JOURNAL OF VIROLOGY, Mar. 1982, p. 821–832
0022-538X/82/030821-12$02.00/0

Vol. 41, No. 3

Synthesis of Vesicular Stomatitis Virus Negative-Strand RNA In Vitro: Dependence on Viral Protein Synthesis

NANCY L. DAVIS AND GAIL W. WERTZ*

Department of Bacteriology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

Received 3 August 1981/Accepted 6 November 1981

An in vitro system is described which supports the synthesis of vesicular stomatitis virus (VSV) negative-strand RNA. The major components of this system are (i) an mRNA-dependent rabbit reticulocyte lysate to carry out cell-free protein synthesis, (ii) the five VSV mRNAs to program VSV-specific protein synthesis, and (iii) nucleocapsids containing positive- and negative-strand genome-length RNA. The protein products synthesized in the system in response to addition of saturating amounts of the five VSV mRNA's included polypeptides which comigrated in acrylamide gels with the five VSV proteins. Approximately 200 pmol of protein per ml was synthesized during a 90-min reaction. The RNA products synthesized in the system included all five of the VSV mRNA's and, in addition, negative-strand, genome-sense RNA. All of the negative-strand RNA, which represented 2 to 5% of the total RNA product synthesized in vitro, banded in CsCl at the position of nucleocapsids. All of the mature mRNA's made in the system pelleted in CsCl. This technique allowed a clear separation of negative-strand product from the mRNA products and facilitated further analysis of the negative-strand product. The amount of negative-strand product produced in the system was shown to be a function of the amount of concurrent protein synthesis in the system. An increase in the level of protein synthesis led to an increase in the amount of negative-strand RNA synthesized, whereas inhibition of protein synthesis by cycloheximide resulted in a 70% inhibition of negative-strand synthesis. In contrast to the negative-strand RNA product, the amount of transcriptive product was decreased by 50% in the presence of maximum levels of viral protein synthesis. This inhibition was reversed by adding cycloheximide. Characterization of the negative-strand product by Northern blot analysis demonstrated that negative-strand product was being synthesized which hybridized to all five of the VSV mRNA's and, hence, that product representing all of the VSV cistrons was being made. This in vitro system offers an opportunity to study factors involved in the promotion of VSV genome replication as well as those responsible for the regulation of transcription.

The negative-strand RNA virus vesicular stomatitis virus (VSV) carries out two distinct RNA synthetic processes. One process, transcription, is the synthesis of RNAs that are complementary to the genome and include a 47-base leader RNA and five individual mRNA's (5, 7, 17). The other RNA synthetic process is that of genome replication, in which a complete genome-length complementary (VC) RNA is made and then functions as template for the replication of progeny negative-strand RNAs (16, 18). The two processes are clearly distinguished on the basis of their requirements for protein synthesis. Transcription can occur in the absence of protein synthesis, whereas replication requires concurrent protein synthesis (10, 20). If protein synthesis is inhibited at any stage during VSV infection, even at late times when replication is established, the production of progeny genomes is rapidly inhibited whereas the synthesis of the complementary mRNA's not only continues but continues at an increased rate (20).

The transcription process has been reproduced in highly purified in vitro systems, and reconstitution experiments executed in these systems have shown that three virion proteins are required for transcription (9, 13). These proteins are N, the nucleocapsid structural protein, L, one component of the RNA-dependent RNA polymerase, and NS, a phosphoprotein. This work has also shown that the active template for VSV RNA synthesis is not free viral RNA but RNA coated with the N protein in an RNase-resistant nucleocapsid structure (8). In contrast to these studies of transcription, direct studies of the requirements for replication have

not been possible because a cell-free, in vitro system for the synthesis of VSV genome RNA has not been described.

In this communication we describe a cell-free, in vitro system which supports the synthesis of VSV negative-sense RNA. This study has focused on the second step in replication only, that is, the synthesis of progeny negative-strand RNA from the genome-size, positive-strand RNA template. This has been done to use RNA-RNA hybridization as a specific assay for replicative events against the background of transcriptive events.

In designing a system to support negative-strand RNA synthesis, we accommodated the two requirements for this process previously identified by studies of replication in infected cells, namely, (i) a source of continuous viral protein synthesis and (ii) the appropriate template for this process, the genome-size, plus-strand RNA in a nucleocapsid structure. Specifically, the components of the system include (i) an mRNA-dependent rabbit reticulocyte lysate, (ii) purified VSV mRNA's which are used to program protein synthesis in the reticulocyte lysate, and (iii) nucleocapsids containing both positive- and negative-strand RNA to serve as the templates for RNA synthesis. This report describes this in vitro system for the synthesis of negative-strand RNA and the initial characterization of its protein and RNA products. Additionally, this work shows that replication in this system is sensitive to the levels of attendant protein synthesis and thereby reflects an important characteristic of authentic VSV genome synthesis. Results presented here also show that the balance between levels of replicative and transcriptive events in the system are altered as a function of protein synthesis in the system.

MATERIALS AND METHODS

Cell cultures and virus. Monolayers of a continuous line of baby hamster kidney cells (BHK-21/13) were grown in Eagle minimal essential medium containing 5% heat-inactivated calf serum. The Indiana serotype of VSV was propagated in BHK cells as described previously (20). Cells were infected at an input multiplicity of 2 to 4 PFU per cell and incubated at 37°C in the presence of 4 μg of actinomycin D per ml. At 5 h postinfection, infected monolayers were harvested as described below for isolation of either mRNA or intracellular nucleocapsids.

Preparation of purified VSV mRNA's. At 5 h postinfection, VSV-infected BHK cells were suspended in HBS solution (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.6, 10 mM NaCl, 1 mM $MgCl_2$) and broken by Dounce homogenization. Nuclei were removed by centrifugation at 2,000 × *g*. The supernatant was made approximately 4.5 M with respect to CsCl and 1.5% in *N*-lauryl sarcosine and was layered over 2 ml of 5.7 M CsCl solution containing HBS, 0.1 M EDTA, and 2% *N*-lauryl sarcosine. After 12 to 24 h of centrifugation in a Beckman SW40 rotor at 25,000 rpm and 22°C, the clear RNA pellet was resuspended in sterile water, brought to 0.2 M NaCl–0.2% sodium dodecyl sulfate (SDS), and ethanol precipitated. After a second precipitation with ethanol, mRNA's were isolated by binding to and elution from oligodeoxythymidylate [oligo(dT)]-cellulose in 0.01 M Tris-hydrochloride, pH 7.5, containing 0.02% SDS. Eluted mRNA's were precipitated with ethanol after addition of rabbit liver tRNA carrier and NaCl to 0.2 M. Before use in the in vitro system, the RNA was precipitated with ethanol out of 0.2 M potassium acetate with no SDS.

