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Initiation and replication of vesicular stomatitis virus genome RNA in a cell-free system

(defective interfering particles/transcription)

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ABSTRACT A system for studying the *in vitro* replication of the RNA genomes of both wild-type vesicular stomatitis virus (VSV) and its defective interfering particle MS-T has been developed. After lysolecithin treatment of cells infected with VSV or VSV plus MS-T, a cell-free cytoplasmic extract is prepared which will support VSV mRNA synthesis and the synthesis of the 42S wild-type or 19S MS-T genome RNAs. The genome-length RNAs synthesized *in vitro* are assembled into RNase-resistant nucleocapsids. The level of 42S RNA synthesis *in vitro* (6–13% of total RNA synthesis) reflects the level of replication *in vivo*. Although the extracts of VSV-infected cells can also support the synthesis of VSV proteins, RNA replication is not dependent on *de novo* protein synthesis but utilizes the preformed soluble proteins present in the infected cell at the time the extract is prepared. The initiation of genomic RNA during *in vitro* replication can be demonstrated because detergent-disrupted, purified MS-T particles will replicate their RNA when added to either a total cytoplasmic extract from VSV-infected cells or the soluble protein fraction derived from such an extract.

Reproduction of the rhabdovirus vesicular stomatitis virus (VSV) requires both transcription and replication of the (−)-strand genome RNA. Transcription, but not replication, can be carried out by purified detergent-disrupted virus and, after initiation at the 3′ end, results in the sequential synthesis of first leader RNA and then the monocistronic mRNAs for the N, NS, M, G, and L proteins (1). The intracellular replication of the VSV RNA involves the synthesis of a genome-length (+)-strand RNA which then serves as the template for the production of progeny (−)-strand virion RNA (2, 3). Both of these single-stranded templates are found within the cell as nucleocapsids containing the viral proteins L, N, and NS (2). Earlier studies (4, 5) have suggested that VSV RNA replication, in contrast to transcription, requires viral protein synthesis. In this regard, a replication model has been proposed by Kolakofsky and his co-workers (6–8) which suggests that it is the VSV N protein association with the nascent RNA chains—i.e., nucleocapsid formation—that is specifically required for replication to proceed.

A complication in the study of (−)-strand RNA virus replication is that the genome RNA serves as the template for both mRNA synthesis and replication. In order to focus exclusively on the process of replication, we have used for these studies a defective interfering (DI) particle of VSV designated MS-T. This DI particle contains a genome of 1×10^6 daltons (19 S) that consists only of the 5′ terminus of the virus, representing a portion of the L cistron with a 3′ terminus complementary to the 5′ terminus (7, 9, 10). Because MS-T virion RNA lacks the 3′ initiation signal for transcription as well as most of the VSV genetic information, MS-T particles can neither synthesize mRNAs nor replicate their genome autonomously *in vitro*, although a 46-nucleotide DI leader RNA is produced (11). For its reproduction, the defective MS-T particle requires the presence of wild-type VSV as helper to provide the mRNAs and proteins necessary for RNA replication and particle maturation, while simultaneously inhibiting the replication of the helper VSV genome RNA in the mixed infection (10, 12). The use of intracellular MS-T nucleocapsids therefore should provide templates with 19S RNAs of both the (+)- and (−)-strand sense involved specifically in the process of replication.

MATERIALS AND METHODS

Growth and Purification of Virus. Monolayer cultures of baby hamster kidney (BHK) cells were used for all experiments. The HR strain of VSV (Indiana) (13) was grown as described (14). The MS-T (9) was propagated by using HR-VSV as helper virus and was purified as described (14).

