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Self-Annealing of Sendai Virus RNA

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Both complementary strands are found in 50*S* Sendai virion RNA. 50*S* Sendai virion RNA has been shown to consist of unequal amounts of a single population of plus and minus strands by annealing studies.

Sendai virions contain a large single-stranded (ss) RNA genome of approximately 5×10^6 daltons found in a rod-like nucleocapsid (2, 3, 13, 15). This RNA, which sediments in sucrose gradients at 48 to 50*S* (relative to 18 and 28*S* rRNA) has been shown to self-anneal from 16 to 60% in a concentration-dependent fashion (18, 19). However, as Portner and Kingsbury pointed out (18), no firm conclusion could be drawn from these studies as to whether the sequences which annealed belonged originally to the same or to complementary strands because of the extensive thermal degradation of the RNA which had taken place during the annealing reaction. Moreover, Robinson (20) was unable to separate Sendai RNA into populations with complementary base composition by isopychnic centrifugtion in cesium density gradients.

This communication reports that the self-annealing of Sendai RNA is indeed intermolecular, and that Sendai virions contain a mixture of complementary strands. A full-length double-stranded (ds) RNA has been prepared from 50*S* virion RNA. This ds-RNA, and a ribonuclease-resistant RNA core (obtained from ribonuclease-treated 50*S* RNA which had been extensively self-annealed) have been used to show that the incomplete self-annealing of Sendai RNA is the result of an unequal distribution of a single population of complementary strands.

In this work we have used the Obhayashi strain of Haemagglutinating Virus of Japan, an isolate of Sendai virus passaged only in embryonated chicken eggs (M. Homma, personal communication), since the RNA of this strain was found to self-anneal to a higher level (30 to 35%) than the RNA of the Harris strain of Sendai virus (15 to 20%). Hybridization studies using both the virus-specific 18*S* RNA found in infected cells and the 48 to 50*S* virion RNA revealed no detectable sequence differences between these strains of Sendai virus.

MATERIALS AND METHODS

Preparation of Sendai virus. The Obhayashi strain of Sendai virus, obtained from M. Homma, Sendai, Japan, was grown in 9-day-old embryonated chicken eggs. After 72 h of incubation at 32 C, the allantoic fluid was removed and centrifuged for 10 min at $5,000 \times g$ to remove debris, and the virus was pelleted through a 100-ml cushion of 25% glycerol in TNE (25 mM Tris-Cl, pH 7.4, 50 mM NaCl, 1 mM EDTA) for 3.5 h at 18,000 rpm (8 C) in the Spinco 19 rotor.

To prepare [^{3}H]uridine-labeled Sendai virus, 3-day-old confluent cultures of MDBK cells in 75-cm^2 Falcon tissue culture flasks were infected with 10 EID_{50}/cell of Sendai virus (allantoic fluid). After 45 min at 30 C, the infecting medium was removed and replaced with 8 ml of Dulbecco's modified Eagle medium containing 0.5% lactalbumin hydrolysate and 10 μCi of [5-^{3}H]uridine per ml (25 C/mmol). At 18 h postinfection (30 C), the medium was removed and discarded, and was replaced with 8 ml of the above medium containing 2 μCi of [^{3}H]uridine per ml. At 42 h postinfection, the medium was harvested and centrifuged for 10 min at $5,000 \times g$ to remove debris, and the virus was pelleted through a 10-ml cushion of 25% glycerol in TNE for 90 min at 25,000 rpm (8 C) in the Spinco SW 27 rotor. Sendai 50*S* RNA, isolated from virus as prepared above, contained approximately 200,000 counts per min per μg. To lower the specific activity of the RNA for the experiments reported here, [^{3}H]Sendai virus was mixed with a 20-fold excess of non-radioactive egg-grown virus after dissolving each virus in buffer containing SDS (see legend to Fig. 1). Undiluted [^{3}H]Sendai RNA (200,000 counts per min per μg) was found to self-anneal to the same extent as diluted RNA (10,000 counts per min per μg).

[^{32}P]Sendai virus was grown in tissue culture as previously described (15). The RNA contained approximately 400,000 counts per min per μg on isolation.

