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Cell-Free Translation of RNA Synthesized In Vitro by a Transcribing Nucleoprotein Complex Prepared from Purified Vesicular Stomatitis Virus

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The RNA species synthesized in vitro by a transcribing nucleoprotein (TNP) complex of vesicular stomatitis virus (VSV) were translated with high efficiency in a fractionated cell-free system derived from reticulocytes. The use of TNP complexes isolated from VSV Indiana, VSV New Jersey, and Chandipura viruses showed that in each case the predominant polypeptides synthesized had electrophoretic mobilities identical to their virion N, NS, and M polypeptides in proportions reflecting those found in infected cells rather than purified virions. A minor polypeptide corresponding to unglycosylated polypeptide G was also observed, but the in vitro synthesis of polypeptide L was not detected. The addition of RNase inhibitor to transcription mixtures markedly increased the rate of RNA synthesis. Furthermore, the messenger activity of the RNA was significantly enhanced. The inclusion of *S*-adenosyl L-methionine during transcription substantially increased the messenger activity of the product RNA, suggesting a requirement for methylation. Fractionation by oligodeoxythymidylic acid-cellulose chromatography revealed that the RNA required a polyadenylic acid tract for messenger activity.

Vesicular stomatitis virus (VSV) is a rhabdovirus containing a single negative strand of RNA as the genome (11). This RNA codes for the five known virus-specified polypeptides: the large (L), the glycosylated (G), nucleocapsid (N), minor structural (NS), and matrix (M) polypeptides (26).

The use of cell-free systems active in the translation of added mRNA provides an extremely critical method of analyzing individual RNA species. This technique has been used in the study of VSV to identify the size and intracellular location of the mRNA's for the five known virus-specified polypeptides (8, 12, 15). Furthermore, it has been possible to transcribe and translate in vitro the mRNA's for the N, NS, and M polypeptides using purified VSV virions as the source of the transcriptase (9). The synthesis of unglycosylated G polypeptide has also been demonstrated (8), but the transcription and translation in vitro of polypeptide L has not been achieved so far. Subviral particles have also been shown to be active in the synthesis of mRNA's for the polypeptides N, NS, M, and G (10, 24), and recently the use of disrupted virions in a coupled in vitro transcription and translation system has given evidence for the order of the VSV cistrons (2).

A transcribing nucleoprotein (TNP) derived from VSV virions which is active in transcription in vitro was described by Szilágyi and Urvayev (23). This particle is infectious and therefore can probably perform accurate mRNA synthesis in infected cells. It is known that TNP contains only polypeptides L, N, and NS in detectable amounts (22, 23); hence it was of interest to determine whether this subviral particle was capable of the same functions in vitro as previously reported for purified virus.

We report that a commercially available "RNase inhibitor" preparation greatly stimulates transcription by the TNP as well as the messenger activity of the RNA product. We have developed a cell-free system derived entirely by fractionation of mouse and rabbit reticulocytes for the translation of in vitro synthesized RNA. Linking these in vitro systems, we have investigated the transcription and translation of RNA made on TNP templates.

MATERIALS AND METHODS

Chemicals and isotopes. Oligodeoxythymidylic acid [oligo(dT)]-cellulose type 7 was obtained from P-L Biochemicals, Inc., Milwaukee, Wis. L-[^{35}S]methionine (specific activity, 300 to 500 Ci/mmol) and [5,6-^{3}H]uridine triphosphate (specific activity, 42 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks., England.

Mouse reticulocytes were the generous gift of G. Lanion.

RNase inhibitor preparation was the rat liver RNase inhibitor supplied by Searle, High Wycombe, Bucks., England. A 500-U/ml solution of the RNase inhibitor was made by dissolving it in a solution containing 20 mM Tris-hydrochloride (pH 8.0), 1 mM dithiothreitol, and 1 mM EDTA, and it was stored at −70°C.