Preparation of Cell Extracts and *in Vitro* RNA Synthesis. Subconfluent monolayers of BHK cells, 3.5×10^6 or 1×10^7 cells in 60- or 100-mm dishes, respectively, were infected with VSV at 20 plaque-forming units/cell with or without stock suspension of MS-T particles (5 μl/10^7 cells) which gives 70–80% inhibition of VSV production. At 4 hr after infection, the cells were treated with L-α-lysophosphatidylcholine, palmitoyl (lysolecithin) (Sigma), at 250 μg/ml for 1 min at 4°C as described by Miller *et al.* (15). [Infected BHK cell monolayers approaching or at confluency were made permeable only poorly by use of this concentration of lysolecithin.] After this treatment the cells were scraped, with a rubber policeman, into 300–500 μl of reaction mixture containing 0.1 M Hepes adjusted to pH 7.4 with KOH, 0.2 M NH_4Cl, 7 mM KCl, 4.5 mM Mg acetate, 1 mM dithiothreitol, 1 mM ATP, 1 mM CTP, 1 mM GTP, the 20 amino acids at 40 μM each, 100 μM S-adenosylmethionine, 1 mM spermidine, creatine phosphokinase at 40 units/ml, 10 mM creatine phosphate, and actinomycin D at 2 μg/ml. The cells were gently disrupted by pipetting with a Pasteur pipette and then centrifuged at 800 × g for 5 min to remove nuclei and cell debris. The resulting cell-free supernatant fluid was used for *in vitro* RNA synthesis after the addition of 200 μCi (1 Ci = 3.7×10^{10} Bq) of [^{3}H]UTP (10 μM; 35–40 Ci/mmol; Amersham) to each reaction in a final volume of 0.5 ml. Samples were incubated at 30°C for various periods of time as described in the text. Purified MS-T particles were disrupted with 0.1% Triton N101 for 5 min at 4°C prior to their addition to *in vitro* reaction mixtures such that the final Triton concentration was <0.006%.

Subviral nucleocapsids were isolated from extracts by centrifugation in an SW 65 rotor at 50,000 rpm for 90 min at 4°C

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Abbreviations: VSV, vesicular stomatitis virus; DI, defective interfering; MS-T, Mudd–Summers DI particle of VSV.

in 0.7-ml tubes that contained 25 μl of 20% (vol/vol) glycerol atop a 25-μl cushion of 96% (vol/vol) glycerol as described by Abraham and Banerjee (16). The viral nucleocapsid fraction was collected from the top of the 96% glycerol cushion, and the remaining supernatant fluid constituted the soluble protein fraction.

Product Analysis. After the *in vitro* reaction, the nucleocapsid product was separated from mRNA by banding in CsCl gradients (17). The nucleocapsid peaks were pelleted and resuspended in 0.1 M NaCl/0.05 M Na acetate, pH 5.1/0.01 M EDTA/0.5% $NaDodSO_4$ containing 50 units of heparin per ml; proteinase K was added to 500 μg/ml and the mixture was incubated at 37°C for 1 hr. After phenol extraction, the RNA was precipitated by the addition of 2.5 vol of ethanol. RNA was analyzed by electrophoresis on either acid/urea/agarose gels (18) or formaldehyde/agarose gels (19) after denaturation at 90°C for 5 min. The gels were processed for fluorography (20) and exposed to x-ray film at −70°C.

RESULTS

Preparation and Characterization of Cell-Free Extracts. A procedure has been developed for the preparation of extracts from VSV-infected cells that will support all virus-associated macromolecular synthesis *in vitro*, including RNA transcription and replication as well as translation. Protein synthesis in such an extract was linear for 30–45 min and then increased at a slower rate up to 2 hr of incubation (data not shown). All five of the VSV proteins—L, G, M, NS, and N—were synthesized *in vitro*, although the synthesis of proteins G and L was diminished.

Total RNA synthesis, as measured by [^{3}H]UTP incorporation *in vitro*, in extracts of VSV-infected cells was linear for 1 hr and increased at a reduced rate thereafter (Table 1). The amount of synthesis of 42S RNA, as determined by its encapsidation into nucleocapsids, could be quantitated by analysis of the products on CsCl gradients. *In vitro* genome replication increased for the first 90 min of incubation, and the newly formed nucleocapsids appeared to be stable to further incubation. In this and other experiments, nucleocapsid synthesis (i.e., replication) represented 6–13% of the total RNA synthesized *in vitro*, a level similar to that occurring *in vivo* (3, 4).

