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Asynchronous Synthesis of the Complementary Strands of the Reovirus Genome

MICHAEL SCHONBERG, SAMUEL C. SILVERSTEIN*, DANIEL H. LEVIN, AND GEORGE ACS

The Institute for Muscle Disease, and *The Rockefeller University, New York, N.Y. 10021

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ABSTRACT **The mechanism of replication of the double-stranded RNA genome of reovirus has been analyzed by tracing the fate of the parental double-stranded RNA genome and by determining whether the complementary strands, which comprise the progeny double-stranded RNA, are synthesized simultaneously or sequentially. The results indicate that the parental double-stranded RNA is conserved as the original duplex molecule within a subviral particle throughout the viral replicative cycle. The complementary strands, which form the progeny double-stranded RNA, are produced asynchronously. Minus strands are synthesized on preformed plus-strand templates, whereas plus strands appear to be synthesized on double-stranded RNA templates.**

The mechanism by which viruses containing double-stranded RNA (dsRNA) replicate their genomes is unresolved. If the dsRNA replicates by a semiconservative mechanism, then the complementary strands of the parental RNA duplex would be displaced into separate progeny genomes. Alternatively, the parental duplex could be conserved or degraded. In the present studies, the fate of the infecting parental genome was followed. We observed that the parental genome was preserved intact in the original duplex and did not appear in the progeny virus, confirming a conservative mode of replication. Furthermore, we determined whether the two complementary RNA strands, which comprise the progeny dsRNA, are replicated simultaneously as has been shown for DNA (1), or sequentially, as demonstrated in the formation of double-stranded replicative intermediates after infection with single-stranded RNA viruses (2–4). The results indicate that the two complementary RNA strands of the reovirus progeny genome are synthesized in an asynchronous manner; one strand is formed on the dsRNA template and then serves as the template for the synthesis of the complementary strand.

MATERIALS AND METHODS

Cells and virus

Reovirus Type 3 was grown and assayed in L-929 mouse fibroblasts (5). The preparation of RNA-labeled reovirus and subviral particles has been described (5, 6).

Preparation of RNA plus strands

Single-stranded products (plus strands) of the RNA transcriptase enzyme were prepared and purified as described (5).

Preparation of [^{3}H]uridine-labeled dsRNA from infected cells

RNA was extracted from infected cells by the method of Tavitian *et al.* (7) and was treated with pancreatic DNase (4 μg/ml) for 10 min at 37°C. The entire mixture was added to a Whatman CF11 cellulose column (4), and the RNA was eluted stepwise from STE (0.1 M NaCl–50 mM Tris–1 mM EDTA (pH 6.85 at 25°C) buffer (4) containing, in order, 35, 20, and 0% ethanol. The dsRNA, which elutes in the third fraction, was precipitated with 2.5 vol of ethanol containing 2% potassium acetate and resuspended in 0.02 M sodium acetate (pH 5.5)–0.001 M EDTA; the RNA was incubated at 65°C for 10 min to remove contaminating fragments, and rechromatographed on CF11 columns. The dsRNA fraction was collected as before.

Annealing of RNA

Unlabeled plus-strands were prepared *in vitro* (5) and added in an 8-fold excess (based on absorbance at 260 nm) to ^{3}H-labeled dsRNA isolated from infected cells. The mixture was placed in a boiling-water bath for 9 min, quickly chilled on ice, and brought to a final concentration of 0.3 M NaCl and 0.01 M sodium phosphate (pH 7.2). The mixture was heated to 95°C and allowed to cool slowly as follows: 95°C for 30 min, 95–72°C for 1 hr, 72°C for 15 hr, and 72–45°C over a period of 1 hr.

RESULTS

Fate of the parental genome

Previous studies have established that the parental genome is conserved within subviral particles in a double-stranded form throughout the replicative cycle (8, 9). However, it was not determined whether the two parental RNA strands remained in the original duplex or whether the complementary strands were displaced into separate progeny genomes. It was possible to distinguish between these two possibilities since the subviral particles have a different buoyant density from that of newly-synthesized virions.

