In Vitro Synthesis of Triphosphate-Initiated *N*-Gene mRNA Oligonucleotides Is Regulated by the Matrix Protein of Vesicular Stomatitis Virus

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Wild-type Indiana virus transcribed four 11- to 14-nucleotide-long, 5' *N*-gene mRNA sequences in vitro. The amount of oligonucleotides synthesized relative to leader by wild-type virions varied inversely with the salt concentration of the transcription reaction. Reduced oligonucleotide synthesis by nucleocapsids at all salt concentrations tested and a comparison of the proteins remaining bound to the template of nucleocapsids and virions transcribed in different NaCl concentrations suggested that the matrix (M) protein regulates oligonucleotide synthesis. Examination of the transcription products synthesized in no NaCl and 0.144 M NaCl by an M-protein mutant and an increase in oligonucleotide synthesis by nucleocapsids when purified M-protein was added to transcription reactions confirmed M-protein's role in oligonucleotide synthesis. Wild-type virion mRNA synthesis was inhibited, and oligonucleotide synthesis was greater than leader synthesis at high virus concentrations. As the virus was diluted, inhibition of mRNA synthesis was relieved and oligonucleotide synthesis was reduced. The M-protein mutant *tsG33* exhibited neither transcription inhibition at high virus concentrations nor the reciprocal synthesis of mRNA and the oligonucleotides seen with wild-type virions. These results are entirely consistent with the stop-start model of transcription and suggest a model for the control of transcription by M-protein.

In vitro transcription of wild-type (wt) vesicular stomatitis virus (VSV) is inhibited when virions are present at high protein concentrations (>0.2 mg/ml). Previous reports indicate that the virally coded matrix (M) protein is responsible. In contrast to virions, inhibitor activity is absent from ribonucleoprotein cores containing only RNA and the L, N, and NS proteins. Purified M-protein inhibits transcription by ribonucleoprotein cores (3). Data from in vivo experiments comparing the amounts of mRNA produced by group III temperature-sensitive (*ts*) mutants and wt virus correlate with the in vitro data; group III mutants, which have a lesion in the M-protein, produce two- to five-fold more mRNA than wt virus at the nonpermissive temperature. This increased production of mRNA is abolished when cells are coinfectd with group III mutants and wt virus (5). Collectively, the data strongly implicate M-protein as an inhibitor of viral mRNA synthesis.

M-protein can apparently interact with both the nucleocapsid and the membrane fractions of the virions. Salts, such as NaCl, as well as nonionic detergents are necessary to remove M from the nucleocapsid when virions are dissociated. This suggests that M, which is highly basic

($pI, \approx 9.1$), has an electrostatic association with either the template or a template-bound protein(s) (3). Previous experiments have not conclusively demonstrated how M-protein inhibits transcription. However, recent reports indicating that nucleocapsids exhibit inhibition only if they retain M-protein were interpreted to mean that M inhibits transcription by binding to the nucleocapsid (15).

Previously, we described a discrete group of small transcripts that corresponded to the 11 to 14 nucleotides of the uncapped 5' terminus of *N*-gene mRNA. These oligonucleotides were consistently synthesized 8 to 10 times more than leader RNA by every wt Indiana stock tested. In all cases, the transcriptions were performed at the standard salt concentration (0.144 M NaCl), using high protein concentrations of virus (>0.4 mg/ml). From experiments presented in this paper, we have determined that the amounts of the oligonucleotides relative to leader can be varied dramatically by changing the salt concentration or the virus concentration in the transcription reaction. Using these data and the data obtained from transcription assays with nucleocapsids and M-protein mutants, we show that M-protein regulates the synthesis of the 5'

N-gene mRNA oligonucleotides. Further, we demonstrate that there is a reciprocal synthesis of mRNA and oligonucleotides that can be correlated with the presence or absence of wt M-protein. Finally, we propose a model to explain inhibition of transcription by M-protein in vitro and extrapolate this to explain M-protein's function in vivo.

