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Overexpression of the Influenza Virus Polymerase Can Titrate Out Inhibition by the Murine Mx1 Protein

TAOSHENG HUANG,[1] JOVAN PAVLOVIC,[2] PETER STAEHELI,[3] AND MARK KRYSTAL[1]*

Department of Microbiology, Mt. Sinai School of Medicine, One Gustave Levy Place, New York, New York 10029-6574[1]; Institut für Immunologie und Virologie, Universität Zürich, CH-8028, Zürich, Switzerland[2]; and Department of Virology, University of Freiburg, D-7800, Freiburg, Germany[3]

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The murine Mx1 protein is an interferon-inducible protein which confers selective resistance to influenza virus infection both in vitro and in vivo. The precise mechanism by which the murine Mx1 specifically inhibits replication of influenza virus is not known. Previously, sensitive replication systems for influenza virus ribonucleoprotein, in which a synthetic influenza virus-like ribonucleoprotein is replicated and transcribed by influenza virus proteins provided in *trans*, have been developed. With these systems, the antiviral activity of the murine Mx1 protein was examined. It was found that continued expression of influenza polymerase polypeptides via vaccinia virus vectors can titrate out the inhibitory action of the murine Mx1 protein. This titration of inhibitory activity also occurs when the viral PB2 protein alone is overexpressed, suggesting that an antiviral target for the murine Mx1 polypeptide is the viral PB2 protein.

Interferon (IFN) treatment of cells usually results in an antiviral state against a large number of viruses. Although the mechanism of antiviral activity is not well characterized for most viruses, there are clearly multiple inhibitory pathways by which it could act on any given virus. For influenza virus, a proven pathway involves the IFN-inducible murine Mx1 protein, which is a 72-kDa nuclear protein (4, 9). This Mx1 protein has been shown to mediate specific resistance to influenza virus (independent of other IFN-mediated effects) both in vitro and in vivo (2, 13, 25, 28, 29). Mx proteins with related antiviral activities have also been found in other diverse species (1, 19, 30).

The mechanism by which the murine Mx1 protein selectively inhibits the replication of influenza virus is unclear. Initially, it was reported that treatment of macrophages obtained from Mx^+ mice with IFN-α/β prior to influenza virus infection led to the inhibition of translation but not the synthesis of viral mRNAs in the nucleus (20). However, this observation has been controversial, and conflicting data have also been reported. In another study, it was shown that when Mx^+ mouse embryo fibroblasts were treated with IFN-α/β, viral mRNA synthesis in the nucleus was severely inhibited at the level of primary transcription (13). Furthermore, by using transfected cell lines expressing Mx1 protein in a constitutive manner, a strong inhibitory effect on influenza virus primary transcription was observed (24a).

The ability to reconstitute active influenza virus ribonucleoprotein (RNP) from purified proteins and synthetic RNA has contributed to new approaches in the study of negative-strand RNA viruses (23). The use of a synthetic viruslike RNA encoding the chloramphenicol acetyltransferase (CAT) gene allows for sensitive and quantitative estimates of replication and gene expression when introduced into virus-infected cells (16, 17). Previously, we extended this system by establishing an artificial replication system, in which the synthetic model CAT gene is replicated and expressed by influenza virus proteins supplied in *trans* via infection with vaccinia virus recombinant vectors (10). In the present study, these systems have been used to examine the antiviral activity of the murine Mx1 in cells constitutively expressing different steady-state levels of these proteins (25). Using influenza virus as helper, we find that the presence of the murine Mx1 protein completely inhibits expression of the synthetic CAT gene. Use of recombinant vaccinia virus-expressed proteins as helper permits a more detailed examination of the molecular mechanism of action of the murine Mx1 protein. We find that Mx1 is an inhibitor whose activity can be competed out through continued expression of either the influenza virus polymerase (PB2, PB1, and PA) or, to a lesser extent, the PB2 protein alone.

MATERIALS AND METHODS

Viruses and cells. Influenza virus A/PR/8/34 was grown in the allantoic cavities of 10- to 11-day-old embryonated chicken eggs (31). Influenza A virus strain A/WSN/33 was grown and titrated in Maden-Darby bovine kidney cells. The stock of the FPV-B strain (11) of influenza A virus (3×10^8 PFU/ml) was prepared from supernatants of virus-infected Swiss 3T3 cells (25). Virus-yield reduction assays were performed as previously described (25). Recombinant vaccinia viruses Vac-PB2, Vac-PB1, Vac-PA, Vac-NP, and VTF3 (6, 26) were propagated in monolayer Hela cells and titrated in CV-1 cells. Transfected Swiss 3T3 cells constitutively expressing the murine Mx1 protein and control cells lacking Mx proteins were as previously described (8, 25) or were produced by using the same technology. These cell cultures were passaged in 10% fetal serum-supplemented Dulbecco modified essential medium with 400 μg of G418 per ml as described previously (25). Mouse embryo cells of the Mx^+ phenotype were maintained as previously described (28).