Preparation of [^{3}H]ds-Sendai RNA core. [^{3}H]50*S* Sendai RNA (93.5 μg, 1.03×10^6 counts/min, prepared as described in Fig. 1) was annealed in 0.75 ml of 50% formamide and 3× buffer A (1× buffer A: 150 mM NaCl, 20 mM Tris-Cl, pH 7.4, 1 mM EDTA) at 37 C for 90 min. The RNA was precipitated by the addition of 2 volumes of ethanol, recovered by centrifugation, dissolved in 0.35 ml of 2× buffer A, and digested with 14 μg of ribonuclease A for 15 min at

25 C. The solution was then made 0.2% in SDS, 100 μg of Proteinase K was added, and the solution was incubated for a further 10 min at 25 C. The sample was then chromatographed on a 20-ml column of Sephadex G-100 in 0.25 M LiCl, 20 mM Tris-Cl, pH 7.4, 4 mM EDTA, and 0.1% SDS, and the excluded RNA or core, representing 35% of the load, was recovered by ethanol precipitation and disolved in buffer A.

Preparation of other RNAs. [^{32}P]QB RF (10^6 counts per min per μg on isolation), the kind gift of M. Billeter, Zurich, was constructed from [^{32}P]UTP-labeled minus strands synthesized in vitro by using QB replicase and unlabeled virion-plus strands.

[^{14}C]adenosine-labeled reovirus was the kind gift of J. Skehel, London. The RNA was isolated by incubating the virus for 1 min at 50 C in 50 mM sodium acetate, pH 5.3, 10 mM EDTA, and 1% SDS, followed by sedimentation in sucrose gradients as described in Fig. 1. The 10 to 14S RNA (7,900 counts per min per μg) was recovered from the gradient by ethanol precipitation and dissolved in buffer A.

[^{14}C]uridine-labeled mouse kidney cell RNA was prepared as previously described (15).

RESULTS

As previously shown (18, 19), the 50S RNA of Sendai virus partially self-anneals. Approximately 30% of the viral RNA becomes resistant to ribonuclease after annealing, whereas only 2 to 3% of the RNA is resistant before annealing (Fig. 1). Since previous attempts to determine whether this self-annealing was inter- or intramolecular by following its concentration dependence were difficult to interpret due to the thermal degradation of the viral RNA (18), we have repeated these experiments by using low temperature annealing conditions (50% formamide and 3× SSC at 37 C) (16). The results of these experiments showed that at least 75% of the self-annealing was concentration dependent and therefore intermolecular under conditions where no degradation could be detected by sucrose gradient sedimentation analysis (data not shown).

We therefore attempted to isolate a completely double-stranded form of Sendai RNA from self-annealed 50S viral RNA by sucrose gradient centrifugation (1, 8) and cellulose chromatography (9) but we were able to find only partially double-stranded forms of the viral RNA (data not shown, see Discussion). However, if 50S Sendai RNA is simply recovered from the preparative sucrose gradient by ethanol precipitation without previously having been self-annealed, approximately 10% of the RNA is now found to sediment at 25S, a position expected for a completely ds-RNA of this size, at the ionic strength used (1, 8). This RNA is completely resistant to digestion with high concentrations of ribonuclease (Fig. 2). Moreover, as expected for a completely ds-RNA (1) the 25S RNA is soluble in 2 M LiCl, whereas the viral RNA which still sedimented at 50S is not (Fig. 3).

