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Parental Influenza Virion Nucleocapsids Are Efficiently Transported into the Nuclei of Murine Cells Expressing the Nuclear Interferon-Induced Mx Protein

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The interferon-induced murine Mx1 protein, which is localized in the nucleus, most likely specifically blocks influenza virus replication by inhibiting nuclear viral mRNA synthesis, including the mRNA synthesis catalyzed by inoculum (parental) virion nucleocapsids (R. M. Krug, M. Shaw, B. Broni, G. Shapiro, and O. Haller, J. Virol. 56:201–206, 1985). We tested two possible mechanisms for this inhibition. First, we determined whether the transport of parental nucleocapsids into the nucleus was inhibited in murine cells expressing the nuclear Mx1 protein. To detect the Mx1 protein, we prepared rabbit antibodies against the Mx1 protein with a CheY-Mx fusion protein expressed in bacteria. The fate of parental nucleocapsids was monitored by immunofluorescence with an appropriate dilution of monoclonal antibody to the nucleocapsid protein. The protein synthesis inhibitor anisomycin was added to the cells 30 min prior to infection, so that the only nucleocapsid protein molecules in the cells were those associated with nucleocapsids of the parental virus. These nucleocapsids were efficiently transported into the nuclei of murine cells expressing the Mx1 protein, indicating that this protein most likely acts after the parental nucleocapsids enter the nucleus. The second possibility was that the murine Mx1 protein might act in the nucleus to inhibit viral mRNA synthesis indirectly via new cap-binding activities that sequestered cellular capped RNAs away from the viral RNA transcriptase. We show that the same array of nuclear cap-binding proteins was present in Mx-positive and Mx-negative cells treated with interferon. Interestingly, a large amount of a 43-kDa cap-binding activity appeared after interferon treatment of both Mx-positive and Mx-negative cells. Hence, the appearance of new cap-binding activities was unlikely to account for the Mx-specific inhibition of viral mRNA synthesis. These results are most consistent with the possibility that the Mx1 protein acts directly to inhibit the viral transcriptase in the nucleus.

The interferon (IFN)-induced murine Mx1 protein mediates selective resistance to influenza virus (12, 23, 34). Only mouse cells possessing this Mx-positive allele develop an efficient antiviral state against influenza virus after exposure to alpha/beta interferon (IFN-α/β), whereas the antiviral state against other viruses is independent of the Mx gene (13). The murine Mx1 gene product is a 72-kDa protein that accumulates in the nucleus (8, 19). The antiviral activity of the Mx protein does not require other IFN-induced proteins: when the cloned DNA encoding the murine Mx1 protein was transfected into Mx-negative mouse 3T3 cells, an antiviral state specifically directed against influenza virus was established (35).

A key issue is the determination of the mechanism by which the Mx protein selectively inhibits the replication of influenza viruses. Two studies have established that influenza virus mRNA synthesis catalyzed by the parental (inoculum) viral transcriptase, i.e., primary transcription, is severely inhibited in Mx-expressing cells. For Mx-positive mouse embryo cells treated with IFN-α/β, it was shown that the steady-state levels of the viral mRNAs as well as the rate of viral mRNA synthesis per se, as measured by in vitro RNA synthesis catalyzed by permeabilized cells, were strongly inhibited (24). In contrast, IFN treatment of Mx-negative mouse embryo cells had little or no effect on either the steady-state level or the rate of synthesis of viral mRNA made by the inoculum transcriptase. These observations were confirmed by studies of rat cells. Homologs to the murine Mx1 protein have been identified in several other species (1, 16–18, 26, 27, 33), and direct evidence has been obtained that the antiviral state induced by IFN-α/β against influenza virus in rat cells is mediated by an Mx-related protein (3). Nuclear as well as cytoplasmic Mx homologs have been identified in rat cells, and as in Mx-positive mouse embryo cells, IFN-α/β treatment caused a severe inhibition in the accumulation of viral mRNAs synthesized by the parental viral transcriptase (26).

