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Mechanism of human MxA protein action: variants with changed antiviral properties

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Cells respond to treatment with interferons by synthesizing several induced proteins, including one or more structurally related proteins collectively called Mx. Nuclear and cytoplasmic forms of Mx have been described, some of which inhibit virus replication. Human MxA is a cytoplasmic protein that specifically inhibits the multiplication of influenza virus and vesicular stomatitis virus. Here, we describe a mutant MxA protein, MxA(R_{645}), which inhibited influenza virus but was inactive against vesicular stomatitis virus. It differs from wild-type MxA by a Glu to Arg substitution near the carboxy terminus. Like wild-type MxA, and as expected for an Mx protein acting in the cytoplasm, MxA(R_{645}) blocked influenza virus at a step after primary transcription. When moved to the nucleus of transfected cells with the help of a foreign nuclear transport signal, its mode of action changed. Like mouse Mx1, nuclear MxA(R_{645}) interfered with primary transcription of influenza virus, which is a nuclear process. Our results thus define an MxA region that determines antiviral specificity and further demonstrate that nuclear forms of MxA can mimic the action of mouse Mx1 whose natural location is the cell nucleus.

Key words: antiviral activity/influenza virus resistance/Mx proteins/nuclear transport/vesicular stomatitis virus resistance

Introduction

The human MxA protein is a powerful antiviral agent that specifically blocks the multiplication of both influenza virus and vesicular stomatitis virus (VSV) (Aebi *et al.*, 1989; Pavlovic *et al.*, 1990; Pavlovic and Staeheli, 1991; Samuel, 1991). It accumulates to high levels in the cytoplasm of interferon-treated cells (Staeheli and Haller, 1985; Horisberger and Hochkeppel, 1987). It contains sequence motifs typically found in GTP-binding proteins (Horisberger *et al.*, 1990; Pavlovic *et al.*, 1990; Staeheli, 1990), suggesting that MxA acts against viruses via a GTP-dependent biochemical activity.

The viruses susceptible to MxA have little in common and the antiviral specificity of MxA has been poorly understood to date. Influenza viruses are members of the family *Orthomyxoviridae*, whereas VSV is a member of the family *Rhabdoviridae*. Both are enveloped viruses with negative-stranded RNA genomes, but their multiplication strategies are very different. For example, transcription of influenza virus takes place in the nucleus and is dependent on host cell RNA synthesis (Krug *et al.*, 1989). In contrast, transcription and replication of VSV takes place entirely in the cytoplasm of infected cells (Banerjee, 1987). MxA inhibits VSV by interfering with normal virus mRNA synthesis (Staeheli and Pavlovic, 1991). However, its inhibitory effect on influenza virus is not at the level of mRNA synthesis; rather, an undefined multiplication step of influenza virus which follows primary transcription but precedes genome amplification is sensitive to the inhibitory action of MxA (Pavlovic *et al.*, 1992).

The related mouse Mx1 protein accumulates in the nucleus of interferon-treated cells (Horisberger *et al.*, 1983; Dreiding *et al.*, 1985). It blocks the multiplication of influenza virus but not VSV (Staeheli *et al.*, 1986; Pavlovic *et al.*, 1990). In contrast to MxA, the nuclear mouse Mx1 protein inhibits mRNA synthesis of influenza virus (Krug *et al.*, 1985; Pavlovic *et al.*, 1992), presumably via interaction with the viral PB2 polymerase subunit (T.Huang, J.Pavlovic, P.Staeheli and M.Krystal, submitted).

