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Vesicular Stomatitis Virus Matrix Protein Inhibits Host Cell-Directed Transcription of Target Genes In Vivo

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Infection by vesicular stomatitis virus (VSV) results in a rapid inhibition of host cell transcription and translation. To determine whether the viral matrix (M) protein was involved in this inhibition of host cell gene expression, an M protein expression vector was cotransfected with a target gene vector, encoding the target gene, encoding chloramphenicol acetyltransferase (CAT). Expression of M protein caused a decrease in CAT activity in a gene dosage-dependent manner, and inhibition was apparent by 12 h posttransfection. The inhibitory effect of M protein was quite potent. The level of M protein required for a 10-fold inhibition of CAT activity was less than 1% of the level of M protein produced during the sixth hour of VSV infection. Northern (RNA) analysis of cotransfected cells showed that expression of M protein caused a reduction in the steady-state level of the vector-encoded mRNAs. Expression of both CAT and M mRNAs was reduced in cells cotransfected with a plasmid encoding M protein, indicating that expression of small amounts of M protein from plasmid DNA inhibits further expression of both M and CAT mRNAs. Nuclear runoff transcription analysis demonstrated that expression of M protein inhibited transcription of the target genes. This is the first report of a viral gene product which is capable of inhibiting transcription in vivo in the absence of any other viral component.

One important manifestation of cytopathology for many viruses is the inhibition of host cell macromolecular synthesis. Because of its importance in viral disease, inhibition of host cell transcription has been widely studied and has been observed for members of many different classes of viruses, including poxviruses (5), reoviruses (29), picornaviruses (24), paramyxoviruses (34), and rhabdoviruses (4, 32, 35). While all these groups of viruses are known to reduce host transcriptional activity, it has been difficult to define the mechanism of action and the viral components responsible for the observed inhibition. The inhibition of host cell transcription by the prototype rhabdovirus, vesicular stomatitis virus (VSV), has been studied extensively. Previous studies have shown that viral transcription is required for inhibition of host transcription by VSV (33) and have primarily implicated leader RNA as being involved. The leader RNA of VSV is a 45-nucleotide, positive-sense RNA encoded at the 3′ end of the VSV genome and is transcribed by the viral RNA polymerase prior to transcription of the viral mRNAs. The evidence for a role of leader RNA in the inhibition of cellular transcription was obtained by UV inactivation studies of the VSV genome (14, 31) and by assaying the effects of the leader RNA on host transcription in vitro (15, 16, 25). However, other studies have indicated that leader RNA alone is not sufficient to inhibit host transcription and that either a host factor or a viral protein is required for inhibition (10, 26). The matrix (M) protein of VSV is a good candidate for being involved in the shutoff of host transcription for several reasons. M protein is the only VSV protein which is found in the cell nucleus in substantial quantity (21). Nuclear localization by M protein is not required for virus replication, since VSV can replicate in enucleate cells (12), but may play a role in the inhibition of host cell nuclear function. Second, the VSV temperature-sensitive mutant *ts*O82, which is defective in host cell shutoff, has a mutant M gene (9). Third, M protein has been reported to be difficult to express in mammalian cells by using recombinant vectors (6, 20), and when it can be expressed, it causes the cell rounding that is characteristic of VSV cytopathology (6). We tested the effect of M protein on host transcription in a cotransfection assay. Plasmid DNA encoding the M protein was cotransfected with a plasmid encoding the gene for chloramphenicol acetyltransferase (CAT). Expression of M protein in the absence of other VSV gene products inhibited transcription of the target gene. This is the first evidence of a viral gene product which, independent of any other viral component, is sufficient to inhibit host cell-directed gene expression by reducing transcriptional activity.

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MATERIALS AND METHODS

Plasmids. The VSV M gene from plasmid pM309 (27) was subcloned into the *Pst*I site of M13mp18 with the 5′ end of the M gene oriented near the *Eco*RI site of M13mp18. The oligonucleotide 5′-GAACTCATGATGAATGGATTGGGA TAACAAGCTTGTCGACTCTAGAGGATC-3′ was used as a primer for oligonucleotide-directed mutagenesis (17), to delete the homopolymer tail and *Pst*I site and introduce a *Hin*dIII site at the 5′ end of the M gene. The M gene was removed from M13mp18 by digestion with *Hin*dIII and was subcloned into the *Hin*dIII site of plasmid pSV2.Neo (30). Two clones containing the M gene in either the protein-encoding [pSV2.M(+)] or noncoding [pSV2.M(−)] orientation were isolated. In both cases, transcriptional control of the M gene is under control of the simian virus 40 (SV40) early promoter. Plasmid pSV2.CAT (13) was provided by Rosanne Spolski (Wake Forest University) and contains the CAT gene under control of the SV40 early promoter.

