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Inducible and Conditional Inhibition of Human Immunodeficiency Virus Proviral Expression by Vesicular Stomatitis Virus Matrix Protein

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Besides its role in viral assembly, the vesicular stomatitis virus (VSV) matrix (M) protein causes cytopathic effects such as cell rounding (D. Blondel, G. G. Harmison, and M. Schubert, J. Virol. 64:1716–1725, 1990). DNA cotransfection assays demonstrated that VSV M protein was able to inhibit the transcription of a reporter gene (B. L. Black and D. S. Lyles, J. Virol. 66:4058–4064, 1992). We have confirmed these observations by using cotransfections with an infectious clone of human immunodeficiency virus type 1 (HIV-1) and found that the amino-terminal 32 amino acids of M protein which are essential for viral assembly were not required for this inhibition. For the study of the potential role of M protein in the shutoff of transcription from chromosomal DNA, we have isolated stable HeLa T4 cell lines which encode either a wild-type or a temperature-sensitive (*ts*) VSV M gene under control of the HIV-1 long terminal repeat promoter. Transcription of the M mRNA was transactivated after HIV-1 infections. A cell line which encodes the wild-type M protein was nonpermissive for either HIV-1 or HIV-2. A cell line that encodes the *ts* M gene was transfected with the infectious HIV-1 DNA or was infected with HIV-1 or HIV-2. In all cases, at 32°C, the permissive temperature for M protein, the cells were nonpermissive for HIV replication. At 40°C, the *ts* M protein was nonfunctional and both HIV-1 and HIV-2 were able to replicate at high levels. A comparison of the amounts of proviral HIV-1 DNAs and HIV-1 mRNAs at 10 and 36 h after HIV-1 infection demonstrated that proviral insertion had not been prevented by M protein and that the block in HIV-1 replication was at the level of proviral expression. The severe reduction of HIV-1 proviral transcripts demonstrates that the VSV M protein alone can inhibit expression from chromosomal DNA. These results strongly support the hypothesis that the VSV M protein is involved in the shutoff of host cell transcription. M protein was able to attenuate HIV-1 infections and protect the cell population from HIV-1 pathogenesis. The temperature-dependent switch from a persistent to a lytic HIV-1 infection in the presence of *ts* M protein could be useful for studies of HIV-1 replication and pathogenesis.

The matrix (M) protein of vesicular stomatitis virus (VSV) plays a central role in viral assembly (for reviews, see references 12 and 41). As a multivalent protein, M protein interacts with several viral and cellular components, including the genomic ribonucleocapsid, cellular membranes, and tubulin (8, 10, 25, 26, 28–32, 42). Earlier attempts to select a cell line that expresses a temperature-sensitive (*ts*) VSV M protein under control of a eukaryotic promoter (6a) were not successful. Li et al. (18) were unable to select a recombinant vaccinia virus that expresses a wild-type (wt) VSV M protein. This indicated that M protein may be toxic to the cell and/or to vaccinia virus. Transient expression of M protein from plasmid DNA by T7 RNA polymerase encoded by a recombinant vaccinia virus, however, was efficient and allowed to complement M protein mutants of VSV (18, 19). A recombinant vaccinia virus that expresses M protein of the closely related rabies virus has been isolated (14). VSV M protein was expressed at high levels by a recombinant baculovirus (17), suggesting that the viral source of the M protein as well as the host cell and the expression vector may influence its pathogenicity.

We have previously shown that the expression of the VSV M protein alone, in the absence of the other viral proteins, causes cytopathic effects, as evidenced by the rounding of polygonal host cells (6). Cell rounding is a result of a disorganization of the cytoskeleton which often occurs within a few hours after VSV infections (38). Initially, we did not detect M protein expression from a simian virus 40 (SV40) late promoter after DNA transfection into Cos cells by using immunofluorescent staining. This differed from the high-level expression of the other four VSV proteins under the same conditions (6, 15, 34, 36, 40). One explanation was that M protein may be cytotoxic and/or that it may have inhibited its own expression.

This latter interpretation was based on observations by Lyles et al. (20), who initially detected VSV M protein in nuclei of infected cells. Black and Lyles (4) subsequently found that DNA cotransfections of a chloramphenicol acetyltransferase reporter gene with excess amounts of plasmid DNA that encodes M protein resulted in an inhibition of reporter gene expression at the level of transcription initiation. These observations suggested that M protein could also be responsible for the shutoff of cellular RNA synthesis. Detailed comparative studies by Marcus et al. (24) with several VSV M protein mutants, however, seem to exclude a direct link between M protein and the shutoff of interferon gene expression. These

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studies raised the question whether the inhibitory effects of M protein as measured by DNA cotransfections could be extrapolated to transcriptions from chromosomal DNA. For these reasons, it was important to study the effect of M protein on chromosomal transcription directly. The levels of M protein and reporter gene expression from transfected DNAs could be affected by different mechanisms that may not relate to host shutoff after VSV infection. Therefore, to study this aspect without having to resort to DNA transfections, it was desirable to be able to induce the expression of this potentially pathogenic protein in every cell and to study its effects on chromosomal transcription.

In this report, we focus on the transcriptional transactivation of human immunodeficiency type 1 (HIV-1) as a method for the induction of M protein expression because of its high induction efficiency from low basal levels. As a target gene, we have chosen the integrated HIV-1 provirus itself. With this approach, we anticipated gaining valuable information on direct effects of M protein on chromosomal DNA transcription from the HIV provirus. In addition, if M protein is inhibitory to HIV-1 under these conditions, this approach may also allow us to evaluate the potential use of the VSV M protein as an inducible antiviral agent in the context of an antiviral gene therapy against HIV.

The presented data demonstrate that low levels of VSV M protein can inhibit expression of the integrated HIV-1 provirus DNA. This is the first demonstration that the M protein of VSV, in the absence of all other viral proteins, can inhibit gene expression from chromosomal DNA. The findings strongly support the hypothesis that VSV M protein alone is sufficient for the shutoff of cellular transcription after VSV infection. The studies also show that a stable cell line which encodes a Tat-inducible M protein can be protected by M protein from the characteristic cytopathic effects caused by either HIV-1 or HIV-2.

MATERIALS AND METHODS

Cells and viruses. Cells were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum. HIV-1 and HIV-2 stocks were prepared either after transfections of HeLa T4 cells with the infectious DNA clone pNL4-3 for HIV-1 (1) or by infection with HIV-2 CBL-20/H9 (37). Cell supernatants were harvested 5 days after transfection or infection and were used as viral stocks. Infections were carried out overnight in the presence of 20 μg of DEAE-dextran per ml. Reverse transcriptase was inhibited by 22 μM zidovudine (AZT) present at all times shortly before and after the infections. Infectious center assays for the titrations of HIV-1 and -2 were carried out by using HeLa-CD4-LTR-β-gal cells as described by Kimpton and Emerman (16). These cells and viruses as well as HeLa T4 cells (21) were obtained from the AIDS Research and Reference Reagents Program of the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Plasmid DNA constructs. Plasmid pCDM8M contained the VSV wt M gene (35) under control of a cytomegalovirus promoter. The VSV wt M gene was inserted into the pCDM8 vector by using the unique *Hin*dIII and *Pst*I sites (InVitrogen, San Diego, Calif.). The deletion mutant M gene clones pMΔ1 and pMΔ2 contain the truncated M gene under control of the SV40 late promoter as described earlier (6).