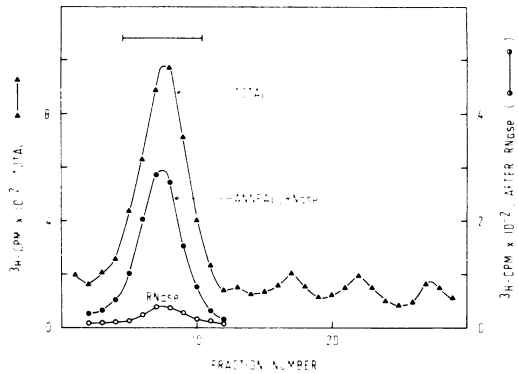

FIG. 1. *Self-annealing of 50S Sendai RNA. Approximately 3 mg of [^{3}H]uridine-labeled Sendai virus was dissolved in 0.6 ml of TNE containing 0.5% SDS and digested with 0.12 mg of Proteinase K (Merck) for 15 min at room temperature. The solution was then layered onto three 5 to 23% linear sucrose gradients containing 50 mM LiCl, 10 mM Tris-hydrochloride, pH 7.4, 4 mM EDTA, and 0.1% SDS, and centrifuged for 105 min at 50,000 rpm (7 C) in the Spinco SW 56 rotor. The gradients were fractionated by puncturing the bottom of the tubes, and 6-μliter portions were taken to determine (i) total trichloroacetic acid-precipitable radioactivity (▲); (ii) acid-precipitable radioactivity after digestion with 33 μg of ribonuclease A in 1.0 ml of 2× SSC for 30 min at 25 C (○); (iii) acid-precipitable radioactivity after annealing for 1 h at 37 C in 50% formamide and 3× SSC, in a total volume of 18 μliters, followed by ribonuclease digestion as above (○). The bar indicates the fractions pooled and recovered by ethanol precipitation as 50S Sendai RNA.*

The 25S Sendai RNA was also examined in the electron microscope. Figure 4 shows a micrograph of a mixed sample of 25S Sendai RNA and circular, single-stranded φX 174 DNA. Note that the linear 25S Sendai RNA, which is approximately 5 μm long, appears thicker and less convoluted than the single-stranded φX 174 circles. Identical structures for 25S Sendai RNA were also visualized by using an aqueous spreading technique in which ss-RNA remains collapsed and is visible only as "bushes" (6). By using circular SV40 DNA (3.6×10^6 daltons) (22) as an internal standard, 25S Sendai RNA was found to be 4.84 ± 0.16 μm long (29 molecules measured) relative to SV40 DNA at 1.81 ± 0.07 μm long (34 molecules measured), yielding a molecular weight estimate of 9.63 ± 0.33 × 10^6 for Sendai ds-RNA. This estimate is in good agreement with the value of 5.2 × 10^6

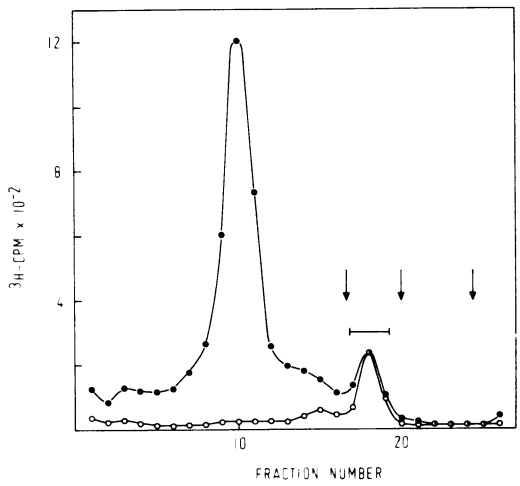

FIG. 2. *Isolation of completely double-stranded Sendai RNA by sucrose gradient centrifugation. A sample of [^{3}H]50S Sendai RNA isolated by ethanol precipitation as described in Fig. 1 and containing approximately 140,000 counts/min was dissolved in 0.1 ml of 1 mM sodium acetate, pH 5.3, 1 mM EDTA, and 0.05% SDS, and was sedimented on a 5 to 23% linear sucrose gradient as described in the legend to Fig. 1. After fractionation of the gradient, 7-μliter portions were taken for (i) total acid-precipitable radioactivity (●), and (ii) acid-precipitable radioactivity after ribonuclease digestion as described in the legend to Fig. 1 (○). The bar indicates the fractions pooled and recovered by ethanol precipitation as double-stranded 25S Sendai RNA. The arrows indicate the positions of the peaks of 4, 18, and 28S ^{14}C-mouse RNA sedimented in a parallel gradient.*

daltons obtained for ss-Sendai RNA determined previously by using the same spreading method (15).

