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Mumps Virus Replication in Chick Embryo Lung Cells: Properties of Ribonucleic Acids in Virions and Infected Cells[1]

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Ribonucleic acid (RNA) species in mumps virions and in infected cells were compared. The predominant RNA species in virions labeled with ^{3}H-uridine sedimented at 50*S*; RNA species sedimenting at 28, 18, and about 10*S* were also present. The virion-associated RNA species sedimenting slower than 50*S* contained some nucleotide sequences similar to 50*S* virion RNA. Although mumps virus replication was severely inhibited by high concentrations of actinomycin D, some virus was made, and virus-specific RNA species accumulated in infected cells. Mumps virus resembled other paramyxoviruses in inducing, in infected cells, synthesis not only of 50*S* RNA but also of slower sedimenting RNA species with a peak distribution at about 18*S*, complementary in base sequences to 50*S* virion RNA. In addition, base sequences of the parental type were relatively abundant in the RNA species sedimenting slower than 50*S*; these may represent precursors of the slowly sedimenting RNA species associated with virions. Ribonuclease-resistant RNA was detected in infected cells; this may represent replicative or transcriptive intermediates. Inhibition of protein synthesis with cycloheximide severely depressed accumulation of labeled 50*S* RNA in infected cells but did not interfere with accumulation of RNA species sedimenting slower than 50*S*. Actinomycin D treatment had a similar effect. Annealing of genomes and virus-induced complementary RNA species of Newcastle disease virus, Sendai virus, and mumps virus did not reveal any base sequence homologies.

The availability of systems for growing relatively large amounts of some paramyxoviruses [Newcastle disease virus (NDV), Sendai virus, and simian virsus 5] in large part accounts for the fact that most of what is presently known about paramyxovirus structure and replication comes from work with these viruses (1, 18, 27). The much poorer growth, especially in cell culture systems, of paramyxoviruses more pertinent to human disease probably explains the paucity of data on their structure and replication (6, 21). It is important to learn more about these neglected paramyxoviruses not only because of their relevance to medicine but because the biological significance of phenomena like the synthesis of single-stranded ribonucleic acid (RNA) species complementary to virus genomes in infected cells (2, 3, 16) and the occurrence of "incomplete" or defective virions containing viral genome fragments (20) may not be fully understood until it is known how general they are among the paramyxovirus group.

We began study of mumps virus as a model of the more recalcitrant paramyxoviruses because an egg-adapted strain which formed plaques in avian cells was available (11, 12), offering prospects of growing useful amounts of virus in ovo conveniently and economically and of studying virus replication in chick embryo cell culture by using methods with which we were familiar (10). The long growth cycle of mumps virus and reported lack of virus-induced cytopathology in avian tissues (6) indicated that we might compensate for small virus yields by long-term radioisotopic labeling.

Our results, reported here, reveal that mumps virus resembles other paramyxoviruses in containing a 50*S* viral genome and in inducing large amounts of complementary (minus) RNA in infected cells. In addition, our strain of mumps virus contained genome fragments which may be similar to the "subgenomic" RNA species seen

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in some strains of Sendai virus (20). Infected cells contained relatively large amounts of slowly sedimenting RNA species with genomic base sequences which may be precursors of the small RNA species found in virions.

MATERIALS AND METHODS

Virus. We obtained the chick embryo-adapted Enders strain of mumps virus (11) at the 34th allantoic passage from Allan Granoff, St. Jude Children's Research Hospital, Memphis, Tenn. Best yields of virus were obtained by inoculating allantoic cavities of 8-day-old embryonated hens' eggs with 300 to 2,000 plaque-forming units (PFU) per egg and incubating at 37 C for 7 days. In agreement with Cantell (6), we found that more concentrated inocula induced less virus and that about half of the inoculated embryos produced no virus detectable by hemagglutination. We centrifuged the allantoic fluid at 1,000 × *g* for 20 min to remove cells and debris and then collected virus by centrifugation at 35,000 × *g* for 45 min. Pellets were resuspended at one-tenth the original volume in phosphate-buffered saline (PBS) containing 1% gelatin. Routinely, we subjected the suspended virus to sonic treatment for 1 min to break up possible aggregates; however, this never increased infectivity titers more than twofold. Concentrated virus was sterilized by filtration through a type HA membrane filter (Millipore Corp.) and was stored at −70 C for as long as 6 months without any loss in titer. When titrated as described below, such virus stocks had 1.0×10^8 to 1.8×10^8 PFU and 2.7 to 5.4 $\times 10^2$ hemagglutinating units (HAU) per ml. This was a recovery of about 40% of the virus activity in the original allantoic fluid, indicating that the Enders strain of mumps virus is not inactivated or irreversibly aggregated by centrifugation [in contrast to the data of Cantell (6)].

Cells. Methods for preparing chick embryo fibroblasts (CEF) and chick embryo lung (CEL) cells have been described elsewhere (10). Confluent monolayer cultures, obtained by incubating at 37 C in an atmosphere of 5% CO_2 in air for 48 hr, were used.

Virus titrations. Virus infectivity was measured by the plaque assay of Frothingham and Granoff (12) in CEF cell cultures. Maximum plaque counts were obtained at 10 to 12 days after plating. Viral hemagglutinin was assayed in a 0.5% suspension of chicken erythrocytes.

Infection. Although CEF cells were useful for plaque titrations, we obtained better growth of virus in CEL

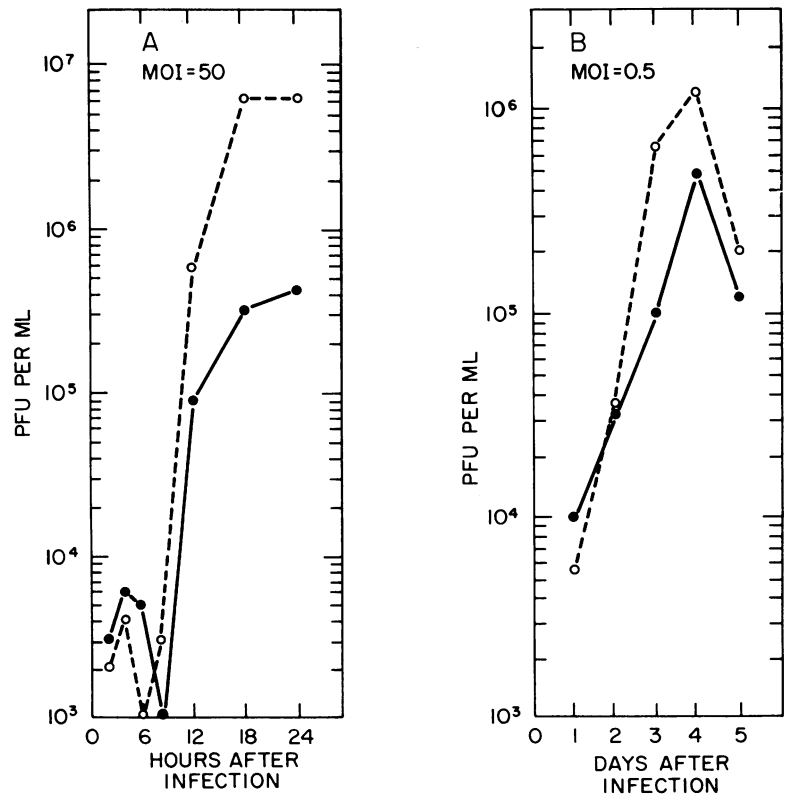

FIG. 1. *Growth of mumps virus in CEL cells after infection with(A) 50 PFU/cell or (B) 0.5 PFU/cell. Symbols:* ○, *released virus;* ●, *cell-associated virus from cells disrupted by sonic treatment.*