Growth and purification of viruses. The growth and purification of VSV Indiana, VSV New Jersey, and Chandipura virus in BHK cells (13) were performed by the method previously described for VSV Indiana (21).

TNP preparation. TNP complex from the purified virus suspensions was prepared by the method of Szilágyi and Uryvayev (23) using the modifications described by Szilágyi and Pringle (22). The TNP preparation contained 1.33 mg of protein per ml.

Transcription mixtures. The conditions for RNA transcriptase of VSV (3, 21-23) were modified to resemble more closely those of the cell-free translation system.

The 0.2-ml transcription mixtures contained (in order of addition) the 20 mM Tris-hydrochloride buffer (pH 7.4) needed to make the final volume up to 0.2 ml, 0.04 ml of TNP preparation, 0.01 ml of 20 mM dithiothreitol, 0.02 ml of 500 U of RNase inhibitor per ml, 0.01 ml of 2 M KCl, 0.01 ml of "reagent mixture" (containing 20 mM Tris-hydrochloride [pH 7.4], 2.56 mM CTP, 2.56 mM GTP, 1 mM UTP, 60 μCi of [5,6-^{3}H]uridine 5'-triphosphate per ml, which was evaporated immediately before use, and 64 μg of actinomycin D per ml), and 0.005 ml of 1 mM *S*-adenosyl L-methionine (SAM). After mixing, a zero-time 0.01-ml sample was taken, the transcription mixture was raised to 31°C, and after 1 min RNA synthesis was started with 0.005 ml of a solution containing 80 mM $MgCl_2$ and 32 mM spermidine. During the incubation, 0.01-ml samples were placed on Whatman no. 1 filter paper disks. The disks were dried and washed seven times with 5% trichloroacetic acid containing 0.04 M sodium pyrophosphate, twice in ethanol, and twice in diethyl ether. After drying the disks were placed in toluene-based scintillant, and radioactivity was counted in an Intertechnique SL 30 liquid scintillation spectrometer. The washed zero-time samples gave approximately 35 cpm, and unwashed 0.01-ml samples gave approximately 20,000 cpm.

In the washed samples 1,000 cpm represent approximately 160 ng of newly synthesized RNA, on the assumption that 25% of bases in the RNA are uracil.

Preparation of the cell-free translation system. For the in vitro protein synthesis we used the fractionated reticulocyte system of Schreier and Staehelin (19), except that rat liver pH 5 fraction and purified reticulocyte ribosomal subunit were replaced by a mouse reticulocyte pH 5 fraction. The ribosomal salt-wash fraction was obtained from either mouse or rabbit reticulocytes.

Preparation of pH 5 and ribosomal salt-wash fractions. Reticulocytosis was induced in New Zealand White rabbits by subcutaneous injection of 1 ml of 2.5% phenylhydrazine on four consecutive days. After 2 further days, the animals were bled and the blood was centrifuged at 3,000 × *g* for 15 min. All subsequent steps were performed at 4°C. The reticulocytes were washed four times with a buffered salt solution (10 mM Tris-hydrochloride, pH 7.4, 130 mM NaCl, 7.4 mM $MgCl_2$, 5 mM KCl) and lysed by resuspending in 3 volumes of a hypotonic solution (3 mM $MgCl_2$ and 1 mM dithiothreitol). After 1 min, 0.25 volume (relative to the lysate) of a hypertonic solution (1.5 M sucrose and 150 mM KCl) was added, and the lysate was centrifuged at 30,000 × *g* for 20 min. The clarified lysate was centrifuged at 100,000 × *g* for 30 min, and the supernatant and polyribosomal pellets were separated.

To the supernatant, 2 volumes of 1 mM dithiothreitol was added, the pH of the solution was adjusted to 5.1 by dropwise addition of 1 M acetic acid, and the formed precipitate was collected by centrifugation at 2,000 × *g* for 10 min and then resuspended in buffer A (0.2 M sucrose, 0.1 M NH_4Cl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 20 mM Tris-hydrochloride buffer, pH 7.4). This "pH 5 fraction" has been shown to contain ribosomal subunits and monomers, elongation factors, tRNA, and activating enzymes (20). The pH fraction was stored at −70°C.

