Replication of Vesicular Stomatitis Virus Defective Interfering Particle RNA In Vitro: Transition from Synthesis of Defective Interfering Leader RNA to Synthesis of Full-Length Defective Interfering RNA

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The replication of the RNA of vesicular stomatitis virus (VSV) defective interfering (DI) particles was established in a defined cell-free system. The transition from synthesis of only the DI-leader RNA to replication of the full-length DI RNA was effected in the system by newly synthesized VSV proteins and occurred in the absence of VSV helper virus. Both positive- and negative-polarity full-length DI RNA were synthesized. Furthermore, the products of RNA replication associated with newly synthesized viral proteins to form complexes that were indistinguishable from authentic DI particle nucleocapsids on the basis of buoyant density and resistance to ribonuclease digestion. The DI-leader RNA did not form ribonuclease-resistant structures. We conclude that this in vitro system successfully executes many of the reactions of VSV DI particle replication and assembly.

One of the major differences between the two RNA synthetic reactions, transcription and replication, that are carried out by the negative-strand RNA virus, vesicular stomatitis virus (VSV), is that replication requires viral protein synthesis, whereas transcription does not. The template for both RNA synthetic reactions is the negative-strand genomic RNA (4×10^6 daltons) in the form of a nucleocapsid structure, that is, coated with the nucleocapsid protein, N, and associated with the phosphoprotein, NS, and the large protein, L, which are components of the RNA polymerase. This structure is capable of carrying out transcription of the genome to yield leader RNA and the five VSV mRNAs (2, 10).

At present, we do not know precisely what protein or proteins are required to effect and maintain the transition from the synthesis of leader RNA and the discrete mRNAs (transcription) to the synthesis of a complete read-through product to yield a full-genome-sized plus strand RNA, which is subsequently used as the template for synthesis of the progeny negative-strand RNA genomes (replication). In cells, the full-length genomic RNA products of replication are found only in the form of nucleocapsids and are therefore resistant to digestion by ribonucleases, whereas the mRNA products of transcription are completely sensitive to digestion by nucleases (30). Since the products of replication are protein-coated RNAs, it has been

postulated by numerous workers that a requirement for the nucleocapsid structural protein, N, may constitute the need for continuous protein synthesis in negative-strand virus RNA replication. It has been proposed that N protein may play a specific role in catalyzing the transition from transcription to replication by binding to a site in leader RNA which may be the nucleation site for encapsidation (1, 14, 21). This event would be dependent on the availability of N protein and would determine the balance between replication and transcription. It has been proposed specifically that N functions to suppress a termination signal at the end of the leader gene and that the binding of N within leader simultaneously starts nucleocapsid assembly (5, 21).

It is also possible that other newly synthesized proteins are required to promote replication. For example, the switch to replication may require a newly synthesized L molecule, an L molecule that has been modified by association with a newly synthesized form of the phosphoprotein NS, which has been shown to exist in two distinct phosphorylated forms (6, 15), or both. Additionally, it is possible that host factors may play a role in replication (24).

To investigate the requirement for protein synthesis in RNA replication, a defined in vitro system has been designed that supports both transcription and replication of the negative-

strand genomic RNA of the rhabdovirus, VSV (8). The system consists of three components: (i) purified VSV nucleocapsids as templates for RNA synthesis; (ii) an mRNA-dependent rabbit reticulocyte lysate to support protein synthesis; and (iii) purified VSV mRNAs to direct protein synthesis, if required. By using this combination of components, the level of viral protein synthesis can be controlled as desired by omission or addition of various amounts of viral mRNA. In this system, replication of the genomic RNA is a function of the level of viral protein synthesis, thereby allowing us to investigate the protein requirements for replication.