L-cells were infected with either subviral particles (ρ = 1.45 g/cm^3; Fig. 1*a*) or reovirions (ρ = 1.37 g/cm^3; see Fig. 2*a*) that had been previously labeled in their respective RNAs with [^{3}H]uridine. 15 hr after infection with RNA-labeled subviral particles, the L-cells were collected and a cytoplasmic extract was prepared and analyzed by equilibrium centrifugation in CsCl. As indicated in Fig. 1*b*, the ^{3}H-labeled parental RNA remained within the subviral particles at its original density of 1.45 g/cm^3, while the progeny virus, as measured by plaque-forming activity, was unlabeled and had a density of 1.37 g/cm^3.

L-cells infected with subviral particles were assayed for their ability to form infectious centers. This analysis indicated that essentially every cell in the culture had been infected. Thus, the cells that contained subviral particles and those that were producing progeny virus did not represent mutually exclusive populations.

Abbreviations: PFU, plaque-forming units; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA.

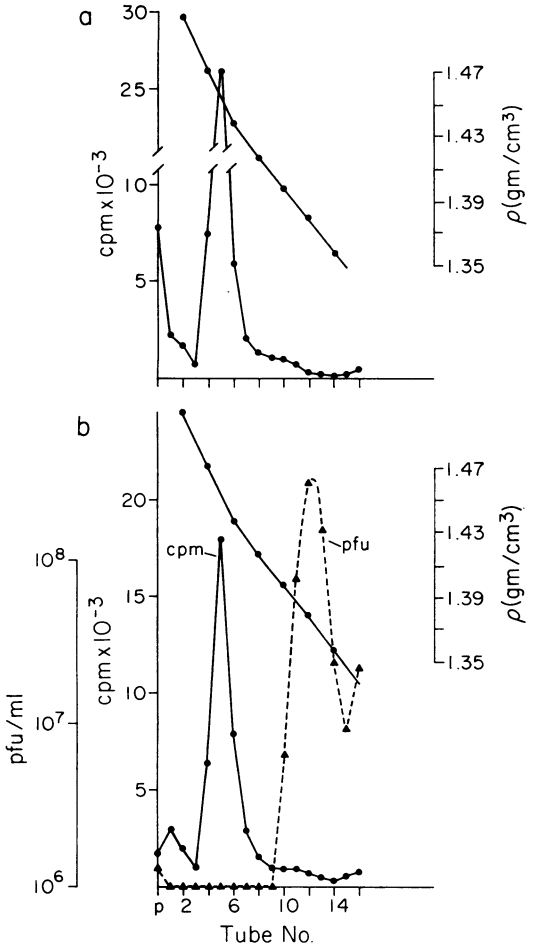

FIG. 1. (*a*) [³H]RNA-labeled subviral particles were analyzed by equilibrium centrifugation in CsCl. The gradient fractions were analyzed for acid-insoluble radioactivity and density (9). (*b*) 7.6×10^7 PFU of [³H]RNA-labeled subviral particles were adsorbed to 6.5×10^6 L-cells for 2 hr at 37°C. The unadsorbed inoculum was removed, and the cells were overlaid with fresh medium and incubated at 37°C for an additional 13 hr. The cells were then harvested and a cytoplasmic fraction was prepared and analyzed in a CsCl gradient (9). Aliquots of each gradient fraction were assayed for acid-insoluble radioactivity, plaque-forming activity, and density (9). The peak of cpm corresponds to a density of 1.45, that of PFU to a density of 1.37.

The fate of the parental genome after infection with RNA-labeled reovirus was examined in a second experiment. 10 hr after infection, cytoplasmic extracts were prepared and analyzed in CsCl gradients. As indicated in Fig. 2*b*, the parental RNA appeared in a particle with a density of 1.39 g/cm³ (9), while the progeny virus, as measured by A_{260}, was not labeled and had a density corresponding to that of mature virus (1.37 g/cm³).

Hence, regardless of whether the infection was initiated with reovirus or subviral particles, there was no detectable transfer of the labeled parental RNA templates to the progeny viral particles. The labeled parental duplex RNA remained intact in parental subviral particles, a result consistent with a conservative mode of dsRNA replication.