The polyribosomal pellet was resuspended in buffer C (0.25 M sucrose, 40 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM EDTA, and 20 mM Tris-hydrochloride, pH 7.4) at a concentration of 200 absorbancy units at 260 nm per ml, and 4 M KCl was added dropwise to give a final concentration of 0.5 M. The suspension was stirred for 30 min and centrifuged at 100,000 × *g* for 2 h. The supernatant was collected and solid ammonium sulfate was added gradually to a final concentration of 30% (wt/vol). The precipitate obtained after centrifugation at 15,000 × *g* for 15 min was discarded. Solid ammonium sulfate was added to the supernatant to give a final concentration of 70% (wt/vol). The precipitate was collected by centrifugation at 15,000 × *g* for 15 min and dissolved in buffer D (10% glycerol, 0.16 M KCl, 1 mM dithiothreitol, 0.2 mM EDTA, 20 mM Tris-hydrochloride, pH 7.4). This fraction, the "ribosomal salt-wash" fraction, was dialyzed overnight against 1,000 volumes of buffer D and stored at −70°C.

The pH 5 fraction and ribosomal salt-wash fraction were both necessary for full activity of the in vitro protein-synthesizing system. For the experiments reported here, the pH 5 fraction was derived only from mouse reticulocytes, but both rabbit and mouse reticulocytes were used as a source of the ribosomal salt-wash fraction. Each preparation of ribosomal salt-wash fraction was titrated to determine the optimum concentration for the stimulation of protein synthesis.

In vitro protein synthesis. The cell-free translation system (25 μl) contained (final concentration): 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.6; 70 mM KCl; 2 mM magnesium acetate; 0.8 mM spermidine trihydrochloride (adjusted to pH 7.6); 1 mM ATP; 0.1 mM GTP; 0.6 mM CTP; 4 mg of creatine phosphate per ml; 200 μg of creatine phosphokinase per ml; 50 μM

each of the 19 common amino acids except methionine; 300 to 500 μCi of L-[^{35}S]methionine per ml; 2.5 μl of pH 5 fraction (containing 220 μg of protein); 1.5 to 2.5 μl of ribosomal salt-wash (containing 20 to 40 μg of protein).

To this cell-free translation system samples (ranging from 1.0 to 4.0 μl) of transcription mixtures were added and incubated at 30°C for 2 h, when 25 μl of a terminating solution (100 mM EDTA, 100 mM L-methionine, 300 μg of RNase A per ml) was added. Incubation at 30°C was continued for 15 min, and 2.5-μl samples were spotted onto Whatman no. 1 disks (2.5 cm in diameter), which were then immersed in 5% trichloroacetic acid containing 100 mM L-methionine. The disks were washed three times with 10% trichloroacetic acid, twice with 5% trichloroacetic acid, and twice with ethanol, dried, and counted using a toluene-based scintillant.

Proteins were precipitated from the remainder of the reaction mixtures by the addition of 80% acetone and kept for 30 min at 0°C. The precipitates were collected by centrifugation at 2,000 × *g* for 10 min, dried, and dissolved in 25 μl of electrophoresis buffer (50 mM Tris-hydrochloride, pH 6.7, 5% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, and 6 M urea). Samples were heated at 100°C for 3 min, and 25 μl of electrophoresis diluent buffer (50 mM Tris-hydrochloride, pH 6.7, 10% glycerol, 0.05% bromophenol blue) was added.

Polyacrylamide gel electrophoresis. Samples were analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis as described by Marsden et al. (14). Gels were analyzed either by autoradiography, using Kodak KD54T film, or by fluorography (6), using Kodak RP Royal X-Omat film.