Influenza virus mRNA synthesis occurs in the nucleus (15, 23, 32). This synthesis is initiated by host cell primers, specifically, capped (m^7GpppNm-containing) RNA fragments derived from host cell RNA polymerase II transcripts (4, 22, 23, 30). These host cell primers are generated by a viral cap-dependent endonuclease that cleaves the capped cellular RNAs 10 to 13 nucleotides from their 5′ ends, preferentially at a purine residue. Viral mRNA synthesis is catalyzed by viral nucleocapsids, which consist of each of the eight virion RNAs associated with four viral proteins: the nucleocapsid (NP) protein and the three polymerase (P)

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proteins (PB1, PB2, and PA) (20, 23, 36). The NP protein constitutes the majority (92%) of the protein and is situated along the virion RNA chains at approximately 20-nucleotide intervals (6). The P proteins, which are in the form of complexes that are initially located at the 3′ ends of the virion RNA templates, catalyze viral mRNA synthesis (5, 22, 23, 36). The PB2 protein in this complex recognizes and binds to the cap of the primer RNA, and the PB1 protein most likely catalyzes the addition of nucleotides to the growing mRNA chains.

The murine Mx1 protein could operate at various points to block influenza virus mRNA synthesis catalyzed by the inoculum viral transcriptase. One possibility was that the Mx1 protein might block the transport of inoculum (parental) viral nucleocapsids into the nucleus of infected cells. In the present study, we showed that parental virion nucleocapsids are efficiently transported into the nuclei of murine cells expressing the nuclear Mx1 protein. Hence, the murine Mx1 protein acts after the virion nucleocapsids enter the nucleus. In the nucleus, the Mx1 protein could act indirectly to inhibit the viral RNA transcriptase by making host cell RNA primers unavailable for the viral transcriptase. For example, the Mx1 protein, which contains putative guanine nucleotide-binding sites (18), might be a cap-binding protein or might activate (or induce) a cellular cap-binding protein, and this cap-binding activity might sequester cellular capped RNA primers. As shown here, the same array of nuclear cap-binding activities was observed in both Mx-positive and Mx-negative mouse cells treated with IFN. Interestingly, a large amount of a 43-kDa cap-binding activity appeared in both Mx-positive and Mx-negative murine cells after IFN treatment.

Preparation of antibody directed against the Mx protein. The transport of parental virion nucleocapsids into the nuclei of Mx-expressing cells was assayed by immunofluorescence. For these experiments, an antibody directed against the Mx protein was needed. This antibody was generated against an Mx fusion protein expressed in *Escherichia coli*. Mx cDNA was synthesized from poly(A)-containing RNA from mouse A2G (Mx-positive) cells treated with IFN-α/β and cloned into lambda gt10. The clones were screened by hybridization with synthetic oligonucleotide probes derived from the published Mx1 sequence (35) and subcloned into vector pJC264 (10) for expression in *E. coli* as a CheY-Mx fusion protein under the control of the *E. coli lac* promoter. The 83-kDa CheY-Mx fusion protein was induced with IPTG (isopropyl-β-D-thiogalactopyranoside), solubilized from inclusion bodies with guanidine hydrochloride and dithiothreitol, purified by hydroxyapatite chromatography, and injected into rabbits. The resulting serum specifically immunoprecipitated the 72-kDa Mx protein synthesized in Mx-positive A2G mouse cells treated with IFN-α/β (Fig. 1, lane 5). This protein was not immunoprecipitated from extracts of A2G mouse cells not treated with IFN or from extracts of Mx-negative BALB/c mouse cells either with or without IFN treatment (lanes 6 to 8). The same specificity was seen with immunoblots (data not shown). Consequently, this antiserum was specific for the IFN-induced murine Mx1 protein.

Transport of parental viral nucleocapsids into the nuclei of murine cells expressing nuclear Mx protein. To test the possibility that the Mx protein blocks the transport of parental influenza virus nucleocapsids into the nucleus of infected cells, it was necessary to be able to monitor the fate of these nucleocapsids. This was accomplished by immunofluorescence using high concentrations of monoclonal anti-nucleocapsid (NP) protein antibodies (1:100 dilution) (kindly provided by Robert Webster [37]) and of a rhodamine-conjugated second antibody (1:25 dilution). To ensure that we were detecting only those NP protein molecules that were associated with parental viral nucleocapsids, infection of Mx-positive cells was carried out in the presence of the protein synthesis inhibitor anisomycin, which was added 30 min before infection. No detectable synthesis of NP protein occurred under these conditions (Fig. 2). Consequently, the only NP protein molecules in the infected cells were those associated with parental viral nucleocapsids. In Mx-positive cells not treated with IFN, this immunofluorescence procedure clearly detected parental viral nucleocapsids, which were predominantly in the nucleus (Fig. 3d). The detection of parental nucleocapsids was dependent on the use of high concentrations of NP antibodies. Higher dilutions of the NP antisera, which readily detected the NP protein synthesized in the absence of anisomycin, failed to detect parental nucleocapsids.