Since VSV nucleocapsid templates carry out both RNA transcription and replication in this system, we chose to extend our studies of replication by using a defective interfering (DI) particle as the template for RNA synthesis to focus solely on the process of RNA replication. The RNA of the DI particle we selected (called DI-T, VSI-DI 0.25; genome molecular weight, 0.9×10^6) contains only the 5' 25% of the standard virus genome; it completely lacks genetic information for the N, NS, M, and G proteins and contains approximately half of the information for the L protein (19, 31). This DI particle, therefore, does not direct mRNA synthesis. The major RNA product made *in vitro* by the DI particles generated from the 5' end of the genome is a 46-base RNA encoded by the extreme 3' end of the DI particle RNA and called the DI-leader RNA (9, 28, 29). In cells coinfecting with DI particles and standard helper virus, both the DI-leader and genome-length DI RNA of positive and negative polarity are synthesized (20, 25, 26). An important feature of the DI particle RNA, for our purposes, is that the 3' terminus of the positive strand (the template for negative-strand replication) is identical to the 3' terminus of the standard VSV positive strand (13, 18). In addition, the 5' and 3' termini of the DI RNAs are complementary. Therefore, the 3'-terminal sequences of both positive and negative RNA strands, which are the initiation sites for RNA synthesis, are identical. Thus, this DI particle constitutes an excellent template for investigating replication, since it is small, containing only a quarter of the genome, and does not direct mRNA synthesis, and yet it has the correct sites for initiation of replication and can replicate efficiently in cells coinfecting with infectious VSV. It is assumed that the requirement for helper virus is as a source of mRNAs to direct viral protein synthesis.

In this report we describe the replication *in vitro* of full-length DI particle RNA of both positive and negative polarity. The replication of the DI RNA occurs in the absence of helper virus and only requires viral protein synthesis.

Additionally, the full-length DI RNAs produced in this system are encapsidated with protein to form nucleocapsids that are indistinguishable from authentic DI nucleocapsids on the basis of buoyant density and resistance to ribonuclease digestion.

MATERIALS AND METHODS

Cell cultures and virus. Virus was propagated in monolayer cultures of BHK-21/13 cells as described previously (33). The Indiana serotype (San Juan strain) of VSV was used as standard VSV. Stocks of the DI particle, DI-T (VSI-DI 0.25; 5' 25% of VSV genome [31]), were generously provided by R. Lazzarini (National Institutes of Health, Bethesda, Md.).

Preparation of nucleocapsids. Intracellular DI particle nucleocapsids were prepared by infection of BHK cells with standard VSV at a multiplicity of infection of 1 and VSV DI particles (VSI-DI 0.25 was used in all cases) at multiplicities indicated. DI nucleocapsids were extracted from mixedly infected cells by Dounce homogenization, and DI particle nucleocapsids were separated from standard virus nucleocapsids by velocity sedimentation in 15 to 30% sucrose gradients (2 h at 38,500 rpm; Beckman SW40 rotor). The band of DI nucleocapsids was collected and sedimented through 25% sucrose (2 h at 44,000 rpm; Beckman SW50 rotor), and the pelleted nucleocapsids were suspended in HGD buffer (10% glycerol, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.6, 2 mM dithioerythritol).

Analysis of template RNA. The amount of unlabeled nucleocapsid template RNA added to each reaction was quantitated by parallel preparation of [³H]uridine-labeled templates with each preparation of unlabeled templates. The specific activity of labeled template RNA was quantitated, and this figure was used to calculate the amount of template RNA in unlabeled preparations made under identical conditions. Similarly, the ratio of positive to negative strand full-length DI RNA in template preparations was determined by densitometric scanning of fluorograms or by excision of appropriate bands from gels as described below. In all experiments where DI particle nucleocapsid templates were used, approximately 1,000 pg of DI particle template RNA was added per 25- μ l reaction.

In vitro synthesis of viral RNA and proteins. Viral RNA and protein synthesis were carried out in the presence of a micrococcal nuclease-treated rabbit reticulocyte lysate as described previously (8) except that the incubation times were varied as indicated in the figure legends.