MATERIALS AND METHODS

The procedures used in these experiments were identical to those previously described (11), with the following exceptions.

Virus. *tsG33*, an M-protein mutant of the Indiana serotype (12), was grown in BHK-21 cells as described previously (9) except that the incubation temperature was 31°C. Radiolabeled virions were prepared by including 5 μ Ci of [3 H]leucine per ml of the growth medium.

In vitro transcription assays. Reactions of 15 μ l were carried out for 3 h, and the RNA products were processed as described previously. When salt titrations were performed on viruses, the solubilizer contained 10 times the final NaCl concentration; when nucleocapsids were titrated, 3 μ l of purified nucleocapsids was added to 12 μ l of reaction mix containing the appropriate concentration of NaCl. Virus protein concentration was determined by the method of Lowry et al. (8), and the stock virus was diluted with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.9, before use.

Nucleocapsid preparation. Ribonucleoprotein cores were prepared as previously reported (3). A 1.8-ml portion of virus in 10 mM Tris-hydrochloride, pH 7.4, was mixed with 1.8 ml of solubilizer (10 mM Tris-hydrochloride, pH 7.4, 10% glycerol, 0.72 M NaCl, 2% Triton X-100, and 0.8 mM dithiothreitol). The solubilized virus was immediately layered over 1 ml of 20% glycerol underlaid with 0.2 ml of 100% glycerol in a cellulose nitrate tube (0.5 by 2 in. [1.27 by 5.08 cm]). Centrifugation was at 40,000 rpm for 2 h at 5°C in an SW50.1 rotor. The supernatant was removed with a Pasteur pipette down to the 20 to 100% glycerol interface, which contained a visible nucleocapsid band. The nucleocapsids in glycerol were mixed with 2 volumes of 10 mM HEPES, pH 7.9, for use in transcription assays. To prepare nucleocapsids and soluble proteins for gel analyses, 50 μ l of [3 H]leucine-labeled virus was mixed with 50 μ l of solubilizer (82 mM HEPES, pH 7.9, 10 mM dithiothreitol, 2% Triton X-100, 75 mM MgCl₂) containing 1.44, 0.36, or 0 M NaCl. After 5 min at room temperature, each sample was layered into a cellulose nitrate tube (0.1875 by 1.6563 in. [ca. 0.476 by 4.21 cm]) containing 500 μ l of a solution consisting of 20% glycerol and 50% solubilizer so that the final concentration of NaCl was 0.72, 0.18, or 0 M, respectively. Centrifugation was at 40,000 rpm for 90 min at 4°C in an SW50.1 rotor. The top 100 μ l contained the solubilized proteins, whereas the nucleocapsid was in the pellet.

Protein gel analysis. Solubilized proteins or virions were mixed with an equal volume of sample buffer (6.25 mM Tris-hydrochloride, pH 6.8, 4% sodium dodecyl sulfate [SDS], 4% dithiothreitol, 0.01% bromophenol blue), whereas the nucleocapsid pellets

were resuspended directly in the sample buffer. After the samples were heated at 100°C for 3 min, they were electrophoresed at 200 V on a discontinuous Tris-glycine-buffered acrylamide slab gel containing SDS (7). A 12.5% running gel and a 5% stacking gel were used. Protein bands were located by staining with 0.5% Coomassie brilliant blue in 50% methanol–10% acetic acid. For quantitation, bands were excised from the gel, incubated with 1 ml of Nuclear-Chicago solubilizer, and counted in complete toluene-based scintillation fluid.

M-protein preparation. Purified M-protein was a generous gift of J. McGowan and was prepared, as described, by chromatography on phosphocellulose columns (16). The column-purified M-protein was dialyzed into 10 mM Tris-hydrochloride, pH 7.4. Either 3 or 6 μ l of M-protein suspension at 150 μ g of protein per ml was substituted for the same volume of HEPES in the transcription assays.