For the inducible expression of the VSV wt and *ts* M proteins in HeLa T4 cells, the insertion vectors pHywtM and pHytsM were assembled (see Fig. 2). The coding sequence of the VSV wt or *ts* M gene (820 bp), which had been cloned earlier (6) from the VSV mutant *ts*G33 (13, 33), was excised as a *Xho*I-*Bss*H2 fragment from pKOM1 or ptsM20, respectively. The DNA fragment was blunt ended and ligated into the blunt-ended *Bss*H2-*Hpa*I site of pHyDI3 to yield either pHywtM or pHytsM, respectively (13a). With this vector, the wt and *ts* M coding sequences were placed under control of the HIV-1 long terminal repeat (LTR) promoter. For the selection of cell clones, these insertion vectors also contain the hygromycin B resistance gene HmB under control of the Moloney sarcoma virus LTR (5).

DNA transfection and p24 antigen release. HeLa T4 cell monolayers (5×10^5 cells) were transfected with approximately 1 μg of the infectious HIV-1 DNA clone pNL4-3 or cotransfected with 1 μg of pCDM8M or an unrelated control DNA, using Lipofectin as specified by the manufacturer (Gibco BRL Life Technologies Inc., Gaithersburg, Md.). In cotransfections with the deletion mutant M gene clones pMΔ1 and pMΔ2, a 10- to 20-fold excess of DNA relative to pNL4-3 DNA was used. Media were replaced daily, and the concentration of viral p24 antigen in cell supernatants was determined by using an antigen capture enzyme-linked immunosorbent assay (ELISA; SRA Technologies Inc., Rockville, Md.). Syncytium formation was evaluated daily and photographed 5 days after transfection.

Selection of stable HeLa T4 cell clones that express the VSV M gene. Twenty micrograms of plasmids pHywtM and pHytsM containing the HmB gene was transfected into 10^7 HeLa T4 cells. After 48 h, the cells were trypsinized and diluted 1:4 into Dulbecco's modified Eagle medium containing 200 μg of hygromycin B (Boehringer Mannheim, Indianapolis, Ind.) per ml. Resistant clones were picked after 12 to 14 days and analyzed for M gene insertion.

Southern and Northern (RNA) blot analyses. High-molecular-weight cellular DNA was isolated as specified for the Genomic DNA Isolation System (Qiagen, Chatsworth, Calif.) and analyzed by Southern blotting. For Northern blot analyses, polyadenylated RNA was isolated by the guanidine thiocyanate method followed by oligo(dT)-cellulose chromatography. Ten micrograms of mRNA was analyzed by standard techniques (22). Hybridization probes were made by nick translating the VSV wt and *ts* M genes in the presence of [α-^{32}P]dCTP (Amersham, Arlington Heights, Ill.). Unincorporated nucleotides were removed by Strata Clean Resin (Stratagene, La Jolla, Calif.). Hybridizations were carried out for 18 h at 60°C.

A 509-bp PCR DNA product of the human glyceraldehyde phosphodehydrogenase (hGAPHD) gene was obtained by using the primers hGAPDH-5′ (ACC ACC ATG GAG AAG GCT GG) and hGAPDH-3′ (CTC AGT GTA GCC CAG GAT GC). For HIV-1 transcripts, part of the *nef* region, positions 8887 to 9200 of pNL4-3, was used as a probe. To verify that equal amounts of mRNA had been applied to each lane of the gel, the blots were washed and rehybridized with a probe specific for the hGAPDH mRNA.

RT-PCR. Total cellular RNA was isolated from infected cells as described by Chirgwin et al. (9) and subsequently treated with RNase-free DNase I (Stratagene). One microgram of RNA was transcribed by using 2.5 U of reverse transcriptase and 0.5 μg of primer as specified for the reverse transcriptase-PCR (RT-PCR) kit (Perkin Elmer Cetus, Norwalk, Conn.). The cDNA was amplified by using 30 cycles. The oligonucleotide primers for DNA amplification were M402 (GCG GTA TTG GCA GAT CAA GGT) and M713 (AGA ATC CAG GAC CCA CGC TCC AGA) to amplify a 312-bp fragment of the M gene DNA. A nick-translated, ^{32}P-labeled 820-bp complete M gene fragment was used as a probe for Southern blot hybridization of the RT-PCR product.

RESULTS

Effect of M protein on the nuclear expression of plasmid DNA. To study the potential inhibitory effect of M protein on cellular transcription, we initially confirmed the findings by Black and Lyles (4), who used DNA cotransfection assays. We expressed wt M protein under control of a cytomegalovirus promoter together with the infectious HIV-1 clone pNL4-3 (1), which served as a reporter gene. As shown in Fig. 1A, cotransfection of both DNAs into HIV-1-susceptible HeLa T4 cells led to a dramatic reduction in p24 antigen released from the cells. Viral spread was also inhibited as measured by syncytium formation. In contrast, cotransfections of HIV-1 DNA with an unrelated control DNA, pGem3Z, resulted in massive syncytium formation that correlated with high levels of p24 antigen release.

We have previously reported that M proteins with truncations of the amino-terminal 32 amino acids (pMΔ1) and the carboxyl-terminal 56 amino acids (pMΔ2) drastically differed in their levels of expression in Cos cells when expressed under control of the SV40 late promoter. In contrast to wt M protein, the carboxyl-terminal deletion protein MΔ2 allowed expression of the truncated protein whereas expression of the amino-terminal deletion protein MΔ1 remained undetected, similar to expression of the wt M protein. However, both MΔ1 and MΔ2 could be translated in vitro and could be immunoprecipitated by specific monoclonal antibodies (6). To determine whether the inability to express the amino-terminal truncated protein was the result of transcription inhibition and whether it was also effective in *trans*, both truncated M protein genes were cotransfected with the infectious HIV-1 DNA into HeLa T4 cells. As shown in Fig. 1B, HIV-1 p24 antigen release was reduced only after transfection with the amino-terminal deletion protein MΔ1. These results demonstrate that the amino-