IFN and IFN treatment. Mouse IFN-α/β (specific activity, 4.7×10^5 U/mg of protein) was purchased from Lee Biomolecular (San Diego, Calif.). Mx^+ mouse embryo fibroblasts were treated with IFN at a final concentration of 1,000 U/ml in Dulbecco modified essential medium containing 10% fetal calf serum. The cells were incubated for 15 h before trans-

* Corresponding author.

fection of synthetic RNP and subsequent influenza virus infection.

Preparation and transfection of synthetic RNP. Influenza virus core proteins were purified through a series of glycerol and CsCl-glycerol gradients (23). This resulted in a pool of viral proteins consisting mainly of the nucleoprotein (NP protein) along with minor amounts of active polymerase (PB2, PB1, and PA). Synthetic viruslike RNA encoding the CAT gene was generated through runoff transcription by bacteriophage T7 polymerase off *Hga*I-digested pIVACAT-1 plasmid in standard reaction conditions (10, 17).

The appropriate amounts of viral protein needed to assemble RNP was determined empirically. Synthetic RNP was assembled and transfected via calcium phosphate precipitation. In a typical experiment, 1 μg of RNA was suspended in 100 μl of 0.25 M $CaCl_2$ along with 10 μl of NP-polymerase preparation and 2 μl (60 U) of RNasin (Promega). This solution was mixed with an equal volume of 2× HeBS (350 mM NaCl, 100 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 1 mM Na_2HPO_4, pH 7.05) and allowed to sit at room temperature for 20 min before being added to cells. Cells were left at room temperature for 15 min, and then 2 ml of the appropriate growth medium was added. Following a 4-h incubation at 37°C, cells were infected with either influenza virus or recombinant vaccinia viruses (multiplicity of infection [MOI] = 3, for each vector) and incubated at 37°C overnight. Cells were harvested, and equivalent amounts of viral proteins (15) were assayed for CAT activity via standard procedures (7).

Immunoprecipitation analysis. Confluent monolayer cells were infected with recombinant vaccinia viruses at an MOI of approximately 3. At 3 h postinfection, medium was removed, and the cells were washed twice with PBS and then labeled with 100 μCi of [^{35}S]methionine in methionine-free medium. Following 2 h of incubation at 37°C, cells were harvested and lysed in lysis buffer (0.5% Nonidet P-40–25 mM deoxycholate in phosphate-buffered saline [PBS]). Corresponding antibodies were incubated with the sample in buffer A (190 mM NaCl, 50 mM Tris-Cl [pH 7.4], 6 mM EDTA, 2.5% Triton X-100, 1 to 2 mM phenylmethylsulfonyl fluoride [PMSF], 0.1% bovine serum albumin [BSA], and 1.5 mg of methionine per ml) at 4°C overnight. Protein A-Sepharose beads (Sigma) were added to each sample and shaken for an additional 1.5 to 3 h at 4°C. The protein A-Sepharose beads were washed three times with buffer B (150 mM NaCl, 0.1% Triton X-100, 100 mM Tris-Cl [pH 8.3], 5 mM EDTA, 1 to 2 mM PMSF, 0.1% BSA, 1.5 mg of methionine per ml) and once with PBS. After treatment with iodoacetamide at 37°C for 20 min, the samples were boiled in sodium dodecyl sulfate (SDS) loading buffer and resolved by SDS–10% polyacrylamide gel electrophoresis. The gel was fixed in glacial acetic acid, treated with 22% PPO (2,5-diphenyloxazole) in glacial acetic acid, and dried before autoradiography was performed at −70°C.

Western immunoblot analysis. Protein extracts were prepared by lysing cells in protein gel sample buffer (14). Samples (200 μg of protein per lane) were electrophoresed through 10% polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes. Immunostaining was carried out as previously described (1) with 0.5% AP5 antiserum (18). Bound antibodies were visualized with 0.1% peroxidase-conjugated protein A.