RESULTS

In the accompanying paper (11), we identified four 11- to 14-nucleotide-long transcripts as the uncapped 5' termini of *N*-gene mRNA. These oligonucleotides were consistently synthesized 8 to 10 times more frequently than leader. We showed that the majority of the oligonucleotides were released from the template and, therefore, could not serve as precursors to mature mRNA. However, only one set of reaction conditions was used, and it was feasible that an alteration in these conditions would affect oligonucleotide production. Therefore, we have manipulated the in vitro transcription conditions to determine the relationship between oligonucleotide and mRNA synthesis.

Effect of NaCl concentration on oligonucleotide production. Baltimore et al. demonstrated that in vitro transcription reactions have an absolute need for Mg²⁺, nonionic detergent, and nucleotides, but only a partial dependence on salt and reducing agent (1). Further, several researchers have shown that M-, L-, and NS-proteins are released from the template above specific salt concentrations (2, 6). To determine whether salt concentration affected oligonucleotide production, VSV virions were transcribed under various NaCl concentrations ranging from 0 to the reported optimum, 0.144 M. Analysis of the transcription products on 20% polyacrylamide gels (Fig. 1) revealed that, at 0.072 and 0.144 M NaCl, leader and mRNA were produced in addition to the 5' *N*-gene mRNA oligonucleotides and several other short RNA species. Below 0.072 M NaCl, the only detectable transcription products were the 5' *N*-gene mRNA oligonucleotides and leader.

At low salt concentrations, total RNA synthesis (Fig. 2) was drastically reduced; however, synthesis of the oligonucleotides did not decrease proportionally. As the salt concentration

FIG. 1. Autoradiograph of a 20% polyacrylamide gel containing the $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ -labeled transcripts synthesized by equal amounts of wt Indiana in vitro in various salt concentrations. RNA synthesized for 3 h at 31°C was phenol extracted, ethanol precipitated, and lyophilized. Samples were resuspended in 8 M urea with xylene cyanol and bromophenol blue before electrophoresis. Each lane contains the RNA transcribed in the following final NaCl concentrations: A (0.144 M); B (0.072 M); C (0.036 M); D (0.018 M); E (0.009 M); F (0.005 M); G (0.0 M). The location of leader (L), the 11- to 14-nucleotide-long 5' *N*-gene mRNA oligonucleotides (11–14), and the dyes (arrows) are noted.

was lowered, the synthesis of mRNA was abolished, whereas the oligonucleotides increased relative to leader (Fig. 3). Since both the leader and the 5' *N*-gene mRNA oligonucleotides reflect initiation of RNA synthesis, these data

indicate that initiation occurs preferentially at the *N*-gene under low salt conditions.

To determine whether preferential transcription of the oligonucleotides at low salt was due to an effect on the polymerase, nucleoprotein cores were prepared. SDS-polyacrylamide gel electrophoresis (Fig. 4) of the nucleocapsid proteins showed that >92% of the G- and M-proteins were removed whereas essentially all of the N-, NS-, and L-proteins remained template bound (Table 1). Nucleocapsids were transcribed under the same salt conditions used for virions. Electrophoresis of the transcripts revealed that, regardless of the salt concentration, transcription of leader and mRNA species predominated (Fig. 5). Of the four 5' *N*-gene mRNA oligonucleotides, only the 13- and 14-nucleotide-long bands could be seen. The intensities of these bands were reduced relative to leader when compared with whole virus and were not responsive to salt concentration (Fig. 3). In contrast to the results with whole virus, total RNA synthesis by nucleocapsids was reduced only gradually as the salt concentration was lowered (Fig. 2). These data indicate that the polymerase proteins, L and NS, are not greatly affected by salt concentration. Therefore, some other virion component removed during nucleocapsid preparation must control production of the oligonucleotides.