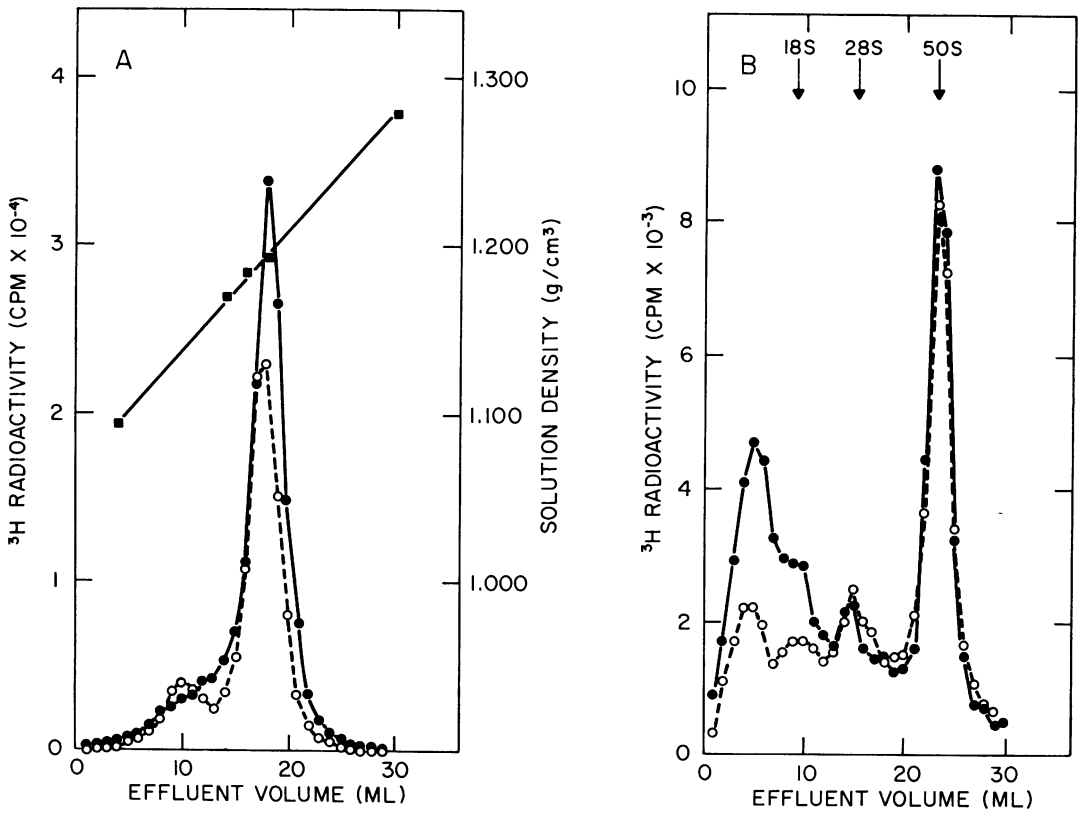

FIG. 2. *(A) Isopycnic centrifugation in a 25 to 60% (w/w) sucrose density gradient of ^{3}H-uridine-labeled (50 μCi/ml, 3 × 24 hr) mumps virions from cells infected with 0.5 PFU/cell. Centrifugation was at 20,000 rev/min, 20 C, for 19 hr in an SW 25.1 rotor. (B) Sucrose gradient rate zonal centrifugation of ^{3}H-uridine-labeled mumps virion RNA species. Virions prepared like those shown in part A were made 0.5% in sodium dodecyl sulfate (SDS) and layered on a 30-ml linear 15 to 30% (w/w) sucrose gradient in 0.005 M Tris-hydrochloride, 0.001 M ethylenediaminetetraacetic acid, 0.1 M NaCl, 0.5% SDS (pH 7.4). Centrifugation was at 20,000 rev/min in an SW 25.1 rotor for 16 hr at 20 C. The arrows show peak positions of ribosomal RNA species and 50S RNA from Sendai virions centrifuged in parallel. Symbols:* ○, *virions treated with 10 μg of micrococcal nuclease per ml at 37 C for 2 hr;* ●, *untreated.*

cells and used these cells for studying virus replication and RNA synthesis and for preparing radioisotopically labeled virions. Virus, diluted in PBS, was added to cultures and they were incubated at 37 C for 1 hr. Virus inocula were removed and replaced by Eagle's minimal essential medium, bicarbonate-buffered, and supplemented with 2% horse serum. Greater concentrations of serum inhibited virus growth, and little or no virus was produced in media containing chicken, newborn calf, or immunoprecipitated fetal calf serum.

Other methods and sources of materials. Micrococcal nuclease (staphylococcal nuclease) was "grade IV, chromatographically purified, essentially homogeneous electrophoretically, from strain SA-B," supplied by Sigma Chemical Co., specific activity about 100 to 200 μM units/mg. Virions were treated with micrococcal nuclease at 10 μg/ml in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.01 M NaCl, 0.5 mM $CaCl_2$ (pH 8.4) at 37 C for 2 hr. Pancreatic ribonuclease A was obtained from Worthington Biochemical Corp. Schwarz BioResearch was the supplier of uridine-5-^{3}H (20 Ci/mmole) which was used at 50 μCi/ml in all experiments. Acid-insoluble radioactivity was measured as described earlier (20). Also described previously were methods for preparing unlabeled NDV 50S RNA (15), labeled NDV 50S RNA (17), labeled and unlabeled NDV-specific (16) or Sendai virus-specific (24) RNA species from infected cells and the method for RNA hybridization (16). Other experimental details are given in the legends of figures and tables.

RESULTS

Virus growth in CEL cells. The growth of mumps virus in cell culture after primary infection of all cells (single-step growth) has apparently not been described previously. In CEL cells, after infection with 50 PFU/cell, there was an apparent latent period of about 8 hr, similar to

TABLE 1. *Hybridization of labeled RNA species from mumps virions*[a]

Labeled RNA	Unlabeled RNA added	Counts/min added	Per cent ribonuclease-resistant
50S	None	244	3.3
	Plus[b]	304	11
	Minus[c]	266	59
28S	None	214	4.3
	Plus[b]	256	15
	Minus[c]	220	30
18S	None	162	4.5
	Plus[b]	202	11
	Minus[c]	158	17
10S	None	312	5.8
	Plus[b]	320	9.4
	Minus[c]	278	12

[a] Labeled RNA species were obtained from micrococcal nuclease-treated virions, isolated from a gradient like that shown in Fig. 2B, and recentrifuged separately on sucrose gradients. The final materials showed no evidence of cross-contamination on sedimentation analysis.

