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Biochemistry

Avian reovirus mRNAs are nonfunctional in infected mouse cells: Translational basis for virus host-range restriction

(gene expression/nonpermissive cell infections/posttranscriptional regulation/viral cytopathogenesis)

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ABSTRACT **Avian reovirus S1133 penetrates and uncoats in suspension cultures of mouse L cells. The multiple species of viral transcripts are produced in the cytoplasm of the infected cell, but they fail to associate with polysomes, consistent with the absence of viral protein synthesis. The selective block in avian virus mRNA translation is not overcome by coinfection with mammalian reovirus type 3, which replicates in mouse L cells, or by hypertonic shock or exposure to a low concentration of cycloheximide. Although the avian viral transcripts are inactive *in vivo*, RNA extracted from infected, nonpermissive L cells directs the synthesis of a normal spectrum of viral proteins in rabbit reticulocyte lysates. These results indicate that avian viral transcription is not restricted in mouse cells and that viral replication is prevented at the level of initiation of protein synthesis.**

Avian reoviruses are nearly identical in structure and molecular composition to the well-studied mammalian reoviruses (1, 2). The genomes of both consist of 10 segments of double-stranded RNA that are transcribed by a virion-associated RNA polymerase. Like most animal viruses, avian reoviruses have a restricted host range; they fail to replicate in mammalian cells. In many virus-cell systems, the basis for host-range restriction is a lack of cell-surface receptors that mediate productive attachment and entry of parental virions (3, 4). However, it has been shown with avian reovirus S1133 and mouse L cells growing in suspension that the virus penetrates and uncoats, but progeny virions are not formed (5). In this heterologous system, transcription of the parental viral genome was reported to be limited to 4 of the 10 RNA segments, suggesting a transcriptional basis for the nonpermissiveness of mammalian cells for avian reoviruses (5). It was reported further that the partial block in avian virus transcription in infected L cells could be overcome by coinfection with mammalian reovirus, yielding transcripts from all segments of both viral genomes (5). Other studies have indicated that in reovirus type 3-infected permissive cells, transcription is temporally regulated (6–8).

Although viral protein synthesis in the heterologous system was not assayed previously, avian reovirus mutants capable of replicating in L cells were detected (5), implying that avian viral transcripts can be translated in mammalian cells. To explore further the molecular basis for avian reovirus restriction, mouse L cells growing in suspension were infected and analyzed for the production of virus-specific gene products. Although viral protein synthesis was not detectable, all viral transcripts were formed in the infected L cells. Furthermore, the purified RNAs were shown to have messenger activity *in vitro*. This striking example of translational control represents an unusual basis for host-range restriction of an animal virus.

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MATERIALS AND METHODS

Cells and Virus. Chicken embryo fibroblasts (CEF) were purchased from SPAFAS (Storrs, CT) and grown in medium 199 supplemented with 10% tryptose phosphate broth and 10% (vol/vol) fetal calf serum. Mouse L cells were grown in suspension in Eagle's minimal essential medium (MEM) containing 5% fetal calf serum. The S1133 strain of avian reovirus and mammalian reovirus serotype 3 Dearing strain were grown in primary CEF and in L cells, respectively. Virus purification and plaque assays were as described (1, 5, 8).