Preparation of nucleocapsids. At 5 h postinfection, actinomycin-treated, VSV-infected BHK cells were suspended in 3.3 mM Tris-hydrochloride, pH 8.5, 3 mM NaCl, and 1.5 mM $MgCl_2$ and broken by Dounce homogenization. Nuclei and large cell debris were removed by centrifugation. In some cases, a high-speed pellet fraction containing viral nucleocapsids as well as viral polysomes, cellular membrane structures, and other organelles was prepared by centrifugation in a Beckman SW50.1 rotor at 35,000 rpm for 1 h at 4°C. The pellet was gently resuspended in 10 mM HEPES, pH 7.7, 2 mM dithioerythritol, and 10% glycerol. In other experiments the intracellular nucleocapsids were partially purified as follows. The lysed cells were centrifuged at 10,000 × *g* for 10 min, the pellet was rehomogenized in fresh buffer and centrifuged, and the two 10,000 × *g* supernatants were combined. In a siliconized tube, Tris-hydrochloride, pH 8.1, was added to 50 mM, $CaCl_2$ was added to 1 mM, and micrococcal nuclease was added to 10 μg/ml. After 10 min of incubation at 23°C, the nuclease activity was inhibited by addition of 2 mM ethylene glycol-bis(β-aminoethyl ether-*N,N*-tetraacetic acid (EGTA), and the nucleocapsids were sedimented through a 3-ml 15 to 30% (wt/vol) sucrose gradient containing 3 mM Tris-hydrochloride, pH 8, 3 mM $MgCl_2$, 66 mM NH_4Cl, 14 mM KCl, and 2 mM dithioerythritol in a Beckman SW50.1 rotor for 2 h at 45,000 rpm and 4°C. The pellet was gently resuspended and added to the in vitro system as indicated below.

Reticulocyte lysate preparation. Reticulocytes from a single rabbit were purchased from Green Hectares (Oregon, Wis.) and lysed with an equal volume of water. The lysates were clarified by centrifugation at 17,000 × *g* for 10 min, and the supernatants were stored in small portions at −70°C. Immediately before use, the lysate was thawed, brought to 20 mM Tris-hydrochloride, pH 8.1, 50 μg of creatine phosphokinase per ml, 1 mM $CaCl_2$, 30 μM hemin, and 10 μg of micrococcal nuclease per ml, and was incubated for 10 min at 23°C. EGTA was then added to a concentration of 2 mM (14).

In vitro synthesis of viral RNA and proteins. The components of the system for VSV RNA synthesis were combined in a final volume of 50 μl to give the following concentrations: micrococcal nuclease-treated rabbit reticulocyte lysate, 70% by volume; purified viral mRNA's, approximately 100 μg/ml; nucleocapsids, 12 to 20% by volume; HEPES, pH 7.6, 50 mM; creatine-phosphate, 10 mM; ATP, 1 mM; GTP, 0.6 mM; CTP, 0.6 mM; UTP, 0.1 mM; a mixture of 20 amino acids, each at 0.05 mM; magnesium acetate, 2 mM; dithioerythritol, 2 mM; ammonium chloride, 66

mM; potassium acetate, 14 mM; and rabbit liver tRNA, 50 to 60 μg/ml.

Viral proteins were labeled with [^{35}S]methionine in reaction mixtures in which the concentration of unlabeled methionine was reduced to 12.5 μM and to which 3 to 5 μCi of [^{35}S]methionine was added. Viral RNA was labeled in vitro by adding [^{3}H]UTP (1 mCi/ml) or [α-^{32}P]UTP (0.5 to 0.7 mCi/ml) to the complete reaction mixture. The reactions were incubated at 30°C, and the synthesis of RNA or proteins was assayed by counting radioactivity present in a trichloroacetic acid precipitate of 1- to 2-μl samples removed during the incubation. ^{35}S-labeled samples were heated to 95°C for 15 min in 5% trichloroacetic acid before processing to destroy ^{35}S-labeled methionine tRNA. Acrylamide gel electrophoresis of ^{35}S-labeled protein products was performed by use of an SDS-containing 7.5 to 18% gradient polyacrylamide slab gel as described previously (2, 7a). ^{3}H- or ^{32}P-labeled RNA products were purified by phenol extraction and were electrophoresed on 1.5% agarose gels containing 6 M urea (19).

Analysis of reaction products by centrifugation in CsCl gradients. In vitro reaction mixtures were diluted into 2.5 ml of a solution containing 3.3 mM Tris, pH 8.5, 3 mM NaCl, 1.5 mM $MgCl_2$, and 0.5% Nonidet P-40 detergent in siliconized tubes. The solution was pipetted onto a 1.5-ml layer of 5% sucrose covering an 8-ml preformed gradient of 20 to 40% (wt/wt) CsCl. The gradient was centrifuged in a Beckman SW40 rotor at 35,000 rpm for 14 to 16 h at 4°C. Gradients were fractionated by removing 0.5-ml portions from the top. The visible nucleocapsid band was removed and transferred to a siliconized tube. The pellet was resuspended in 0.5 ml of 10 mM Tris-hydrochloride, pH 7.4. The distribution of ^{3}H- or ^{32}P-labeled RNA products was determined by trichloroacetic acid precipitation and scintillation counting of samples of the fractions.

A portion of the nucleocapsid-containing fraction was diluted approximately 30-fold into 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate) for assay of RNase resistance as follows. Identical samples were diluted either into 2× SSC and immediately trichloroacetic acid precipitated or were diluted into 2× SSC containing 25 μg of pancreatic RNase per ml, incubated at 23°C for 30 min, and then trichloroacetic acid precipitated. The remaining CsCl-banded product was desalted by chromatography on Sephadex G-25, phenol extracted, and ethanol precipitated. The purified CsCl-banded RNA was then used in hybridization experiments with unlabeled VSV virion RNA or mRNA's (18).

Affinity chromatography of reaction products on oligo(dT)-cellulose. Reaction products labeled with [α-^{32}P]UTP were solubilized in 1% SDS and extracted with phenol. After precipitation with ethanol, the RNA was dissolved in 10 mM Tris-hydrochloride, pH 7.4, 1 mM EDTA, and 0.2% SDS, heated at 100°C for 90 s, chilled, and brought to 0.4 M NaCl. After passage through oligo(dT)-cellulose, the unbound products were diluted to give 0.2 M NaCl and were precipitated with ethanol. The bound RNAs were eluted with 10 mM Tris-hydrochloride–0.02% SDS and ethanol precipitated. Recovery of RNA products was 90 to 100%, and 40% of the acid-insoluble RNA product was recovered in the unbound fraction. This nonadenylated fraction, which contained all of the negative-strand RNA product, was incubated in 0.2 N NaOH–0.1 N NaCl for 15 min at 4°C, neutralized with 1 M HEPES, and used to probe Northern blots of electrophoretically separated unlabeled VSV mRNA's immobilized on diazobenzyloxymethyl paper (1, 19a).

Materials. Micrococcal nuclease was purchased from Sigma Chemical Co. Rabbit liver tRNA (Sigma) was phenol extracted and precipitated with ethanol before use. Oligo(dT)-cellulose was purchased from Bethesda Research Laboratories. [^{3}H]UTP and [α-^{32}P]UTP were obtained from New England Nuclear, and [^{35}S]methionine, from Amersham Corp.

RESULTS

Components of the in vitro system. The synthesis of genome RNA in the VSV system requires ongoing protein synthesis. Therefore, a primary consideration in establishing a cell-free, in vitro system for genome RNA synthesis was to provide an efficient, continuous source of VSV proteins. Rabbit reticulocyte lysates can be made mRNA dependent by treatment with micrococcal nuclease. These lysates then synthesize proteins efficiently when programmed with exogenous mRNA's (14). Saturating amounts of VSV mRNA's were added to the mRNA-dependent lysates to program viral protein synthesis. The kinetics and products of this reaction will be described in detail below.