FIG. 2. Total RNA synthesis by wt virions and ribonucleoprotein (RNP) cores in vitro in various salt concentrations. Total RNA synthesis was determined as $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ -containing trichloroacetic acid-precipitable material per microliter of transcription mixture after a 3-h incubation at 31°C .

FIG. 3. Oligonucleotide synthesis relative to leader RNA synthesis by wt virions and ribonucleoprotein (RNP) cores in vitro in various NaCl concentrations. The 11- to 14-nucleotide-long 5' N-gene mRNA oligonucleotides and leader were isolated from 20% polyacrylamide gels containing the [α - 32 P]UTP-labeled transcripts synthesized at 31°C for 3 h. The radioactivity in each band was determined by the method of Cerenkov. Since there are four times more UMP residues in leader than in each of the N-oligonucleotides, the molar ratio of oligonucleotide to leader was calculated by the formula: (counts per minute in oligonucleotide band/counts per minute in leader band) $\times$ 4. Symbols: $\bullet$, virion 11 mer; $\blacksquare$, virion 12 mer; $\bullet$, virion 13 mer; $\blacktriangle$, virion 14 mer; $\circ$, RNP core 13 mer; Δ , RNP core 14 mer.

Oligonucleotide production is correlated with presence of M-protein. Studies have shown that nonionic detergent alone is capable of removing the viral envelope and G-protein, but that the salt concentration must be raised to remove M-protein (3, 6). To determine which proteins remained template bound during transcription at different NaCl concentrations, virions were treated with the same solutions used as part of the whole-virus transcription assays, and the template was separated from solubilized proteins by centrifugation. SDS-polyacrylamide gel electrophoresis (Fig. 4) of the template-bound and solubilized proteins showed that, under all conditions of solubilization, >94% of the G-protein was removed (Table 1) whereas all N-protein remained template bound. In contrast,

FIG. 4. Protein gel analysis of the proteins solubilized by treating virions with 0.72 M (lane 1), 0.18 M (lane 2), and 0.0 M (lane 3) NaCl; the proteins remaining template bound after virions were treated with 0.72 M (lane 4), 0.18 M (lane 5), and 0.0 M (lane 6) NaCl; the proteins in whole virus (lane 7); the proteins remaining bound to the template of ribonucleoprotein cores (lane 8); and the proteins solubilized during the preparation of ribonucleoprotein cores (lane 9). Proteins were electrophoresed on a 12.5% polyacrylamide slab gel containing SDS and visualized by Coomassie brilliant blue staining.

the amount of M-protein removed was proportional to the NaCl concentration. Comparison of the proteins remaining template bound and the transcription patterns produced by virus at dif-

TABLE 1. Ratio of proteins relative to N-protein^a

Sample	Ratio				
	M	N	NS	G	L
Whole virus ^b	0.65	1	0.15	0.47	0.25
Nucleocapsids ^c	0.05	1	0.15	0.03	0.21
Virus pellets ^d					
0.72 M NaCl	0.03	1	0.06	0.02	0.03
0.18 M NaCl	0.04	1	0.11	0.03	0.18
0.0 M NaCl	0.10	1	0.11	0.03	0.22

^a Stained protein bands were cut out of a 12.5% SDS-polyacrylamide slab gel (see Fig. 4) and incubated for 2 h at 50°C with 1.0 ml of Nuclear-Chicago solubilizer. The radioactivities of the [3 H]leucine-labeled proteins were determined in complete liquid scintillation fluid and reported as a ratio relative to the radioactivity of the N-protein band.

^b wt virions in 10 mM Tris, pH 7.4, were diluted with sample buffer and heated at 100°C for 3 min before electrophoresis.

^c Ribonucleoprotein cores were prepared as described in the text. The ratios of the proteins remaining template bound were determined.