Since no *in vitro* systems are available at present for monitoring the antiviral activity of Mx proteins, the mechanistic details of Mx protein action remains unresolved. In particular, the molecular basis of the dual antiviral specificity of MxA towards influenza virus and VSV is unknown. Furthermore, as human MxA and mouse Mx1 proteins are located in different cell compartments and block different steps of influenza virus, it is unclear whether MxA could also function in the nucleus and whether nuclear forms of MxA could interfere with primary transcription of influenza virus like mouse Mx1 protein. Here we addressed these topics by generating clonal lines of 3T3 cells constitutively expressing mutant MxA cDNAs. We describe a nuclear form of MxA which mimics the action of mouse Mx1 and which blocks influenza virus mRNA synthesis. We describe further a cytoplasmic mutant MxA protein with altered antiviral specificity. It is inactive against VSV but fully active against influenza virus. This mutant protein differs from wild-type MxA by a single amino acid substitution near the carboxy terminus, indicating that this region of MxA determines antiviral specificity.

Results

MxA(R_{645}), a cytoplasmic protein that blocks influenza virus but not VSV

Our initial goal was to determine whether nuclear forms of MxA would inhibit influenza virus, therefore we examined whether MxA could be moved to the nucleus by a Glu to Arg substitution at position 645 (Figure 1). This modification creates a sequence motif in MxA that closely resembles the nuclear translocation signal of mouse Mx1 (Noteborn *et al.*, 1987). We anticipated that the resulting mutant protein, MxA(R_{645}), would accumulate in the nucleus of transfected

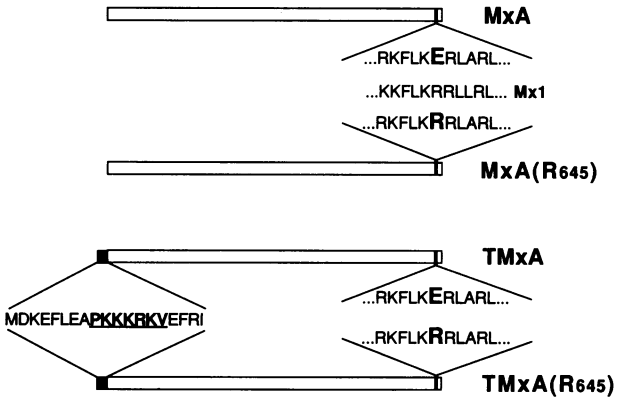

Fig. 1. Schematic drawing showing the primary structures of MxA, mutant MxA(R_{645}) carrying an Arg (R) residue at position 645 (bold) in place of Glu (E), TMxA containing 18 extra amino acids at the amino terminus of MxA that constitute the SV40 large T nuclear translocation signal (underlined) and TMxA(R_{645}) containing this SV40-derived sequence at the amino terminus of MxA(R_{645}).

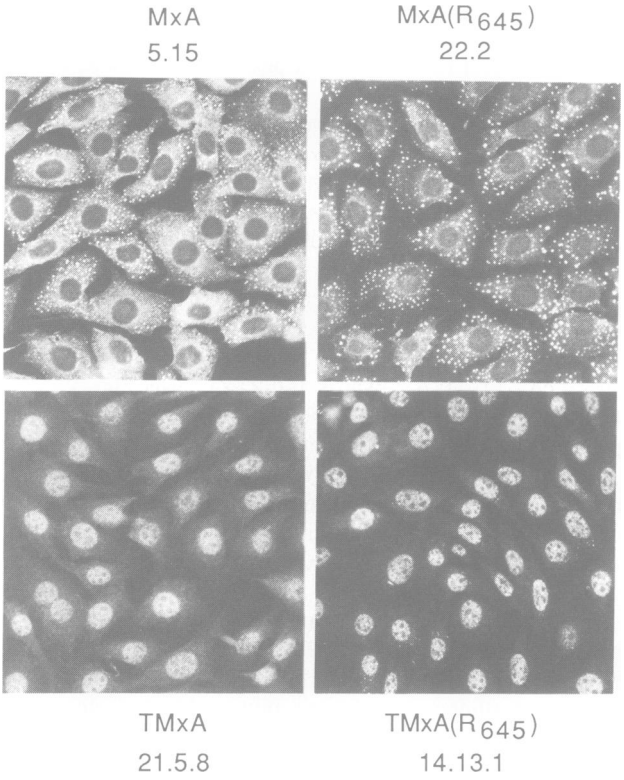

Fig. 2. Visualization of indirect immunofluorescence analysis of MxA, MxA(R_{645}), TMxA and TMxA(R_{645}) in permanently transfected mouse 3T3 cell lines. MxA variants and cell clone numbers are indicated.

