Large-Scale Chromatin Immunoprecipitation with Promoter Sequence Microarray Analysis of the Interaction of the NSs Protein of Rift Valley Fever Virus with Regulatory DNA Regions of the Host Genome

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Rift Valley fever virus (RVFV) is a highly pathogenic Phlebovirus that infects humans and ruminants. Initially confined to Africa, RVFV has spread outside Africa and presently represents a high risk to other geographic regions. It is responsible for high fatality rates in sheep and cattle. In humans, RVFV can induce hepatitis, encephalitis, retinitis, or fatal hemorrhagic fever. The nonstructural NSs protein that is the major virulence factor is found in the nuclei of infected cells where it associates with cellular transcription factors and cofactors. In previous work, we have shown that NSs interacts with the promoter region of the beta interferon gene abnormally maintaining the promoter in a repressed state. In this work, we performed a genome-wide analysis of the interactions between NSs and the host genome using a genome-wide chromatin immunoprecipitation combined with promoter sequence microarray, the ChIP-on-chip technique. Several cellular promoter regions were identified as significantly interacting with NSs, and the establishment of NSs interactions with these regions was often found linked to deregulation of expression of the corresponding genes. Among annotated NSs-interacting genes were present not only genes regulating innate immunity and inflammation but also genes regulating cellular pathways that have not yet been identified as targeted by RVFV. Several of these pathways, such as cell adhesion, axonal guidance, development, and coagulation were closely related to RVFV-induced disorders. In particular, we show in this work that NSs targeted and modified the expression of genes coding for coagulation factors, demonstrating for the first time that this hemorrhagic virus impairs the host coagulation cascade at the transcriptional level.

During the last 2 decades, efforts have been made to better understand the interactions between viruses and their vertebrate hosts. The use of large-scale approaches has been proven to be very helpful to address this issue but contrary to protein-protein interactions, the analysis of interactions of viral protein with the host’s genome has remained almost totally unexplored in spite of the presence of viral proteins in the nuclei of infected cells. The nonstructural NSs protein encoded by Rift Valley fever virus (RVFV) is one of these proteins. Even though the entire life cycle of RVFV takes place in the cytoplasm, NSs protein, which is the major virulence factor, is present in the nuclei of infected cells where it forms filamentous structures interacting with cellular transcription factors and cofactors (48, 24, 25). In previous work, we have shown that in nuclei of infected cells, NSs protein interacted with the promoter region of the beta interferon (IFN-β) gene abnormally maintaining IFN-β gene expression in a repressed state (25). In this work, we have carried out a genome-wide analysis of the interaction of NSs protein with the host promoter regions with the aim to uncover new cellular pathways targeted by RVFV through NSs.

Rift Valley fever is a mosquito-borne zoonotic viral disease affecting livestock and humans (44). The causative agent, RVFV, is a Phlebovirus of the Bunyaviridae family (34). Initially confined to sub-Saharan regions of Africa, RVFV spread to Egypt in 1977 and to the Middle East in 2000, representing a high risk to other regions (35, 3). RVFV is responsible for high fatality rates in sheep and cattle with the mortality rate reaching 100% in neonates. A characteristic feature of RVFV infection observed in naturally or experimentally infected sheep is a focal or generalized hepatic necrosis and hemorrhages in several organs (6, 42). In humans, mild cases manifest a self-limiting febrile illness, while more serious cases develop myalgia, arthralgia, photophobia, and severe headaches. In a small proportion of cases, which was estimated to be 1 or 2% during the 1977 Egyptian outbreak but appeared to increase up to 20% in recent outbreaks, the disease evolved to hepatitis, encephalitis, retinitis, or fatal hemorrhagic fever (15) with case fatality rates reaching 28% during the 2007 Tanzanian outbreak (31). A new attenuated vaccine for animals (8, 46) is now available. However, there is no vaccine or appropriate treatment for RVF in humans.

Like all the members of the family, RVFV possesses a single-stranded tripartite RNA genome of negative/ambisense polarity. The L and M segments code, respectively, for the L protein and the precursor to the Gn and Gc glycoproteins which also generates two nonstructural proteins of 78 kDa and 14 kDa. The S segment utilizes an ambisense strategy and codes for two proteins in opposite polarities, the nuprotein N and the nonstructural NSs protein (21).
Although this virus replicates in the cytoplasm, the NSs protein, which is considered one of the main factors responsible for RVFV pathogenesis, is the only viral protein present in the nucleus of the host cell where it interacts with several cellular proteins inducing a variety of events involved in RVFV pathogenicity. The interaction of NSs with the TFIH (transcription factor IHI) general transcription factor subunits p44 and p62 leading to sequestration of p44 within NSs filaments and degradation of p62 (24, 19) is responsible for the inhibition of general cell transcription, whereas its interaction with the cellular corepressor SAP30 (Sin3A-associated protein, 30 kDa) (25) is directly involved in the earlier and more specific inhibition of the beta interferon (IFN-β) gene expression. Also through SAP30, NSs interacts with pericentromeric chromosomal sequences causing chromosomal segregation defects in mouse fibroblasts and sheep kidney cells (27).

Through a yet-unknown mechanism, NSs also posttranscriptionally downregulates protein kinase PKR (14), inducing its specific degradation through the proteasome pathway (9).

In this work, using a genome-wide chromatin immunoprecipitation (ChIP) combined with promoter sequence microarray (ChIP-on-chip) approach, we have identified host DNA regions interacting with the viral NSs protein. Host regulatory DNA regions identified here as statistically significantly interacting with NSs revealed new cellular pathways targeted by RVFV that are closely related to RVFV-induced disorders. Particularly striking was the capacity of the NSs protein to interact with the regulatory regions of several genes coding for coagulation factors affecting their expression at the transcriptional level.