[b] Unlabeled plus RNA sedimenting at 50S was isolated from egg-grown mumps virions and was present at 40 μg/ml in each annealing reaction.

[c] Unlabeled RNA containing minus strands was from cells infected with 5 PFU/cell and was extracted at 22 hr after infection. This RNA was centrifuged on a sucrose gradient and only species sedimenting slower than 50S were selected. In each annealing reaction, 448 μg/ml was used.

what Northrup (23) found after infection of human conjunctiva cells with a small inoculum. A large amount of new virus appeared at 12 hr after infection, and titers about 1,000-fold above background were reached by 18 hr (Fig. 1A). Although only the first 24-hr period is shown, virus titers remained at the 18-hr level for at least 3 days after infection, probably reflecting a balance between continued virus production and thermal inactivation (6). Viral hemagglutinin accumulated in parallel with virus infectivity and PFU/HAU ratios were about 10^6.

Microscopic examination showed only slight cytopathology, with less than 5% of the cells becoming refractile and rounded by 3 days after infection. The monolayers remained confluent and no syncytia were seen. Hemadsorption tests revealed that 95% of the cells were producing virus at 3 days; cultures of CEL cells did not manifest the variability in virus production seen in embryonated eggs (*see above*).

Cells infected at a 100-fold lower multiplicity (0.5 PFU/cell) made somewhat less virus, at a slower rate, with maximum titers being reached only by 3 or 4 days after infection (Fig. 1B). The low multiplicity of infection gave a much better ratio of yield to input and was used in many experiments to conserve relatively scarce virus. Since released virus was more abundant than cell-associated virus at periods of peak virus production, it was practical to use released virus exclusively for study of virion RNA species.

RNA species in mumps virions. Although the relatively small yields of mumps virions from CEL cells made it difficult to obtain useful amounts of ^{3}H-uridine-labeled virus, early experiments showed that exposing infected cells to three consecutive 24-hr labeling periods with 50 μCi of ^{3}H-uridine per ml gave satisfactory labeling. Labeled virions were collected from culture medium by centrifugation after adding unlabeled mumps virus as carrier. Even after isopycnic centrifugation in sucrose (Fig. 2A), these virions, which banded at a buoyant density of 1.19 g/cm^3, contained several RNA species (Fig. 2B). The most abundant RNA species sedimented at the same rate as the largest RNA in NDV or Sendai virions and was therefore designated as 50S (15, 20). Substantial amounts of radioactivity were present in slower-sedimenting components with peak distributions at about 28, 18, and 10S, relative to cell ribosomal RNA species.

In view of the likelihood that the RNA species sedimenting slower than 50S, especially the 18 and 28S species, which sedimented like ribosomal RNA species, were contaminants, we tried to remove them by treating virions with nuclease. Since the more commonly employed pancreatic ribonuclease has a narrow specificity and is not easily inactivated, we employed micrococcal nuclease for this purpose. Micrococcal nuclease degrades single- and double-stranded RNA as well as deoxyribonucleic acid (DNA) and has an absolute dependence upon calcium ions, allowing complete inactivation after enzyme treatment by means of a chelating agent (25, 28). When applied to virus preparations purified by isopycnic centrifugation in sucrose, micrococcal nuclease solubilized about 20% of the associated radioactivity (Fig. 2A), without changing virus infectivity. Radioactivity in 50 or 28S RNA was not affected by the enzyme, but half of the counts in the 18 and 10S species were solubilized (Fig. 2B). Annealing studies showed that the slower sedimenting RNA species remaining after nuclease treatment contained some nucleotide sequences like those in 50S RNA (Table 1). Such sequences were most abundant in the 28S species and least abundant in the 10S species. The apparent inefficiency of hybridization with

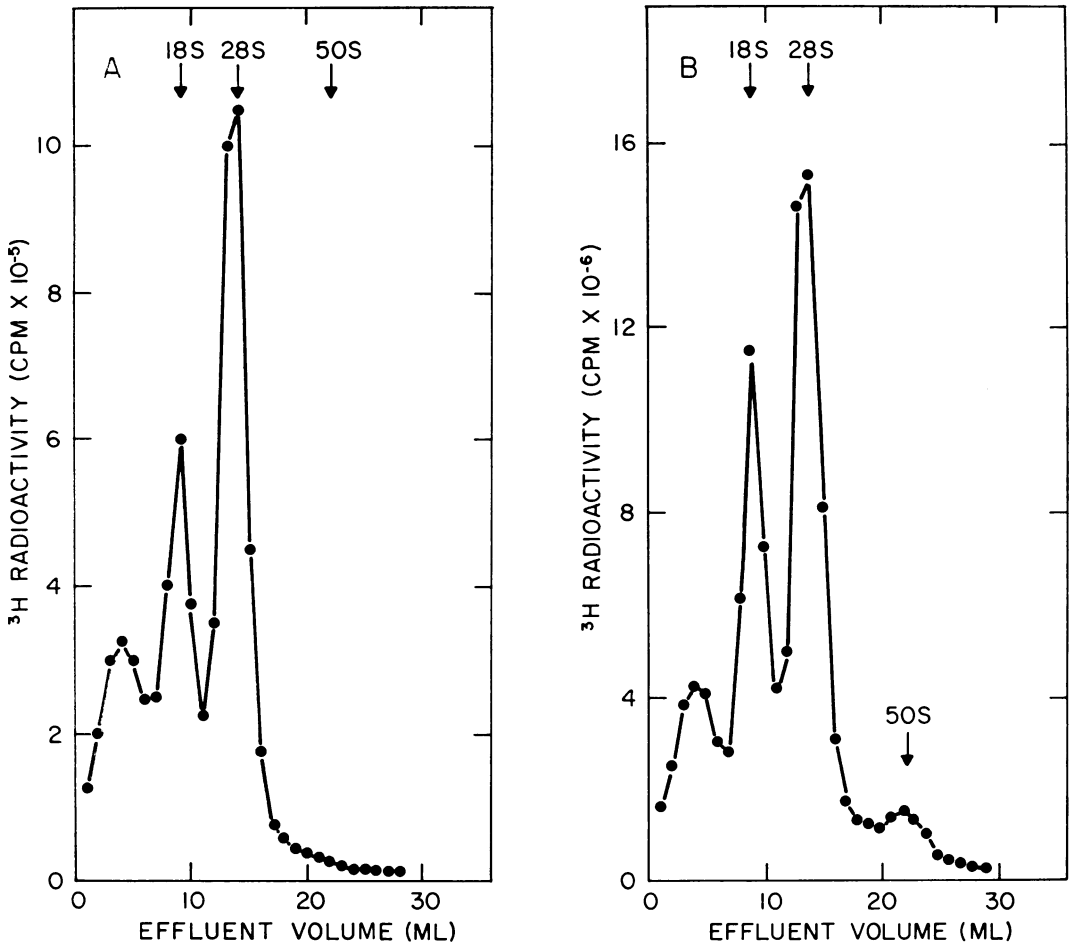

FIG. 3. *Rate zonal centrifugation of RNA species from uninfected and mumps virus-infected CEL cells. (A) Uninfected cells were labeled with ^{3}H-uridine for 24 hr. (B) Cells were infected with 0.5 PFU/cell and labeled for 3 × 24 hr.*

unlabeled "minus" RNA appears to be due to the presence of large amounts of plus base sequences in this reagent, as discussed below. Nevertheless, much of the base sequences in the virus-associated RNA sedimenting slower than 50*S* appear to be nonviral, and they presumably reside in nonvirion structures that protect them from the enzyme. The same may be true for the virus-specific base sequences, but it is also possible that these reside in virus particles. Some annealing was also detected with unlabeled 50*S* (plus) virion RNA (Table 1), which may reflect self-complementarity or the presence of some minus strands in virions (24, 26).