The second major consideration in establishing an in vitro system was the provision of the appropriate template for the replication process. The establishment of a system whose goal was the synthesis of negative-strand RNA made it necessary to provide the genome-size, positive-strand RNA in the form of a nucleocapsid. Since positive-strand nucleocapsids exist only in the infected cell (16), intracellular nucleocapsids were isolated for use as templates in this system. Thus, by programming the system with positive-strand as well as negative-strand templates, it was expected that progeny negative-strand RNA could be synthesized directly from the positive-strand templates. Therefore, these experiments do not require that the system first make a complete positive-strand nucleocapsid template for the synthesis of negative-strand RNA.

It was our aim to design a system that could be made dependent on protein synthesis programmed by addition of VSV mRNA's to the messenger-dependent reticulocyte lysate. Therefore, it was necessary to devise a method for separating the intracellular nucleocapsid templates from contaminating cellular debris and, in particular, any viral mRNA's or polyribosomes which our early experiments demonstrated could copurify with the nucleocapsids. This was accomplished by treating cytoplasmic extracts from VSV-infected cells with micrococ-

cal nuclease in the presence of calcium and then inactivating the nuclease by addition of EGTA. This treatment digests mRNA's (14). The genome-size VSV RNAs, however, are present in the cytoplasm only as protein-coated nucleocapsids and were thus protected from digestion by this enzyme under the conditions used (see below and Fig. 1).

The nucleocapsids present in the micrococcal nuclease-treated extract were concentrated and separated from soluble cellular components by pelleting through a 15 to 30% sucrose gradient. Nucleocapsids prepared in this manner were compared with untreated control nucleocapsids and found to cosediment in a 15 to 30% sucrose gradient (data not shown). A comparison of the RNA present in nucleocapsid preparations made either with or without micrococcal nuclease treatment is shown in Fig. 1. Only RNA that comigrates in an agarose-urea gel with marker virion RNA is present in the treated preparation (Fig. 1, lane 3). In contrast, in the untreated preparation viral mRNA's are also present which, presumably, have pelleted with the nucleocapsids as polysomes (Fig. 1, lane 2). Analyses of the polarity of the RNA in the treated nucleocapsid preparation showed approximately 15% positive-strand and 85% negative-strand RNA. Comparison of the levels of [^{3}H]UTP incorporation programmed by parallel preparations of micrococcal nuclease-treated and untreated templates in the in vitro system showed no loss of RNA synthetic activity after nuclease treatment. These results showed that this procedure for the preparation of intracellular nucleocapsids yields intact genome-length templates with full enzymatic activity which are free from viral mRNA molecules.

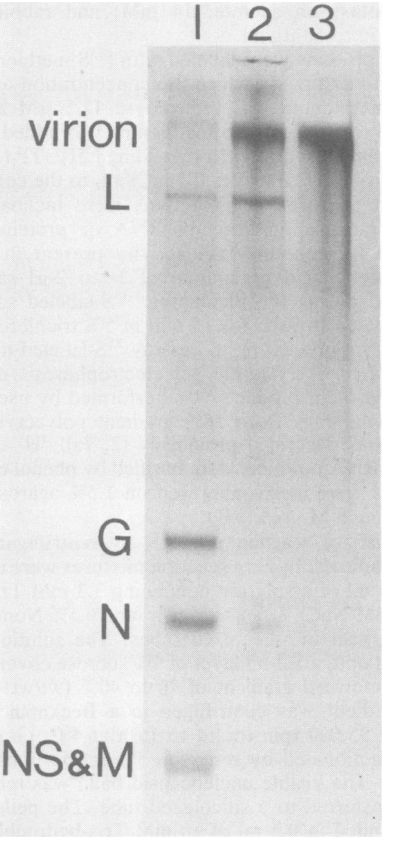

FIG. 1. Agarose-urea gel electrophoresis of template RNA. Micrococcal nuclease-treated intracellular nucleocapsids were prepared as described in Materials and Methods from VSV-infected BHK cells which had been labeled with 10 μCi of [^{3}H]uridine per ml between 4 and 5 h postinfection. An untreated control was prepared from the identical 10,000 × *g* supernatant to which micrococcal nuclease was not added. The RNAs present in the control and treated nucleocapsid pellets were purified by phenol extraction, precipitated with ethanol, and analyzed by electrophoresis in a 1.5% agarose gel containing 6 M urea and 0.025 M sodium citrate, pH 3.0 (19). The gel was processed by fluorography as described (19). Lane 1: ^{3}H-labeled VSV total intracellular RNA marker. Lane 2: RNA in untreated nucleocapsid pellet. Lane 3: RNA in nucleocapsid pellet treated with micrococcal nuclease (10 μg/ml) for 10 min at 23°C.

Protein synthesis. The kinetics of protein synthesis in the in vitro system were assayed by incorporation of [^{35}S]methionine into acid-insoluble material (Fig. 2). Reaction mixtures containing the mRNA-dependent reticulocyte lysates to which 60 to 120 μg of purified VSV mRNA's per ml and the intracellular nucleocapsid templates had been added gave a nearly linear incorporation rate for 90 min at 30°C (Fig. 2, curve A). Addition of this amount of mRNA gave up to a 50-fold stimulation of [^{35}S]methionine incorporation as compared with a reaction without added mRNA. These conditions result in the synthesis of an estimated 10 pmol of viral protein during 90 min in a 50-μl reaction. Addition of cycloheximide (or omission of templates and purified mRNA's, data not shown) to the reaction resulted in 97% inhibition of [^{35}S]methionine incorporation (Fig. 2, curve C).

The nuclease-treated templates, when added to the reaction in the absence of VSV mRNA's, promoted a very low rate of protein synthesis during the first 30 min of incubation (Fig. 2, curve B). However, the rate of protein synthesis began to increase in this reaction after 30 min, and by 90 min the amount of protein synthesized reached a level that was approximately 50% of that made in the complete reaction mixture.

To determine what viral proteins were made in the cell-free system in response to the ad-

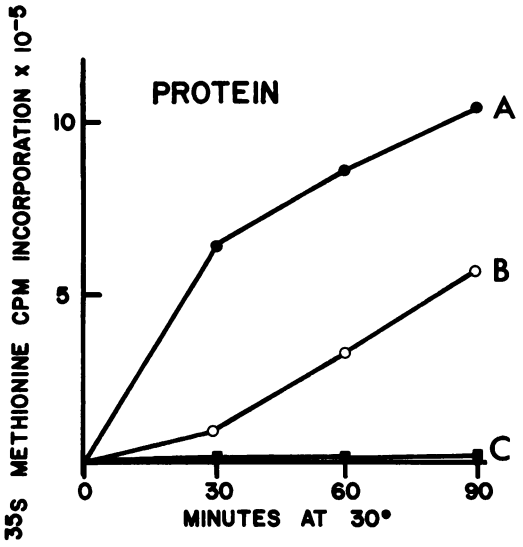

FIG. 2. Kinetics of in vitro protein synthesis. Reaction mixtures (25 μl) were adjusted to contain 12.5 μM methionine, 50 μM of the remaining 19 amino acids, and 3 to 5 μCi of [^{35}S]methionine, and were incubated at 30°C. At various times during the incubation, 2-μl samples were diluted into 10 mM Tris, pH 7.4, 0.2 M NaCl, 1 mM EDTA, and 1% SDS, and were trichloroacetic acid precipitated. Samples were heated in 5% trichloroacetic acid at 95°C for 15 min to hydrolyze ^{35}S-labeled tRNA, collected onto glass-fiber filters, and assayed for ^{35}S counts per minute by liquid scintillation spectroscopy. Total counts per minute of [^{35}S]methionine incorporated in 25 μl is plotted versus time. Reaction mixtures contained: (A) mRNA-dependent reticulocyte lysate, intracellular nucleocapsid templates, and purified VSV mRNA's; (B) reticulocyte lysate and nucleocapsid templates; and (C) same as in A, with cycloheximide (100 μg/ml).