**MATERIALS AND METHODS**

**Viruses and cells.** Stocks of RVFV ZH548 and RVFV ZHΔNSs were produced under biosafety level 3 (BSL3) conditions by infecting Vero cells at a multiplicity of infection (MOI) of 10−3 and by harvesting the medium at 72 h postinfection (p.i.). Murine fibroblastic L929 cell lines have been described previously (25). The murine hepatocyte cell line H2.35 was from ATCC (CRL-1995).

**Antibodies.** Mouse anti-NSs and rabbit anti-N polyclonal antibodies were raised against the entire NSs and N protein, respectively (48). Rabbit polyclonal anti-acetyl-histone H3 (Lys14) (catalog no. 06-911) from Upstate was used for immunofluorescence. Secondary antibodies used for immunofluorescence were Alexa Fluor 488-conjugated chicken anti-mouse antibody from Invitrogen (catalog no. 11034) and Alexa Fluor 555-conjugated donkey anti-rabbit antibody from Invitrogen (catalog no. A-31572).

**Immunofluorescence.** For immunofluorescence, cells grown in 24-well plates on coverslips were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 15 min and permeabilized with 0.5% Triton X-100 in PBS for 30 min. Then the cells were incubated for 1 h at 37°C with the corresponding primary antibodies diluted in PBS containing 1% bovine serum albumin (BSA). The cells were then washed with PBS and incubated for 45 min at room temperature with the corresponding secondary antibodies.

**Image acquisition and manipulation.** Samples were analyzed at room temperature by confocal laser scanning microscopy using an Axiovert 200M (Zeiss LSM510 confocal system) at the Service Commun de Microscopie (SCM) of Université Paris Descartes. This system is equipped with a 63×, 1.4 numerical-aperture oil immersion lens (Plan Neofluor). For oil immersion microscopy, we used oil with a refractive index of 1.518 (Zeiss). Images were collected in the direction of the z axis corresponding to the optical axis of the microscope at 0.37-μm intervals with the z axis going through the image planes. LSM 510 imaging software was used for image capture (512 × 512 pixels; 8-bit data). The images were analyzed using either LSM5 Image browser or Image J software program. Double-labeled pixels were displayed in yellow on the merge images.

**ChIP-on-chip experiments.** Chromatin immunoprecipitation followed by the microarray hybridization (ChIP-on-chip) experiments were performed on three independent series of infections with each series consisting of murine fibroblastic L929 cells infected by either the RVFV ZH strain (expressing the nonstructural NSs protein) or the RVFV ZHΔNSs strain (not expressing the NSs protein). Chromatin immunoprecipitation experiments were performed as previously described (25). For each experiment, 20 μg of sonicated genomic DNA was immunoprecipitated with mouse polyclonal anti-NSs antibody using protein A agarose-salmon sperm DNA beads from Upstate. After elution from the beads, immunoprecipitated chromatin and input DNA were tagged with primer A (5′-G TTTCCAGATCGCGTCTCNNNNN-3′), which carries eight random nucleotides (N), using the DNA sequencing from USB/Affymetrix, and then tagged DNA was amplified using Taq polymerase and primer B (5′-GTTTCCAGATCGCGTCTC-3′) under linear conditions. Labeling of the linearly amplified DNA, hybridization on Affymetrix mouse promoter 1.0R arrays, and array scanning were conducted at the Department of Translational Research of Institut Curie (France). Data from .cel files (GCOS 1.3 software) were imported into the Partek Genomic Suite software (Partek Inc., St. Louis, MO). Analysis was done using the tiling regulation study in Partek genomic suite protocols. The imported data were prenormalized using the Robust Multichip Averaging algorithm and converted to log2. To obtain “true” antibody-specific intensities, an analysis of variance (ANOVA) analysis was performed on the mean values of signals from RVFV ZHΔNSs arrays (which were considered background) and the mean values of signals from the RVFV ZH arrays.

**RT-qPCR.** Total RNA was extracted using TriPure isolation reagent (Roche) according to the manufacturer’s protocol. One microgram of total RNA was digested with RQI RNase-free DNase (Promega) to remove contaminated DNA. Reverse transcription was carried out with random primers (Promega) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer’s recommendations. Reverse transcription-quantitative PCR (RT-qPCR) was performed using SYBR green (Thermo Scientific) reagents as follows: 15 min at 95°C; then 40 cycles, with 1 cycle consisting of 15 s at 95°C and 1 min at 60°C, followed by a dissociation step. Relative quantification of mRNA expression was calculated using the ΔΔCt method and the REST software (Qiagen). The threshold cycle (Ct) values for the different genes tested in Fig. 2 and 5 were first normalized to the Ct values of the housekeeping gene (18S rRNA) analyzed in the same samples, and then the values for RVFV ZH-infected cells were further normalized to the Ct values from RVFV ZHΔNSs-infected cells in Fig. 2 and 5 to determine the relative changes in gene expression after RVFV ZH compared to RVFV ZHΔNSs. The result is a fold change value that indicates whether the expression level of a gene after RVFV ZH infection is higher or lower than the expression level of the same gene after infection with RVFV ZHΔNSs. For fold changes lower than 1 (indicating that the expression of the gene was repressed), the inverted values were determined such that a 0.5-fold change corresponds to −2. A minimum of two independent series of infection, with each time point carried out in triplicate, were used to allow statistical reliability of the results.

**Statistical analysis.** Statistical analysis was carried out using the REST software that uses a Pair-Wise Fixed Reallocation Randomization Test to determine P values. In Fig. 2, fold changes with P values of ≤0.1 were taken as statistically significant. In Fig. 5, fold changes with P values of ≤0.05 were taken as statistically significant.