Plasmid pF1s/G, containing the VSV G gene under control of the vaccinia virus 11K promoter, was constructed by

using the VSV G gene from plasmid pG1 (27). *Eco*RI sites were added to both the 5′ and 3′ ends of the VSV G gene by polymerase chain reaction using appropriate oligonucleotide primers. This G gene was then subcloned into the *Eco*RI site of plasmid pTKgptF1s (11) to create pF1s/G.

DNA transfection. BHK-21 cells were transfected by using the Lipofectin reagent (GIBCO BRL) according to the manufacturer's directions, with minor modifications. Briefly, cell monolayers were washed once with serum-free Dulbecco modified Eagle medium (Flow Laboratories, Inc.); then 15 μg of Lipofectin reagent and 3 μg of DNA were added in 1.0 ml of Optimem reduced-serum medium (GIBCO BRL) to 10^6 BHK-21 cells in a 60-mm dish, and the cells were incubated for 5 h. Two volumes of Dulbecco modified Eagle medium with 15% fetal calf serum was then added to the monolayers, and the cells were incubated for the indicated times before harvesting.

Transfection efficiency of plasmid DNA into the BHK-21 cells used in this study was determined by transfection of plasmid pF1s/G by the method described above. After the 5-h incubation with Lipofectin, cells were lifted from the culture dishes and allowed to reattach to glass coverslips. The cells were then infected with wild-type vaccinia virus at a multiplicity of 50 PFU per cell and incubated overnight. The cells were then processed for immunofluorescence labeling, using anti-G antibody I1 (19) as described previously (21) except that the cells were labeled prior to fixation with paraformaldehyde, and the cells were not permeabilized with Triton X-100.

CAT assays. Cells were harvested by being scraped from the monolayer in the presence of 40 mM Tris-HCl (pH 7.5)–1 mM EDTA–150 mM NaCl at the indicated times posttransfection and then pelleted and resuspended in 250 mM Tris-HCl (pH 7.5) (3×10^7 cells per ml). The cells were lysed by three freeze-thaw cycles and centrifuged at approximately $10,000 \times g$ to remove cellular debris. The extracts were then incubated at 65°C for 5 min and frozen at −70°C. A 40-μl volume of cell lysate (normalized for total protein) was assayed for CAT activity as described by Gorman et al. (13), using 0.1 μCi of [^{14}C]chloramphenicol (58.2 mCi/mmol; DuPont-New England Nuclear Corp.) and 20 μl of 40 mM acetyl coenzyme A in a final reaction volume of 150 μl. Reactions were conducted at 37°C for 5 h. The reaction was stopped and analyzed by thin-layer chromatography as previously described (13) and then subjected to autoradiography or radioanalytic imaging (AMBIS Systems, Inc., San Diego, Calif.).

Northern (RNA) and Southern blot analysis. Isolation of total cellular DNA and Southern blotting were done as described previously (7). Polyadenylated RNA was isolated by the guanidine thiocyanate method followed by oligo(dT)-cellulose chromatography, and 5 μg of RNA was analyzed by Northern blot, using standard techniques (22). Northern and Southern blots were hybridized with DNA probes labeled with ^{32}P by nick translation (DuPont-New England Nuclear).

Isolation of nuclei and runoff transcription assays. A total of 10^8 nuclei were isolated from BHK-21 cells by solubilization with Triton X-100 and Dounce homogenization, using a modified version of the method described by Marzluff and Huang (23) except that after solubilization in Triton X-100, the cells were homogenized in a Dounce homogenizer with a type B pestle, and the nuclei were separated from cellular debris by being spun onto a cushion of 68% sucrose. The nuclei were then spun on a discontinuous gradient of 35 and 92.5% glycerol in 5 mM magnesium acetate–0.1 mM EDTA–5 mM dithiothreitol–50 mM Tris-HCl (pH 8.0). The nuclei were then removed from the glycerol cushion in 400 μl of the 35% glycerol buffer described above, frozen in a dry ice-ethanol bath, and stored at −70°C.

The nuclei were thawed at room temperature and then assayed in an elongation reaction mixture containing [α-^{32}P]UTP as described previously (2) at 30°C for 10 min. The nuclei were then digested with DNase I and proteinase K as described previously (2), and the labeled extract was solubilized in 9 ml of guanidine thiocyanate (GTC) buffer and precipitated with 0.6 volume of ethanol. The precipitate was pelleted, resolubilized in 0.9 ml of guanidine thiocyanate buffer, and ethanol precipitated. This pellet was resuspended in 100 μl of NET buffer (0.3 M sodium acetate, 5 mM EDTA, 50 mM Tris-HCl [pH 7.5]) and chromatographed on a G-50 NICK column (Pharmacia). The radioactive fractions corresponding to the incorporated label were precipitated with 2.5 volumes of ethanol. The RNA pellets were resuspended in 3.0 ml of hybridization solution (50% deionized formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50 mM sodium phosphate [pH 6.5], 50 μg of heparin per ml, 0.5% sodium dodecyl sulfate), and an aliquot from each sample was counted in a scintillation counter (Beckman). An equal number of incorporated counts of radiolabeled RNA from each sample was hybridized at 42°C for 48 h to unlabeled DNA probes (1.5 μg) corresponding either to the VSV M gene or to a 551-bp fragment of the CAT gene. The DNA probes had been fixed to a nylon membrane filter as described previously (2) and then prehybridized overnight in hybridization solution prior to incubation with the labeled RNA.