dition of the five purified VSV mRNA's, polypeptides were labeled with [^{35}S]methionine and analyzed by SDS-acrylamide gradient gel electrophoresis. Polypeptides synthesized in a reaction mixture containing the mRNA-dependent reticulocyte lysate and the five mRNA's are shown in Fig. 3. They include the VSV M, N, and NS proteins, two polypeptides which migrate in the region of precursors of G protein, and a high-molecular-weight protein which comigrates with the virion L protein. This result demonstrates that this system for translation of VSV proteins supports the synthesis of proteins which comigrate with each of the five viral polypeptides.

The ^{35}S-labeled protein products synthesized in a reaction mixture containing reticulocyte lysate plus nucleocapsid templates with no added mRNA were analyzed by SDS-polyacrylamide gel electrophoresis after various times of incubation. After 15 min, only a small amount of N protein was detectable; at 30 min, small amounts of NS and M proteins were detectable in addition to increased levels of N protein. By 90 min, a band at the position of the G protein precursor was visible (data not shown). We interpret this finding as showing that newly synthesized mRNA's made in the system are functional and that, in the absence of added mRNA, protein synthesis can be programmed by these new transcripts.

RNA synthesis. The kinetics of RNA synthesis in the in vitro system as assayed by incorporation of [^{3}H]UTP into acid-insoluble material are shown in Fig. 4. The highest levels of RNA synthesis were obtained in a reaction mixture containing nucleocapsid templates and the mRNA-dependent reticulocyte lysate to which no exogenous mRNA was added (Fig. 4, curve A). RNA synthesis was completely dependent on the presence of templates which, when added

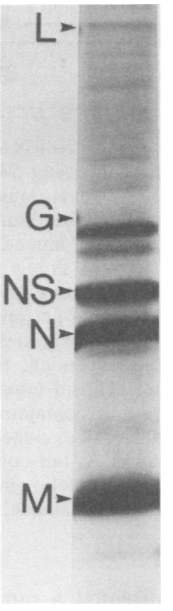

FIG. 3. SDS-acrylamide gradient gel electrophoresis of protein products. A reaction mixture containing the mRNA-dependent reticulocyte lysate, purified VSV mRNA's, and [^{35}S]methionine was incubated as described in Fig. 2 for 90 min. A 2-μl portion of the 25-μl reaction mixture was diluted into sample buffer containing 2% SDS and 0.1 m dithioerythritol, heated at 100°C for 2 min, and electrophoresed in an SDS-containing 7.5 to 18% gradient polyacrylamide gel cross-linked with *N,N'*-diallyltartardiamide (2, 7a). The stacking gel and discontinuous buffer system were as described by Laemmli (11). The gel was assayed by fluorography. The positions of the five VSV proteins were determined by electrophoresis of ^{14}C-amino acid-labeled virion proteins in an adjacent lane.

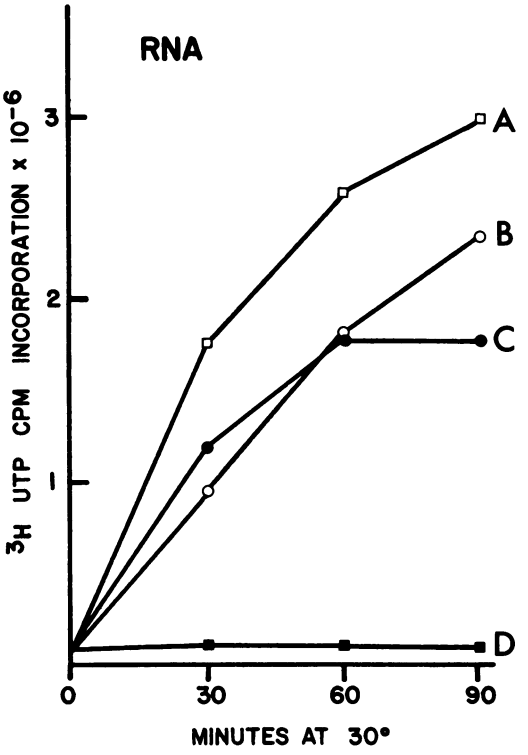

FIG. 4. Kinetics of in vitro RNA synthesis. Reaction mixtures (50 μl) containing 50 μCi of [^{3}H]UTP were incubated at 30°C. At various times during the incubation, 2-μl samples were diluted into SDS-containing buffer as in Fig. 2 and immediately precipitated with 5% trichloroacetic acid. Precipitates were collected onto glass-fiber filters and assayed for ^{3}H counts per minute. Total ^{3}H counts per minute incorporated in 50 μl is plotted versus time. (A) A reaction mixture containing the mRNA-dependent reticulocyte lysate and intracellular nucleocapsid templates; (B) a reaction mixture as in A, but containing 100 μg of VSV mRNA per ml and 100 μg of cycloheximide per ml; (C) a reaction mixture as in A, but containing 100 μg of VSV mRNA per ml; (D) a reaction mixture as in A, but without intracellular nucleocapsids.

to the reaction, directed a rapid rate of RNA synthesis for at least 90 min. The addition of the VSV mRNA's to the system containing the nucleocapsid templates and the mRNA-dependent reticulocyte lysate to promote high levels of protein synthesis at early times led, reproducibly, to a 30 to 50% reduction in the rate of RNA synthesis (Fig. 4, curve C) as compared with the reaction to which no mRNA was added (Fig. 4, curve A). Addition of equivalent amounts of mRNA from uninfected cells did not show this inhibitory effect on RNA synthesis (data not shown). When the translation of the added mRNA's was inhibited by the addition of cycloheximide to the system, the amount of total RNA synthesis reached an intermediate level (Fig. 4, curve B).

RNA products were analyzed by electrophoresis in 1.5% agarose gels containing 6 M urea. As shown in Fig. 5, the predominant products were the five VSV mRNA's, which comigrated with mature mRNA's synthesized in the VSV-infected cell. In addition to the mRNA's, ^{3}H-labeled product was also present which migrated in the region of genome-length RNA. A product with the electrophoretic migration rate of the 47-