**RESULTS**

**Genome-wide identification of cellular DNA regulatory regions targeted by NSs.** For ChIP-on-chip experiments, three independent series of infections were carried out, each series consisting of murine fibroblastic L929 cells infected by either the pathogenic virulent strain expressing the (wild-type) nonstructural NSs protein (RVFV ZH) or the nonpathogenic viral strain with the NSs sequence deleted and therefore not expressing the NSs protein.
(RVFV ZHΔNSs) used as a negative control. In agreement with previous results, NSs filaments were observed in the nuclei of cells after infection with RVFV ZHΔNSs (ΔNSs) or RVFV ZH (ZH) 8 h p.i. The leftmost panels show cellular DNA counterstained with ToPro3 (blue). The middle panels show distribution of the nonstructural NSs protein detected with anti-NSs mouse polyclonal antibody (green), and the merged images of ToPro3 and NSs are shown in the right panels. (B) Single confocal sections of nuclei of murine L929 cells either not infected (NI) or in cells infected with RVFV ZHΔNSs (ΔNSs) or RVFV ZH (ZH) at 8 h p.i. The left panels show cellular DNA counterstained with ToPro3 (blue). The middle panels show N distribution detected with anti-N polyclonal antibody (red), and the merged images of ToPro3 and N are shown in the right panels. (C) General characteristics of regions identified as statistically significantly interacting with NSs as determined after analysis of ChIP-on-chip results using Partek Genomic Suite (PGS). Nb, number. (D) Single confocal section of a nucleus of a murine L929 cell 8 h p.i. with RVFV ZH. (a) Distribution of cellular DNA carrying the acetylated form of lysine 14 of histone H3 (H3K14Ac) as an epigenetic mark detected with anti-H3K14Ac polyclonal antibodies (red), (b) NSs distribution detected with anti-NSs mouse polyclonal antibody (green); (c) merged image, with colocalization displayed in yellow. (E) The profile of the line scan quantifying the relative intensities of the fluorochromes for H3K14Ac-DNA (red) and NSs (green) along the indicated line coincide halfway through the graph over a distance of >1 μm indicative of a colocalization. Bars, 10 μm.

FIG 1 Identification of cellular promoter regions interacting with RVFV NSs protein as determined by ChIP-on-chip. (A) Single confocal sections of nuclei of murine L929 cells either not infected (NI) or in cells infected with RVFV ZHΔNSs (ΔNSs) or RVFV ZH (ZH) 8 h p.i. The leftmost panels show cellular DNA counterstained with ToPro3 (blue). The middle panels show distribution of the nonstructural NSs protein detected with anti-NSs mouse polyclonal antibody (green), and the merged images of ToPro3 and NSs are shown in the right panels. (B) Single confocal sections of nuclei of murine L929 cells either not infected (NI) or in cells infected with RVFV ZHΔNSs (ΔNSs) or RVFV ZH (ZH) at 8 h p.i. The left panels show cellular DNA counterstained with ToPro3 (blue). The middle panels show N distribution detected with anti-N polyclonal antibody (red), and the merged images of ToPro3 and N are shown in the right panels. (C) General characteristics of regions identified as statistically significantly interacting with NSs as determined after analysis of ChIP-on-chip results using Partek Genomic Suite (PGS). Nb, number. (D) Single confocal section of a nucleus of a murine L929 cell 8 h p.i. with RVFV ZH. (a) Distribution of cellular DNA carrying the acetylated form of lysine 14 of histone H3 (H3K14Ac) as an epigenetic mark detected with anti-H3K14Ac polyclonal antibodies (red), (b) NSs distribution detected with anti-NSs mouse polyclonal antibody (green); (c) merged image, with colocalization displayed in yellow. (E) The profile of the line scan quantifying the relative intensities of the fluorochromes for H3K14Ac-DNA (red) and NSs (green) along the indicated line coincide halfway through the graph over a distance of >1 μm indicative of a colocalization. Bars, 10 μm.

### Table 1: Partek Genomic Suite analysis of ChIP-on-chip assays

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nb of regions</td>
<td>3,728</td>
</tr>
<tr>
<td>Regions length</td>
<td>335 to 3,013 bp</td>
</tr>
<tr>
<td>P-values</td>
<td>10^{-3} to 10^{-2}</td>
</tr>
<tr>
<td>MAT score</td>
<td>12.18 to 3.99</td>
</tr>
<tr>
<td>Nb of genes</td>
<td>2,786</td>
</tr>
<tr>
<td>Genes with:</td>
<td></td>
</tr>
<tr>
<td>one region/gene</td>
<td>78.71 %</td>
</tr>
<tr>
<td>two regions/gene</td>
<td>15.72 %</td>
</tr>
<tr>
<td>≥ 3 regions/gene</td>
<td>5.36 %</td>
</tr>
</tbody>
</table>

Analysis of ChIP-on-chip experiments (Fig. 1C) led to the identification of a total of 3,728 regions statistically significantly interacting with the NSs protein (listed in Table S1 in the supplemental material) that overlapped 2,786 annotated genes. These 3,728 NSs-interacting regions represented 1.56% of the total number of probes present within the array (see Fig. S1B in the supplemental material) and were distributed among all autosomal chromosomes. A maximum of 2.3-fold difference separated the least represented and the most represented autosomal chromosomes with chromosomes 5 and 11 identified as the two chromosomes with the highest proportion (2.2 and 2.03%, respectively) of their probes present within the array interacting with NSs (Fig. S1B). Among the sex chromosomes, the Y chromosome was strongly underrepresented, with only 0.087% of its probes present on the array interacting with NSs, whereas the X chromosome was represented similarly to the autosomal chromosomes, with 1.42% of its probes present on the array interacting with NSs.