Sedimentation analysis of virus-specific RNA species synthesized in actinomycin D-treated cells. Continued incorporation of radioactive precursor into cell RNA after mumps virus infection (Fig. 3; *unpublished data*) made it necessary to use actinomycin D to determine if virus-specific RNA species sedimenting slower than 50*S*, similar to the complementary RNA species seen in other paramyxovirus infections (2, 3), were induced by mumps virus. Although 5 μg of actinomycin D per ml inhibited RNA synthesis 95% in uninfected CEL cells, 99% inhibition was achieved with 50 μg/ml, making identification of virus-induced RNA species less ambiguous. There were no obvious differences between virus-specific RNA species isolated from cells treated with 5 or 50 μg/ml. When actinomycin D was added to cells immediately after infection and left in the medium during single-step growth of the virus, virus replication was severely inhibited, but some virus was made, with essentially the same kinetics as in untreated

cells (Fig. 4). To examine virus-specific RNA species, actinomycin D was added to cells 1 hr before ^{3}H-uridine in all subsequent experiments. As shown in Fig. 5A, cells labeled for 3 hr in the presence of the drug at 1, 2, or 3 days after infection contained 50*S* RNA, but more radioactivity was in components sedimenting slower than 50*S*, with a major peak at about 18*S*. When, at 2 days after infection, cells were labeled for various periods of time, the same RNA components were seen consistently, and they all appeared to accumulate (Fig. 5B).

Most of the mumps virus-specific RNA accumulating in infected cells was ribonuclease-sensitive (Fig. 6), indicating that it was single-stranded. A small amount of ribonuclease-resistant RNA sedimented heterogeneously with a peak distribution at about 30*S*. When RNA preparations were treated with ribonuclease before centrifugation, the enzyme-resistant radioactivity no longer sedimented as rapidly but was recovered in the top third of the gradient, with a peak distribution at about 10*S* (Fig. 6). All of this ribonuclease-resistant radioactivity was in RNA since all of the counts were rendered acid-soluble by ribonuclease treatment in the absence of salt. Conversion of the ribonuclease-resistant material to slower sedimenting species by pretreatment with the enzyme is consistent with the idea that this material represents partially double-stranded replicative or transcriptive intermediate forms of mumps virus-specific RNA similar to the ribonuclease-resistant forms seen in NDV infection (4, 29).

Hybridization of RNA species from infected cells. Annealing experiments showed that labeled mumps virus-specific RNA species sedimenting slower than 50*S* were converted to ribonuclease-resistant form by unlabeled 50*S* RNA from virions (Fig. 7). Labeled 50*S* RNA from infected cells, isolated from gradients like that shown in Fig. 3, did not react with 50*S* RNA from virions nor did labeled RNA from uninfected cells (Fig. 7). The ribonuclease-resistant material produced by annealing behaved like double-stranded hybrids when its melting behavior was examined (Fig. 8). These results did not establish that all the RNA sedimenting slower than 50*S* was complementary (minus) with respect to base sequences in viral genomes, since this RNA self-hybridized as much as 60% (Fig. 9). This result indicated that a large fraction of the nucleotide sequences in this material, including unlabeled virus-specific RNA which accumulated before actinomycin D and labeled precursor were added, were of the genomic (plus) type.

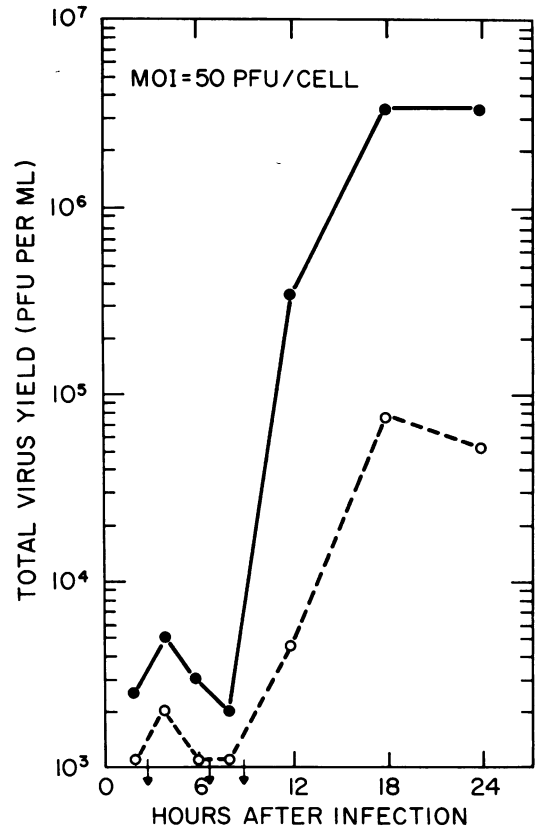

FIG. 4. *Growth of mumps virus in CEL cells* (○) *in 5 μg of actinomycin D per ml added 1 hr after infection or* (●) *in the absence of actinomycin D. Plotted points represent the sum of released and cell-associated virus.*

Data bearing on this interpretation came from experiments in which unlabeled RNA sedimenting slower than 50*S* from infected cells was annealed with labeled 50*S* (plus) RNA. Contrary to results obtained when similar experiments had been performed with virus-specific RNA species from NDV or Sendai virus infections, where 80% or more of the genomic RNA was made ribonuclease-resistant (17, 24), saturating concentrations of unlabeled mumps virus-specific RNA converted no more than about 50% of the 50*S* RNA to hybrid form (Table 1, Table 2). Only if no competing plus strands were present in the unlabeled RNA preparation could these data be taken as showing that only half the mumps virus genome is represented by complementary RNA counterparts in infected cells. A more probable explanation is that this result reflects competition by unlabeled plus strands (in the unlabeled RNA preparations) with