RESULTS

Effect of M protein on expression of a target gene. Previously, the M protein of VSV was reported to be difficult to detect in eukaryotic cells by using recombinant vectors encoding the M gene (6, 20). One possible explanation for this difficulty might be that very low levels of M protein are capable of inhibiting host gene expression. Thus, M protein might shut off its own expression, thereby making it difficult to detect. This hypothesis suggested a strategy to test the effect of M protein on host gene expression by using a cotransfection assay. In this assay, an M protein expression vector, pSV2.M(+), which contains the M gene under control of the SV40 early promoter (30), was cotransfected with the reporter gene, CAT, also under control of the SV40 early promoter (13) (Fig. 1). Both genes were placed under control of the same promoter so that if M protein were to inhibit its own expression, then it would have a similar effect on the production of CAT. A plasmid, pSV2.M(−), containing the M gene in the noncoding orientation under control of the SV40 early promoter was used as a negative control.

To test the effect of M protein on CAT gene expression, BHK cells were cotransfected with pSV2.M(+), hereafter called the M(+) plasmid, and a constant amount of pSV2.CAT (CAT plasmid) at ratios of 20:1, 10:1, 5:1, 3:1, and 1:1 (Fig. 2A). The total amount of plasmid DNA transfected in each sample was held constant by adding the appropriate amount of pSV2.Neo, the parent plasmid of the M(+) plasmid. Cells were harvested 48 h posttransfection, and CAT activity was assayed by measuring the conversion of radiolabeled chloramphenicol to its acetylated forms. Expression of M protein inhibited CAT activity in a gene dosage-dependent fashion (lanes 3 to 7), with maximum inhibition present when the M(+) plasmid was cotransfected

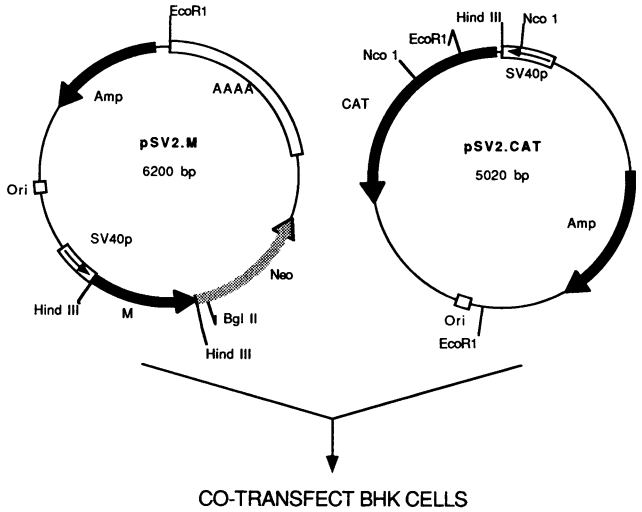

FIG. 1. Expression vectors used in the cotransfection assays. In plasmid pSV2.M, the complete coding region of the VSV M gene (27) was cloned into the *Hin*dIII site of plasmid pSV2.Neo (30), placing the M gene under the control of the SV40 early promoter. AAAA in plasmid pSV2.M denotes the SV40 polyadenylation signal present in pSV2.Neo. Two different M plasmid constructs were used in the cotransfection assays: pSV2.M(+), which contains the M gene in the protein-encoding orientation, and the control plasmid pSV2.M(−), which contains the M gene in the opposite orientation. Plasmid pSV2.CAT (13) contains the target gene, CAT, also under control of the SV40 early promoter. BHK cells were cotransfected with the two plasmids to assay the effect of M protein expression on the expression of CAT.