To examine the size of the nucleocapsid-associated RNA synthesized *in vitro*, products derived from extracts of cells infected with either VSV or VSV plus MS-T were analyzed by CsCl gradient centrifugation. In each case, a peak of RNA banded in the position of viral nucleocapsids (Fig. 1 *A* and *B*) which was collected and pelleted. The nucleocapsid-associated RNA was purified and analyzed by electrophoresis on a formaldehyde/agarose gel, conditions under which RNAs are fully denatured (21). Genomic-length 42S VSV RNA was found in nucleocapsids synthesized *in vitro* in extracts from VSV-infected cells, but the

Table 1. VSV RNA synthesis *in vitro*

	^{3}H incorporated, cpm		
Time, min	Nucleocapsid RNA	Total RNA	% replication
0–30	15,246	188,055	8.1
0–60	21,633	363,105	6.0
0–90	30,879	499,725	6.2
0–120	31,593	606,645	5.2

VSV RNA was synthesized *in vitro* in 500-μl extracts derived from 3.5×10^6 VSV-infected cells in the presence of [^{3}H]UTP (200 μCi) for the indicated times. Total RNA synthesis was measured by the incorporation of radioactivity into acid-precipitable material. Nucleocapsid formation was determined by quantitating the product banding as nucleocapsids at 1.31 g/ml in CsCl.

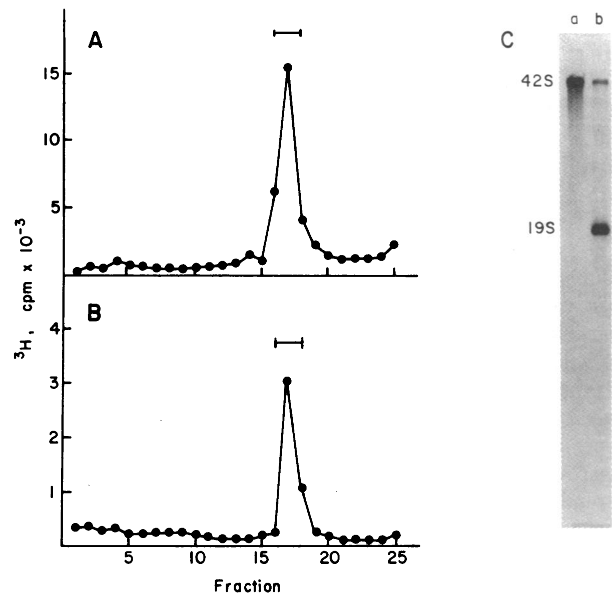

FIG. 1. Analysis of products of *in vitro* VSV RNA synthesis in cell-free extracts from cells infected with VSV alone (*A*) or with VSV plus MS-T (*B*) in the presence of [^{3}H]UTP for 90 min. The products were analyzed separately by centrifugation on 20–40% (wt/wt) CsCl gradients at 33,000 rpm for 17 hr at 4°C in the SW 41 rotor (sedimentation is from left to right). The acid-precipitable radioactivity in a sample of each fraction was determined. The nucleocapsid peaks were pooled as indicated, and the nucleocapsid RNA was purified and analyzed by formaldehyde/agarose gel electrophoresis (*C*). Lanes: a, peak from *A*; b, peak from *B*.

RNA found within nucleocapsids from extracts of cells infected with VSV plus MS-T was predominantly genomic 19S MS-T RNA with lesser amounts of 42S VSV RNA (Fig. 1*C*). These data show that the *in vitro* replication system described here will synthesize and encapsidate both full-length VSV and MS-T particle genomic RNAs.