Since only approximately 10% of the 50S Sendai RNA could be isolated in a completely double-stranded form, and upon self-annealing only 30 to 35% of the RNA was converted to a ribonuclease-resistant form, we next examined the possibility that the RNA which did self-anneal represented a different population of strands from those which did not self-anneal, as opposed to an unequal distribution of complementary strands of the same population. To aid in these studies, a ribonuclease-resistant core obtained after exhaustive self-annealing of 50S Sendai RNA was isolated (see Materials and Methods). This core RNA should represent all complementary sequences present in 50S Sendai RNA. Both this core and the 25S ds-RNA, after heated denaturation, were tested for their ability to hybridize to unfractionated [^{32}P]50S Sendai RNA under conditions where self-annealing of the [^{32}P]RNA was minimal due to its low concentration. Since the Sendai core and 25S RNA were also labeled, but with tritium at relatively low specific activity, the appearance of both ribonuclease-resistant ^{3}H- and ^{32}P-labeled RNA was followed as the concentration of [^{3}H]ds-Sendai RNA was increased. The results, shown in Fig. 5, demonstrate that both the core and 25S RNA were equally as effective in annealing to the unfractionated [^{32}P]50S Sendai RNA as they were in reannealing to themselves, converting 91 and 75%, respectively, of the [^{32}P]RNA to a ribonuclease-resistant form. Since these values are minimum values, it is likely that all sequences contained in 50S Sendai RNA are also present in both the core and 25S Sendai RNA. We therefore conclude that the limited self-annealing of 50S Sendai RNA is the result of an unequal distribution of complementary strands.

From the above data, however, one cannot conclude that the sequences present in 50S Sendai RNA are unique, i.e., that only one population of complementary strands is present in the viral RNA. Such information can only come from direct chemical analysis or from analysis of reannealing kinetics (5). [^{3}H]Sendai core RNA was therefore heat denatured, and its rate of reassociation was measured in parallel with that of QB RF and reovirus RNA. The

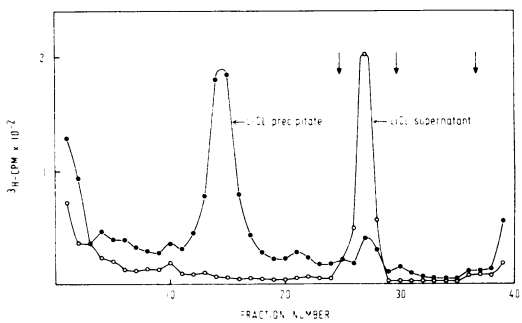

FIG. 3. *Isolation of completely double-stranded Sendai RNA by 2 M LiCl fractionation. A sample of [^{3}H]50S Sendai RNA, isolated as described in Fig. 1 and containing 240,000 counts/min, was dissolved in 0.25 ml of TNE buffer, and an equal volume of 4 M LiCl was added. After 16 h at 0 C, the solution was centrifuged for 45 min at 15,000 × g, the supernatant (32,000 counts/min) was removed, and the precipitates (163,000 counts/min) were dissolved in 0.5 ml of water. Both solutions were ethanol precipitated, the RNA was dissolved in TNE, and portions containing 1,000 counts/min of the LiCl-soluble RNA and 2,000 counts/min of the LiCl-insoluble RNA were made 0.1% in SDS and sedimented in 5 to 23% sucrose gradients as described in Fig. 1. The arrows indicate the positions of 4, 18, and 28S ^{14}C-mouse cell RNA sedimented in a parallel gradient.*