Analysis of Protein Synthesis. Samples of 10^7 L cells were mock-infected or infected with 80 plaque-forming particles of avian reovirus per cell and, where indicated, with the same multiplicity of type 3 reovirus 1 hr later. After virus adsorption to 5×10^6 cells per ml for 2 hr at 4°C, cultures were diluted 1:9 and incubated at 37°C in MEM containing 2% fetal calf serum and 0.5 μg of actinomycin D (ActD) per ml to inhibit host-cell RNA synthesis selectively (9). At the indicated times, cells were pelleted, resuspended to 10^7 per ml in methionine-free MEM containing 80 μCi (1 Ci = 37 GBq) of [^{35}S]methionine (specific activity, 1000 Ci/mmol), and incubated for 2 hr at 37°C. Cultures were then chilled, harvested by low-speed centrifugation, washed three times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, and lysed at 4°C in 0.1 ml of lysis buffer (10 mM Tris·HCl, pH 8.6/0.14 M NaCl/1.5 mM $MgCl_2$/0.5% Nonidet P-40). Nuclei were pelleted ($800 \times g$ for 10 min), and samples of the supernatant fraction (2×10^6 acid-precipitable cpm) were immunoprecipitated with rabbit antibody to purified avian reovirus S1133 (10) or were directly diluted with an equal volume of double-strength PAGE loading buffer (11), heated for 5 min in a boiling water bath, and analyzed by $NaDodSO_4$/PAGE followed by autoradiography (11).

Isolation of Cytoplasmic RNA. Samples of 10^7 mock-infected and virus-infected L cells collected at the indicated times were lysed in 0.6 ml of lysis buffer; after nuclei were pelleted, the supernatant was extracted with 1 ml of phenol followed by 1 ml of phenol/chloroform/isoamyl alcohol, 24:24:1 (vol/vol). The aqueous phase was made 0.3 M in NaOAc and 80% in ethanol; after 15 min in dry ice, RNA was pelleted, dried, dissolved to 5 mg/ml in water, and stored at −80°C.

Preparation of Reovirus mRNA. Viral cores were prepared by incubating 1 mg of purified reovirus type 3 at 42°C for 45 min in 1 ml of 70 mM Tris·HCl (pH 8.0) containing 90 mM KCl and 0.5 mg of α-chymotrypsin. The resulting cores were pelleted ($10,000 \times g$ for 20 min), washed three times with 50 mM Tris·HCl (pH 8.0) containing 50 mM KCl, and resus-

Abbreviations: pi, postinfection; CEF, chicken embryo fibroblasts; ActD, actinomycin D.

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pended in 0.1 ml of the same buffer. Reaction mixtures for the synthesis of viral mRNA included the viral cores in 1 ml of 70 mM Tris·HCl (pH 8.0) containing 18 mM $Mg(OAc)_2$; 4 mM each of ATP, CTP, and GTP; 2 mM UTP; 0.1 mCi of [^{3}H]UTP (specific activity, 47 Ci/mmol); 5 mM phosphoenolpyruvate; 0.1 mM *S*-adenosylmethionine; 17 international units of pyruvate kinase; 2 μg of inorganic pyrophosphatase; and 0.2 mg of macaloid. After incubation at 42°C for 2 hr, cores were pelleted and washed twice with 20 mM Tris·HCl (pH 7.5). The washes combined with the supernatant were extracted twice with phenol/chloroform/isoamyl alcohol, and the aqueous phase was passed through a column of Sephadex G-50 equilibrated with 20 mM Tris·HCl (pH 7.5). Fractions containing the radioactive product were pooled, and the RNA recovered by ethanol precipitation had a final specific activity of 33,000 cpm/μg.

Protein Synthesis in Rabbit Reticulocyte Lysates. Incubation mixtures in a final volume of 15 μl consisted of 5 μl of lysate (Bethesda Research Laboratories, no. 8111), 1.2 mM $MgCl_2$, 0.1 M KOAc, 50 μM of each amino acid except methionine, 5 μC of [^{35}S]methionine, 8 μM hemin, 10 mM creatine phosphate, 17 μg of creatine kinase per ml, 0.17 mM dithiothreitol, 0.3 mM $CaCl_2$, 0.66 mM EGTA, and mRNA as indicated. After 2 hr at 30°C, samples were immunoprecipitated or analyzed directly by PAGE.