^d wt virions were mixed with an equal volume of 10 $\times$ solubilizer containing 1.44, 0.36, or 0 M NaCl. After 5 min at room temperature, each preparation was centrifuged to separate the template-bound and soluble proteins. The ratios of the proteins remaining template bound were determined.

FIG. 5. Autoradiograph of a 20% polyacrylamide gel containing the [α - 32 P]UTP-labeled transcripts synthesized by ribonucleoprotein cores in various NaCl concentrations, in the absence or presence of purified M-protein. Ribonucleoprotein cores were prepared and transcribed as described in the text. Column-purified M-protein in 10 mM Tris-hydrochloride, pH 7.4, was added to some of the reaction mixtures. Each lane contains transcripts synthesized for 3 h at 31°C under the following conditions: A, 0.144 M NaCl; B, 0.072 M NaCl; C, 0.036 M NaCl; D, 0.018 M NaCl; E, 0.0 M NaCl; F, 0.144 M NaCl–30 μ g of M-protein per ml; G, 0.036 M NaCl–30 μ g of M-protein per ml; H, 0.018 M NaCl–30 μ g of M-protein per ml; I, 0.036 M NaCl–60 μ g of M-protein per ml; J, 0.018 M NaCl–60 μ g of M-protein per ml; K, 0.072 M NaCl–60 μ g of M-protein per ml; L, wt virions control–0.144 M NaCl. Arrows indicate the positions of the dye markers.

ferent salt concentrations indicates a direct correlation between the amount of M-protein remaining bound and the extent of oligonucleotide production.

If the M-protein is involved in oligonucleotide production, adding it back to nucleocapsids should result in an increase in the appearance of oligonucleotides. M-protein from wt virus was purified by column chromatography and added to nucleocapsid transcription reactions conducted under various salt concentrations. Autoradiographs of polyacrylamide gels (Fig. 5) showed that oligonucleotide production by nucleocapsids increased with the addition of M-protein when the NaCl concentration was below 0.072 M.

A temperature-sensitive M-protein mutant is

unable to synthesize standard amounts of the oligonucleotides. A temperature-sensitive mutant of Indiana virus, *tsG33*, has been characterized as a group III (M-protein) mutant (12). As additional evidence that M-protein is involved in the production of the oligonucleotides, we compared in vitro transcription by wt virus and *tsG33* at the two extreme salt concentrations, 0 and 0.144 M NaCl, at 31 and 39°C. Total RNA synthesis and the ratio of the 13-nucleotide-long oligomer (13 mer) and 14 mer to leader was determined (Table 2). Only these two oligonucleotides were quantitated because the 11 mer and 12 mer were not detected. As determined previously, total RNA synthesis by wt virus was drastically reduced, whereas oligonucleotide production increased relative to leader at 31°C and 0 M NaCl compared with that at 31°C and 0.144 M NaCl. In contrast, total RNA synthesis by *tsG33* increased by >2.5-fold at the lower salt concentration at 31°C. Relative oligonucleotide synthesis of the mutant remained constant at both salt concentrations at 31°C. At the higher temperature, wt virus incorporated poorly at both salt concentrations, although preferential production of the oligonucleotides at 0 M NaCl compared with 0.144 M NaCl was still apparent (data not shown). Temperature studies have shown that polymerase is rapidly inactivated in vitro at 39°C. Total RNA synthesis by *tsG33* at 0.144 M and 39°C was low, comparable to wt virus at 39°C. Notably, total RNA synthesis by the mutant at 0 M NaCl and 39°C was greater, approaching the level noted at 31°C. Again, relative oligonucleotide production remained low at both salt extremes at 39°C (data not shown).

