation of the p44 subunit of the TNFIIH complex (4). Bunyamwera virus NSs binds to MED8 and inhibits the phosphorylation of CTD at serine 2 followed by Rpb1 degradation (24). Finally, La Crosse virus NSs appears to induce Rpb1 degradation by an unknown mechanism (36). Similarly, several members of *Picornaviridae* family globally inhibit cellular transcription (38). Thus, at least three groups of the viruses, alpha-, bunya-, and picornaviruses, have evolved different mechanisms of inhibition of the same process: transcription of cellular mRNAs. This implies that the inhibition of cellular transcription is a highly efficient means of downregulating the cellular antiviral response and is utilized effectively by rapidly replicating cytoplasmic RNA viruses.

In conclusion, we have defined the mechanism of the global transcriptional inhibition utilized by the OW alphaviruses. We have demonstrated that nsP2 protein induces ubiquitination of Rpb1, a catalytic subunit of the RNAPII complex, which leads to its rapid degradation. By 6 h postinfection, no Rpb1 can be detected in infected cells. This Rpb1 degradation occurs before other cellular modifications, such as apoptosis, autophagy, or inhibition of STAT1 phosphorylation, can be detected. Thus, nsP2-mediated Rpb1 degradation appears to be the main mechanism utilized by OW alphaviruses for evasion of the cellular antiviral response.

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REFERENCES

1. **Agapov EV, et al.** 1998. Noncytopathic Sindbis virus RNA vectors for heterologous gene expression. Proc. Natl. Acad. Sci. U. S. A. **95**:12989–12994.
2. **Anindya R, Aygun O, Svejstrup JQ.** 2007. Damage-induced ubiquitylation of human RNA polymerase II by the ubiquitin ligase Nedd4, but not Cockayne syndrome proteins or BRCA1. Mol. Cell **28**:386–397.
3. **Atasheva S, Fish A, Fornerod M, Frolova EI.** 2010. Venezuelan equine encephalitis virus capsid protein forms a tetrameric complex with CRM1 and importin alpha/beta that obstructs nuclear pore complex function. J. Virol. **84**:4158–4171.
4. **Billecocq A, et al.** 2004. NSs protein of Rift Valley fever virus blocks interferon production by inhibiting host gene transcription. J. Virol. **78**:9798–9806.
5. **Breakwell L, et al.** 2007. Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response. J. Virol. **81**:8677–8684.
6. **Burnham AJ, Gong L, Hardy RW.** 2007. Heterogeneous nuclear ribonuclear protein K interacts with Sindbis virus nonstructural proteins and viral subgenomic mRNA. Virology **367**:212–221.
7. **Dubois MF, Nguyen VT, Bellier S, Bensaude O.** 1994. Inhibitors of transcription such as 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole and isoquinoline sulfonamide derivatives (H-8 and H-7) promote dephosphorylation of the carboxyl-terminal domain of RNA polymerase II largest subunit. J. Biol. Chem. **269**:13331–13336.
8. **Egloff S, Murphy S.** 2008. Cracking the RNA polymerase II CTD code. Trends Genet. **24**:280–288.
9. **Frick DN, Lam AM.** 2006. Understanding helicases as a means of virus control. Curr. Pharm. Des. **12**:1315–1338.
10. **Frolov I, et al.** 1999. Selection of RNA replicons capable of persistent noncytopathic replication in mammalian cells. J. Virol. **73**:3854–3865.
11. **Frolov I, Garmashova N, Atasheva S, Frolova EI.** 2009. Random insertion mutagenesis of sindbis virus nonstructural protein 2 and selection of variants incapable of downregulating cellular transcription. J. Virol. **83**:9031–9044.
12. **Frolova EI, et al.** 2002. Roles of nonstructural protein nsP2 and alpha/beta interferons in determining the outcome of Sindbis virus infection. J. Virol. **76**:11254–11264.
13. **Frolova EI, Gorchakov R, Pereboeva L, Atasheva S, Frolov I.** 2010. Functional Sindbis virus replicative complexes are formed at the plasma membrane. J. Virol. **84**:11679–11695.
14. **Garmashova N, Gorchakov R, Frolova E, Frolov I.** 2006. Sindbis virus nonstructural protein nsP2 is cytotoxic and inhibits cellular transcription. J. Virol. **80**:5686–5696.
15. **Garmashova N, et al.** 2007. The Old World and New World alphaviruses