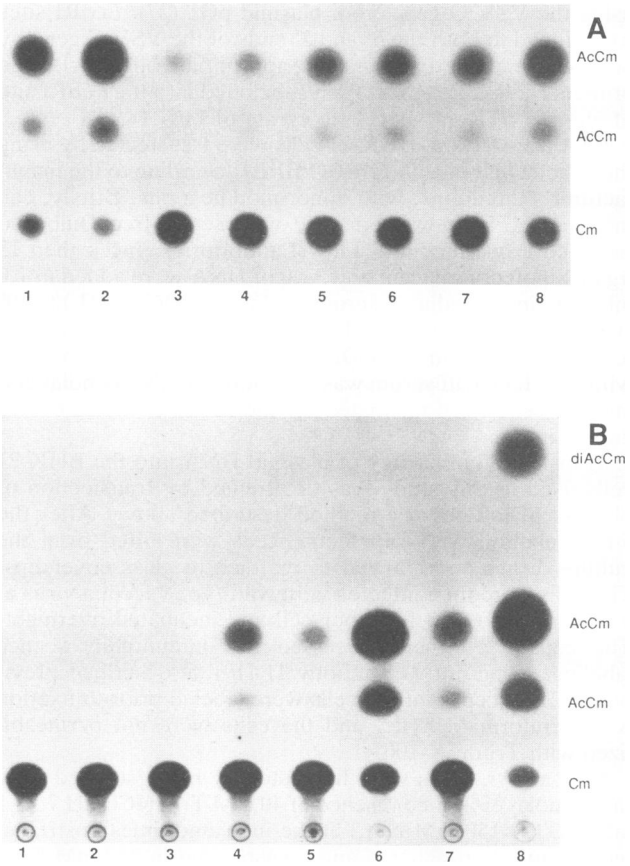

FIG. 2. Analysis of the effect of M protein on CAT gene expression. (A) Titration of M(+) plasmid to a constant amount of CAT plasmid. BHK-21 cells were cotransfected with M(+) plasmid to CAT plasmid at various ratios and harvested 48 h posttransfection. The total amount of plasmid transfected was held constant by using pSV2.Neo to bring the total to 3 μg of DNA per 10^6 cells. Lanes: 1, Neo-CAT, 20:1; 2, M(−)-CAT, 20:1; 3, M(+)-CAT, 20:1; 4, M(+)-CAT, 10:1; 5, M(+)-CAT, 5:1; 6, M(+)-CAT, 2:1; 7, M(+)-CAT, 1:1; 8, 1 U of purified CAT enzyme. (B) Time course of inhibition of CAT activity by M protein. BHK cells were cotransfected at a 20:1 ratio with either M(+)-CAT (lanes 1, 3, 5, and 7) or M(−)-CAT (lanes 2, 4, 6, and 8) and harvested at 6 h (lanes 1 and 2), 12 h (lanes 3 and 4), 24 h (lanes 5 and 6), and 48 h (lanes 7 and 8) posttransfection. CAT activity of cell extracts was determined by the conversion of [^{14}C]chloramphenicol (Cm) to acetylated forms (AcCm and diAcCm) and analyzed by thin-layer chromatography, autoradiographs of which are shown.

at a 20:1 ratio to the CAT plasmid (lane 3). Radiometric scanning of the plate revealed that M protein caused a 10-fold decrease in the percent conversion of chloramphenicol when equivalent amounts of the M(+) plasmid and the M(−) control plasmid, pSV2.M(−), were cotransfected with the CAT plasmid at a 20:1 ratio (lanes 2 and 3).

Figure 2B shows the time course of M protein-induced inhibition of CAT gene expression. In this experiment, cells were cotransfected at a 20:1 ratio with either M(+) plasmid-CAT plasmid or M(−) control plasmid-CAT plasmid. The cells were then harvested at 6, 12, 24, and 48 h posttransfection, and CAT activity was assayed. Inhibition of CAT gene expression by the M(+) plasmid was clearly present by 12 h posttransfection (lanes 3 and 4) as well as at 24 h (lanes 5 and 6) and 48 h (lanes 7 and 8) posttransfection. Longer exposures showed that inhibition of CAT activity by M protein was present by 6 h as well (lanes 1 and 2). These results demonstrate that M protein inhibited CAT gene expression at times early after transfection and that this inhibition was maintained for at least 48 h.

The plasmids in the transfected cells were analyzed in Southern blots to determine whether the differences in CAT expression in Fig. 2 might be due to differences in transfection efficiency. In this experiment, 2×10^7 BHK cells were cotransfected at a 20:1 ratio with either M(+) plasmid-CAT plasmid or M(−) control plasmid-CAT plasmid. The attached cells were harvested 48 h posttransfection, and total cellular DNA was isolated. The isolated DNA was digested with *Eco*RI, which produces a 6.2-kb linear DNA fragment from the M plasmids and cleaves pSV2.CAT into two fragments of 2.9 and 2.1 kb. The DNA was subjected to Southern analysis and was then probed with a ^{32}P-labeled DNA probe specific for M plasmid sequences (Fig. 3). Lanes 1 and 2 show that equal amounts of the 6.2-kb M plasmid DNA are present in cells cotransfected with both M(+) and M(−) plasmids, indicating that both cell populations are uniformly transfected. The control lanes 3 to 7 contained total cellular DNA from untransfected cells. Lanes 4 to 7 had exogenous plasmid DNA added to the *Eco*RI digestion reaction mixture to demonstrate that this technique is quantitative for the DNA range involved. The probe used also weakly hybridized to vector sequences of the 2.9-kb fragment from the CAT plasmid, and longer exposures showed that the two cell populations also contained similar amounts of the CAT plasmid. Lanes 1 and 2 also show the presence of degraded intracellular M plasmid DNA, indicated by a smear that occurs only below the full-length plasmid. This degra-