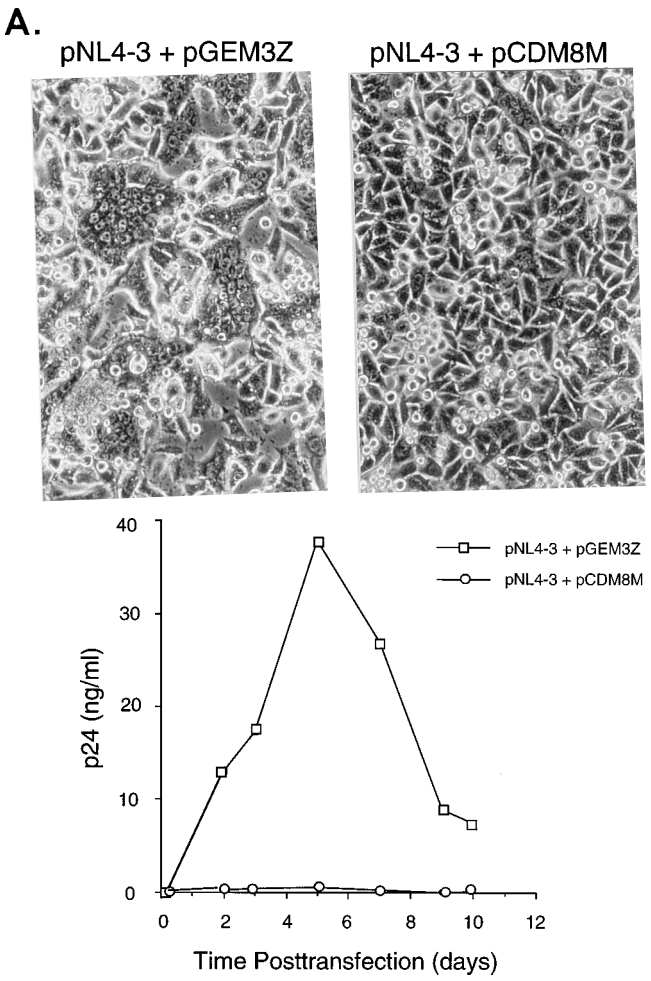

FIG. 1. (A) Inhibition of HIV-1 replication by VSV wt M protein. HeLa T4 cells were cotransfected with equal amounts of the infectious HIV-1 clone pNL4-3 and either pCDM8M DNA, which encodes the wt M protein under control of the cytomegalovirus promoter, or pGem3Z, an unrelated control DNA. Syncytium formation was examined 5 days after transfections. HIV-1 p24 antigen released into the medium was quantitated by using an antigen capture ELISA. (B) Inhibition of HIV-1 replication by a truncated M protein MΔ1. HeLa T4 cells were cotransfected with pNL4-3 DNA and a 20-fold excess of either pMΔ1 or pMΔ2 DNA, which encode MΔ1 or MΔ2 protein under control of the less efficient SV40 late promoter (6). pMΔ1 has the 32-amino terminal amino acids of M protein deleted, and pMΔ2 lacks the carboxyl-terminal 56 amino acids of the M protein, as indicated by the shaded areas.

terminal domain of M protein, which is required for membrane and nucleocapsid binding (10, 30, 43), neither participates in the inhibition of HIV-1 DNA plasmid transcription nor reduces its own expression, as we have described earlier (6).

Inhibition of HIV-1 replication in stable cell lines expressing an inducible wt or *ts* M protein. High levels of constitutive M protein expression in a cell line would most likely be toxic to cells. Basal expression levels from the HIV-1 LTR are often very low but can be greatly increased in the presence of the transactivating HIV-1 Tat protein (7, 11, 27, 39). It was uncertain, however, whether basal levels of M protein expression from the HIV-1 LTR would even be tolerated by cells and whether the cells would express the gene after transactivation by HIV-1. Resolution of this question was important to determine whether M protein can inhibit chromosomal gene expression. If M protein inhibited transcription from chromosomal DNA, it may also shut off expression of an integrated HIV-1 provirus and possibly its own expression.

We constructed two insertion vectors, pHywtM and pHytsM, which contain the wt VSV San Juan strain M gene and the *ts* VSV *ts*G33 M gene, respectively, under control of the HIV-1 LTR. In addition, they contained a hygromycin B resistance gene for selection (Fig. 2). HIV-1-susceptible HeLa T4 cells were transfected, and hygromycin B-resistant cell clones were isolated after 2 weeks. Selection of the cell clones was carried out at 37°C. This is the normal temperature for the wt M protein, and it is partially permissive for the *ts* M protein with respect to VSV replication. We selected several cell clones which all grew at rates similar to those of their parental cells. There was no visual indication for cytotoxicity such as cell rounding. Chromosomal insertion of the complete plasmid DNAs was confirmed by Southern blot hybridizations of the cell lines (Fig. 2).

The two cell clones 7 and 17, which encode the wt and *ts* M proteins, respectively, were used for the following studies together with the control HeLa T4 cell clone 5, which contained the insertion vector but without the M gene insert. The three cell clones were initially challenged by transfection with the infectious HIV-1 clone pNL4-3. After transfection, cell clones 7 and 5 were cultured at 37°C. Compared with the control cell line 5, the release of p24 antigen was drastically reduced in the culture fluid of clone 7 cells. Syncytium formation was virtually absent during several days in culture (Fig. 3).

After similar transfections of cell clones 17 and 5 (Fig. 4), the cells were incubated at temperatures both permissive (32°C) and nonpermissive (40°C) for the *ts* M protein. Cell clone 5 was highly permissive for HIV-1 growth at both 32 and 40°C, as indicated by syncytium formation and p24 antigen release. At 32°C, the cells showed reduced but still very efficient syncytium formation and viral spread. In contrast, with cell clone 17 at the temperature (32°C) permissive for M protein, there was only a very low level of HIV-1 replication compared with the control cells, and no syncytium formation was observed. At the temperature (40°C) nonpermissive for the M protein, cell clone 17 was highly permissive for HIV-1. These results clearly demonstrate a temperature-dependent inhibition of HIV-1 replication caused by the presence of the *ts* M protein.

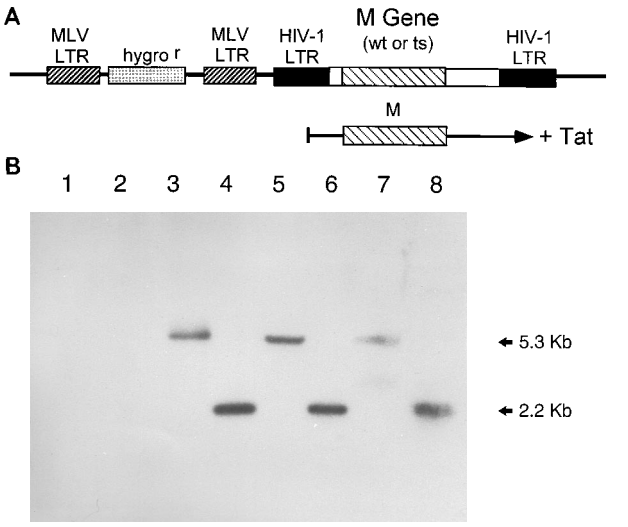

FIG. 2. (A) Map of pHywtM and pHytsM DNAs used for the selection of HeLa T4 cells expressing an inducible wt or *ts* M protein. Coding regions of both the wt and *ts* M genes were placed under control of the HIV-1 LTR promoter. (B) Southern blot analysis of the genomic DNA of selected cell clones 7 (wt M) and 17 (*ts* M). Eight micrograms of genomic DNA from each cell line was digested with *Eco*RI (lanes 3 and 7) or *Hin*dIII (lanes 4 and 8). A ^{32}P-labeled 820-bp double-stranded DNA fragment of the *ts* M gene was used for hybridization. A DNA fragment of 5.3 kb was detected after *Eco*RI digestion, and a 2.2-kb fragment was visible after *Hin*dIII digestion, corresponding to the predicted size fragments. The same amount of genomic DNA from parental HeLa T4 cells was digested with *Eco*RI (lane 1) or *Hin*dIII (lane 2). About 100 pg of pHytsM DNA digested with *Eco*RI (lane 5) or *Hin*dIII (lane 6) was used as markers.