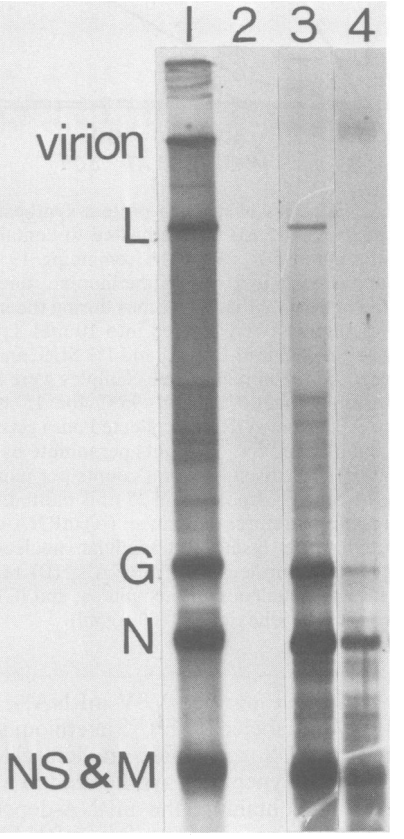

FIG. 5. Agarose-urea gel electrophoresis of RNA products. RNAs synthesized in cell-free reaction mixtures containing [^{3}H]UTP were purified by phenol extraction and analyzed by electrophoresis in 1.5% agarose gels containing 6 M urea (19). ^{3}H-labeled RNAs were visualized by fluorography of the dried gel. Lane 1: ^{3}H-labeled total viral RNA from infected cells. Lane 2: ^{3}H-labeled RNA products of a reaction mixture containing reticulocyte lysate without intracellular nucleocapsid templates. Lane 3: ^{3}H-labeled RNA products of a reaction mixture which included mRNA-dependent reticulocyte lysate and a nucleocapsid-containing high-speed pellet from infected cells. Lane 4: ^{3}H-labeled RNA products of a reaction mixture identical to that used for lane 3 except that purified viral mRNA's were added to program protein synthesis.

base leader RNA was also observed when the products were analyzed by electrophoresis on a 15% acrylamide gel (data not shown). Denaturation of RNA products by heating in the presence of glyoxal reduced the amount of product which migrated in the region of virion RNA, but a detectable amount of this product remained (data not shown). Therefore, although some small products might migrate in this region in hydrogen-bonded structures with unlabeled template RNA, a detectable quantity retained this migration rate after stringent denaturation.

It was possible to increase the rate of protein synthesis in the reaction by adding saturating amounts of viral mRNA. Comparison of the products synthesized in a reaction which contained no added mRNA (Fig. 5, lane 3) with those in a reaction to which mRNA had been added to increase the rate of protein synthesis (Fig. 5, lane 4) shows two marked differences. Addition of mRNA to the reaction resulted in (i) a decrease in the amount of mRNA synthesis and (ii) an increase in the amount of product migrating in the region of genome-length RNA. The intracellular templates used in the experiment shown in Fig. 5 were not treated with micrococcal nuclease. The template fraction therefore contained viral mRNA in polysomal aggregates, and possibly intracellular viral proteins. All other experiments described in this communication were carried out with intracellular templates prepared by treatment with micrococcal nuclease to make the system dependent on the addition of exogenous mRNA for programming of protein synthesis.

This analysis of RNA products yielded valuable information concerning the ability of the system to synthesize bona fide viral RNA products and demonstrated a striking effect of the level of protein synthesis on the RNA products synthesized. However, the analysis of products by agarose gel electrophoresis can give no information regarding the polarity of RNA products. A different method of analysis was needed, therefore, to analyze possible negative-strand products of this system.

Analysis of RNA products by CsCl gradient centrifugation. One of the difficulties inherent in the attempt to analyze VSV genome RNA synthesis is that transcription is by far the predominant RNA synthetic event in this system. Even in infected cells under presumably optimal conditions, replication comprises only 10% of the viral RNA synthetic events. Therefore, to focus on negative-strand RNA synthesis, we had to select for replicative products of the system. Studies of VSV replication in infected cells have shown that genome-size RNA of both positive and negative polarity is present in the cell only in the form of a nucleocapsid structure (16). It seemed likely, therefore, that replicative products might be distinguished from transcriptive products by their association with protein.

To test the possibility that replicative RNA products synthesized in the in vitro RNA synthesis system were being coated with protein, we analyzed the products by equilibrium centrifugation in CsCl. This technique cleanly separates the protein-coated viral nucleocapsids, which band at a density of 1.3 g/cm^3, from the mRNA's, which pellet to the bottom of the CsCl gradient. The products synthesized in reaction mixtures to which purified nucleocapsid templates were added in the presence or absence of added mRNA were analyzed by centrifugation in CsCl. The distribution of the ^{3}H-labeled RNA products after centrifugation is shown in Fig. 6. Approximately 95% of the total product of the in vitro system pelleted. However, a distinct peak comprising 2 to 5% of the ^{3}H-labeled RNA product banded in the CsCl. This peak of labeled product banded exactly at the position of intracellular nucleocapsids when the two were analyzed by cosedimentation. The data in Fig. 6 represent analyses of reactions programmed with templates alone (Fig. 6A) or templates plus added mRNA (Fig. 6B). The amount of material which pelleted through the CsCl was reduced by approximately 50% in the presence of added message. In contrast, in the presence of increased levels of protein synthesis, the amount of material which banded in CsCl never decreased and frequently increased. These results showed that RNA product could be recovered at the position of nucleocapsids and thereby separated from the mature mRNA's which, as subsequent results confirmed, were all in the pellet. This CsCl-banded RNA product was a likely candidate for the presence of negative-strand sequences.

Analysis of RNA products: hybridization. The RNA products separated by centrifugation in CsCl were analyzed by RNA-RNA hybridization, a sensitive assay for the presence of negative-sense replicative products. It was found that all of the negative-sense RNA made in the system was in the material that banded in CsCl (Table 1). The material that pelleted contained no negative-strand RNA product, only positive-sense RNAs.

Analysis by electrophoresis in agarose gels showed that all of the pelleted material comigrated with mature mRNA's from infected cells; no material the size of mature mRNA banded at the position of nucleocapsids (data not shown). The finding that all of the negative-strand product made in the system banded in CsCl in a manner that clearly separated it from the completed RNA transcriptive products which pelleted provided an excellent means of distinguishing and

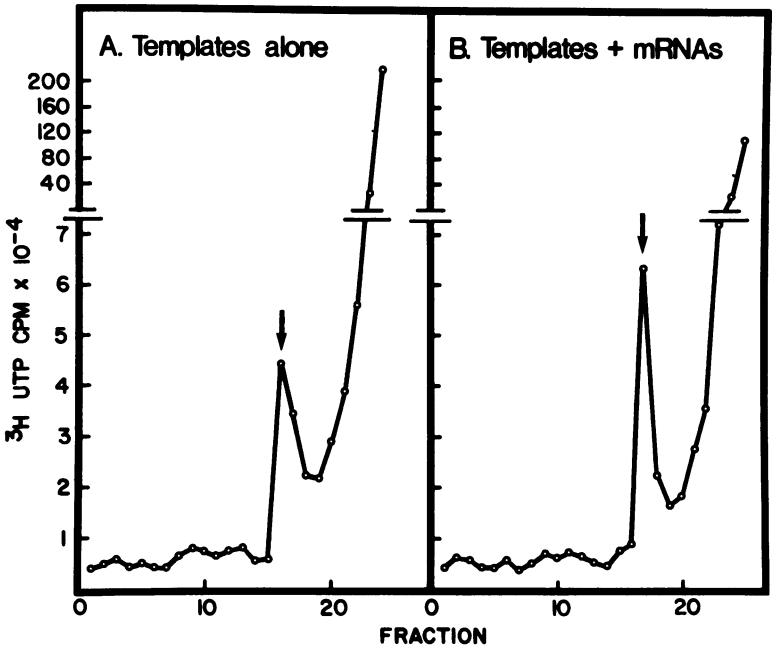

FIG. 6. Equilibrium centrifugation of RNA products in CsCl gradients. Reaction mixtures containing [^{3}H]UTP were incubated for 90 min at 30°C, diluted into buffer containing 0.5% Nonidet P40 and layered onto preformed 20 to 40% CsCl gradients (Materials and Methods). The gradients were centrifuged to equilibrium and fractionated, and the pelleted material was suspended in 10 mM Tris, pH 7.4, and 1 mM EDTA. A portion of each fraction was assayed for trichloroacetic acid-insoluble counts per minute. The arrows indicate the fraction containing cosedimented intracellular nucleocapsid markers. (A) A reaction mixture containing mRNA-dependent reticulocyte lysate and intracellular nucleocapsids. The total number of ^{3}H counts per minute recovered at nucleocapsid density in this reaction was 69,000. The pellet contained 2,607,000 cpm. (B) A reaction mixture as in A, to which approximately 120 μg of purified VSV mRNA's per ml was added. In this reaction 76,000 ^{3}H counts per minute were recovered at the position of nucleocapsids, and 1,146,000 ^{3}H counts per minute were recovered in the pellet.

separating the products of transcriptive and replicative events in this system.