Polysome Preparation. Suspension cultures of mock-infected, avian reovirus-infected, or type 3 reovirus-infected L cells (10^9 cells each) were rapidly cooled at 17 hr postinfection (pi) by adding ice-cold polysome buffer (12), and washed cells were homogenized in 4 ml of polysome buffer containing 1 mM dithiothreitol. Polysomes were resolved by centrifugation of the postnuclear supernatant fraction in 36-ml 15–50% sucrose gradients in 20 mM Tris·HCl, pH 7.4/0.1 M KCl/5 mM $Mg(OAc)_2$ (in a SW 28 rotor at 26,000 rpm for 200 min at 4°C), and 2.5-ml fractions monitored automatically at 254 nm were collected in 6 ml of cold ethanol. After 30 min on dry ice, polysomes and ribonucleoproteins were pelleted, resuspended in 3 ml of 0.1 M Tris·HCl, pH 7.6/0.2 M LiCl/2 mM EDTA/2% $NaDodSO_4$, and extracted with phenol followed by phenol/chloroform/isoamyl alcohol. After precipitation with ethanol, RNA was dissolved in water to 5 mg/ml for cell-free translation studies. To measure the distribution of viral mRNA, 10^8 cells from the same cultures were radiolabeled for 4 hr with 40 μCi of [^{3}H]uridine (specific activity, 50 Ci/mmol) per ml before harvesting. The postnuclear supernatant was layered onto a 12-ml 15–50% sucrose gradient and centrifuged in the SW 41 rotor at 36,000 rpm for 2 hr at 4°C. Fractions of 1 ml were collected, and 0.1 ml of each was assayed for radioactivity in 10 ml of Hydrofluor.

RESULTS

Protein Synthesis in Avian Reovirus-Infected Cells. The report that some mutants of avian reovirus can replicate in mouse L cells implied that avian viral transcripts are translated in mammalian cells (5). To investigate this possibility directly, L cells growing in suspension were infected with avian reovirus and radiolabeled with [^{35}S]methionine at 24 hr pi; cytoplasmic extracts were compared to the corresponding samples of infected CEF by immunoprecipitation. Radiolabeled, newly synthesized large (λ), medium (μ), and small (σ) avian reovirus-specific polypeptides were readily discernible in infected CEF (lanes I in Fig. 1) as compared to mock-infected cells (lanes U in Fig. 1). By contrast, no avian viral proteins were evident in the L-cell cultures. The protein patterns obtained from extracts of infected and mock-infected mouse cells were essentially identical, and no proteins were detectable by immunoprecipitation. The results suggest that any avian viral mRNAs transcribed from the parental genome in infected mouse L cells failed to direct viral protein synthesis.

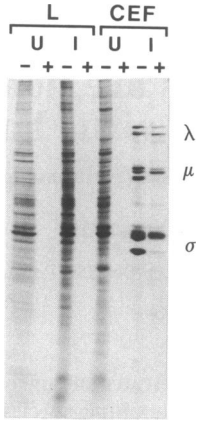

FIG. 1. Analysis of protein synthesis in avian reovirus-infected cells. L cells growing in suspension culture or CEF in monolayers were mock-infected (lanes U) or infected with avian reovirus (lanes I) and after 24 hr were radiolabeled with [^{35}S]methionine for 2 hr. Cytoplasmic extracts were analyzed by electrophoresis in a $NaDodSO_4$/15% polyacrylamide gel either directly (lanes −) or after immunoprecipitation with antibody to purified avian reovirus (lanes +). Positions of large (λ), medium (μ), and small (σ) avian viral polypeptides were determined by using purified virions.