Analysis of RNA products. ³H-labeled products were analyzed by electrophoresis on 1.75% agarose-6 M urea gels as described previously (32) or on 20% polyacrylamide slab gels according to the method of Laemmli and Faure (16). DI-leader RNA (characterized and kindly provided by L. A. Ball, University of Wisconsin, Madison, Wis.) and DI particle template RNA were electrophoresed as internal markers in all gels, and their positions are indicated. After electrophoresis, gels were fixed with 10% acetic acid and subjected to fluorography according to the method of Laskey (17). The resulting fluorograms were scanned to quantitate the intensity of bands by using an LKB

2202 UI trascan densitometer interfaced with an Apple II computer. Preflashed film was used to ensure a linear response to low levels of radioactivity. Additionally, a calibration curve of the linear response range for film darkening was constructed and used to ensure that intensity scans were always carried out in the linear range. To measure the amount of radioactivity in ^3H -labeled RNA species, appropriate bands were cut out of a dried gel, using the fluorogram as a guide. The dried gel fragments were dissolved in NCS solubilizer (Amersham Corp.) at 60°C for 16 h before analysis by liquid scintillation spectroscopy.

Materials. $[5,6\text{-}^3\text{H}]\text{UTP}$ (specific activity, 35 Ci/mmol) was from ICN Pharmaceuticals.

RESULTS

Preparation of DI particle nucleocapsids. VSV DI particles lack complete information for the structural genes, so it is necessary to propagate them by mixed infection of cells with standard VSV helper virus. The following method was used to prepare DI particle nucleocapsid preparations that did not contain standard virus nucleocapsids to use as templates for RNA replication reactions. Mixed infections to propagate DI particles were carried out at multiplicities that yielded the greatest amount of DI particle nucleocapsids while giving minimum yields of standard virus without totally inhibiting the infection. Optimum multiplicities were determined by assaying the types of RNA synthesized in BHK cells infected with standard virus at a multiplicity of infection of 1 while the input of DI particles varied. As shown in Fig. 1, decreasing dilutions of the DI particle preparation gave high yields of DI particle RNAs, whereas synthesis of standard 40S virion RNA was greatly diminished. These RNAs were analyzed on 1.75% agarose-urea gels, which can resolve the positive and negative strands of the DI particle genomic RNAs (11). The dilution of DI particles which we subsequently employed in our experiments was 1:3,800, a dilution which reduced the 12-h yield of standard virus by 98.6%, using the Cooper and Bellett assay (7).

DI particle nucleocapsids were extracted from mixedly infected cells and separated from standard virus nucleocapsids by velocity gradient sedimentation. The purity of the RNAs isolated by this procedure is shown in Fig. 2. Only the positive- and negative-strand RNAs of DI particle nucleocapsids were detectable even after overexposure of fluorograms of gels on which these RNAs were analyzed. These results indicated that our preparations of DI particle nucleocapsids were essentially free of standard VSV nucleocapsids and mRNA. Further evidence that the preparations were devoid of detectable standard VSV nucleocapsids came from an analysis of the RNA products synthesized by these preparations in vitro (see below).

FIG. 1. Agarose-urea gel electrophoresis of RNA from BHK cells infected with standard VSV and VSV DI particles. BHK cells were infected with standard VSV at a multiplicity of infection of 1 and with VSV DI particles at dilutions of: lane 1, 1:10,000; 2, 1:7,500; 3, 1:5,000; and 4, 1:2,500. RNA labeled with $[^3\text{H}]\text{uridine}$ from the start of infection was harvested at 12 h postinfection from cytoplasmic extracts and analyzed by electrophoresis in 1.75% agarose gels containing 6 M urea.

