JOURNAL OF VIROLOGY, Feb. 1996, p. 915–923 Vol. 70, No. 2
0022-538X/96/$04.00+0

Inhibition of Bunyaviruses, Phleboviruses, and Hantaviruses by Human MxA Protein

MICHAEL FRESE,[1] GEORG KOCHS,[1] HEINZ FELDMANN,[2] CHRISTIAN HERTKORN,[1] AND OTTO HALLER[1]*

Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene, Universität Freiburg, D-79104 Freiburg,[1] and Institut für Virologie, Universität Marburg, D-35037 Marburg,[2] Germany

Received 11 August 1995/Accepted 3 November 1995

Viruses of the *Bunyaviridae* family cause a variety of diseases ranging from uncomplicated fever to potentially lethal encephalitis and hemorrhagic fever. Little is known about the factors determining pathogenicity in the vertebrate host. Interferons have been reported to be inhibitory, but their mode of action against members of the *Bunyaviridae* has not yet been elucidated. The interferon-induced MxA protein encoded on human chromosome 21 is a large GTPase with antiviral activity against distinct negative-strand RNA viruses, notably influenza viruses. Here we show that MxA inhibits representative members of the *Bunyaviridae* family by interacting with an early step of virus replication. When constitutively expressed in stably transfected Vero cells, MxA prevented the accumulation of viral transcripts and proteins of Hantaan virus (genus *Hantavirus*). Other members of the family such as La Crosse virus (genus *Bunyavirus*) and Rift Valley fever virus and sandfly fever virus (both genus *Phlebovirus*) were likewise inhibited, and virus titers were reduced up to 10^4-fold. Our data indicate that humans have evolved a mechanism of controlling these viruses irrespective of differences in viral coding strategies.

Bunyaviridae is a family of over 300 viruses divided into five genera, namely, *Bunyavirus*, *Phlebovirus*, *Hantavirus*, *Nairovirus*, and *Tospovirus* (24). Although only a few members are known to be human pathogens, some have now been recognized as significant health problems. Currently, the prospect of newly emerging viruses in this family is a cause of public concern. These viruses are transmitted to humans from infected animals, which constitute a huge natural reservoir. Some viruses cause sudden and severe epidemics that are always unpredictable. For example, Rift Valley fever virus (RVFV), a phlebovirus (24), causes epizootics among domestic animals in many parts of sub-Saharan Africa and also in Egypt. During such outbreaks, the virus can be transmitted to humans by contact with sick animals or by mosquito bite. In the 1977 to 1978 epidemic in Egypt, over 200,000 people contracted the disease and at least 600 died (22). In contrast, sandfly fever Sicilian virus (SFSV) and related viruses cause a self-limiting disease with high fever and, occasionally, aseptic meningitis (8). They are transmitted by phlebotomus flies (sandflies) and are enzootic in geographic regions surrounding the Mediterranean Sea and India. La Crosse virus (LACV), belonging to the California encephalitis virus group of bunyaviruses, is transmitted by mosquitoes and is an important cause of summer encephalitis in children in the midwestern United States (10), while Tahyna virus causes outbreaks of an influenza-like illness in central Europe (18). Hantaviruses are exceptional, because they are not transmitted by arthropods. Humans are infected by contact with contaminated excretions of infected rodents and develop a variety of diseases. Thus, Hantaan virus (HTNV) causes Korean hemorrhagic fever (KHF) with a reported mortality rate of 10 to 15%, while Puumala virus (PUUV) causes a mild form of hemorrhagic fever with renal syndrome, which is observed throughout the Eurasian continent. Moreover, Sin Nombre virus, a newly discovered hantavirus, is responsible for hantaviral pulmonary syndrome, a severe and often fatal form of adult respiratory distress first recognized in 1993 in the Four Corners region of the southwestern United States (26).

Much progress has been made in recent years in elucidating the molecular biology and taxonomy of this large family of viruses. However, a better understanding of virus-host cell interactions and of the various factors determining virus virulence and pathogenicity is necessary to design successful strategies for disease control. Early studies by Peters et al. (31) indicated that human alpha interferon (IFN-α) is effective in protecting RVFV-infected rhesus monkeys from viremia and fatal liver damage, and it has been suggested but not proven that IFNs may be beneficial in treating human RVFV infections (23). In fact, RVFV (31), HTNV (45), and PUUV (47) are sensitive to the antiviral action of human IFN-α. However, the mode of IFN action against members of the *Bunyaviridae* has so far not been elucidated. The antiviral effect of IFNs is mediated by IFN-induced proteins which inhibit the multiplication of viruses by distinct mechanisms (34, 39). We have previously shown that some negative-strand RNA viruses are blocked by IFN-induced Mx proteins (39, 41), which are large GTPases related to dynamin (43). Human MxA protein accumulates in the cytoplasm, where it interferes with the multiplication of influenza virus and Thogoto-like viruses by an as yet unknown mechanism (6, 28). In addition, MxA protein selectively blocks the multiplication of vesicular stomatitis virus (VSV) at the level of primary transcription (37, 42) and, in some host cells, blocks the multiplication of measles virus (35, 36) and human parainfluenza virus type 3 (50). Importantly, human MxA protein is also a powerful antiviral agent in vivo, as demonstrated by enhanced virus resistance of transgenic mice expressing the human MxA protein in a constitutive manner (27). Human cells harbor a second IFN-regulated Mx gene on chromosome 21, which encodes human MxB protein. MxB protein is related to MxA protein, but no antiviral activity has

* Corresponding author. Phone: 49-761-2036534. Fax: 49-761-2036626. Electronic mail address: haller@sun1.ukl.uni-freiburg.de.

been found so far (6, 29). Here we show that human MxA protein is inhibitory to bunyaviruses, phleboviruses, and hantaviruses by interacting with an early step of virus replication.

MATERIALS AND METHODS

Cell cultures. Permanently transfected African green monkey kidney (Vero) cells constitutively expressing human MxA protein (clones VA8, VA9, and VA12) or human MxB protein (VB22, VB33, and VB72) and control cells expressing only the neomycin resistance gene were the same as described previously (6). Untransfected Vero cells, baby hamster kidney cells (BHK-21 cells), and Swiss mouse 3T3 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Transfected Vero cells were grown in the same medium plus 2.0 mg of G418 (GIBCO-BRL, Berlin, Germany) per ml.

Viruses. The original strain of LACV (49) was kindly provided by Raju Ramasamy, Meharry Medical College, Nashville, Tenn. Stock virus grown in BHK-21 cells contained 1.6×10^8 50% tissue culture infective doses ($TCID_{50}$) per ml as determined on Vero cells. HTNV and SFSV were obtained from Tino F. Schwarz, Max-von-Pettenkofer-Institut, Munich, Germany. SFSV stock virus grown in Vero cells contained 1.2×10^7 $TCID_{50}$/ml. The 76-118 strain of HTNV was grown in Vero E6 cells, and virus stocks contained 10^6 PFU/ml as determined on our control VN5 Vero cells. The attenuated MP12 strain of RVFV (2) was kindly provided by Michèle Bouloy, Institut Pasteur, Paris, France, and stock virus grown in Vero cells contained 2.5×10^7 $TCID_{50}$/ml. A mammalian cell-adapted variant of influenza A/FPV/Dobson/34 (H7N7) virus, called FPV-B (16), was grown in Swiss mouse 3T3 cells as described previously (29). Stock virus contained 6.0×10^7 $TCID_{50}$/ml.