Protein Synthesis in L Cells Coinfected with Avian and Mammalian Reoviruses. Mixed infection of L cells with avian and mammalian reoviruses has been reported to "derepress" transcription of the avian virus genome, leading to synthesis of all 10 avian virus mRNAs (5). To test for the synthesis of avian viral proteins under these conditions, L cells were infected with 80 plaque-forming particles each of avian and type 3 reovirus per cell, and protein synthesis was analyzed as above. No avian viral proteins were detected by immunoprecipitation of extracts of doubly infected L cells radiolabeled either at 18 hr (Fig. 2, lane 5) or 24 hr pi (Fig. 2, lane 8) as compared to avian reovirus-infected CEF (Fig. 2, lane 11). However, reovirus type 3 proteins were evident by specific immunoprecipitation of extracts of the same doubly infected cells (Fig. 2, lanes 6 and 9) and in singly infected L cells (Fig. 2, lane 10). These results indicate that the productive replication of reovirus type 3 does not lead to avian virus protein synthesis in doubly infected mouse L cells.

Hypertonic and Cycloheximide Treatment of Reovirus-Infected L Cells. Brief treatment of infected L cells with hypertonic medium (13) or a low concentration of cycloheximide (12) has been shown to enhance the translation of

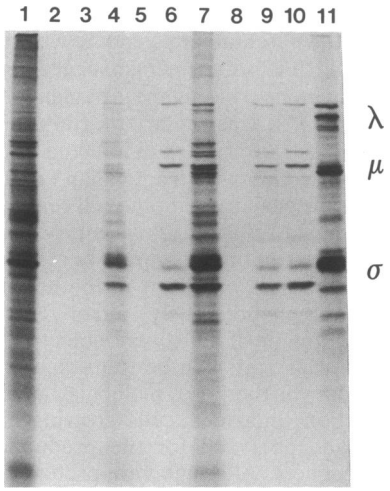

FIG. 2. Effect of mammalian reovirus superinfection on protein synthesis in avian reovirus-infected L cells. Cultures were mock-infected (lanes 1–3) or infected with avian reovirus followed 1 hr later by reovirus type 3 (lanes 4–9). Cells were radiolabeled after 18 hr (lanes 1–6) or 24 hr (lanes 7–9) and processed as in Fig. 1. As controls, L cells infected with type 3 reovirus (lane 10) and CEF infected with avian reovirus (lane 11) were labeled at 12 hr pi. Samples were immunoprecipitated with antibody to avian reovirus (lanes 2, 5, 8, and 11) or to reovirus type 3 (lanes 3, 6, 9, and 10).

reovirus type 3 mRNAs relative to cellular mRNAs. The same approaches were used in an effort to detect avian viral protein synthesis in infected mammalian cells. Hypertonic treatment of reovirus type 3-infected L cells reduced drastically and differentially host protein synthesis without affecting the pattern of radiolabeled viral products (Fig. 3, lanes H). Similar results were obtained when type 3 reovirus-infected cells were exposed to cycloheximide, although viral protein synthesis was decreased (Fig. 3, lanes C). As in uninfected cultures, the same treatments of avian reovirus-infected L cells diminished host protein synthesis but failed to reveal any viral translation products.

Transcription of the Avian Viral Genome in Avian Reovirus-Infected L Cells. To investigate whether the absence of avian virus protein synthesis in infected mammalian cells is due to a transcriptional restriction, we assayed for viral mRNA synthesis in ActD-treated, infected L cells. Cultures radiolabeled with [^{3}H]uridine for 4 hr at the indicated times showed an initial decrease followed by an increase in RNA synthesis beginning at 16–20 hr pi; this increase coincided with the *in vitro* messenger activity of RNAs isolated from the same culture (Fig. 4*A*). Sedimentation analysis of the extracted RNAs indicated that the radiolabel incorporated at early times pi was mainly in slowly sedimenting material (Fig. 4*B*), consistent with ActD-resistant tRNA 3′-end radiolabeling (14). At 28 hr pi the radioactivity was predominantly in viral mRNAs that sedimented in the same positions as the three size classes of *in vitro* synthesized avian viral transcripts (Fig. 4*B*).