Analysis of RNA products: effects of protein synthesis. RNA synthesis was directed by the DI particle templates in the in vitro system, either in the absence or presence of concomitant viral protein synthesis programmed by added VSV mRNA. The products of the reaction were analyzed by electrophoresis on a 1.75% agarose gel containing 6 M urea (Fig. 3). This gel system separated the DI particle genome-size positive- and negative-strand RNA as well as retaining the 46-base DI-leader RNA and thereby allowed simultaneous quantitation of both DI particle-specific products.

The major product synthesized by the VSV DI particle nucleocapsids in the absence of viral protein synthesis was the 46-base DI-leader RNA (Fig. 3, lane 2). This finding confirmed previous reports that the DI-leader is the major product transcribed from the genomes of purified DI particles (9). In the presence of VSV mRNA translation, two distinct changes in the pattern of RNA products were observed: (i) full-length DI particle RNA of both positive and

FIG. 2. Analysis of RNA from DI particle nucleocapsid preparations. DI particle nucleocapsids were isolated from cytoplasmic extracts of BHK cells infected with standard VSV at a multiplicity of infection of 1 and DI particles at a dilution of 1:3,800 by two cycles of velocity sedimentation. RNA was extracted from nucleocapsids and analyzed as described in the legend to Fig. 1. Lane 1, Marker RNA from DI particle virions; 2, RNA from DI particle nucleocapsids (fluorogram exposed 3 days); 3, same as lane 2 except that the fluorogram was exposed for 7 days.

negative polarity appeared (Fig. 3, lane 3), which comigrated with marker template DI particle RNA (Fig. 3, lane 5); and (ii) there was a marked reduction in the amount of DI leader that was produced. The appearance of the full-length RNAs was dependent on the presence in the reaction mixture of all four ribonucleoside triphosphates. This finding indicated that these products were synthesized *de novo* and that they were not generated by terminal incorporation or exchange of labeled nucleotide.

At high concentrations of DI particle nucleocapsids, the synthesis of full-length DI RNA became less efficient and, in some experiments, was not completely dependent on the addition of viral mRNA. The reasons for this are unknown, but one possibility is that the high concentration of viral proteins contributed by the nucleocapsid preparation was able to support a limited amount of RNA replication.

The data presented above suggested that protein synthesis directed by VSV mRNAs was responsible for the transition from synthesizing only leader RNA to synthesizing full-length DI

particle genomic RNA as well. Analysis of protein synthesis in this system (data not shown) (8) has demonstrated that all five of the VSV-specific proteins are synthesized. Synthesis of the VSV proteins is linear with time for up to 3 h and approximately 5 to 10 pmol of protein is made during 180 min in a 25- μ l reaction. To test directly whether this transition in the pattern of RNA products was due to viral protein synthesis or was a direct effect of the addition of viral mRNA, protein synthesis was inhibited by the addition of cycloheximide or cycloheximide and anisomycin. Under these conditions, where protein synthesis was inhibited by more than 99%, no DI full-length RNA was synthesized and the amount of DI-leader RNA made increased (Fig. 3, lane 4). These results showed that viral protein synthesis is responsible for enabling the DI

FIG. 3. Agarose-urea gel electrophoresis of RNA products synthesized by DI particle nucleocapsids *in vitro*. RNA products were labeled with [3 H]UTP in the cell-free system programmed with the following components: lane 1, no DI templates; 2, DI templates, no VSV mRNA; 3, DI templates, VSV mRNA; 4, DI templates, VSV mRNA, and cycloheximide (50 μ g/ml); 5, marker DI template RNA. Incubation was for 180 min at 4°C. RNA was extracted and analyzed as described in the legend to Fig. 1.

particle nucleocapsids to carry out the synthesis of full-length RNA copies.

