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The Vesicular Stomatitis Virus Matrix Protein Inhibits Transcription from the Human Beta Interferon Promoter

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In cells infected by wild-type (wt) vesicular stomatitis virus (VSV) Indiana, host transcription is severely inhibited. DNA cotransfection studies have implicated the VSV matrix (M) protein in this process (B. L. Black and D. S. Lyles, J. Virol. 66:4058–4064, 1992). The M protein inhibited transcription not only from viral promoters in plasmids but also from the chromosomally integrated human immunodeficiency virus type 1 (HIV-1) provirus promoter (S.-Y. Paik, A. C. Banerjea, G. G. Harmison, C.-J. Chen, and M. Schubert, J. Virol. 69:3529–3537, 1995). In this study, we investigated the effect of wt VSV M protein on expression of a reporter gene under control of a cellular promoter (beta-interferon [IFN-β] promoter), using double transient transfections in BHK and COS-1 cells. The cellular IFN-β promoter was as susceptible to the inhibitory effect of the M protein as the viral promoters used previously. Viral proteins N, P, and G had no significant effect on reporter gene expression. The M protein gene from VSV mutant T1026R1, which is defective in host transcription inhibition, was cloned and sequenced, and its effect on reporter gene expression was tested. The mutant M protein had a methionine-to-arginine change at position 51 in the protein sequence and did not inhibit transcription from either the IFN-β promoter or viral promoters. This VSV mutant is a good inducer of IFN, as opposed to the wt virus, which suppresses IFN induction. These results show that the M protein inhibits transcription from cellular as well as viral promoters and that the M protein does not regulate the IFN promoter any differently from viral promoters. While the M protein may play a role in IFN gene regulation, other viral or cellular factors that provide specificity to the induction process must also be involved.

Infection of mammalian cells with wild-type (wt) vesicular stomatitis virus (VSV), Indiana serotype, results in the inhibition of host RNA and protein synthesis (reviewed in reference 40). wt VSV isolates in general do not induce interferon (IFN) (22, 39, 41), but certain mutants of this virus, such as T1026R1 (36), are very good inducers of IFN (24, 32). It is thought that the wt virus produces an inhibitor or suppressor of IFN induction and that those VSV mutants that are good inducers of IFN are defective in the gene coding for the suppressor (23). The suppressing phenotype is dominant, for cells coinfected with IFN-suppressing and -inducing virus do not induce IFN (25, 26).

The matrix (M) protein of VSV is a potent inhibitor of transcription. Using double transient transfection experiments, Black and Lyles (4) showed that M gene expression caused the inhibition of transcription of the cotransfected chloramphenicol acetyltransferase (CAT) reporter gene. Northern blot and nuclear runoff transcription analyses demonstrated a reduction in the level of the vector-encoded reporter mRNAs and transcription of the target gene, respectively. Recently, Paik et al. (29) demonstrated that VSV M protein can inhibit transcription from the chromosomally integrated human immunodeficiency virus type 1 (HIV-1) provirus, suggesting that the effect of M protein is not limited to transcription from plasmids. The data also supported the role of the M protein in shutoff of host cell transcription.

The ability of M protein to inhibit transcription is only one of the various functions of this multifunctional protein. It plays a role in virus assembly (reviewed in reference 40), causes the disorganization of the cytoskeleton resulting in cytopathic cell rounding (5, 34), and down-regulates viral RNA transcription (8, 11). It also interacts with the viral genomic nucleocapsid and cellular components such as tubulin and the plasma membrane (9, 10, 27, 28, 42). It has been detected both in the cytoplasm and in the nucleus of infected cells (20) and is a phosphoprotein (2).

The M protein appears to be quite cytotoxic. Several laboratories have reported that while the VSV L, G, N, and P genes are expressed in high levels in transfected cells when controlled from a simian virus 40 (SV40) late promoter, it has been difficult to detect expression of the M gene when driven by the same promoter (4). This is believed to occur because M protein inhibits its own transcription as well as cellular transcription (4). This inhibition is not noted when the M gene is driven by the T7 bacterial virus promoter in a system that does not rely on the host transcription machinery (3). Its involvement in cytopathic cell rounding may also contribute to its low level of expression (5).

Until now, the effects of M protein on expression of target genes from only a few viral promoters, including the SV40 early promoter, the cytomegalovirus (CMV) promoter, and the HIV-1 long terminal repeat, have been examined (4, 29). In this study, we determined whether the M protein had the same effect on a cellular promoter, the human IFN-β promoter, as on the aforementioned viral promoters. The IFN promoter was of particular interest because it could possibly have been regulated differently by the M protein, given the role of IFN in antiviral defense.

We also examined the effects of cotransfection of VSV genes N, P, L, and G on reporter gene expression controlled by the IFN and two (SV40 early and CMV) viral promoters. The

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results were expected to provide information about whether the N, P, L, or G protein had any regulatory effect on the IFN promoter. Finally, we cloned the M gene from the IFN-inducing VSV mutant T1026R1 and studied the effect of its expression on reporter gene transcription from both the IFN and SV40 promoters, using double transient transfections. The data showed that transcription from the cellular IFN promoter, like transcription from viral promoters, was inhibited by the M protein from wt VSV but not by the VSV G, N, and P proteins. On the other hand, the M protein from mutant T1026R1 did not inhibit transcription from either the IFN or SV40 promoter. The role of the M protein in IFN gene regulation is discussed.