The VSV genome RNA associated with the nucleocapsids isolated either from virus particles or from the infected cell has been shown to be resistant to RNase digestion, whereas the intracellular viral mRNAs are sensitive to RNase (12). In order to determine if the RNA synthesized and assembled into nucleocapsids *in vitro* was similarly RNase resistant, extracts were prepared from either VSV or VSV plus MS-T coinfected cells and incubated in the presence of [^{3}H]UTP. Samples of the total product were then incubated in the absence or presence of RNases A and T1, and the mRNA and nucleocapsid fractions remaining were quantitated after separation by CsCl gradient centrifugation. In contrast to the virtually complete RNase sensitivity (>98%) of viral mRNA, in each case the *in vitro* synthesized VSV or MS-T nucleocapsid RNAs, like those synthesized *in vivo*, were largely (80–100%) resistant to RNase digestion and thus appear to have been encapsidated.

Initiation of RNA Replication *in Vitro*. Although efficient replication of VSV or MS-T RNAs occurred in the cell-free extracts described here, we considered it of primary importance to establish whether this system is capable of the *de novo* chain initiation of genomic RNA in addition to the elongation and termination of chains that had been initiated in the cell prior to the preparation of the extract. We addressed the question of genome-length RNA chain initiation by utilizing purified MS-T particles which can neither transcribe nor replicate their RNA genome *in vitro* after detergent activation (10). When detergent-disrupted MS-T particles are added to an extract of VSV-infected cells and the synthesis of 19S MS-T genome RNA can be demonstrated in this reaction mixture, then both the ini-

tiation of RNA replication and its elongation must occur *in vitro*.

RNA was synthesized *in vitro* in extracts of VSV-infected cells in the absence or presence of added MS-T particles. Equilibrium centrifugation of the products on CsCl gradients showed that the addition of either 95 or 250 μg of detergent-disrupted MS-T particles to an extract of VSV-infected cells resulted in a 35–40% inhibition of overall nucleocapsid synthesis (Fig. 2). The synthesis of VSV mRNAs found in the pellet of these gradients was only decreased by 7–8% with the addition of MS-T particles (data not shown). These data suggest that the MS-T particles can specifically interfere *in vitro* with the replication of wild-type VSV RNA.

The RNA species present in the nucleocapsids synthesized *in vitro* in the absence or presence of added MS-T particles were analyzed in two different gel systems. Formaldehyde/agarose gels completely denature RNA so that the (+) and (−) strands of both 42S and 19S RNAs migrate as single bands. The less-stringent denaturing conditions of acid/urea/agarose gels, however, allow the separation of the (+) and (−) strands of 19S MS-T RNA (22). Acid/urea/agarose gel electrophoresis of nucleocapsid RNA synthesized *in vitro* in an extract of cells infected with VSV plus MS-T showed the presence of both (−) and (+) sense 19S product RNAs (Fig. 3, lane IA), indicating that replication of intracellular MS-T nucleocapsids with RNA of both strand senses occurred. In this sample, no 42S RNA was synthesized because the mixed infection with VSV plus MS-T severely suppressed the formation of the 42S nucleocapsid template *in vivo*. Lane IB shows nucleocapsid RNA synthesized *in vitro* from a reaction in which 250 μg of purified MS-T particles was added to the extract of VSV-infected cells (from Fig. 2). In addition to the synthesis of genomic 42S RNA from the intracellular VSV nucleocapsid templates, full-length 19S RNA of only the (+)-strand sense was synthesized (Fig. 3, lane IB). This experiment demonstrates that cell-free extracts prepared as described are capable of supporting the initiation and complete elongation of genome-length MS-T RNA *in vitro*.