Quantitation of RNA products. The reduction in DI-leader synthesis under conditions where the replication of full-length RNA was occurring prompted us to quantitate the amounts of these two products. The relative amounts of full-length DI RNA and DI-leader RNA were quantitated by scanning laser densitometry of fluorograms of gels of the separated products. The data represented in Table 1 show that for each 1 mol of full-length DI product (both positive and negative strand) produced in the presence of protein synthesis, approximately 100 mol of DI-leader was synthesized. In the absence of protein synthesis, threefold more DI-leader was synthesized than in the presence of protein synthesis. If cycloheximide was added to inhibit protein synthesis, more leader was made, although less than was seen in the absence of added mRNA and inhibitor. These results are in agreement with the observation that the amount of negative-strand leader in infected cells is increased in the presence of cycloheximide (5).

The ratio of positive to negative strand for full-sized DI RNA product was compared to this ratio for the input template nucleocapsid RNAs. Averaged data from five separate experiments showed that after 90 min of synthesis, the product DI full-length RNA was composed of approximately 42% negative-strand RNA and 58% positive-strand RNA. Input template preparations contained an average of 57% negative and 43% positive strands. These data showed that synthesis of progeny full-sized RNA in this system closely reflected the ratio of input template RNAs at early times in the reaction. This is the result one would predict if the full-length DI product was copied from the input template. At later times in the reaction (3 h), the ratio of positive- to negative-strand products became approximately equal. This finding may suggest that RNAs synthesized at early times can serve as new templates for replication at later times. Additionally, the absolute amount of input template RNA was calculated and compared to the amount of DI full-length RNA product by excision of appropriate bands from gels. Approximately 1,000 pg of template RNA was added per 25- μ l reaction and approximately 400 to 700 pg of DI genome-length RNA was produced.

Kinetics of RNA synthesis. To investigate the decrease in DI-leader synthesis with the onset of RNA replication, we analyzed the kinetics of appearance of both DI-leader and full-length DI RNA. For these experiments, the synthesis of DI-leader RNA was analyzed by electrophoresis of samples on 20% acrylamide gels followed by densitometric scanning of fluorograms of dried gels (Fig. 4). Initially, DI-leader was produced at

TABLE 1. Relative molar amounts of DI particle RNA products

Reaction	Molar amt	
	DI genome-sized RNA ^a	DI-leader RNA
DI nucleocapsids alone	0	328
DI nucleocapsids + VSV mRNA	1	93
DI nucleocapsids + VSV mRNA + cycloheximide	0	114

^a Relative molar amounts were calculated by densitometric scanning of fluorograms of [³H]UTP-labeled products separated by gel electrophoresis. Uridine contents of 6.5% for DI-leader (28, 29) and 31% for DI particle genome RNA (27) were used in making calculations.

^b Both positive- and negative-strand genome-sized RNAs were included in this calculation.

the same rate whether mRNA had been added to program viral protein synthesis or not. However, after 20 to 40 min, while DI-leader continued to accumulate at a linear rate in the absence of protein synthesis, there was a marked decrease in its rate of accumulation in the presence of ongoing protein synthesis. Indeed, there was little additional DI-leader RNA accumulation after 40 min in reactions with ongoing protein synthesis.

A corresponding kinetic analysis of full-length DI RNA synthesis showed that full-size RNA was not detectable until approximately 60 to 90 min after the start of the reaction (Fig. 5). The accumulation of this product was not linear with time, and the major accumulation of full-length DI RNA occurred abruptly 2 to 3 h after the start of the reaction.

Association of RNA products with protein. Studies of RNA replication in infected cells have shown that replication is dependent on protein synthesis and that both positive- and negative-strand genome-sized RNAs are found only in the form of nucleocapsids, never as naked RNA. Having demonstrated that full-sized DI particle RNA could be synthesized in vitro and that its production was dependent on protein synthesis, we next examined the ability of the RNA products to associate with newly synthesized proteins. The association was assayed in two ways. First, the behavior of the RNA products in CsCl density gradients was analyzed. The ³H-labeled DI full-length product banded in CsCl gradients at the same position as marker DI nucleocapsids (data not shown), indicating that the RNA was not naked but was associated with protein and, also, that the RNA/protein ratio was approximately the same as that of authentic nucleocapsids. Next, the susceptibility of the RNA product to digestion with ribonuclease was

