

## MxA-Dependent Inhibition of Measles Virus Glycoprotein Synthesis in a Stably Transfected Human Monocytic Cell Line

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Received 8 February 1993/Accepted 10 May 1993

**The alpha/beta (type I) interferon-inducible human MxA protein confers resistance to vesicular stomatitis virus (VSV) and influenza A virus in MxA-transfected mouse 3T3 cells (3T3/MxA). We investigated the inhibitory effects of the MxA protein on measles virus (MV) and VSV in the human monocytic cell line U937. In transfected U937 clones which constitutively express MxA (U937/MxA), the release of infectious MV and VSV was reduced approximately 100-fold in comparison with control titers. Transcription of VSV was inhibited similar to that observed for 3T3/MxA cells, whereas no difference was detected for MV in the rates of transcription or the levels of MV-specific mRNAs. In contrast, analysis of MV protein expression by immunofluorescence and immunoprecipitation revealed a significant reduction in the synthesis of MV glycoproteins F and H in U937/MxA cells. These data demonstrate a virus-specific effect of MxA which may, in the case of MV, contribute to the establishment of a persistent infection in human monocytic cells.**

The interaction of measles virus (MV) with peripheral blood mononuclear cells (PBMCs) in acute measles is of pathogenetic importance since this virus not only replicates in these different cell populations (19, 30, 45) but also alters their function (25). In particular, the immunosuppression that accompanies acute measles, and contributes to opportunistic infections which account for most of the morbidity and mortality associated with measles, is of interest (8, 25). It is evident that these effects are in part dependent on the replication strategy of the virus as well as on host factors. So far, the mechanisms by which MV interferes with PBMC functions are unknown.

During acute measles, MV can be isolated from PBMC fractions of patients (22, 37), whereas after disease recovery infectious MV has disappeared (22, 27, 37, 38). Only occasionally is it possible to demonstrate the presence of MV in PBMCs of healthy donors who have previously experienced acute MV infections, suggesting a state of viral persistence (3, 13, 36). In tissue culture experiments, productive as well as silent MV infections have been observed in PBMCs, depending on the activation stage of the cells, providing evidence of a direct influence of cellular factors in MV replication (6, 25). In lymphocytes, for example, MV can replicate only after these cells have been stimulated by a mitogen, whereas in unstimulated cells MV RNA persists. Subsequent stimulation of infected lymphocytes leads to viral replication without lymphocyte proliferation and differentiation (6, 8, 19, 25). For permanent cell lines of lymphocytic origin, a lytic infection, as well as the establishment of persistent infections, has been described, allowing the investigation of cellular factors contributing to MV persistence (5, 9, 21).

The observed downregulation of MV multiplication in PBMCs appears to be mediated by cytokines that are in-

duced upon infection. In particular, type I interferon (IFN) has been shown to be released from MV-infected PBMCs (20) and, more recently, to interfere with MV replication in these cells (24). In response to IFN, a variety of IFN-inducible proteins with different antiviral activities are produced, but it is not clear which of these interferes with MV gene expression. In other virus-host cell systems, the human cytoplasmic MxA protein, an IFN-inducible protein, has been shown to restrict the replication of vesicular stomatitis virus (VSV) and influenza A virus in stably transfected mouse 3T3 cells. In the case of VSV infection, a downregulation of primary transcription occurred, whereas a later step of viral replication was blocked in influenza virus-infected cells (32, 43).

Since the MxA protein has been shown to be expressed to high levels in monocytes following IFN treatment *in vivo* (44), we analyzed the *in vitro* replication of MV in comparison with that of VSV in an MxA-expressing stably transfected human mononuclear cell line, U937. Production of infectious virus particles was blocked by approximately 2 logs for MV and VSV in MxA-expressing U937 cells compared with nonexpressing cells. However, in contrast to VSV, MV-specific transcription was not altered in the presence of MxA. Analysis of protein expression showed the synthesis of MV-specific glycoproteins F and H to be inhibited, indicating that the mechanism of MxA-mediated restriction of MV gene expression occurs at a posttranscriptional level. These data suggest that MxA-dependent specific inhibition of glycoprotein expression may contribute to the establishment of measles virus persistence in PBMCs.

### MATERIALS AND METHODS

**Cell lines, viruses, IFNs, and antibodies.** The human histiocytic lymphoma cell line U937 was grown in RPMI 1640 containing 10% fetal calf serum, 2.0 g of NaHCO<sub>3</sub> per liter, 0.1 g of penicillin per liter, and 0.1 g of streptomycin per

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liter. For the human B-cell line BJAB, this medium contained in addition 1 mM sodium pyruvate, 1× nonessential amino acids, 1× Eagle basal medium vitamins, and 2 mM L-glutamine (all from Seromed). Vero (African green monkey kidney) cells were grown in minimal essential medium supplemented with 5% fetal calf serum. Stocks of measles virus, Edmonston strain, were prepared from infected Vero cells. Stocks of VSV, serotype Indiana, were prepared from infected Swiss mouse 3T3 cells as previously described (12). Recombinant human alpha interferon (IFN- $\alpha$ ) and beta interferon (IFN- $\beta$ ) were obtained from Merck. The p68 antibody was a generous gift from G. Barber. For immunoprecipitation, a measles-specific human serum with a hemagglutination inhibition titer of 1:1,024 was used.

**Plasmid construction and transfection.** The entire coding sequence of the human MxA gene was excised by a *Sma*I digest of pHG327/MxA (1). The 2.2-kb MxA fragment was cloned into the blunt-ended *Bam*HI site of the expression vector pH $\beta$  Apr-1-neo (15) containing the human  $\beta$ -actin promoter, generating the vector pH $\beta$  Apr-1-neo/MxA.