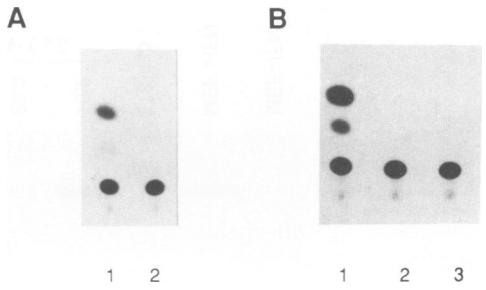

FIG. 1. (A) The murine Mx1 protein inhibits expression of IVACAT-1 RNP. The 80% confluent monolayer Mx^+ cells (A2G mouse embryo fibroblast cells) were used for IFN induction and subsequent transfection-infection as described in the text. After overnight incubation, cells were harvested and assayed for CAT activity by standard procedures (7). The assays contained 0.05 μCi of [^{14}C] chloramphenicol, 20 μl of 40 mM acetyl coenzyme A, and cell extract in 0.25 M Tris-HCl buffer (pH 7.5). Incubation times were 2 h. Lane 1, untreated cells; lane 2, cells treated with IFN. (B) Control 6.1 cells (lane 1) and Mx1-expressing cell lines 23.3 and 27.2 (lanes 2 and 3, respectively) were transfected with IVACAT-RNP and infected with WSN at an MOI of 3. CAT activity was examined as described in the text.

RESULTS

Examination of the antiviral activity of the murine Mx1 protein. In order to study the mechanism of inhibition by Mx1 at the molecular level, we have used a system in which a model RNA gene segment encoding the bacterial CAT gene (IVACAT-1) is assembled into RNP and transfected into influenza virus-infected cells. The infecting influenza virus proteins provide help in *trans* as the synthetic RNP is expressed, replicated, and packaged into infectious virions (17). Monolayer Mx^+ fibroblasts originating from A2G-Mx mice (29) were left untreated or were treated with 1,000 U of mouse IFN-α/β per ml for 15 h to induce the expression of the Mx1 protein. The cells were then transfected with reconstituted IVACAT-1 RNP through standard calcium phosphate transfection followed by influenza virus infection 2 h later. Replication and expression of the transfected synthetic RNA were assayed by analyzing cells for CAT activity after overnight incubation. Figure 1A (lane 1) shows that Mx^+ cells not treated with IFN were able to express significant levels of CAT activity, whereas the same cells pretreated with IFN do not support the expression of CAT (lane 2).

Recently, we have developed an artificial replication system in which recombinant vaccinia vectors expressing the influenza virus PB2, PB1, PA, and NP proteins can substitute for helper function previously supplied through influenza virus infection (10). Since the murine Mx1 protein is specific only for influenza virus (25, 28), the influenza virus proteins expressed by these vaccinia virus recombinants should not be inhibited and would be expected to continually be transcribed and translated in the presence of the Mx1 protein. The possible effects of this continued expression on Mx1 activity can then be assayed by examining the expression of the synthetic CAT-RNP. In the absence of IFN treatment, the four vaccinia virus vectors supported CAT expression after transfection in Mx^+ and Mx^- cells (data not shown). However, when the transfection-infection experiment was repeated in the presence of IFN treatment, neither Mx^+ nor Mx^- cells generated CAT activity (data not