Virus plaque and virus yield reduction assays. For plaque assays, cell monolayers in six-well macroplates were infected for 1 h at 37°C with serial 10-fold dilutions of LACV, RVFV, or HTNV stocks in medium containing 2% fetal calf serum and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.3). The virus inoculum was removed, and medium containing 2% fetal calf serum, 20 mM HEPES (pH 7.3), 0.4% Noble agar, and 0.002% DEAE-dextran was added. The plates were further incubated at 37°C for 4 days (LACV), 5 days (RVFV), or 12 days (HTNV). The agar overlay was removed; the cells were stained with a solution of 1% crystal violet, 3.6% formaldehyde, 1% methanol, and 20% ethanol; and the number of plaques was counted. For virus yield reduction assays, confluent cell monolayers were infected with a multiplicity of 0.1 $TCID_{50}$ of LACV, SFSV, RVFV, or FPV-B per cell for 1 h at 37°C. The virus inoculum was removed by washing the infected cells with phosphate-buffered saline (PBS) four times, and the cultures were further incubated at 37°C in DMEM containing 10% fetal calf serum. Samples of the culture supernatants were harvested at the times indicated, and the virus titers were determined on Vero cells. The virus titers were calculated as reciprocals of the $TCID_{50}$.

Immunofluorescence analysis. Cells were prepared and stained for MxA protein and viral antigens by indirect immunofluorescence, essentially as described previously (29). To visualize the MxA protein, monoclonal mouse antibody 2C12 was used (40). To label HTNV antigens, a convalescent-phase serum sample from a patient with KHF or the GB04-BF07 mouse monoclonal antibody (33) against the nucleocapsid protein was used. Both antibodies were obtained from the Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, Ga. (kindly provided by Thomas G. Ksiazek and Suyu L. Ruo). A polyclonal rabbit antibody against the LACV nucleocapsid protein was obtained from Raju Ramasamy, Meharry Medical College, Nashville, Tenn. A mixture of three monoclonal antibodies (807-25, 807-33, and 807-35) directed against the G1 envelope glycoprotein (9) was obtained from Francisco González-Scarano, University of Pennsylvania, Philadelphia. The preparations were then stained with fluorescein (DTAF)- or rhodamine (TRITC)-conjugated goat antibodies against mouse, rabbit, or human immunoglobulin G (Dianova, Hamburg, Germany). All antibodies were used in an appropriate dilution in PBS containing 5% normal goat serum. The stained cells were viewed and photographed on a Reichert-Jung Polyvar or a Zeiss Axiophot microscope equipped with epifluorescence.

Western blot (immunoblot) analysis. Total cell extracts were prepared by lysing cells in sample buffer (20). Proteins were separated onto 10% polyacrylamide–sodium dodecyl sulfate gels (20) and transferred to nitrocellulose by standard procedures. Filters were blocked for 30 min in PBS containing 5% nonfat dry milk and 0.1% Tween 20. Monoclonal antibody 2C12 (40) was used to immunostain MxA protein, and a polyclonal mouse antiserum against a β-galactosidase–MxB fusion protein (1) was used to label MxB. To stain HTNV antigens, a convalescent-phase serum sample from a patient with KHF was used (see above). To detect RVFV proteins, mouse polyclonal antibodies against the Zinga strain of RVFV (a gift from Michèle Bouloy) were used. All antibodies were diluted in PBS plus 5% nonfat dry milk and 0.1% Tween 20. Filters were incubated with the diluted antisera for 2 h, washed in PBS containing 0.1% Tween 20, and then incubated for 1 h with alkaline phosphatase-conjugated goat anti-human or goat anti-mouse antisera diluted 1:5,000 in PBS containing 0.1% Tween 20. The filters were washed in PBS containing 0.1% Tween 20 and stained with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (BCIP-NBT).

Northern (RNA) blot analysis. Total cellular RNA was prepared by the guanidinium thiocyanate-phenol-chloroform procedure (3). RNA was subjected to electrophoresis through 1.2% agarose gels containing formaldehyde, blotted to nytran (NY-13) membranes (Schleicher & Schuell, Dassel, Germany), and hybridized to radiolabeled probes as described previously (1).

cDNA and RNA probes. The cDNA probes specific for HTNV RNAs were generated as follows. Total RNA from HTNV-infected Vero cells was prepared, and cDNA was synthesized with random primers and Superscript RT as specified by the manufacturer (GIBCO-BRL, Berlin, Germany). PCR was performed for each of the three reverse-transcribed RNA segments. An L RNA fragment of 347 bp was amplified with oligonucleotides HTNL1 (5′-CTATACAGAAGAC CAAGGGCAATTCC-3′) and HTNL2 (5′-GGATAAACTCCACTTGCACC AACAG-3′). An M RNA fragment of 205 bp was amplified with oligonucleotides HTNM1 (5′-TGTATAATTGGGACAGTATCTAA-3′) and HTNM2 (5′-GCAAAGTTACATTTCTTCCT-3′). An S RNA fragment of 353 bp was amplified with oligonucleotides HTNS1 (5′-AGGCTATACGCCAGCATGCAGA AGC-3′) and HTNS2 (5′-CTAAGTGGAAGTTGTCCACAGCCTC-3′). The amplification products were cloned into the pCRII vector (Invitrogen, Leek, The Netherlands) and partially sequenced. *Eco*RI restriction fragments were radiolabeled by nick translation (Boehringer, Mannheim, Germany) and used as cDNA probes.

The cDNA probes specific for LACV RNAs were generated as follows. The L RNA-specific probe was made as described above with oligonucleotides LACL1 (5′-GGTATGGATCACATGTACCATTC-3′) and LACL2 (5′-AGTAGTGTG CTCCTATCTAC-3′). For the M RNA-specific probe, the *Bam*HI insert of the pM.ORF plasmid (corresponding to the entire LACV M open reading frame) (17) was radiolabeled by nick translation (Boehringer). The plasmid was kindly provided by Francisco González-Scarano. For the S RNA-specific probe, the *Xba*I insert of the pGEM3-LACS plasmid (corresponding to the full-length LACV S genome) was radiolabeled as described above. The plasmid was kindly provided by Raju Ramasamy. To generate the positive- and negative-sense LACV S RNA probes, the pGEM3-LACS plasmid was linearized with *Bam*HI or *Hin*dIII and radiolabeled mRNA was transcribed with SP6 or T7 polymerase, respectively.

The cDNA probe specific for the L RNAs of RVFV consisted of the radiolabeled *Eco*RV insert of plasmid pL (corresponding to the full-length RVFV L genome). The M RNA-specific cDNA probe consisted of the radiolabeled *Sac*II insert of plasmid pM (corresponding to the antigenomic-sense nucleotide positions 1200 to 2340 of RVFV M segment). Both pL and pM were derived from the $pBSK^+$ vector. In addition, strand-specific riboprobes for the S segment of RVFV were generated. To obtain a radiolabeled negative-sense S RNA probe (detecting S cRNA and N mRNA), the pN plasmid ($pBSK^+$ containing an RVFV S cDNA corresponding to the genomic-sense nucleotide positions 1190 to 1691) was linearized with *Not*I and radiolabeled mRNA was transcribed with T3 polymerase. To generate a radiolabeled positive-sense riboprobe specific for the S viral RNA (vRNA) and NSs mRNA, the pNSs plasmid (pGem3 containing an RVFV S cDNA corresponding to the genomic-sense nucleotide positions 62 to 729) was linearized with *Bam*HI and radiolabeled mRNA was transcribed with T7 polymerase. All plasmids with RVFV nucleotide sequences were kindly provided by Agnès Billecocq, Institut Pasteur, Paris, France.

The human β-actin probe consisted of the complete coding region of the β-actin cDNA cut from the plasmid pCDV1 by *Bam*HI digestion (12).