MATERIALS AND METHODS

Cell culture, viruses, and infection. BHK cells were grown in Dulbecco's modified essential medium (DME) containing 10% calf serum and 10% tryptose phosphate broth (Difco) on plastic petri dishes at 37°C. COS-1 cells were grown in DME containing 10% fetal bovine serum as described above. The heat-resistant (HR-C) strain of the Indiana serotype of VSV was used as the wt virus. Mutant T1026R1, a temperature-stable revertant of T1026 (36), was supplied by C. P. Stanners. This mutant was originally derived from the Indiana HR strain of VSV by chemical mutagenesis (36).

Plasmids. Plasmid pTWU54 contains the CAT reporter gene regulated by the human IFN-β promoter region and was generously provided by R. Schloemer, Indiana University School of Medicine (see reference 6 for a detailed description of this plasmid). The expression vectors containing the different VSV genes regulated by the SV40 late promoter were previously cloned into pJC119 (35) and were generously provided by M. Schubert (National Institutes of Health). They were as follows: pSVGL containing the G gene (30), pSV-VSL1 containing the L gene (31), pKOM1 containing the M gene (5), pJS223 containing the N gene (35), and pLH7 containing the P gene (18). pJC119 was used as a control in many of the experiments and to keep the amount of DNA per transfection constant. pSV2CAT contains the CAT gene driven by the SV40 early promoter and was the generous gift of M. Sekellick (University of Connecticut). pCEP4/CAT (Invitrogen) contains the CAT reporter gene regulated by the CMV constitutive promoter. Plasmids were prepared by using Qiagen Maxi Prep kits according to the manufacturer's instructions. DNA was quantitated by Hoechst staining.

Cloning and sequencing of the T1026R1 M gene. Confluent cultures of BHK-21 cells grown on 100-mm-diameter plates were infected with T1026R1 at a multiplicity of 10 for 6 h. Cytoplasmic RNA was isolated by standard techniques (21).

The M gene of mutant T1026R1 was amplified by reverse transcription-PCR (RT-PCR) in a single tube (Perkin Elmer). The synthesis of the first single-stranded DNA copy of the M mRNA was initiated by using primer A (5′ CCCTCGAG$(dT)_{14}$CATAGG 3′) consisting of oligo$(dT)_{14}$ flanked at its 3′ end with six nucleotides complementary to those preceding the poly(A) tail and flanked at the 5′ end with six nucleotides representing a *Xho*I cloning site plus two additional C residues. The reverse transcription reaction was carried out at 42°C for 15 min, followed by enzyme inactivation by heating at 99°C for 5 min and cooling to 5°C for 5 min (one cycle) in a Perkin Elmer Gene Amp PCR System 9600 Thermocycler.

The complementary plus-strand DNA was synthesized by PCR by addition of synthetic oligonucleotide primer B (5′ CCGGATCCCAATCCATTCATCATG AGTTCC 3′), which is identical in sequence to positions 27 to 50 at the 5′ end of the M mRNA preceded at its 5′ end by six nucleotides representing the *Bam*HI cloning site and two additional C residues. The double-stranded DNA was amplified by PCR with *Taq* polymerase, using both oligonucleotides A and B (94°C for 30 min, 55°C for 30 min, and 72°C for 30 min; 25 cycles). The amplified PCR product was purified from a 1% agarose gel and ligated into vector pCR3 according to the manufacturer's instructions (Eukaryotic TA Cloning Kit-Bidirectional; Invitrogen). Two microliters of the ligation reaction was transformed into One Shot TOP10F′ competent cells the following day, and 10 colonies were screened for the presence of the M cDNA fragment. One colony containing the mutant M gene in the proper orientation was identified by restriction mapping [pCR1-M (+)]. A second colony that contained the mutant gene in the opposite orientation for comparison [pCR1-M (−)] was also selected.

The DNA sequence of a clone containing the T1026R1 M gene cDNA in the coding [pCR1-M (+)] and noncoding [pCR1-M (−)] orientations was determined. The wt M sequence from VSV Indiana, HR-C serotype, kindly provided by S. Beausoleil and L. Poliquin (l'Univerité de Québec à Montréal), was used for comparison with the sequence of the T1026R1 M gene.

Transient transfection using calcium phosphate and poly(I)-poly(C) induction of the IFN promoter. BHK cells (2×10^5) were passed approximately 24 h prior to transfection into 60-mm-diameter plates. Cells were transfected by the calcium phosphate method (Stratagene Mammalian Transfection kit) according to the manufacturer's directions, with minor modifications. Briefly, the cells were refed approximately 1 to 3 h before transfection with 3 ml of complete medium minus antibiotics. The transfection was carried out with 10 μg of DNA/60-mm-diameter plate, 5 μg of pTWU54, and 5 μg of the second plasmid. The transfection mix was added to the cells dropwise. After exposure of the cells to the mixture for 5 h, the solution was removed and the cells were subjected to a 1-min glycerol (20% [vol/vol] shock. Following three washes with DME, the cells were refed with 5 ml of complete medium and incubated for approximately 18 to 24 h.

To induce the IFN promoter, cells were treated with 5 ml of complete medium containing 50 μg of poly(I)-poly(C), 10 μg of DEAE-dextran, and 50 μg of cycloheximide per ml for 6 h. The cells were washed twice with DME, refed with complete medium, incubated for an additional 24 h, and harvested for CAT assay (see below).