To test the functional integrity of the avian viral transcripts, cytoplasmic RNAs extracted from infected CEF and mouse L cells were translated in rabbit reticulocyte lysates. The *in vitro* products were compared to virus-specific polypeptides made in avian reovirus-infected CEF that could be detected above the background of host-cell proteins by 4 hr pi (Fig. 5, lanes 1 and 3). RNA preparations from both

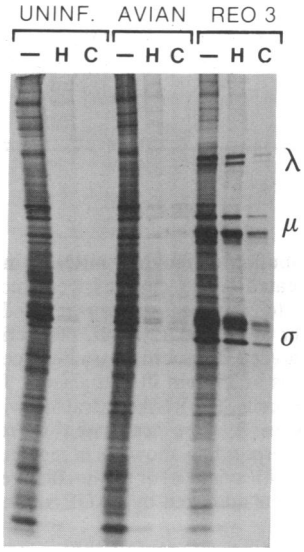

FIG. 3. Effect of hypertonic shock or cycloheximide on avian reovirus-infected L cells. At 7 hr pi, suspension cultures of mock-infected (lanes Uninf.), avian reovirus-infected (lanes Avian), or type 3 reovirus-infected (lanes Reo 3) L cells were plated on 60-mm dishes (10^7 cells per plate). When all of the cells were attached 1 hr later, each culture was "pulsed" for 15 min with 1 ml of methionine-free medium supplemented with 100 μCi of [^{35}S]methionine (lanes −), 200 μCi of [^{35}S]methionine and 125 mM NaCl (lanes H), or 200 μCi of [^{35}S]methionine and 0.75 μg of cycloheximide (lanes C). After a 15-min "chase" in 5 ml of the corresponding medium containing 75 μg of methionine in place of the [^{35}S]methionine, cytoplasmic fractions were prepared and analyzed by PAGE.