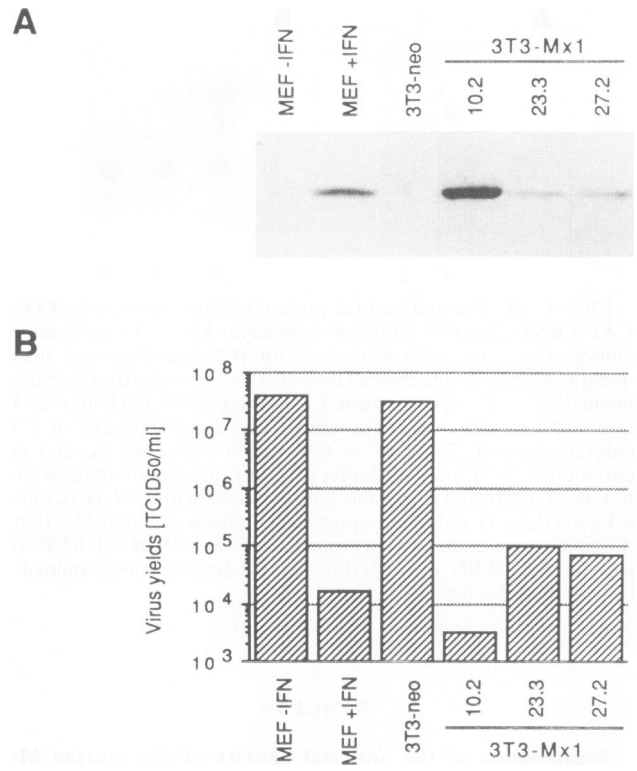

FIG. 2. Western blot analysis of Mx1 protein in transfected Swiss 3T3 cells. Protein extracts of the indicated Mx1-expressing cells lines were subjected to Western blot analysis by using AP5 antiserum (18). Protein extracts of control (3T3-neo; the 6.1 cell line) and BALB.A2G-Mx mouse embryo fibroblasts (MEF) treated with IFN (+ IFN) or left untreated (− IFN) were included as controls. (B) Inhibition of influenza virus multiplication by Mx1 protein. The indicated cell lines were infected with 1 PFU of influenza virus (FPV-B) per cell. Samples of the culture supernatants were removed 24 h postinfection, and the viral titers were determined by the 50% tissue culture infective dose method.

shown). This inhibition of RNP expression is presumably caused by IFN-induced inhibition of vaccinia virus.

In an effort to obviate the need for IFN induction, stable cell lines constitutively expressing the Mx1 proteins were used in subsequent experiments. We previously described the production of such cell lines (25) which varied with respect to Mx1 expression (Fig. 2). The degree with which each cell line inhibits influenza virus correlates with the level of Mx1 protein present (Fig. 2). When reconstituted IVACAT-1 RNP particles were transfected into these Mx1-expressing cell lines and subsequently infected with influenza virus, no detectable CAT activity was obtained (Fig. 1B, lane 2 and 3). Thus, these cells behaved like cells cultured from Mx^+ mice treated with IFN (Fig. 1A, lane 2). When the control cell line (6.1) was used as the host (Fig. 1B, lane 1), high CAT activity was observed.

Overexpression of the influenza virus polymerase can titrate out the inhibitory activity of the murine Mx1 protein. Since the transformed 3T3 cell lines express Mx1 protein in a constitutive fashion in the absence of IFN (Fig. 2A), these cells may be suitable hosts for the vaccinia virus-driven influenza virus replication system. Murine Mx1-expressing and control cell lines were transfected with IVACAT-RNP and subsequently infected with recombinant vaccinia viruses expressing the PB2, PB1, PA, and NP proteins. As can be seen in Fig. 3A (lanes 2 to 4), all three Mx1-expressing cells could now support the replication and subsequent expression of RNP-mediated CAT protein. However, the three cell lines still exhibited a certain degree of inhibition compared with control cells (lane 1; 60% acetylation in control cells). Also, the three cell lines consistently exhibited different levels of CAT activity compared with each other, with cell line 10.2 always displaying the lowest CAT activity (3% acetylation in this experiment), while cell line 23.3 displayed slightly higher activity (9.3% acetylation) than cell line 27.2 (7.5% acetylation). Thus, this observation correlates well with the steady-state levels of Mx1 and the reduction of inhibition of virus titer obtained after influenza virus infection. Cell line 10.2 exhibited the highest level of Mx1 expression and the most elevated degree of inhibition of virus growth titers, while cell line 27.2 exhibited the least inhibition (Fig. 2). To rule out the possibility that the