Mx protein in Mx-positive cells treated with IFN was identified by using the rabbit polyclonal antibody generated against the CheY-Mx fusion protein expressed in *E. coli* and then goat anti-rabbit antibody coupled to fluorescein (Fig. 3a). There was little or no nonspecific cross-reactivity between the Mx and NP antisera: little or no rhodamine staining occurred with the NP antiserum in the absence of infection (Fig. 3c), and little or no fluorescein staining

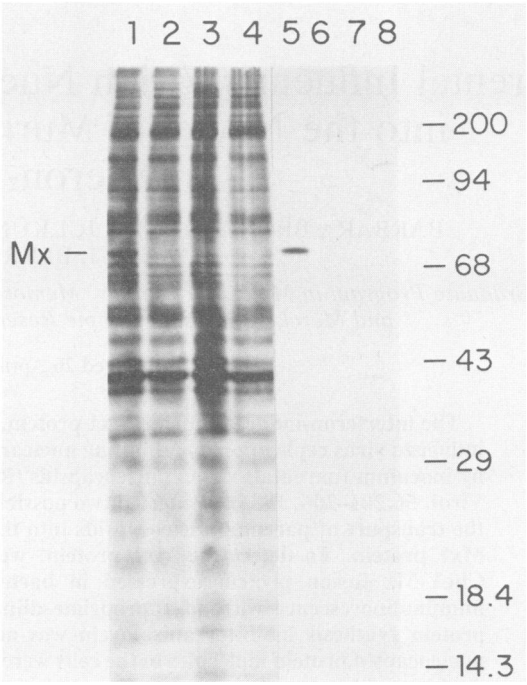

FIG. 1. Characterization of the rabbit antiserum directed against the purified 83-kDa CheY-Mx fusion protein synthesized in *E. coli*. Mx-positive A2G cells (lanes 1, 2, 5, and 6) and Mx-negative BALB/c cells (lanes 3, 4, 7, and 8) were either not treated (lanes 2, 4, 6, and 8) or treated with 1000 U of IFN-α/β per ml for 18 h (lanes 1, 3, 5, and 7). The cells were then labeled with [^{35}S]methionine for 2 h. A 50-μl aliquot of the total cell lysate was analyzed on a 14% polyacrylamide gel (lanes 1 and 4), and a 100-μl aliquot was immunoprecipitated with protein A-Sepharose beads precoated with 5 μl of the anti-fusion protein antiserum by a procedure described previously (2), and then the immunoprecipitate was analyzed on the same gel (lanes 5 to 8). Molecular masses are indicated in kilodaltons on the right.