RESULTS

Human MxA protein inhibits members of the *Bunyaviridae* family. The *Bunyaviridae* family consists of diverse viruses, which have been assigned to five genera. Some viruses replicate well in Vero cells, growing to high titers and causing a cytopathic effect. Also, Vero cells are devoid of type I IFN genes because of a chromosomal deletion in their genome (4). Hence, they are unable to produce IFN-α/β upon viral infection and, as a consequence, do not express IFN-regulated genes, including those encoding endogenous Mx proteins. We therefore used MxA-transfected Vero cells to test the antiviral potential of human MxA protein against this group of viruses. Under the control of the constitutive 3-hydroxy-3-methylglutaryl coenzyme A reductase promoter, these Vero cells permanently express MxA protein at high levels in close to 100% of the cell populations. Transfected Vero cells expressing only the neomycin resistance gene served as susceptible controls. Monolayer cultures were infected with suitable dilutions of LACV (genus *Bunyavirus*) or, for comparison, influenza A virus FPV-B (an orthomyxovirus). Virus yields were assayed in supernatants obtained 24 h after FPV-B infection or 48 h after LACV infection. Figure 1A shows that the virus titers of the MxA cell cultures infected with LACV were 1,000- to 10,000-

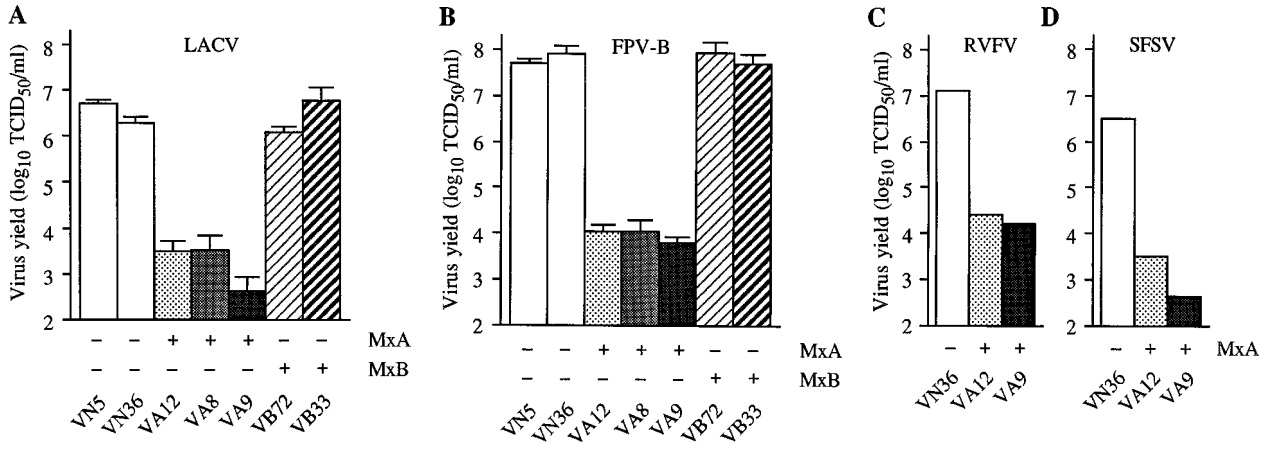

FIG. 1. Antiviral activity of human MxA protein against LACV (A), influenza A virus FPV-B (B), RVFV attenuated strain MP12 (C), and SFSV (D). Permanently transfected Vero cells expressing human MxA protein (clones VA8, VA9, and VA12) or MxB protein (clones VB33 and VB72) and control cell clones expressing only the neomycin resistance gene (clones VN5 and VN36) were infected with the viruses indicated at a multiplicity of infection of 0.1. Virus titers of culture supernatants were determined at 24 h (for FPV-B) or 48 h (for LACV, RFVF, and SFSV) after infection by the $TCID_{50}$ assay as indicated. The mean log titer for three independent experiments is shown for LACV (A) and FPV-B (B). Error bars indicate standard deviations.

fold lower than the titers of the control cultures. Influenza A virus titers were reduced to a similar degree, as expected (Fig. 1B). Vero cells expressing human MxB protein were fully permissive to LACV and, as shown previously (6), to influenza A virus (Fig. 1A and B). Likewise, plaque formation by LACV was severely inhibited in MxA-expressing cells (Table 1). These results demonstrate that human MxA protein has anti-LACV activity while MxB is inactive. Similar results were obtained with Tahyna virus, which, like LACV, belongs to the California encephalitis virus group (data not shown). Next, we tested two members of the genus *Phlebovirus*, RVFV (Fig. 1C) and SFSV (Fig. 1D). For practical purposes, we used the attenuated MP12 strain of RVFV. Growth of both viruses was severely inhibited in two independent MxA-expressing Vero cell clones, and virus yields were reduced by 3 orders of magnitude compared with those in control cells. Sandfly fever Toscana virus behaved in the same way (data not shown). To corroborate these findings, plaque formation of RVFV was analyzed. The MP12 strain of RVFV formed plaques in MxA-negative control Vero cells but not in Vero cells expressing MxA protein (Table 1). Finally, we determined the capacity of human MxA protein to prevent plaque formation by HTNV 76-118 (genus *Hantavirus*). Monolayer cultures of MxA-expressing and control cells were infected with suitable dilutions of HTNV, and the viruses were allowed to form plaques under soft agar for 12 days. No plaques were visible in the presence of MxA, whereas plaques were detectable in control cells up to high dilutions (Table 1). In conclusion, human MxA protein was inhibitory to all members of the *Bunyaviridae* family tested, irrespective of differences in the coding strategies among some of these viruses.

TABLE 1. Inhibition of virus plaque formation

Cell line[a]		No. of plaques[b] formed by:		
Clone	MxA	LACV	RVFV	HTNV
VA9	+	$\leq 10^3$	$\leq 10^3$	$\leq 10^3$
VN5	−	1×10^7	2×10^7	1×10^6

[a] Monolayers of permanently transfected Vero cells expressing human MxA (clone VA9) and control cells expressing only the neomycin resistance gene (clone VN5) were infected with stock virus of LACV (original strain), RVFV (strain MP12), or HTNV (strain 76-118).

[b] The number of plaques was counted 4 days (LACV), 5 days (RVFV), or 12 days (HTNV) after infection.

HTNV growth is blocked at an early step of virus replication. The inhibition of HTNV growth observed in MxA-expressing Vero cells was remarkable, because a previous report had suggested that MxA in stably transfected monocytic cells (U937) does not mediate resistance to PUUV, another member of the *Hantavirus* genus (47). We therefore analyzed the mechanism of inhibition in more detail. To determine whether HTNV protein synthesis was inhibited, MxA-expressing and control cells were infected with 3 PFU of HTNV 76-118 per cell for 24 h, fixed, and then stained by indirect immunofluorescence for HTNV proteins with a high-titer convalescent-phase serum sample from a patient with KHF (detecting predominantly the nucleocapsid protein). Human MxA protein was stained with monoclonal antibody 2C12. Figure 2A shows that viral proteins were barely detectable in the presence of MxA whereas HTNV proteins were clearly visible in the cytoplasm of control cells lacking MxA. Likewise, monoclonal antibody GB04-BF07, recognizing the nucleocapsid protein of HTNV, easily stained infected control cells but hardly stained MxA-expressing cells. Synthesis of viral proteins in infected cells was further assessed by the Western blot technique. Cell monolayers were infected with 3 PFU per cell, and total cell extracts were analyzed 24 and 48 h later. At both time points after infection, HTNV nucleocapsid proteins were barely visible in MxA-containing extracts (Fig. 2B and C), confirming the immunofluorescence data. In contrast, nucleocapsid protein bands were prominent in infected control cell extracts, and their intensity increased with time. Moreover, comparable amounts of nucleocapsid proteins were also detectable in cells expressing human MxB protein, indicating that MxB had no or at best a marginal effect against HTNV (Fig. 2B and C). As a control, aliquots of these samples were tested for their MxA or MxB protein content with MxA-specific antibody 2C12 or a polyclonal mouse serum directed against human MxB (Fig. 2D). Finally, we monitored virus growth by determining the accumulation of viral RNAs in infected cells by Northern blot analysis with cDNA probes specific for the L, M, and S segments of HTNV (Fig. 3). The susceptible control cells contained high concentrations of all three viral RNA species 24 h