U937 cells were transfected with this construct by the lipofectin method in accordance with the manufacturer's instructions (GIBCO/BRL). For selection, cells were cultured in the presence of 1 mg of G418 per ml and examined for MxA expression after 4 weeks by immunofluorescence. Transfectants were cloned by limiting dilution and assayed for MxA expression by immunofluorescence and Western blot (immunoblot) analysis.

**Titration.** A total of  $10^6$  U937 cells or U937/MxA transfectants were infected with  $10^7$  PFU of MV or VSV at 37°C for 1 h. The virus inoculum was removed by centrifugation, and infected cells were incubated at 37°C for the time intervals indicated. Tenfold dilutions of the supernatants were analyzed on Vero cells by endpoint titration after 3 or 4 days of incubation.

**RNA analysis.** A total of  $10^7$  U937 cells or U937/MxA transfectants were infected with MV at a multiplicity of infection (MOI) of 1 for 4 days. Cells were lysed in 4 M guanidinium isothiocyanate buffer, and RNA was purified by centrifugation through a CsCl cushion as described previously (11). A total of 30  $\mu$ g of total RNA samples and 1  $\mu$ g of pA<sup>+</sup> Vero control RNA, which was enriched by 1 cycle of oligo(dT)-cellulose chromatography (Sigma), was analyzed by quantitative Northern (RNA) blot assay as described earlier (39). Briefly, RNA was separated on a 1.5% agarose gel containing formaldehyde. After blotting onto nitrocellulose, filters were cut into strips and hybridized to strand-specific, [<sup>32</sup>P]CTP-labelled RNA probes specific for the MV genes N, P, M, F, and H. The concentrations of the added synthetic standard RNAs were as follows: N, 13 fmol; P, 7 fmol; M, 7 fmol; F, 10 fmol; and H, 5 fmol.

For the *in vivo* [<sup>32</sup>P]<sub>i</sub> labelling of MV-specific RNA synthesis,  $10^7$  cells of U937/MxA clones 5, 6, and 8 were infected with MV at an MOI of 1 for 20 h. Cells were starved for 1 h in phosphate-free medium (ICN-Flow) containing 20  $\mu$ g of actinomycin D per ml before being labelled with 0.6 mCi of [<sup>32</sup>P]<sub>i</sub> in the presence of actinomycin D (10  $\mu$ g/ml) for 7 h. RNA was prepared as described above, separated on a 1.5% agarose gel containing formaldehyde, transferred to a nitrocellulose filter, and analyzed by autoradiography.

For the pulse-chase experiment,  $3 \times 10^7$  U937 cells,  $3 \times 10^7$  U937/MxA clone 5 cells, and  $6 \times 10^6$  Vero cells were infected with MV at an MOI of 1. Cells were labelled after 20 h, following 1 h of phosphate starvation, with 1.1 mCi of [<sup>32</sup>P]<sub>i</sub> in the presence of actinomycin D for 2.5 h. Cells were

lysed at the indicated time points, and RNA preparation and analysis were performed as described above.

For experiments with VSV, U937/MxA cells were infected at 37°C with VSV at an MOI of 5 PFU per cell. Following removal of the inoculum, the cells were further incubated in growth medium at 37°C. At 3 h postinfection, cells were harvested and RNA was prepared as described by Chomczynski and Sacchi (12). Experiments with cycloheximide (CHX) required that cells be pretreated with CHX at a concentration of 25  $\mu$ g/ml for 30 min before infection and kept in growth medium containing 25  $\mu$ g of CHX per ml for the duration of the whole experiment. The RNA preparations were analyzed for the presence of VSV N and L mRNAs by the Northern blotting technique as described earlier (38). The hybridization probes were the *Hind*III-*Eco*RI fragment of plasmid pGN2 (14), for detection of N mRNA, and an equimolar mixture of the 1,200- and the 1,400-bp *Pst*I fragments of plasmid pLH661 (42), for detection of L mRNA.

**Immunofluorescence and flow cytometry.** For total cell staining,  $5 \times 10^5$  cells were fixed with 3.5% formaldehyde in phosphate-buffered saline (PBS) for 30 min and permeabilized in PBS containing 0.25% Triton X-100 for 10 min at room temperature. After being blocked for 15 min in washing buffer (PBS without Mg<sup>2+</sup> and Ca<sup>2+</sup>, containing 0.4% bovine serum albumin and 0.02% sodium azide), the cells were incubated for 1 h with the specific antibody and, after three washing steps, incubated for a further hour with the second antibody (goat anti-mouse phycoerythrin; Dianova). All steps were carried out at room temperature. Cells were analyzed in a fluorescence-activated cell scanner (FACScan; Becton Dickinson).

For surface immunofluorescence staining, cells were fixed for 5 min without permeabilization and incubated with the appropriate antibodies for 30 min on ice.

**Immunoprecipitation.** Pulse-chase experiments were made with  $2 \times 10^6$  cells infected with MV at an MOI of 1 for 15 h. Cells were starved for 30 min in methionine-free medium before 0.2 mCi of [<sup>35</sup>S]methionine was added. Labelling was performed for 3 h, and cell extracts were prepared by lysis in RIPA detergent (150 mM sodium chloride, 10 mM Tris, 1% deoxycholic acid, 1% Triton X-100, 1% sodium lauryl sulfate [SDS], 10 mM phenylmethylsulfonyl fluoride). Equal amounts of protein were immunoprecipitated with human hyperimmune serum and separated by SDS-10% polyacrylamide gel electrophoresis (PAGE). Pulse-labelling of infected cells was performed as described above except for an increase in the pulse-labelling period to 5 h. Lysates were immunoprecipitated with either human hyperimmune serum or a monoclonal antibody against the MV H protein.

**Western blot analysis.** Cell extracts were prepared as described above in RIPA detergent. A total of 10  $\mu$ g of total cell extracts was separated on 10% PAGE and transferred to nitrocellulose filters with a semidry blotter. Western blots were immunostained with a monoclonal anti-human MxA antibody or an anti-human p68 antibody. A peroxidase-conjugated rabbit anti-mouse immunoglobulin was used as the second antibody and developed with the enhanced chemiluminescence (ECL) system (Amersham).