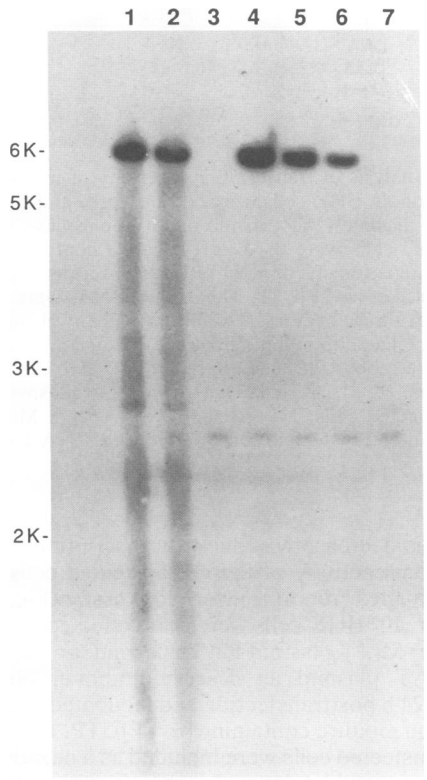

FIG. 3. Southern blot analysis of transfected plasmids. Total cellular DNA from cotransfected cells was isolated, *Eco*RI digested, and analyzed by Southern blot. Southern blots were probed for M plasmid sequences. DNA was obtained at 48 h posttransfection from cells cotransfected at a 20:1 ratio with either M(+) plasmid-CAT plasmid (lane 1) or M(−) plasmid-CAT plasmid (lane 2) or from untransfected cells (lanes 3 to 7). Lanes 4 to 7 contain DNA from untransfected cells mixed with exogenous plasmid DNA prior to the *Eco*RI digestion reaction. Lane 4, 1,600 M(+) plasmid copies per cell added; lane 5, 500 M(+) plasmid copies per cell added; lane 6, 160 M(+) plasmid copies per cell added; lane 7, 200 CAT plasmid copies per cell added. Molecular sizes in kilobase pairs (K) are indicated at the left.

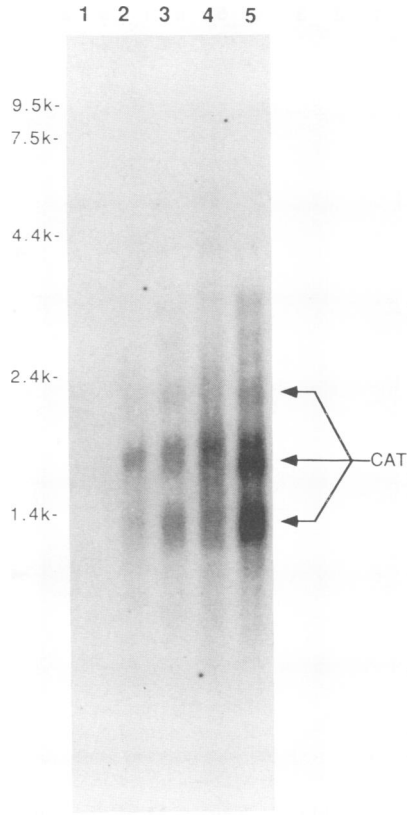

FIG. 4. Northern blot analysis of CAT plasmid-encoded mRNA. BHK cells were cotransfected at a 20:1 ratio with either M(+) plasmid-CAT plasmid (lanes 2 and 4) or M(−) plasmid-CAT plasmid (lanes 3 and 5); polyadenylated mRNA was isolated at 12 h (lanes 2 and 3) and 24 h (lanes 4 and 5) posttransfection and analyzed by Northern blot probed with a CAT message-specific probe. Lane 1 is a control containing untransfected cell mRNA. Molecular sizes in kilobases (K) are indicated at the left.

dation occurs intracellularly during the 48 h between transfection and harvesting of the cells, since it is not present in control lanes (3 to 7). No significant difference in the amount of full-length or degraded M(+) versus M(−) plasmid DNA was detected by radiometric scanning of the blot.