Virus concentration affects oligonucleotide synthesis. Several researchers have shown that VSV exhibits transcription inhibition at high protein concentrations (3, 10). It was postulated that this inhibition is due to M-protein. With these studies in mind, wt virus was tested at various concentrations in transcription assays at the standard (0.144 M) salt concentration. Polyacrylamide gel analysis (Fig. 6) showed that as virus concentration was reduced from the undiluted stock at 1.042 mg/ml to 0.261 mg/ml, the 13 mer-to-leader molar ratio dropped linearly from 13.16 to 2.59, and the 12 mer-to-leader molar ratio dropped linearly from 2.8 to 0.7 (data not shown). Total [α - 32 P]GTP incorporation into trichloroacetic acid-precipitable material indicated that there was inhibitory activity when the virus concentration was above 0.347 mg of protein per ml (Fig. 7). These results suggest that, as inhibition of transcription is relieved by diluting the virus, the amount of oligonucleotides synthesized relative to leader is reduced.

The effect of virus concentration on *tsG33* was

TABLE 2. Oligonucleotide synthesis relative to leader and total RNA synthesis by wt Indiana and *tsG33* at 31°C in 0.144 and 0 M NaCl

Virus	NaCl concn (M) ^a	Total incorporation (cpm) ^b	Oligonucleotide-to-leader molar ratio ^c	
			13 mer	14 mer
Indiana wt	0.144	61,764	1.3	0.6
	0.0	1,265	11.8	34.0
<i>tsG33</i>	0.144	67,307	0.7	0.5
	0.0	171,836	0.6	0.4

^a wt virions and *tsG33* were allowed to transcribe, using [α -³²P]UTP as the labeled substrate in either 0.144 or 0 M NaCl for 3 h at 31°C. The products were phenol extracted, ethanol precipitated, and lyophilized. Each sample was resuspended in sample buffer and electrophoresed on a 20% polyacrylamide gel.

^b Total RNA synthesis was determined as the radioactivity contained in each microliter of the final reaction mixture found as trichloroacetic acid-precipitable material.

^c Oligonucleotide bands and leader RNA, visualized by autoradiography, were cut out, and the radioactivities were determined by the method of Cerenkov. Ratios were determined by the formula: (counts per minute in each oligonucleotide band/counts per minute in leader RNA) $\times$ 4.

similarly examined. The mutant did not exhibit transcription inhibition at high protein concentration (Fig. 7). Paralleling this, oligonucleotide synthesis relative to leader remained low and essentially constant at all virus concentrations (Fig. 6); the 13 mer-to-leader molar ratio varied from 0.14 to 0.42, and the 12 mer-to-leader molar ratio varied from 0.42 to 0.56 (data not shown). These ranges did not correlate with the virus dilution. Only the 13 mer and 12 mer were quantitated because they were the only oligonucleotides synthesized in detectable amounts.

DISCUSSION

The results presented demonstrate that the M-protein of wt VSV regulates the synthesis of the 11- to 14-nucleotide-long 5' *N*-gene mRNA sequences observed in vitro. wt nucleocapsids which lack M-protein and an M-protein mutant synthesized mainly leader and mRNAs regardless of the transcription conditions. Significant amounts of *N*-gene mRNA oligonucleotides were synthesized only when wt M-protein was present and transcription conditions such as low salt or high protein concentrations were used to increase M-protein interaction with the template.

The effect of salt concentration was informative for a number of reasons. The optimum NaCl concentration for VSV Indiana virions is generally considered to be around 0.1 to 0.15 M. However, the M-protein mutant synthesized more RNA as the NaCl concentration was lowered below 0.1 M, whereas nucleocapsids lacking M were relatively insensitive to NaCl concentration. These results indicate that the transcriptase is as active or more active at intermediate or low salt concentrations than at

0.144 M NaCl. Therefore, the normal in vitro NaCl concentration of 0.1 to 0.15 M reflects the requirement for M-protein removal from template for optimal transcription.