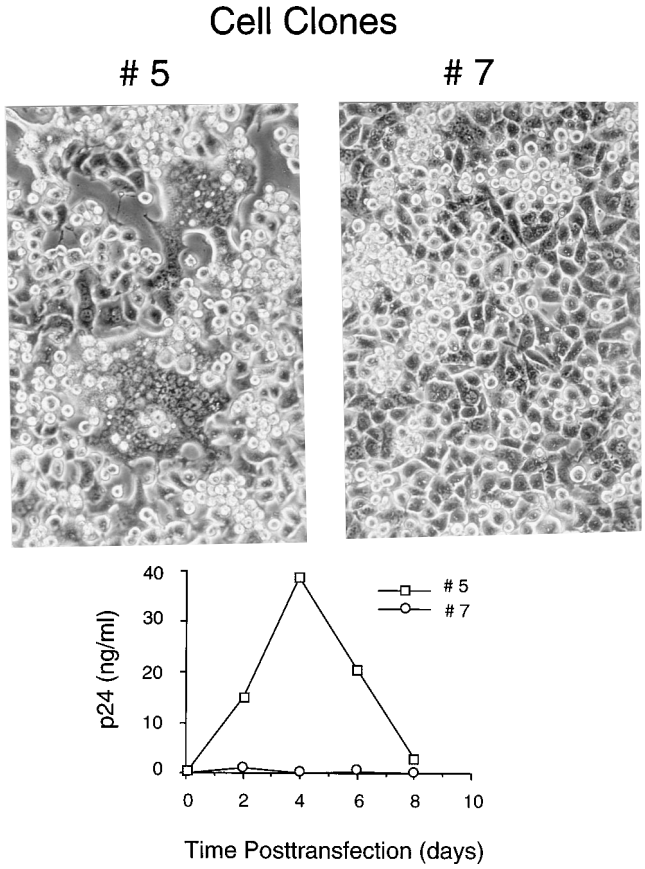

FIG. 3. Inhibition of HIV-1 replication in HeLa T4 cells encoding a wt VSV M gene under control of the HIV-1 LTR. Selected HeLa T4 cells with (clone 7) and without (clone 5) the wt M gene were transfected with the infectious HIV-1 clone pNL4-3 and incubated at 37°C. The amount of HIV-1 p24 antigen in the medium harvested at the indicated times was determined by ELISA. Syncytium formation was evaluated 5 days after transfection.

To rule out the possibility that the inhibition of HIV-1 replication and spread did not only affect expression from transfected vector DNA, we also challenged the two cell clones by infection with HIV-1 as well as HIV-2 particles. Results of infection of the *wt* M (clone 7) and *ts* M (clone 17) cell lines with either HIV-1 or HIV-2 at 32 and 40°C are shown in Fig. 5. The amounts of released p24 antigen and infectious units released from the cells are shown. The data indicate that similar to transfections with the infectious HIV-1 clone, infections by HIV-1 and HIV-2 were strongly inhibited. It is important to point out that HIV-2, although it differs greatly in its nucleotide and amino acid sequences from HIV-1, is able to transactivate expression from the HIV-1 LTR. In fact, infectious units of HIV-2 were quantitated by transactivation of the HIV-1 LTR in HeLa-CD4-LTR-β-gal cells (16).

Proviral transcription but not proviral integration is inhibited by M protein. To identify which stage in HIV-1 replication was blocked, cell clones 7 (with wt M) and 5 (without wt M) were infected with HIV-1. Infections were carried out in the presence or absence of AZT to inhibit reverse transcription and integration by the infecting virus. High-molecular-weight chromosomal DNA was isolated 10 and 36 h postinfection and analyzed for the presence of HIV-1 proviral inserts by PCRs of a 420-bp region from the HIV-1 *pol* gene. In parallel reactions, a 509-bp cellular hGAPDH gene fragment was amplified to ensure that PCR products from the same amounts of chromosomal DNAs were compared.

At 10 h after infection, no proviral integration was observed in the presence of AZT (Fig. 6A). Without AZT, cell clones 5 and 7 showed very similar amounts of integrated proviral DNA after this single round of viral infection. Integration was independent of the presence of the M gene. After 36 h, a slightly greater amount of proviral DNA was observed in cell clone 5 than in cell clone 7. This finding was consistent with the observed viral spread in clone 5. We conclude that the presence of the wt M gene in clone 7 did not inhibit proviral integration or any processes prior to integration such as viral adsorption, entry, uncoating, reverse transcription, and the translocation of the preintegration complex to the nucleus. A very low amount of a PCR product was often detected in clone 7 after 36 h even in the presence of AZT that was not seen after 10 h. This low level may possibly be the result of a partial escape of HIV from the inhibitory effects of AZT.

Northern blot hybridization of RNA transcripts from similar amounts of integrated HIV-1 provirus DNAs, however, showed a severe reduction of all HIV-1 transcripts at both 10 and 36 h after infection (Fig. 6B). The *nef*-specific probe identified three spliced and unspliced mRNAs including up to full-length HIV-1 genomic RNA (arrows). These results demonstrated that transcription of the HIV-1 provirus was very much reduced in the presence of M protein. This reduction can account for the observed inhibition of viral replication. As expected, the level of cellular hGAPDH mRNA was the same. It was not reduced because only a small number of cells were infected by HIV-1 and transactivated M gene expression.

Several hybridizations for the detection of M mRNA transcripts were carried out but were not successful, in part because of an unspecific RNA band that was present even in cell

