Down-regulation of vesicular stomatitis virus transcription by the matrix protein of influenza virus

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The matrix (M1) protein isolated from influenza A/WSN/33 virus, when reconstituted with ribonucleoprotein (RNP) cores of vesicular stomatitis virus (VSV), resulted in inhibition of VSV transcription in vitro. The presence of endogenous wild-type (wt) or mutant (tsO23) VSV matrix (M) protein on RNP cores did not prevent down-regulation of VSV transcription by reconstituted influenza virus M1 protein. In fact, endogenous VSV wt M protein augmented transcription inhibition by M1 protein reconstituted with RNP/M protein cores, whereas mutant tsO23 M protein endogenous to RNP cores had no effect on down-regulation of VSV transcription by M1 protein. These data suggest that VSV M protein and influenza virus M1 protein recognize two different sites on RNP cores responsible for down-regulation of VSV transcription. Monoclonal antibodies (MAbs) directed to epitope 2 of M1 protein had been previously shown to reverse transcription inhibition by M1 protein on influenza virus RNP cores, but the same epitope 2-specific MAb had little effect on transcription inhibition by M1 protein reconstituted with VSV RNP cores. VSV M protein bears a striking resemblance biologically and genetically to the M1 protein, including, as shown here, their capacity to bind viral RNA. However, the VSV wt M protein exhibited no capacity to down-regulate transcription by influenza virus RNP cores. The significance of these studies is the identification on VSV RNP templates of at least two separate sites for recognition of protein factors that repress VSV transcription.

Influenza virus and vesicular stomatitis virus (VSV) are enveloped negative-strand viruses of helical configuration with protein constituents of similar composition and function. A major structural protein of both virus families is the matrix (M) protein which serves to assemble the virion by binding to the helical ribonucleoprotein (RNP) core and to the surface membrane at the site of insertion of virus-coded glycoproteins (Lamb & Choppin, 1983; Wagner, 1987). Another property of the matrix proteins of both VSV and influenza virus is the capacity to down-regulate viral transcription upon binding to RNP templates (Carroll & Wagner, 1979; Zvonarjev & Ghendon, 1980). By RNA sequence analysis, the VSV M protein can be determined to be composed of 229 amino acids (Rose & Gallione, 1981), whereas the M1 protein of influenza virus contains 252 amino acids (Winter & Fields, 1980). These two matrix proteins exhibit a certain degree of sequence similarity based on a computer analysis which shows them sharing a moderate number of conserved key amino acids (Rose et al., 1982), suggesting a common ancestor for myxoviruses and rhabdoviruses. In the case of influenza virus, the M gene has a second open reading frame which codes for a minor separate M2 protein which also binds to the surface membrane (Lamb & Zebedee, 1985) but which appears to have functions distinct from those of influenza virus M1 protein or VSV M protein.

There are a number of significant differences between the RNP cores and major matrix proteins of influenza virus and VSV. The RNP core of influenza virus is segmented (Lamb & Choppin, 1983) and the encapsidated RNA genome is sensitive to ribonuclease digestion (Pons & Hirst, 1969; Ye et al., 1989), whereas the RNA encapsidated by the unsegmented RNP core of VSV is completely inaccessible to ribonuclease (Emerson, 1987). Compared to the integral M1 protein of influenza virus, which partitions into neutral phospholipid vesicle membranes (Gregoriades & Frangione, 1981; Ye et al., 1987), the peripheral M protein of VSV is devoid of lengthy hydrophobic amino acid sequences (Rose & Gallione, 1981) and appears to interact by means of basic amino acids with superficial headgroups of acidic phospholipids in vesicle membranes (Wiener et al., 1983). By indirect studies using epitope-specific monoclonal antibodies (MAbs), we presented tentative evidence that the RNP-binding site and the transcription-
inhibition site of the M₁ protein of influenza virus is located carboxy-distal to amino acid residue 89 (Ye et al., 1987, 1989). In contrast, similar experiments with the wild-type (wt) VSV M protein and its temperature-sensitive (ts) mutant tsO23 indicated that the VSV M protein transcription inhibition site is located in the amino-terminal region between residues 19 and 43 (Ogden et al., 1986). However, Kaptur et al. (1991) have provided convincing evidence that the VSV M protein complexes to the RNP core at a binding site located between residues 34 and 50.

The similar size, virion location, functions and possible evolutionary origin of the matrix proteins of VSV and influenza virus prompted us to test the capacity of influenza virus M₁ protein to down-regulate transcription by VSV RNP cores in a reconstituted system. It was also of interest to test the M protein group III VSV mutant tsO23, and to compare the RNA-binding affinity of M₁ protein with that of wt and mutant VSV M proteins.