## RESULTS

**MxA expression is not inducible in the human monocytic cell line U937.** To assess the inducibility of MxA, the monocytic cell line U937 and the B-cell line BJAB were treated exogenously with 1,000 U of IFN- $\beta$  per ml or infected with MV at

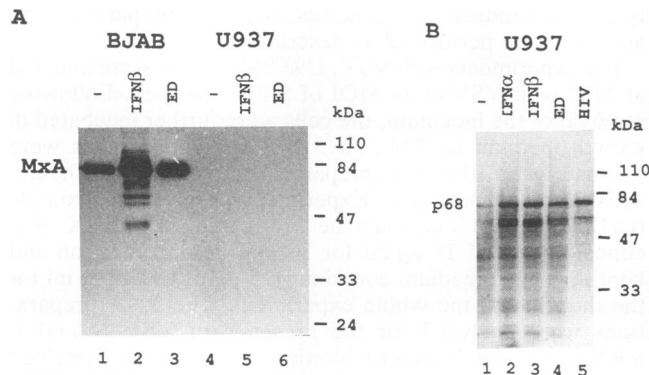


FIG. 1. (A) Western blot analysis of cell lysates (10  $\mu$ g per lane) prepared from BJAB cells (lanes 1 to 3) and U937 cells (lanes 4 to 6). Cells were either kept untreated (lanes 1 and 4), stimulated with 1,000 U of IFN- $\beta$  per ml for 48 h (lanes 2 and 5), or infected with MV at an MOI of 1 for 48 h (lanes 3 and 6). The Western blot was immunostained with a monoclonal antibody against the human MxA protein, and the ECL system was used for detection of specific signals. The position of the MxA band is indicated. (B) U937 cells were left untreated (lane 1), stimulated with 1,000 U of IFN- $\alpha$  (lane 2) or IFN- $\beta$  (lane 3) per ml, infected with MV at an MOI of 1 for 48 h (lane 4), or infected with human immunodeficiency virus at an MOI of 0.1 for 4 days (lane 5). A total of 10  $\mu$ g of total cell lysates per lane was separated on a 10% polyacrylamide gel. The Western blot was stained with a monoclonal antibody against the IFN-inducible p68 protein, the position of which is indicated.

an MOI of 1 for 48 h. Whole-cell protein extracts were prepared and analyzed for the expression of the 78-kDa MxA protein by Western blotting (Fig. 1A). In BJAB cells, MxA was induced by both IFN- $\beta$  (Fig. 1A, lane 2) and infection with MV (Fig. 1A, lane 3). However, in protein extracts of U937 cells no MxA-specific signals could be detected (Fig. 1A, lanes 4 to 6). In contrast to the inability to detect MxA protein, expression of a second IFN-inducible gene, p68, was clearly stimulated in U937 cells by IFN treatment (Fig. 1B, lanes 2 and 3) or viral infection (Fig. 1B, lanes 4 and 5). This indicates that the inability of U937 cells to express MxA was specific for this particular protein rather than reflecting a general defect in the IFN pathway of these cells.

**Stably transfected U937 cells expressing MxA protein.** The 2.0-kb *Sma*I cDNA fragment containing the entire coding region for the MxA protein, but lacking 397 nucleotides of the 3' untranslated 450-bp sequence, was cloned into the multiple cloning site of plasmid pH $\beta$  APr-1-neo between the  $\beta$ -actin promoter and the polyadenylation sequence as described in Materials and Methods. In addition, the vector contained a neomycin resistance gene driven by the simian virus 40 promoter as a selection marker. Following lipofection, stably transfected U937 cells were selected in medium containing G418 for a period of approximately 4 weeks, after which surviving cells were cloned by limiting dilution. Nine independent cell lines were obtained after 7 weeks and continuously cultured in the selection medium. The U937 cell clones were assayed for MxA expression by immunofluorescence staining and subsequent flow cytometry analysis. As shown in Table 1, all clones, with the exception of clone 8, expressed high levels of MxA protein. The mean fluorescence intensity in these populations was comparable to the intensity for infected BJAB cells which were used as a control (data not shown). Clone 8 did not express MxA and was therefore used in addition to the nontransfected cell line as a negative control for the following experiments. Express-

TABLE 1. Expression of MxA protein in cloned U937 transfectants

Cell line	% Fluorescence-positive cells <sup>a</sup>
BJAB .....	90.1
U937 .....	1.7
U937 clones	
1 .....	89.3
2 .....	89.3
3 .....	87.8
4 .....	93.8
5 .....	91.3
6 .....	93.6
8 .....	1.4
9 .....	92.5
10 .....	89.6

<sup>a</sup> Percent fluorescence-positive cells was estimated by fluorescence-activated cell scanner analysis of cells stained with a monoclonal antibody against the MxA protein. For negative controls, cells were stained with a nonrelated monoclonal antibody whose fluorescence signal was set to 0%.

sion of MxA remained stable for all U937/MxA clones for more than 1 year.

**VSV and MV multiplication in stably transfected U937/MxA clones.** To test the biological activity of the transfected MxA gene, the multiplication of VSV in U937 clones expressing MxA was compared with that in the parental cell line. Cells were infected at an MOI of 10 and supernatants were harvested after 24 h and titrated on Vero cells. In control cells, VSV replicated to a titer of 10<sup>7</sup> PFU/ml; however, in the transfectants VSV titers were reduced, indicating a reduction of at least 30- to 100-fold. This was comparable to the data described for 3T3/MxA cells (Table 2) (33). By repeating the experiment under the same conditions with MV, essentially the same results were obtained, with titers of infectious MV released after 48 h from U937 MxA-expressing clones being significantly lower than those of the nonexpressing control cells (Table 2).