The peak detection parameters used for this analysis were as follows: P-value threshold of 0.01, minimum number of 10 probes for each region, and average 600-bp length for the ChIP fragment.
On the one hand, ChIP-on-chip results showed that despite the huge impact NSs filaments impart through their occupation of most of the nuclear space 8 h.p.i., only a small proportion of genes interact via their promoters with NSs. This observation agrees with previously published results indicating that the cellular genome is mostly excluded from NSs filaments (27). On the other hand, in agreement with the capacity of the NSs protein to interact with specific promoter regions, we show in Figs. 1D and E that cellular chromatin carrying the acetylated form of lysine 14 of histone H3 (H3K14Ac), which is an epigenetic mark predominantly present over promoter regions of genes either poised for transcription or actively transcribed, was not excluded from and partially colocalized with NSs filaments.

RVFV infection affected the expression of several of the genes interacting with NSs. In previous experiments, we have shown that NSs interaction with the IFN-β promoter leads to inhibition of IFN-β gene expression (25). Thus, we investigated by RT-qPCR the effect of the NSs protein on the expression of several genes whose promoter regions were identified during ChIP-on-chip experiments as interacting with NSs. We have previously shown that infection with RVFV induced a general NSs-dependent inhibition of the transcriptional rate of the host cell (24). Since this inhibition was characterized as starting 8 h.p.i., it was not expected to influence the rate of transcription measured here using RT-qPCR, since RNAs from cells infected with RVFV ZH and RVFV ZHΔNSs were collected 8 h.p.i. (as for ChIP-on-chip assays) before the establishment of the general NSs-dependent inhibition of transcription. Nevertheless, in order to confirm that at this time there was no general NSs-dependent inhibitory effect, we also investigated here the expression of several housekeeping genes whose promoter regions were not identified during ChIP-on-chip experiments as interacting with NSs.

The expression of 33 NSs-interacting genes and three non-NSs-interacting genes, whose expression could be detected in L929 fibroblasts, was tested. The NSs-interacting genes tested were randomly chosen as belonging to different gene families and cellular pathways. The results presented in Fig. 2 correspond to those obtained from a minimum of two independent series of infections, with each series carried out in triplicate. Ten of these genes had their expression statistically significantly affected (Mapk8p3, Fbxo3, Stat2, Il3, Il10rb, Tyk2, Casp9, Phj21, Ncoa3, and Notch4) with all of them being downregulated after RVFV ZH infection compared to RVFV ZHΔNSs infection. The expression of neither one of the three, non-NSs-interacting genes tested was affected after RVFV ZH infection compared to RVFV ZHΔNSs infection, confirming that at this time after infection, the general NSs-dependent inhibition of transcription induced by NSs was not yet effective.

Genes whose expression was significantly affected were functionally related to immune response, protein phosphorylation, ubiquitination, apoptosis, RNA polymerase II (RNA Pol II) transcription or development. It is noteworthy that we cannot exclude the possibility that among genes whose expression appeared not to be significantly affected, some could code for mRNAs with long half-lives for which an eventual inhibition of their expression could not be detected 8 h after RVFV ZH infection.

Biological functions regulated by genes whose regulatory regions interacted with the NSs protein are closely related to RVFV-induced pathological disorders. In order to determine whether a correlation exists between NSs interactions with the host genome and RVFV-induced pathological disorders, we analyzed the biological functions and molecular processes enriched among NSs-interacting genes using the Partek Genome Suite (PGS) (Fig. 3A) and Ingenuity Pathway Analysis (IPA) (Fig. 3B and C) software programs. Interestingly, biological processes and functions identified by both software programs as significantly enriched among NSs-interacting genes were closely related to either earlier or recently reported RVFV-induced pathologies.

Neurological disorders are the most common clinical features among RVFV-infected patients (31, 35), and indeed, the neurological system appeared as the most significantly overrepresented function through IPA (Fig. 3B) and as the most enriched multicellular organismal process using PGS (Fig. 3A).

Coagulation and development processes were also found enriched using PGS (Fig. 3A). Again, this is closely related with RVFV-induced pathology. Severe hemorrhagic fever associated with hepatitis being the first cause of RVFV-induced mortality in humans or primates, whereas developmental disorders such as abortion and teratogenesis are frequently observed in ruminants.

Cell adhesion (cell-cell and cell-substrate adhesion) was the most enriched biological process identified using PGS (Fig. 3A), and cell-to-cell signaling and interaction was the fifth most overrepresented function identified through IPA (Fig. 3B). Cell-cell and cell-substrate adhesion play essential roles in the neurological system during development, coagulation, and immune and inflammatory processes, all of which were affected during RVFV infection.

Concerning the immune response, it is interesting to note that the innate response appeared much more enriched than the adaptive response (Fig. 3A), an observation that is once again closely related with RVFV-induced disorders, since the innate response is strongly affected, whereas the adaptive response remains functional during RVFV infection.

In Fig. 3C are listed 5 of the 10 top canonical pathways identified through IPA for which at least 20% of the genes intervening in the pathway significantly interacted with the NSs protein. Axonal guidance and docosahexaenoic acid (DHA) signaling pathways regulate neuronal signaling and survival. Thrombin signaling regulates platelet aggregation and blood coagulation, and phosphatase and tensin homolog (PTEN) signaling is essential for embryonic development as well as cell migration and adhesion.

