Rabies virus matrix protein regulates the balance of virus transcription and replication

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RNA synthesis by negative-strand RNA viruses (NSVs) involves transcription of subgenomic mRNAs and replication of ribonucleoprotein complexes. In this study, the envelope matrix (M) protein of rabies virus (RV) was identified as a factor which inhibits transcription and stimulates replication. Transcription, but not replication, of RV minigenomes or of full-length RV was decreased by expression of heterologous M. Since RV assembly involving M and the glycoprotein G renders virus synthetically quiescent, an RV was generated with the M and G genes substituted by placeholders. Surprisingly, RNA synthesis by this recombinant was characterized not only by an increased transcription rate but also by a diminished accumulation of replication products. This phenotype was reversed in a dose-dependent manner by providing M in trans, showing that M is a replication-stimulatory factor. The role of M in determining the balance of replication and transcription was further exploited by generation of a recombinant RV with attenuated M expression, which is highly active in transcription. Regulation of RNA synthesis by matrix proteins may represent a general mechanism of nonsegmented NSVs, which is probably obscured by the silencing activity of M during virus assembly.

INTRODUCTION

Genome and antigenome RNAs of rhabdoviruses such as rabies virus (RV) or vesicular stomatitis virus (VSV) are enwrapped with nucleoprotein N to form a typical ribonucleoprotein (RNP) complex. Only RNPs can be used as a template for the viral RNA polymerase, which is made up of the enzymatically active L protein and phosphoprotein P. The polymerase is active in two different modes of RNA synthesis, transcription and replication. Transcription involves sequential synthesis of free, subgenomic RNAs, including a short leader RNA, and five mRNAs in the order 3'-le-N-P-M-G-L-5'. Replication, in contrast, gives rise to RNPs, i.e. N-encapsidated full-length RNAs (Abraham & Banerjee, 1976; Ball & White, 1976). In contrast to transcription, replication requires ongoing N synthesis, suggesting that replicative elongation is coupled to encapsidation of the newly generated RNA (Arnheiter et al., 1985; Patton et al., 1984).

RNPs entering a cell are thus not able to replicate until virus transcription and subsequent translation provide a critical concentration of N and P protein, allowing a switch or shift to the processive replication mode. According to a widely accepted model, newly synthesized N–P complexes bind to nascent leader RNA and, if present in sufficient amounts, prevent recognition of the leader/N gene junction and other cis-active sequences defining cistrons in the transcription mode. According to this model, the coupling of RNP assembly and RNA elongation leads to a self-regulatory system for controlling the balance of transcription and replication (Blumberg et al., 1981, 1983).

Experimental evidence for intracellular N levels switching the polymerase to replication is not available for any nonsegmented negative-strand RNA virus. In minigenome systems in which all RNP proteins are supplied in trans, alteration of the amounts and ratios of N, P and L proteins did not change the ratio of transcription and replication (unpublished results; Fears et al., 1997; Wertz et al., 1998), and VSV transcription initiation may be independent of leader RNA synthesis at the leader–N junction (Whelan & Wertz, 2002; Chuang & Perrault, 1997). This strongly argues in favour of a situation where the availability of N is just a prerequisite for replication and that other factors determine whether an RNP is a template for transcription or replication. These may include modifications of P and L proteins (Das et al., 1998; Pattnaik et al., 1997; Hwang et al., 1999) or involvement of other viral and cellular factors. Indeed, in the more complex negative-strand RNA virus (NSV) respiratory syncytial virus (RSV) the absence of the viral M2-2 gene product resulted in enhanced transcription and reduced replication (Bermingham & Collins, 1999).

How transcription and replication of the rhabdoviruses are regulated is unknown. Apart from the RNP proteins N,
P and L, RV encodes only two more viral proteins, a matrix protein, M, and the spike glycoprotein. RV M protein is a multifunctional protein, playing a crucial role in virus assembly and budding. M is responsible for recruiting RNPs to the cell membrane, their condensation into tightly coiled ‘skeleton’-like structures and the budding of enveloped virus particles (Mebatsion et al., 1999). The long-known inhibitory activity of M proteins of rhadoviruses and other NSVs on viral RNA synthesis in vitro (Carroll & Wagner, 1979) has been attributed to the function of M in condensing RNPs prior to virus export. Here, we have used a reverse genetics approach involving defective and non-defective recombinant RV to investigate the effect of M protein expression on RV RNA synthesis. We present several lines of evidence that M is a key factor in the regulation of RV polymerase functions that exerts opposite effects on transcription and replication, and thereby tips the balance toward replication.