**MV-specific transcription in transfected and nontransfected U937 cells.** Since the inhibition of primary transcription of VSV genes has been described for 3T3/MxA cells (43), MV-specific transcription in U937 cells and the MxA-transfected cell lines was analyzed. Vero cells were included as additional controls. Cells were infected with MV at an MOI of 1; total RNA was prepared after 48 h and assayed for the expression of the monocistronic MV-specific transcripts by quantitative Northern blotting (Fig. 2). Signals specific for N-, P-, M-, F-, and H-specific monocistronic transcripts and the corresponding synthetic standard RNAs were quantitated, and the transcription gradients were determined in

TABLE 2. Virus production in MxA-expressing U937 cells

Cell line	Amt of virus (PFU/ml) <sup>a</sup>	
	MV <sup>b</sup>	VSV <sup>c</sup>
U937	2 $\times$ 10 <sup>5</sup>	1 $\times$ 10 <sup>7</sup>
U937/5	3 $\times$ 10 <sup>3</sup>	1 $\times$ 10 <sup>5</sup>
U937/6	2 $\times$ 10 <sup>3</sup>	3 $\times$ 10 <sup>5</sup>

<sup>a</sup> The data result from two independent experiments.

<sup>b</sup> A total of 10<sup>6</sup> cells were infected with MV for 2 days at an MOI of 10 and cultivated in 1 ml of medium. Supernatants were analyzed in 10-fold dilutions on Vero cells in endpoint titration assays.

<sup>c</sup> Cells were infected with VSV at an MOI of 10 for 24 h, and supernatants were analyzed as described above.

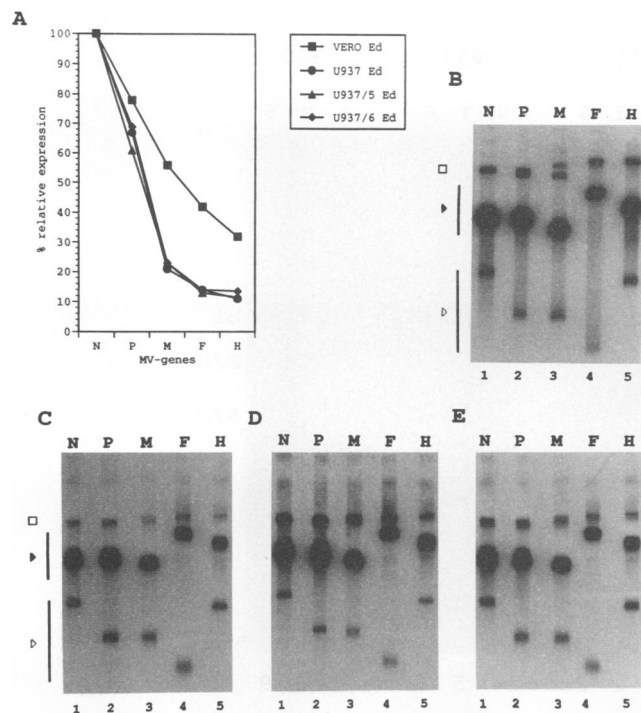


FIG. 2. (A) Results of the quantitative analysis of the Northern blots (D to E). The values are the relative expression of the MV genes in comparison with that of the individual N gene whose expression was set at 100%. (B to E) Northern blot analysis of 1 µg of pA<sup>+</sup> RNA derived from MV-infected Vero cells (B) and of 30 µg of total RNA from U937 (C), U937/5 (D), and U937/6 (E) cells infected with MV at an MOI of 1. Probes were mixed with synthetic standard RNAs and separated on a formaldehyde-containing 1.5% agarose gel. Filter strips were hybridized to <sup>32</sup>P-labeled strand-specific RNA probes and ordered according to the MV gene order. Open squares, positions of the polycistronic RNAs; closed triangles, positions of the monocistronic RNAs; open triangles, positions of the standard RNAs.

relation to the individual N-gene transcript (N = 100%). Compared with Vero cells (Fig. 2A and B), untransfected U937 cells showed a significantly steeper transcription gradient, characterized by a low abundance of all monocistronic MV-specific transcripts downstream of the N gene (Fig. 2A and C). Comparison of the amounts of MV-specific transcripts in the MxA-expressing U937 clones 5 and 6 (Fig. 2D and E) and in untransfected control cells revealed no significant differences (Fig. 2A, C, D, and E). The same results were obtained with U937/MxA clone 8 as the negative control (data not shown). In order to exclude the possibility that transcriptional downregulation was missed because of the accumulation of viral transcripts during the 48 h of infection, pulse-labelling of the viral RNAs in the presence of [<sup>32</sup>P]P<sub>i</sub> was performed for U937 clones 5, 6, and 8. As shown in Fig. 3A, considerable amounts of all MV-specific transcripts were produced. For clones 5 and 6 (Fig. 3A, lanes 1 and 2), which both expressed high levels of MxA, again no differences from the control cells were observed (Fig. 3A, lane 3).

To assess the stability of MV-specific transcripts, a pulse-chase experiment was performed for MV-infected Vero, U937, and U937/MxA clone 5 cells (Fig. 3B). The rate of synthesis of MV-specific transcripts in U937 cells was generally lower than that in Vero cells during the 90-min

pulse-labelling period (Fig. 3B, lanes 4 and 7). No differences in the stability of the viral transcripts were observed for infected U937/MxA transfectants (Fig. 3B, lanes 1 to 3) compared with U937 parental cells. We conclude from these experiments that the restriction observed for MV growth in U937/MxA cells is not due to regulation at the level of RNA transcription or at the level of mRNA stability.