Among the other canonical pathways identified through IPA as significantly represented, albeit not in the top 10, was the Janus kinase (JAK)/signal transducer and activator of transcription factor (STAT) signaling pathway. Genes involved in this pathway were identified as statistically significantly interacting with the NSs protein as shown in Fig. 4 (gray ovals). Several of these genes were already discernible in Fig. 2 as downregulated following RVFV ZH infection compared to RVFV ZHΔNSs infection (Fig. 4, gray ovals outlined in red). Activation of this pathway by type I (IFN-α/β) and type I-like (IFN-λ) IFNs regulates establishment of the cellular innate antiviral response. IFN-β and IFN-λ are both induced after infection by a number of viruses, leading in turn to induction of IFN-stimulated genes (ISGs) through activation and phosphorylation of Jak1 (Janus kinase 1), Tyk2 (tyrosine kinase 2), Stat1 (signal transducer and activator of transcription factor 1), and Stat2 (20, 41). Interestingly, the genes coding for Tyk2 and Stat2 were identified as interacting with the NSs protein as well as those coding for the two subunits of the IFN-λ receptor (IL28Ra [interleukin 28 receptor, alpha subunit] and IL10Rb), that in response to IFN-λs induce the phosphorylation of Stat1, Stat2, Stat3, and Stat5. Besides Stat2, the gene coding for Stat5 was...
also found here to interact with NSs (Fig. 4). In association with interferon regulatory factor 9 (IRF9), Stat1 and Stat2 form the IFN-stimulated gene factor 3 (ISGF3) that regulates ISG expression after binding to the IFN-stimulated response element (ISRE) present within the promoter region of ISGs. The Med14 gene, which has also been identified as interacting with NSs, codes for a subunit of the Mediator complex that has been shown to enhance Stat2-dependent regulation of ISGs (22). Among the ISGs playing a critical role in setting up the innate antiviral response are the genes of the 2′-5′-oligoadenylate synthetase (Oas) family, which activate latent RNase L, which in turn degrades viral RNA. Precisely, we also identified Oasl2, Oasl1b, and RNaseL as NSs-interacting genes (Fig. 4).

Overall, these results indicated that the NSs protein targeted...
FIG 3 Top biological processes, functions, and canonical pathways represented among NSs-interacting genes. (A) Enrichment score of biological processes determined from NSs-interacting genes listed in Table S1 in the supplemental material using PGS. The enrichment score shows that the biological process was an overrepresented functional group from a list of significant genes. It is calculated using $-\log P$ value, where the $P$ value is determined using a chi-square statistic looking at the observed value versus the expected value. (B) Six functions from the Ingenuity Pathway Analysis (IPA) library that were among the top 10 functions most significant in our set of NSs-interacting genes. (C) Five canonical pathways from the IPA library that were among the 10 top pathways most significant in our set of NSs-interacting genes. The red lines in panels B and C indicate the threshold of $P$ value of $<0.05$. 
By targeting members of the Jak/Stat signaling pathway, RVFV did not target solely the innate antiviral response. The IL10Rβ subunit of the IFN-γ receptor, whose gene was identified here as targeted by the NSs protein, also associates with the interleukin 22 (IL-22) receptor subunit IL22R as well as with the IL-10 receptor subunit IL10Ra. These receptors regulate inflammatory responses through Stat1, Stat2, Stat3, and Stat5 (23, 37). Therefore, through interaction with IL10Rb, NSs also targets the IL-22 and IL-10 responses, a phenomenon that could explain in part the recently described effect of RVFV infection on proinflammatory response in mice and humans (17, 29).

The IL-22 response includes activation of the mitogen-activated protein (MAP) kinase pathway in which Mapk8ip3 (also known as JIP3 or JSAP1), whose promoter region interacts with NSs and whose expression is downregulated (Fig. 2), is predominantly expressed in the brain where it regulates vesicle transport in neurons (12, 30).

Certain gene families were overrepresented among NSs-interacting genes. One of the characteristics of the NSs-interacting genes was the recurrent presence of genes belonging to the same gene family as defined by the HUGO Gene Nomenclature Committee.
TABLE 1 Gene families overrepresented in the list of NSs-interacting genes

<table>
<thead>
<tr>
<th>Name of gene family</th>
<th>Function(s) of proteins encoded by gene family</th>
<th>% of total genes in the gene family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic helix-loop-helix (BHLH)</td>
<td>Transcription factors</td>
<td>15.4</td>
</tr>
<tr>
<td>Cadherins (CDH) and protocadherins (PCDH)</td>
<td>Cell-cell adhesion (Ca(^{2+}) dependent)</td>
<td>17.4</td>
</tr>
<tr>
<td>Cluster of differentiation (CD)</td>
<td>Membrane proteins present on cell surfaces that play a role in immunity, cell signaling, and development of the nervous system</td>
<td>16.4</td>
</tr>
<tr>
<td>DNAJ (Hsp40)</td>
<td>Protein folding and cell response to stress</td>
<td>25.6</td>
</tr>
<tr>
<td>F-box (FBX)</td>
<td>Proteins playing a role in the E3 ubiquitin ligase complex</td>
<td>29.4</td>
</tr>
<tr>
<td>Forkhead box (FOX)</td>
<td>Transcription factors involved in embryonic development</td>
<td>16.2</td>
</tr>
<tr>
<td>G-protein-coupled receptors (GPR)</td>
<td>Membrane proteins that relay chemical signals from outside the cell to the interior of the cell, triggering complex networks of signaling pathways through activation of G proteins. They regulate olfaction, vision, the immune system, and autonomous nervous system.</td>
<td>15.8</td>
</tr>
<tr>
<td>Homeobox (HOX)</td>
<td>Transcription factors involved in morphogenesis during development</td>
<td>18.4</td>
</tr>
<tr>
<td>Krüppel-like transcription factor (KLF)</td>
<td>Transcription factors</td>
<td>19.4</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase (MAPK)</td>
<td>Kinases playing a critical role in signal transduction of extracellular signals</td>
<td>15.0</td>
</tr>
<tr>
<td>PHD finger protein (PHF)</td>
<td>Regulators (corepressor or coactivator) of transcription</td>
<td>26.0</td>
</tr>
<tr>
<td>Plexins (PLX)</td>
<td>Membrane-bound proteins participating in axon guidance; coreceptors of semaphorins</td>
<td>33.3</td>
</tr>
<tr>
<td>Protein phosphatase 1 regulatory (inhibitor) subunit (PPP1R)</td>
<td>Regulatory subunits of serine/threonine phosphatases; signal transduction</td>
<td>20.0</td>
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<td>Protein tyrosine phosphatase (PTP)/class I</td>
<td>Class I tyrosine phosphatases. Regulation of signal transduction pathways</td>
<td>30.0</td>
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<tr>
<td>Regulators of G-protein signaling (RGS)</td>
<td>Regulators of signaling cascades triggered by G-coupled proteins (GPRs)</td>
<td>28.7</td>
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<tr>
<td>Semaphorin (SEMA)</td>
<td>Axon guidance ligands</td>
<td>15.0</td>
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<tr>
<td>Sex-determining region Y-box (SOX)</td>
<td>Sex-determining region Y-box (SOX)</td>
<td>30.0</td>
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<tr>
<td>Sulfo transferase, membrane bound (ST)</td>
<td>Participate in the biosynthesis of selectins. Play a role in inflammation and coagulation</td>
<td>22.8</td>
</tr>
<tr>
<td>Ubiquitin-specific peptidases (USP)</td>
<td>Deubiquitinating enzymes. Specifically cleave ubiquitin from ubiquitin-conjugated protein</td>
<td>24.5</td>
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<tr>
<td>Voltage-gated cation channels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wingless-type mouse mammary tumor virus (MMTV) integration site family (WNT)</td>
<td>Ligands of Frizzled receptors regulating signaling pathways associated with embryonic development, proliferation, and stem cell renewal</td>
<td>21.0</td>
</tr>
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</table>