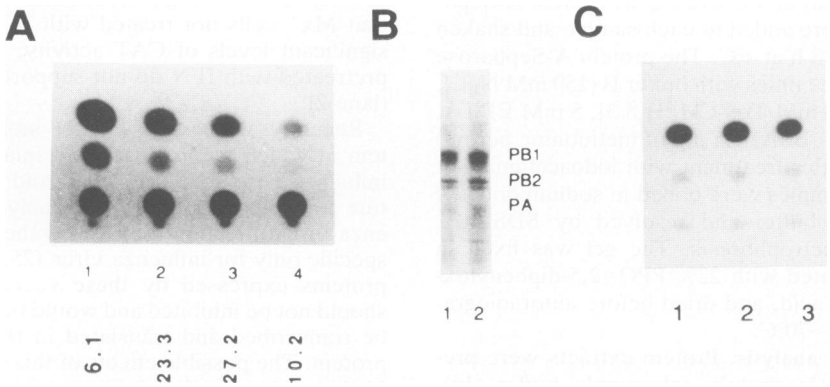

FIG. 3. Overexpression of polymerase can titrate out Mx1 inhibition. (A) Control 6.1 and Mx 1-expressing cell lines 3T3 23.3 (lane 2), 27.2 (lane 3), and 10.2 (lane 4) were transfected with IVACAT-1 RNP and infected with the mixture of recombinant vaccinia viruses expressing the three polymerase and NP proteins. After overnight incubation, CAT activity was assayed after 2 h of incubation at 37°C. (B) Both 6.1 and 23.3 cell lines were infected with the mixture of recombinant vaccinia vectors expressing the influenza virus proteins PB2, PB1, and PA. Cells were analyzed for expression of these proteins by immunoprecipitation with antibody to PB1 and electrophoresis on a 10% polyacrylamide–SDS gel. (C) Cells in 35-mm-diameter dishes were transfected with 2 μg of pIVACAT-9 DNA (10) by standard calcium phosphate transfection and infected with VTF-3 at an MOI of 3 PFU per cell. The CAT activity was assayed after overnight incubation.

variation in CAT activity between control and Mx1 expressing cell lines is due to differences in vaccinia virus-mediated expression of influenza virus polypeptides, these proteins were examined by immunoprecipitation analysis. The murine Mx1 protein expressing cell line (23.3) and the control cell line (3T3 SV2neo 6.1) were infected with the mixture of 5 PFU each of the recombinant vaccinia virus vectors expressing the PB2, PB1, and PA proteins. Cell extract was incubated with antibody raised against the influenza virus PB1 protein, and immunocomplexes were precipitated and analyzed in a 10% polyacrylamide–SDS gel. As shown in Fig. 3B, these two cell lines produce similar amounts of the influenza virus polymerase proteins through infection by the recombinant vaccinia virus vectors. In addition, since the three polymerase proteins coprecipitate with antibody against PB1, the complex of the PB2, PB1, and PA proteins can be formed in these Mx1-expressing cells. Thus, continued expression of the influenza virus polymerase complexes in the face of the murine Mx1 protein can titrate out the inhibitory activity, but the more Mx1 protein present, the less active the polymerase is.

Although control cells (6.1) and the Mx1-expressing cells were all derived from Swiss 3T3 cells, it was formally possible that clonal variation may have accounted for some of the results presented above. We had previously shown that different cell lines can vary in their ability to replicate and express the synthetic viruslike RNP and that this may correlate with transfection efficiency (10). To exclude this possibility, relative transfection efficiencies were measured by using a plasmid containing the CAT gene downstream (in the sense orientation) of a T7 promoter. After transfection with this plasmid, these cells were then infected with 5 PFU per cell of a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase (VTF-3) (6). The T7 polymerase expressed by the recombinant vaccinia virus should allow for the expression CAT protein of the transfected plasmid. Thus, relative transfection efficiencies can be measured via measurements of CAT activity. As shown in Fig. 3C, all cell lines behaved similarly. In dilution experiments, the variation seen between any two lines was less than 10% (data not shown).

Identification of a viral protein inhibited by Mx1. As described above, the murine Mx1 protein normally does not allow for expression of the synthetic CAT-RNP. Even when the recombinant vaccinia virus system was used to express viral proteins, CAT activity was inhibited to a certain extent (Fig. 3A). This suggested that Mx1 is inhibiting the function of one or more of the four viral proteins which make up the viral polymerase (PB2, PB1, PA, and NP). Therefore, the artificial replication system was next used to determine which of the polymerase protein(s) is targeted by Mx1. Influenza virus (WSN strain) was used along with single recombinant vaccinia virus vectors to coinfect the Mx1-expressing cell line 23.3 which had been previously transfected with IVACAT-1 RNP. By itself, WSN virus would not provide enough helper proteins to enable detection of CAT activity, due to the Mx1-induced block (Fig. 1B). Alternatively, infection of cells by less than the full complement of recombinant vaccinia viruses expressing the PB2, PB1, PA, and NP proteins would not support replication and expression of RNP (10). However, if a single recombinant vector or a mixture of recombinant vectors expressing the protein affected by Mx1 is added to WSN helper-infected cells, then the vaccinia virus-expressed influenza virus protein(s) may titrate out the Mx1 inhibitory activity. This would restore the replication and expression of RNP through