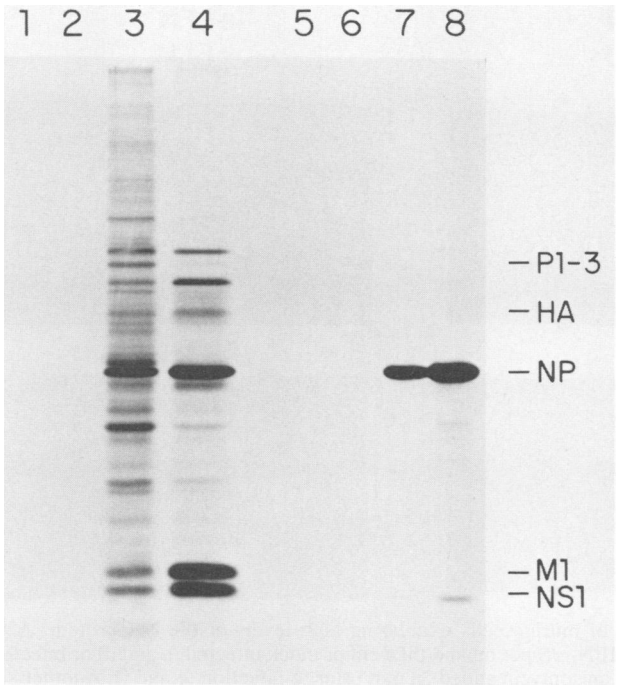

FIG. 2. Anisomycin (100 μM) added 30 min prior to influenza virus infection inhibits the synthesis of detectable NP protein. A2G (Mx-positive) cells were infected with 100 PFU of influenza virus per cell in the presence (lanes 1, 2, 5, and 6) or absence (lanes 3, 4, 7, and 8) of 100 μM anisomycin. Cells were labeled with 100 mCi of [^{35}S]methionine per ml either 2 to 3 h postinfection (lanes 1, 3, 5, and 7) or 4 to 5 h postinfection (lanes 2, 4, 6, and 8). After lysis in 0.05 M Tris hydrochloride (pH 7.4)–0.15 M NaCl–0.02 M EDTA–1% Triton X-100–1% sodium deoxycholate–1% sodium dodecyl sulfate, either 50-μl aliquots were analyzed directly on a 14% polyacrylamide gel (lanes 1 to 4) or 100-μl aliquots were first subjected to immunoprecipitation with protein A-Sepharose precoated with 2 μl of the mouse monoclonal antibody pool against the virus NP protein (2) (lanes 5 to 8). The positions of viral proteins are indicated on the right.

occurred with the Mx antiserum in the absence of IFN treatment (Fig. 3b).

To determine whether the Mx protein inhibited the transport of parental nucleocapsids into the nucleus, Mx-positive A2G embryo fibroblasts treated with IFN were infected with influenza virus in the presence of anisomycin. Transcription catalyzed by the parental nucleocapsids was severely inhibited under these conditions (24). Two hours postinfection, the presence of the Mx protein (Fig. 3c) and of the parental nucleocapsids (Fig. 3f) was determined by double immunofluorescence using rhodamine and fluorescein staining. All the cells expressing high levels of the Mx protein in the nucleus also contained parental virus nucleocapsids in the nucleus. The intensity of NP-specific rhodamine staining was indistinguishable from that observed in A2G cells not treated with IFN (Fig. 3e), indicating that the transport of parental nucleocapsids into the nucleus of A2G cells in the presence of the Mx protein was similar to the transport in its absence.

The same array of nuclear cap-binding proteins was present in Mx-positive and Mx-negative cells treated with IFN. These results indicated that the murine Mx1 protein most likely acts after the parental nucleocapsids enter the nucleus. One possibility was that the Mx protein caused the sequestration of capped host cell RNA primers away from the viral RNA transcriptase, thereby inhibiting viral mRNA synthesis. This might occur if the Mx protein itself were a cap-binding protein or if it activated or induced one or more cellular cap-binding proteins in the nucleus. Several nuclear cap-binding proteins in cells not treated with IFN have been described (31). We determined whether Mx-positive cells treated with IFN contained nuclear cap-binding activities not present in Mx-negative cells treated with IFN. As controls, Mx-positive and Mx-negative cells not treated with IFN were also analyzed. UV light-induced cross-linking was used to identify proteins in nuclear extracts from these cells that specifically interact with the cap structure on an mRNA molecule, alfalfa mosaic virus RNA 4, containing ^{32}P label only in its cap structure (5, 36). Purified virion nucleocapsids were also subjected to this cross-linking. After cross-linking took place, the samples were treated with pancreatic RNase and then analyzed on protein gels for the presence of ^{32}P label in proteins.

In nuclear extracts from both Mx-positive and Mx-negative cells treated with IFN, seven proteins were consistently cross-linked to the labeled cap; these proteins had molecular masses of 115, 80, 65, 43, 30, 24, and 18 kDa (Fig. 4, lanes 2 and 8). Four of these proteins—the 115-, 80-, 43- and 18-kDa proteins—could be classified as cap-recognizing proteins on the basis of the inhibition of their cross-linking by the addition of increasing concentrations of the cap analog m^7GDP (Fig. 4; compare lanes 3 to 6 with lane 2, lanes 3′ to 6′ with lane 2′, and lanes 9 to 12 with lane 8). The cross-linking of the 80- and 18-kDa proteins was inhibited by lower concentrations of m^7GDP than the cross-linking of the 115- and 43-kDa proteins; the lowest tested concentration (165 μM) of m^7GDP inhibited the cross-linking of the 80- and 18-kDa proteins by about 80 to 90%, whereas a comparable inhibition of the crosslinking of the 115- and 43-kDa proteins occurred with 660 μM m^7GDP. The inhibition of the last two proteins was similar to that of the cap-recognizing PB2 protein in purified virion nucleocapsids (lanes 13 to 17). In some but not all experiments, m^7GDP stimulated the cross-linking of the 65-kDa protein (lanes 3 to 6); this stimulation occurred with nuclear extracts from both Mx-positive and Mx-negative cells.