Oligo(dT)-cellulose column chromatography. A transcription mixture was incubated for 2 h and placed in a 0.8-ml cellulose nitrate centrifuge tube on top of a preformed sucrose gradient made by layering 0.05 ml each of 50, 45, and 40% sucrose in transcription mixture buffer. After centrifugation at 100,000 × *g* for 5 h at 2°C, the supernatant was collected. This procedure removed TNP and template-bound RNA.

To the supernatant fraction, 4 M KCl was added to give a final concentration of 0.4 M. This was then applied to a column containing 0.2 g of oligo(dT)-cellulose, equilibrated at 0°C with a high-salt buffer (0.4 M KCl, 25 mM HEPES, pH 7.4, 2 mM magnesium acetate, and 0.8 mM spermidine). The column was washed with high-salt buffer, and 0.1-ml samples were collected. When no further labeled RNA was eluted, the column was transferred to 37°C. The polyadenylic acid [poly(A)]-containing RNA was eluted with 5 mM HEPES, pH 7.4, 0.1-ml fractions were collected, and the fractions containing the RNA peak were stored at −20°C.

RESULTS

RNA synthesis by TNP Indiana serotype. Modification of the conditions for in vitro RNA synthesis by TNP has permitted the direct addition of samples of transcription mixture to the cell-free translation system without the need for prior extraction of RNA. Under these reaction conditions RNA synthesis was similar to that obtained with the optimal conditions (23), but only about half as much RNA was synthesized.

Cell-free protein synthesis by various rhabdoviruses. Without the addition of transcription mixture, the cell-free translation system produced globin as the only major polypeptide (Fig. 1, track 7). Since globin has a much lower molecular weight than any of the virus polypeptides, its production did not interfere with the results. Other polypeptides were synthesized in such small amounts that they provided only a very low background.

When samples from transcription mixtures containing TNP of VSV Indiana, VSV New Jersey, or Chandipura virus were added, the predominant polypeptides synthesized had sim-

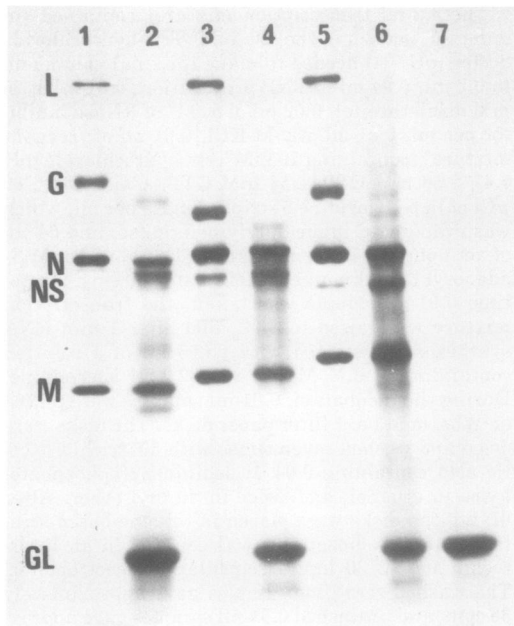

FIG. 1. *Polypeptides synthesized in response to in vitro produced RNAs by TNP of various rhabdoviruses. Samples (0.004 ml) from the transcription mixtures were taken after 2 h of incubation. Track 1, Virion polypeptides of Chandipura virus; track 2, in vitro synthesized polypeptides in the presence of transcription mixture containing TNP Chandipura; track 3, virion polypeptides of VSV New Jersey; track 4, in vitro synthesized polypeptides in the presence of transcription mixture containing TNP New Jersey; track 5, polypeptides of the virion of VSV Indiana; track 6, in vitro synthesized polypeptides in the presence of transcription mixture containing TNP Indiana; track 7, polypeptides synthesized in vitro without the addition of any transcription mixture.*

ilar electrophoretic mobilities to the N, NS, and M polypeptides of their respective virions (Fig. 1). The relative proportion, however, resembled more closely those found in infected cells than in purified virions (27). In each case there was a minor polypeptide approximately 5,000 daltons smaller than polypeptide G of the virions. Presumably these are the unglycosylated precursors of polypeptide G characterized by Both et al. (8) and Knipe et al. (12). Additional minor polypeptides were detected, but in no case was there any evidence for in vitro synthesis of polypeptide L.