The Indiana serotype (Orsay strain) of wt VSV and its mutant tsO23 were plaque-purified, grown in BHK-21 cells and purified by rate-zonal centrifugation (Pal et al., 1985). Stocks of influenza A/WSN/33 virus were prepared and purified as previously described (Ye et al., 1987) by infecting the allantoic sac of 10-day-old chicken embryos and harvesting the allantoic fluid 48 h after infection. Nucleocapsids (N) (RNP) of wt or tsO23 VSV complexed with endogenous M protein (RNP/M) were prepared by treatment of virions for 30 min with 10 mM-Tris-HCl pH 8-0 containing 1% Triton X-100 in the absence of any salt (Pal et al., 1985). To prepare VSV RNP cores devoid (80 to 90%) of M protein (RNP), purified virions were treated with Triton X-100 for 30 min in the same Tris–HCl buffer containing 0-25 mM-NaCl (Emerson, 1987). RNP or RNP/M cores were purified by centrifugation for 30 min through a glycerol pad in an SW 50.1 rotor at 37000 r.p.m. The pelleted RNP or RNP/M cores were resuspended in 10 mM-Tricine pH 8-0; the ratio of M protein to N protein (M/N) in purified nucleocapsids was measured following electrophoresis on a 12-5% polyacrylamide–SDS gel stained with Coomassie blue before scanning and integrating by laser densitometry. As previously described (Ye et al., 1987), the M₁ protein of influenza A/WSN/33 virus was purified from virions by the acidic chloroform extraction procedure of Gregoriades (1973) and was devoid of viral or cellular contaminants as determined by gel electrophoresis. Preparation and transcription of influenza virus RNP cores have been described in detail (Ye et al., 1987).

As previously described (Pal et al., 1985), in vitro transcription of VSV nucleocapsid templates (RNP or RNP/M cores) was carried out in duplicate using 0-1 ml of transcription buffer containing 10 mM-Tris–HCl pH 8-0, 1 mM-DTT, 80 mM-NaCl, 7-5 mM-MgCl₂, 1 mM each of ATP, GTP and CTP, and 0-1 mM-[α-³²P]UTP (3 µCi). Following incubation for 2 h at 31 °C, the transcription reaction was terminated by the addition of an equal volume of 0-067 mM-sodium pyrophosphate. ³²P-Labelled RNA in the presence of 0-05 mg of carrier yeast RNA was precipitated with 10% TCA and the radioactivity measured by liquid scintillation spectrometry.

Purified MAbs and their epitope specificity for M₁ protein were produced in the University of Virginia Lymphocyte/Hybridoma Center, whereas those secreting MAb M2-1C6, MAb 289/4 and MAb 904/6 were kindly supplied by Kathleen van Wyke (van Wyke et al., 1984). By studies of competitive binding to influenza A/WSN/33 virus M₁ protein, MAB M2-1C6 has been assigned to epitope 1, MAB 289/4 and MAB 7E5 to epitope 2, MAB 904/6 to epitope 3 and MAB 5C9 to epitope 4 (Ye et al., 1989). The method for studying binding of ³²P-labelled viral RNA to M proteins separated by PAGE and electroblotted onto nitrocellulose paper has also been described previously in some detail (Ye et al., 1989).

Prior to studying the transcription-inhibitory effect of influenza virus M₁ protein reconstituted with VSV RNP templates, it was necessary to determine the amount of endogenous M protein released from VSV RNP cores with increasing salt concentrations. Purified virions of wt VSV and its M gene mutant tsO23 were treated with 1% Triton X-100 in the presence of various concentrations of NaCl and the residual amount of M protein present in gradient-purified RNP cores was measured by laser densitometry after PAGE and Coomassie blue staining; residual M protein in RNP cores was determined in relationship to N protein (M/N ratio). These experiments showed that 100 mM-NaCl removed 60% of M protein from wt VSV RNP/M cores, whereas 80% M protein was released from tsO23 RNP/M cores. With increasing salt concentrations (up to 400 mM-NaCl), 80% of M protein was released from wt RNP cores, compared with tsO23 RNP cores which retained only 10 to 15% of their endogenous M protein at NaCl concentrations from 200 to 400 mM (data not shown). Somewhat different results were reported by Kaptur et al. (1991) who, using the non-ionic detergent octylglucoside (rather than Triton X-100), found no significant difference between wt and tsO23 VSV RNP/M cores in their degree of retention of endogenous M protein on exposure to increasing NaCl concentrations. Our salt dissociation studies, however, suggest that the M protein of the mutant tsO23 has a slightly, but significantly, reduced ionic bond affinity for RNP cores than does the wt M
protein. Under any circumstances, as much as 20% of endogenous M protein cannot be released from wt RNP/M cores even at salt concentrations that remove considerable amounts of the large and non-structural (NS) polymerase proteins (Emerson, 1987).