To eliminate any possible artifact of the acid/urea gel system, nucleocapsid RNA synthesized in a separate experiment was analyzed by formaldehyde/agarose gel electrophoresis. In extracts from either VSV plus MS-T coinfected- or VSV-infected cells, genome-length 42S and 19S or 42S nucleocapsid RNA, respectively, was synthesized *in vitro* (Fig. 3, lanes IIA and IIB, respectively). Furthermore, this second gel system confirmed the *in vitro* initiation of replication of the RNA of added MS-T particles in the VSV-infected cell extract (Fig. 3, lane IIC). When the same MS-T particle preparation was added to an extract of uninfected cells, however, no MS-T RNA replication occurred (Fig. 3, lane IID). In this case, the entire unfractionated RNA product was analyzed to detect any 19S RNA synthesis that might have occurred in the absence of encapsidation whereas only 20% of the nucleocapsid RNA produced in the other reactions was analyzed. These experiments show that extracts of VSV-infected cells, but not of uninfected cells, are fully capable of VSV RNA replication in terms of RNA initiation and elongation. Furthermore, the product genomic RNAs are isolated as nucleocapsids, not as free RNA, indicating that encapsidation of the replicating RNA takes place.

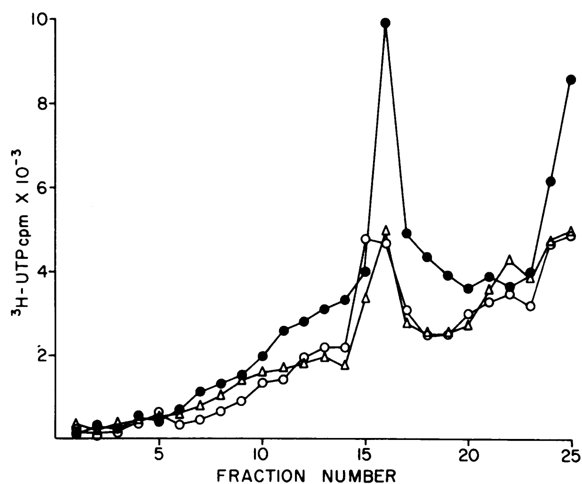

FIG. 2. Equilibrium CsCl centrifugation of *in vitro* RNA products synthesized in extracts of VSV-infected cells in the presence of MS-T particles. RNAs were synthesized *in vitro* in the presence of [^{3}H]UTP in an extract of VSV-infected cells (3×10^7 cells) in the absence or presence of exogenous detergent-disrupted purified MS-T particles. The replication products were analyzed separately by banding on 20–40% (wt/wt) CsCl gradients (sedimentation is from left to right). The acid-precipitable radioactivity in samples of each fraction was determined. ●, in the absence of MS-T; ○, in the presence of 95 μg of MS-T; △, in the presence of 250 μg of MS-T.

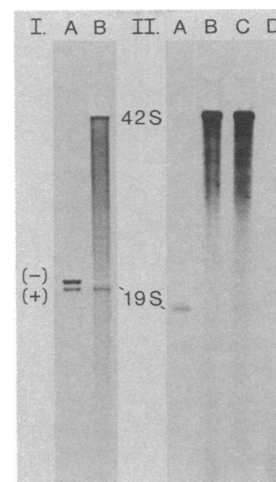

FIG. 3. Agarose gel analysis of viral nucleocapsid RNA synthesized *in vitro* in extracts of infected or uninfected cells (3×10^7 cells) in the absence or presence of added detergent-disrupted purified MS-T. The nucleocapsid products were isolated after equilibrium banding in CsCl as in Fig. 2. The nucleocapsid RNA was purified and analyzed by acid/urea/agarose (I) or formaldehyde/agarose (II) gel electrophoresis. Lanes: IA, cells infected with VSV plus MS-T; IB, cells infected with VSV and 250 μg of exogenous purified MS-T added as in Fig. 2; IIA, cells infected with VSV plus MS-T; IIB, cells infected with VSV; IIC, cells infected with VSV and 65 μg of purified MS-T added; IID, total RNA from an extract of uninfected cells with 65 μg of exogenous purified MS-T added. The samples in lanes IIA, IIB, and IIC represent one-fifth of the total nucleocapsid RNA from each reaction mixture.