*Olfactory receptors were not taken into account here.*

(HGNC) (http://www.genenames.org/genefamily.html). A small number of these families (corresponding to the 21 gene families listed in Table 1) were overrepresented, whereas a majority of the gene families (111) had either 0 or only 1 of their genes identified as a NSs-interacting gene. The genes belonging to the 21 gene families overrepresented among NSs-interacting genes regulate neurological functions, development, coagulation, immune response, RNA Pol II-dependent transcription, and protein phosphorylation (Table 1), which all correspond to biological functions affected after RVFV infection.

Among the families coding for genes regulating the nervous system was the family coding for potassium (KCN) and calcium (CACN) voltage-gated cation channels that play a critical role in transmitting electrical signals within and between nerve cells. Also playing important roles on the nervous system were the families of semaphorin and plexin genes that code for proteins regulating axonal guidance and the family of cadherin genes that code for Ca\(^{2+}\) cell-cell adhesion proteins, which includes the Cdh22 gene coding for a protein predominantly expressed in the developing and adult brain (28). Genes belonging to the protocadherin gene family also code for proteins that intervene in the establishment and function of specific cell-cell connections in the brain (32).

Directly related with the regulation of the coagulation cascade was the F3 gene that belongs to the cluster of differentiation (CD) gene family. The F3 gene codes for coagulation factor III, better known as tissue factor (TF), that is essential for the initiation of the extrinsic pathway of the blood coagulation cascade. Interestingly, TF also regulates innate immunity and inflammatory responses (38, 7).

Six out of the 21 gene families overrepresented among NSs-interacting genes coded for proteins regulating RNA Pol II transcription among which several corresponded to transcription factors playing a critical role during early embryonic development such as the Forkhead box (FOX), homeobox (HOX), and sex-determining region Y-box (SOX) gene families. Also playing an important role during development were genes belonging to the Wingless-type mouse mammary tumor virus (MMTV) integration site family (WNT) gene family.

Two of the gene families listed in Table 1 (F-box [FBX] and ubiquitin-specific peptidase[USP]) coded for proteins implicated in the ubiquitin pathway, which is often affected by viruses (16).

Genes regulating protein phosphorylation cascades were also abundantly present among NSs-interacting genes. Several of these genes belonged to gene families listed in Table 1, such as mitogen-activated protein kinase (MAPK), protein tyrosine phosphatase (PTP), and protein phosphatase 1 regulatory subunit (PPP1R).
The abundance of genes regulating protein phosphorylation among NSs-targeted genes could explain the recently described effect of RVFV on global phosphorylation of cellular proteins (36).

**NSs affected the expression of genes regulating the coagulation cascade.** Lethality caused by RVFV is often associated with virus-induced hemorrhages, but until now, the molecular mechanisms responsible for these virus-induced hemorrhagic syndromes have remained unknown. Among the regions identified as interacting with NSs were the regulatory regions of genes coding for several main factors of the coagulation cascade, such as prothrombin, TF, or factors VII, VIII, and X as well as of tissue factor pathway inhibitor (TFPI) (see Table S1 in the supplemental material), suggesting that RVFV-induced hemorrhages could result from virus-induced transcriptional deregulation of the expression of genes coding for main coagulation factors.

In order to analyze the effect of NSs on the expression of the corresponding genes, murine hepatocytes were infected with pathogenic RVFV ZH or nonpathogenic RVFV ZHΔNSs strain. Hepatocytes were chosen for these experiments because, in contrast with fibroblasts, they express the main factors of the coagulation cascade. As shown in Fig. S2 in the supplemental material, the hepatocyte cell line used in this work was susceptible to RVFV, displaying after infection the same general characteristics previously described for other RVFV-infected cell types.