Transient transfection using LipofectAMINE and poly(I)-poly(C) induction of the IFN promoter. BHK or COS-1 cells were transiently transfected by using the LipofectAMINE reagent (Gibco-BRL) according to the manufacturer's directions. A total of 2×10^5 to 2.5×10^5 cells per 35-mm-diameter plate were seeded the day before transfection in complete medium. Cells were transfected with the amounts of plasmid DNA indicated in the figure legends. COS cells were transfected with 6 μl of LipofectAMINE, and BHK cells were transfected with 8 μl of LipofectAMINE. Cells were exposed to the lipid-DNA complex for 5 h. An equal volume of medium containing twice the amount of serum was added, and the cells were incubated for 18 to 24 h. Fresh complete medium was then added, and the cells were incubated for an additional 18 to 24 h. DNA and LipofectAMINE volumes were scaled up based on surface area when 60-mm-diameter plates were used.

After approximately 18 to 24 h of transfection, COS cells transiently cotransfected with the plasmids were treated with poly(I)-poly(C) as described above. After transfection for 37 to 41 h, BHK cells transiently transfected with the same plasmids were treated with poly(I)-poly(C) as described above except that cells were incubated for 4.5 h instead of 6 h before being refed and incubated for 2.5 h. Cells were collected and prepared for CAT analysis. All incubations were done at 37°C.

CAT analysis. CAT activity was determined by the method of Gorman et al. (16), with slight modifications. The cells were disrupted by three freeze-thaw cycles and spun in a microcentrifuge for 15 min at 4°C. One-tenth microcurie of [^{14}C]chloramphenicol (59.50 mCi/mmol; New England Nuclear Corp.) was used per sample. The separated acetylated chloramphenicol forms were visualized by autoradiography for approximately 24 h. The results were quantitated with a Packard Instruments InstantImager. The percent acetylation was calculated as follows: (cpm in acetylated forms of [^{14}C]chloramphenicol/total label in all bands in the lane) × 100.

Immunoprecipitation. Cells were infected with wt VSV at a multiplicity of 20 for 3 to 4 h followed by labeling for 2 h in a mixture containing prewarmed DME minus amino acids, 1× amino acid mix without methionine, and 80 μCi of [^{35}S]methionine per ml. Cells for transfection were seeded on 60-mm-diameter wells and transiently transfected by using LipofectAMINE. After 28 to 30 h of transfection, cells were labeled with 1.5 ml of prewarmed DME minus amino acids, 2% fetal bovine serum (COS cells) or 1% calf serum, 1% tryptose phosphate broth (BHK cells), 1× amino acid mix without methionine, and 80 μCi of [^{35}S]methionine per ml. Proteins were labeled for 12 to 16 h at 37°C. After labeling, cells were washed twice with ice-cold phosphate-buffered saline, and 900 μl of radioimmunoprecipitation assay buffer (10 mM Tris-HCl [pH 7.4], 1% Triton X-100, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride) was added to the monolayer. Cells were scraped into a microcentrifuge tube and incubated on ice for 30 min. Cell debris was removed by centrifugation for 3 min. The clarified supernatant was adjusted to 0.2% sodium dodecyl sulfate and incubated with VSV polyclonal antibody 6794 (generously provided by E. Kretzschmar and J. Rose, Yale University) for 30 min at 37°C. Protein A-agarose was added to each sample, and the samples were incubated for 2 h at 37°C while being rotated on a revolving wheel. The pelleted beads were washed three times with radioimmunoprecipitation assay buffer, resuspended in 1× sample buffer, boiled, and applied to a sodium dodecyl sulfate–10% polyacrylamide gel (19). After electrophoresis, the fixed and dried gel was autoradiographed.

RESULTS

Inducibility of the IFN promoter in transiently transfected cells. The goal of this study was to determine whether the wt VSV M protein inhibits transcription from a cellular (IFN) promoter in addition to transcription from the SV40, CMV, and HIV-1 viral promoters studied previously (4, 29). The general experimental strategy was to cotransfect cells with pTWU54, the IFN promoter-CAT reporter gene construct described in previous reports (6, 38), and one of the five VSV genes attached to the SV40 late promoter. These transfected cells were then subjected to poly(I)-poly(C) treatment to activate the IFN promoter. After a certain time period (see Materials and Methods), cells were lysed and the effect of the cotransfecting viral gene on IFN promoter activity was measured by CAT assays.

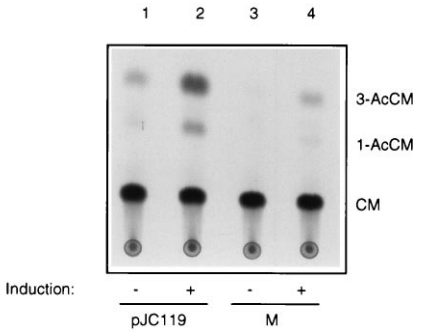

FIG. 1. Dependence of IFN promoter transcription on inducer. BHK cells were cotransfected by the calcium phosphate method with 5 μg of pTWU54 and 5 μg of the plasmid mentioned below. After transfection for approximately 24 h, cells in lanes 2 and 4 were induced by poly(I)-poly(C) treatment for approximately 24 h as described in Materials and Methods. Cell lysates were prepared, and their CAT activity was determined by thin-layer chromatography. The cotransfected viral plasmids and the amount of conversion of [^{14}C]chloramphenicol to acetylated forms are as follows: lanes 1 and 2, pJC119, 3.1 and 15.6%, respectively; and lanes 3 and 4, pKOM 1 (M), 0.5 and 2.2%, respectively. Abbreviations: CM, chloramphenicol; 1-AcCM, 1-acetyl chloramphenicol; 3-AcCM, 3-acetyl chloramphenicol.