Effect of protein synthesis on negative-strand RNA synthesis. The ability to separate the negative-strand RNA product made in the in vitro system from the transcriptive products and to measure the amount of negative-strand RNA by hybridization made it possible to examine the effect of altered levels of protein synthesis on the synthesis of negative-strand RNA.

The relationship between protein synthesis and negative-strand RNA synthesis in this in vitro system was tested by altering the levels of protein synthesis in the reaction. In these experiments the amount of negative-strand RNA synthesized in the complete system (consisting of nucleocapsid templates and reticulocyte lysate programmed with saturating amounts of exogenous VSV mRNA) was compared with the products synthesized in a parallel reaction to which cycloheximide had been added to prevent protein synthesis, or in a reaction in which exogenous VSV mRNA had not been added at time zero to program protein synthesis. RNA products from the above reactions were labeled with [^{3}H]UTP and separated by banding in CsCl. The products which banded and those which pelleted were quantitated and analyzed for their content of negative and positive sequences by hybridization (Table 1). It was found that 45% of the material that banded in CsCl from the complete reaction carried out in the presence of the highest levels of protein synthesis was negative stranded. In comparison, when protein synthesis was reduced by the omission of mRNA, the percentage of negative-strand sequences dropped to 28%. When 97% of protein synthesis was inhibited by the addition of cycloheximide, only 15% of the product which banded in CsCl was of negative-strand polarity. The increase in the proportion of negative-strand product in the presence of increased protein synthesis represents a net increase in the amount of negative-strand RNA made, since the amount of material banding of CsCl and used for hybridization in that case was always equal to or greater than that in the absence of added mRNA. The decrease in the proportion of negative-strand prod-

TABLE 1. Analysis of RNA products separated by CsCl gradient centrifugation

Prepn	Total cpm	cpm in banded RNA[a]	Analysis of CsCl-banded RNA (cpm)[b]			cpm in pelleted RNA[d]
			Negative strand[c]	Positive strand[c]	RNase resistant	
A. Templates + mRNA added to the reaction	1,002,200	68,780 (6.7)	30,050 (45)	28,720 (43)	27,380 (41)	935,400 (93)
B. Templates alone	1,443,100	68,180 (4.7)	19,090 (28)	49,170 (73)	18,910 (27)	1,375,000 (95)
C. Templates + mRNA + cycloheximide	1,429,400	70,000 (4.9)	10,500 (15)	60,900 (87)	11,200 (16)	1,359,400 (95)

[a] ^{3}H-labeled RNA products which banded at the position of nucleocapsids in CsCl were either tested directly for RNase resistance (Materials and Methods) or desalted, purified by phenol extraction, and used in hybridization reactions (18). Numbers in parentheses are the percentage of the total counts per minute.

[b] Numbers in parentheses are the percentage of banded RNA counts per minute.

[c] Hybridization analysis of RNA products. Values shown are the amount of product protected with saturating amounts of unlabeled RNA probe. Purified mRNA and virion RNA probes were prepared as previously described (18). Each hybridization reaction contained 2,000 to 4,000 ^{3}H counts per minute in RNA product. The reason that 100% protection could not be achieved in all cases may be a result of the fact that the mRNA probe was isolated by velocity sedimentation followed by chromatography on cellulose CF-11 to ensure that no negative-strand RNA was present. Therefore, the entire complement of the genome (i.e., the small "leader" and "trailer" RNAs) probably is not represented.

[d] ^{3}H-labeled RNA products which sedimented to the bottom of the CsCl gradient were ethanol precipitated and used in hybridization reactions. Greater than 95% of pelleted RNA products were rendered RNase resistant by hybridization to purified virion RNA. Purified unlabeled mRNA was unable to protect any counts from RNase digestion in a parallel hybridization. Numbers in parentheses are the percentage of the total counts per minute.

uct in the presence of cycloheximide also represents a net decrease in the production of negative-stranded RNA, since the amount of CsCl-banded RNA product was never greater than that produced in the reaction without cycloheximide.

These results show: (i) that significant quantities of negative-strand, genome-sense RNA are being synthesized in this in vitro system, (ii) that all of the progeny negative-strand RNA is banding in CsCl at the position of nucleocapsids, and (iii) that the level of negative-strand products synthesized in the system is a function of the amount of concurrent protein synthesis in the system. An increase in the amount of viral protein synthesis leads to an increase in the amount of negative-strand RNA that is being made.

In contrast to the increase in negative-strand RNA synthesis in the presence of increased protein synthesis, it was observed that the total amount of RNA synthesis decreased under these same conditions (Fig. 4, curves A and C). That this was a specific inhibition of transcription is supported by the data presented in Fig. 5, lanes 3 and 4, and by the analysis of product on CsCl gradients (Fig. 6), which showed a 50% decrease in the amount of pelleted material, containing only mRNA products, in the presence of increased protein synthesis. In other experiments it was shown that the addition of cycloheximide to the system partially reversed this inhibition (Fig. 4, curve B). Cycloheximide treatment increased the amount of ^{3}H-labeled mRNA products which pelleted through CsCl (1.2×10^6 cpm) as compared with the amount of pelleted RNA product made in a parallel reaction mixture containing exogenous mRNA but no cycloheximide (0.8×10^6 cpm). Thus, the inhibition of total RNA synthesis by increased levels of protein synthesis reflects a specific inhibition of transcription which is partially reversed in the presence of cycloheximide.

The banding of the negative-strand RNA products in CsCl at the position of nucleocapsids and the stimulation of genome RNA synthesis by increased protein synthesis suggested that these RNAs might be associated with viral protein in an RNase-resistant structure similar to that of the mature nucleocapsid. To test this hypothesis, we tested the CsCl-banded RNA products for their resistance to digestion with pancreatic RNase (Table 1). A control preparation of naked, purified virion RNA analyzed at the same time was completely digested under the conditions used in these experiments. A comparison of the CsCl-banded RNA products from the three different reaction mixtures described in Table 1 demonstrated a close correlation between the percentage of product which is negative stranded and the percentage which is RNase resistant. Taken together, these data show that higher levels of protein synthesis in the system were associated with an increased

amount of negative-strand RNA synthesis and an increased amount of RNase-resistant product.