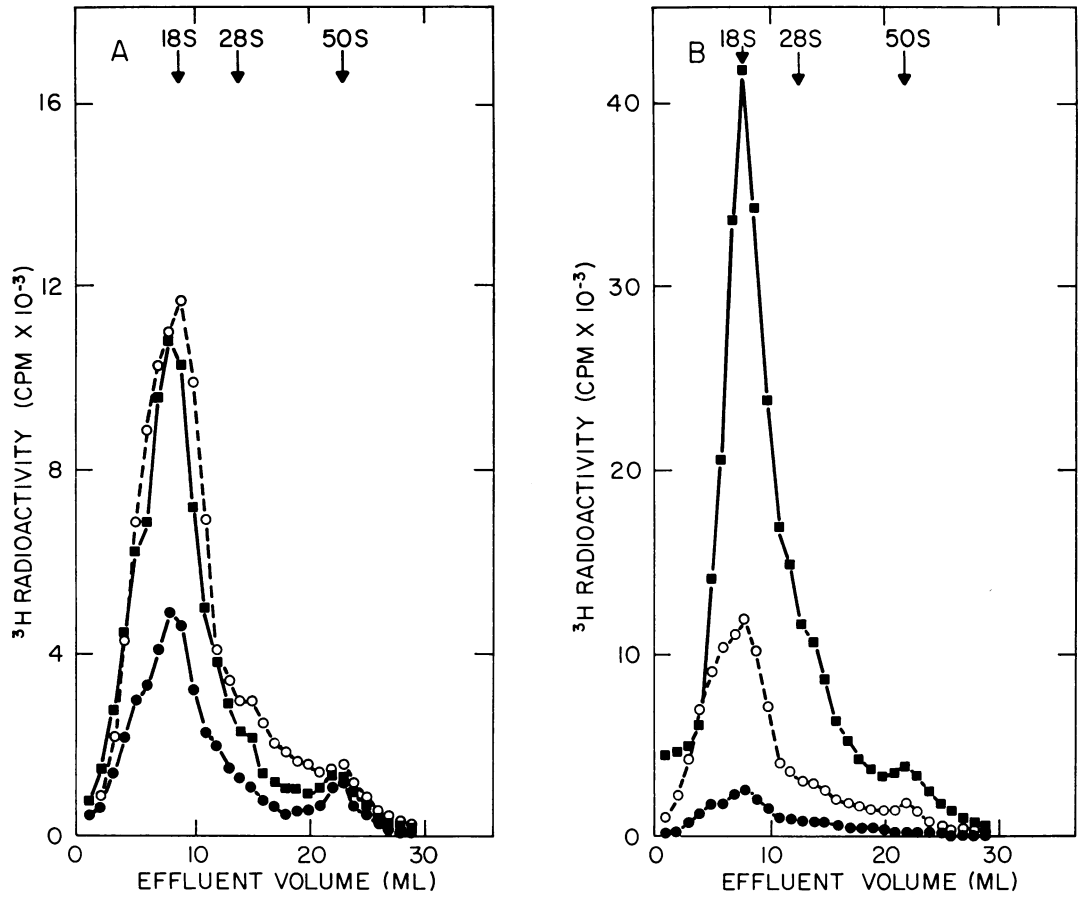

FIG. 5. *Rate zonal centrifugation of mumps virus-specific RNA species from CEL cells infected with 5 PFU/cell. (A) At (●) 1, (○) 2, or (■) 3 days after infection, cells received 50 μg of actinomycin D per ml 1 hr before addition of 50 μCi of ^{3}H-uridine per ml for 3 hr. (B) At 2 days after infection, cells received 50 μg of actinomycin D per ml 1 hr before addition of 50 μCi of ^{3}H-uridine for (●) 1, (○) 3, or (■) 12 hr. RNA species, extracted with hot phenol-sodium dodecyl sulfate (16), were centrifuged as described in the legend of Fig. 2B.*

labeled plus strands. Indeed, preannealing (24) of virus-specific RNA sedimenting slower than 50S decreased its ability to hybridize with labeled plus strands, confirming the presence of a large proportion of slowly sedimenting plus strands in infected cells (Table 2). Moreover, the very steep slopes of dilution assays indicated that slowly sedimenting plus strands were actually more abundant than minus strands (Fig. 10). If minus strands had been in excess, the plus strands added (abscissa values) would have dominated, giving slopes of 1, like those obtained with NDV-specific RNA species (17). The efficient hybridization of labeled slowly sedimenting RNA from infected cells (Fig. 7) with added plus RNA can be reconciled with the idea that plus strands predominate in cells, if most of the RNA labeled in the presence of actinomycin D is of the minus type (*see below*). Preannealing (Table 2) decreased the concentration of free minus strands but did not remove them completely. Since the preannealing was performed at RNA concentrations which appeared to be saturating (Fig. 9), the residual hybridization with labeled plus strands indicates that some of the minus-strand sequences are not represented by small plus strand counterparts in infected cells.

Preferential inhibition of mumps virus genome replication by cycloheximide and actinomycin D. Amounts of radioactivity in 50S RNA from cells treated with actinomycin D were markedly less than in 50S RNA from cells not treated with the drug (Fig. 12). Minus-strand accumulation, however, not only continued but appeared to have been enhanced by actinomycin D; dilution

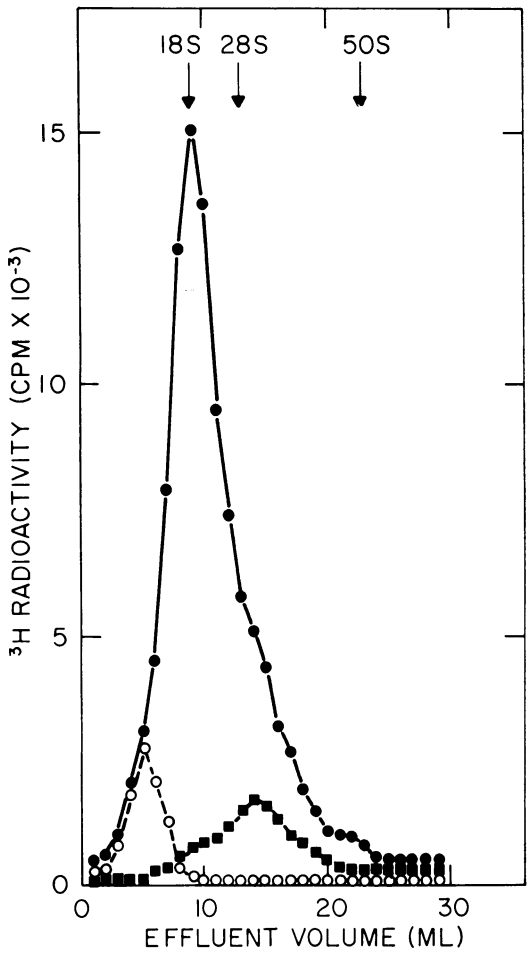

FIG. 6. *Rate zonal centrifugation of ribonuclease-resistant mumps virus-specific RNA. At 2 days after infection with 5 PFU/cell, cells received 50 μg of actinomycin D per ml and 50 μCi of ^{3}H-uridine per ml for 12 hr. RNA was isolated (16) and a portion (○) was treated with 10μg of pancreatic ribonuclease per ml for 30 min at 24 C before sucrose gradient centrifugation. Another portion was centrifuged in a sucrose gradient and divided into 1-ml fractions, and the sodium dodecyl sulfate was removed by ethanol precipitation. Symbols: ●, half of each fraction was acid precipitated; ■, the remainder of each fraction was treated with 10 μg of ribonuclease per ml and then acid-precipitated.*

assays (17) revealed significantly more minus strands in cells treated for 12 hr with actinomycin D than in untreated cells (Fig. 10).