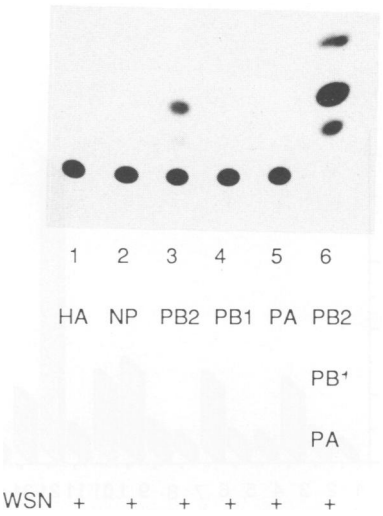

FIG. 4. Rescue of IVACAT-1 expression in the Mx1 protein-expressing cells. The Mx1 protein-expressing cells (cell line 23.3) was transfected with IVACAT-1 RNP; 4 h later, cells were coinfected with WSN and recombinant vaccinia virus expressing either hemagglutinin (HA; lane 1), NP (lane 2), PB2 (lane 3), PB1 (lane 4), PA (lane 5), or the three polymerase proteins (lane 6). Cells were incubated overnight, and CAT activity was examined by assay after a 2-h incubation.

the rescue of WSN helper functions. The results of these experiments using single recombinant vaccinia virus vectors or a mixture of PB2, PB1, and PA vectors are shown in Fig. 4. Simultaneous infection with the three recombinant vectors expressing PB2, PB1, and PA was able to rescue CAT activity (lane 6). In addition, CAT activity was also partially rescued when the recombinant vector expressing the PB2 protein was used in coinfection experiments with WSN virus (lane 3). Therefore, the continued expression of the PB2 protein alone could compete out the Mx1-induced inhibition.

Additional experiments were performed to determine if any other protein mixtures were able to titrate out the Mx1-induced block. Confluent monolayers of the Mx1-expressing cell line 23.3 were transfected with the reconstituted IVACAT-1 RNP and coinfected 4 h later with influenza A virus (WSN) and recombinant vaccinia viruses expressing the three polymerase (PB2, PB1, and PA) and NP proteins in a variety of combinations. The rescue effect was assayed by analyzing cells for CAT activity, and results were quantitated through scintillation counting of reaction products. The results are shown in Fig. 5A. A positive control (lane 17), in which cell line 6.1 was used in lieu of the Mx-expressing cells, and a negative control, in which 23.3 cells were infected with a recombinant vaccinia virus vector expressing the influenza virus hemagglutinin (lane 1), were also included. Significant increase in CAT activity (8- to 12-fold increase over that in lane 1) was present in only those cells coinfected with WSN and any combination including Vac-PB2 (Fig. 5A, lanes 3, 6, 9, 10, 12, 13, 15, 16). Cells coinfected with WSN and other recombinant vaccinia virus vectors did not produce such a notable rescue effect (two- to threefold at best), irrespective of mixtures used. Also, infection of cells with vaccinia virus vectors expressing complete polymerase complexes (PB2, PB1, and PA; lanes 12 and 16) was able to neutralize Mx1 inhibition better than infection with vectors expressing incomplete PB2-containing mixtures.