use different virus-specific proteins for induction of transcriptional shutoff. J. Virol. **81**:2472–2484.
16. **Gomez de Cedron M, Ehsani N, Mikkola ML, Garcia JA, Kaariainen L.** 1999. RNA helicase activity of Semliki Forest virus replicase protein NSP2. FEBS Lett. **448**:19–22.
17. **Gorbalenya AE, Blinov VM, Donchenko AP, Koonin EV.** 1989. An NTP-binding motif is the most conserved sequence in a highly diverged monophyletic group of proteins involved in positive strand RNA viral replication. J. Mol. Evol. **28**:256–268.
18. **Gorbalenya AE, Koonin EV, Donchenko AP, Blinov VM.** 1989. Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. Nucleic Acids Res. **17**:4713–4730.
19. **Gorchakov R, Frolova E, Frolov I.** 2005. Inhibition of transcription and translation in Sindbis virus-infected cells. J. Virol. **79**:9397–9409.
20. **Haaf T, Ward DC.** 1996. Inhibition of RNA polymerase II transcription causes chromatin decondensation, loss of nucleolar structure, and dispersion of chromosomal domains. Exp. Cell Res. **224**:163–173.
21. **Hanawalt PC, Spivak G.** 2008. Transcription-coupled DNA repair: two decades of progress and surprises. Nat. Rev. Mol. Cell Biol. **9**:958–970.
22. **Huibregtse JM, Yang JC, Beaudenon SL.** 1997. The large subunit of RNA polymerase II is a substrate of the Rsp5 ubiquitin-protein ligase. Proc. Natl. Acad. Sci. U. S. A. **94**:3656–3661.
23. **Kujala P, et al.** 1997. Monoclonal antibodies specific for Semliki Forest virus replicase protein nsP2. J. Gen. Virol. **78**:343–351.
24. **Leonard VH, Kohl A, Hart TJ, Elliott RM.** 2006. Interaction of Bunyamwera Orthobunyavirus NSs protein with mediator protein MED8: a mechanism for inhibiting the interferon response. J. Virol. **80**:9667–9675.
25. **Lin JY, et al.** 2009. hnRNP A1 interacts with the 5′ untranslated regions of enterovirus 71 and Sindbis virus RNA and is required for viral replication. J. Virol. **83**:6106–6114.
26. **Michael WM, Eder PS, Dreyfuss G.** 1997. The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein. EMBO J. **16**:3587–3598.
27. **Nakielny S, Dreyfuss G.** 1999. Transport of proteins and RNAs in and out of the nucleus. Cell **99**:677–690.
28. **Nguyen VT, et al.** 1996. In vivo degradation of RNA polymerase II largest subunit triggered by alpha-amanitin. Nucleic Acids Res. **24**:2924–2929.
29. **Petrakova O, et al.** 2005. Noncytopathic replication of Venezuelan equine encephalitis virus and eastern equine encephalitis virus replicons in mammalian cells. J. Virol. **79**:7597–7608.
30. **Pinol-Roma S, Dreyfuss G.** 1992. Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. Nature **355**:730–732.
31. **Russo AT, White MA, Watowich SJ.** 2006. The crystal structure of the Venezuelan equine encephalitis alphavirus nsP2 protease. Structure **14**:1449–1458.
32. **Siomi MC, et al.** 1997. Transportin-mediated nuclear import of heterogeneous nuclear RNP proteins. J. Cell Biol. **138**:1181–1192.
33. **Strauss JH, Strauss EG.** 1994. The alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. **58**:491–562.
34. **Svejstrup JQ.** 2003. Rescue of arrested RNA polymerase II complexes. J. Cell Sci. **116**:447–451.
35. **Vasiljeva L, Valmu L, Kaariainen L, Merits A.** 2001. Site-specific protease activity of the carboxyl-terminal domain of Semliki Forest virus replicase protein nsP2. J. Biol. Chem. **276**:30786–30793.
36. **Verbruggen P, et al.** 2011. Interferon antagonist NSs of La Crosse virus triggers a DNA damage response-like degradation of transcribing RNA polymerase II. J. Biol. Chem. **286**:3681–3692.
37. **Volkova E, Gorchakov R, Frolov I.** 2006. The efficient packaging of Venezuelan equine encephalitis virus-specific RNAs into viral particles is determined by nsP1-3 synthesis. Virology **344**:315–327.
38. **Weidman MK, et al.** 2003. The interaction of cytoplasmic RNA viruses with the nucleus. Virus Res. **95**:75–85.