The most striking result of the salt concentration effect was the demonstration of an inverse relationship between synthesis of the oligonucleotides and mRNAs. As oligonucleotide synthesis increased, mRNA synthesis decreased and vice-versa. At the extremes, the oligonucleotides were virtually the only RNAs made by virions in no salt, whereas under the same transcription conditions, nucleocapsids lacking M made only trace amounts of oligonucleotides but synthesized large quantities of mRNAs. This demonstration of a reciprocal relationship between the synthesis of triphosphate-containing *N*-gene mRNA oligonucleotides and capped, full-length mRNAs suggests a precursor-product relationship. Unfortunately, since the oligonucleotides do not remain template bound, we have not yet been able to chase them into capped mRNA. However, the very low ratio of leader to oligonucleotide synthesis under low salt or high protein concentrations shows that the transcriptase can initiate extremely efficiently at the beginning of the *N*-gene. Although others have demonstrated low levels of internal initiation relative to leader (4, 13, 14), we found that under low salt conditions initiation at the *N*-gene occurred up to 25 times as often as at the leader. These results are difficult to reconcile with the RNA-processing model that has been proposed, but are completely consistent with the stop-start model of transcription in which initiation occurs independently at each gene. From our results, we cannot conclude directly that genes distal to the *N*-gene are also transcribed

FIG. 6. Autoradiograph of a 20% polyacrylamide gel containing the [α - 32 P]GTP-labeled transcripts synthesized by *tsG33* and wt virions in vitro at various virus concentrations. Virus concentrations were determined as milligrams of protein per milliliter. The RNA synthesized in standard transcription reaction mixes for 1 h at 31°C was phenol extracted, ethanol precipitated, and lyophilized. Samples were resuspended in 8 M urea with xylene cyanol and bromophenol blue before electrophoresis. Each lane contains RNA synthesized by *tsG33* or wt virions at the following concentrations: *tsG33*—A (1.042 mg/ml); B (0.78 mg/ml); C (0.521 mg/ml); D (0.346 mg/ml); E (0.261 mg/ml); F (0.064 mg/ml). Samples A to C were diluted and approximately equal numbers of counts per minute were applied to the gel. wt—G (1.042 mg/ml); H (0.782 mg/ml); I (0.521 mg/ml); J (0.347 mg/ml); K (0.261 mg/ml); L (0.064 mg/ml). Arrows indicate the positions of dye markers.

by a start-stop mechanism. Although other small triphosphate-starting RNAs were made, their concentrations were too low for us to identify from which gene(s) they were transcribed. However, Testa et al. have reported that triphosphate-initiated oligonucleotides 28, 40, and 72 nucleotides long, which represent the uncapped 5' termini of the *NS*-, *N*-, and *M*-gene mRNAs, respectively, are synthesized in vitro (14).

FIG. 7. Total RNA synthesis in vitro by wt virions and *tsG33* at various virus concentrations. Transcriptions were for 1 h at 31°C in 0.144 M NaCl, using [α - 32 P]GTP as the labeled substrate. Total RNA synthesis was determined as the radioactivity in 4 μ l of final reaction mixture found as trichloroacetic acid-precipitable material. Virus concentrations are reported as milligrams of protein per milliliter.

Therefore, initiations can also occur at the other VSV genes, and the low levels of respective oligonucleotide synthesis may reflect a different degree of M-protein interaction with these genes.

Since M-protein regulates the synthesis of a discrete class of oligonucleotides, it must inhibit transcription by binding to a specific site on the template. Since transcription is sequential, binding to the beginning of the *N*-gene would inhibit transcription of all distal genes as well. Our data also show that the oligonucleotides are released from the template. Therefore, we propose that the inhibition of transcription by M-protein is a necessary step in preparing the viral nucleocapsid for budding. According to this model, M-protein interacts with the nucleocapsid, causing the release of nascent RNA chains and preventing further movement of the polymerase along the template. In this way, the polymerase would remain bound to the template but any RNA chains would be cleared so that packaging could proceed efficiently. When the released virus particle infected a cell, removal of M-protein from the template during the normal uncoating process would release the transcription block and RNA synthesis could begin immediately.

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