IFN induction in virus-infected cells requires synthesis or activation of an IFN inducer. In wt VSV-infected cells, one or more suppressors of IFN induction that effectively prevent expression of the IFN gene are also presumably synthesized (23). By providing an IFN inducer [poly(I)-poly(C)] in the transfection experiments, we measured only the suppressor activity of the product of the viral gene under analysis on the target gene.

BHK cells were selected for these studies because of their high level of transient transfection. In addition, this cell line is a good host for VSV and was used by Black and Lyles (4) in their studies on transcriptional inhibition by VSV M protein. To be able to compare our results to theirs, we used the same cell line. Cells were transfected by using either calcium phosphate or LipofectAMINE. The two reagents gave similar results and were used interchangeably in these studies.

The dependence of the IFN-CAT gene on poly(I)-poly(C) for induction was tested because of the possibility that one of the plasmid preparations contained residual RNA contaminants that could induce the IFN promoter. BHK cells were cotransfected by the calcium phosphate method with plasmid pTWU54 and a second plasmid carrying the VSV M gene. The control sample was cotransfected with pTWU54 and pJC119, the parental plasmid containing the VSV genes (35). As shown in Fig. 1, cells cotransfected with pTWU54 and pJC119 (lane 1) produced only very small amounts of CAT in the absence of poly(I)-poly(C). The addition of poly(I)-poly(C) (lane 2) resulted in a substantial increase in CAT activity. These results indicated that transcription from the IFN gene promoter was dependent on the presence of an inducer under the conditions of our experiments and that if there were RNA contaminants in the plasmid preparations, their contribution to IFN induction was minimal.

Cotransfection of BHK cells with plasmid pTWU54 and the plasmid containing the VSV M gene resulted in the inhibition of CAT synthesis (Fig. 1; compare lanes 2 and 4), indicating that the M protein inhibited transcription from a cellular promoter as well as from the viral promoters previously reported.

Effect of cotransfection of each of the five VSV genes on IFN promoter activity and cell type specificity of the effect. To determine whether cotransfection of the L, N, G, and P genes with pTWU54 in BHK cells influenced CAT synthesis, we repeated the previous experiment except that LipofectAMINE was used for the transfections instead of calcium phosphate. Figure 2A shows that the plasmid containing the M gene was the most inhibitory in terms of CAT synthesis. Cotransfection with plasmids containing the G, L, N, and P genes had considerably less effect on CAT synthesis. Similar results were obtained when cells cotransfected with pTWU54 and one of the five VSV gene-containing plasmids for 42 h were induced by infection with T1026R1 for 6 h instead of by poly(I)-poly(C) (data not shown).

The VSV genes used in these experiments were under regulation of the SV40 late promoter. Although most of these genes were expressed in BHK cells (as shown in Fig. 3), we also tested their effect on the IFN gene promoter in COS-1 cells, which express the SV40 large T antigen. As in BHK cells, IFN induction was dependent on the addition of poly(I)-poly(C) (Fig. 2B, lanes 1 and 2). Cotransfection of these cells with pTWU54 and the M gene resulted in a substantial decrease in CAT activity (Fig. 2B, lane 5). Cotransfection of these cells with pTWU54 and the G, L, N, and P genes had no significant inhibitory effect on CAT activity (Fig. 2B, lanes 3, 4, 6, and 7).