The above results suggested that DNA-dependent RNA synthesis was somehow involved in mumps virus genome replication but not in minus-strand synthesis. Inhibition of protein synthesis had similar effects on these virus functions. Concentrations of cycloheximide which inhibited cell protein synthesis more than 90% did not prevent the accumulation of slowly sedimenting viral RNA (Fig. 11) but did inhibit accumulation of 50*S* RNA (Fig. 12). Cycloheximide and actinomycin D together inhibited 50*S* RNA accumulation more severely than either inhibitor alone (Fig. 12). These data indicate that protein involved in minus-strand production is markedly more stable or more abundant than protein involved in virus genome replication.

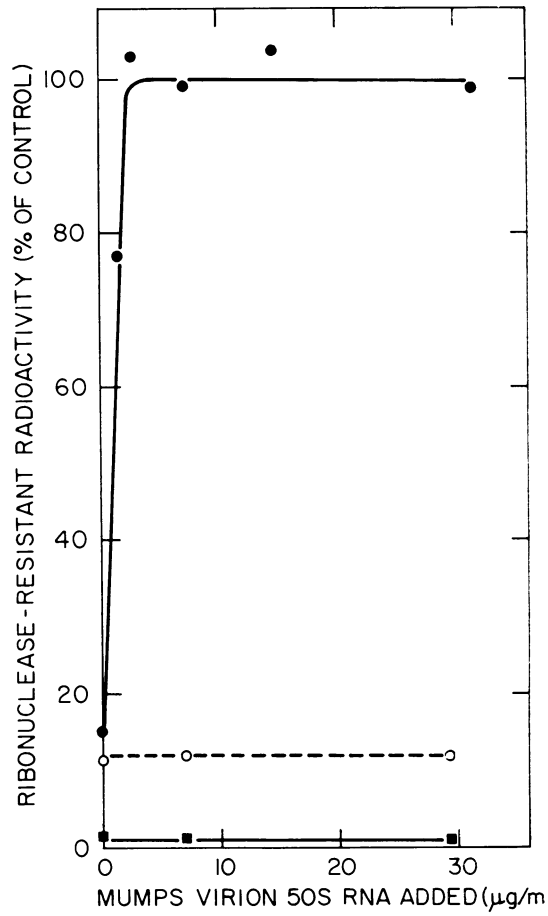

FIG. 7. *Hybridization of mumps virus-specific RNA species. Various concentrations of unlabeled 50S RNA from virions were annealed with ^{3}H-uridine-labeled RNA species. (●) Virus-specific RNA (12 μg/ml) from actinomycin D-treated, infected cells, labeled for 12 hr at 2 days after infection. Fractions 5 to 17 were selected from a gradient like those shown in Fig. 5, pooled, and concentrated by precipitation with ethanol. Symbols: ○, 50S RNA from infected cells, selected from a gradient like that shown in Fig. 12; ■, total RNA from uninfected cells labeled for 24 hr with ^{3}H-uridine.*

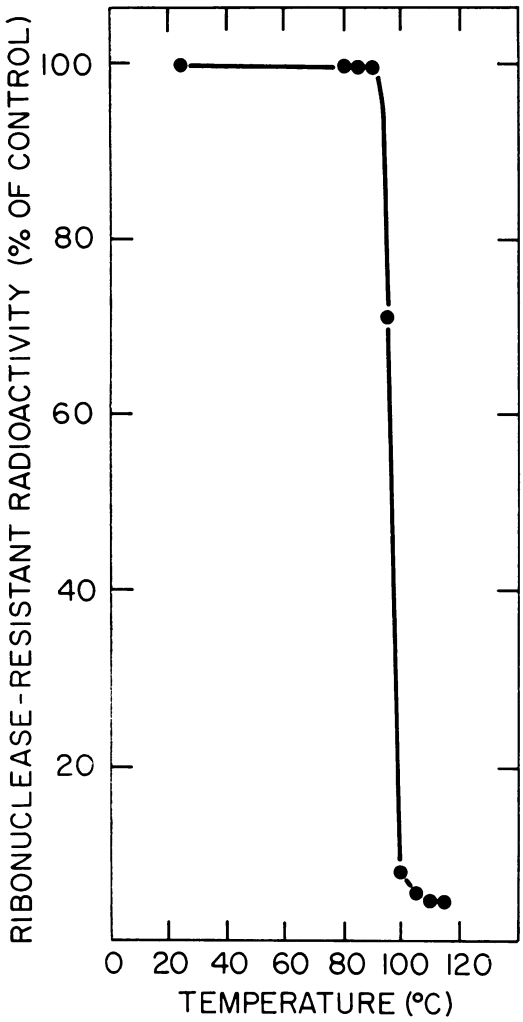

FIG. 8. *Thermal denaturation of in vitro hybrid of unlabeled 50S virion RNA and ^{3}H-RNA (less than 50S) from actinomycin D-treated, infected cells. After heating portions of an annealed mixture at the indicated temperatures for 10 min, samples were rapidly cooled and treated for 30 min with 10 μg of pancreatic ribonuclease per ml.*

Search for homologies among paramyxovirus RNA species. To determine if 50S RNA species from mumps virus, NDV, and Sendai virus had any common base sequences, we annealed 50S RNA from virions of each type with labeled minus strands from cells infected with each virus and treated with actinomycin D. Significant hybridization occurred in all of the homologous reactions between virion RNA and virus-induced RNA, but no increase in ribonuclease-resistant counts over self-annealing backgrounds was observed in any of the heterologous tests (Table 3). These results indicate that mumps virus, NDV, and Sendai virus have dissimilar base sequences in their genomes, at least with respect to the sequences represented in each minus-strand preparation. The possibility that any of the virion 50S RNA species was complementary to another virion 50S RNA was ruled out by annealing unlabeled 50S RNA from each virus with labeled 50S RNA from each virus. No increase over self-annealing values was obtained in any of these reactions.