**Specific inhibition of MV glycoprotein expression in U937/MxA transfectants.** U937 cells and transfected U937/MxA clones 5, 6, and 8 were infected with MV at an MOI of 1, and the expression of MV-specific structural proteins was analyzed after 48 h by indirect immunofluorescence with monoclonal antibodies. The quantitative analysis was performed by flow cytometry with an N-specific antibody. No significant differences in the expression of this structural protein could be observed for all cell lines tested. The histograms for U937/MxA clones 8 and 5, which are representative of all cell clones, are shown in Fig. 4 (Fig. 4, N). Similar results were obtained for the accumulation of P and M proteins (data not shown). In contrast, the expression of the viral glycoproteins F and H was significantly reduced at the surface of U937/MxA cell clones 5 and 6 compared with the nontransfected cells and U937/MxA clone 8, which does not express MxA. As an example, the immunofluorescence intensity for clone 5 in comparison to clone 8 is reduced on average by 1 log (Fig. 4, F and H). The considerably reduced expression of the viral glycoproteins in MxA-expressing U937 cells was confirmed by performing indirect immunofluorescence staining of MV F and H in permeabilized cells.

In order to determine whether or not the restricted accumulation of the glycoproteins was due to their reduced stability, U937/MxA clones 5 and 8 were infected with MV at an MOI of 1 for 15 h, pulse-labelled with [<sup>35</sup>S]methionine for 3 h, and subsequently chased for the time intervals indicated (Fig. 5). MV-specific proteins were immunoprecipitated with human hyperimmune serum (Fig. 5, lanes 1 to 8). As in Fig. 4, no differences in N-protein expression between the cell clones were seen (Fig. 5, lanes 1 and 5), whereas the H-protein synthesis in U937/MxA clone 5 was reduced, in comparison with that in U937/MxA clone 8, at each time point tested (Fig. 5). In contrast, no differences in stability were observed for N and H proteins in U937 clone 5 and clone 8 cells (Fig. 5, lanes 1 to 8).

In order to assess differences in the rate of synthesis of MV glycoproteins, U937 clones 5, 6, and 8, plus the untransfected U937 cells, were infected as described in the previous experiment. Pulse-labelling was performed for 5 h, beginning at 15 h postinfection, and MV-specific proteins were immunoprecipitated with human hyperimmune serum (Fig. 6, lanes 1 to 4) or a monoclonal antibody against H (Fig. 6, lanes 5 to 8). Precipitation with human hyperimmune serum against MV revealed that the synthesis of N protein was not significantly different in U937 clones 5 and 6 compared with clone 8 and the untransfected control (Fig. 6, lanes 1 to 4). In contrast, the synthesis of H protein was strongly reduced in clone 5 cells and less, but still somewhat, significantly reduced in clone 6 cells compared with the controls (Fig. 6, lanes 1 to 4). This finding was confirmed with the monoclonal anti-H antibody (Fig. 6, lanes 5 to 8). Therefore, we investigated the MxA expression of clone 6 cells. A considerable loss of the mean expression level of MxA was observed, which most probably accounted for the lesser ability to downregulate H protein synthesis compared with that of clone 5 (Fig. 6, lanes 6 and 7).

Our data indicate that the primary target for the regulation of MV gene expression by MxA may be a specific inhibition

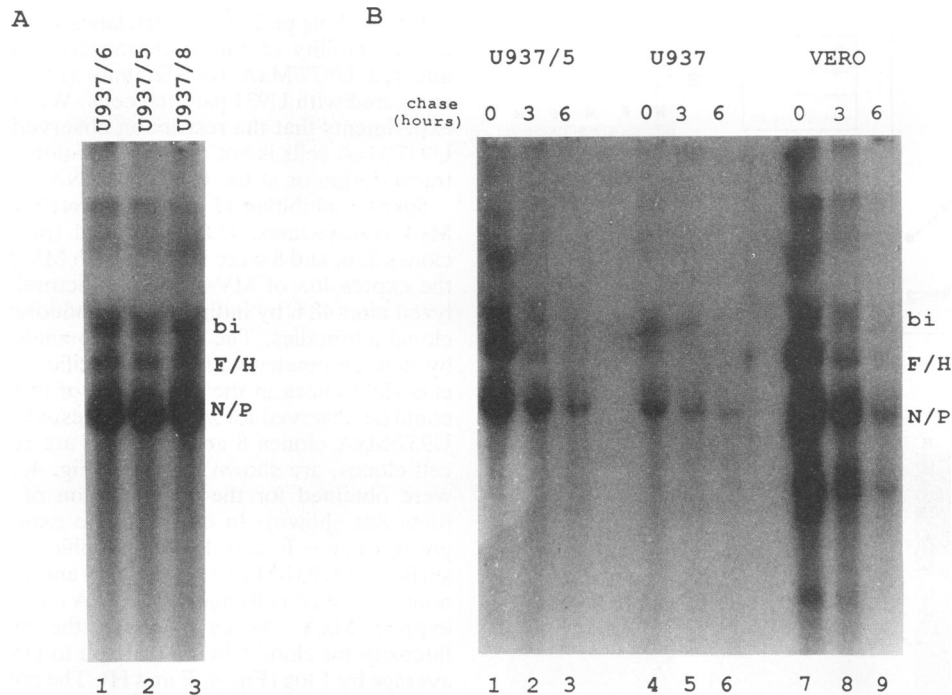


FIG. 3. In vivo [ $^{32}\text{P}$ ]P<sub>i</sub> labelling of MV-specific RNAs (A). U937/6 (lane 1), U937/5 (lane 2), and U937/8 (lane 3) cells were infected with MV (MOI of 1) for 20 h. Cells were labeled with [ $^{32}\text{P}$ ]P<sub>i</sub> for 7 h in the presence of actinomycin D. RNA was prepared, separated on a formaldehyde-containing 1.5% agarose gel, transferred to nitrocellulose, and analyzed by autoradiography. (B) Pulse-chase experiment to compare the rates of RNA degradation in the MxA-expressing clone U937/5 (lanes 1 to 3), the parental cell line U937 (lanes 4 to 6), and MV-infected Vero cells (lanes 7 to 9). Cells were labeled with [ $^{32}\text{P}$ ]P<sub>i</sub> for 2.5 h, and RNA was prepared at the indicated time points after the start of the chase. Positions of the virus-specific RNAs are indicated. "Bi" means the polycistronic MV-specific RNAs.

of viral glycoprotein synthesis, leaving the expression of other viral structural proteins unaffected.