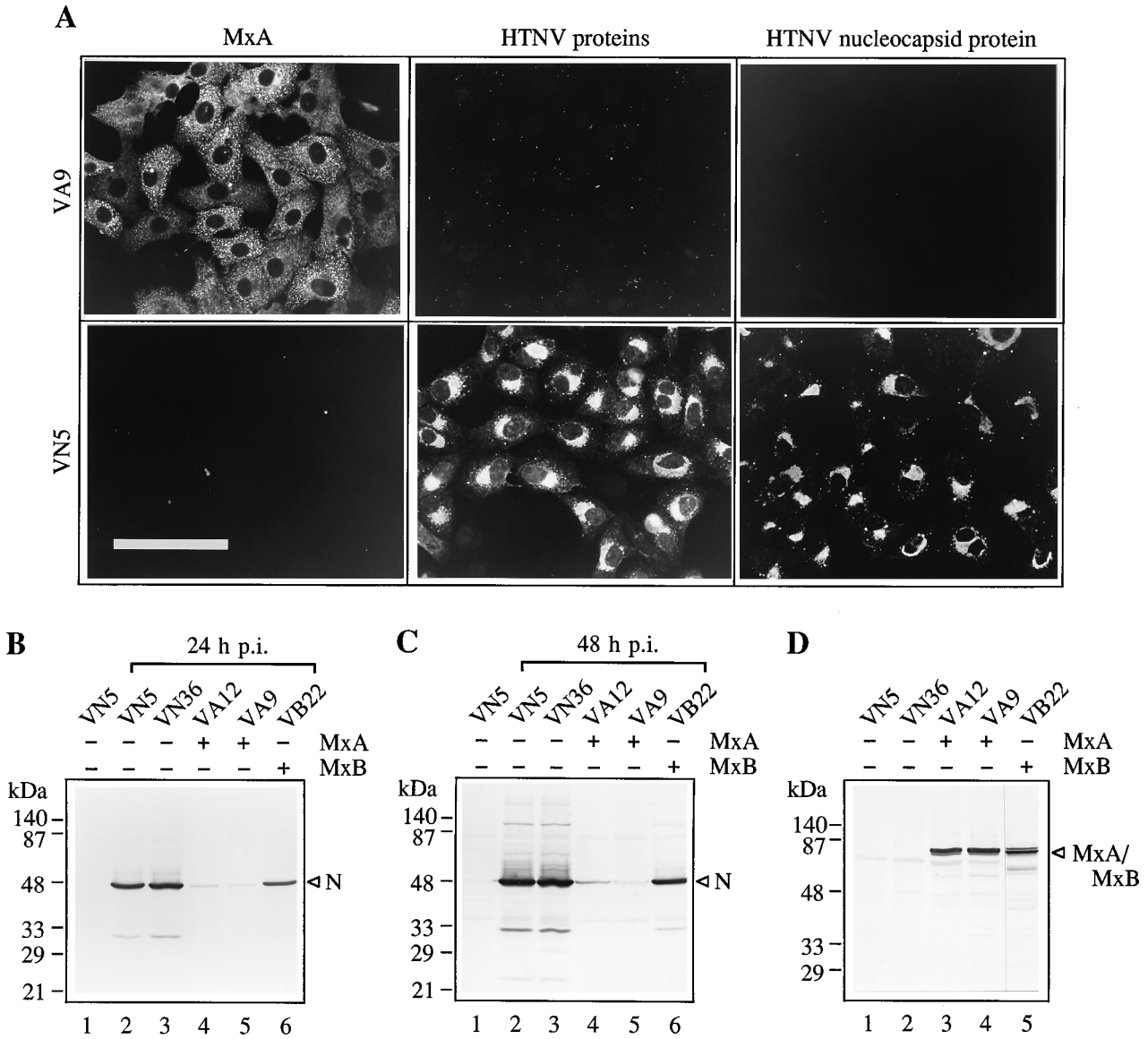

FIG. 2. Inhibition of HTNV protein synthesis by human MxA protein. (A) Monolayers of MxA-expressing Vero cells (clone VA9) or control cells expressing only the neomycin resistance gene (clone VN5) were infected with HTNV 76-118 at a multiplicity of infection of 3 PFU per cell. Cells were fixed 24 h after infection and were analyzed by indirect immunofluorescence for the presence of human MxA protein with monoclonal antibody 2C12 (left) and simultaneously for synthesis of HTNV proteins (middle) or nucleocapsid protein (right) with a convalescent-phase serum sample from a patient with KHF or monoclonal antibody GB04-BF07, respectively. Bar, 100 μm. (B and C) Detection of HTNV proteins by Western blot analysis. Control Vero cells (clones VN5 and VN36 [lanes 1 to 3]) and permanently transfected Vero cells expressing either the human MxA protein (clones VA12 and VA9 [lanes 4 and 5]) or the human MxB protein (clone VB22 [lane 6]) were infected (lanes 2 to 6) as described above or were left uninfected (lane 1). Cell extracts were prepared 24 h (B) and 48 h (C) after infection (p.i.), and samples of 15 μg of protein were analyzed. Western blots were developed with a convalescent-phase serum sample from a patient with KHF. Molecular mass markers are shown on the left. (D) Detection of human MxA and MxB protein in permanently transfected Vero cell clones by Western blot analysis. Extracts of the cell lines indicated were prepared, and samples of 15 μg of protein per lane were analyzed with MxA-specific monoclonal antibody 2C12 (lanes 1 to 4) or a polyclonal mouse serum directed against human MxB protein (lane 5). Molecular mass markers are shown on the left.

after infection. In contrast, both MxA-expressing cell clones lacked detectable viral RNAs, although low levels of S RNA transcripts coding for the nucleocapsid protein were expected to accumulate, given the Western blot data shown above. These results demonstrate that HTNV is highly sensitive to the antiviral action of human MxA protein, which acts at an early step of the virus replication cycle.

Inhibition of LACV by human MxA protein. LACV belongs to the California encephalitis virus group and is among the best-studied bunyaviruses. Bunyaviruses differ from hantaviruses in many respects, notably also in the size of the S genome segment, which is considerably smaller. For comparison, we studied LACV growth in MxA-expressing Vero cells by immunofluorescence and Northern blot techniques. Monolayers of MxA-expressing and control cells were infected with a high input multiplicity of 15 $TCID_{50}$ of LACV per cell to obtain a maximal percentage of infected cells. Monolayers were stained 9 h later with a polyclonal antibody for the nucleocapsid protein N and, in parallel cultures, with monoclonal antibodies specific for the envelope glycoprotein G1. The clearest difference between the two cultures was seen with G1 staining (Fig. 4A). Most control cells exhibited the characteristic G1 staining

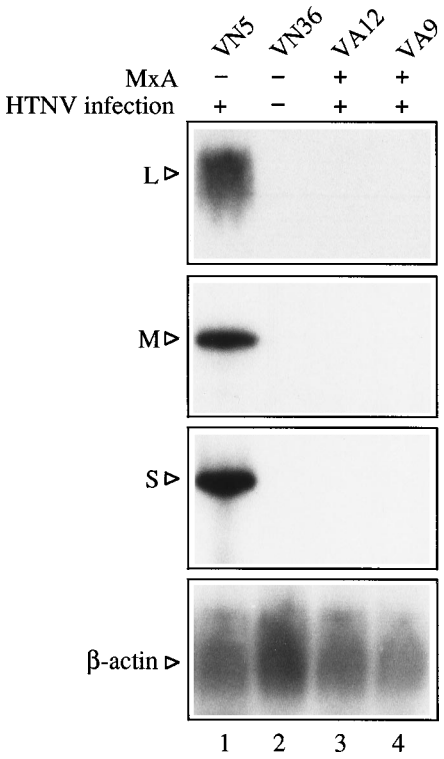

FIG. 3. Detection of HTNV transcripts in infected cells by Northern blot analysis. Control Vero cells (clones VN5 and VN36 [lanes 1 and 2]) and MxA-expressing Vero cells (clones VA12 and VA9 [lanes 3 and 4]) were infected with 3 PFU of HTNV 76-118 per cell (lanes 1, 3, and 4) or were left uninfected (lane 2). Total RNA was prepared 24 h after infection, and samples of 20 μg of RNA per lane were analyzed with specific cDNA probes for the L, M, and S RNAs. Hybridization signals obtained with a human β-actin probe are shown at the bottom. The blot was exposed to X-ray films for different times to give strong signals for all bands.