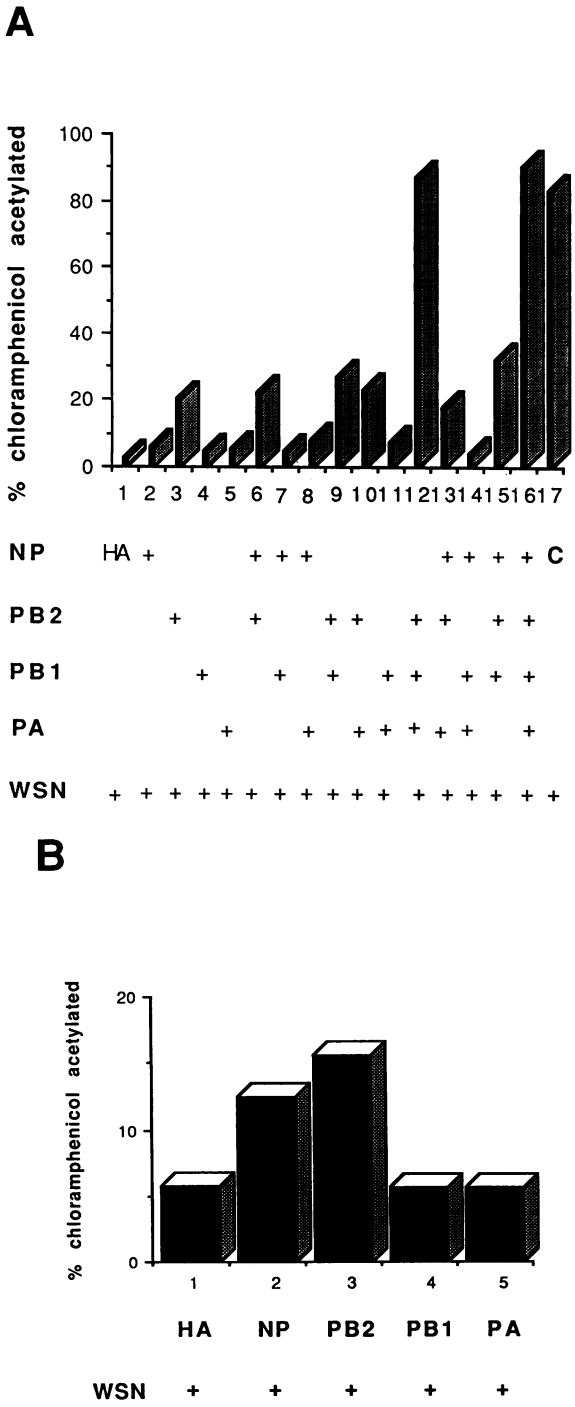

FIG. 5. (A) Effect of various mixtures on CAT expression. Cell line 23.2 was transfected with IVACAT-1 RNP for 4 h and then coinfected with WSN and recombinant vaccinia viruses expressing influenza virus proteins in a variety of combinations. Cells were assayed for CAT activity, products were excised from thin-layer chromatography plates, and conversion rates were calculated by scintillation counting. Labels (+) under the lane numbers indicate the treatment of cells. Control cell line 6.1 was used in lane 17. (B) Effect of vector-expressed proteins during normal influenza virus infection. Confluent cells (cell line 6.1) were transfected with IVACAT-1 RNP as described above. Cells were then infected with WSN virus and recombinant vaccinia virus vectors as indicated. Cells were harvested after overnight incubation, and dilutions of extracts were used for CAT assays. Results are presented in graphic form.

A final control experiment examines the general effect of overexpression of individual viral proteins during normal influenza virus infection. The 6.1 cell line (Mx^-) was infected with WSN and a recombinant vector expressing either one of the four polymerase proteins or hemagglutinin. The cells were then transfected with reconstituted CAT-RNP and assayed for CAT expression after overnight incubation. Small aliquots were used to correspond to activity levels measured for the constitutive $Mx1^+$-expressing cells. Overexpression of PB2 or NP resulted in a two- to threefold increase in CAT activity above that measured for vaccinia virus-hemagglutinin-infected cells, while overexpression of PB1 or PA did not significantly alter the CAT expression levels (Fig. 5B). Therefore, both PB2 and NP are slightly limiting during normal virus infection, whereas PB1 and PA are not.

DISCUSSION

The development of reverse genetics for the study of negative-strand viruses has opened new avenues for the molecular analysis of these viruses (22–24). In terms of influenza virus, this development has allowed the examination of *cis*-acting sequences within the genome (16, 23), the study of the viral proteins needed for replication (10), and the construction of new viruses (5). A feature of this influenza virus system is the use of a synthetic RNP molecule coding for the marker CAT gene. In this report, we use these systems to analyze the mechanism of inhibition of a specific inhibitor of influenza virus, the murine Mx1 protein. Although the antiviral activity of the IFN-inducible murine Mx1 protein to influenza virus has been documented for decades, the mechanism is still poorly understood. The murine Mx1 protein does not affect attachment or penetration of influenza virus, as parental virion nucleocapsids are efficiently transported into the nucleus in the presence of the murine Mx1 protein (3). Previous work has suggested that the murine Mx1 protein inhibits the accumulation of primary transcripts off the parental nucleocapsid (12, 24a).

We first examined whether the introduction of additional polymerase through expression by a noninfluenza vector system could titrate out the inhibition by Mx1. Since the murine Mx1 protein is specific for influenza virus and does not inhibit the expression of vaccinia virus proteins, recombinant vaccinia virus vectors expressing influenza virus proteins were employed (26). Stable cell lines constitutively expressing the murine Mx1 were used to avoid the IFN-stimulated but non-Mx-related inhibition of vaccinia virus expression. Continued expression of the PB2, PB1, PA, and NP proteins via vaccinia virus vectors was indeed able to titrate out the inhibition by Mx1 in all three cell lines tested. These facts are consistent with a model in which, during wild-type influenza virus infection of Mx1-expressing cells, the virus is inhibited at such an early step (primary transcription) that replication and subsequent amplification of viral polymerase proteins do not occur (13). Normally, the high concentration of Mx1 protein in IFN-induced cells (27) is presumably more than enough to overwhelm the incoming parental nucleocapsid and block its multiplication. However, the experiments described show that this inhibition was reversible, as continued expression of polymerase complexes eventually titrated out the inhibitory activity of Mx1. The significant decrease in CAT activity observed in Mx1-expressing versus control cell lines (Fig. 3) indicates that a large proportion of vaccinia virus-expressed influenza polymerase is being inactivated by the Mx1 present in cells. This

result is supported by the observation that the degree of inhibition was also concentration dependent, as the levels of CAT activity observed in transfected-infected Mx1 cell lines were inversely proportional to the levels of Mx1 present in each cell line.