The linked RNA-protein-synthesizing system appears to be very efficient, since 160 ng of in vitro produced RNA gave maximum protein synthesis.

The demonstration of serotype-specific differences in electrophoretic mobility of in vitro synthesized polypeptides strongly suggests that the cell-free systems are accurate in the transcription and translation of mRNA for the N, NS, M, and unglycosylated G polypeptides of three different rhabdoviruses.

Effect of RNase inhibitor preparation on cell-free RNA and protein synthesis. A component of the RNA transcription mixture, which, as far as we know, has never previously been used, is the so-called RNase inhibitor prepared from rat liver by G. D. Searle and Co. Ltd. The addition of this preparation even at low concentrations (12.5 U/ml) doubled the rate of RNA synthesis (Fig. 2). Increasing further the concentration of the RNase inhibitor resulted in a lesser increase in RNA synthesis, until a plateau was reached at a concentration of about 100 U/ml. Further addition of this preparation (e.g., 150 U/ml) reduced the total amount of RNA synthesized. Separate experiments established that this large increase was due to an active stimulation of the RNA transcriptase and not merely to the inhibition of the action of RNase, only trace amounts of which could be detected in the TNP (manuscript in preparation).

When a sample of RNA transcription mixture containing no RNase inhibitor was added to the cell-free translation system, only small quantities of virus polypeptides were produced (Fig. 3, track 2). However, when the transcription mixture contained 12.5 U of RNase inhibitor per ml, there was a large increase in the protein synthesis (track 3), which is presumably due to the increased messenger activity of the in vitro synthesized RNA since the two

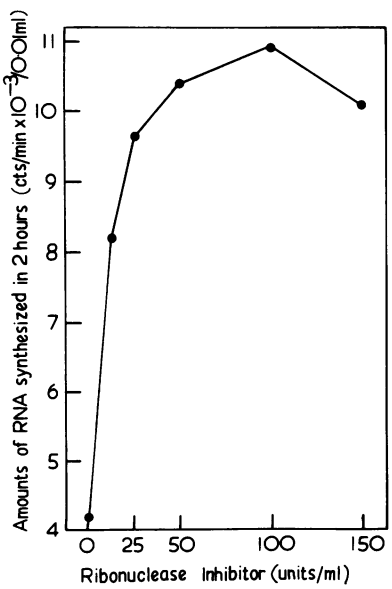

FIG. 2. *Effect of the RNase inhibitor on the in vitro RNA synthesis by TNP of VSV Indiana serotype. Transcription mixtures containing TNP Indiana and increasing amounts of Searle's rat liver RNase inhibitor were incubated for 2 h and 0.01-ml samples were taken.*

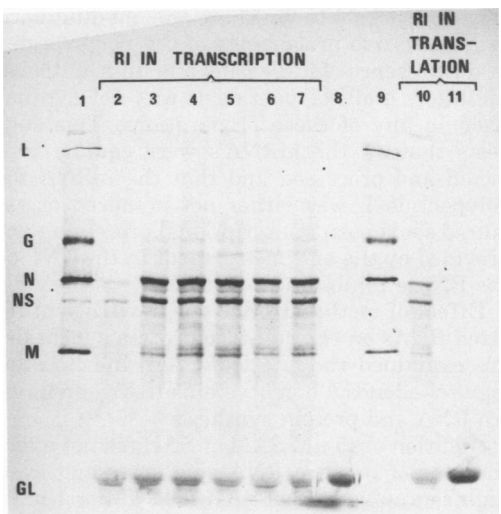

FIG. 3. *In vitro protein synthesis in response to transcription mixtures containing increasing amounts of RNase inhibitor. Samples, each containing 1,700 cpm of the transcription mixtures of Fig. 2, were added to the translation systems. The transcription mixture contained 0 units (track 2), 12.5 (track 3), 25 (track 4), 50 (track 5), 100 (track 6), and 150 (track 7) U of RNase inhibitor (RI) per ml. In track 8 no transcription mixture was added to the translation system. Tracks 10 and 11 are identical to tracks 2 and 8, respectively, except that 150 U of RNase inhibitor per ml was added in addition to the other ingredients. Tracks 1 and 9 show the virion polypeptides of VSV (Indiana).*