pattern as observed in productive infections. In contrast, MxA-expressing cells did not show specific fluorescence, indicating that G1 envelope glycoprotein had not accumulated to detectable levels. Similarly, nucleocapsid protein staining was most prominent in infected control cells, whereas N expression was reduced overall but still detectable in several MxA-containing cells (Fig. 4A). These results were confirmed by Western blot analysis showing that LACV nucleocapsid protein accumulated in resistant cells, albeit at lower levels than in susceptible cells (data not shown). To test whether the quantitative differences between the expression of the G1 envelope glycoprotein (a product of the M genome segment) and that of the nucleocapsid protein (a product of the S genome segment) corresponded to different levels of M and S transcripts, Northern blot analysis was performed. Two distinct MxA-expressing Vero cell clones were infected with LACV at a multiplicity of 3 $TCID_{50}$ per cell, together with two control cell clones, and total RNA was isolated 9 h later. Northern blots containing these RNAs were hybridized with radiolabeled cDNA probes of the three LACV genome segments. Clearly, in cells expressing MxA protein, synthesis of L- and M-specific RNAs was much more strongly inhibited than was synthesis of S-specific RNAs (Fig. 4B). To distinguish between positive-sense RNA (mRNA and cRNA) and negative-sense RNA (vRNA), the membranes were stripped and rehybridized with radiolabeled in vitro-transcribed RNA probes of the appropriate orientation corresponding to the S genome segment (Fig. 4C). All three RNA species, including mRNA, full-length cRNA, and vRNA, were detectable irrespective of the presence or absence of MxA protein. These data indicated that both transcription and replication of the input S genomic RNA still occurred in MxA-expressing cells. However, the relative levels of the full-length RNA species were estimated to be 5- to 10-fold lower in MxA-expressing cells than in control cells, and those of the mRNA species were at best 2-fold lower. Taken together, these results suggest that MxA protein inhibits the multiplication of LACV at the level of transcription and/or replication, two processes that both occur in the cytoplasm.

Early block of RVFV multiplication in MxA-expressing cells. RVFV differs from HTNV and LACV in its ambisense coding strategy. We therefore studied RVFV inhibition by MxA in more detail by Western and Northern blot analyses (Fig. 5). Control Vero cells (clones VN5 and VN36) and permanently transfected Vero cells expressing the human MxA protein (clones VA9 and VA12) were infected with 3 $TCID_{50}$ of RVFV MP12 per cell or were left uninfected. Cell extracts were prepared 12 h after infection, and viral proteins were reacted with a polyclonal mouse antibody specific for the Zinga strain of RVFV. This antibody detected distinct protein bands in extracts of infected control cells corresponding to the viral glycoproteins, the nucleocapsid protein, and the nonstructural protein NSs (Fig. 5A). In MxA-expressing cells, levels of viral proteins were much reduced (Fig. 5A). To assess the effect of MxA on viral RNA accumulation, total RNA was prepared 9 h after infection and analyzed by Northern blotting with either nick-translated cDNA probes specific for the L and M RNAs or strand-specific RNA probes able to detect the various S RNA species (Fig. 5B). Clearly, accumulation of L and M RNA transcripts was inhibited in MxA-expressing cells. L gene products remained undetectable in both cell lines tested (Fig. 5B, upper left panel), and only trace amounts of M transcripts were visible (Fig. 5B, upper right panel). In contrast, the susceptible control cells contained large amounts of both L and M RNA species. However, the relative intensities of both viral RNA bands differed slightly between the two clonal cell lines VN5 and VN36, indicating that additional factors may influence the efficacy of infection. The ambisense S gene segment of RVFV gives rise to four distinct RNA species. On the one hand, positive-sense N mRNA and S cRNA are produced and are detectable with antisense riboprobes (Fig. 5B, lower left panel). On the other hand, S vRNA and NSs mRNA are formed and are detectable with sense riboprobes (Fig. 5, lower right panel). Accumulation of all four RNA species was affected in MxA-expressing cells, indicating that transcription and replication of the S segment is also inhibited irrespective of its ambisense nature. However, inhibition was far from complete, as also observed in the case of the S segment of LACV (Fig. 4).

DISCUSSION

The family *Bunyaviridae* consists of a large number of species which have certain molecular and biological characteristics in common. Here, we add a new feature by demonstrating their susceptibility to the antiviral action of human MxA protein. Members of the *Bunyaviridae* contain a single-stranded RNA genome of negative polarity, although ambisense coding segments are present in some genera. The genome is divided into large (L), middle-sized (M), and small (S) segments. Most viruses are arboviruses and thus are able to replicate in arthropod as well as in vertebrate cells (24, 25). MxA-sensitivity is obviously a property common to all genera tested so far, irrespective of their coding strategy. It will be of great interest to