FIG. 4. Kinetics of accumulation of DI-leader RNA. (A) DI-leader RNA synthesis was compared in cell-free reactions programmed with DI templates to which VSV mRNA was (b,d, and f) or was not (a,c, and e) added. RNA products synthesized in the presence of [^3H]UTP added at the start of the reaction were analyzed at 20 (a,b), 40 (c,d), or 80 (e,f) min by electrophoresis on 20% acrylamide gels. ^3H -labeled product comigrated exactly with marker DI-leader (position indicated). (B) Fluorograms of the dried gels were scanned with a laser densitometer, and the relative amounts of product synthesized during each time interval were plotted.

examined. RNA products were synthesized in vitro in the presence or absence of viral protein synthesis as described for Fig. 3. After 90 min of incubation, the reaction mixtures were divided in half. Half of each reaction was immediately terminated by the addition of sodium dodecyl sulfate. The RNA was extracted and analyzed by gel electrophoresis. The other half of each reaction mixture was digested with micrococcal nuclease in the presence of calcium chloride.

After 10 min at 23°C ethylene glycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid was added to inhibit further nuclease activity. The reactions were terminated, and the RNA was extracted and analyzed in the same manner as the undigested samples. Nuclease digestion of mRNA transcription products synthesized in the in vitro system by VSV virion nucleocapsids was performed under identical conditions to test whether the micrococcal nuclease was active in these conditions. The results of this experiment are presented in Fig. 6. Only the full-length DI RNA products were resistant to nuclease digestion (Fig. 6, lane 6). The DI-leader RNA was digested by micrococcal nuclease even when made in the presence of ongoing protein synthesis. As expected, the VSV mRNAs synthesized by nucleocapsids from infectious virions were also

FIG. 5. Kinetics of appearance of full-length DI particle RNAs. (A) [^3H]UTP-labeled products synthesized in the in vitro system programmed with DI templates and added VSV mRNA were analyzed at 60, 90, 120, and 180 min after the start of the reaction by electrophoresis as described in the legend to Fig. 1. A small amount of positive- and negative-strand full-length product was detectable at 60 min on the original fluorogram, but was not of great enough quantity to be discerned on subsequent photographic exposure. Lane M shows marker DI template RNAs. (B) Fluorograms of the dried gel were scanned as described in the legend to Fig. 4, and the kinetics of appearance of full-length product (both positive and negative strand) are shown.

FIG. 6. Nuclease sensitivity of products synthesized by DI particle nucleocapsids. [^3H]UTP-labeled RNA products synthesized by DI particle nucleocapsids in the in vitro system during a 90-min incubation in the presence of no added VSV mRNA (lanes 1 and 5), added VSV mRNA (lanes 2 and 6), added VSV mRNA plus cycloheximide (50 $\mu\text{g}/\text{ml}$) (lanes 3 and 7). [^3H]UTP-labeled products synthesized by standard VSV nucleocapsids with no added VSV mRNA (lanes 4 and 8) were analyzed before (lanes 1 through 4) or after lanes 5 through 8) digestion with micrococcal nuclease, (10 $\mu\text{g}/\text{ml}$) for 10 min at 23°C by gel electrophoresis as described in the legend to Fig. 1.

completely digested by the nuclease (Fig. 6, lane 8).

These results demonstrate not only that full-length DI positive- and negative-strand RNAs are synthesized in this system in a protein synthesis-dependent reaction, but also that the proteins made are able to associate with these newly synthesized RNAs to form structures that are stable in 7 M CsCl, that coband in CsCl density gradients with authentic DI particle nucleocapsids, and that are completely resistant to digestion with nuclease. These properties are characteristic of nucleocapsids and suggest that such structures are formed in this in vitro system. Furthermore, analyses of the viral proteins found in these newly synthesized structures

indicate that they contain N, NS, and L, the three viral proteins found in authentic VSV and DI particle nucleocapsids (22).