samples were adjusted to contain equal amounts of RNA. Furthermore, when RNase inhibitor was added to the cell-free translation system after the addition of a RNA transcription mixture containing no RNase inhibitor (track 10), there was a substantial increase in protein synthesis, but this was not nearly so marked as when the inhibitor was present during RNA synthesis. Thus, it appears that the RNase inhibitor not only increased the rate of RNA synthesis, but also improved the messenger activity of in vitro synthesized RNAs. This may be partly due to the protection of the mRNA's from inactivation by RNases, but it may also be facilitated by enzymes present in the RNase inhibitor preparation responsible for processing of the RNAs into active messages (e.g., capping, methylation). The RNase inhibitor when added to the cell-free translation system without transcription mixtures did not appear to contain any mRNA activity of its own (track 11).

Increasing the RNase inhibitor concentration above 12.5 U/ml in the transcription mixture did not result in further increase in the messenger activity of the in vitro synthesized RNA (Fig. 3, tracks 4 to 7). There was no difference in the relative proportions of the polypeptides in the presence of increasing amounts of RNase inhibitor, and polypeptide L was not synthesized in any of these preparations. This suggests that all the mRNA's were equally protected and processed and that the mRNA for polypeptide L was either not produced or required some post-transcriptional processing not provided by the enzymes present in the TNP or the RNase inhibitor preparation.

Effect of methylation of the in vitro synthesized RNAs on the cell-free protein synthesis. We examined the effect of SAM and its analogue *S*-adenosyl homocysteine (SAH) on in vitro RNA and protein synthesis.

Addition of 25 μM SAM or SAH did not affect the rate of in vitro RNA synthesis, and even high concentrations of SAH (200 μM or 1 mM) decreased the rate of RNA synthesis only to a small extent (results not shown).

Samples from transcription mixtures containing various concentrations of SAM or SAH were assayed for messenger activity in the presence or absence of 200 μM SAH. A moderate amount of virus protein was made when the transcription mixture contained neither SAM nor SAH (Fig. 4, track 2), but addition of 25 μM SAM greatly increased in vitro protein synthesis (track 6). There was slightly less protein synthesized when identical samples of the same transcription mixtures were added to cell-free

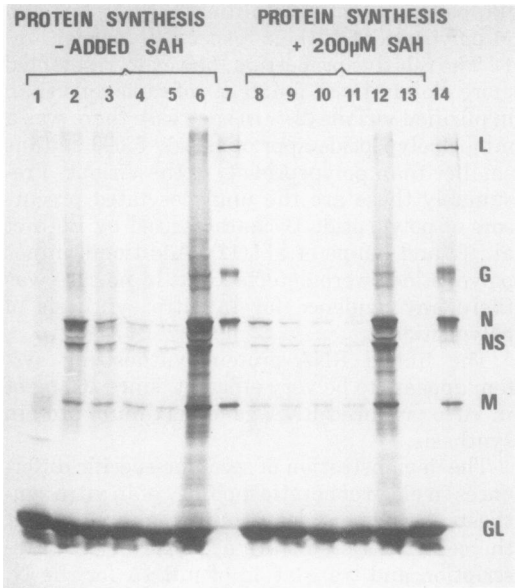

FIG. 4. *In vitro protein synthesis in response to transcription mixtures containing SAM or SAH. Samples of transcription mixtures taken after 2 h of incubation and containing 2,600 cpm of in vitro synthesized RNA were added to two sets of translation systems. The second set (tracks 8 to 12) was like the first (tracks 2 to 6), except that it also contained 200 μM SAH. The added samples of the transcription mixtures contained either no SAM or SAH (tracks 2 and 8) or 25 μM SAH (tracks 3 and 9), 200 μM SAH (tracks 4 and 10), 1 mM SAH (tracks 5 and 11), or 25 μM SAM (tracks 6 and 12). Tracks 1 and 13 contained no transcription mixture, whereas tracks 7 and 14 show the polypeptides of purified virions.*