cells. However, MxA(R_{645}) remained cytoplasmic in transfected Swiss 3T3 cells. A characteristic granular cytoplasmic staining pattern was observed when cells expressing MxA(R_{645}) were analysed with specific antibodies (Figure 2). Compared to wild-type MxA, the staining of MxA(R_{645}) was less diffuse and the granules were slightly larger.

Although MxA(R_{645}) was not nuclear, we wanted to determine its antiviral activity. To do this, we created permanently transfected mouse 3T3 cell lines that constitutively expressed wild-type MxA or MxA(R_{645}). The MxA expression levels were assessed by Western blot

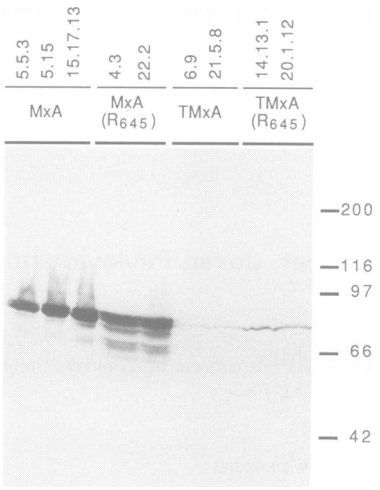

Fig. 3. Detection by Western blot analysis of the different MxA variants in clonal lines of transfected 3T3 cells. MxA variants and cell clone numbers are indicated. The relative gel positions of marker proteins are shown.

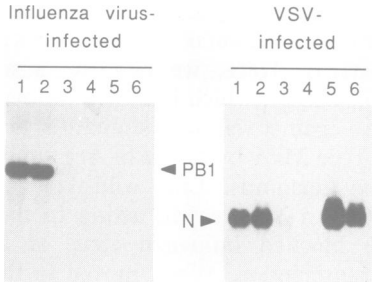

Fig. 4. Detection of influenza virus *PB1* and VSV *N* gene transcripts in virus-infected cells by Northern blot analysis. The cultures were infected with three plaque-forming units per cell of either influenza virus or VSV and the cells were harvested 5 h later. Total RNA (10 μg of RNA per lane) of two pools of transfected control cells lacking Mx proteins (lanes 1 and 2), cell clones 5.15 and 15.17.13 expressing MxA (lanes 3 and 4), and cell clones 22.2 and 4.3 expressing MxA(R_{645}) (lanes 5 and 6) were analysed.

analysis (Figure 3). The cell lines that we selected for subsequent studies contained comparably high concentrations of either MxA(R_{645}) or wild-type MxA (Figure 3). To our surprise, we found that the antiviral potential of MxA(R_{645}) differed from that of wild-type MxA: its antiviral activity against vesicular stomatitis virus (VSV) was lost. We infected 3T3 control cells expressing the neomycin resistance gene and permanent cell lines expressing either MxA(R_{645}) or wild-type MxA at a high level with three plaque-forming units per cell of influenza virus or VSV, respectively, and monitored virus growth by determining the levels of specific viral mRNAs at 5 h post-infection by Northern blot analysis (Figure 4). The infected control cells contained high concentrations of the influenza virus *PB1* mRNA, whereas only traces of *PB1* mRNA (at least 50 times less) were present in the infected cell lines expressing either wild-type MxA or MxA(R_{645}). In contrast, VSV *N* mRNA was detected at high concentrations not only in the infected control cells but also in the cell lines expressing MxA(R_{645}), whereas infected cells expressing wild-type MxA contained strongly reduced levels of VSV *N* mRNA.