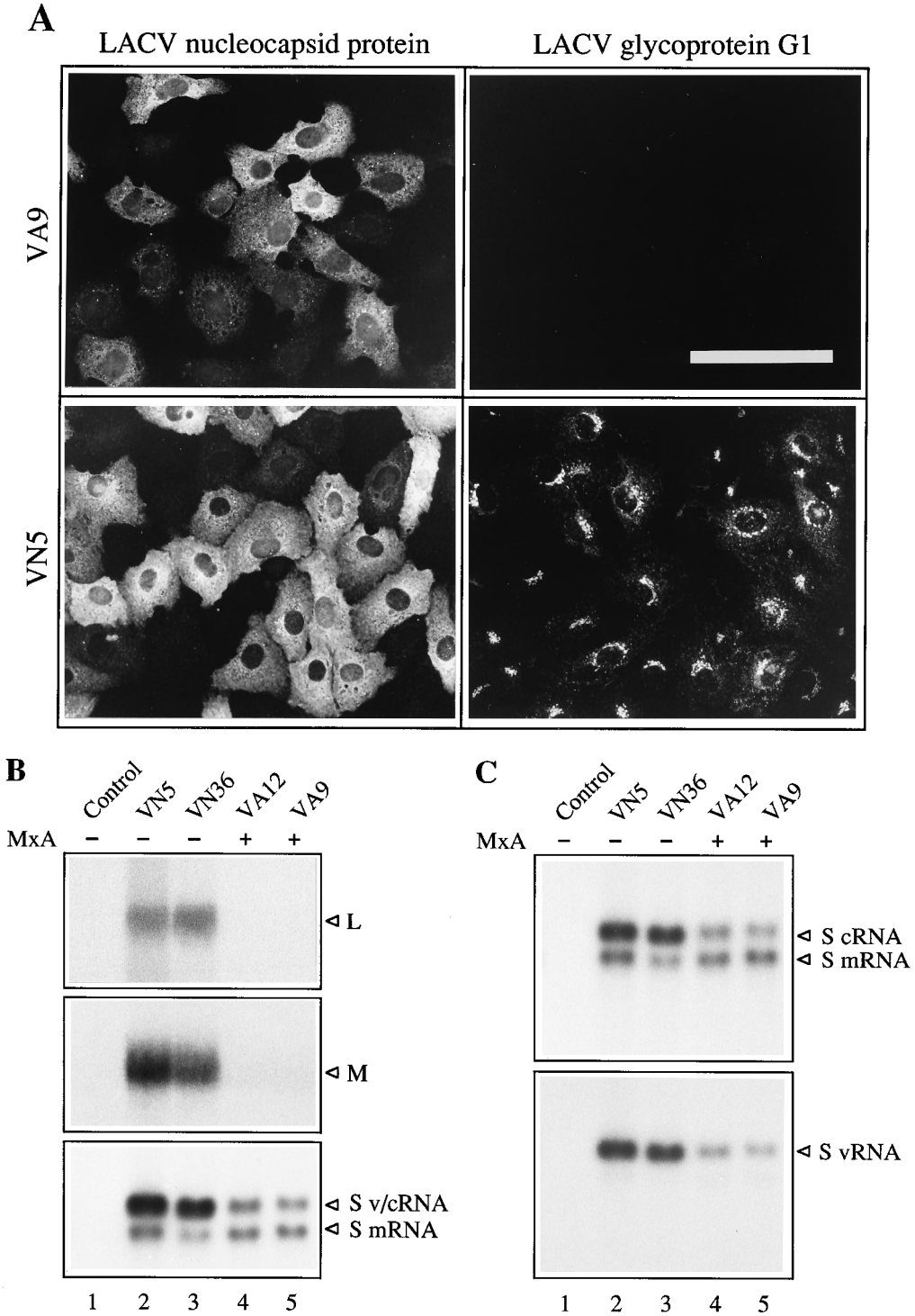

FIG. 4. Inhibition of LACV multiplication by human MxA protein. (A) Detection of LACV proteins by immunofluorescence analysis. Monolayers of MxA-expressing Vero cells (clone VA9) and control Vero cells lacking MxA (clone VN5) were infected with LACV at a multiplicity of infection of 15 $TCID_{50}$ per cell. Cells were fixed at 9 h after infection and were stained for LACV nucleocapsid protein N or envelope glycoprotein G1 with specific antibodies. Bar, 100 μm. (B and C) Detection of LACV transcripts by Northern blot analysis. Vero cells expressing only the neomycin resistance gene (clones VN5 and VN36 [lanes 1 to 3]) and MxA-expressing Vero cells (clones VA12 and VA9 [lanes 4 and 5]) were infected with 3 $TCID_{50}$ of LACV per cell (lanes 2 to 5) or were left uninfected (lane 1). Total RNA was prepared 9 h after infection, and samples of 20 μg of RNA per lane were analyzed with nick-translated cDNA probes specific for the L, M, and S RNAs (B). The Northern blot was stripped and reprobed with a radiolabeled negative-sense S RNA probe detecting S cRNA and S mRNA (C, top) and also with a positive-sense S RNA probe detecting S vRNA (C, bottom). The blot was exposed to X-ray films for different times to give strong signals for all bands.

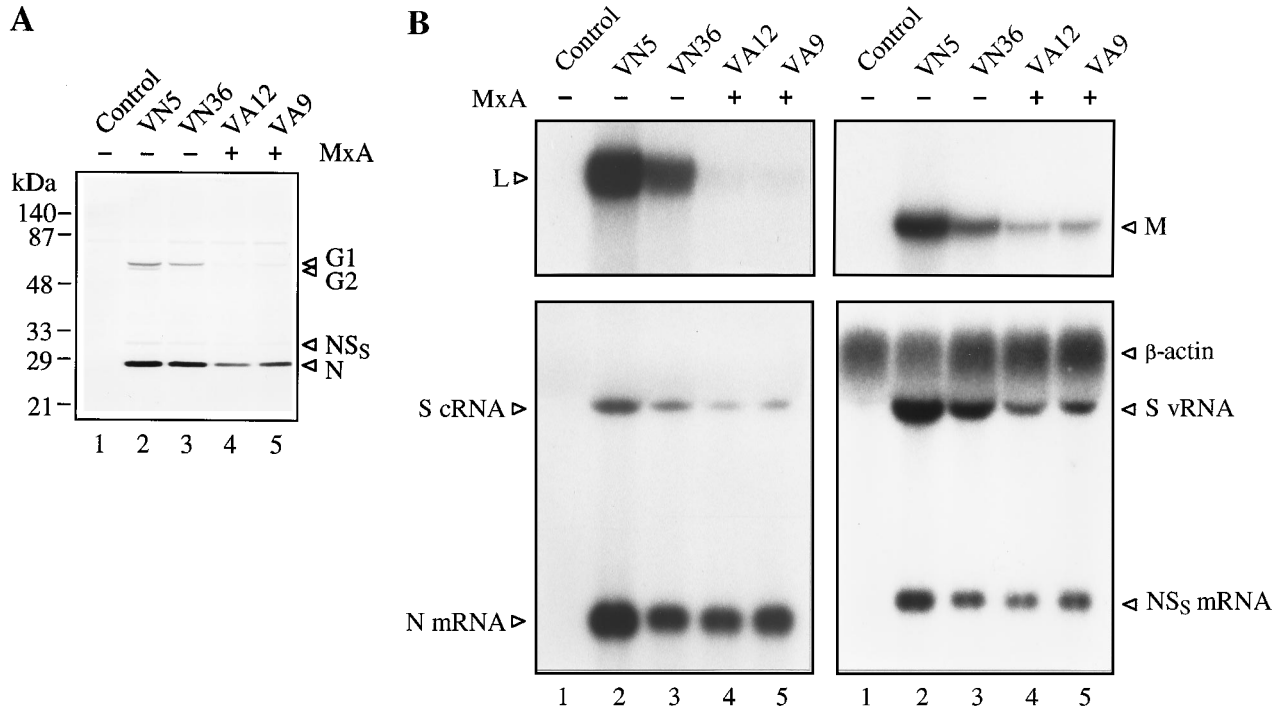

FIG. 5. Inhibition of RVFV multiplication by human MxA protein. (A) Detection of RVFV proteins by Western blot analysis. Control Vero cells (clones VN5 and VN36 [lanes 1 to 3]) and permanently transfected Vero cells expressing the human MxA protein (clones VA12 and VA9 [lanes 4 and 5]) were infected with RVFV MP12 at a multiplicity of infection of 3 (lanes 2 to 5) or were left uninfected (lane 1). Cell extracts were prepared 12 h after infection, and samples of 10 μg of protein were analyzed. Western blots were developed with mouse polyclonal antibodies against RVFV (Zinga strain). Molecular mass markers are shown on the left. (B) Detection of RVFV transcripts by Northern blot analysis. Vero cells expressing only the neomycin resistance gene (clone VN5 and VN36 [lanes 1 to 3]) and MxA-expressing Vero cells (clones VA12 and VA9 [lanes 4 and 5]) were infected with 3 $TCID_{50}$ of RVFV MP12 per cell (lanes 2 to 5) or were left uninfected (lane 1). Total RNA was prepared 9 h after infection, and samples of 10 μg of RNA per lane were analyzed with nick-translated cDNA probes specific for the L RNA (top left) or M RNA (top right). The blot was stripped and reprobed with a radiolabeled negative-sense S RNA probe detecting S cRNA and N mRNA (bottom left) or with a positive-sense S RNA probe detecting S vRNA and NS_S mRNA (bottom right). Hybridization signals obtained with a human β-actin probe are shown in the bottom right panel. The blot was exposed to X-ray films for different times to give strong signals for all bands.

determine MxA susceptibility of tospo- and tenuiviruses, which are plant pathogens of major economic importance (7). If MxA or related proteins are protective, it should be possible to generate transgenic plants with increased resistance, e.g., to tomato spotted wilt virus or rice stripe tenuivirus.