Identification of genomic sequences present in negative-strand product. After we had determined that negative-strand RNA was being synthesized in the system, the next important question was to identify what regions of the genome were being replicated so that we could determine whether the negative-strand product represented sequences from all regions of the genome. To test this possibility, we labeled RNA products synthesized in a 90-min reaction in the complete system with [α-^{32}P]UTP. The labeled negative-strand product was separated from the majority of the transcriptive products by affinity chromatography on oligo(dT)-cellulose. The unbound RNA, which contained all of the negative-strand sequences, was subjected to limited treatment with NaOH to produce molecules no larger than 500 to 1,000 nucleotides. This labeled product was then hybridized to the five VSV mRNA's which had been separated by electrophoresis and transferred by Northern blotting techniques to diazobenzyloxymethyl paper. As shown by the data presented in Fig. 7 (lane 1), labeled product annealed to all five of the VSV mRNA's. The observation that the amount of probe hybridized to the L mRNA appears less than that annealed to the other messages does not indicate that there is less product from that region. Rather, this is a result of the fact that less L mRNA than the other mRNA's was transferred to diazobenzyloxymethyl paper. This is confirmed by the control (Fig. 7, lane 2), which shows that similar proportions of uniformly labeled ^{32}P-virion RNA are hybridized to the mRNA's under identical conditions of hybridization. A control experiment demonstrating the specificity of hybridization in this system is shown in Fig. 7, lane 3, where the ^{32}P-labeled oligo(dT)-bound products made in the system were annealed to diazobenzyloxymethyl-bound unlabeled mRNA. As is clearly shown, no hybridization occurred.

The results of these experiments show that RNA product complementary to all five of the VSV mRNA's, and, hence, product from all of the genes is being synthesized in vitro.

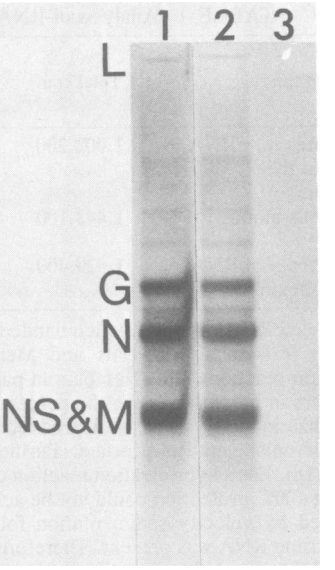

FIG. 7. ^{32}P-labeled RNA product used to probe a Northern blot of purified VSV mRNAs. RNA was labeled with [α-^{32}P]UTP in cell-free reactions containing mRNA-dependent reticulocyte lysate, intracellular nucleocapsids, purified VSV mRNA's, and other components as described in Materials and Methods. ^{32}P-labeled RNA products were purified by phenol extraction, precipitated with ethanol, and chromatographed on oligo(dT)-cellulose. Lane 1: hybridization of RNAs which did not bind to oligo(dT)-cellulose, and which contained all of the negative-strand RNA product, to a Northern blot of electrophoretically separated VSV mRNAs. Lane 2: hybridization of excess ^{32}P-uniformly labeled VSV virion RNA to an identical blot of VSV mRNA as a control to demonstrate the relative amounts of the VSV mRNA's bound to the diazobenzyloxymethyl paper. Lane 3: hybridization of ^{32}P-labeled oligo(dT)-bound RNA synthesized in vitro which contained only mature polyadenlated VSV mRNA's to an identical blot.

DISCUSSION

The results presented in this communication describe the synthesis of VSV negative-strand RNA in an in vitro system. This is the first report of a cell-free system in which negative-strand RNA synthesis is controlled, as it is in the infected cell, by the level of attendant protein synthesis. The major components of the system are an mRNA-dependent rabbit reticulocyte lysate, the five VSV mRNA's, and VSV nucleocapsids. The system is able to synthesize all five viral proteins in response to addition of the five viral mRNA's. Template-dependent RNA synthesis in this system produces the five polyadenylated VSV mRNA's and, in addition, negative-strand RNA.

The experiments described show that increased viral protein synthesis in the in vitro reaction leads to increased negative-strand RNA synthesis. This same increase in protein synthesis also results in a 30 to 50% reduction in mRNA synthesis. Conversely, the reduction of protein synthesis by 97% in the presence of cycloheximide leads to a 70% decrease in the amount of negative-strand RNA synthesized while stimulating mRNA synthesis by 30 to 50%. These results show that factors involved in the regulation of transcription and the promotion of

negative-strand genome RNA synthesis are operating in this system.

A striking finding is that not only are negative-strand, genome-sense sequences made in this system, but, in contrast to the mRNA products, all of the negative-strand RNA product bands in CsCl gradients at the position of viral nucleocapsids. RNA products which band at the equilibrium density of nucleocapsids represent 2 to 5% of the total RNA made in the system, and as much as 45% of this RNA is in negative-strand sequences. The finding that the genome-sense RNA products, unlike the mRNA products, band at the position of nucleocapsids and that their synthesis is dependent on protein synthesis suggests that they have associated, in vitro, with the viral proteins also being synthesized in the system. Support for this suggestion comes from the finding that a significant fraction of these banded RNA products are resistant to RNase. Indeed, the percentage of RNase resistance of the RNA products in the nucleocapsid band correlates very closely with the percentage of RNA products which are negative-stranded. Further experiments are required, however, to determine whether all of the negative-strand RNA which bands at the position of nucleocapsids is present in an RNase-resistant structure containing viral proteins. Positive-stranded RNA sequences are also found in the material which bands in CsCl. At present, it has not been determined whether this material represents immature template-associated mRNA's which are susceptible to RNase digestion or, possibly, the synthesis of positive-strand nucleocapsid templates. We do know, however, that the products which band in CsCl do not include material which migrates as mature mRNA when this material is analyzed by gel electrophoresis. All mature RNA that is synthesized in this system pellets in CsCl.

We have estimated that an average of 10 pmol of viral protein is synthesized in the 50-μl reaction mixture. This estimate is derived from the specific activity of the [^{35}S]methionine (assuming the lysate to contain 9.2 μM methionine [15]), the relative amounts of each mRNA added (assuming that each mRNA is translated with equal efficiency), and the known methionine content of the four small viral proteins (21). A similar calculation leads to an estimate of 0.5 to 1.3 pmol for the amount of mRNA synthesized in the 50-μl reaction mixture. Data presented here have shown that negative-strand RNA synthesis represents approximately 2.5% of mRNA synthesis. Therefore, viral protein is made in much larger quantities than the estimated amounts of negative-strand RNA. Studies of viral nucleocapsid structure have indicated that approximately 1,000 to 2,000 N proteins are associated with the genome-length RNA (12). Our rough calculations serve to point out that the ratio of newly made viral proteins to negative-strand RNA synthesized in the system is sufficiently high to permit their association in a nucleocapsid-type structure.

Northern blot analysis of the negative-strand RNA products made in the system showed that this product contained sequences complementary to all of the five viral mRNA's including the L mRNA which is complementary to the 5′ region of the genome RNA. These results demonstrate that negative-strand product is being synthesized from all cistrons of the genome and that the negative-strand product is not confined to a particular region. The product does not merely represent, for example, synthesis of only a small region of the genome.