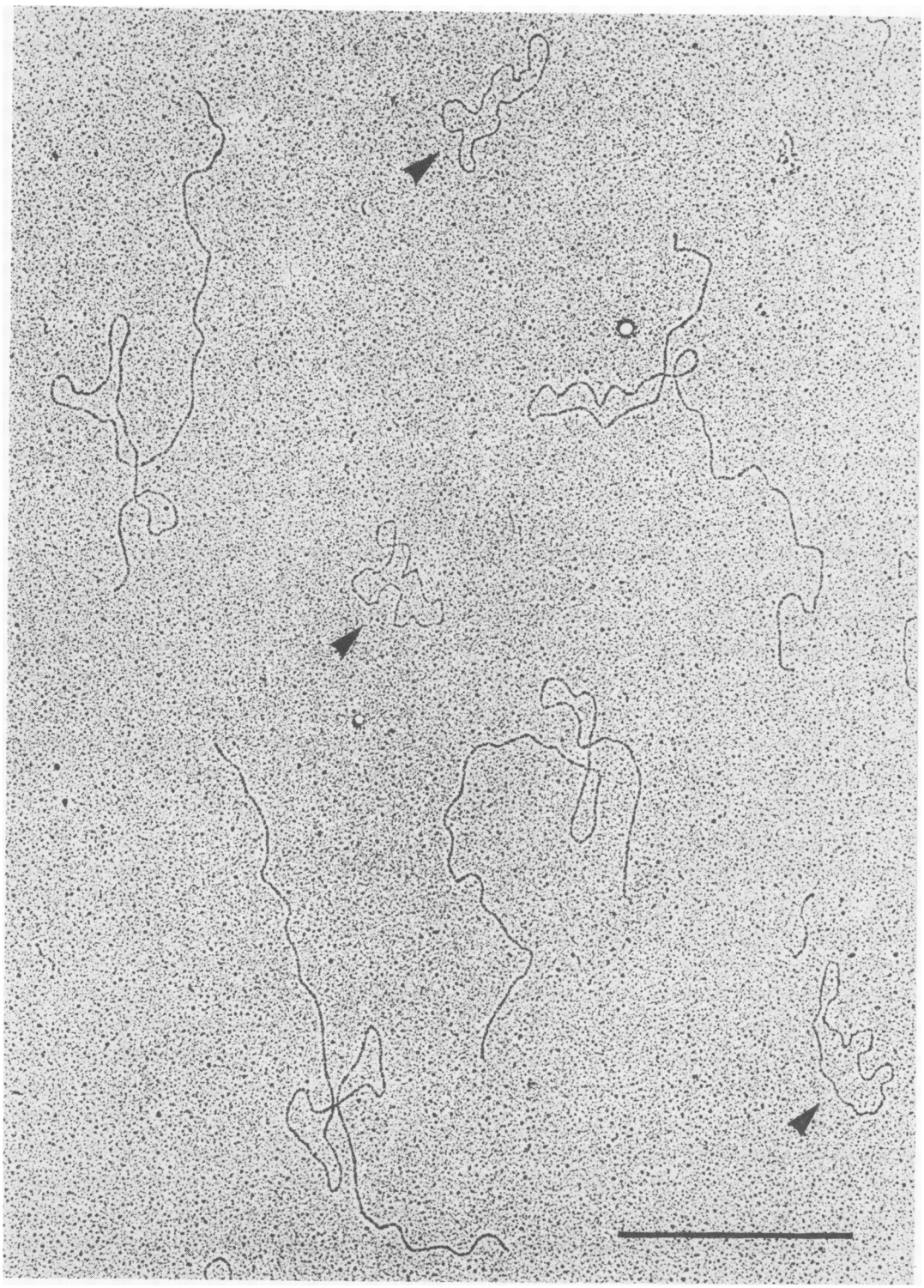

FIG. 4. *Examination of Sendai 25S RNA in the electron microscope. A 5-ng amount of 25S Sendai RNA (isolated as described in Fig. 2) and 8 ng of circular φX 174 ss-DNA in 14 μliters of 20 mM Tris-Cl, pH 8.5, 2 mM EDTA, and were mixed with 10 μliters of formamide and 2 μliters of 0.1% cytochrome c. A 5-μliter amount of the above solution was then spread onto a 1-ml drop of water and processed for microscopy as previously described (10). To allow maximum resolution between single-stranded and double-stranded nucleic acids, the grids were only lightly stained with uranyl acetate and shadowed with platinum. The arrows indicate the φX 174 ss-DNA circles. The bar in the micrograph represents a length of 1 μm.*