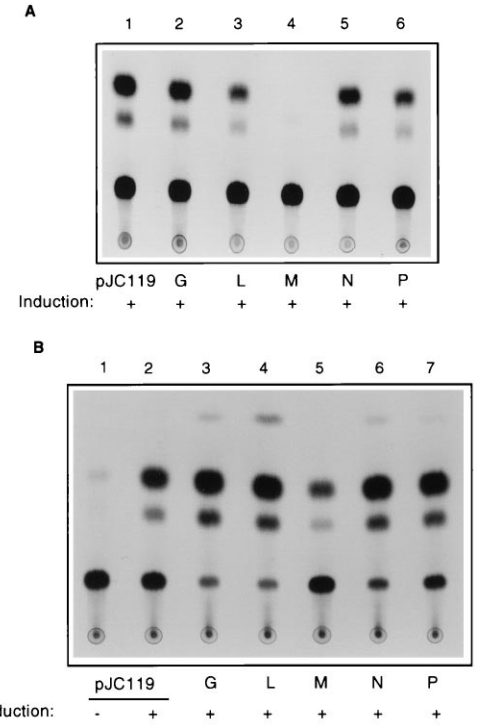

FIG. 2. Effect of the VSV proteins on IFN promoter activity in LipofectAMINE-transfected BHK and COS-1 cells. (A) BHK cells were cotransfected with 1 μg of pTWU54 and 2 μg of the viral expression vector indicated below, using the LipofectAMINE reagent. After transfection for approximately 40 h, cells were induced with poly(I)-poly(C) as indicated in Materials and Methods and cell lysates were prepared. CAT activity of cell extracts was determined by thin-layer chromatography. The cotransfected viral plasmids and the amounts of chloramphenicol acetylation are as follows: lane 1, pJC119, 23.5%; lane 2, pSVGL (G), 21.3%; lane 3, pSV-VSL1 (L), 10.6%; lane 4, pKOM 1 (M), 1.8%; lane 5, pJS223 (N), 17.7%; and lane 6, pLH7 (P), 9.8%. (B) COS-1 cells were cotransfected with 0.75 μg of pTWU54 and 2.25 μg of viral plasmid for 44 h. Cells were induced with poly(I)-poly(C) as indicated in Materials and Methods, except for one sample that was not induced (lane 1). The cotransfected viral plasmids and the amounts of chloramphenicol acetylation are as follows: lane 1, pJC119, 3.0%; lane 2, pJC119, 40.7%; lane 3, pSVGL (G), 93.8%; lane 4, pSV-VSL1 (L), 94.4%; lane 5, pKOM 1 (M), 18.1%; lane 6, pJS223 (N), 90.8%; and lane 7, pLH7 (P), 78.7%.

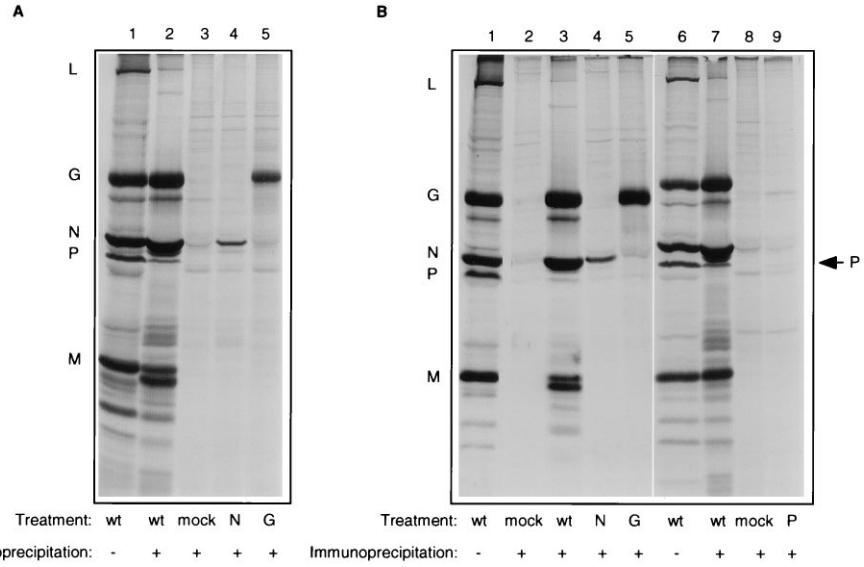

FIG. 3. Immunoprecipitation of VSV proteins in transiently transfected BHK and COS-1 cells. Cells were transfected with 1.25 μg of pTWU54 and 5 μg of a second plasmid (indicated below) by the LipofectAMINE method. Following incubation for 33 to 36 h, the cells were incubated for 12 to 15 h in medium containing 80 μCi of [^{35}S]methionine per ml. Lysates were immunoprecipitated with polyclonal VSV antibody (see Materials and Methods). The method for infecting and labeling cells with wt VSV was described in Materials and Methods. (A) BHK cells. Lane 1, infected cell lysate not immunoprecipitated; lane 2, wt VSV-infected cells immunoprecipitated; lane 3, pJC119 immunoprecipitated; lane 4, pJS223 (N) immunoprecipitated; lane 5, pGL (G) immunoprecipitated. (B) COS-1 cells. Lanes 1 and 6, wt VSV-infected cell lysate not immunoprecipitated; lanes 2 and 8, pJC119 immunoprecipitated; lanes 3 and 7, wt VSV-infected cells, immunoprecipitated; lane 4, pJS223 (N) immunoprecipitated; lane 5, pSVGL (G) immunoprecipitated; lane 9, pLH7 (P) immunoprecipitated.

These results demonstrated that in these two different cell lines, the M protein was the most inhibitory VSV protein.

Immunoprecipitation of cells transiently transfected with VSV genes. Immunoprecipitation was used to determine whether the transfected BHK and COS cells were expressing the intended viral gene product. Cells were transfected for a total of 48 h, and the proteins were labeled with [^{35}S]methionine during the last 12 h of transfection as described in Materials and Methods. After the transfections, cell lysates were prepared and the incorporation of radioactive methionine into virus-specific protein was determined by using immunoprecipitation with a polyclonal antibody to total VSV proteins.

In some cases, cells were infected with wt VSV and labeled with [^{35}S]methionine. Lysates from these cells served as markers for viral proteins and also showed which VSV proteins were the most antigenic. Figure 3A, lane 2, shows that the polyclonal antibody precipitated proteins G, N, and M best, followed by proteins P and L. When BHK cells were transfected with pTWU54 and the plasmid containing the N or G gene followed by immunoprecipitation with the polyclonal antibody, both the N and G genes appeared to be expressed, since both G and N proteins were immunoprecipitated (Fig. 3A, lanes 4 and 5). No L nor P protein was detected in similar experiments (data not shown), probably because the polyclonal antibody used did not immunoprecipitate these proteins very effectively.