**METHODS**

**Cells and viruses.** Viruses were grown on BHK-21 clone BSR cell monolayers maintained in Glasgow MEM supplemented with 10% newborn calf serum. The BSR T7/5 cell clone (Buchholz et al., 1999), which was used for virus rescue, was maintained under selection conditions (1 mg G418 (ml cell culture supernatant)\(^{-1}\)). BSR cell clones that express M or M and G after induction with doxycycline (M-on and MG-on, respectively) were grown under selection conditions by adding G418 (1 mg ml\(^{-1}\)) and hygromycin (1 mg ml\(^{-1}\)).

**Recombinant genomes and minigenomes.** The minigenome cDNA plasmid pSDI-CL(NP) (Finke & Conzelmann, 1999) comprises the 3’-terminal genome promoter (GP) sequence followed by the chloramphenicol acetyltransferase (CAT) reporter gene, the non-coding RV N/P gene border sequence, the firefly luciferase reporter gene and, finally, the 5’-terminal antigenome promoter (AGP) sequence. A 5’-copy-back version of pSDI-CL(NP) was generated by replacement of the 3’-terminal GP sequence with a reversed copy of the 5’-terminal 67 nt of the RV genome containing the AGP sequence. This resulted in a minigenome that exclusively contains replication promoters.

The pNPrL cDNA plasmid was constructed by replacement of the M and G genes in pSAD L16 (Schnell et al., 1994) with the EGF (g) and DSred (r) reporter genes (Clontech) resulting in the genome organization 3’-N-P-EGFP-DSred-L-5’ (see Fig. 3).

In pSAD LM, the M gene was translocated from its original position between the P and the G genes to the most distal position on the viral genome, i.e. downstream of the L gene. This was achieved by replacement of the L gene transcription stop signal in the previously described M-deficient pSAD ΔM (Mebatsion et al., 1999) with a copy of the N/P-gene border sequence and subsequent insertion of the M-coding sequence downstream of the new transcription signal, resulting in a genome organization of 3’-N-P-GL-M-5’. A detailed description of all cloning steps and the final sequences are available from the authors by email.

**cDNA expression plasmids.** Expression plasmids pTITT-N, pTITT-P and pTITT-L have been described previously (Finke & Conzelmann, 1999). pTITT-M and pTITT-G were generated by insertion of the coding sequences into pTIT (Buchholz et al., 1999) after PCR amplification from pT7T-M and pT7T-G (Conzelmann & Schnell, 1994). The resulting plasmids encoded the RV genes under the control of the bacteriophage T7 RNA polymerase promoter and the internal ribosome entry site (IRES) of encephalomyocarditis virus.

**cDNA rescue experiments.** cDNA plasmids were transfected into cells after calcium phosphate precipitation (mammalian transfection kit; Stratagene) according to the supplier’s instructions. To enable T7 RNA polymerase-driven expression, either the transfected cells were infected with T7 polymerase-expressing vaccinia virus vTF7-3 (Fuerst et al., 1986) or the cDNAs were transfected into BSR T7/5 cells that constitutively express T7 polymerase.

Rescue of recombinant viruses was performed as described previously (Finke & Conzelmann, 1999) by cotransfection of 1 × 10⁶ BSR T7/5 cells grown in 8 cm² culture dishes with 10 µg of full-length cDNA and plasmids pTITT-N (5 µg), pTITT-P (2·5 µg) and pTITT-L (2·5 µg). Fresh culture medium was added 3 days post-transfection and, after another 3 days, cell culture supernatants were harvested and transferred to BSR cells. Two days after passage, infectious viruses were detected by immunostaining for RV N protein.

Minigenome virus-like particles (VLPs) were generated from cDNAs as described previously (Conzelmann & Schnell, 1994) by coexpression of minigenome RNA and virus proteins N, P, M, G and L in vaccinia virus vTF7-3-infected cells. In the complementation experiments, VLPs were transferred to vaccinia virus vTF7-3-infected BSR cells that were cotransfected with pT7T-N, pT7T-P, pT7T-L (5·0 µg, 2·5 µg and 2·5 µg, respectively) and with increasing amounts of pT7T-M (0·0 to 2·0 µg).