At present, we do not know whether initiation of negative-strand synthesis is occurring in this system. The templates used are intracellular nucleocapsids which have been prepared by using micrococcal nuclease treatment to remove exogenous non-protein-coated RNAs. It is possible that these templates may contain nascent progeny RNA molecules which remain attached to the template or that the nascent chains may have been clipped off during template purification. Therefore, it is possible that the product made could represent (i) de novo synthesis from the 3′ end of the template, (ii) elongation of attached nascent strands, (iii) synthesis of RNA by polymerase molecules that remain attached along the template even though nascent tails may have been removed during template purification, or (iv) any combination of the above. Although it is not possible to discriminate among these possibilities at this time, data presented here show that label is incorporated into material that migrates in agarose gels at the position of genome RNA, and Northern blot analysis of product demonstrates that product is being synthesized from all cistrons of the genome. Further experiments will be required to demonstrate whether initiation, elongation, and completion of genome-size, negative-stranded RNA are occurring in this system.

The system described here is similar in concept to cell-free systems described previously for the synthesis of VSV RNAs and proteins (3, 4, 6). In contrast to previous systems, however, there are three differences in the present system that should be noted. First, intracellular nucleocapsids containing both negative-strand and positive-strand RNA are used as templates for viral RNA synthesis instead of mature virions or virion nucleocapsids. The 10 to 20% of these nucleocapsids which contain positive-strand RNA are capable of serving directly as templates for the synthesis of progeny genomes.

This is in contrast to former situations in which negative-strand RNA synthesis could not be initiated until an active positive-strand nucleocapsid template was made. Second, previous reports described "coupled" systems in which the synthesis of viral proteins relied on newly synthesized mRNA to program the translational machinery. Data presented here show that the addition of purified mRNA's to the in vitro system, especially at early times in the incubation, greatly increases the level of viral protein synthesis. The levels of protein synthesis in the system directly correlate with the levels of negative-strand RNA that can be made in the system. Third, the separation of the RNA products by equilibrium centrifugation in CsCl has allowed a significant enrichment for negative-strand products in the nucleocapsid band. Quantitative studies and molecular characterization of the genome-sense RNA can now be carried out in the absence of the large background of transcriptive products.

The system described here demonstrates not only that negative-strand RNA synthesis can be carried out in vitro but also that the synthesis of negative-strand RNA and the relative levels of transcription and replication are dependent upon the level of viral protein synthesis programmed in the system. The negative-strand product made bands in CsCl at the position of viral nucleocapsids and contains sequences from all five viral genes. This translation-assisted, cell-free system for VSV RNA synthesis provides a unique opportunity to study the factors involved in the promotion and regulation of RNA synthesis. Each of the viral proteins can be synthesized in response to purified mRNA's added individually to the in vitro system. Therefore, this system provides a means of testing the relationship of each of the viral proteins to RNA synthesis by programming the in vitro system with the individual mRNA's. These studies are currently underway.

ACKNOWLEDGMENTS

We thank Dennis Hruby for preparation of reticulocyte lysates.

This work was supported by Public Health Service grants AI-12464 and AI15134 from the National Institute of Allergy and Infectious Disease and grant CA 19014 from the National Cancer Institute.

LITERATURE CITED

1. **Alwine, J. C., D. J. Kemp, and G. R. Stark.** 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. U.S.A. **74**:5350–5354.
2. **Anker, H. S.** 1970. A solubilizable acrylamide gel for electrophoresis. FEBS Lett. **7**:293.
3. **Ball, L. A., and C. N. White.** 1978. Coupled transcription and translation in mammalian and avian cell-free systems. Virology **84**:479–495.
4. **Batt-Humphries, S., C. Simonsen, and E. Ehrenfeld.** 1979. Full-length viral RNA synthesized *in vitro* by vesicular stomatitis virus-infected HeLa cell extracts. Virology **96**:88–99.
5. **Both, G. W., S. A. Moyer, and A. K. Banerjee.** 1975. Translation and identification of the viral mRNA species isolated from subcellular fractions of vesicular stomatitis virus-infected cells. J. Virol. **15**:1012–1019.
6. **Breindl, M., and J. J. Holland.** 1976. Studies on the in vitro transcription and translation of vesicular stomatitis virus mRNA. Virology **73**:106–118.
7. **Colonno, R. J., and A. K. Banerjee.** 1976. A unique RNA species involved in initiation of vesicular stomatitis virus RNA transcription in vitro. Cell **8**:197–204.

7a. **Davis, N. L., and G. W. Wertz.** 1981. Preliminary characterization of a cell-free system for vesicular stomatitis virus negative-strand RNA synthesis, p. 789–795. *In* D. H. L. Bishop and R. Compans (ed.), The replication of negative strand viruses. Elsevier/North-Holland, Inc., New York.

8. **Emerson, S. U., and R. R. Wagner.** 1972. Dissociation and reconstitution of the transcriptase and template activities of vesicular stomatitis B and T virions. J. Virol. **10**:297–309.
9. **Emerson, S. U., and Y. Yu.** 1975. Both NS and L proteins are required for in vitro RNA synthesis by vesicular stomatitis virus. J. Virol. **15**:1348–1356.
10. **Huang, A. S., and E. K. Manders.** 1972. RNA synthesis of vesicular stomatitis virus. IV. Transcription by standard virus in the presence of defective interfering particles. J. Virol. **9**:909–916.
11. **Laemmli, U. K., and M. Faure.** 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. J. Mol. Biol. **80**:575–599.
12. **Mellon, M. G., and S. U. Emerson.** 1978. Rebinding of transcriptase components (L and NS proteins) to the nucleocapsid template of vesicular stomatitis virus. J. Virol. **27**:560–567.
13. **Naito, S., and A. Ishihama.** 1976. Function and structure of RNA polymerase from vesicular stomatitis virus. J. Biol. Chem. **251**:4307–4314.
14. **Pelham, R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**:247–256.
15. **Shih, D. S., C. T. Shih, D. Zimmern, R. R. Rueckert, and P. Kaesberg.** 1979. Translation of encephalomyocarditis virus RNA in reticulocyte lysates: kinetic analysis of the formation of virion proteins and a protein required for processing. J. Virol. **30**:472–480.
16. **Soria, M., S. P. Little, and A. S. Huang.** 1974. Characterization of vesicular stomatitis virus nucleocapsids. I. Complementary 40S RNA in nucleocapsids. Virology **61**:270–280.
17. **Stampfer, M., D. Baltimore, and A. S. Huang.** 1969. RNA synthesis of vesicular stomatitis virus. I. Species of RNA found in Chinese hamster ovary cells infected with plaque-forming and defective particles. J. Virol. **4**:154–161.
18. **Wertz, G. W.** 1978. Isolation of possible replicative intermediate structures from vesicular stomatitis virus. Virology **85**:271–285.
19. **Wertz, G. W., and N. L. Davis.** 1979. RNase III cleaves vesicular stomatitis virus genome-length RNAs but fails to cleave viral mRNA's. J. Virol. **30**:108–115.

19a. **Wertz, G. W., and N. Davis.** 1981. Characterization and mapping of RNase III cleavage sites in vesicular stomatitis virus genome RNA. Nucleic Acids Res. **9**:6487–6503.

20. **Wertz, G. W., and M. Levine.** 1973. RNA synthesis by vesicular stomatitis virus and a small-plaque mutant: effects of cycloheximide. J. Virol. **12**:253–264.
21. **Rose, J. K., and C. J. Gallione.** 1981. Nucleotide sequences of the mRNA's encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. J. Virol. **39**:519–528.