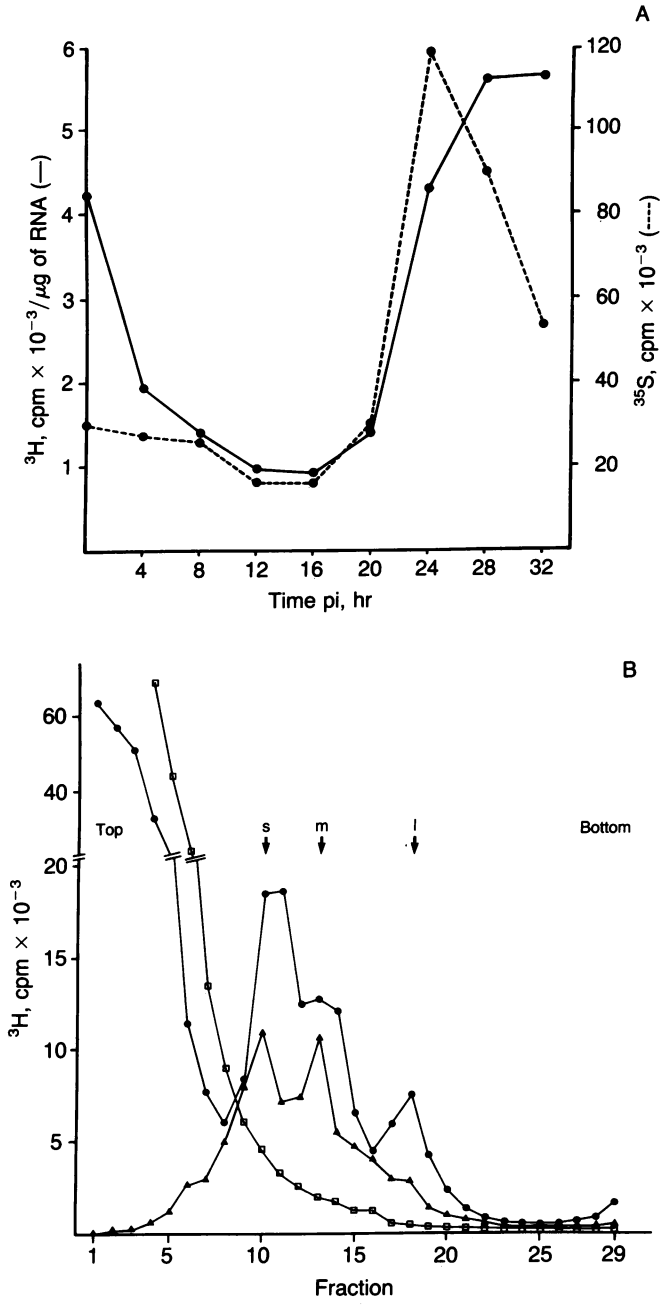

FIG. 4. Viral mRNA synthesis in avian reovirus-infected L cells. (*A*) Cultures treated with 0.5 μg of ActD per ml were labeled with [^{3}H]uridine for 4 hr at the times indicated, and the specific radioactivity of phenol-extracted cytoplasmic RNA was estimated from the acid-precipitable radioactivity. The same RNA samples were used to program a reticulocyte lysate for [^{35}S]methionine incorporation into acid-precipitable products (----). (*B*) [^{3}H]Uridine-labeled RNA samples extracted at 8 hr (□) and 28 hr pi (●) were compared by glycerol gradient centrifugation (8) to labeled transcripts made *in vitro* by purified avian reovirus cores (▲). The positions of the small (s), medium (m), and large (l) mRNA size classes are indicated.

infected L cells and CEF directed the synthesis of a similar spectrum of ^{35}S-labeled proteins including products in the λ, μ, and σ size classes (Fig. 5, lanes 5 and 7). The same results were obtained with mRNAs synthesized by purified avian reovirus cores (Fig. 5, lane 9). In each case the virus-specific products were confirmed by immunoprecipitation with antibody against avian reovirus S1133 (Fig. 5, lanes 2, 4, 8, and 10). The results demonstrate that avian reovirus genome segments in all three size classes are transcribed in infected

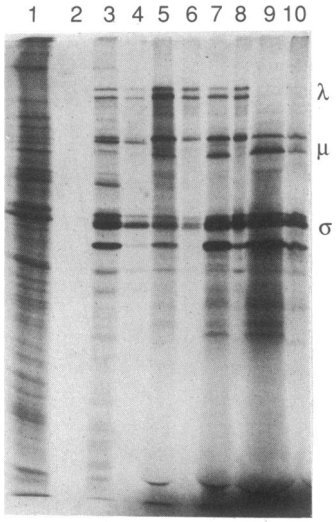

FIG. 5. Identification of avian reovirus polypeptides by immunoprecipitation. Mock-infected (lane 1) and avian reovirus-infected CEF (lane 3) were labeled with [^{35}S]methionine for 1 hr before harvesting at 4 hr pi. RNAs extracted from another L cell culture at 28 hr pi (lane 5) and from avian reovirus-infected CEF at 12 hr pi (lane 7) as well as transcripts made *in vitro* by purified avian viral cores (lane 9) were translated in reticulocyte lysates. Samples in lanes 1, 3, 5, 7, and 9 also were immunoprecipitated with antibody against purified avian reovirus S1133 as shown in lanes 2, 4, 6, 8, and 10, respectively.

L cells, yielding mRNAs that direct the synthesis of viral proteins *in vitro*. Although viral transcription is not restricted, the resulting mRNAs apparently are inactive in the nonpermissive mammalian cell. As a consequence, viral replication is blocked at the translational level.