Similar results were obtained when COS cells were used in the double transient transfections instead of BHK cells. The N and G genes were obviously expressed (Fig. 3B, lanes 4 and 5), and a small amount of P protein (Fig. 3B, lane 9) was also detected in these cells. It is not known whether the L gene was expressed in these cotransfection experiments, since the L protein could not be detected. Therefore, the question of whether the L protein affects IFN transcription is still open. However, the L clone used in these experiments has been shown to complement and rescue L gene temperature-sensitive mutants of VSV at the nonpermissive temperature (31). No M gene expression was detected in either BHK or COS cells by using immunoprecipitation methods, confirming similar observations reported by other laboratories (3, 4, 29).

Effect of each of the VSV proteins on viral promoters. In their studies, Black and Lyles (4) examined the role of M gene expression on CAT reporter gene expression, using the SV40 early (pSV2CAT) promoter. We extended these studies by determining the effect of cotransfection with viral genes L, N, P, and G on the CAT target gene under control of the CMV (pCEP4/CAT) and SV40 promoters. As shown in Fig. 4, lanes 1 to 3, 5, and 6, cotransfection of the CAT reporter gene controlled by the CMV promoter with pJC119 or viral gene G, L, N, or P did not inhibit CAT production. Cotransfection with

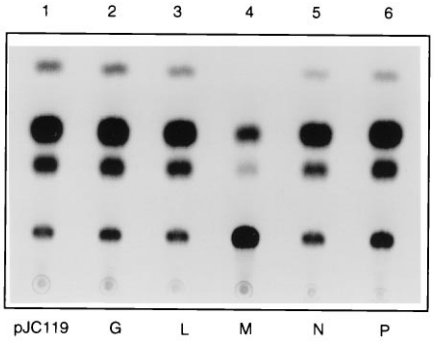

FIG. 4. Effect of the VSV proteins on CAT gene expression controlled by the CMV promoter. BHK cells were cotransfected with 8 ng of pCEP4/CAT and 3 μg of the viral expression vector indicated below, using the LipofectAMINE reagent. After transfection for approximately 40 h, cell lysates were prepared and their CAT activity was determined as described in Materials and Methods. The cotransfected viral plasmids and the amount of conversion of chloramphenicol to acetylated chloramphenicol were as follows: lane 1, pJC119, 90.8%; lane 2, pSVGL (G), 89.6%; lane 3, pSV-VSL1 (L), 88.9%; lane 4, pKOM 1 (M), 14.2%; lane 5, pJS223 (N), 85.5%; and lane 6, pLH7 (P), 83.3%.

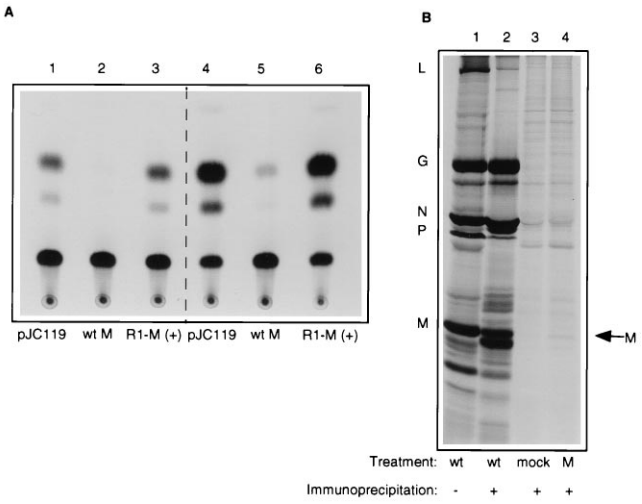

FIG. 5. Effect of the M protein from T1026R1 on the IFN and SV40 early promoters. (A) In lanes 1 to 3, BHK cells were transiently cotransfected with 0.4 μg of pTWU54 and 1.6 μg of a viral plasmid, using LipofectAMINE. After approximately 40 h of transfection, cells were induced by poly(I)-poly(C) treatment as described in Materials and Methods. Cells were collected, lysed, and analyzed by CAT assay. In lanes 4 to 6, BHK cells were transiently cotransfected with 25 ng of pSV2CAT and 3 μg of the indicated viral plasmid. The cotransfected viral plasmids and the amounts of conversion of chloramphenicol to acetylated forms are as follows: lanes 1 and 4, pJC119, 10.1 and 68.0%, respectively; lanes 2 and 5, pKOM 1 (wt M), 1.0 and 3.2%, respectively; and lanes 3 and 6, pCR1-M (+) (R1 M), 12.8 and 70.4%, respectively. (B) Immunoprecipitation of T1026R1 M protein in transfected BHK cells. Cells were cotransfected with 1.25 μg of pTWU54 and 5 μg of pCR1-M (+) for 30 h, using the LipofectAMINE method. Cells were then labeled with 80 μCi of [^{35}S]methionine per ml for 12 h following immunoprecipitation with VSV polyclonal antibody. Lane 1, wt virus-infected cells not immunoprecipitated; lane 2, wt virus-infected cells immunoprecipitated; lane 3, cells transfected with PJC119 and immunoprecipitated; lane 4, cells transfected with pCR1-M (+) and immunoprecipitated.

the M gene, on the other hand, caused a large reduction in CAT activity (Fig. 4, lane 4). The same results were obtained when the target CAT gene was controlled by the SV40 early promoter instead of the CMV promoter (data not shown). These results demonstrated that the five VSV proteins affected viral and cellular promoters similarly.