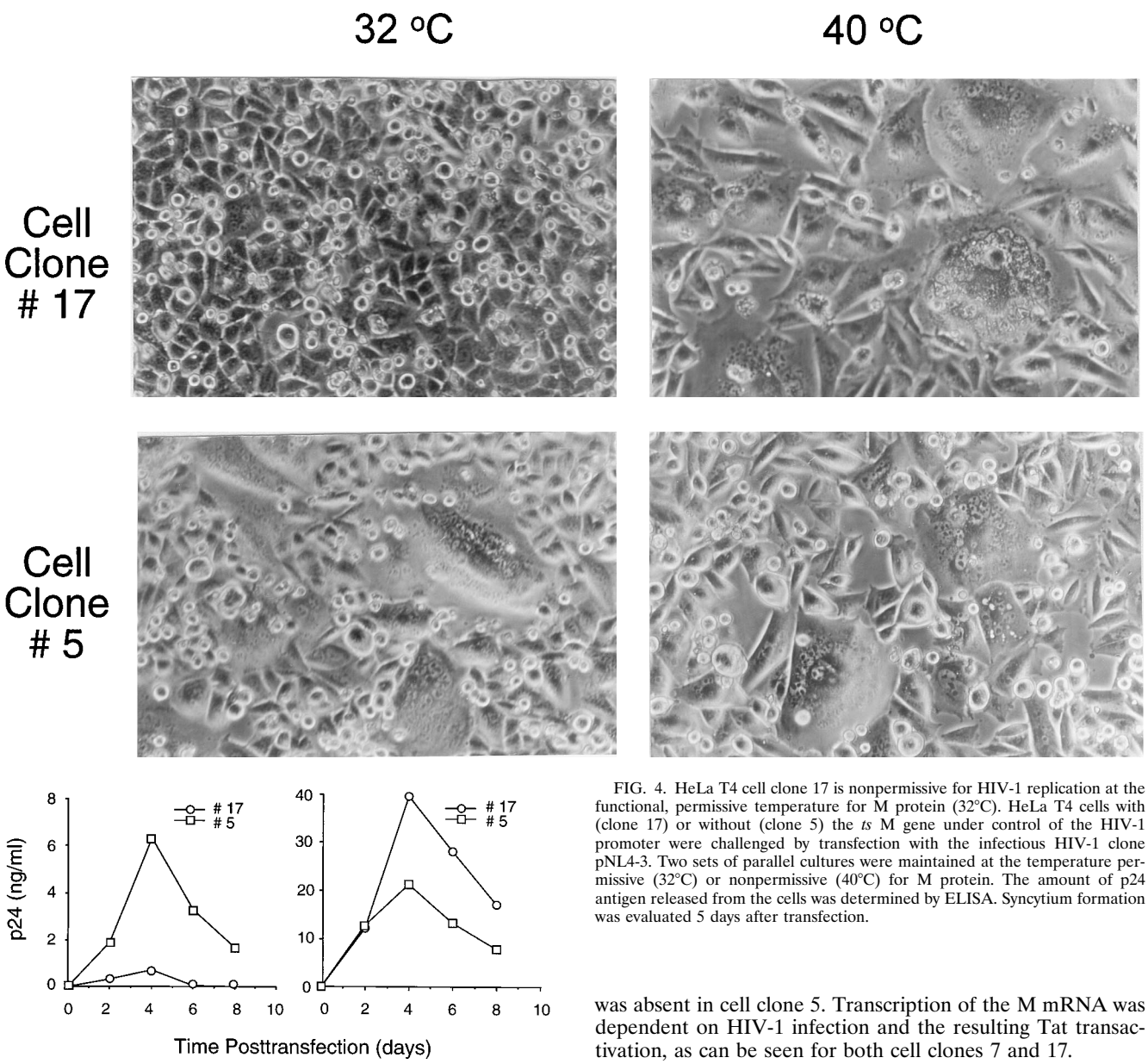

FIG. 4. HeLa T4 cell clone 17 is nonpermissive for HIV-1 replication at the functional, permissive temperature for M protein (32°C). HeLa T4 cells with (clone 17) or without (clone 5) the *ts* M gene under control of the HIV-1 promoter were challenged by transfection with the infectious HIV-1 clone pNL4-3. Two sets of parallel cultures were maintained at the temperature permissive (32°C) or nonpermissive (40°C) for M protein. The amount of p24 antigen released from the cells was determined by ELISA. Syncytium formation was evaluated 5 days after transfection.

clone 5 which masked low levels of the expected size transcript (data not shown). Western immunoblot analyses as well as immunofluorescent staining of HIV-1-infected or Tat-transfected cells did not detect M protein when we used a polyclonal antibody against M protein that normally detects M protein in VSV-infected cells very well by both methods. We also did not detect M protein even after transactivation of the *ts* M protein at the nonpermissive temperature. These conditions should favor the expression of larger amounts of M protein.

To demonstrate that M gene expression was indeed responsible for the observed antiviral effect against HIV-1, we carried out RT-PCRs of total RNA from HIV-1-infected and uninfected cells of cell clones 5, 7, and 17 (Fig. 7). Subsequent hybridization of the RT-PCR product from M mRNA with an M-gene-specific DNA clearly identified a DNA fragment of the expected size. The product was detected only in cells containing either the wt or the *ts* M protein gene. As expected, it was absent in cell clone 5. Transcription of the M mRNA was dependent on HIV-1 infection and the resulting Tat transactivation, as can be seen for both cell clones 7 and 17.

With clone 17, mRNA encoding the *ts* M protein was most abundant in HIV-1-infected cells at the nonpermissive temperature. These were conditions which could potentially have resulted in a more efficient M protein staining. But as indicated above, M protein was not detected. M mRNA was not detected at 32°C or was detected only at very low levels even after HIV-1 infection. This may be the combined result of a lower transactivation and expression efficiency at 32°C, which, however, still resulted in a very effective inhibition of HIV-1 replication compared with cell clone 5. We conclude that very low levels of VSV M protein can inhibit HIV-1 replication by blocking the transcription of the integrated HIV-1 provirus.

Temperature-controlled switch from a persistent to a lytic HIV-1 infection. Cell clone 17, infected with HIV-1 at 32°C, the functional temperature for M protein, did not show any visible evidence of HIV-1 infection. At days 3, 6, 9, and 12 after infection, parallel cell cultures were shifted to 40°C, the nonpermissive temperature for M protein. Every time the cells were shifted to 40°C, the cells produced high levels of HIV-1 within 1 or 2 days followed by massive syncytium formation

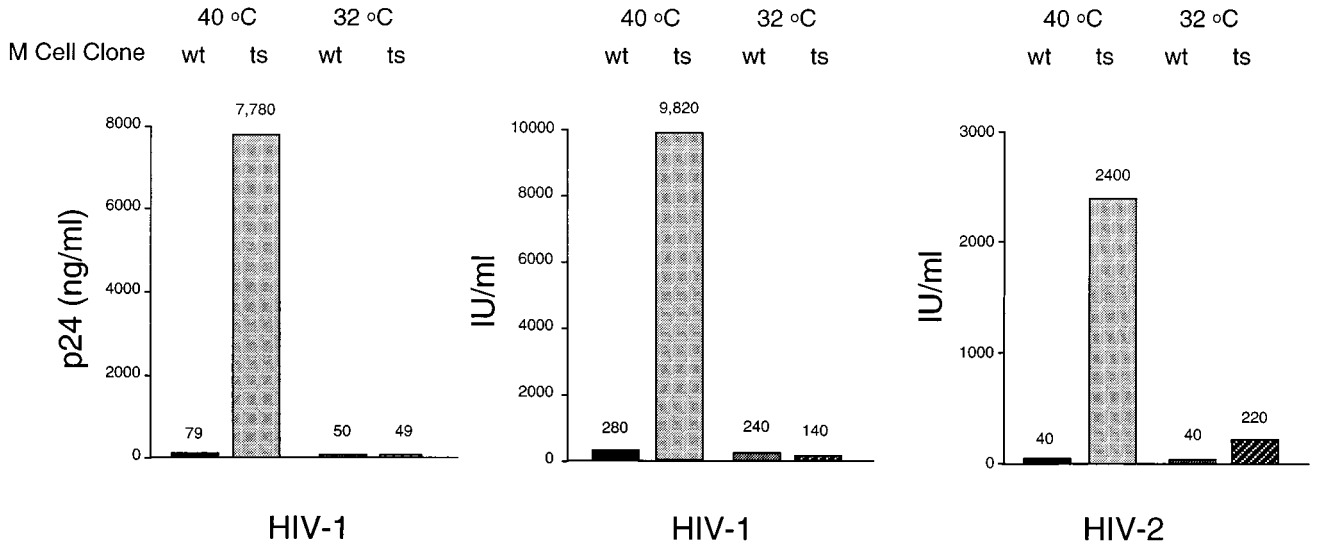

FIG. 5. Inhibition of HIV-1 and HIV-2 replication after infection of cell clones 7 (wt M) and 17 (*ts* M). Both cell lines were infected with either HIV-1 or HIV-2 and incubated at either 32 or 40°C as indicated. The amount of p24 antigen released into the medium during 5 days in culture was determined by ELISA for HIV-1. Infectious units were determined by an infectious center assay using HeLa-CD4-LTR-β-gal cells for HIV-1 and HIV-2 (16).

similar to those shown in Fig. 3 and 4. These observations suggest that the initial HIV-1 infection of cell clone 17 at 32°C is maintained as a persistent infection with very low levels of p24 released. This infection can quickly be activated to become a very productive and lytic infection by simply shifting the temperature to 40°C, which inactivates VSV M protein. We did not observe a delay in the spread of HIV-1 that increased with later times of the temperature shift. This indicated that at least during the 12 days of the experiment, there was no major decrease in the number of HIV-1-infected cells within the cell population.