DISCUSSION

Mumps virus presents the investigator with severe technical problems: it is difficult to obtain large numbers of virions from eggs or from cell cultures, the virus grows slowly, and virus-specific RNA species appear to be synthesized in infected cells at a slow rate. A better system for studying mumps virus biochemistry might be

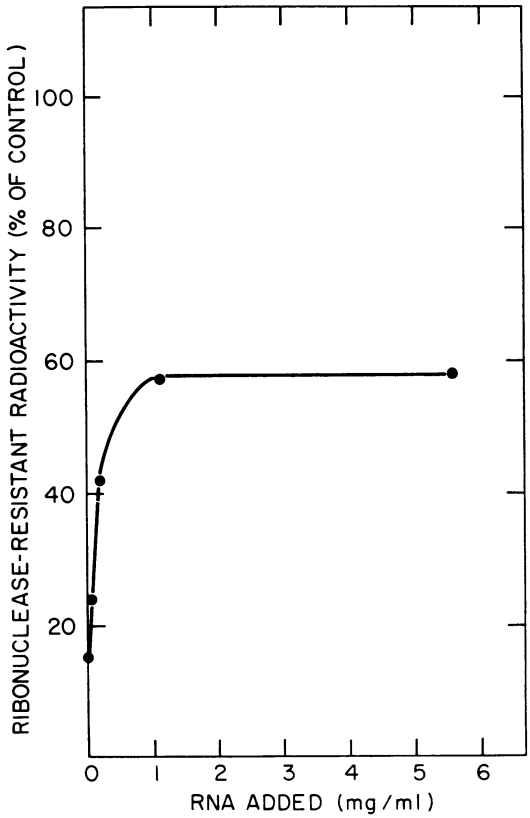

FIG. 9. *Self-hybridization of virus-specific (less than 50S) RNA from actinomycin D-treated, infected cells labeled for 12 hr at 2 days after infection. RNA from fractions 5 to 17 of a gradient like those shown in Fig. 5 was annealed alone at the indicated concentrations.*

TABLE 2. *Hybridization of labeled 50S mumps virus RNA with unlabeled RNA from infected cells*[a]

Unlabeled RNA added[b] (μg/ml)	Counts/min added	Per cent ribonuclease resistant
None	1,396	8.3
464	1,300	43
928	1,024	42
Preannealed[c]		
464	1,284	19
928	1,122	18

[a] Each reaction mixture contained ^{3}H-uridine-labeled 50S RNA from infected cells obtained as described in the legend of Fig. 12.

[b] Unlabeled RNA was extracted from infected cells at 2 days after infection and centrifuged on a sucrose gradient; RNA species sedimenting slower than 50S were selected.

[c] After preannealing the unlabeled RNA at the indicated concentrations (24), labeled 50S RNA was added and annealing was repeated.

developed; in our studies, the Enders egg-adapted mumps virus strain (11) grew better in avian cell cultures than a commercially available egg-adapted vaccine strain (5), CEL cells made more virus than CEF cells, whereas the mammalian MDBK bovine kidney cell line (8) did not support virus replication (East and Kingsbury, *unpublished data*). Compensating somewhat for the slower pace of events, the growth cycle of mumps virus in CEL cells was prolonged, making it possible to achieve useful levels of radioisotopic labeling in virions and virus-specific RNA species by long-term exposure to radioactive precursor. Furthermore, cytopathology was slight, probably reducing the contamination of released virus by cellular debris in the cell culture fluid.

In view of evidence that mumps virus nucleocapsids have the same dimensions as other paramyxovirus nucleocapsids (9, 13), it is not surprising that the major RNA component in mumps virions sedimented at the same rate as other paramyxovirus RNA species. However, this finding is of more than taxonomic interest, since it implies that this characteristic of paramyovirus genomes is fundamentally important, despite a widely divergent evolutionary history among these viruses. It suggests that all of these viruses contain the same number of cistrons, coding proteins of similar size, and that genome redundancy, if it exists for any one of the group, like NDV (19), is a universal property of the group.

There are several possible explanations for the relatively large amounts of RNA with plus-base sequences sedimenting slower than 50S in mumps virions and infected cells. The slowly sedimenting plus strands might arise from breakdown of 50S viral genomes, either artifactually during RNA extraction or metabolically during the course of infection. This is at once the least interesting possibility and the most difficult to rule out. Arguing against random breakdown was the finding of discrete sizes of RNA species sedimenting slower than 50S in mumps virions. Another possibility is that small plus strands are generated during infection to serve as templates for production of small minus strands. If this

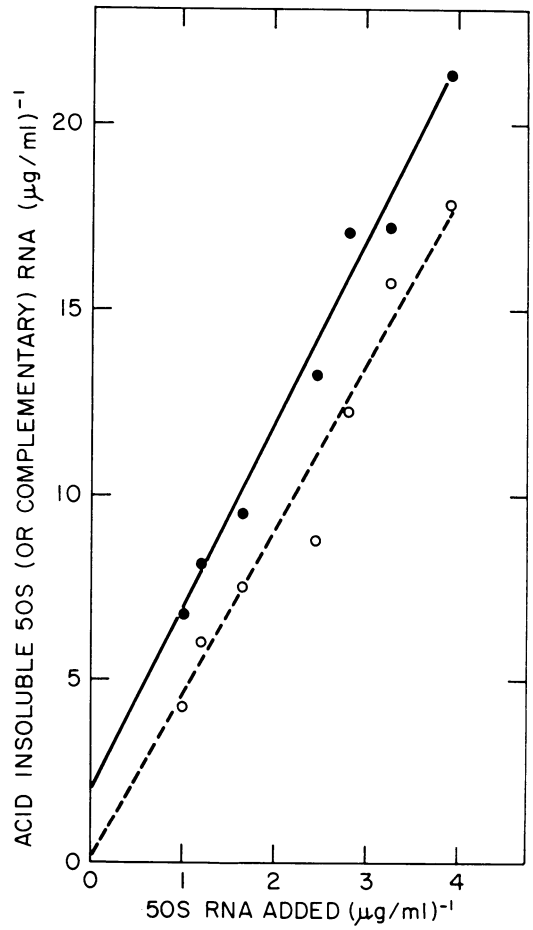

FIG. 10. *Dilution assays of complementary (minus) RNA species from infected cells. Indicated concentrations of ^{3}H-labeled 50S RNA were annealed with (●) 69 μg of virus-specific (less than 50S) RNA per ml from cells not treated with actinomycin D or with (○) 68 μg of virus-specific (less than 50S) RNA per ml from cells treated with 50 μg of actinomycin D per ml for 12 hr at 2 days after infection. The double-reciprocal plots were made as described previously (17).*

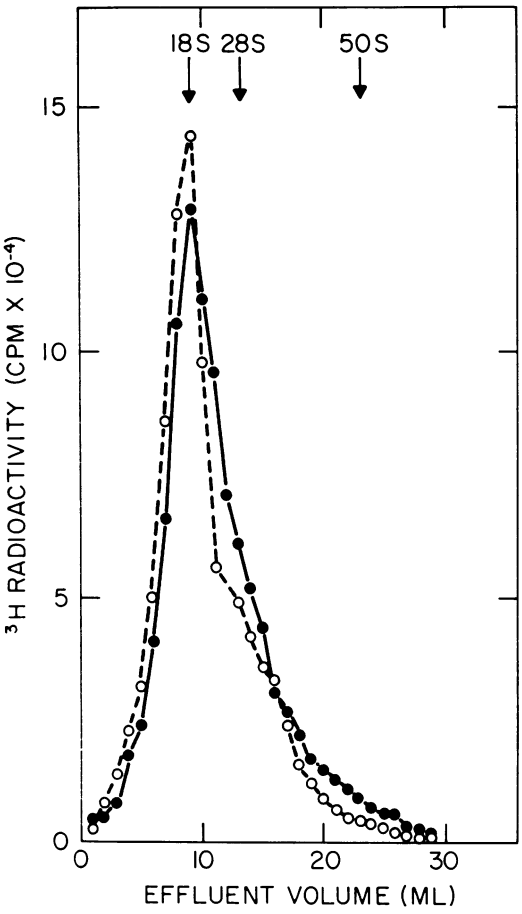

FIG. 11. *Effect of cycloheximide on mumps virus-specific RNA species. At 2 days after infection with 5 PFU/cell, cells were labeled with 50 μCi of ^{3}H-uridine per ml for 12 hr in (●) 50 μg of actinomycin D per ml or (○) 100 μg of cycloheximide per ml and 50 μg of actinomycin D per ml. Extracted RNA species were centrifuged in sucrose gradients.*