**Inhibition of VSV synthesis in U937/MxA cells.** Previous results demonstrated an interference of MxA protein with VSV mRNA synthesis in Swiss 3T3 mouse cells constitutively expressing MxA (43). This is in contrast to the data presented here which indicate that the reduction of MV multiplication by MxA protein occurs by inhibition of viral glycoprotein synthesis. The following experiments were done to define the VSV multiplication step blocked in U937/MxA cells. In Western blotting experiments, a strong reduction of viral proteins was found for VSV-infected

U937/MxA cells which expressed high levels of MxA compared with control cells (data not shown). To examine RNA synthesis,  $10^7$  cells of the U937/MxA clones 1, 5, 6, and 9, and of clone 8 as a control, were infected with VSV at 5 PFU per cell. After 30 min at 37°C, the inoculum was removed and the cells were incubated in medium at 37°C. At 3 h postinfection, cells were harvested and RNA was prepared and analyzed for the presence of N and L mRNAs. As shown in Fig. 7A, at least 50-fold-lower concentrations of N and L mRNAs were found for the clones expressing MxA at high levels (lanes 2 to 5) compared with control cells (lane 1). This inhibitory effect of MxA protein on both virus protein

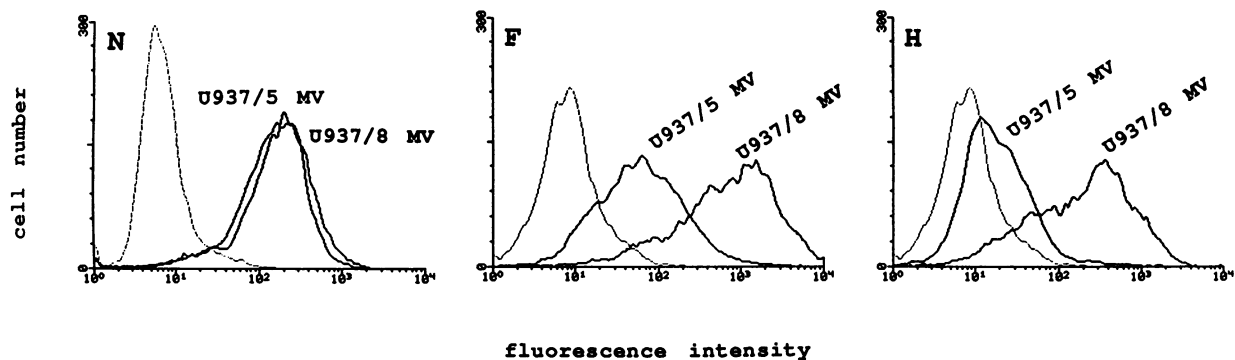


FIG. 4. Fluorescence-activated cell scanner analysis of virus-specific protein expression. Cells were fixed, permeabilized with Triton X-100, and stained with a monoclonal antibody against the N protein (N), or not permeabilized and surface stained with monoclonal antibodies against MV F and H proteins (F and H) as described in Materials and Methods. Infected cells of U937/MxA clone 5, stained with an unrelated antibody (anti-coronavirus S protein), were used as controls and are represented by the unlabelled lines.

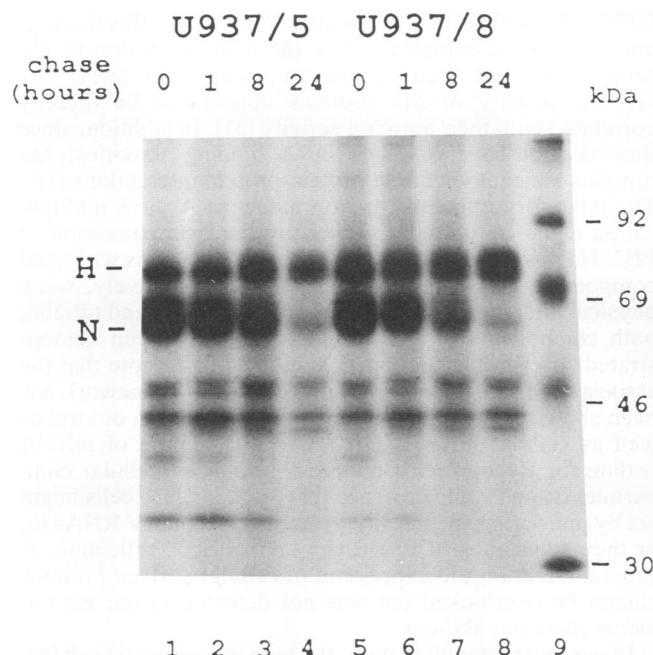


FIG. 5. Time course of virus protein degradation in MxA-expressing U937 cells. U937/5 (lanes 1 to 4) and U937/8 (lanes 5 to 8) cells were infected with MV (MOI of 1) overnight and labelled with [<sup>35</sup>S]methionine for 3 h. Lysates were prepared at the indicated time points following chase. A total of 50  $\mu$ g of every lysate was immunoprecipitated with a human anti-MV hyperimmune serum and separated on a 10% polyacrylamide gel. Positions of MV N and H proteins are indicated. Lane 9, molecular size markers.

and RNA accumulation could be due to direct interference with either VSV mRNA or protein synthesis. To distinguish between primary transcription of the parental viral genome and transcription of the amplified viral genome, which depends on ongoing translation, we carried out a parallel experiment in the presence of the protein synthesis inhibitor CHX. The optimal concentration of CHX to achieve minimal cytotoxicity with inhibition of protein synthesis by more than 95%, as determined by [<sup>35</sup>S]methionine incorporation, for our cell lines was 25  $\mu$ g/ml. CHX (25  $\mu$ g/ml) was added to each culture 30 min before infection, and this concentration was maintained throughout the experiment. As shown in Fig. 7B, under these conditions a pronounced reduction of N mRNA and an even greater reduction of L mRNA concentrations were found in U937/MxA clones (lanes 2 to 5) compared with control cells (lane 1). Thus, MxA protein inhibits the accumulation of primary VSV transcripts in both U937/MxA cells and MxA-transfected 3T3 cells and interferes with different steps of viral replication of VSV and MV in a homologous system.