RNA Replication in Fractionated Cell Extracts. Our original assumption in preparing a cell extract that would support both RNA and protein synthesis was that VSV RNA replication would be coupled to protein synthesis (4, 5). However, in experiments in which replication and encapsidation were ongoing *in vitro*, we could not demonstrate the incorporation of any *de novo* synthesized [^{3}H]leucine-labeled VSV proteins into nucleocapsids. This observation led to the conclusion that, in this system, RNA replication is uncoupled from ongoing protein synthesis and instead is supported by a pool of proteins present in the infected cell at the time the extract is prepared. In support of this hypothesis we found that the *in vitro* replication of VSV RNA proceeds normally in the presence of cycloheximide (100 μg/ml) or puromycin (500 μM) (data not shown), conditions that inhibit virtually all protein synthesis in these cell-free extracts. Moreover, when protein pools within VSV-infected cells were

depleted by treatment with cycloheximide (100 μg/ml) for 30 min prior to the preparation of the extract, subsequent *in vitro* VSV RNA replication in extracts of these cells was reduced by 86% but the level of mRNA synthesis was unaffected (data not shown).

In order to confirm the proposal that VSV RNA replication is supported by a pool of proteins, cell-free extracts were prepared from cells infected with either VSV alone or VSV plus MS-T. The extracts were separated by high-speed centrifugation into nucleocapsid and soluble protein fractions which were used in reconstitution experiments. Fig. 4 shows the total unfractionated RNA products synthesized *in vitro* under various conditions as analyzed by acid/urea/agarose gel electrophoresis, conditions that resolve the MS-T genomic (+) and (−) RNA strands. Markers were the *in vivo* viral RNAs from cells infected with VSV plus MS-T (Fig. 4, lane A). An unfractionated extract of VSV-infected cells synthesized 42S RNA as well as the viral mRNAs (Fig. 4, lane B). RNA synthesized from an unfractionated extract of VSV-plus-MS-T infected cells showed, in addition to the RNA species synthesized in the wild-type extract, significant synthesis of both (+)- and (−)-sense 19S MS-T RNA (Fig. 4, lane C).

To test the competence of subcellular fractions derived from infected cells in genome RNA synthesis, the intracellular nucleocapsid fraction from VSV-plus-MS-T-infected cells was isolated and added to an unfractionated extract of VSV-infected cells. The added nucleocapsids were predominantly MS-T nucleocapsids but also contained a small amount of VSV nucleocapsids. Under these conditions the added intracellular MS-T nucleocapsids also successfully replicated their RNA *in vitro*, producing both (+)- and (−)-strand 19S RNAs (Fig. 4, lane D). Furthermore, both (+)- and (−)-strand 19S MS-T RNAs also were synthesized when intracellular nucleocapsids derived from VSV-plus-MS-T infected cells were added to the soluble protein fraction derived from VSV-infected cells in which the endogenous nucleocapsids of the extract had been removed (Fig. 4, lane E). The mRNA synthesized in this reaction results from transcription of the added VSV nucleocapsids from the mixed infection. The level of MS-T RNA replication in these reconstituted reactions was 90% of the level occurring in the unfractionated reaction. These data show that the soluble protein fraction from nucleocapsid-depleted VSV-infected cell extracts is sufficient to support the replication of MS-T RNA of intracellular MS-T nucleocapsid templates.

The availability of a cell-free system that carries out the synthesis of VSV genomic RNA is a necessary step to dissect the components, both viral and cellular, required for RNA replication. As a first step towards this end we tested the ability of the soluble protein fraction from VSV-infected cells to support the initiation of MS-T RNA replication from the purified MS-T particle template. As controls for this experiment, nucleocapsid RNA synthesized *in vitro* in various unfractionated extracts was analyzed by formaldehyde/agarose gel electrophoresis. An extract from VSV or VSV-plus-MS-T infected cells synthesized 42S RNA (Fig. 5, lane A) or a trace of 42S plus large amounts of 19S RNAs (Fig. 5, lane B), respectively. Furthermore, the genome RNA of purified MS-T particles (70 μg) replicated when it was added to the unfractionated VSV extract (Fig. 5, lane C) as shown above. When this same amount of MS-T particles was added to increasing concentrations of the soluble protein fraction from VSV-infected cells, the lowest level of soluble protein (that from 1×10^7 cells) did not support 19S RNA synthesis (Fig. 5, lane D), but increasing concentrations (3- and 5-fold) of soluble protein did support 19S RNA synthesis (Fig. 5, lanes E and F, respectively). These data show that the initiation and elongation of the MS-T genome RNA and its encapsidation into nucleocapsids can occur *in vitro* by utilizing a preformed soluble pool of proteins from infected cells and that