DISCUSSION

The results presented above demonstrate that it is possible to carry out the replication of full-length VSV DI particle RNAs of both positive and negative polarity in vitro. The ratios of positive to negative strand progeny RNA closely reflected the ratio of input nucleocapsid template RNA at early times in the reaction. At later times (3 h), approximately equal amounts of positive and negative strand RNAs were produced. The DI particle nucleocapsid templates were able to make the transition from synthesizing only the DI-leader RNA in vitro to synthesizing full-length, DI genome-sized RNA by viral protein synthesis. This transition was blocked by inhibitors of protein synthesis. These results show that DI RNA replication is regulated by viral protein synthesis in this system, as it is in infected cells.

The events involved in replication of DI RNA can be considered in more detail. In the absence of protein synthesis, the DI-leader is the only product synthesized by the DI nucleocapsid template. A strong termination signal is present at the end of the leader gene. Theoretically, any read through or elongation of product beyond the DI-leader boundary can be considered replication. The successful elongation of the product to yield a full genome-sized RNA, however, may be a process involving different protein requirements than the initial read-through event. For example, the transition from transcription to replication may require catalytic or stoichiometric (or both) amounts of a certain protein(s) or a short-lived intermediate (see above), whereas the efficient elongation of product to yield full-length progeny may require only a constant supply of the nucleocapsid protein N. We have not assayed directly for the former event in this system at this time. Rather, we chose to assay only for the complete read-through product, a full-length DI molecule. Therefore, based on the studies presented here, we can say that for efficient synthesis of full-length product to occur, a certain concentration of viral protein must be available. The findings reported here are consistent with the requirement for continuous protein synthesis to maintain genome RNA synthesis in VSV-infected cells (23, 33).

A striking aspect of the quantitation of RNA products was the finding that as protein synthesis was initiated in the system and the synthesis of full-length DI RNA occurred, the amount of DI-leader RNA decreased threefold. When the kinetics of appearance of the DI-leader RNA

were compared in the presence and absence of viral protein synthesis, it was observed that the rate of accumulation was similar for the first 20 min of the reaction. After that time, however, while DI-leader continued to accumulate at a linear rate in the absence of protein synthesis, there was a marked decrease in its accumulation in the presence of protein synthesis. The appearance of full-length DI RNA could not be detected until approximately 60 to 90 min in reactions in which protein synthesis was initiated at time 0. These data showed that the sharp decrease in the rate of accumulation of DI-leader RNA was followed by the appearance of full-sized DI RNA. These two events, however, did not occur until approximately 40 min after the start of the reaction.

In considering these results, it is important to note that viral protein synthesis was being programmed from the start of the reaction and, therefore, that no preexisting pool of free virus proteins existed. One explanation for the results of these kinetic studies may be as follows. After protein synthesis is initiated in the system and proteins begin to accumulate, a necessary concentration of protein is achieved such that the transition can be made from termination at the end of the DI-leader to production of full-length product. As this occurs, one might predict a decrease in the appearance of discrete DI-leader RNA as this molecule is elongated to produce the full genome-sized product. However, this explanation can account for only a small part of the decrease in accumulation of discrete DI-leader (Table 1). We do not know what other factors may be involved. It is possible that the rate of initiation in the system slows down as the result of production of some new viral protein, or perhaps the involvement of polymerase molecules in elongation of the DI product restricts the amount of polymerase available for initiation. Another possibility is that there is a pause at the junction between DI-leader and the rest of the DI genome, which may result in a slowing of polymerase movement and new initiation events. Iverson and Rose (12) have shown that there is a distinct pause of approximately 5 min in the rate of VSV RNA transcription at each intercistronic junction. Taken together, the simplest explanation of these results and one that is consistent with our knowledge of replication in the infected cell is that the transition to replication of full-length RNA in this system requires not simply ongoing protein synthesis but rather the availability of a certain concentration of viral protein before it is possible to detect the synthesis of the full-sized RNA.