It also seemed essential to compare the temperature lability of the binding affinity of M protein complexed with RNP/M cores of the wt and mutant tsO23. To this end 0-05 mg of purified wt or tsO23 virions was exposed to temperatures of 20 °C to 40 °C for 30 min in a buffer of Tris–HCl pH 8-0 containing 1% Triton X-100 in the absence of NaCl or any other salt. After PAGE and staining with Coomassie blue, the M/N protein ratios were quantified by laser densitometry. These experiments showed no difference in residual binding to RNP/M cores of wt or tsO23 M protein incubated at 25 °C. Incubation of RNP/M cores at increasing temperatures revealed greater dissociation of tsO23 M protein than that of wt M protein; incubation of RNP/M cores at 40 °C resulted in 45% dissociation of tsO23 M protein compared with loss of 25% of wt M protein from RNP/M cores (data not shown). These findings indicate that the tsO23 M protein is also thermolabile with respect to its affinity for RNP cores as well as being more readily dissociable by increasing ionic strength.

The next step was the major thrust of this study, i.e. to determine whether the M1 protein of influenza virus could down-regulate RNA synthesis when reconstituted with VSV RNP templates. To this end, influenza A/WSN/33 virus M1 protein purified by the method of Gregoriades (1973) was added in a transcription mixture to RNP cores derived from VSV wt and tsO23, as described previously (Pal et al., 1985) and as outlined above. RNP cores prepared from tsO23 were chosen for comparison with wt VSV RNP cores because the M protein of this mutant has been shown repeatedly to be completely devoid of transcription inhibition activity (Carroll & Wagner, 1979). In the first set of experiments, the wt and mutant tsO23 RNP cores serving as transcription templates were prepared in the presence of 0-25 m-NaCl to remove the respective endogenous M proteins by about 80% from wt RNP cores and by about 90% from mutant RNP cores. Also, in order to determine whether a full complement of endogenous VSV M protein on RNP/M cores would compete with reconstituted M1 protein, the potential transcription inhibition activity of M1 protein was tested on both wt and tsO23 RNP/M cores prepared in the absence of NaCl in order to retain on the nucleocapsid template almost all of the endogenous wt and tsO23 VSV M protein.

Fig. 1 shows the results of incorporation of [α-32P]UMP into TCA-precipitable RNA made on RNP or RNP/M VSV templates incubated for 2 h at 31 °C after reconstitution with concentrations of M1 protein varying from 0 to 0-3 mg/ml. As noted, increasing concentrations of M1 protein gradually reduced the transcriptional activity of both wt and tsO23 RNP cores (Fig. 1a). At an M1 protein concentration of 0-3 mg/ml, the transcriptional activity of both wt and tsO23 RNP cores was reduced by about 60% when compared to that of naked RNP cores in the absence of any M1 protein. The consistently lower transcription of wt RNP cores at each concentration of M1 protein is presumably due to the well documented transcription inhibition activity of residual (approx. 20%) endogenous wt VSV M protein which is non-existent for the tsO23 endogenous mutant M protein (Carroll & Wagner, 1979).

It was of interest to determine whether the endogenous wt and mutant M proteins on wt and tsO23 RNP/M cores occupied and competed for the same site on VSV RNP cores at which influenza virus M1 protein exhibits
its capacity to inhibit VSV transcription. For this purpose, the same transcription inhibition experiments were done with M1 protein reconstituted, in this case, with VSV wt and tsO23 RNP/M cores (0-12 mg/ml) containing full complements of endogenous wt and mutant M proteins. As shown in Fig. 1(b), wt RNP/M template in the absence of added M1 protein exhibited 80% of the transcriptional activity of comparable tsO23 RNP/M cores, a finding consistent with that of earlier results (Carroll & Wagner, 1979). A similar increasingly greater degree of transcription inhibition was exhibited by wt RNP/M cores reconstituted with increasing amounts of M1 protein. The decline in transcription caused by M1 protein at concentrations of 0-15 and 0-3 mg/ml reconstituted with tsO23 RNP/M cores was about 60%, a reduction in transcription comparable to that of tsO23 RNP essentially devoid of mutant M protein after reconstitution with M1 protein (compare Fig. 1a with Fig. 1b). On the other hand, a considerably greater degree of transcription inhibition was evident by wt RNP/M cores reconstituted with M1 protein, about 80% down-regulation of transcription compared to approx. 60% transcription inhibition by wt RNP cores depleted of endogenous M protein but reconstituted with M1 protein. These data strongly suggest additive transcription inhibition effects of the wt matrix proteins of VSV and influenza virus acting in concert on VSV RNP templates. It would also seem likely that the VSV M protein and the M1 protein have different binding sites on the VSV RNP template, a postulate supported by the fact that the endogenous mutant M protein present on the tsO23 RNP/M core does not compete out the transcription inhibitory activity of reconstituted M1 protein.