We also determined the potential of MxA(R_{645}) to prevent influenza virus and VSV plaque formation

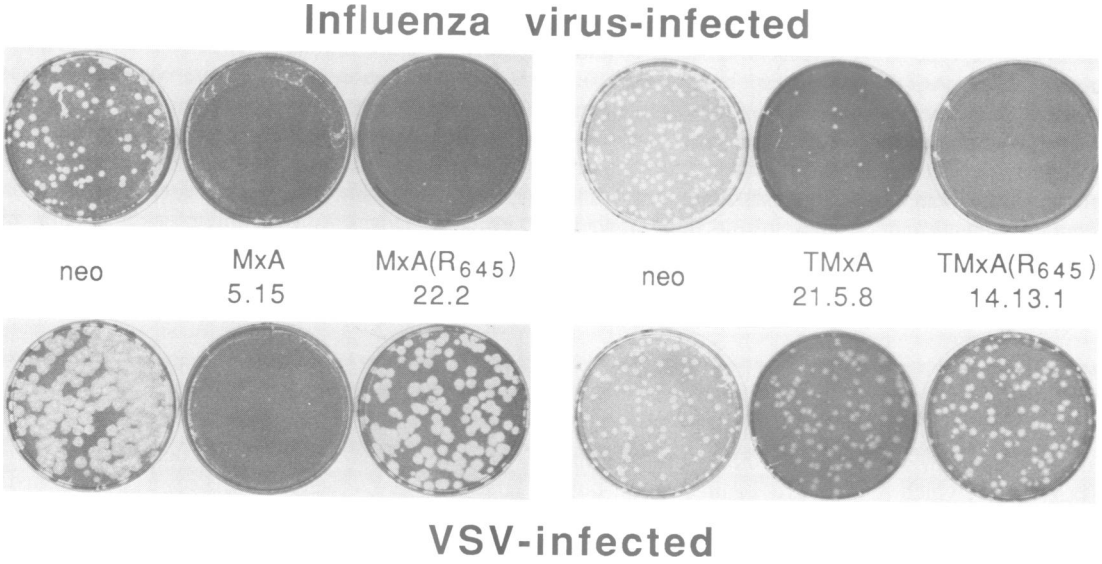

Fig. 5. Formation of influenza virus and VSV plaques on monolayers of transfected cells. Experiment 1: pool of transfected cells lacking Mx proteins (neo), cell clone 5.15 expressing MxA and cell clone 22.2 expressing MxA(R_{645}). Experiment 2: pool of transfected cells lacking Mx proteins (neo), cell clone 21.5.8 expressing TMxA and cell clone 14.13.1 expressing TMxA(R_{645}).

(Figure 5). Both viruses readily formed plaques on monolayers of control 3T3 cells lacking Mx proteins. Neither influenza virus nor VSV formed visible plaques on monolayers of cells expressing wild-type MxA. However, VSV formed plaques of normal size on monolayers of cells expressing MxA(R_{645}), whereas influenza virus failed to replicate and form visible plaques on these cells. Thus, viral RNA analyses and viral plaque assays showed that MxA(R_{645})-expressing cells are highly resistant to influenza virus but not to VSV, indicating that Glu 645 of MxA is part of a region that determines VSV specificity.

Nuclear forms of MxA are active against influenza virus

Another strategy to direct MxA to the cell nucleus took advantage of the simian virus 40 (SV40) large T nuclear translocation signal that is known to mediate nuclear transport of nonkaryophilic proteins (Kalderon *et al.*, 1984). Using recombinant DNA technology, we prepared a cDNA expression construct that encodes the mutant protein TMxA (Figure 1), which differs from wild-type MxA by a total of 18 additional SV40-derived amino acids at the amino terminus. As anticipated, TMxA accumulated in the nucleus of permanently transfected Swiss mouse 3T3 cells (Figure 2). The permanently transfected cell lines that we obtained expressed only a very low level of TMxA that was barely detectable by Western blot analysis (Figure 3). Nevertheless, these cell lines showed a low but significant degree of influenza virus resistance. About five times fewer influenza virus plaques were observed on TMxA-expressing cells compared with that on control cells lacking Mx proteins (Figure 5).