The effect of RVFV ZH on the expression of genes coding for coagulation factors specifically interacting with NSs was assessed by RT-qPCR from RNAs collected at 8 h p.i. The results shown in Fig. 5A indicated that infection with RVFV ZH statistically significantly affected the expression of all the factors interacting with the NSs protein compared to infection with RVFV ZHΔNSs except for factor VII whose expression remained undetectable under noninfected and infected conditions (data not shown). While the expression of the gene coding for TF was repressed ∼5.8 ± 0.17-fold, surprisingly the expression of the genes coding for prothrombin, factors VIII, X, and TFPI was significantly enhanced. It is noteworthy that the fold change in the expression of coagulation factor genes whose promoter regions did not interact with NSs, such as those coding for factors XI and XII, was not statistically significant and were lower than 1.5-fold (Fig. 5B), and the expression of genes coding for FV and FXIII (also not interacting with NSs) remained undetectable under noninfected, RVFV ZH-, and RVFV ZHΔNSs-infected conditions.

TF enables cells to initiate the extrinsic blood coagulation cascade in response to vascular injury (Fig. 5C). Repression of the expression of the gene coding for TF combined with activation of the expression of TFPI, a potent inhibitor of the TF-dependent coagulation cascade, is expected to lead to the inhibition of the extrinsic pathway that regulates clot formation under physiological conditions, establishing an anticoagulant state that could account for the hemorrhagic syndrome observed after RVFV ZH infection.

In contrast to the extrinsic pathway, the intrinsic pathway has low physiological significance, but under pathological conditions, its activation is associated with the formation of circulating clots and thrombi (47). Activation of the intrinsic pathway is triggered in response to damaged vessel wall surfaces, after contact of vessel walls with lipoprotein particles, bacteria, or other pathogens including viruses (33, 39). Infection with RVFV ZH enhanced the expression of the gene coding for coagulation factor VIII, X, and prothrombin (Fig. 5A) that are common to both pathways (Fig. 5C). Activation of the intrinsic pathway under RVFV ZH-induced pathological conditions could explain the presence of disseminated intravascular coagulation (DIC) observed in RVFV-infected animals and humans.

In contrast to the RVFV ZH-dependent downregulation of NSs-interacting genes, which is an expected result, since transcription corepressors have been found concentrated within NSs filaments (25), it was surprising to observe that in several cases, NSs interaction with a cellular DNA regulatory regions could lead to the statistically significant activation of the expression of the corresponding gene, as observed here for the genes coding for prothrombin, coagulation factors VIII, and X, or TFPI.

Upon initiation of transcription, RNA Pol II, which is hypophosphorylated when poised for transcription, becomes phosphorylated at residues Ser5 and Ser2 (5). In order to further confirm the possibility that RNA Pol II-dependent transcription can take place within NSs filaments, we analyzed using immunofluorescence and confocal microscopy the distribution of RNA Pol II phosphorylated at the Ser5 residue (PolII(Ser5-p)) with respect to NSs filaments in murine fibroblastic cells 8 h after infection by RVFV ZH. The results in Fig. 5D show that PolII(Ser5-p) that marks RNA Pol II initiating transcription colocalized with NSs filaments at some discrete foci (Fig. 5D, white arrows), indicating that transcription initiation could indeed take place within NSs filaments.

Overall, these results demonstrate that pathogenic RVFV ZH infection affects the expression of genes coding for factors involved in the coagulation cascade either down- or upregulating them, opening up exciting new possibilities for deciphering viral strategies responsible for virus-induced hemorrhagic syndromes.

**DISCUSSION**

In the case of RNA viruses, most studies concerning host-pathogen interactions have focused on protein-protein interactions and rarely on the capacity of viral proteins to interact with cellular chromatin. In this work, using genome-wide ChIP-on-chip analysis, we demonstrated that the viral nonstructural NSs protein, present in the nuclei of infected cells, specifically interacted with the promoter regions of 2,786 cellular annotated genes corresponding to 10% of the promoter regions present within the array. Quantitative expression analysis indicated that establishment of NSs interaction with these cellular DNA regulatory regions often affected the expression of the corresponding genes, predominantly downregulating them. Under the same conditions, no effect was observed on the expression of three housekeeping genes whose promoter regions were not characterized as interacting with NSs, indicating that downregulation was not a consequence of the previously described general, NSs-dependent transcription inhibition that is established at later times postinfection (24).

Large-scale analysis of the biological functions enriched among NSs-interacting genes showed a close correlation between the biological functions regulated by the genes whose promoter regions were found to interact with NSs and the pathological effects induced by RVFV. This correlation suggested that NSs interaction with regulatory regions of the cellular genome could be responsible for RVFV-induced disorders, such as neurological complications, hemorrhages, and developmental defects.