A previous study identified two nuclear cap-recognizing proteins with molecular masses close to those of the 115- and 18-kDa proteins and showed that an 80-kDa cap-recognizing protein in nuclear preparations was a cytoplasmic contaminant, the translation initiation factor eIF-4B (31). Consequently, the 43-kDa protein, which was found almost totally in the nuclear fraction, is the only species of nuclear cap-binding protein that has not been reported previously. In the assay whose results are shown in Fig. 4, the 43-kDa cap-binding protein was not detected in the absence of IFN treatment in either Mx-positive or Mx-negative cells (lanes 1 and 7 and lanes 2 and 8, respectively). However, the possibility that a small amount of this protein was present in untreated cells cannot be ruled out. In assays containing 5 to 10 times more nuclear protein from untreated cells, a small amount of a 43-kDa protein cross-linked to the labeled cap, but this cross-linking was not significantly inhibited by m^7GDP (data not shown). Because increased 43-kDa cap-binding activity appeared after IFN treatment of both Mx-positive and Mx-negative cells, it was unlikely to account for the Mx-specific inhibition of influenza virus mRNA synthesis. IFN also stimulated the cap-binding activity of the 115-kDa protein in both Mx-positive and Mx-negative cells; low levels of this activity were detected in the absence of

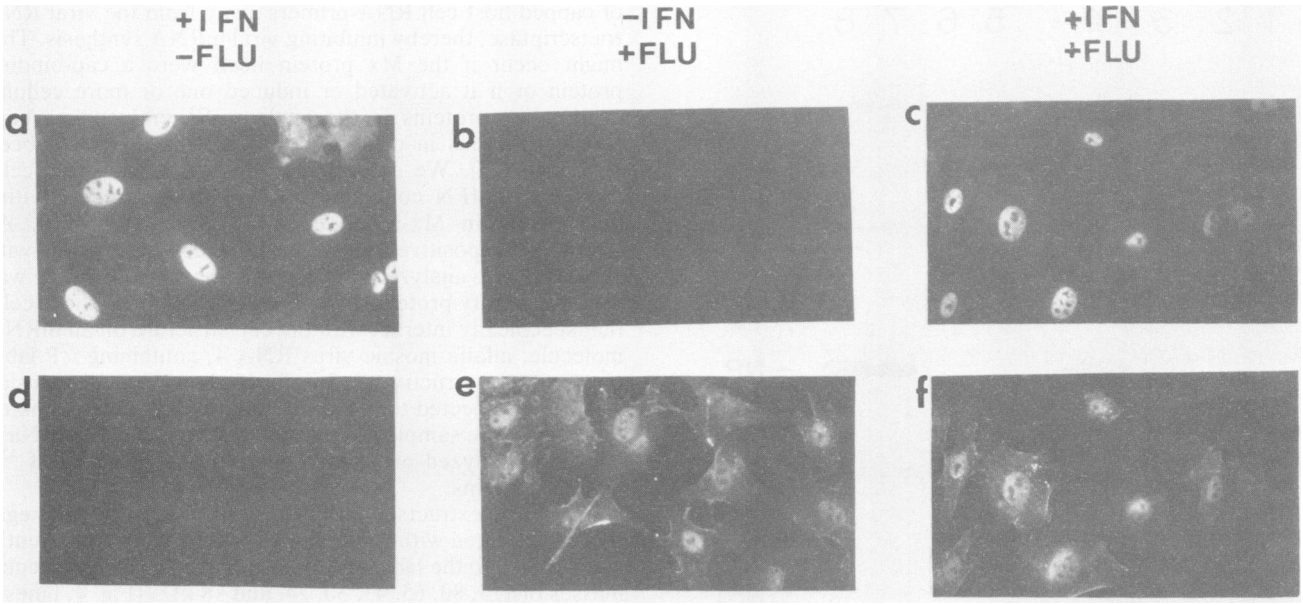

FIG. 3. Parental virion nucleocapsids are transported into the nuclei of murine cells expressing high levels of the Mx protein. A2G (Mx-positive) mouse embryo cells were pretreated for 18 h with 1000 U of IFN-α/β per ml and then either mock infected (a and d) or infected for 2 h with 100 PFU of influenza virus per cell in the presence of 100 μM anisomycin added 30 min prior to infection (c and f). Another set of A2G cells was not treated with IFN and was then infected with influenza virus in the presence of anisomycin (b and e). Immunofluorescence was carried out with either rabbit antibody against the Mx protein and goat anti-rabbit antibody coupled to fluorescein (a and b), mouse monoclonal antibody pool against the viral NP protein and goat anti-mouse antibody coupled to rhodamine (d and e), or both antisera and both conjugates (c and f). Individual cells were analyzed for both fluorescein and rhodamine fluorescence in a Zeiss UV light fluorescent microscope. (c) Anti-Mx rhodamine staining; (f) anti-NP fluorescein staining of the same cells.

IFN treatment (seen in darker exposures of Fig. 4, lanes 1 and 7).

No consistent difference in the cross-linking patterns of IFN-treated Mx-positive and Mx-negative cells was observed (compare lanes 2 and 8 of Fig. 4). In particular, no cap-binding protein in the molecular mass range of the Mx protein (between the 65- and 80-kDa proteins) was detected in the IFN-treated Mx-positive cells, even when the protein gels were run for a longer time than those of Fig. 4 in order to achieve better resolution of the 65- to 80-kDa-molecular-mass species. Mx protein was present in these nuclear extracts, as shown by immunoprecipitation of a [^{35}S]methionine-labeled nuclear extract (Fig. 4, lane 18).