DISCUSSION

In this study, we have addressed two points: the role of the VSV M protein in viral pathogenesis and the potential use of M protein as an antiviral protein against HIV.

Role of M protein in viral pathogenesis. The observation that M protein enters the nuclei of infected cells (20) did not initially appear of obvious relevance for viral replication itself, since VSV is able to replicate in anucleated cells. Cotransfections of vector DNAs encoding the M gene with a reporter gene demonstrated that M protein was able to inhibit transcription initiation from a chloramphenicol acetyltransferase reporter gene plasmid (4). This important finding had obvious implications for the potential role of M protein in the inhibition of cellular transcription. It could provide a rationale for the presence of M protein in the nucleus. The experiments, however, did not directly address the shutoff of chromosomal DNA transcription. To study this aspect, it was necessary to express M protein as well as a chromosomal reporter gene without having to rely on DNA cotransfections.

For this reason, we have selected cell lines which encode inducible wt and *ts* M genes that allowed us to study the effect of M protein on chromosomal expression. We focused on the expression of the HIV-1 provirus, which acted as a chromosomal reporter gene. The isolation of high-molecular-weight chromosomal DNA only 10 h after infection ruled out reinfections and ensured that the HIV-1 transcripts were transcribed from the same number of proviral genomes. Proviral expression was compared in the presence or absence of a Tat-inducible M gene. The results demonstrated that the expression of spliced and unspliced mRNAs from the HIV-1 provirus was drastically reduced in cells encoding the wt M protein without affecting any of the prior steps from viral entry to proviral insertion (Fig. 6). In the presence of functional M protein, a very low level of HIV-1 expression was still detected and was probably necessary for the transactivation of the provirus as well as M gene expression (Fig. 7). In the absence of HIV-1 infection, there was no detectable expression of wt M. With the *ts* M protein-encoding cell line 17, M gene expression was tolerated at low levels even without HIV-1 transactivation.

Several attempts to demonstrate M protein expression by either immunofluorescent staining or Western blotting did not detect M protein, suggesting that very low levels of M protein expression were sufficient to inhibit proviral expression. The observed *ts* phenotype of the *ts* M protein itself and its resulting inhibitory effect on HIV allowed us to rule out any temperature-sensitive antiviral mechanisms by the cell itself which could have affected HIV-1 proviral expression. This temperature-dependent HIV inhibition together with the inducible M mRNA expression strongly suggests that M protein itself is indeed the inhibitor. These results are consistent with earlier studies on the expression of the *ts* and wt M proteins in Cos cells when the SV40 late promoter was used instead or the HIV-1 LTR (6). The coding regions of these DNA clones were used in the study described here to evaluate the potential roles of both wt and *ts* M proteins in cytopathogenesis.

Our data, therefore, strongly support the hypothesis that the VSV M protein is able to inhibit chromosomal expression as measured by the expression of the integrated HIV-1 provirus. Proviral DNA as part of the chromosomal structure is transcribed by RNA polymerase II directed by nuclear transcription factors. The precise mechanism of inhibition by M protein is unknown. It may involve interactions of M protein with one of these components. DNA cotransfection assays demonstrated that the transcription from three different viral promoters can be affected by M protein. This finding suggests that shared factors or mechanisms, which could include the RNA polymerase core proteins themselves, may be affected. It is still

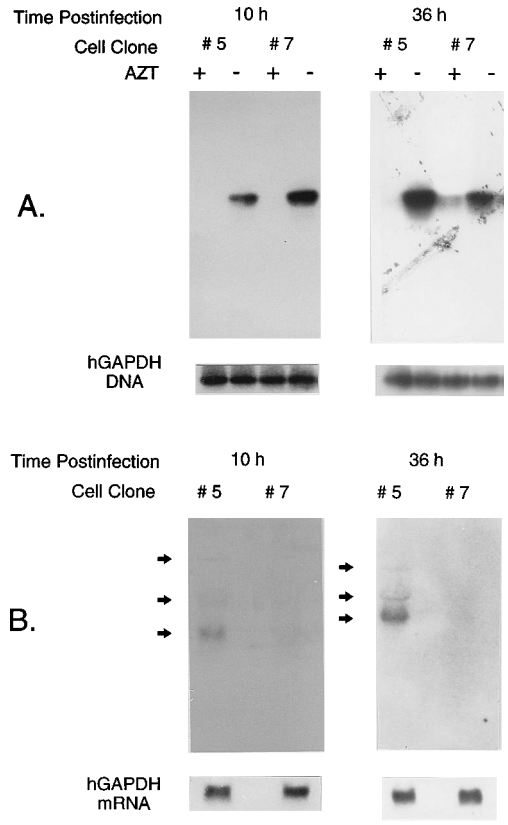

FIG. 6. HIV-1 provirus insertion and expression in cell clones 7 and 5. (A) HIV-1 proviral insertion after infection of cell clone 7 encoding a VSV wt M gene. Cell clones 7 with and 5 without the M gene were infected with HIV-1 in the presence or absence of 22 μM AZT. In parallel cultures and at 10 and 36 h after infection, high-molecular-weight chromosomal DNA was isolated, and the amounts of inserted proviral HIV-1 DNA were compared by semiquantitative PCR of a specific 420-bp fragment of the HIV-1 *pol* region. The PCR products were detected by hybridization using a *pol*-specific probe. A 509-bp PCR product of the cellular hGAPDH gene was used as a control for the amounts of genomic DNA used in the reactions. (B) HIV-1 proviral expression in cell clones 7 and 5. Parallel flasks of the cultures shown in panel A were used to prepare poly(A)$^+$ RNA for Northern blot hybridizations of all HIV-1 mRNAs. A DNA probe of the *nef* region was used as a hybridization probe. The same RNA blot was rehybridized for the detection of hGAPDH mRNA. The arrows mark the three expected and detectable HIV-1 transcripts. The slowest transcript is genome-size RNA.