Further confirming the possible link between ChIP-on-chip results and RVFV-induced disorders, several genes whose expression had recently been described as affected after RVFV infection...
FIG 5 NSs protein targets the coagulation cascade. (A and B) The change in gene expression (fold change) was calculated in cells infected with RVFV ZH with respect to cells infected by RVFV ZHΔNSs by RT-qPCR 8 h p.i. The horizontal broken lines indicate the cutoff value for upregulation (+1.5-fold) and downregulation (−1.5-fold). Values that are significantly different from the value for cells infected with RVFV ZHΔNSs are indicated by asterisks as follows: *, P value <0.05; **, P value <0.01. F2, coagulation factor 2; Tpi, tissue factor pathway inhibitor. (C) The promoter regions of several genes coding for factors involved in the coagulation cascade were identified during ChIP-on-chip experiments as interacting with NSs (gray ovals). Genes identified in panel A whose expression was statistically significantly downregulated after the cells were infected with RVFV ZH compared with cells infected with RVFV ZHΔNSs are shown in gray ovals outlined in red, and genes whose expression was statistically significantly upregulated after the cells were infected with RVFV ZH compared with cells infected with RVFV ZHΔNSs are shown in gray ovals outlined in green. FXII, factor XII; FIX, serine protease factor IX; FVIII, factor VIII. (D) Single confocal section of a nucleus of a murine L929 cell infected with RVFV ZH 8 h p.i. The left panel shows the distribution of RNA Pol II phosphorylated at residue Ser5 (Pol II Ser5p) detected with anti-pPolII (pPolII stands for phosphorylated RNA Pol II) (8A7) rabbit polyclonal antibody (red). The middle panel shows NSs distribution detected with anti-NSs mouse polyclonal antibody (green), and the merged image is shown in the right panel with colocalization displayed in yellow. Bar, 10 μm.
were found present among the NSs-interacting genes listed in Table S1 in the supplemental material. This was the case for genes involved in apoptotic or inflammatory responses recently described as statistically significantly up- or downregulated after RVFV infection in mice (17). In particular, the gene coding for IL-1RA (Illrn) was identified here as interacting with NSs. Up-regulation of this receptor antagonist of IL-1 after wild-type RVFV infection has recently been shown to be associated with an increased risk of mortality in humans (29).

Given the presence of members of the transcriptional corepressor complex SAP30/Sin3a/NCoR (nuclear receptor corepressor)/HDACs (histone deacetylases) (HDACs 1, 2, and 3) within NSs filaments (25), genes interacting with the NSs protein were expected to be downregulated rather than upregulated. Indeed, among the 33 randomly chosen NSs-interacting genes whose expression was compared in RVFV ZH- versus RVFV ZHΔNSs-infected cells, expression of the 10 genes that were significantly affected was downregulated. Nevertheless, the situation was different when we specifically analyzed the effect of NSs on those genes regulating the coagulation cascade whose regulatory regions were found interacting with NSs. In this case, all the genes except for the one coding for TF were significantly upregulated, demonstrating that even though NSs filaments constitute an environment that is predominantly repressive for gene expression, it is not totally refractory to transcription. Under certain yet determined circumstances, association with NSs can lead to transcriptional activation as further ascertained by the presence within NSs filaments of a few foci of RNA Pol II phosphorylated at Ser5, indicative of transcription initiation.

There is little information concerning the transcriptional regulation of genes coding for coagulation factors but in the case of the gene coding for factor X, its expression has been reported to be regulated by transcription factor GATA-4 in conjunction with hepatocyte nuclear factor 4 (HNF-4) (13). Interestingly, GATA-4 has been shown to cooperate with transcription factor Yin Yang 1 (YY1) to induce gene expression activation (2).

Transcription factor YY1, an activator or repressor of numerous cellular genes (1), directly interacts with cofactor SAP30 (11) and was also found strongly concentrated within NSs filaments (25). In the case of RVFV ZH-induced repression of the IFN-β gene, we have shown that NSs interaction with the IFN-β promoter required the presence of functional YY1 binding sites on the promoter region (25). Given the strong concentration of YY1 within NSs filaments, it is possible that as demonstrated in the case of the IFN-β promoter, YY1 could play a major role facilitating the interaction of the NSs protein with the host genome. This appears all the more possible, since 58% of 611 NSs-interacting genes that were chosen among NSs-interacting genes as belonging to different families contained at least one YY1 binding site when analyzed using the ChIP Transcription Factor Search Portal of SABiosciences for the presence of YY1 binding sites on their promoter regions. Since only approximately 8% of human or murine promoters are estimated to contain one or more YY1 binding site (40), promoters containing at least one YY1 binding site appeared 7-fold overrepresented among NSs-interacting regions. Future experiments using small interfering RNAs (siRNAs) directed against YY1 should help us to further analyze the potential role of YY1 on the establishment of NSs interactions with cellular chromatin.

Despite our previous ChIP results showing that the NSs protein interacts with the IFN-β promoter region (25), this region was not found present among the list of regions identified here during ChIP-on-chip experiments as statistically significantly interacting with NSs. This otherwise disturbing result was explained after making a close analysis of the probes present in the array covering the IFN-β locus. The IFN-β gene is transcribed in an antisense orientation with coordinates chromosome 4 (Chr4) 88 168 428 for the start site and Chr4 88 167 929 for the termination site. In the array, the orientation of the gene is mistakenly reversed. As a result, the probes designed to cover the IFN-β promoter region actually covered a region near and beyond the termination site, and probes meant to cover the IFN-β promoter were totally absent from the array.

Hemorrhages are often associated with RVFV-induced lethality in humans and animals, and in this work we have shown for the first time that not only did the NSs protein interact with the regulatory DNA regions of genes coding for coagulation factors but also that NSs affected the expression of the corresponding genes. Future analysis of RVFV ZH infection on other cellular pathways identified here as targeted by RVFV through NSs such as cell adhesion, axonal guidance, protein phosphorylation, and WNT signaling will certainly help to better decipher RVFV host-pathogen interaction and RVFV-induced pathological disorders.

Overall, these results demonstrated that a large-scale analysis of the capacity of a viral protein to interact with the host genome could lead to identification of cellular pathways targeted during virus infection. The approach that we have used here in the case of RVFV could be enlarged to other RNA viruses coding for proteins conveyed to the nuclei of host cells as for example in the case of the VP35 protein encoded by the Ebola virus, which is also a RNA virus inducing hemorrhagic fever and neurological disorders (10), that is detected in the nuclei of infected cells (4). Closer to RVFV, the NSs protein of Bunyamwera virus (26) and of La Crosse virus (45) also display nuclear distributions. Interestingly, it has been recently reported that the capacity of the C protein of the measles virus to inhibit IFN-β gene expression requires the nuclear localization of the viral protein (43).

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