Conclusions. Several possible mechanisms can be proposed for the inhibition of influenza virus mRNA synthesis by the IFN-induced murine Mx1 protein. In the present study, we tested two possible mechanisms for this inhibition. First, we determined whether the transport of parental viral nucleocapsids into the nucleus is blocked. Influenza virus most likely enters cells by endocytosis in coated pits, leading to the subsequent release of the nucleocapsids into the cytoplasm via a hemagglutinin protein-mediated fusion reaction with lysosomal membranes (25). How the parental viral nucleocapsids are subsequently transported from the cytoplasm to the nucleus has not yet been determined. We analyzed the transport of parental nucleocapsids into the nucleus by using indirect immunofluoresence with monoclonal antibodies directed against the viral NP protein. These NP protein molecules were detected predominantly in the nucleus but not the cytoplasm of A2G mouse cells expressing high levels of nuclear Mx1 protein. We can thus conclude that the murine Mx1 protein acts after the parental nucleocapsids are transported into the nuclei of infected cells.

The second possibility was that the murine Mx1 protein might act in the nucleus to inhibit viral mRNA synthesis indirectly, by causing the appearance of new cap-binding proteins that sequestered cellular capped RNA primers away from the virion RNA transcriptase. Interestingly, a large amount of a nuclear cap-binding protein of 43 kDa was found in mouse cells after IFN-α/β treatment. However, this protein, which has not been described previously, appeared both in the absence and in the presence of the Mx protein and hence is not likely to account for the Mx-specific inhibition of influenza virus mRNA synthesis. The role of this 43-kDa cap-binding protein in the actions of IFN remains to be determined. Indeed, the role of the other nuclear cap-binding proteins that are not induced by IFN is also not known. Because some evidence points to the role of the cap in splicing and in 3′ end formation of mRNA (9, 11, 14, 21), it is conceivable that these cap-binding proteins are involved in these processes. Our results show that the arrays of nuclear cap-binding activities in Mx-positive and Mx-negative cells treated with IFN were not significantly different. No cap-binding activity associated with the Mx protein in IFN-induced murine cells was detected. In addition, no cap-binding activity was detected in association with the Mx protein expressed with a baculovirus vector (unpublished data). Though the Mx1 protein contains putative guanine nucleotide-binding sites (18), it does not possess detectable cap-binding activity.