The work described above also demonstrated that the full-length DI positive- and negative-strand RNAs, made in a protein synthesis-de-

pendent reaction, could associate with the proteins synthesized concomitantly in the system. The RNA products banded in CsCl at the same buoyant density as marker DI nucleocapsids, indicating that the RNA was associated with proteins and that the RNA/protein ratio was the same as that of authentic nucleocapsids. Moreover, both the full-length positive- and negative-strand RNA products were completely resistant to digestion with ribonuclease. Our data showed that all of the replicated product was protected from nuclease digestion. Since the electrophoretic migration of the RNAs after nuclease digestion was the same as that before, these data suggest that, within the limits of this assay, the full-length RNAs were fully protected. These findings show that the newly synthesized RNA and proteins associate to form structures that resemble authentic nucleocapsids.

Quantitation of full-length DI product RNA demonstrated that approximately 1.4 to 2.1 pmol of nucleotide was synthesized in a 25- μ l reaction. In this same reaction, approximately 4.8 pmol of viral protein was made, of which 1.7 pmol was N protein. Assuming that the size of the DI genome RNA is approximately 3,000 bases, these data show that there are roughly 2,500 to 3,600 N molecules available per mol of DI product RNA. Bishop and Roy (3) have calculated that there are approximately 2,000 N molecules per standard VSV genome. The DI genome is approximately one-quarter this size and, based on the above figure, should require approximately 500 N molecules to coat each full-size DI genome. Thus, the replication system reported here produces sufficient N protein to coat the progeny DI RNAs. This finding is consistent with the fact that all the DI full-length RNA is nuclease resistant, and it shows that this system can produce viral proteins and RNA in quantities appropriate to successfully carry out RNA replication and nucleocapsid assembly.

The DI-leader RNA, in contrast to the full-length DI RNA, however, was not resistant to digestion with ribonuclease. A model has been put forward (5, 21) which proposes that the availability of N protein controls the balance between replication and transcription by the binding of N protein to a site in leader RNA, which thereby attenuates the termination at the end of leader and allows read through while simultaneously creating a nucleation site for encapsidation; failure to bind N results in termination and release of leader is a discrete molecule. We observed that the accumulation of free DI-leader RNA was decreased in the presence of protein synthesis and full-length RNA replication. Moreover, we have shown that all full-length DI RNA is encapsidated with protein, whereas free leader is not. These findings are

compatible with, but do not prove, the idea that N protein binding to a leader RNA can result in elongation to yield genome-length RNA, whereas failure of N to interact with leader results in termination and release of free leader. In contrast to our finding that free leader was not encapsidated in our system, Blumberg and Kolakofsky (4) reported that approximately three-quarters of the total intracellular leader RNA was encapsidated. In relation to the model proposed, their finding suggests that association of N with leader may not always result in read through or that encapsidation of free leader may occur after release from the template in the infected cell, or both. We suggest that in our in vitro system, RNA synthesis occurs under conditions where protein concentration may not be as great as in the infected cell. Under these circumstances, it may be that free leader is revealed as a less efficient competitor for N protein than a nascent leader molecule still attached to its template.

In conclusion, an in vitro system has been established which supports the synthesis of full-length VSV DI particle RNA and carries out its encapsidation to form nucleocapsids. In this system, the transition from synthesis of only the DI-leader RNA, a transcription event, to the replication of full-length DI RNA is made as a function of viral protein synthesis, which can be controlled by omission or addition of viral mRNA. Thus, individual VSV mRNAs can be added alone or in combination to program protein synthesis, and the effects of these proteins on the transition to RNA replication can be evaluated. This approach provides a method for assaying the involvement of individual proteins in the process of RNA replication.

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