The same approach was used to move MxA(R_{645}) to the nucleus (Figure 1). As expected, the resulting mutant MxA protein, TMxA(R_{645}), accumulated in the nucleus of transfected 3T3 cells (Figure 2). Stable cell lines could be isolated that expressed TMxA(R_{645}) at a level that permitted easy detection by Western blotting (Figure 3). Influenza virus failed to form visible plaques on monolayers of

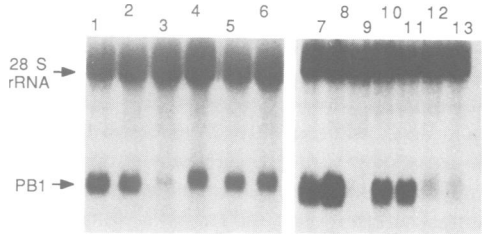

Fig. 6. Levels of primary influenza virus *PB1* transcripts in infected cells expressing different Mx proteins. The protein synthesis inhibitor cycloheximide (50 μg/ml) was added to the cultures 45 min prior to infection with five plaque-forming units of influenza virus FPV-B per cell. Cycloheximide was maintained in the culture media throughout the experiment. At 4 h post-infection, total RNA was prepared, and samples (20 μg of RNA per lane) were analysed for the presence of influenza virus *PB1* mRNA by Northern blotting using radiolabelled negative-strand *PB1* RNA as a hybridization probe. Experiment 1: control cells lacking Mx proteins (lanes 1 and 2), cell clone 27.2 expressing mouse Mx1 (lane 3), cell clone 5.15 expressing MxA (lane 4), and cell clones 22.2 (lane 5) and 4.3 (lane 6) expressing MxA(R_{645}). Experiment 2: control cells lacking Mx proteins (lanes 7 and 8), cell clone 27.2 expressing mouse Mx1 (lane 9), cell clones 5.15 (lane 10) and 5.5.3 (lane 11) expressing MxA, and cell clones 14.13.1 (lane 12) and 20.1.12 (lane 13) expressing TMxA(R_{645}). Cross-hybridization of the RNA probe to 28S ribosomal RNA demonstrated that all lanes contained similar concentrations of total RNA.

TMxA(R_{645})-expressing cells (Figure 5), further indicating that influenza virus can be blocked by nuclear forms of MxA.

Cytoplasmic and nuclear forms of MxA block different multiplication steps of influenza virus

We next determined the steps of the influenza virus multiplication cycle blocked by cytoplasmic MxA(R_{645}) and nuclear TMxA(R_{645}), respectively. As primary transcription of influenza virus takes place in the nucleus of infected cells, MxA(R_{645}) was expected to have no inhibitory effect at this level, like wild-type MxA (Pavlovic *et al.*, 1992). Primary transcription analysis of the influenza viral *PB1* gene, determined in the presence of an inhibitor of protein

synthesis, showed that this was indeed the case. High levels of primary *PB1* transcripts were found in infected cells expressing either wild-type MxA (Figure 6; lanes 4, 10 and 11) or cytoplasmic MxA(R_{645}) (Figure 6; lanes 5 and 6). In contrast, only low levels of primary *PB1* transcripts were detected in infected cells expressing the nuclear TMxA(R_{645}) protein (Figure 6; lanes 12 and 13). A similar result was obtained with infected cells expressing mouse Mx1 protein (Figure 6; lanes 3 and 9). These results thus showed that nuclear TMxA(R_{645}) blocked primary transcription of influenza virus like mouse Mx1 (Krug *et al.*, 1985; Pavlovic *et al.*, 1992) whose natural location is the cell nucleus.