What might be the mechanism of inhibition of members of the *Bunyaviridae* by human MxA protein? Clearly, an early step in virus replication is affected, as with other MxA-susceptible viruses. MxA protein interferes with primary transcription of VSV catalyzed by the parental viral transcriptase in the cytoplasm (36, 42). In contrast, MxA is unable to inhibit primary transcription of influenza A virus, which takes place in the nucleus. Rather, MxA interferes with a poorly defined later step that follows primary transcription but precedes viral genome amplification (28). When MxA protein is artificially moved to the nucleus by means of a foreign nuclear translocation signal, it gains the ability to interfere with primary transcription of influenza virus. Most probably, then, the same viral target is recognized by MxA in both locations, and the subcellular localization of MxA determines whether primary transcription or a subsequent cytoplasmic step is affected (51). This view is supported by recent data on in vitro transcription of VSV and influenza virus. Schwemmle et al. demonstrated that purified MxA protein inhibited the transcriptional activity of VSV ribonucleoprotein complexes (37). Similar findings were obtained by Landis et al. with influenza A virus in an in vitro transcription system (21). These findings and other evidence (14, 44) suggest that the active RNA polymerase complex might be the target of MxA action. The process by which bunyaviruses transcribe their genome shares many features with that used by influenza viruses. However, in contrast to influenza viruses, bunyavirus transcription and replication takes place entirely in the cytoplasm of infected cells (32), which is the very location of MxA protein action. Our results with LACV and RVFV show that synthesis of the small viral RNAs was less impeded than that of the medium-sized or large RNAs. This argues for a block in elongation rather than initiation of viral RNA synthesis and is reminiscent of similar findings with influenza virus. We have previously shown that the synthesis of the smallest viral mRNAs, the NS1 and M1 mRNAs, is also preferentially spared during inhibition of primary transcription of influenza virus (19, 28). Recent results obtained with a cell-free in vitro system indicate that Mx proteins seem not to interfere with the initial cap-recruiting steps of the influenza virus polymerase (involving cap binding and subsequent cleavage of the host mRNA primer) but, rather, with elongation of primed transcripts (21). The present results are compatible with such a model of MxA action.

Bunyaviruses share susceptibility to MxA with certain other negative-strand RNA viruses, namely, influenza virus, Thogoto virus, and VSV (6, 28, 37, 42). It should be emphasized here that a number of negative-strand RNA viruses are not inhibited by MxA, at least not in MxA-expressing Vero cells. These comprise Dhori virus, measles virus, canine distemper virus, Ebola virus, Marburg virus, and Borna disease virus (5, 6, 13,

38, 48). The basis for this difference in MxA susceptibility is presently not understood.

It has been reported that human MxA protein does not mediate resistance to PUUV infection. Permanently transfected monocytic U-937 cells constitutively expressing MxA protein were found to be susceptible to PUUV, as judged by the level of N protein accumulation (47). Since PUUV was inhibited in our MxA-expressing Vero cells (46), the intriguing possibility exists that the antiviral activity of MxA against hantaviruses is modulated by the host cell. Host cellular factors are proposed to influence the antiviral spectrum of MxA proteins against measles virus, which is inhibited at the level of transcription in the neuronal cell line U-87 (35), at the level of viral glycoprotein synthesis in the monocytic cell line U-937 (36), and not at all in Vero cells (38). It will be of great interest to identify the modulating cellular factor(s). Alternatively, the MxA levels in U-937 cells may not have been sufficient to inhibit transcription of the S genome segment of PUUV, because, as shown here with LACV and RVFV, the smaller segments may be preferentially spared.

Our results firmly establish MxA as an intracellular mediator of the antiviral effect of IFNs against members of the *Bunyavirus*, *Phlebovirus*, and *Hantavirus* genera. Human MxA protein or related proteins induced in cells from other species are most probably responsible for the impressive IFN effects against Bunyamwera virus, HTNV, PUUV, and RVFV described in the literature (15, 30, 31, 47). If humans are equipped with such a potent defense system, why are these virus infections sometimes not self-limiting? The reason probably is that, depending on the virus strain, dose, and circumstances, the infecting virus does not induce MxA protein rapidly enough to reach sufficiently high levels in the right place to halt virus spread. A rapid expression of the antiviral protein at precisely the sites where the virus multiplies is probably crucial. Why, then, is IFN-α not established as a therapeutic agent? A possible explanation is that IFN therapy is, by necessity, always given too late (11). When patients are symptomatic, virus growth and spread have already occurred. If IFN were given at the time of the infection, the patients might benefit, as is observed in animal models (23, 31). New laboratory tests of rapid viral diagnosis should allow trials of early IFN treatment in the near future.

ACKNOWLEDGMENTS

We thank Agnès Billecocq, Michèle Bouloy, Francisco González-Scarano, Thomas G. Ksiazek, Suyu L. Ruo, Raju Ramasamy, and Tino F. Schwarz for reagents and advice; Hans-Dieter Klenk, Daniel Kolakowski, and Jonathan F. Smith for helpful discussions; Ilkka Julkunen and Antti Vaheri for communicating data prior to publication; Albrecht E. Sippel for his interest in this work; and Simone Gruber for excellent technical assistance.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Ha 1582/1-1 and FE 286/5-1).