We examined the possibility that the transfected plasmid DNA which was analyzed in the Southern blots may have been adsorbed to the surface of the cells rather than internalized by the cells. Transfected cells were treated with DNase I just before they were harvested as described above. DNase I treatment of the outside of the cells had no effect on the results of the Southern analysis, indicating that all of the plasmid DNA being measured in the Southern blot was intracellular (data not shown). The results of the Southern analysis show that the reduced level of CAT expression in cells cotransfected with the M(+) plasmid is not due to detachment of cells that express M protein from the culture dish, which has been previously proposed to explain the failure to detect M protein in transfected cells (6). These data also indicate that the results in Fig. 2 are not due to differences in transfection efficiency between cells cotransfected with the M(+) versus the M(−) plasmids.

Effect of M protein expression on the level of vector-encoded mRNA. The level of plasmid-encoded mRNAs was analyzed by Northern analysis to determine whether the inhibition of CAT expression by M protein was due to a reduction in mRNA levels. In this experiment, 2×10^7 BHK cells were cotransfected at a 20:1 ratio with either M(+) plasmid-CAT plasmid or M(−) control plasmid-CAT plasmid. Polyadenylated mRNA was then isolated at 12 and 24 h posttransfection. The isolated mRNA was electrophoresed in an agarose gel, transferred to a nylon membrane filter, and hybridized with a ^{32}P-labeled DNA probe specific for CAT mRNA (Fig. 4). The data in Fig. 4 show that by 12 h posttransfection, the level of CAT mRNA was lower in cells cotransfected with the M(+) plasmid than in cells cotransfected with the M(−) plasmid (lanes 2 and 3) and that the difference in CAT mRNA level between cells cotransfected with M(+) versus M(−) plasmids increased by 24 h posttransfection (lanes 4 and 5).

Figure 5 shows a similar Northern analysis of M mRNA isolated at 12, 24, and 48 h posttransfection detected with an M mRNA-specific probe. The level of M message was lower in cells cotransfected with the M(+) plasmid than in cells cotransfected with the M(−) plasmid at all time points. These data show that by 24 h posttransfection, the level of M mRNA was dramatically lower in cells cotransfected with

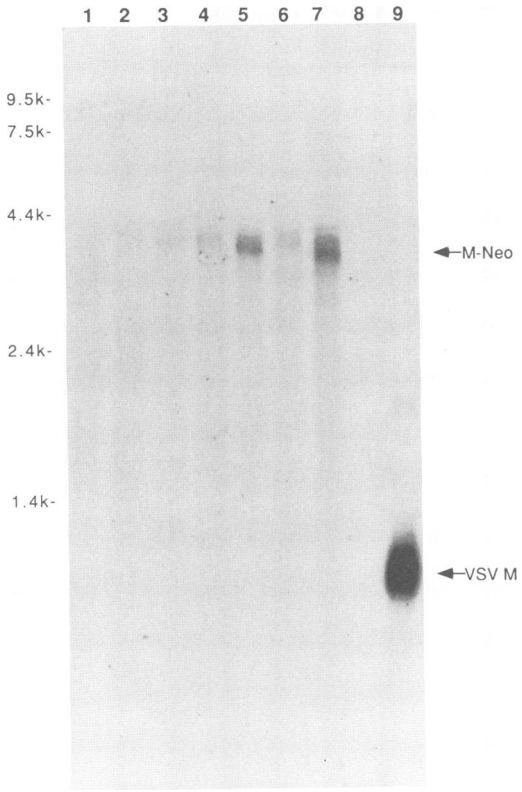

FIG. 5. Northern blot analysis of M plasmid-encoded mRNA. BHK cells were cotransfected at a 20:1 ratio with either M(+) plasmid-CAT plasmid (lanes 2, 4, and 6) or M(−) plasmid-CAT plasmid (lanes 3, 5, and 7); polyadenylated mRNA was isolated at 12 h (lanes 2 and 3), 24 h (lanes 4 and 5), and 48 h (lanes 6 and 7) posttransfection and analyzed by Northern blot probed for the VSV M message. Lane 1, untransfected cell mRNA; lane 8, blank; lane 9, untransfected cell mRNA mixed with 1% mRNA from VSV-infected BHK cells at 4 h postinfection. Molecular sizes in kilobases (K) are indicated at the left.

the M(+) plasmid than in cells cotransfected with the M(−) plasmid (lanes 4 and 5) and that the difference in mRNA level between cells cotransfected with M(+) versus M(−) plasmids was still present at 48 h posttransfection (lanes 6 and 7). Longer exposures also indicated a difference in the level of mRNA by 12 h (lanes 2 and 3), but the data suggest that this difference did not approach its maximum until 24 h. Lane 9 shows a control in which mRNA from VSV-infected cells was mixed with mRNA from uninfected cells at a 1:100 ratio and thus represents 1% of the level of expression of M mRNA in VSV-infected cells. The viral M transcript is smaller than the M message from the transfected cells due to vector SV40 and neomycin (Neo) sequences contained in the plasmid-encoded M mRNA. These data indicate that the reduction in CAT activity seen in Fig. 2 results, at least in part, from an M protein-induced reduction in CAT message level. Furthermore, the data in Fig. 5 show that the original premise for these experiments was correct. The small amount of M protein expressed from the vector is sufficient to inhibit expression of its own mRNA.