Though these results do not establish the mechanism by which the murine Mx1 protein inhibits viral mRNA synthesis, they do narrow the possible modes of inhibition and are most consistent with the possibility that the Mx1 protein acts directly to inhibit the viral transcriptase in the nucleus. One way to test this possibility is to purify the murine Mx1 protein and determine its activity in in vitro systems. These studies are in progress. Finally, it should be noted that the

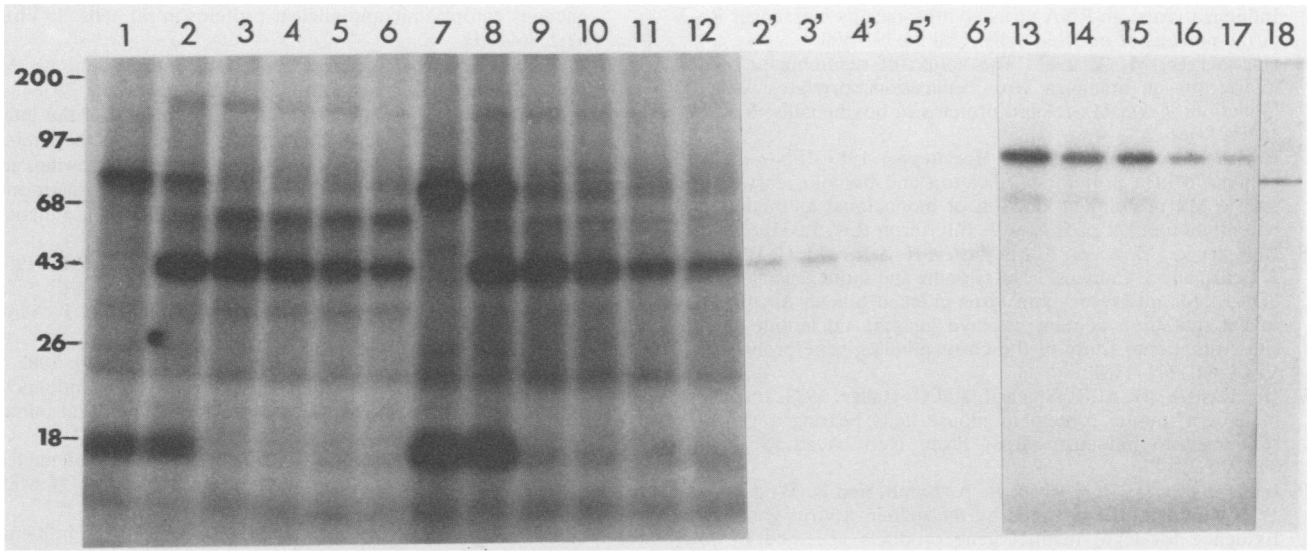

FIG. 4. The array of nuclear cap-binding proteins in Mx-positive and Mx-negative mouse cells after IFN treatment is indistinguishable and includes a large amount of a 43-kDa cap-binding protein. Alfalfa mosaic virus RNA 4 labeled with ^{32}P in its cap structure was incubated for 10 min at 37°C with either 15 μg of nuclear extract from Mx-positive cells (lanes 1 to 6), 15 μg of nuclear extract from Mx-negative cells (lanes 7 to 12), or 1 μg of purified influenza virion nucleocapsids (lanes 13 to 17). Nuclear extracts were prepared as described by Dignam et al. (7), and purified virion nucleocapsids were prepared from lysolecithin-treated virions as previously described (30). The Mx-positive and Mx-negative cells were either treated with IFN for 18 h (lanes 2 to 6 and 8 to 12) or not treated (lanes 1 and 7). m^7GDP was added to the indicated reaction mixtures at concentrations of 165 μM (lanes 3, 9, and 14), 330 μM (lanes 4, 10, and 15), 660 μM (lanes 5, 11, and 16), and 1.3 mM (lanes 6, 12, and 17). After incubation, the reaction mixtures were irradiated with UV light and then digested with pancreatic RNase. Labeled proteins were resolved by gel electrophoresis. Lanes 2' to 6' are lighter exposures of lanes 2 to 6. Lane 18 shows the Mx protein immunoprecipitated from a nuclear extract from [^{35}S]methionine-labeled, IFN-treated A2G cells. Molecular masses are indicated in kilodaltons on the right.

murine Mx1 protein may differ in its mode of action from that of one of the human Mx homologs, the MxA protein. The murine Mx1 protein, which is localized in the nucleus, selectively inhibits only influenza virus replication. Deletion of the nuclear localization signal of the murine Mx1 protein greatly reduced its anti-influenza virus activity (28). In contrast, the human MxA protein, which is localized in the cytoplasm, is less specific in that it inhibits the replication of not only influenza virus but also vesicular stomatitis virus (1, 29).

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