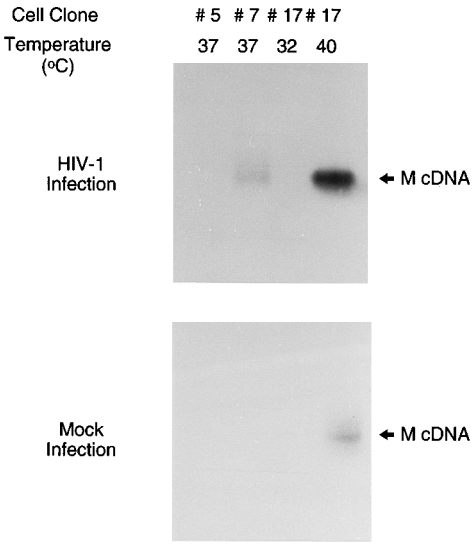

FIG. 7. Transactivation of wt and *ts* M gene expression. Total mRNA of cell clones 5, 7, and 17, cultured at the indicated temperatures, was purified before and 36 h after HIV-1 infection. The RNA was reverse transcribed by using an oligo(dT) primer. The reverse transcript was amplified by PCR as described in Materials and Methods. The 312-bp PCR products had the expected size and were detected by hybridization with a nick-translated probe of the complete VSV M gene.

uncertain, however, whether the expression of all cellular genes is affected to the same extent (see below).

The VSV matrix protein has several structural domains which have been partially characterized, such as the amino-terminal domain that interacts with the nucleocapsid as well as with cellular membranes (30, 43). By deleting the amino-terminal 32 amino acids of M protein, part of these domains were eliminated. Unlike with the deletion of the 56 carboxyl-terminal amino acids, expression of this truncated protein was still able to inhibit HIV-1 replication in cotransfection assays (Fig. 1B). The truncated protein, however, was not detected by immunofluorescence, similar to wt M protein (6), suggesting that the remainder of the molecule was still inhibitory. These observations are also consistent with the recent expression of M protein deletion mutants by Ye et al. (43) and by Black et al. (3), who demonstrated that amino acid positions 4 to 21 of M protein are not required for the inhibition of host-directed transcription and the nuclear localization of the truncated protein. Our earlier observations that the *ts* M protein could be expressed in Cos cells only at the nonpermissive temperature together with the reversibility of *ts* M protein expression in these cells are also consistent with the temperature-dependent HIV inhibition in cell line 17 (Fig. 4 and 5). These findings strongly suggest that M protein also inhibits its own expression.

Marcus et al. (24) sequenced several VSV M gene mutants and their revertants as well as different VSV strains and correlated their sequences with the ability of these viruses to induce and/or suppress interferon expression. The authors concluded that M protein as well as leader RNA do not regulate interferon induction. One of the mutants which was compared by Marcus et al. was *ts*G33. Its M gene was inserted and expressed in cell line 17 used in this study. At the permissive temperature, this *ts* M protein inhibited HIV replication in the cell line as well as its own expression. It was shown earlier that M protein does not affect cellular translation (2). If M protein is involved in the suppression of interferon induction, it would most likely function on the transcriptional level. The fact that a single VSV particle can both induce and suppress interferon production (23) makes it more difficult to assign specific viral gene products for this function. *ts*G33 is a good inducer of interferon, and there was no indication for a suppression of interferon gene expression by this mutant (24). These results could indicate that M protein may not indiscriminately inhibit chromosomal transcription. In fact, it may not affect interferon expression at all.

Until now, the expression from only a few viral promoters such as those of SV40, cytomegalovirus, and HIV have been shown to be inhibited by M protein. A study of the expression of other cellular promoters in the presence of M protein will be needed to distinguish whether M protein affects the main core functions of RNA polymerase II or whether it is selective, for example, through specific transcription factors. For the interferon induction-suppression phenotypes, whether an increased level of interferon induction can overcome transcription inhibition by M protein must also be evaluated. Alternatively, during a VSV infection, interferon induction may, depending

on the mutant used, occur prior to a shutoff of cellular transcription, which would require both an accumulation of M protein and a translocation to the nucleus. Therefore, with the interferon induction-suppression phenotype, the timing and efficiency of interferon induction could be very different for each virus strain and may be affected by other viral gene products in addition to M protein.

VSV M protein as an antiviral against HIV? M protein was able to protect not only the HIV-infected cell but indirectly also the entire cell population. There was no evidence for toxic effects of M protein at basal, uninduced levels of expression even after HIV-1 as well as HIV-2 infections. This indicates that M protein levels which were sufficient for HIV-1 inhibition may be tolerated by the cell. This obviously represents a paradox. It is unknown at this time for how long the cell can tolerate the presence of low levels of functional M protein. Clearly, low levels did not cause a rapid clearance of HIV-1-infected cells from the population.

The highly effective inhibition of HIV suggests that M protein could potentially be used as an HIV-induced antiviral agent against HIV itself. Such a strategy may be used in the context of an antiviral gene therapy. There are obvious limitations with such an approach which are not limited to the delivery of the gene in the first place. For example, an expected immune response against cells expressing low levels of M protein may clear these cells from the patient.

Interestingly, HIV-1 infection of the M gene-containing cell lines 7 and 17 resulted in an attenuation of HIV-1 replication and the establishment of what appears to be a persistent infection with low levels of viral p24 antigen and infectious particle release without evidence of any visible cytopathic effects. Temperature shifts of the HIV-1-infected cell line 17 to 40°C revealed that HIV-1 was continuously present at any time during the 12 days in culture. There was no evidence for a clearance of HIV-1-infected cells during this time at 32°C. These results suggest that a balance between the cytotoxicity of M protein and a low level of M protein expression was established at the permissive temperature that allowed a persistent state of the infection to be maintained. This balance could be altered by a simple change in temperature to inactivate the *ts* M protein, which in turn allowed HIV-1 expression and replication to occur.

In summary, while the potential use of the M gene as an HIV antiviral agent raises several questions, the use of these cell lines or other cell lines which encode inducible M protein genes could be useful not only in the continued detailed study of M protein function but also in experiments that may benefit from a temperature-controlled induction of HIV-1 replication and pathogenesis.

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REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Black, B. L., G. Brewer, and D. S. Lyles.** 1994. Effect of vesicular stomatitis virus matrix protein on host-directed translation in vivo. J. Virol. **68:**555–560.
3. **Black, B. L., R. B. Rhodes, M. McKenzie, and D. S. Lyles.** 1993. The role of vesicular stomatitis virus matrix protein in inhibition of host-directed gene expression is genetically separable from its function in virus assembly. J. Virol. **67:**4814–4821.
4. **Black, B. L., and D. S. Lyles.** 1992. Vesicular stomatitis virus matrix protein inhibits host cell-directed transcription of target genes in vivo. J. Virol. **66:**4058–4064.
5. **Blochlinger, K., and H. Diggelmann.** 1984. Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotic cells. Mol. Cell. Biol. **4:**2929–2931.
6. **Blondel, D., G. G. Harmison, and M. Schubert.** 1990. Role of matrix protein in cytopathogenesis of vesicular stomatitis virus. J. Virol. **64:**1716–1725.

6a. **Blondel, D., and M. Schubert.** Unpublished data.