Distribution of Viral Transcripts in Cytoplasmic Fractions of Reovirus-Infected Cells. To determine if initiation of viral mRNA translation occurs in avian reovirus infected mouse L cells, cytoplasmic fractions from ActD-treated cultures radiolabeled with [^{3}H]uridine from 24 to 28 hr pi were analyzed by sucrose gradient sedimentation. Most of the radiolabeled material, like the sample from mock-infected cells, migrated near the top of the gradient above or close to the peak of 80S ribosomes (Fig. 6*A*, arrow). Under the same conditions, radiolabeled RNA from reovirus type 3-infected L cells sedimented faster than monosomes and was broadly distributed in the polysome region. To confirm that the radioactivity profiles corresponded to viral mRNAs, gradient fractions were phenol-extracted and translated in reticulocyte lysates. The incorporation of [^{35}S]methionine into polypeptides correlated with the distribution of radiolabeled RNA: maximum protein synthesis was obtained with RNAs extracted from fractions 3 and 4 in the case of avian reovirus-infected cells and fractions 7 and below for reovirus type 3-infected cultures (Fig. 6*B*). In addition, the ^{35}S-labeled polypeptide products directed by mRNAs from the peak fractions corresponded to λ, μ, and σ viral polypeptides as determined by PAGE (Fig. 6*B Insets*).

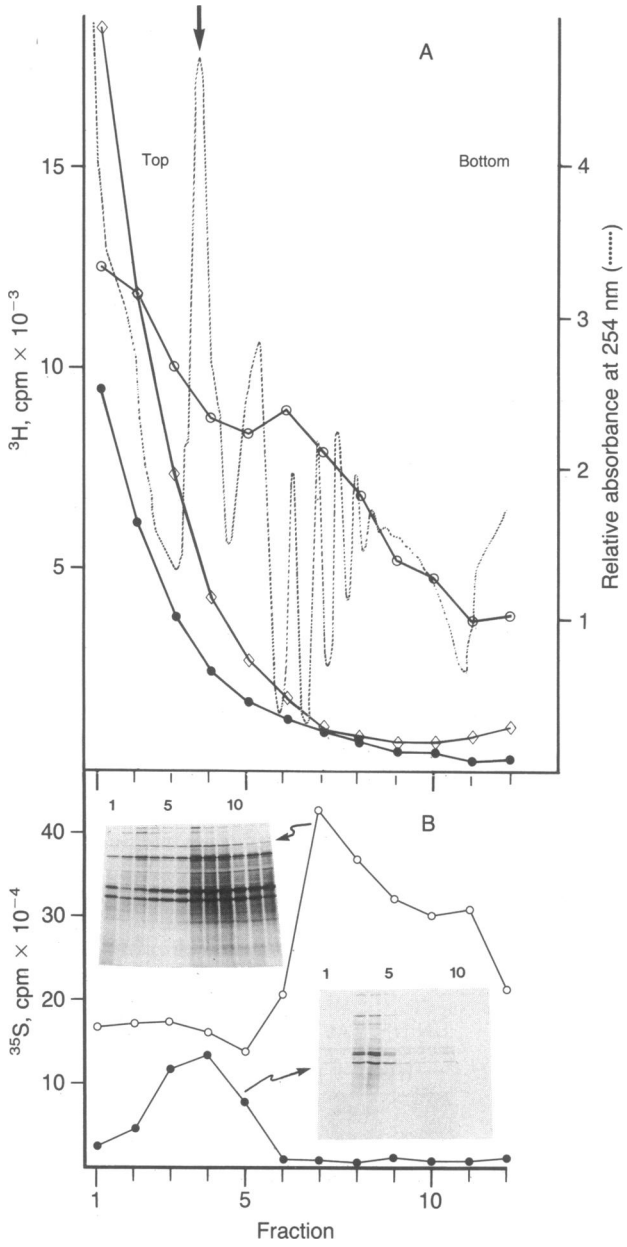

FIG. 6. Distribution of reovirus mRNAs in cytoplasmic fractions. (*A*) ActD-treated L cell cultures were mock-infected (◇) or infected with avian (●) or type 3 reovirus (○) and were labeled with [^{3}H]uridine for 4 hr at 24 and 12 hr pi, respectively. Cytoplasmic fractions were analyzed by sucrose gradient centrifugation. ····, Profile of polysomes at 254 nm in cytoplasmic fraction from uninfected, untreated L cells. (*B*) RNAs extracted with phenol from the gradient fractions in *A* were translated in reticulocyte lysate. [^{35}S]Methionine incorporation shown is in response to RNA samples from cells infected with avian (●) or mammalian reovirus (○). (*Insets*) Radiolabeled products analyzed by PAGE and autoradiography.