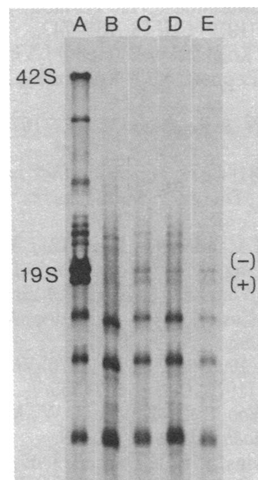

FIG. 4. Acid/urea/agarose gel analysis of the total *in vitro* RNA synthesized in fractionated extracts of virus-infected cells (1×10^7 cells). The nucleocapsid and soluble protein fractions were isolated. After reconstitution in various combinations, product was synthesized *in vitro* in the presence of [^{3}H]UTP, and the total RNA was purified and analyzed by acid/urea/agarose gel electrophoresis. Lanes: A, marker RNA labeled *in vivo* with [^{3}H]uridine from cells infected with VSV plus MS-T; B, C, and D, total *in vitro* RNA product from extracts of VSV-infected cells (B), VSV-plus-MS-T infected cells (C), and VSV-infected cells with added intracellular VSV-plus-MS-T nucleocapsids from 1×10^7 coinfected cells (D); E, total RNA product from a reaction containing soluble protein from 1×10^7 VSV-infected cells and added intracellular VSV-plus-MS-T nucleocapsids from 1×10^7 coinfected cells. The bands migrating faster than the (−) and (+) 19S RNAs represent VSV mRNAs for the G, N, and NS + M proteins, respectively.

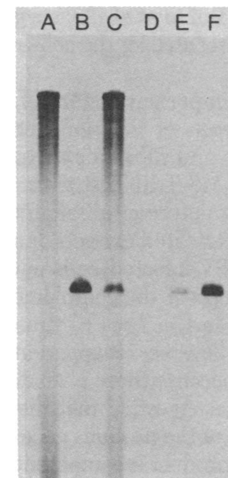

FIG. 5. Effect of VSV soluble protein concentration on the replication of exogenous MS-T genome RNA. Extracts of VSV-infected cells were prepared and the soluble protein fraction was isolated. [^{3}H]UTP-labeled product was synthesized *in vitro* in reactions in the absence or presence of added detergent-disrupted purified MS-T, and nucleocapsids were then isolated by equilibrium banding in CsCl. The nucleocapsid RNA was extracted and analyzed by formaldehyde/agarose gel electrophoresis. Lanes: A–C, *in vitro* nucleocapsid RNA from unfractionated extracts of 3×10^7 cells infected with VSV (A), VSV plus MS-T (B), or VSV plus 70 μg of exogenous purified MS-T (C); D–F, nucleocapsid RNA synthesized in reactions in which 70 μg of purified MS-T was added to the soluble protein fraction from 1×10^7 (D), 3×10^7 (E) or 5×10^7 (F) VSV-infected cells.

there seems to be a threshold concentration of these proteins that is required for relatively high levels of replication to occur.