## DISCUSSION

This study provides experimental evidence for an MxA-dependent regulation of MV gene expression in the stably transfected human monocytic cell line U937. In contrast to findings with VSV in transfected mouse 3T3/MxA and human U937/MxA cells, the primary target of the regulatory action of MxA for MV appeared to be located at the posttranscriptional level for the expression of the virus-specific glycoproteins.

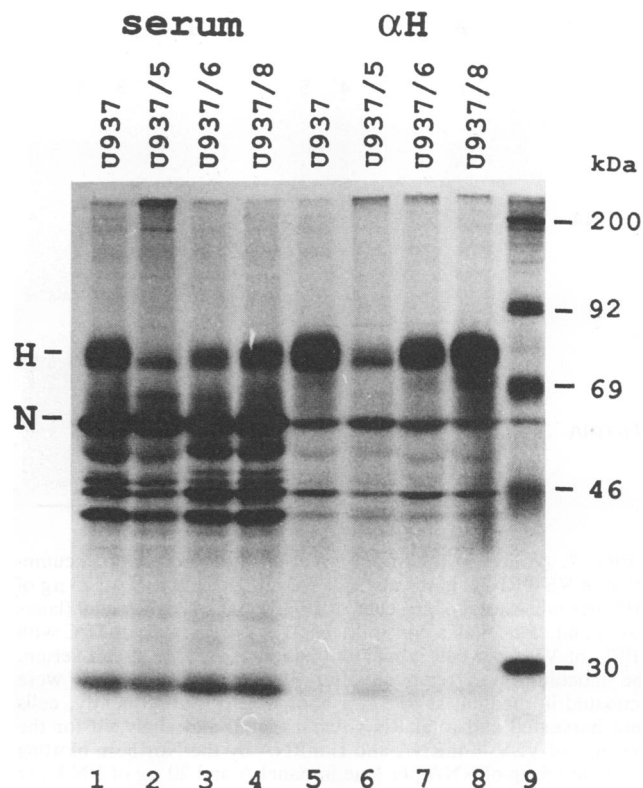


FIG. 6. Immunoprecipitation of MV-specific proteins in the MxA-expressing U937 clones 5 (lanes 2 and 6) and 6 (lanes 3 and 7) in comparison with that in the original cell line (lanes 1 and 5) and the non-MxA-expressing clone 8 (lanes 4 and 8). Cells were labelled with [<sup>35</sup>S]methionine after overnight infection for 5 h. Lysates were prepared and immunoprecipitated with either an anti-MV hyperimmune serum (lanes 1 to 4) or a monoclonal antibody against the MV H protein (lanes 5 to 8). Positions of the MV N and H proteins are indicated. Lane 9, molecular size markers.

The mechanism for the observed restriction of viral glycoprotein expression appeared to act on steps later than transcription, because it was shown that synthesis and stability of the corresponding mRNAs were unaffected (Fig. 2 and 3). Unfortunately, the translational activity of these mRNAs could not be tested *in vitro*. First, the corresponding mRNAs are expressed to relatively low copy numbers, and second, the proteins synthesized *in vitro* are not detected because the glycoprotein-specific antibodies are directed against the glycosylated protein. The possibility that MxA interferes with the processing of the mRNAs for F and H is unlikely since comparable amounts of H protein were detected in both U937/MxA cells and the control cells by short-time-labelling experiments (Fig. 5). In addition, it is difficult to envisage that defects in mRNA maturation would be selective for F- and H-specific transcripts alone. For the same reason, alterations of the secondary structure that might impair the translational efficiency (23) are not anticipated. Whereas for the F-specific mRNA secondary structures were proposed because of the 582-nucleotide-long 5' nontranslated region (35), the H mRNA does not differ from the other MV-specific mRNAs in having just a few nucleotides preceding the coding region (2). However, it is remarkable to note in this context that specific translational inhibition of MV M and F mRNAs has been described with elevation of the temperature (28, 29).