**RNA analysis.** RNA was isolated from cells with the RNeasy Mini kit (Qiagen). For nuclease treatment, the cells were trypsinized and pelleted. Pellets of 1 × 10⁶ cells were lysed in 50 µl buffer (10 mM NaCl, 10 mM Tris pH 7·5, 1·5 mM MgCl₂, 1% Triton). After 30 min incubation at 30°C with 50 µl micrococcal nuclease (20 µg nuclease S7 (Boehringer Mannheim) in 10 mM Tris pH 7·5, 1 mM CaCl₂), RNA was isolated. Northern blots and hybridizations with [α-32P]dCTP-labelled cDNAs were performed as described previously (Conzelmann et al., 1991). Hybridization signals were quantified by phosphorimaging (Molecular Dynamics Storm).

**Immunoblotting.** Western blots were performed as described previously (Finke et al., 2000). Signals were quantified by fluorography (ECF; Amersham) using a phosphorimager (Molecular Dynamics Storm).

**RESULTS**

**Inhibition of minigenome transcription**

The interdependency of replication and transcription in non-deficient NSVs impedes investigation of the individual processes. Therefore, to examine the effect of RV M on intracellular RV RNA synthesis, we first used a minigenome system in which all virus proteins are provided in trans (Conzelmann & Schnell, 1994). To analyse the products of transcription, a previously described transcriptionally active bicistronic minigenome, SDI-CL(NP), was used (Finke & Conzelmann, 1999). SDI-CL(NP) contains two reporter genes CAT and luciferase flanked by the authentic RV 3’ and 5’ ends (Fig. 1A, grey and black boxes, respectively). Accordingly, transcription of SDI-CL(NP) gives rise to a leader RNA and two mRNAs. To determine the effect of M on RV replication, a transcriptionally inactive 5’-Copy-back model genome was generated, by replacing the 3’-end...
of SDI-CL(NP) with a reverse copy of the 5′-terminal 67 nt containing the RV antigenome promoter (Fig. 1B, black boxes). As the antigenome promoter is highly active in replication but unable to direct transcription, this copy-back model genome is replicated at high levels. VLPs were prepared as described (Conzelmann & Schnell, 1994) and were transferred to BSR cells that were transfected with fixed amounts of T7 promoter-driven plasmids encoding RV proteins N, P and L, along with increasing amounts of pT7T-M, encoding RV M protein.

RNA synthesis from the minigenomes was monitored by Northern hybridization 2 days post-infection (p.i.). The probes used in this and in all following hybridizations recognized both positive-stranded mRNAs and antigenomes as well as genomes. Substantial amounts of monocistronic CAT and luciferase mRNAs, as well as bicistronic CAT/luciferase RNAs transcribed from SDI-CL(NP), were present in the absence of M (Fig. 1A). With increasing amounts of pT7T-M, the level of mRNAs decreased in a dose-dependent manner. At maximal amounts of M, mRNA synthesis was reduced to ~10%. A signal corresponding to genome RNA and representing replication products from SDI-CL(NP) was not detectable under these conditions.

RNA synthesis products of the 5′-copy-back genome were almost exclusively full-length RNAs (Fig. 1B). Resistance to nuclease digestion further showed that this RNA is encapsidated in N proteins and represents the product of replication. In striking contrast to SDI-CL(NP) transcription, increasing amounts of M did not have a detectable influence on the accumulation of RNPs, even at the highest M concentration (Fig. 1B).