REFERENCES

1. **Aebi, M., J. Fäh, N. Hurt, C. E. Samuel, D. Thomis, L. Bazzigher, J. Pavlovic, O. Haller, and P. Staeheli.** 1989. cDNA structures and regulation of two interferon-induced human Mx proteins. Mol. Cell. Biol. **9:**5062–5072.
2. **Caplen, H., C. J. Peters, and D. H. L. Bishop.** 1985. Mutagen-directed attenuation of Rift Valley fever virus as a method for vaccine development. J. Gen. Virol. **66:**2271–2277.
3. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
4. **Diaz, M. O., S. Ziemin, M. M. Le Beau, P. Pitha, S. D. Smith, R. R. Chilcote, and J. D. Rowley.** 1988. Homozygous deletion of the alpha- and beta 1-interferon genes in human leukemia and derived cell lines. Proc. Natl. Acad. Sci. USA **85:**5259–5263.
5. **Feldmann, H., M. Frese, and O. Haller.** Unpublished data.
6. **Frese, M., G. Kochs, U. Meier-Dieter, J. Siebler, and O. Haller.** 1995. Human MxA protein inhibits tick-borne Thogoto virus but not Dhori virus. J. Virol. **69:**3904–3909.
7. **Goldbach, R., and D. Peters.** 1994. Possible causes of the emergence of tospovirus diseases. Semin. Virol. **5:**113–120.
8. **Gonzalez-Scarano, F., and N. Nathanson.** 1990. Bunyaviruses, p. 1195–1228. *In* B. N. Fields (ed.), Virology. Raven Press, New York.
9. **Gonzalez-Scarano, F., R. E. Shope, C. E. Calisher, and N. Nathanson.** 1982. Characterization of monoclonal antibodies against the G1 and N proteins of La Crosse and Tahyna, two California serogroup bunyaviruses. Virology **120:** 42–53.
10. **Griot, C., F. Gonzalez-Scarano, and N. Nathanson.** 1994. Molecular determinants of the virulence and infectivity of California serogroup bunyaviruses. Annu. Rev. Microbiol. **47:**117–138.
11. **Gui, X. N., M. Ho, M. S. Cohen, Q. L. Wang, H.-P. Huang, and Q.-X. Xie.** 1987. Hemorrhagic fever with renal syndrom: treatment with recombinant α interferon. J. Infect. Dis. **155:**1047–1051.
12. **Gunning, P., P. Ponte, H. Okayama, J. Engel, H. Blau, and L. Kedes.** 1983. Isolation and characterization of full length cDNA clones for human α, β, and γ actin mRNAs. Mol. Cell. Biol. **3:**787–795.
13. **Hallensleben, W.** 1995. Diploma thesis. Universität Freiburg, Freiburg, Germany.
14. **Huang, T., J. Pavlovic, P. Staeheli, and M. Krystal.** 1992. Overexpression of the influenza virus polymerase can titrate out inhibition by the murine Mx1 protein. J. Virol. **66:**4154–4160.
15. **Isaacs, A., J. S. Porterfield, and S. Baron.** 1961. The influence of oxygenation on virus growth. II. Effect on the antiviral action of interferon. Virology **14:** 450–455.
16. **Israel, A.** 1979. Preliminary characterization of the particles from productive and abortive infections of L cells by fowl plague virus. Ann. Microbiol. (Paris) **130B:**85–100.
17. **Jacoby, D. R., C. Cooke, I. Prabakaran, J. Boland, N. Nathanson, and F. Gonzalez-Scarano.** 1993. Expression of the La Crosse M segment proteins in a recombinant vaccinia expression system mediates pH-dependent cellular fusion. Virology **193:**993–996.
18. **Knuth, T., H. Liebermann, and D. Urbaneck.** 1990. Für die DDR neuartige Virusinfektionen der Haustiere, 4. Mitteilung: Die Tahyna-Virusinfektion—ein Überblick. Arch. Exp. Vet. Med. **44:**265–277.
19. **Krug, R. M., M. Shaw, B. Broni, G. Shapiro, and O. Haller.** 1985. Inhibition of influenza viral mRNA synthesis in cells expressing the interferon-induced Mx gene product. J. Virol. **56:**201–206.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **Landis, H., H. P. Hefti, and J. Pavlovic.** Mx1 and MxA inhibit influenza virus RNA synthesis in vitro independent of the endonuclease activity of PB2. Virology, in press.
22. **Laughlin, L. W., J. M. Meegan, L. H. Strausbaugh, D. M. Morens, and R. H. Watten.** 1979. Epidemic Rift Valley fever in Egypt: observations of the spectrum of human illness. Trans. R. Soc. Trop. Med. Hyg. **73:**630–633.
23. **Morrill, J. C., G. B. Jennings, T. M. Cosgriff, P. H. Gibbs, and C. J. Peters.** 1989. Prevention of Rift Valley fever in rhesus monkeys with interferon-alpha. Rev. Infect. Dis. **11:**(Suppl. 4)**:**815–825.
24. **Murphy, F. A., C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo, and M. D. Summers (ed.).** 1995. Virus taxonomy. Springer-Verlag KG, Vienna.
25. **Nathanson, N., and F. Gonzalez-Scarano.** 1994. Bunyaviruses, p. 185–196. *In* R. G. Webster and A. Granoff (ed.), Encyclopedia of virology. Academic Press Ltd., London.
26. **Nichol, S. T., C. F. Spiropoulou, S. Morzunov, P. E. Rollin, T. G. Ksiazek, H. Feldmann, A. Sanchez, J. Childs, S. Zaki, and C. J. Peters.** 1993. Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness. Science **262:**914–917.
27. **Pavlovic, J., H. A. Arzet, H. P. Hefti, M. Frese, D. Rost, B. Ernst, E. Kolb, P. Staeheli, and O. Haller.** 1995. Enhanced virus resistance of transgenic mice expressing the human MxA protein. J. Virol. **69:**4506–4510.
28. **Pavlovic, J., O. Haller, and P. Staeheli.** 1992. Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle. J. Virol. **66:** 2564–2569.
29. **Pavlovic, J., T. Zürcher, O. Haller, and P. Staeheli.** 1990. Resistance to influenza virus and vesicular stomatitis virus conferred by expression of human MxA protein. J. Virol. **64:**3370–3375.
30. **Pensiero, M. N., J. B. Sharefkin, C. W. Diefenbach, and J. Hay.** 1992. Hantaan virus infection in human endothelial cells. J. Gen. Virol. **66:**5929–5936.
31. **Peters, C. J., J. A. Reynolds, T. W. Slone, D. E. Jones, and E. L. Stephen.** 1986. Prophylaxis of Rift Valley fever with antiviral drugs, immune serum, an interferon inducer, and a macrophage activator. Antiviral Res. **6:**285–297.
32. **Rossier, C., J. Patterson, and D. Kolakofsky.** 1986. La Crosse virus small genome mRNA is made in the cytoplasm. J. Virol. **58:**647–650.
33. **Ruo, S. L., A. Sanchez, L. H. Elliott, L. S. Brammer, J. B. McCormick, and S. P. Fisher-Hoch.** 1991. Monoclonal antibodies to three strains of hantaviruses: Hantaan, R22, and Puumala. Arch. Virol. **119:**1–11.

34. **Samuel, C. E.** 1991. Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities. Virology **183:**1–11.
35. **Schneider-Schaulies, S., J. Schneider-Schaulies, A. Schuster, M. Bayer, J. Pavlovic, and V. ter Meulen.** 1994. Cell-type-specific MxA mediated inhibition of measles virus transcription in human brain cells. J. Virol. **68:**6910–6917.
36. **Schnorr, J. J., S. Schneider-Schaulies, A. Simon-Jödicke, J. Pavlovic, M. A. Horisberger, and V. ter Meulen.** 1993. MxA-dependent inhibition of measles virus glycoprotein synthesis in a stably transfected human monocytic cell line. J. Virol. **67:**4760–4768.
37. **Schwemmle, M., K. C. Weining, M. F. Richter, B. Schumacher, and P. Staeheli.** 1995. Vesicular stomatitis virus transcription inhibited by purified MxA protein. Virology **206:**545–554.
38. **Sester, U.** 1994. Ph.D. thesis. Universität Freiburg, Freiburg, Germany.
39. **Staeheli, P.** 1990. Interferon-induced proteins and the antiviral state. Adv. Virus Res. **38:**147–200.
40. **Staeheli, P., and O. Haller.** 1985. Interferon-induced human protein with homology to protein Mx of influenza virus-resistant mice. Mol. Cell. Biol. **5:** 2150–2153.
41. **Staeheli, P., and O. Haller.** 1987. Interferon-induced Mx protein: a mediator of cellular resistance to influenza virus. Interferon **8:**1–23.
42. **Staeheli, P., and J. Pavlovic.** 1991. Inhibition of vesicular stomatitis virus mRNA synthesis by human MxA protein. J. Virol. **65:**4498–4501.
43. **Staeheli, P., F. Pitossi, and J. Pavlovic.** 1993. Mx proteins: GTPases with antiviral activity. Trends Cell Biol. **3:**268–272.
44. **Stranden, A., P. Staeheli, and J. Pavlovic.** 1993. Function of the mouse Mx1 protein inhibited by overexpression of influenza virus PB2. Virology **197:** 642–651.
45. **Tamura, M., H. Asada, K. Kondo, M. Takahashi, and K. Yamanishi.** 1987. Effects of human and murine interferons against hemorrhagic fever with renal syndrome (HFRS) virus (Hantaan virus). Antiviral Res. **8:**171–178.
46. **Temonen, M., I. Julkunen, and A. Vaheri.** Personal communication.
47. **Temonen, M., H. Lankinen, O. Vapalathi, T. Ronni, I. Julkunen, and A. Vaheri.** 1995. Effect of interferon-alpha and cell differentiation on Puumula virus infection in human monocyte/macrophages. Virology **206:**8–15.
48. **Thimme, R., M. Frese, G. Kochs, and O. Haller.** 1995. Mx1 but not MxA confers resistance against tick-borne Dhori virus in mice. Virology **210:** 296–301.
49. **Thompson, W. H., B. Kalfayan, and R. O. Anslow.** 1965. Isolation of California encephalitis group virus from a fatal human illness. Am. J. Epidemiol. **81:**245–253.
50. **Zhao, H., B. P. De, and A. K. Banerjee.** 1995. Effect of interferon-α on the replication of human parainfluenza virus type 3, abstr. W40-10, p. 196. *In* 14th Annual Meeting of the American Society for Virology.
51. **Zürcher, T., J. Pavlovic, and P. Staeheli.** 1992. Mechanism of human MxA protein action: variants with changed antiviral properties. EMBO J. **11:** 1657–1661.