One question that arises is whether the same domain on the M1 protein is responsible for down-regulating transcription of both VSV and influenza virus. A way to approach this question is to test MAbs that bind four separate epitopes on the M1 protein, one of which reversing transcription inhibition of influenza virus by M1 protein (Ye et al., 1987, 1989), for their capacity to reverse transcription inhibition of VSV RNP by reconstituted M1 protein. In this experiment, increasing concentrations of MAbs to each of the four known epitopes of M1 protein were preincubated for 1 h at room temperature with a constant amount (0-2 mg/ml) of purified M1 protein. The M1 protein–MAb mixtures, with appropriate positive and negative controls, were added to wt VSV RNP cores (depleted of endogenous M protein) in a standard transcription reaction mixture. The results of this experiment showed that MAbs directed to M1 protein epitopes 1, 3 and 4 had no effect on its capacity to inhibit transcription when reconstituted with VSV RNP templates. MAb 7E5, directed to epitope 2, did reverse the transcription inhibition activity of M1 protein reacting on VSV RNP template by a mere 10% and only at the highest MAb concentration of 0-3 mg/ml (data not shown). The same epitope 2-specific MAb 7E5 almost completely reverses transcription inhibition by endogenous M1 protein on RNP cores of influenza virus (Ye et al., 1987). The only valid interpretation of these data is that M1 protein appears to have separate domains for down-regulating transcription of VSV and influenza virus.

The M1 protein had been found to interact with influenza virion RNA, as well as with VSV RNA, in an in vitro RNA–protein binding assay (Ye et al., 1989); in fact, it forms a stable complex with various ssRNAs, possibly by means of a zinc finger motif (Wakefield & Brownlee, 1989). Quite understandably, this finding has led to the hypothesis that transcription inhibition by M1 protein is due to its binding to virion RNA exposed in the influenza virus RNP core segments (Wakefield & Brownlee, 1989; Ye et al., 1989). VSV M protein also inhibits viral transcription (Carroll & Wagner, 1979) but does not possess a zinc finger motif. Moreover, in sharp contrast to RNA in the influenza virus RNP, the RNA in the tightly coiled VSV RNP complex is completely inaccessible to ribonuclease and hence presumably inaccessible to direct binding by the VSV M protein in the RNP/M complex (Emerson, 1987; Ye et al., 1989). It was of interest, not withstanding, to test the potential of VSV M protein to bind influenza virus RNA and to compare this to the capacity of M1 protein to bind influenza virus RNA. For this purpose, as previously described (Ye et al., 1989), influenza virus RNA metabolically labelled with 32P-orthophosphate was extracted from virions by the modified method of Both & Air (1977). The virion proteins of both VSV and influenza A/WSN/33 virus were separated by electrophoresis on 12-5% polyacrylamide–SDS gels, electroblotted onto nitrocellulose membranes and tested for their capacity to bind 32P-labelled RNA of influenza virus by the autoradiographic method of Bowen et al. (1980).

Fig. 2 compares the electrophoretically separated viral proteins of influenza A/WSN/33 virus with those of VSV wt and mutant tsO23 stained with Coomassie blue and autoradiographs after reaction with 32P-labelled influenza virion RNA. As previously described (Pal et al., 1985), the VSV wt M protein migrated faster than the mutant M protein of tsO23. The marked binding affinity for 32P-labelled RNA of the influenza virion M1 protein and nucleoprotein (NP), but not the other proteins of virions (Fig. 2, lane 3) is consistent with earlier observations (Ye et al., 1989). Unexpectedly, the M proteins of VSV wt and tsO23 showed a marked capacity to bind 32P-labelled influenza virus RNA compared to the failure of
the N protein or other VSV structural proteins to bind RNA (Fig. 2, lanes 4 and 5).

The affinity of the VSV M protein to bind influenza virus RNA prompted us to test its capacity to down-regulate transcription of influenza virus RNP cores (devoid of endogenous M1 protein) by reconstitution in a transcription assay with purified VSV wt M protein. We found no significant capacity of VSV wt M protein to inhibit influenza virus RNP transcription under these experimental conditions. In fact, there appears to be no justification for assigning a biological function to the RNA-binding activity of the VSV M protein for still another reason; the mutant tsO23 M protein also strongly binds 32P-labelled influenza virus RNA (lanes 3, 4 and 5) by the method of Ye et al. (1989).

In conclusion, the M1 protein of influenza A/WSN/33 virus down-regulates transcription of VSV RNP cores in a reconstituted in vitro transcription assay system. It seems likely that the binding site on RNP cores for M1 protein differs from the RNP-binding site for endogenous VSV M protein, based on the failure of wt or mutant tsO23 M protein to impede the transcription inhibition activity of reconstituted M1 protein. Moreover, the

References


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