DISCUSSION

We describe here a rapid, simple method for the preparation of cell-free extracts from cells infected with VSV or VSV plus MS-T which initiate and carry out faithful *in vitro* viral RNA replication. The procedure for extract preparation involves the treatment of infected cells with lysolecithin, disruption of the cells by pipetting, and removal of the nuclei by centrifugation. Extracts prepared from VSV-infected cells support the synthesis of the 42S VSV genome RNA and its subsequent encapsidation into RNase-resistant nucleocapsids. We have also shown that the (+) or (−) RNA strands of MS-T nucleocapsid templates present in extracts from VSV-plus-MS-T-infected cells replicate *in vitro* to yield (−)- and (+)-strand 19S genome-length RNA products, respectively, that are found within newly formed nucleocapsids.

There are three notable features of VSV or MS-T genome RNA replication in these extracts. First, the level of replication compared to transcription *in vitro* for extracts of both VSV- and VSV-plus-MS-T-infected cells is similar to that observed *in vivo* (2, 3), showing that these extracts mimic RNA replication *in vivo*. Second, although these extracts catalyze *de novo* protein synthesis, the proteins required for VSV RNA replication are those present within the soluble protein fraction from infected cells at the time the extracts are prepared (Fig. 4). Because extracts of uninfected cells will not support *in vitro* RNA replication (Fig. 3), virus-specified proteins are necessary for this process. The demonstration here that VSV-infected cells contain a pool of soluble protein(s) sufficient to support replication *in vitro* is in agreement with the study of Rubio *et al.* (23) demonstrating that VSV-infected cells contain soluble pools of viral nucleocapsid proteins N, NS, and L, which are sufficient to support *in vivo* RNA replication for 10–30 min in the absence of any additional protein synthesis. Therefore, our data together with those of Rubio *et al.* (23) suggest that ongoing protein synthesis is not essential for the cell-free replication of VSV RNA.

Finally, the most important feature of our study is that we have shown that extracts of VSV-infected cells are capable of the *de novo* initiation and subsequent chain elongation to produce genome-length MS-T RNA strands. RNA chain initiation was shown by adding detergent-disrupted purified MS-T particles to either unfractionated extracts or a soluble protein fraction derived from VSV-infected cells and observing the synthesis and encapsidation of the complementary (+)-strand 19S RNA *in vitro* (Figs. 3 and 5). The (+)-strand MS-T nucleocapsid synthesized *in vitro*, however, is apparently not capable of further RNA synthesis because there is no detectable synthesis of (−)-strand 19S RNA, suggesting that the soluble protein pool may lack one or more of the proteins necessary to make the (+)-strand nucleocapsid product a competent template for further RNA synthesis. Although the *in vitro* replication of the genome of purified MS-T under these conditions demonstrates that the *de novo* initiation of genome RNA synthesis occurs, it is not possible to extrapolate these results to estimate the *in vitro* rate of initiation stemming from the endogenous intracellular viral nucleocapsids.

Several groups have also achieved *in vitro* VSV RNA replication by various methods. Condra and Lazzarini (24) showed previously that intact VSV-infected cells made permeable with lysolecithin would carry out efficient VSV genome RNA replication that was coupled to protein synthesis; however, theirs was not a cell-free system. Batt-Humphries *et al.* (25) and Hill *et al.* (26) prepared cell-free extracts of VSV-infected cells by Dounce homogenization. In those two studies, 42S RNA synthesis occurred *in vitro* in the absence of *de novo* protein synthesis but at a very low rate consisting of only 1–2% of the total RNA product (25, 26). Davis and Wertz (27) have coupled VSV genome replication (2–5% of the total RNA synthesized) to protein synthesis by supplying the VSV proteins necessary for replication; they added intracellular VSV nucleocapsid templates to rabbit reticulocyte lysates programmed with VSV mRNAs. It is not yet known whether any of the systems described above are capable of the initation of RNA replication *in vitro*.

The system we describe yields efficient VSV and MS-T genome RNA replication in cell-free extracts of infected cells. It uses endogenous proteins present within the infected cell extract and, therefore, is not encumbered by any requirements of *de novo* protein synthesis. By using the assay described here for the reconstitution of RNA replication, the specific role of viral and cellular proteins essential for RNA replication can be studied after their purification from the soluble protein fraction.

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