Sequential synthesis of the complementary dsRNA strands

The RNA transcriptase contained in the subviral particles transcribes one strand of each of the ten dsRNA segments of the viral genome (5, 6, 10). The RNA products are single stranded and do not self-anneal. Since ssRNAs of the same polarity appear as messenger RNA on the polyribosomes of reovirus-infected L-cells (manuscript in preparation), we have designated them as plus strands. Consequently, the complementary single strand has been designated the minus-strand. Minus strands are found exclusively in dsRNA and do not appear as free minus-strands within the cytoplasm of infected cells. These observations suggested that the synthesis of the two strands comprising the dsRNA occurs asymmetrically. The availability of large quantities of pure viral plus-strands made it technically possible to test this hypothesis. If dsRNA is formed by the synthesis of a complementary strand upon a preformed single strand, then the dsRNA that is formed during a brief exposure of infected cells to [³H]uridine should be preferentially labeled in one of the two complementary strands (Fig. 3*a*, *b*). If both strands of the newly formed duplex molecule are synthesized simultaneously, then they should be labeled equally regardless of the length of the labeling period (Fig. 3*c*). Each of these models yields a clearly distinguishable experimental result if the labeled dsRNA is purified, denatured by heating, and reannealed with excess unlabeled plus-strands. Hybridization of the *in vivo* labeled dsRNA with excess unlabeled plus-strands yields new duplex molecules containing minus strands derived from the original dsRNA. The excess single strands that remain in the annealing mixture can be removed selectively by digestion with RNase in the presence of a high concentration of salt. A comparison of the radioactivity in the reannealed duplex molecules with that present in the original dsRNA then determines whether the two strands were synthesized simultaneously (Fig. 3*c*) or sequentially (Fig. 3*a*, *b*). L-cells were infected with reovirus and, at the end of the adsorption period,

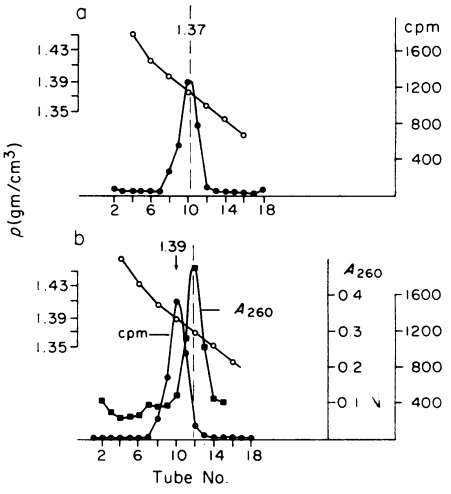

FIG. 2. (*a*) 1.25×10^9 PFU of [³H]RNA-labeled reovirus were analyzed by equilibrium centrifugation in CsCl as described in Fig. 1*a*. (*b*) [³H]RNA reovirus–L-cell complexes were formed at 4°C at a multiplicity of 250 PFU/cell. The unadsorbed virus was removed and the cells were resuspended in fresh medium and incubated for 10 hr at 37°C. The cells were then harvested by centrifugation, and a large-granule fraction was prepared (8). The large-granule fraction was analyzed in a CsCl gradient (9). Aliquots of each gradient fraction were assayed for acid-insoluble radioactivity, A_{260}, and density (9).