**Overexpression of M suppresses transcription of full-length RV**

To determine how RNA synthesis of a non-deficient RV is affected by M, wt RV SAD L16 was grown in cells, providing extra M protein. A BSR-derived cell line which expresses RV M protein after induction with doxycycline in a dose-dependent manner (M-on; to be published elsewhere) was infected with SAD L16 and expression of cell-encoded M (cM) was induced. Total RNA and cell lysates were analysed after 24 h. CM was detected exclusively after induction of cells (Fig. 2). Compared to M expression in virus-infected cells, CM was much less abundantly expressed. Thus, in induced and virus-infected cells, CM contributed little to the total M level. Nevertheless, a significant drop in RV transcription was observed in induced cells. In spite of clearly reduced mRNA levels, a decrease in the accumulation of full-length replication products was not observed (Fig. 2, genome). Since in this system, in contrast to the minigenome system, replication depends on the products of RV N mRNAs, the surprisingly high replication level suggests a positive influence of the additional CM on RV replication. Comparison of the levels of mRNAs and genome RNAs by phosphorimaging revealed that transcription rates (mRNA/genome) were reduced to 53% for the N mRNA and to 35% for the L mRNA after expression of CM. Thus, even low levels of additional M protein exert a distinct regulatory activity on virus RNA synthesis in wt RV infection, resulting in a shift towards replication.

**Deregulation of RV transcription and replication in the absence of M protein**

To further dissect the transcription inhibitory effect of RV M protein, viral RNA production was examined in the absence of M. We have previously described an M gene-deficient RV, SAD-ΔM, which has been used to study aspects of M in virus assembly (Mebatsion et al., 1999). This construct, however, was initially not considered appropriate for the present study, as it lacks an entire cistron. We therefore constructed a novel recombinant genome in which the M gene was replaced with the EGFP reporter gene. In addition, the G gene was replaced with the DSred reporter gene, in order to obviate potentially disturbing interactions of M and G. The cDNA construct pNPgrL (Fig. 3A) produced functional RNPs in cells

![Image](http://vir.sgmjournals.org)
expressing RV N, P and L proteins from transfected plasmids as described previously (Finke & Conzelmann, 1999). To complement for the M and G deficiency, cells were also transfected with M- and G-encoding plasmids pTIT-M and pTIT-G and were mixed with a BSR cell line expressing M and G proteins after induction with doxycycline (MG-on). To select for M- and G-expressing cells, hygromycin was added to the mixture at 1 mg ml$^{-1}$. Expression was repeatedly induced until approximately 40% of the cell culture was infected with NPgrL, as determined by EGFP fluorescence. Cell culture supernatants were transferred to fresh MG-on cells for NPgrL stock production.

RNA synthesis of NPgrL was investigated by Northern blot experiments after infection of BSR cells at an m.o.i. of 5. In the absence of transcription inhibition, a greater accumulation of mRNAs, and, as a result of higher virus gene expression, a higher accumulation of genome RNAs were expected. However, while the mRNA levels were comparable to those of wt RV infection (Fig. 3B, see N mRNA 2 days p.i.), the levels of genome length RNA were strongly reduced. Quantification of hybridization signals revealed an increased transcription rate. Normalized to SAD L16, the transcription rates of NPgrL were 379% for the N mRNA and 501% for the L mRNA at 1 day p.i. At 2 days p.i., the transcription rate of SAD L16 decreased to 42%, whereas that of NPgrL was still 181% and 206% for N mRNA and L mRNA, respectively. In spite of this 4-5-fold increase in transcription rate, which should result in rapid accumulation of N, P and L protein available for replication, total RNA synthesis of NPgrL was always lower as compared to wt RV SAD L16, suggesting that N, P and L proteins were not optimally used for replication. The observation that the complete lack of M is accompanied by a diminished replicase activity again strongly argues in favour of a distinct function of M in enhancing replication.

**Dose-dependent effect of M on RNA synthesis**

To analyse NPgrL RNA synthesis in the presence of increasing levels of RV M protein, M-on cells were infected...
with NPgrL at an m.o.i. of 1. Two days later, doxycycline was added to aliquots of the infected cells at concentrations of 0, 0.1 or 1.0 µg (ml cell culture medium)⁻¹. Two days after induction of M expression, viral RNA was isolated and analysed in Northern blots with RV N- and L-gene-specific DNA probes (Fig. 4A). Upon induction with 0.1 and 1.0 µg ml⁻¹ doxycycline, the transcription rates of the N mRNA decreased to 52% and 22%, respectively (not shown). Similarly, a decrease of the L transcription rate was observed. Since the level of M expression in M-on cells directly correlates with the doxycycline concentration (not shown), the decrease in the transcription rates depends on the level of M protein available.