Further studies of cells coinfected with WSN and single vaccinia virus vectors demonstrated that the PB2 protein alone could partially titrate out the inhibitory effect of the murine Mx1 protein. That this effect is specific is suggested by experiments for which the results are shown in Fig. 5. In Mx^- cells, both PB2 and NP can stimulate CAT expression twofold (Fig. 5B). In $Mx1^+$ cells, only PB2 can stimulate expression (8- to 12-fold), while NP has little effect (Fig. 5A). This increase of stimulation is even more impressive when it is taken into account that a large proportion of the vaccinia virus-expressed PB2 protein is presumably inactivated by the Mx1 protein present in these cells (Fig. 3). This result suggests that a target within the polymerase complex is a function of the PB2 protein; however, nonspecific stimulatory effects of PB2 cannot be totally ruled out. It should be noted that cells producing the three polymerases (Fig. 5A, lane 12) or the three polymerases and NP (Fig. 5A, lane 16) were able to rescue CAT activity at consistently higher levels than subsets containing the PB2 protein (lanes 3, 6, 9, 10, 13, 15). These results indicate that overexpression of the three proteins which make up the polymerase complex are able to titrate out the inhibition of the murine Mx1 protein more efficiently than PB2 alone. This effect may be due to the fact that there are more active polymerase complexes present in cells infected with vectors expressing the three polymerase proteins than in Vac-PB2-expressed cells alone (Fig. 5A, lanes 3, 6, 9, 10, 13, 15). It should also be noted that in any cell expressing an incomplete PB2-containing mixture, the level of CAT activity was roughly equivalent to the level in cells expressing PB2 alone. Therefore, addition of other polymerase proteins had little additive effect on the neutralization of Mx1 activity unless complete complexes were formed. However, since rescue of CAT activity by PB2 alone is inefficient, nontitratable effects on other polymerase proteins cannot be ruled out. Also, the recombinant vaccinia virus assay used in these studies can only examine questions of transcription or replication of viral RNP. It does not address other important aspects of viral infection, such as gene regulation, RNP transport, or virus assembly. Therefore, it is possible that the Mx1 protein has a multivalent effect and also can inhibit at later stages of infection as well.

How can the murine Mx1 protein competitively inhibit a function of the PB2 protein? PB2 protein has been shown to be the protein which recognizes and binds to the cap structures of host cell mRNA (which are needed during transcription) and also probably encodes the endonuclease which cleaves the host primer (12). One possibility is that Mx1 could bind directly to PB2 and thereby inactivate it. If this was the case, a complex of PB2 and Mx1 may be detectable by coimmunoprecipitation with extracts from Vac-PB2-infected Mx1-expressing cells. When we examined this possibility via immunoprecipitation, no evidence of a Mx1-PB2 complex was found (unpublished results). However, this still does not rule out direct interaction between Mx1 and PB2 or the related prospect that the Mx1 induces a second cellular protein to bind to and inactivate PB2. A second inhibitory pathway may involve the Mx1 protein competing with PB2 for a common substrate. A possible substrate may be the capped host message, which is needed for priming of viral mRNA transcripts (12). All Mx-related proteins contain a putative guanine nucleotide-binding (GTP) domain (8), and recently, it has been reported that bacterially expressed Mx1 protein binds to GTP and has GTPase activity (21), although no cap-binding activity was ever detected for Mx protein in vivo (3). A third possible inhibitory pathway may involve interaction with the PB1 and PA proteins rather than direct interaction with the PB2 protein. It is conceivable that Mx1 may bind to a PB1-PA complex and exclude PB2 from the polymerase. However, if this were the case, it would be expected that infection of cells with WSN and vaccinia virus vectors expressing PB1 and PA only would rescue CAT activity to significant levels, and that was not the case (Fig. 5A, lane 11). Since samples of cells expressing only PB2 exhibited significant CAT activity, it can be inferred that Mx1 is acting either directly on PB2 or on a function of PB2 rather than on the other components of the polymerase. It is hoped that further analysis can precisely define the molecular mechanism of inhibition of PB2 by Mx1.

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