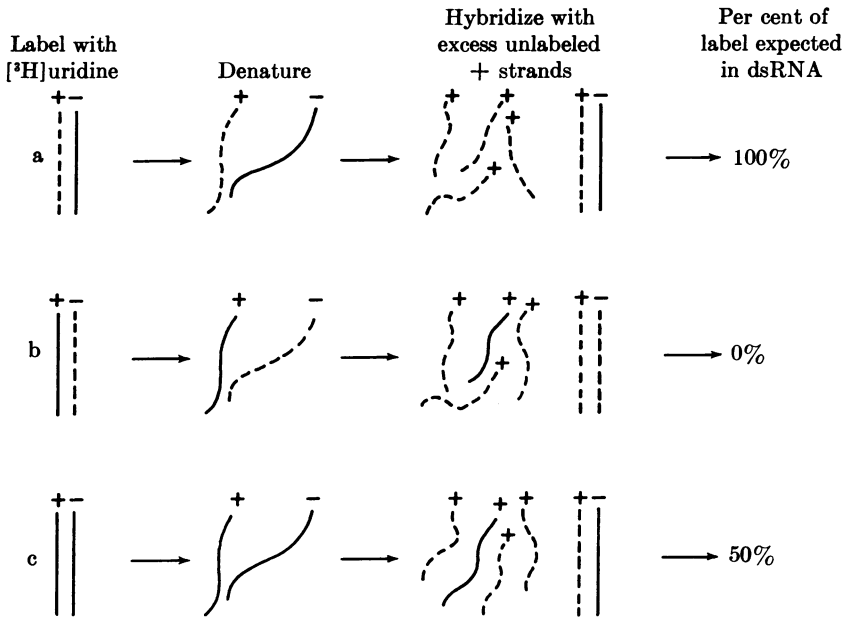

FIG. 3. Models for the synthesis of dsRNA.

the culture was divided into two portions. One aliquot was resuspended in medium containing 10 μCi/ml of [³H]uridine. A second aliquot was resuspended in medium and, at the times indicated, [³H]uridine was added (Table 1). The cultures were collected and processed separately. The dsRNA of each culture was isolated and purified. In the presence of a high salt concentration, the dsRNA was resistant to RNase (Table 1, column *a*). However, after denaturation, the RNA was completely sensitive to RNase (Table 1, column *b*), confirming that the [³H]uridine was contained only in the dsRNA, and that the dsRNA was completely denatured under the conditions specified.

Another aliquot of dsRNA was denatured and then self-annealed; the reannealed dsRNA remained completely resistant to digestion by RNase in the presence of a high salt concentration, indicating the efficiency of annealing was 100%.

However, when a similar sample of the labeled dsRNA was denatured and then reannealed in the presence of excess unlabeled plus-strands, the amount of radioactivity in the annealed product was dependent upon the time of labeling (Table 1, column *c*). In cultures that were continuously labeled for 9 hr or more after infection, the amount of label in the plus- and minus-strands of the dsRNA was the same, as indicated by the fact that annealing of the dsRNA with excess unlabeled plus-strands resulted in an annealed product containing 50% of the label present in the original dsRNA. By contrast, the distribution of label in dsRNA isolated from cells that had been pulse-labeled for 30 min, at a time when dsRNA synthesis was already under way, was asymmetric with regard to the complementary strands. Over 95% of the radioactivity in the pulse-labeled dsRNA was conserved in a double-stranded form after reannealing with excess unlabeled

TABLE 1. *Distribution of [³H]uridine in complementary strands of dsRNA after continuous- and pulse-labeling*

Expt. no.	Period of [³H]uridine labeling (hr)	Acid-insoluble, RNase-resistant radioactivity (cpm)* (*a*)	(*b*) After denaturation	(*c*) After reannealing with excess† unlabeled plus-strands	Ratio (*c*)/(*a*)
1	0–9	50,500	437	28,500	0.55
1	8–8.5	5,450	245	5,440	1.00
2	0–11	52,000	60	28,800	0.56
2	11–11.5	12,400	75	12,400	1.01

L-cell–reovirus complexes were formed at a multiplicity of 100 PFU/cell. The infected cells were resuspended in warmed medium containing 0.5 μg/ml of actinomycin D at a concentration of 3×10^6 cells/ml, divided into two portions, and incubated at 37°C. [³H]uridine (10 μCi/ml) was added to one aliquot of cells at the end of the adsorption period (time = 0) and was present throughout the experiment. The second aliquot of cells was labeled with [³H]uridine (10 μCi/ml) for the half-hour immediately preceding termination of the experiment (time = 8–8.5 or 11–11.5 hr). Incorporation of [³H]uridine was stopped by the addition of an equal volume of frozen isotonic saline and the cells were harvested by centrifugation. The conditions used for RNA extraction, denaturation, and annealing are described in **Methods**.

* Samples were digested with 8 μg/ml of pancreatic RNase in 0.3 M NaCl–0.02 M Tris·HCl (pH 7.4) for 30 min and assayed for acid-insoluble radioactivity (12). The aliquots used for analysis in Expt. 2 were proportionately smaller than those from Expt. 1.

† Eight-fold (A_{260}) excess.