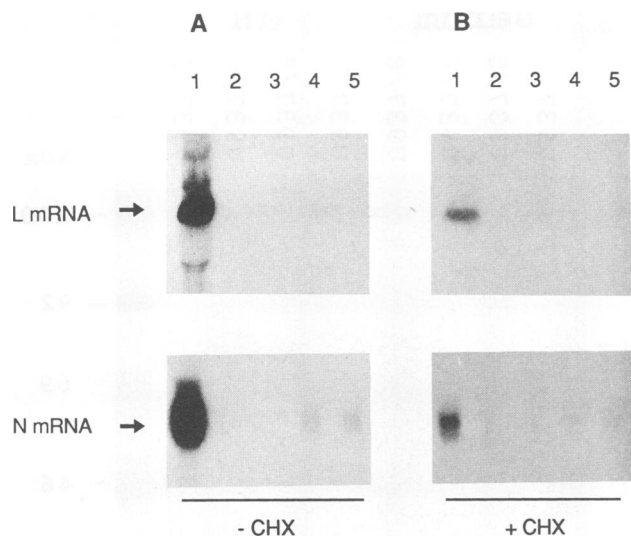


FIG. 7. (A and B) Inhibitory effect of MxA protein on accumulation of VSV RNAs in the absence (A) and presence (B) of 25  $\mu$ g of CHX per ml. A total of  $10^7$  cells of U937 clones 1, 5, 6, and 9 (lanes 2 to 5) and clone 8 as a control (lanes 1) were infected at 37°C with 5 PFU of VSV per cell in medium containing 2% fetal calf serum. The inoculum was removed after 30 min, and the cells were incubated in medium at 37°C for another 3 h. Subsequently, cells were harvested and total RNA was isolated and analyzed for the presence of VSV N mRNA and L mRNA by the Northern blotting technique (5  $\mu$ g of RNA per lane in panel A and 20  $\mu$ g of RNA per lane in panel B). The hybridization probes were the *Hind*III-*Eco*RI fragment of plasmid pGN2 for detection of N mRNA and an equimolar mixture of the 1,200- and the 1,400-bp *Pst*I fragments of plasmid pLH661 for detection of L mRNA.

The finding that the antiviral activity of Mx proteins can be posttranscriptional is not unprecedented (26, 32). In stably MxA-transfected 3T3 cells, the multiplication of influenza A virus proved to be inhibited at a stage later than primary transcription. Maturation and transport of the primary transcripts were unaffected. In that study, the inhibition of viral protein expression was highly pronounced for the larger structural proteins whereas the synthesis of M and NS was merely affected (32). Because of the fact that most of the MV-specific structural proteins, except M and L, are of comparable sizes, it is difficult to relate the translational restrictions to the size of the corresponding proteins. Although we were unable to determine the expression of L protein, because of its low expression levels and the lack of appropriate antibodies, we would not expect this protein to be reduced because transcription of MV-specific mRNAs appeared unaltered qualitatively and quantitatively in MxA-expressing U937 cells.

Our data indicate that the antiviral effect on VSV replication by MxA in U937 cells was directed at the level of viral RNA synthesis. As described previously for mouse 3T3/MxA cells (43), synthesis of VSV primary transcripts was severely inhibited in U937/MxA cells (Fig. 7). Consequently, VSV proteins accumulated to levels barely detectable in U937/MxA cells (data not shown). However, it cannot be ruled out that an additional translational regulation may be imposed on these mRNAs.

Our data confirm that the MxA-dependent inhibition of VSV synthesis in 3T3 and U937 cells is independent of the host cell. However, the inhibitory activity of MxA in human

U937 cells is clearly virus specific. At present, this discrepancy cannot be explained since the mode of action of Mx proteins has not been defined on a molecular level. The GTPase activity of Mx proteins appears to be directly correlated with their antiviral activity (31). In addition, since they do not have RNA or DNA binding activities, Mx proteins may act via direct protein-protein interactions (16). The inhibitory effect of Mx1 on influenza A virus multiplication could be partially titrated by the overexpression of PB2. However, a direct interaction of Mx proteins with viral components could not be found (18). Alternatively, weak physical interactions of MxA protein with actin and tubulin, both components of the cytoskeleton, have been demonstrated (17). In this context, it is interesting to note that the association of mRNAs with the cytoskeletal framework has been shown to play a role in governing translation of viral as well as cellular mRNAs (10). Since translation of mRNA coding for glycoproteins requires specialized cellular compartments, one could envisage that MxA in U937 cells might act by interfering specifically with transport of MV RNAs to, or their translation at, the rough endoplasmic reticulum. A general effect on the expression of cellular surface proteins cannot be overlooked but was not detected in our experiments (data not shown).

In our experimental system, the human monocytic cell line U937 does not express endogenous MxA. Although the general pathway for IFN stimulation did not appear to be defective (Fig. 1B), it has been shown that in vivo monocytes exhibit very high levels of MxA, up to eightfold higher than those in T or B cells, upon exogenous IFN treatment (16). During acute infection, MV infects predominantly PBMCs accompanied by the production of type I IFN (19, 34). Type I IFN, as well as MV infection, also leads to the induction of MxA in PBMCs in vitro. Because of the inhibitory action of MxA on MV glycoprotein synthesis, a powerful mechanism is provided for the establishment of a persistent infection in monocytic cells by a direct interference with virus replication in MxA-expressing cells, accompanied by a reduction in the release of infectious virus. As shown by our experiments, this effect is directly dependent on the expression of MxA rather than additional IFN-inducible proteins, since comparable amounts of IFN are induced in MxA-expressing and nonexpressing U937 cells following MV infection, as indicated by the expression of p68 (Fig. 1B). In addition, following exogenous IFN treatment, no synergistic effects on the inhibition of MV replication in either cell type could be observed (data not shown).

MV is the causative agent of subacute sclerosing panencephalitis and measles inclusion body encephalitis. In both diseases, the occurring MV persistence in brain cells is characterized by particular transcriptional and translational defects (7, 41). It has been observed that, during the establishment of persistent MV infections, factors intrinsic to the neural cells provide regulatory mechanisms restricting viral gene expression (4, 39). Among those, transcriptional downregulation leading to steep transcription gradients for the monocistronic MV-specific mRNAs has been described (39, 40). In accordance with those findings, we have observed a similar transcription pattern in primary infected monocytic U937 cells (Fig. 2). Transcriptional downregulation of MV-specific mRNAs together with an MxA-dependent specific inhibition of glycoprotein expression renders monocytes prime candidates for the establishment of persistent MV infections in PBMCs. Further experiments will help in the elucidation of the molecular mechanisms underlying these particular cell-type-specific regulations of MV.

## ACKNOWLEDGMENTS

We thank J. Schneider-Schaulies, S. A. Moyer, B. K. Rima, and M. A. Billeter for helpful discussions; G. Barber for providing the p68 antibody; M. Bayer for excellent technical assistance; and L. Dunster for correcting the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft and the Swiss National Science Foundation.

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