Normalization with 28S rRNA hybridization signals revealed that with increasing doxycycline concentrations the absolute levels of N mRNA decreased to 50% compared to noninducing conditions (Fig. 4B). In contrast, absolute levels of genome-length RNA were 2-3- and 2-2-fold (N and L hybridization, respectively) increased at a doxycycline concentration of 1.0 µg ml⁻¹, demonstrating that M stimulates RNA replication in NPgrL infected cells. To exclude effects of doxycycline on virus RNA synthesis, transcription rates of NPgrL were also determined in parental Tet-on cells, that only express the reversed Tet-repressor. In contrast to M-on cells, no reduction of transcription rates was detectable with increasing doxycycline concentration (Fig. 4C).

**RVs with attenuated M expression**

According to the above results, RV M has two different functions in the regulation of RV RNA synthesis, namely a pronounced transcription inhibition activity, accompanied by a stimulatory activity on replication. The transient assays with cM already suggested that small amounts of M were sufficient to support replication (Fig. 4), stressing the idea that RV with attenuated M expression might perform better in total RNA synthesis than RV lacking M. To follow this, a recombinant virus cDNA was constructed, in which the M gene was shifted to the very 5′-end, i.e. downstream of the L gene. Due to the steep step in the RV transcription gradient at the G/L gene border (Finke et al., 2000), M gene transcription of this virus should be severely attenuated. Because of the essential function of M during virus assembly (Mebatsion et al., 1999), it was questionable whether viable virus could be recovered. However, autonomously propagating virus, dubbed SAD LM and yielding infectious titres similar to SAD L16, was obtained from cDNA.

BSR cells were infected in parallel with SAD LM and with SAD L16 at an m.o.i. of 1 and the expression of M and N proteins was monitored in Western blots. Signal intensities were determined by fluoroiomaging (Fig. 5). Whereas N protein levels in SAD LM-infected cells were comparable to wt virus, the levels of M protein were always lower than wt M levels, indicating that the gene translocation resulted in attenuated M protein expression. In numbers, the ratio of M to N was decreased 3-2-, 1.4- and 2-0-fold at days 1, 2 and 3 p.i., respectively.

RNA from the same experiment was visualized in Northern hybridizations with an N probe (Fig. 6). Compared to SAD L16, N mRNA in SAD LM-infected cells was much more abundant, whereas the genome RNA levels were lower. The transcription rates of SAD LM were 11-, 33- and 23-fold higher than that of SAD L16 at days 1, 2 and 3, respectively. In addition, and in contrast to the situation with NPgrL in the absence of M (see Fig. 3), total RNA production of SAD LM exceeded that of SAD L16 2-, 5- and 4-fold after 1, 2 and 3 days, respectively. This reflects better accumulation of genome-length RNA by the available M protein. Thus, although sufficient M was produced to promote effective virus particle assembly, the diminished M content tipped the balance of RNA synthesis to ratios that were previously observed only for M deletion mutants.

**DISCUSSION**

Matrix proteins are major components of the envelope of NSVs and are known to shut down transcription from RNP's in vitro, as shown for different NSVs such as influenza virus, measles virus or VSV (Suryanarayana et al., 1994;
The function of M in binding and condensing RNPs during virus assembly and egress provides a plausible explanation for this kind of transcription inhibition. Tightly coiled, ‘freezed’ RNP structures are not assumed to represent a proper template for RNA synthesis. Due to the lack of effective in vitro replication systems for NSVs, data on the effect of M on replication in vitro are not available. Yet, condensation of RNPs should similarly halt transcription and replication in the assembling virus. The present data on intracellular RNA synthesis of recombinant RV constructs reveal that M has another function prior to ‘freezing’ RNPs, namely as a regulatory protein in tipping the balance of virus RNA synthesis towards replication. Most importantly, transcription inhibition activity is accompanied by an opposite stimulatory activity on replication.

The first hint on differential activity of M on transcription and replication of RV was obtained in experiments using reporter minigenome RNAs. All virus proteins were provided in trans, ensuring that at least replication occurs independently of virus transcription. A dose-dependent decrease in mRNA production from SDI-CL(NP) was already observed after expression of very little amounts of M (Fig. 1). The linear decrease of transcription argues against inhibition by RNP condensation, which would be expected to need a threshold level of available M protein.