Expression of M protein inhibits transcription of target genes. The observed decrease in the level of mRNA by M protein could be explained either by inhibition of transcription or by increased mRNA degradation. To determine

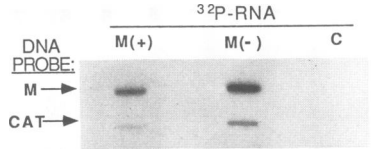

FIG. 6. Analysis of transcriptional activity of transfected genes by nuclear runoff. BHK cells were cotransfected at a 20:1 ratio with either M(+) plasmid-CAT plasmid or M(−) plasmid-CAT plasmid. Untransfected cells were used as a negative control. Nuclei were isolated 24 h posttransfection, and RNA transcripts were elongated in the presence of [α-^{32}P]UTP. The labeled RNAs were isolated and hybridized to the M gene cDNA and a fragment of the CAT gene cDNA immobilized on nylon membranes. Equal amounts of radiolabel (indicated above the lanes) were hybridized to each of the three filters. Lanes: M(+), labeled RNA from M(+) plasmid-CAT plasmid-cotransfected cells; M(−), labeled RNA from M(−) plasmid-CAT plasmid-cotransfected cells; C, labeled RNA from untransfected cells.

whether this decrease was due to transcriptional inhibition, transcriptional activity of the cotransfected cells was measured in a nuclear runoff transcription assay (Fig. 6). In this experiment, 10^8 BHK cells were cotransfected at a 20:1 ratio with either M(+) plasmid-CAT plasmid or M(−) control plasmid-CAT plasmid as described above. Nuclei were isolated at 24 h posttransfection and incubated in a transcription reaction mixture containing [α-^{32}P]UTP. Nuclei isolated from untransfected cells were included as a negative control. Only transcripts which have already initiated at the time that the nuclei are isolated are elongated in the runoff reaction. Labeled RNAs were hybridized to M and CAT DNA probes which had been immobilized on nylon membrane filters in slots and then were analyzed by autoradiography. Labeled RNA from nuclei containing the M(+) plasmid hybridized less extensively to both the M and CAT DNA probes than did RNA from nuclei containing the M(−) plasmid. These data demonstrate that expression of M protein dramatically inhibited transcription of the vector-encoded genes.

The effect of M protein on total radiolabel incorporation into RNA was examined in these nuclear runoff experiments by comparing radioactivity precipitable in 10% trichloroacetic acid. No significant difference was observed between M(+)- and M(−)-transfected cells. There were also no significant differences in in vivo incorporation of [^{3}H]uridine into total cellular polyadenylated RNA in M(+)- versus M(−)-transfected cells (data not shown) due to the fact that the efficiency of transfection in these experiments is not high enough to affect most of the cells in the culture. The transfection efficiency under the conditions of the experiments in this study ranged between 15 and 25% of cells, as determined by transfection of a plasmid encoding the VSV G protein followed by immunofluorescence staining. By increasing the DNA to cell ratio and decreasing the cell density, the efficiency of transfection could be increased to a maximum of 25 to 30% of cells in the culture. This efficiency is still too low to detect a significant change in total cellular RNA synthesis, which is dominated by the untransfected cells in the culture.

DISCUSSION

The study of viral gene products with potent inhibitory activity against cellular functions presents unusual experimental difficulties. Establishment of cell lines in which all of the cells are capable of expressing significant quantities of M

protein is hampered by powerful negative selection pressures, even with the M gene under the control of inducible promoters. Standard transient transfection experiments suffer from the fact that not all of the cells take up DNA, and therefore the cells which are not subjected to M protein-induced inhibition rapidly grow to dominate the culture. Even relatively high transfection efficiencies of 15 to 30% hamper transient assays measuring global effects of M protein. We have attempted to overcome these difficulties by using a cotransfection assay in which the target gene being studied, CAT, is expressed only in cells that also express M protein. The data obtained by using this cotransfection assay indicate that the VSV M protein is sufficient to inhibit host cell-directed transcription of plasmid-encoded target genes independent of any other viral component. The inhibition of gene expression by M protein was not specific for the SV40 promoter. The level of inhibition by M protein that occurred when the CAT gene was under control of the human immunodeficiency virus long terminal repeat was similar to the level of inhibition seen in Fig. 2 (unpublished observations).