DISCUSSION

The reovirus genome consists of 10 segments of double-stranded RNA that are transcribed to form viral mRNAs by an RNA polymerase that is an integral part of the parental virion (2). Transcriptional regulation in reovirus-infected cells has been a matter of controversy; some studies indicated that in permissive cells viral mRNA synthesis is restricted to four genome segments (L1, M3, S3, and S4) during the first few hours after infection (6–8). A model to explain this regulation suggested that a preexisting cellular protein blocks transcription of the six "late" genes and subsequently is inactivated by viral protein(s) encoded by one or more of the four "early" transcripts. However, other studies of mammalian cells productively infected with reovirus failed to demonstrate transcriptional regulation and suggested instead that the pattern of viral mRNA synthesis remains constant and essentially unrestricted throughout most of the infectious cycle (15–17). Avian reovirus S1133 enters mouse L cells and is uncoated but, unlike its mammalian counterpart, does not multiply productively. In the nonpermissive host, transcription of the avian viral genome

was reported to be limited to the 4 "early" gene segments, suggesting that host range restriction occurs at the level of mRNA synthesis (5).

We have investigated gene expression in avian reovirus-infected mouse L cells and found that all of the viral transcripts are apparently produced without detectable viral protein synthesis. A full spectrum of reovirus type 3 proteins was synthesized in L cells coinfected with avian and mammalian reoviruses, but again no avian virus proteins were observed. Similarly, hypertonic shock or cycloheximide treatment failed to demonstrate avian viral protein synthesis in infected mouse cells. The combined results suggest that, in contrast to mammalian reovirus, avian viral transcripts are inactive in L cells. Contrary to previous findings (5), the lack of avian virus replication in L cells was apparently not due to restriction of transcription; all classes of avian virus mRNAs were present in the cytoplasm of infected cells. Although the viral transcripts in the nonpermissive cell were maintained in a nonfunctional state, after deproteinization they actively directed synthesis of a normal spectrum of virus-specific polypeptides *in vitro*.

mRNA molecules not engaged in protein synthesis have been observed in a variety of cell types. They occur in cytoplasmic ribonucleoprotein particles (mRNPs) not associated with ribosomes and presumably contribute to regulation of gene expression at the level of mRNA utilization (18, 19). Some mRNPs can be translated *in vitro* after deproteinization, suggesting that the bound proteins are inhibitory. Activation of these "masked" mRNAs has been shown to occur in response to several different conditions, including fertilization in oocytes (20–23), induction of ferritin synthesis by Fe^{2+} in rat liver (24, 25), insulin stimulation in CEF (26), photoactivation in *Volvox carteri* (27), low level cycloheximide treatment in somatic tissue culture cells (12), and dimethyl sulfoxide exposure in mouse erythroleukemia cells (28).

Avian viral mRNAs in the cytoplasm of nonpermissive infected L cells in suspension culture are apparently not associated with polysomes and may be bound to inhibitory proteins since they are accurately translated *in vitro* after deproteinization. However, attempts to promote translation of the viral mRNAs *in vivo* by coinfection of L cells with reovirus type 3 or by applying conditions that enhance viral protein synthesis in other systems were unsuccessful. The possibility that the 5′- or 3′-terminal, untranslated sequences of avian reovirus mRNAs are inhibitory (29, 30) for translation in mouse cells seems unlikely because the isolated transcripts are functional in rabbit reticulocyte lysates. Further studies of the molecular mechanisms governing ribosome binding and translation initiation of avian reovirus mRNAs in permissive vs. nonpermissive cells should provide new insights into host-range restriction of animal viruses.

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