The low replication activity of SDI-CL(NP), which is also observable in wt helper virus-infected cells (Finke & Conzelmann, 1999), prompted us to use the 5'-copy-back minigenome, which allows reliable quantification of replication products. In striking contrast to transcription, even high levels of M did not abrogate replication of 5'-copy-back RNPs. As condensation of RNPs is expected to shut down not only transcription but also replication, this result also favours pre-condensation effects of M.

Effects of additional M were monitored in experiments in which non-deficient RV was grown in the presence of additional M expressed from the cell (cM). Relative to RV-expressed M, cM expression was low and increased the amount of available M only a little. Nevertheless, a marked inhibition of RV transcription was noted (see Fig. 2), which is most probably due to higher cM than viral M expression in the early stages of infection. The more important finding was that the reduced levels of mRNA observed did not result in a similar decrease of genome RNA levels. The latter was rather expected since reducing viral N mRNA expression below a critical limit might in turn limit N-dependent replication. An explanation for this not taking place is that levels of N in wt virus infection are abundant and far from limiting replication. Since N is not

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the limiting factor, other factors should account for keeping replication in wt RV infections low. The most probable explanation is a stimulatory effect of M on virus RNA replication.

A role of M in stimulating replication was further strongly supported by analysis of RV RNA synthesis in the absence of M. With respect to the resistance of RV replication to M in the minigenome system, we expected that replication of the M- and G-defective virus NPgrL would be at least comparable to wt RV, or, rather, augmented as a consequence of enhanced virus gene expression. This was not the case, as the levels of genome RNA were clearly decreased in the absence of M. Though sufficient levels of N and other virus proteins were obviously available, less full-length replication products accumulated. The lack of M totally abolishes RV RNP condensation and decreases by magnitudes the export of RNPs from infected cells through budding of virus particles (Mebatsion et al., 1999). Thus, considering the lack of RNP export, the absence of M in NPgrL must have an even greater effect on replication than that prevented by the values obtained from phosphor-imaging of Northern blots. Therefore, in spite of roughly comparable N levels (see Fig. 3B), the lack of M is hampering the use of this available N for replication. Thus, M in wt virus is not only exerting a selective inhibition on virus transcription but also has a clear stimulatory effect on replication.

This was further underlined by complementation of the M- and G-defective virus NPgrL, with different amounts of M. Again, in the presence of M, the opposite effects on transcription and replication products were obvious. Importantly, with expression of cM the RNPs gain the ability to bud off the infected cell as spikeless particles (Mebatsion et al., 1996). Although in the absence of G the budding efficiency is approximately 30-fold lower than in wt RV, a certain efflux of RNPs has to be taken into account. Nevertheless, increasing amounts of genome RNAs were observed in cells expressing increasing amounts of M (see Fig. 4).

As suggested by complementation of defective RV in trans and as confirmed by the non-deficient virus SAD LM, RV possesses an M-directed fine-tuning mechanism of RNA synthesis. In SAD LM-infected cells, M protein expression was not much less than with wt RV, but the relative ratios of N and M were changed. In view of this, SAD LM RNA synthesis showed characteristic and predicted features. SAD LM grew well, with a short lag at early time points of infection (not shown), indicating an initial handicap in replication. This was obviously compensated by a highly enhanced virus gene expression, resulting in a total RNA synthesis exceeding that of wt RV. Whereas in wt RV gene expression is downregulated in favour of genome replication, in SAD LM unrestricted transcription was coupled with reduced replication activity. The shift of the L gene to an upstream position probably leads to overexpression of the polymerase in SAD LM. However, this affects only the overall RNA synthesis but not the ratio of replication and transcription, as shown previously for other RVs overexpressing L (Finke et al., 2000). The high transcription phenotype of SAD LM was therefore exclusively due to the attenuated M gene expression.

For regulatory functions of M in RNA synthesis, two different targets of M are conceivable: the L polymerase and the RNP template. Recent models suggest that the polymerase might exist as a nonprocessive transcriptase and as processive replicase. In contrast to replication, Sendai virus transcription requires special reaction conditions to promote processivity on the RNP, indicating that the processivity of transcriptase and replicase is differentially regulated (Gubbay et al., 2001). Moreover, recombinant ambisense RVs indicated that individual replicase and transcriptase forms of the polymerase complex exist (Finke & Conzelmann, 1997).