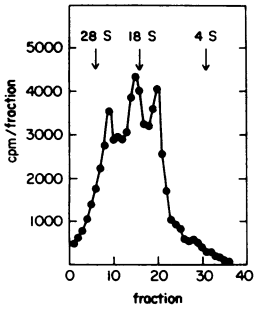

FIG. 4. The [^{3}H]uridine pulse-labeled dsRNA was denatured, rapidly cooled, and layered on a 5–20% sucrose gradient containing 1 mM EDTA–0.1 M potassium acetate (pH 5.5), and centrifuged at 98,000 × *g* in a SW-39 rotor for 4.5 hr at 12°C. The gradient fractions were assayed for acid-insoluble radioactivity.

plus-strands. Several conclusions may be drawn from these data: (*a*) the complementary strands that comprise the duplex molecules are synthesized asynchronously; (*b*) the minus strands that appear in duplex molecules are synthesized later in time than their complementary plus strands; and (*c*) preformed plus-strands appear to serve as templates for minus strands.

It should be noted here that the labeled dsRNA comprises about 15% of the total labeled RNA formed during the [^{3}H]-uridine pulse. The major proportion of labeled RNA molecules formed during this period are viral plus-strands, as judged by their failure to hybridize with unlabeled plus-strands and their ability to anneal with denatured dsRNA. Failure of this pulse-labeled ssRNA to anneal with plus-strands indicates that no free minus-strands are formed during the period of labeling.

These data exclude the mechanisms described in Fig. 3*b* and *c* as potential models for the synthesis of reovirus dsRNA; they provide strong evidence for the mechanism described in Fig. 3*a*. Hence, we conclude that dsRNA is formed by the synthesis of minus strands on preformed plus-strand templates (Fig. 3*a*).

Characterization of the minus strands of pulse-labeled dsRNA

From the RNA hybridization data presented above, it cannot be determined whether all, or only some, of the double-stranded segments of the viral genome were synthesized during the period of pulse labeling. To clarify this point, pulse-labeled double strands, which were labeled exclusively in their minus strands (Table 1), were denatured and analyzed by velocity sedimentation in a sucrose gradient (Fig. 4). As expected, the labeled minus-strands exhibited sedimentation coefficients (24, 19, and 14S) characteristic of the three molecular-weight classes of reovirus ssRNA. Moreover, the pulse-labeled dsRNA was analyzed by electrophoresis on polyacrylamide gels (5). All ten segments of the viral genome were labeled indicating that the complementary strands of each segment of the viral genome are synthesized asynchronously.

DISCUSSION

Shortly after infection the reovirion is converted to a subviral particle (9). This conversion is due to viral uncoating *in vivo*, a process that activates the RNA transcriptase of the viral core (manuscript in preparation). The enzyme transcribes one strand of each segment of the dsRNA template by a mechanism similar to the synthesis of ssRNA on a DNA template; in both cases, the original parental duplex molecules are conserved.

The parental reovirus genome is preserved as the original duplex molecule within the subviral particles throughout the replicative cycle. Since the particle-bound RNA transcriptase synthesizes and exports multiple ssRNA copies of one strand of each of the dsRNA templates, the release of parental dsRNA from the subviral particles is not required for full expression of the viral genome. Plus strands function as messengers, as indicated by their presence in the polyribosomes of reovirus-infected cells; they also serve as templates on which the complementary minus-strands are formed.

Minus strands are not found free within the cytoplasm of infected cells but are synthesized exclusively on plus-strand templates to form progeny dsRNA. These observations are inconsistent with other studies that suggested that ssRNA strands are not precursors of dsRNA (11).

The asynchronous synthesis of dsRNA suggests that there is a heterogeneity in the cellular pool of plus strands since the majority of labeled RNA molecules do not appear in the dsRNA formed during this period.

Although no direct confirmation is available, several lines of evidence suggest that, in contrast to the synthesis of minus strands on plus-strand templates, the plus strands are synthesized on dsRNA templates. This hypothesis is supported by the observations that: (*a*) subviral particles produced after uncoating of the virus *in vivo* (manuscript in preparation) or *in vitro* synthesize only plus strands; and (*b*) plus strands are not synthesized on minus-strand templates, as indicated by the experiments described in this report.

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