Discussion

Our results resolve two questions concerning the mode of antiviral action of human MxA protein. First, our mutant protein MxA(R_{645}), which is inactive against VSV but active against influenza virus, helped to identify the region of MxA that determines specificity for VSV. This region includes Glu645 near the carboxy terminus. VSV replicated unhindered in cells expressing MxA(R_{645}) which has position 645 changed to an Arg residue, whereas influenza virus failed to replicate in such cells (Figures 4 and 5). The immunofluorescence staining patterns of wild-type MxA and mutant MxA(R_{645}) were similar but distinct (Figure 2); the mutant protein appeared more granular. We do not know whether this reflects a discrete change in its intracellular localization and whether this change relates to its inability to block VSV. Interestingly, mouse and rat Mx2 proteins which are specific for VSV (Meier *et al.*, 1990; Zürcher *et al.*, 1992) also have a Glu residue at the corresponding position, whereas mouse and rat Mx1 which are specific for influenza virus have an Arg at this position. Thus, a picture is emerging which shows that the carboxy-terminal parts of MxA and other Mx proteins determine their antiviral specificities, whereas regions closer to the amino termini might constitute the catalytic domains. Indeed, the former regions show a low degree of conservation, whereas the latter regions are highly conserved, as expected for a catalytic domain and they contain a GTP-binding consensus motif (Horisberger *et al.*, 1990). Accordingly, single point mutations affecting the GTP-binding consensus motif of MxA simultaneously abolished the antiviral activity against both influenza virus and VSV (F.Pitossi, J.Pavlovic and P.Staeheli, unpublished results).

The second conclusion from our results is that the mechanistic details of MxA and Mx1 action against influenza virus may not be so different after all, as artificial nuclear forms of human MxA could perfectly mimic the action of mouse Mx1 protein. When moved to the nucleus with the help of a foreign nuclear transport signal, MxA retained its activity against influenza virus. Compared to cytoplasmic wild-type MxA protein, lower concentrations of nuclear mutant MxA were required for a high degree of resistance to influenza virus (Figures 3 and 5), suggesting that MxA is actually most effective against influenza virus in the nucleus.

It was unexpectedly difficult to establish permanently transfected 3T3 cell lines that expressed high levels of nuclear MxA. Eventually, a nuclear variant of MxA, TMxA(R_{645}), could be expressed sufficiently well in 3T3 cells in order to investigate its mode of action against influenza virus. Unlike cytoplasmic wild-type MxA and the cytoplasmic variant MxA(R_{645}), nuclear TMxA(R_{645}) blocked primary transcription of influenza virus (Figure 6). Thus, depending on its intracellular localization, MxA can inhibit different steps of the viral multiplication cycle. In the nucleus, it behaves like nuclear mouse Mx1 and it blocks primary transcription of influenza virus which is a nuclear process. In the cytoplasm, it cannot prevent primary transcription of influenza virus, instead it blocks a later cytoplasmic multiplication step (Pavlovic *et al.*, 1992). It is unclear how interaction of MxA with a viral component could lead to these two alternative modes of action.

We find it remarkable that both cytoplasmic and nuclear forms of MxA were active against influenza virus. Recently, we showed that the reverse is untrue: cytoplasmic forms of the mouse Mx1 protein were inactive against both influenza virus and VSV, although significant amounts of these proteins were produced by the transfected cells (T.Zürcher, J.Pavlovic and P.Staeheli, submitted). It is difficult to understand why Mx1 should not be able to mimic the antiviral effects of MxA in the cytoplasm. To account for these findings, we now postulate that the activities of Mx proteins are modulated by some unknown cellular factors. The putative activator of mouse Mx1 might be a strictly nuclear protein, whereas the activating accessory molecule of MxA might be present in the cytoplasm as well as in the nucleus. Alternatively, only the action of Mx1 might depend on accessory proteins, whereas MxA might function in the absence of such co-factors. Elucidating the molecular nature of putative Mx-associated proteins might eventually help to understand a very efficient natural host defence system against an important human pathogen.