Association of VSV L with different host cell or virus factors might affect L polymerase activity (Barik & Banerjee, 1992; Gupta & Banerjee, 1997; Das et al., 1998). Indeed, VSV P phosphorylation mutants able to support either replication or transcription of defective interfering particles (Pattnaik et al., 1997; Hwang et al., 1999) and P antibodies inhibiting replication but not transcription have been described (Richardson & Peluso, 1996). The RV P protein is phosphorylated by two kinases (Gupta et al., 2000); however, no effect of P phosphorylation mutants on RV RNA synthesis has been described so far. Moreover, no differential activity on replication and transcription could be demonstrated with RV N protein phosphorylation mutants (Yang et al., 1999). As temporal changes in the modification of P, L or N protein during rhabdovirus infection have not been shown, their relevance with respect to a physiological shift between replication and transcription remains unclear.

With the binding of M to the polymerase, either the formation of replicase could be favoured, or the formation of transcriptase could be prevented, both provoking effects on RNA synthesis as described above. In addition, M might act on cellular factors, which in turn influence the polymerase complex composition or activity. For instance, borna disease virus polymerase is differentially influenced by interferon (Staeheli et al., 2001). For the present regulation of RV RNA synthesis, however, a role for interferon can be ruled out since all cells used in this study were interferon incompetent (Schlender et al., 2000).

Another possible mechanism for regulation by M could involve modification of the RNP such that it is better suited as a template for replication than for transcription. Indeed, RV M is able to alter the structure of the RNP by condensing it into tight coils and keeping it in these ‘skeleton’-like forms (Mebatsion et al., 1999). So, at least for assembly, M causes a structural transition of RNPs to, most probably, synthetically inert bodies. To explain the regulatory function of M exerting opposite effects on transcription and replication, a model involving an action
of M on intracellular, decoiled RNPs must be favoured. Indeed, for VSV, M binding to intracellular nucleocapsids prior to virion assembly has been shown (Flood & Lyles, 1999). Interestingly, the affinity of cytosolic M in binding intracellular RNPs was approximately 8-fold less than in binding to virion-derived RNPs. Moreover, membrane-derived M exhibited little or no binding to intracellular RNPs. These findings are not only in favour of different activities of M in virus assembly and in virus RNA synthesis, but also suggest that the M regulatory activity acts on the RNP.

Binding of low amounts of M to intracellular RNPs could affect the function of transcriptase and replicate differently. The higher processivity of the replicase, which is also able to read through transcription stop signals, could override bound M proteins, whereas a more sensitive, non-processive transcriptase might be blocked by the bound M. Whereas selective inhibition of transcription could be easily explained this way, stimulation of replication cannot. Assuming that transcriptase and replicate exist in an equilibrium, M-modified RNPs might change this equilibrium by increasing the concentration of free transcriptase, thereby increasing intracellular replicate levels. This could also explain why in our nontranscribing minigenome system a significant stimulation of replication by M was not observed, in contrast to all transcribing systems. Another possibility might be differential action on genome and antigenome promoters.

The availability of reverse genetics systems for defective and non-defective RV allowed us to dissociate functions of M related to virus assembly and regulation of RNA synthesis. These systems certainly provide tools to further address the mechanism of M action in regulating RV RNA synthesis. A promising approach that will be substantially facilitated by the recently solved crystal structure of the VSV M protein (Gaudier et al., 2002) is the genetic analysis of M proteins to identify relevant domains. It also appears worthwhile to extend these investigations to other NSVs. In spite of ostensible differences in M structure and the location of M proteins in VSV and RV virions (Barge et al., 1993; Mebatson et al., 1999), it is likely that regulation mechanisms in rhabdoviruses are similar. Notably, in the late seventies, a regulatory role of VSV M was suggested after the analysis of RNA synthesis of temperature sensitive VSV mutants (Martinet et al., 1979; Clinton et al., 1978). It will be interesting to also include paramyxoviruses, which encode nonessential, accessory gene products able to modulate RNA synthesis, such as the RSV M2-2 (Bermingham & Collins, 1999), or the Sendai virus C proteins (Curran et al., 1992), in order to determine whether regulation of RNA synthesis by M is a general feature of nonsegmented NSVs.

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