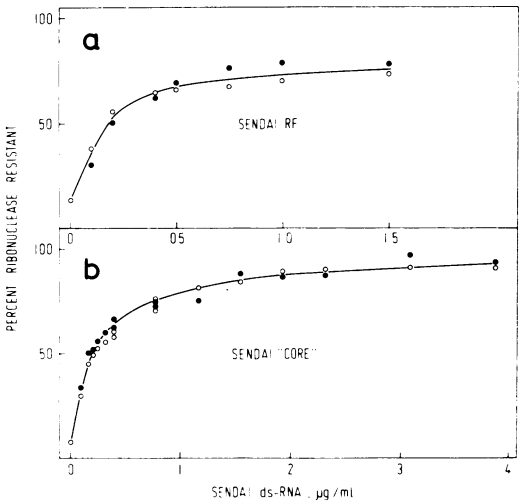

FIG. 5. *Annealing of [^3H]25S Sendai RNA and [^3H]Sendai core RNA to [^{32}P]50S Sendai RNA. [^{32}P]50S Sendai RNA (1,800 counts/min, 290,000 counts per min per μg) was mixed with varying amounts of (a) [^3H]25S Sendai RNA (10,200 counts per min per μg) in a total volume of 0.1 ml, and (b) [^3H]Sendai core RNA (8,150 counts per min per μg) in a total volume of 0.2 ml of 2.5× buffer A, as indicated. All tubes were then sealed, heated for 3 min at 120 C in a glycerol bath, quick cooled in a dry ice-ethanol bath, and annealed for 90 min at 80 C. The tubes were then opened, made up to 1 ml with 2.5× buffer A and incubated for 30 min at 25 C with 33 μg of ribonuclease A, and the remaining RNA was precipitated with 6% trichloroacetic acid, collected on Millipore filters, and counted by liquid scintillation. [^{32}P]RNA is represented by open circles; [^3H]RNA is represented by closed circles.*

results, plotted as C_rt curves (5) in Fig. 6, show that the Sendai core RNA is considerably more complex than QB RF (approximately 4,500 base pairs) (14) but only slightly less complex than reovirus RNA (approximately 22,000 base pairs in all) (21). Since the sequences present in Sendai core are representative of the total sequences found in Sendai RNA (see Fig. 5b), which is approximately 15,000 bases long (5×10^6 daltons as ss-RNA), we conclude that only one population of complementary strands is present in Sendai virions.

We have also investigated the possibility that only one of the complementary strands is contained in the nucleocapsid. Nucleocapsids were isolated from intact virions by the method of Mountcastle et al. (17) and dialyzed against TNE buffer, and the RNA was isolated by phenol extraction in the presence of 1% SDS. RNA prepared as above was found to self-anneal to the same extent as RNA isolated from whole virions (35%). Nucleocapsids therefore contain the same proportion of complementary strands as whole virions.

DISCUSSION

Attempts to isolate a completely double-stranded form of Sendai RNA from 50*S* viral RNA annealed at low temperature in formamide yielded only partially double-stranded structures as defined by ribonuclease resistance. For example, self-annealed Sendai RNA still sedimented in sucrose gradients at 50*S*, although as a broader or more heterogenous band, and the ribonuclease-resistant RNA followed the sedimentation pattern of the total RNA. Furthermore, when self-annealed Sendai RNA was chromatographed on cellulose col-