Materials and methods

Construction of mutant MxA cDNAs

The codon GAG encoding E_{645} of MxA (Aebi *et al.*, 1989) was converted to the Arg codon CGG in MxA(R_{645}) cDNA using the oligonucleotide 5′ CCGTGCAAGCCGCCGCTTCAGGAACTTCC 3′ and standard site-directed mutagenesis techniques (Aebi *et al.*, 1986). The SV40-derived sequences of TMxA and TMxA(R_{645}) were introduced as follows: the first two codons ATG GTT (Met, Val) of the MxA open reading frame were converted to the *Cla*I restriction enzyme recognition site ATC GAT (Ile, Asp) using the oligonucleotide 5′ CACTTCGGAAACATCGATCTTC-CTTCTTTG 3′. A 63 bp oligonucleotide with *Cla*I adapters (T.Zürcher, J.Pavlovic and P.Staeheli, submitted) that codes for the indicated SV40 sequence was then inserted into the newly created *Cla*I restriction sites of the cDNAs encoding MxA and MxA(R_{645}). The manipulated MxA cDNAs were introduced into the eukaryotic expression vector pCL642 (Gautier *et al.*, 1989) that permits constitutive high level expression under the control of the mouse 3-hydroxymethyl-glutaryl-coenzyme A reductase gene promoter in transfected mouse Swiss 3T3 cells (Pavlovic *et al.*, 1990).

Establishing and characterization of transfected 3T3 cell lines expressing MxA variants

Cotransfection of the MxA expression constructs with the SV2neo plasmid, selection of permanently transfected 3T3 cells with G418, MxA expression analysis of individual cell clones by indirect immunofluorescence and Western blotting using a polyclonal mouse antiserum to a β-galactosidase−MxA fusion protein were as previously described (Pavlovic *et al.*, 1990).

Determining virus susceptibility of 3T3 cells expressing Mx variants

Stocks of influenza A virus strain FPV-B and VSV serotype Indiana were prepared in Swiss mouse 3T3 cells as described (Pavlovic *et al.*, 1990). For virus plaque assays, cell monolayers in 60 mm-diameter dishes were infected with ~50−100 plaque-forming particles of either influenza virus or VSV, and the viruses were allowed to form plaques for 36−48 h under soft agar (Pavlovic *et al.*, 1990). To measure virus resistance as a function

of viral RNA synthesis, the cultures were infected with three plaque-forming units per cell of either influenza virus or VSV and the cells were harvested 5 h later. RNAs were extracted and subjected to Northern blot analysis as described (Staeheli and Pavlovic, 1991; Pavlovic *et al.*, 1992). Radiolabelled cDNAs derived from the *PB1* gene of influenza virus strain PR8 (Young *et al.*, 1983) and of the *N* gene of VSV serotype Indiana (Gallione *et al.*, 1981) were used as hybridization probes.

Analysis of influenza virus primary transcription

To distinguish between primary transcription of the parental viral genome by the associated RNA polymerase and transcription of the amplified viral genome, which is dependent on *de novo* protein synthesis, we maintained the cultures throughout the experiment in medium containing 50 μg/ml of the protein synthesis inhibitor cycloheximide. Cycloheximide was added to the cultures 45 min before infection with five plaque-forming units of influenza virus FPV-B per cell. At 4 h post-infection, total RNA was prepared and samples (20 μg of RNA per lane) were analysed for the presence of influenza virus *PB1* mRNA by Northern blotting using radiolabelled negative-strand *PB1* RNA as a hybridization probe (Pavlovic *et al.*, 1992). The conditions for virus infection, RNA extraction and Northern blot analysis were exactly as previously described (Pavlovic *et al.*, 1992).

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