translation systems, in which further methylation was inhibited by the presence of 200 μM SAH (tracks 8 and 12). This suggests that the translation system requires methylation of the in vitro synthesized RNA and that this process occurs predominantly during transcription of the TNP, although a lesser extent of methylation of the RNA can take place in the translation system. The transcription mixture therefore must contain a methylase activity introduced by the TNP or the RNase inhibitor preparation.

High concentrations of SAH in the transcription mixture did not completely suppress protein synthesis (Fig. 4, tracks 3 to 5 and 9 to 11), indicating that unmethylated RNA possesses a small amount of messenger activity.

In vitro protein synthesis by poly(A)-rich and -deficient RNA. The in vitro synthesized RNAs were separated by oligo(dT)-cellulose column chromatography into two fractions. In

four separate experiments, the fraction that was eluted represented 30 to 35% of the RNA, whereas the remaining 65 to 70% was retained by the column. This indicated that two-thirds of the in vitro synthesized RNA had poly(A)-rich regions. The polypeptides synthesized in the presence of equal amounts of the RNA species separated by this procedure are shown in Fig. 5.

Only small amounts of polypeptides were synthesized in the presence of RNA that was not retained by the column (tracks 3 and 4), whereas a much larger amount of protein was made in the presence of RNA containing poly(A) (tracks 5 and 6). The amounts of polypeptides synthesized in response to poly(A)-rich RNA were similar to that produced by the RNA before addition to the column (tracks 9 and 10) and by a 2:1 mixture of the two fractions (tracks 7 and 8).

Therefore, only that fraction of the released RNA that contained poly(A) tracts, presumably attached to the 3' end, were good templates for protein synthesis. Thus, the transcription mixture must contain enzyme activity responsible for the synthesis of the poly(A) tracts at the end of the mRNA's made in vitro. This enzyme activity must be introduced either by the TNP itself or by the RNase inhibitor preparation.

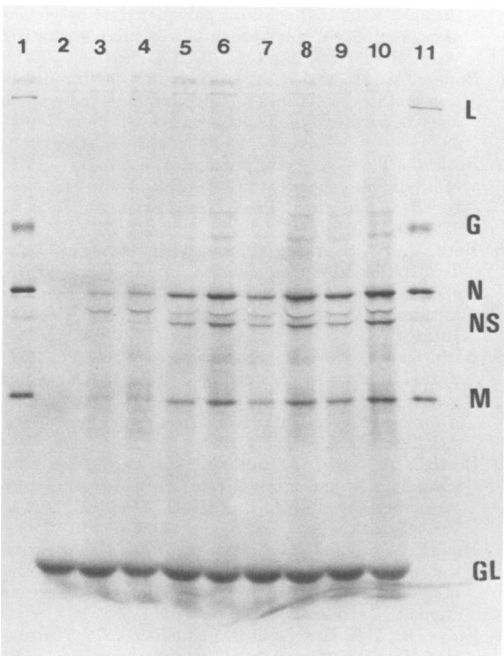

FIG. 5. *Translation of RNA separated by oligo(dT)-cellulose column chromatography. RNA released from TNP was separated into poly(A)-rich and -deficient fractions. In each translation system the amounts of in vitro synthesized RNA were adjusted to represent approximately the same amounts of [^{3}H]UMP. The samples added to the translation systems were either those RNAs that did not bind to column (tracks 3 and 4, representing 200 and 500 cpm) or the RNA bound to the column (tracks 5 and 6, representing 200 and 500 cpm), or a mixture of these two fractions in the proportions of 1:2 (tracks 7 and 8, representing 200 and 500 cpm). For comparison, two translation systems contained 200 and 500 cpm of released RNA (tracks 9 and 10), whereas another translation system did not contain any RNA (track 2). Tracks 1 and 11 show the polypeptides of purified virions.*