The M protein inhibited CAT activity in a gene dosage-dependent manner (data not shown), as previously demonstrated (4).

Cloning and expression of the T1026R1 M gene. If the VSV M protein were responsible for the inhibition of IFN expression in wt virus-infected cells and our cotransfection experiments were an accurate reflection of the events occurring in infected cells, then cotransfection of pTWU54 and the M gene from the IFN-inducing, host transcription inhibition-negative mutant T1026R1 should not suppress CAT activity. The effect of cotransfecting BHK cells with plasmids containing the wt VSV M gene or the mutant M gene, cloned into vector pCR3 containing a CMV promoter, on CAT expression from either the IFN or the SV40 early promoter was examined. The results (Fig. 5A) demonstrated that expression of the M gene from the mutant did not result in the inhibition of CAT transcription from either the IFN (lanes 1 to 3) or the SV40 (lanes 4 to 6) promoter. The same results were obtained with plasmid pCR3 containing the T1026R1 M gene in the noncoding orientation (data not shown). These results indicated that the mutant M protein was defective in its ability to suppress expression from both a cellular and a viral promoter.

Expression of the T1026R1 M gene during cotransfection was verified by immunoprecipitation (Fig. 5B) and RT-PCR (data not shown). T1026R1 M protein was detected but at a rather low level. While this mutant protein was defective in transcription inhibition, it was like the wt protein in causing cytopathic cell rounding (14). The low amount of M protein detected may have resulted from this function of the protein.

The nucleotide sequence of the cloned T1026R1 M gene. The nucleotide sequence of the cloned mutant M gene was determined as described in Materials and Methods and aligned to that of the M gene from the wt Indiana HR strain of VSV. Two nucleotide changes were detected. Each led to amino acid changes. One was a methionine-to-arginine change in amino acid 51, and the other was an aspartic acid-to-glycine change in amino acid 92. Beausoleil and Poliquin (1) sequenced two independent *ts*T1026 revertants and found only the one change in amino acid 51. As discussed later, the mutation in position 51 is the same as that found by Coulon et al. (12) in mutant *ts*082, which is defective in host transcription inhibition. Whether the mutation in amino acid 92 is an independent mutation of T1026R1 arising in our laboratory after many passages of the original stock virus or an artifact produced during the PCR step has yet to be determined.

DISCUSSION

In this study, three points were considered: the effect of the wt VSV M protein on transcription of a target gene from a cellular (IFN) promoter, the cloning and sequencing of the M gene from the IFN-inducing VSV mutant T1026R1, and the effect of this mutant M protein on transcription from both cellular and viral promoters.

Lack of promoter specificity. The M protein has been shown to be a very powerful inhibitor of transcription of target genes controlled by various viral promoters (4, 29). These studies demonstrated that expression of the CAT reporter gene from a cellular promoter was as susceptible to this inhibitor of transcription as expression from viral promoters. This finding suggests that the M protein acts on the general transcription apparatus and does not possess specificity in the regulation of viral and cellular (at least the IFN) promoters. This result was of particular interest because the IFN promoter could have been regulated differently from viral promoters as a result of the major role that IFN plays in host cell defense against viral infection.

This finding, however, does not preclude the possibility that VSV affects host transcription differentially from specific promoters by other mechanisms. Indeed, a nuclear protein that binds specifically to IFN-stimulated response elements (ISRE) is activated shortly after L929 cells are infected with VSV or are treated with poly(I)-poly(C) (7). Activation in the former case requires VSV primary transcription and tyrosine kinase activity (7). What is interesting about this finding is that while transcription of a reporter gene not containing ISRE in its promoter was severely inhibited in VSV-infected cells as a result of host transcription shutoff (presumably by the M protein), transcription of the same reporter gene attached to ISRE was much less reduced (7). Presumably, the VSV-activated ISRE-binding protein was responsible for the differential sensitivity of these two genes to transcription inhibition.

The viral G, N, and P proteins did not possess the potent transcriptional inhibitory properties of the M protein, as measured by CAT assays (Fig. 2 and 4). This was true even when equal molar concentrations of the viral DNAs were used in the transfections (data not shown).

The human IFN-β promoter used in these experiments contained positions −286 to +67 of the regulatory DNA region (38). This region encompasses all known major promoter ele-

ments, including the high-mobility-group binding sites (13). Positions −125 to −38 of the IFN-β promoter region (relative to the cap site) are considered to comprise the minimal sequence required for full IFN induction by virus infection or poly(I)-poly(C) treatment (15).

Cloning of the T1026R1 M gene. The cloning and sequencing of the T1026R1 M gene provided interesting results as to which amino acid was responsible for the lack of inhibition of transcription in cells infected by this virus. Comparison of the sequence of the cloned T1026R1 M gene with that of its wt parent indicated two amino acid changes. Interestingly, the methionine-to-arginine change in amino acid 51 was the same as that found in mutant *ts*082, which is defective in transcription inhibition in chicken embryo fibroblasts at the nonpermissive temperature (12). This mutant is also an excellent inducer of IFN at the nonpermissive temperature in chicken embryo fibroblasts and other cell lines (24). The importance of amino acid 51 in transcription was demonstrated by the isolation of temperature-resistant revertants of *ts*082 in which the wt phenotype in terms of host transcription inhibition and IFN induction was reestablished (12, 24). The M gene from the revertant had the same sequence as the original wt M gene from which the *ts*082 mutant was derived (12).