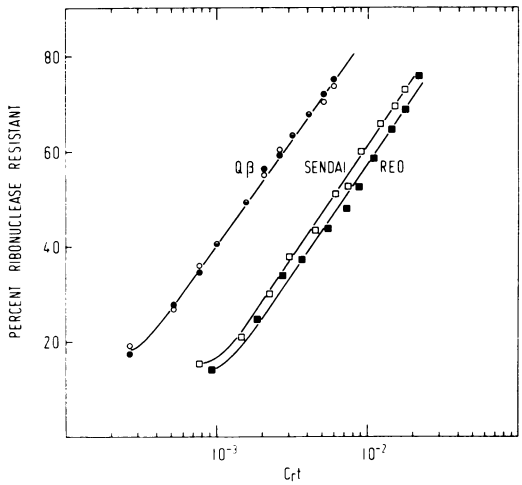

FIG. 6. *Reassociation kinetics of QB, Sendai, and reovirus double-stranded RNA. [^{32}P]QB RF RNA (830,000 and 485,000 counts per min per μg), [^3H]-Sendai core RNA (8,150 counts per min per μg) and [^{14}C]reovirus RNA (7,900 counts per min per μg) at concentrations of 0.25, 0.76, and 0.91 μg/ml, respectively in 2.5 × buffer A were heated separately for 3 min at 120 C in sealed tubes and quick cooled in an ethanol:dry ice bath. The tubes were then opened, the annealing solutions were layered with paraffin oil and annealed at 80 C. Portions were taken over a 2-h period, made up to 1 ml with 2.5× buffer A, and digested with 33 μg of ribonuclease A for 30 min at 25 C. The remaining RNA was precipitated with 6% trichloroacetic acid, collected on Millipore filters, and counted by liquid scintillation. The reannealing kinetics of Sendai RNA (□, 1,060 counts per min per sample) and reovirus RNA (■, 482 counts per min per sample) were compared with that of QB RNA (○, 10,440 counts per min per sample; ●, 6,053 counts per min per sample) on separate occasions. RNA concentrations were determined assuming one optical density unit at 258 nm being equivalent to 50 μg of ds-RNA. C_rt values were calculated on the basis of 1 h of annealing at 83 μg/ml being equivalent to a C_rt of 1.0 for all RNAs.*

umns (9), approximately 75% of the RNA eluted in the position expected for ds-RNA (0% ethanol). However, this RNA was only 50 to 60% resistant to ribonuclease. In view of our findings that Sendai virions contain a single population of full-length complementary strands, it seems most likely that self-annealing of Sendai RNA under the conditions used leads to the formation of multistranded structures, with very few if any completely double-stranded forms. Examination of self-annealed Sendai RNA in the electron microscope supports this conclusion (data not shown). Moreover, we have so far been unable to convert multistranded structures to completely double-stranded forms by annealing under conditions where multistranded forms would be expected to be less stable, e.g., 66% formamide and 3× SSC at 37 C. On the other hand, when Sendai RNA is ethanol precipitated from the preparative sucrose gradient and redissolved in water, completely double-stranded molecules with few if any multistranded structures are formed (see Fig. 2). We can offer no explanation for these events.

In all cases so far examined (Sendai [18, 19], NDV [4, 18, 19], mumps [7]), parainfluenza virion RNA has been found to self-anneal, but to considerably variable extents. For example, Sendai RNA has been found to self-anneal up to 60% by Robinson (19) and only up to 30% by Portner and Kingsbury (18), and in our hands, the Harris strain RNA self-anneals from 15 to 20%, whereas the Ohbayashi strain RNA self-anneals from 30 to 35%. In the case of NDV, Bratt and Robinson (4) and Portner and Kingsbury (18) could find little if any self-annealing of the virion RNA, but Robinson (19) reported NDV RNA to self-anneal up to 22%, and in our hands 50S NDV RNA (California strain) self-anneals from 8 to 10%. Although the self-annealing is fairly reproducible for a given preparation of virus, it appears to vary considerably among different virus preparations and the significance of this variation remains unclear. Assuming that the partial self-annealing of all parainfluenza RNAs is due to an unequal distribution of complementary strands as is the case for Sendai RNA, all parainfluenza viruses contain both complementary strands, but in considerably different proportions.

Although the relative amount of complementary strands in different parainfluenza viruses is variable, 50S virion RNA in all cases is still able to hybridize to the majority of virus-specific 18S RNA found in infected cells (3, 4). This 18S virus-specific RNA has now been shown to function in vitro as mRNA (12) and is therefore, by convention, plus strand; hence the majority species of 50S RNA in parainfluenza virions must therefore be minus strands and the minority species, plus strands. Full length plus strands have previously been demonstrated in infected cells (4), where they presumably function as templates in the replication of virus RNA. Since parainfluenza virions can contain as little as 1% and as much as 30% plus strands, it seems unlikely that the presence of plus strands in the virion is required for infectivity. Although 50S RNA has so far not been shown to be infectious (11), this may have been due to structural reasons, e.g., the minority plus strands being present only in multistranded structures. It would therefore be interesting to ask whether pure plus strands or completely ds-RNA are infectious. Similarly, can full length plus strands function as a template for protein synthesis? We are presently attempting to purify 50S plus strands to investigate these points.

ACKNOWLEDGMENTS

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