DISCUSSION

We have described in vitro protein synthesis, by a fractionated reticulocyte system containing TNP complex, of three different rhabdoviruses. TNP was chosen because we wanted to determine whether it is capable of proper mRNA synthesis.

Only small amounts of viral proteins were synthesized in the cell-free system in response to RNA synthesized in vitro by TNP. However, addition of the RNase inhibitor to the transcription mixture not only increased the rate of RNA synthesis, but also greatly enhanced the messenger activity of the in vitro synthesized RNAs. This effect was so marked that 160 ng of in vitro synthesized RNA saturated the cell-free translation system and as little as 16 ng could be easily detected. Thus, the sensitivity of our system is approximately 10-fold higher than previously reported (9).

Three predominant polypeptides were synthesized corresponding to the N, NS, and M polypeptides of the virion, but since NS was a major product the relative proportions of the in vitro synthesized polypeptides resembled more closely the virus polypeptides in infected cells than those in the virion.

The use of TNP from VSV Indiana, VSV New Jersey, or Chandipura virus resulted in the in vitro synthesis of polypeptides corresponding to their respective N, NS, and M and unglycosylated G in similar proportions, emphasizing the accuracy of the in vitro synthesis of virus mRNA's and polypeptides. Besides these polypeptides, very little else except globin was synthesized, which illustrates the very low background of our cell-free translation system and the high messenger activity of the TNP product RNA.

These properties have enabled an investigation of the requirements for messenger activity of the in vitro synthesized RNA to be carried

out. First, we presume that the mRNA has to be a full-length copy of the cistron. It is relevant that one function of the RNase inhibitor is the inhibition of the traces of RNase known to be present in the TNP preparations.

Second, the mRNA has to be methylated. Since it is known that mRNA's of VSV are capped and the nucleotides involved are methylated both in vivo (17) and in vitro (1, 7, 24), we assume that the mRNA's synthesized in vitro by the TNP are also capped and methylated at the 5′ end. The strong stimulation of message activity in the presence of SAM indicates that methylation occurs mostly during transcription, and therefore modification of the 5′ end may be the first step in in vitro RNA synthesis. It is not established whether the TNP or the RNase inhibitor is the source of the methylase. The conclusion that methylation of mRNA greatly stimulates its messenger activity is in agreement with previous studies using a wheat germ cell-free translation system (7). A recent report, however, shows that the crude rabbit reticulocyte lysate is capable of efficiently translating VSV mRNA which has no capped structure at the 5′ terminus (18).

Third, the in vitro synthesized RNA needs a poly(A) tract in order to act as messenger. The synthesis of poly(A) at the 3′ end of mRNA by virus and subviral particles has been demonstrated previously (4, 5, 16, 25), and two-thirds of the RNA synthesized by our TNP contained such tracts.

Besides the inhibition of RNases and enhancement of messenger activity, the RNase inhibitor may also have other, so far unrecognized effects on the transcription process, for the increase in the rate of RNA synthesis in its presence is far greater than would be expected by the inhibition of trace amounts of RNases in the TNP preparations.

We do not know why polypeptide L was not synthesized, since in other experiments (manuscript in preparation) it was found that a 30S RNA is synthesized in the in vitro transcription system. It is possible that translation of this RNA has requirements that are not met by the components of our transcription and translation systems.

The use of TNP for mRNA synthesis in the presence of the RNase inhibitor preparation resulted in efficient in vitro synthesis of viral polypeptides. This is in good agreement with the infectious nature of the TNP. This system should be readily applicable to the study of other viruses.

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