7. **Buchschacher, G. L., and A. T. Panganiban.** 1992. Human immunodeficiency virus vectors for inducible expression of foreign genes. J. Virol. **66:**2731–2739.
8. **Carroll, A. R., and R. R. Wagner.** 1979. Role of membrane (M) protein in endogenous inhibition of in vitro transcription by vesicular stomatitis virus. J. Virol. **29:**134–142.
9. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5297.
10. **Chong, L. D., and J. K. Rose.** 1993. Membrane association of functional vesicular stomatitis virus matrix protein in vivo. J. Virol. **67:**407–414.
11. **Cullen, B. R.** 1986. Trans-activation of human immunodeficiency virus occurs via a bimodal mechanism. Cell **46:**973–982.
12. **Dubois-Dalcq, M., K. V. Holmes, and B. Rentier.** 1984. Assembly of rhabdoviridae, p. 21–43. *In* Assembly of enveloped RNA viruses. Springer-Verlag, Vienna.
13. **Gopalakrishna, Y., and J. Lenard.** 1985. Sequence alterations in the temperature-sensitive M-protein mutants (complementation group III) of vesicular stomatitis virus. J. Virol. **48:**655–659.

13a. **Harmison, G. G., and M. Schubert.** Unpublished data.

14. **Hiramatsu, K., K. Mannen, K. Mifune, A. Nishizono, and Y. Takita-Sonoda.** 1993. Comparative sequence analysis of the M gene among rabies virus strains and its expression by recombinant vaccinia virus. Virus Genes **7:**83–88.
15. **Hudson, L. D., C. Condra, and R. A. Lazzarini.** 1986. Cloning and expression of a viral phosphoprotein: structure suggests vesicular stomatitis virus NS may function by mimicking an RNA template. J. Gen. Virol. **67:**1571–1579.
16. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virol. **66:**2232–2239.
17. **Li, Y., L. Luo, M. Schubert, R. R. Wagner, and C. Y. Kang.** 1993. Viral liposomes released from insect cells infected with recombinant baculovirus expressing the matrix protein of vesicular stomatitis virus. J. Virol. **67:**4415–4420.
18. **Li, Y., L. Luo, R. M. Snyder, and R. R. Wagner.** 1988. Expression of M gene of vesicular stomatitis virus cloned in various vaccinia virus vectors. J. Virol. **62:**776–782.
19. **Li, Y., Luo, L., and R. R. Wagner.** 1989. Transcription inhibition site on the M protein of vesicular stomatitis virus located by marker rescue of mutant *ts*O23(III) with M-gene expression vectors. J. Virol. **63:**2841–2843.
20. **Lyles, D. S., L. Puddington, and B. J. McCreedy.** 1988. Vesicular stomatitis virus M protein in nuclei of infected cells. J. Virol. **62:**4387–4392.
21. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333–348.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Marcus, P. I., and M. J. Sekellick.** 1985. Interferon induction by viruses. XIII. Detection and assay of interferon induction-suppressing particles of vesicular stomatitis virus. Virology **142:**411–415.
24. **Marcus, P. I., M. J. Sekellick, C. F. Spiropoulou, and S. T. Nichol.** 1993. Interferon induction by viruses. XXII. Vesicular stomatitis virus-Indiana: M-protein and leader RNA do not regulate interferon induction in chicken embryo cells. J. Interferon Res. **13:**413–418.
25. **McCreedy, B. J., and D. S. Lyles.** 1989. Distribution of M protein and nucleocapsid protein of vesicular stomatitis virus in infected cell plasma membrane. Virus Res. **14:**189–206.
26. **Melki, R., Y. Gaudin, and D. Blondel.** 1994. Interaction between tubulin and the viral matrix protein of vesicular stomatitis virus: possible implications in the viral cytopathic effect. Virology **202:**339–347.
27. **Muesing, M. A., D. H. Smith, and D. J. Capon.** 1987. Regulation of mRNA accumulation by a human immunodeficiency virus transactivator protein. Cell **48:**691–701.
28. **Newcomb, W. W., and J. C. Brown.** 1981. Role of vesicular stomatitis virus matrix protein in maintaining the viral nucleocapsid in the condensed form found in native virions. J. Virol. **39:**295–299.
29. **Odenwald, W. F., H. Arnheiter, M. Dubois-Dalcq, and R. A. Lazzarini.** 1986. Stereo images of vesicular stomatitis virus assembly. J. Virol. **57:**922–932.
30. **Ogden, J. R., R. Pal, and R. R. Wagner.** 1986. Mapping regions of the matrix

protein of vesicular stomatitis virus which bind to ribonucleocapsids, liposomes, and monoclonal antibodies. J. Virol. **58:**860–868.
31. **Ono, K., M. E. Dubois-Dalcq, M. Schubert, and R. A. Lazzarini.** 1987. A mutated membrane protein of vesicular stomatitis virus has an abnormal distribution within the infected cell and causes defective budding. J. Virol. **61:**1332–1341.
32. **Pal, R., B. W. Grinnell, R. M. Snyder, and R. R. Wagner.** 1985. Regulation of viral transcription by the matrix protein of vesicular stomatitis virus probed by monoclonal antibodies and temperature-sensitive mutants. J. Virol. **56:**386–394.
33. **Pringle, C. R.** 1970. Genetic characteristics of conditional lethal mutants of vesicular stomatitis virus induced by 5-fluorouracil, 5-azacytidine, and ethyl methane sulfonate. J. Virol. **5:**559–567.
34. **Rose, J. K., and J. E. Bergmann.** 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. Cell **34:**513–524.
35. **Rose, J. K., and C. J. Gallione.** 1981. Nucleotide sequences of the mRNAs encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. J. Virol. **39:**519–528.
36. **Schubert, M., G. G. Harmison, C. D. Richardson, and E. Meier.** 1985. Expression of a cDNA encoding a functional 241-kilodalton vesicular stomatitis virus RNA polymerase. Proc. Natl. Acad. Sci. USA **82:**7984–7988.
37. **Schulz, T. F., D. Whitby, J. G. Hoad, T. Corrah, H. Whittle, and R. A. Weiss.** 1990. Biological and molecular variability of human immunodeficiency virus type 2 isolates from the Gambia. J. Virol. **64:**5177–5182.
38. **Simon, K. O., P. A. Whitaker-Dowling, J. S. Youngner, and C. C. Widnell.** 1990. Sequential disassembly of the cytoskeleton in BHK21 cells infected with vesicular stomatitis virus. Virology **177:**289–297.
39. **Sodroski, J., C. Rosen, F. Wong-Staal, S. Z. Salahuddin, M. Popovic, S. Arya, R. C. Gallo, and W. A. Haseltine.** 1985. Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat. Science **227:**171–173.
40. **Sprague, J., J. H. Condra, H. Arnheiter, and R. A. Lazzarini.** 1983. Expression of recombinant DNA gene coding for the vesicular stomatitis virus nucleocapsid protein. J. Virol. **45:**731–781.
41. **Wagner, R. R.** 1987. Rhabdovirus biology and infection: an overview, p. 9–74. *In* R. R. Wagner (ed.), The rhabdoviruses. Plenum Publishing Corp., New York.
42. **Wilson, T., and J. Lenard.** 1981. Interaction of wild type and mutant M protein of vesicular stomatitis virus with nucleocapsids in vitro. Biochemistry **20:**1349–1354.
43. **Ye, Z., W. Sun, K. Suryanarayana, P. Justice, D. Robinson, and R. R. Wagner.** 1994. Membrane-binding domains and cytopathogenesis of the matrix protein of vesicular stomatitis virus. J. Virol. **68:**7386–7396.