were true, it should be possible to isolate "transcriptive intermediates" (14) containing small plus-strand templates from infected cells. The labeled partially double-stranded heterogeneously sedimenting RNA species seen in NDV (4, 29) and mumps virus infections probably represent chiefly transcriptive intermediates (14) rather than replicative intermediates, since most of the labeled single-stranded RNA which accumulates in infected cells is minus strands (3, 16, 17). A third possibility is that the mumps virus genome and perhaps Sendai virus and NDV genomes are partially self-complementary, as the self-annealing data suggest (24, 26). If this were true, minus strands transcribed from these genomes could be partially self-complementary as well. Finally, the RNA species sedimenting slower than 50*S* in mumps virions and infected cells could be "subgenomic" RNA species like those seen in "incomplete" Sendai virions (20). We searched for defective virions by centrifuging our mumps virus preparations in sucrose gradients (East and Kingsbury, *unpublished data*). No virus particles sedimenting slower than 1,000*S*, where defective virions might be expected by analogy with the behavior of defective Sendai virus

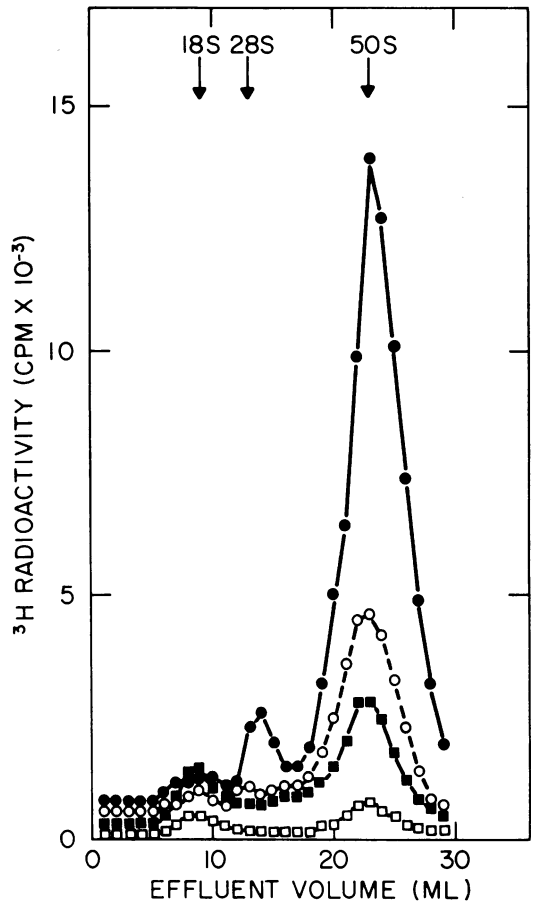

FIG. 12. *Effects of actinomycin D and cycloheximide on 50S RNA accumulation in infected cells. Cultures were infected with 5 PFU/cell and labeled at 2 days after infection for 12 hr with 50 μCi of ^{3}H-uridine per ml in the presence of (○) 100 μg of cycloheximide per ml; (■) 50 μg of actinomycin D per ml; (□) 100 μg of cycloheximide per ml and 50 μg of actinomycin D per ml. (●) Drug-free control. To obtain the data in the figure, each RNA preparation was centrifuged in a sucrose gradient and fractions 20 to 30 from each gradient were pooled, concentrated by ethanol precipitation, and centrifuged in another sucrose gradient.*

TABLE 3. *Hybridization of paramyxovirus plus and minus RNA species*[a]

Labeled minus RNA	Unlabeled plus RNA added	Per cent ribonuclease resistant
Mumps (4,300 counts/min)	None	15
	Mumps	98
	NDV	14
	Sendai	13
NDV (4,088 counts/min)	None	6.1
	Mumps	6.2
	NDV	100
	Sendai	4.7
Sendai (2,636 counts/min)	None	12
	Mumps	13
	NDV	12
	Sendai	77

[a] Each sample of labeled RNA was from actinomycin D-treated cells and was selected from a sucrose gradient to eliminate labeled 50S plus strands. Each annealed mixture contained 28 to 60 μg of virion 50S RNA per ml.

particles (20), were seen. However, neither were 1,000S virions seen; rather, mumps virions sedimented too rapidly and heterogeneously to be resolved. Although these difficulties, presumably technical, precluded obtaining reliable information on this last possibility, we favor it since a von Magnus phenomenon has been reported for mumps virus (6). In this connection, moreover, we have found slowly sedimenting plus strands in CEL cells infected with mixtures of defective and infectious Sendai virus particles (Portner and Kingsbury, *unpublished data*). In both Sendai virus and mumps virus infections, the small RNA species with plus-base sequences may be precursors of small plus RNA species which are observed in virus particles.

As with other paramyxoviruses, actinomycin D was useful for unmasking mumps virus-specific RNA synthesis in infected cells. However, it severely impaired mumps virus replication and 50S RNA accumulation. It must therefore be considered that continued transcription of the host cell genome is involved in mumps virus genome replication and virion production. On the other hand, the long-term studies we have done would be especially likely to have elicited many secondary effects. Whatever the mechanism by which actinomycin D inhibits mumps virus genome replication, it is clear that virus genome transcription is not impaired, and may even be enhanced, by the drug. Inhibition of protein synthesis by cycloheximide had similar differential effects on virus genome replication and transcription, indicating that different enzymes are involved in these two functions and perhaps these functions are subject to different controls. This situation does not appear to be unique for mumps virus, since recent work shows that cycloheximide differentially inhibits genome replication in infections by NDV and Sendai virus (A. Portner, C. Pridgen, and D. W. Kingsbury, *unpublished data*).

Our finding that there were no detectable common nucleotide sequences among three paramyxovirus genomes with respect to those portions of the genomes represented by minus strands isolated from infected cells confirms the results of Blair and Robinson (2) for the NDV-Sendai virus pair. Perhaps this should not be surprising in view of the likelihood that the avian pathogen, NDV, the human pathogen, mumps virus, and Sendai virus, apparently of murine origin, diverged long ago in evolutionary history. If the host cell specifies the carbohydrates in paramyxovirus envelope glycoproteins (22), the antigenic relatedness of the NDV-parainfluenza-mumps virus triad (7) may not be significant at the level of polypeptide sequences. On the other hand, portions of the genome represented in the minus-strand populations may not be messengers for virus envelope proteins. It would be interesting to compare peptide maps of analogous proteins (hemagglutinin, nucleocapsid, etc.) from different paramyxoviruses (22) to learn how much their amino acid sequences have diverged.

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