The observed inhibitory effect of M protein on CAT gene expression (Fig. 2) is quite potent, as the data from Northern analyses demonstrate that less than 0.2% of the amount of M mRNA produced by 4 h in a VSV infection is required to cause transcriptional inhibition (Fig. 4). If the efficiency of transfection is taken into account, the results in Fig. 4 still indicate that an upper limit on the level of M mRNA expressed is less than 1% of that in VSV-infected cells. Expression of M protein in cotransfected cells was below the limit of detection in an immunoprecipitation assay that could readily detect 1% of the M protein produced during the sixth hour of VSV infection (data not shown). The low level of M protein and mRNA in transfected cells resulted from M protein-induced inhibition of its own expression and accounts for previous failures to express detectable quantities of M protein by using recombinant vectors that require host nuclear function.

The extremely low level of M protein required to mediate the observed inhibition of host cell nuclear function may explain previous studies which suggested that M protein was not involved in inhibition of host gene expression. The group III temperature-sensitive M protein mutants, *ts*G33 (6) and *ts*O23 (33), inhibit host transcription at the nonpermissive temperature. The low level of functional M protein required to inhibit gene expression could easily be generated by the temperature-sensitive mutants at the nonpermissive temperature as a result of the leakiness of the mutants.

In contrast to the group III mutants, the temperature-sensitive mutant *ts*O82 fails to inhibit host RNA synthesis at the nonpermissive temperature in chicken embryo fibroblast cells (9). The genetic lesion that leads to the temperature-sensitive phenotype of this mutant has not yet been assigned to a particular VSV gene, since it complements mutants of all five VSV complementation groups. However, this mutant was found to contain a point mutation in the M gene and does not contain one in the NS gene (9). Preliminary data from our laboratory indicate that the mutation in *ts*O82 abolishes the cytopathic effect of M protein at the nonpermissive temperature (26a).

The VSV function required to inhibit host transcription is highly resistant to inactivation by UV irradiation (14, 31). This result led to the suggestion that the VSV leader RNA was responsible for host transcription inhibition, since the leader RNA gene has the smallest target size for UV inactivation of any viral transcript. Our data support the idea that even a very low level of M protein produced by UV-inactivated virus may be sufficient to inhibit host cell-directed transcription, since only very low levels of M protein are required to mediate the inhibitory effect.

Other data have shown that VSV leader RNA can inhibit host transcription in in vitro transcription assays (15, 16, 25). However, leader RNA alone is not capable of inhibition of host transcription in vivo. VSV infection in the presence of cycloheximide does not inhibit host transcription despite the accumulation of substantial amounts of leader RNA (26), suggesting that either viral or host protein synthesis is necessary for the inhibition of host RNA synthesis. Furthermore, inhibition of host transcription by the T1026R1 mutant of VSV is delayed compared with that of wild-type VSV despite normal rates of leader RNA accumulation (10), suggesting that other viral factors may be involved. While our data demonstrate that M protein alone is capable of inhibiting transcription in transfected cells in vivo, it is quite possible that during a VSV infection, both leader RNA and M protein may be involved in the inhibition of host-directed transcription. Such a cooperative effect on host nuclear function would be consistent with the observation that M protein (21) and leader RNA (18) are the only VSV components found in substantial quantities in nuclei of infected cells. Further studies are required to determine the nature of the interaction of M protein and leader RNA with nuclear components and to investigate what role, if any, these nuclear interactions play in the inhibition of host cell transcription.

M protein has been shown previously to play a role in the cytopathology of VSV infection by inducing the rounding of polygonal cells which is typical of VSV infection (6). It is not known whether this effect of M protein is related to the effects described here. It is possible that M protein induces a program of cytopathic effects, of which these are two manifestations. Alternatively, M protein may have multiple effects on the host cell, inhibiting gene expression at very low concentrations and causing cell rounding at higher concentrations. The latter hypothesis is supported by the observation that *ts*G33 inhibits host gene expression but does not induce cell rounding at the nonpermissive temperature (6).

Mutations in viral genes required for cytopathic effects are often involved in the establishment of persistent infections. The σ3 protein of reovirus, for example, has been implicated in viral cytopathology (29) and has also been observed to be hypermutated in persistently infected cells (1). Similar mechanisms may also apply to the many negative-strand RNA viruses known to establish persistent infections. Studies of virus isolates from persistent Sendai virus infection (28) and measles virus from cases of subacute sclerosing panencephalitis have often shown the M proteins of these viruses to be hypermutated, unstable, or even absent (3, 8). It remains to be determined whether the M proteins of these other negative-strand RNA viruses have the same effects as does the VSV M protein. It is an attractive hypothesis that reduced cytopathic effect resulting from changes in the M proteins of these viruses may be one of several factors involved in the establishment of persistence.

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