Black et al. (3) reconstructed the methionine-to-arginine change in the M gene from mutant *ts*082 in an experimental M gene and found that in cotransfections with the CAT reporter gene, it had no effect on transcription of the reporter gene but behaved like the wt gene in its ability to function in virus assembly. Together, these data suggest that amino acid 51 in the M protein plays an important role in the ability of the M protein to inhibit host transcription.

Lack of inhibition of transcription by the cloned T1026R1 M gene. The M protein from mutant T1026R1 did not inhibit expression of CAT from either the IFN promoter or the SV40 promoter (Fig. 5). This finding is consistent with the finding that this mutant does not inhibit host cell transcription until relatively late in the infection. Although expression of the T1026R1 M gene was detected by both RT-PCR and immunoprecipitation, it was not as abundantly expressed as some of the other VSV genes, which was rather surprising since it had little effect on host transcription. We attributed this to the cytopathic cell rounding function of the M protein, which is like that of the wt protein in this mutant.

Relationship of the inhibition of IFN gene expression by M protein to the regulation of IFN induction in wt VSV-infected cells. The results reported in this study demonstrated that the M protein inhibited gene expression from the IFN promoter in BHK and COS-1 cells. The question that we pose is whether the M protein regulates IFN gene expression in wt virus-infected cells. Virus induction of the IFN-β promoter is a multistep process requiring the synergistic interactions between distinct virus-inducible elements and several different transcription factors, including HMG1(Y), NF-κB, and ATF-2 (13). In theory, any of the steps leading to activation of this promoter could be modified or inactivated by virus infection even before the full transcription complex is assembled. The experimental approach used in this study bypassed an essential step in the virus-directed activation of the IFN gene. The IFN gene promoter was induced with poly(I)-poly(C) or by infection with VSV mutant T1026R1. While IFN is induced under these conditions, the control of IFN induction in cells infected by the wt virus is more complex.

In wt VSV-infected cells, viral gene products may interact with cellular factors to provide levels of IFN promoter regulation that would not have been evident in the experiments in this report. For example, activation of transcription factor NF-κB, which entails its translocation to the nucleus (reviewed in reference 17), is one of the first steps in IFN gene induction (13). In mouse L cells infected with VSV mutant T1026R1, NF-κB is activated within the first 30 min to 1 h after virus adsorption (6). In contrast, there is a delay of up to at least 4 h in cells similarly infected with wt VSV. By the time NF-κB is activated in this latter case, host transcription is inhibited to an extent that any gene transcription, including IFN gene transcription, is precluded. Some evidence exists that cells infected with wt VSV may contain an inhibitor of NF-κB activation. Preliminary data suggest that the inhibitor may be different from the general transcriptional inhibitor (M protein) discussed in this report, based on UV target size analysis (6). The nature and role of this NF-κB activation inhibitor is not known at this time, but it may contribute to the overall regulation of IFN induction. These results suggest that the IFN gene may be regulated at both induction and transcription levels. Mutant T1026R1 may have defective genes that act at both of these levels.

A great deal of our knowledge about the regulation of IFN by VSV derives from studies done in aged primary chicken embryo cells. Because there are significant differences in how VSV affects the process of IFN induction in avian and mammalian cells (26, 37), it is particularly difficult to compare results obtained in these two cell types. Indeed, studies by Marcus et al. (24) suggest that the M protein is not involved in the regulation of IFN gene expression in primary chicken embryo cells. They compared the abilities of different field isolates of VSV to induce IFN in these avian cells and found that one isolate (22-20) was an excellent inducer of IFN whereas another isolate (22-25) did not induce IFN, yet the M gene sequence was identical in the two isolates. In mouse L cells, isolate 22-25 also did not induce IFN, but in contrast to avian cells, isolate 22-20 induced only a very small amount of IFN (33).

Since chicken embryo fibroblasts are sensitive to the effects of M protein on host transcription (12), the fact that IFN induction occurs in cells infected with isolate 22-20, which has a wt M gene, is a paradox. These data could be explained if, indeed, two VSV genes, including the M protein gene, are involved in the control of IFN gene transcription. While VSV mutant T1026R1 may be defective in both genes, as argued above, isolate 22-20 may be defective in only one, the one responsible for limiting IFN induction at an early step of transcription. Why the M protein does not prevent transcription of the IFN gene after this step in chicken embryo fibroblasts could be explained if the rate of host transcription inhibition were slow in comparison to the rate of transcription initiation complex formation (induction). In mouse L cells, host transcription inhibition is so rapid that even if the early steps of transcription were to take place, transcription of the IFN gene would be precluded. As mentioned above, very little IFN was induced by isolate 22-20 in these cells (33).

In sum, this report represents the first demonstration that the M protein from wt VSV indiscriminately inhibits reporter gene transcription from cellular and viral promoters and that a mutant M protein from VSV T1026R1 with a methionine-to-arginine change in position 51 of the protein is no longer active in this capacity. While the results presented are consistent with a role of M protein in IFN gene regulation in VSV-infected mammalian cells, specific regulation of the IFN gene promoter probably occurs at another step in transcription.

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