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Virus Interference by Cellular Double-Stranded Ribonucleic Acid

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Ribonuclease-resistant ribonucleic acid (RNA) was isolated from uridine-labeled cultures of rabbit kidney, chicken embryo, and HeLa cells. This RNA, regardless of its source, was found to induce interference with virus growth in either rabbit kidney or chicken embryo cultures. Nuclease-treated cellular nucleic acids exhibited interference-inducing activity which eluted with a small fraction of RNA in the exclusion volume of a 6% agarose gel column. Besides resistance to ribonucleases, the interference inducer and RNA isolated from partially digested nucleic acids have in common two properties of double-stranded RNA: (i) similar sharp melting profiles were obtained for inducer and ribonuclease-resistant RNA, with T_m dependent on NaCl concentration; (ii) ribonuclease-resistant inducer and RNA banded together in Cs_2SO_4 density gradients at a density characteristic of known double-stranded RNA. After melting at low ionic strength, the labeled RNA shifted to a higher density and its capacity to inhibit virus replication was lost. Velocity sedimentation analysis of the cellular ribonuclease-resistant RNA indicated that the majority sedimented between 7 and 11*S*, but only RNA sedimenting at ≥ 8 to 20*S* had a high specific activity of interference induction. Without prior ribonuclease treatment, the ribonuclease-resistant RNA can be precipitated with 2 M LiCl and thus appears to exist in purified cellular nucleic acids as part of molecular complexes with both single- and double-stranded regions of RNA. The biosynthesis of cellular double-stranded RNA is inhibited by actinomycin D.

Interferon is induced by a variety of viruses, often under conditions where little or no viral multiplication occurs (16). It was suggested, therefore, that some part of the virus particle itself, perhaps the "foreign" nucleic acid, could be the stimulus for induction (18). Early experiments designed to test this theory by treatment of a given species of animal or cells in culture with diverse nucleic acids supported the notion that nucleic acids from heterologous animal species could induce interferon (20–22, 27, 30), whereas those from homologous cells were less potent or inactive (20, 22, 27) unless chemically modified (20).

Consequently, the discoveries of several inducers which apparently contained no nucleic acid (15, 17) resulted in questioning of the theory that a foreign nucleic acid was the essential stimulus for interferon induction (19). Conflicting results in further tests (3, 7) have left the issue about the "foreign" origin of inducing ribonucleic acid (RNA) unresolved. Recently, it became clear that synthetic or natural double-stranded RNA species which are not endogenous to the animal system used for assay are extremely potent interferon inducers (11), whereas pure deoxyribonucleic acid (DNA), DNA-RNA hybrids, and single-stranded RNA were found to be inactive (4, 5, 15).

These findings and the discovery that from about 0.01 to 1.0% of RNA in animal cells is ribonuclease-resistant (6, 24) or reactive with antibodies specific for double-stranded RNA (28) led us to suspect that traces of the endogenous ribonuclease-resistant RNA could be the interferon inducer in cellular nucleic acids previously thought to contain RNA only in single-stranded form. Furthermore, the suspected presence of double-stranded RNA in normal cells raised the question of whether homologous as well as foreign double-stranded RNA can induce interferon in a given species of cells. We present here further characterization of cellular ribonuclease-resistant RNA and show that it is indeed double-stranded. Part of this RNA is capable of inducing interference with virus growth in a given species of cells, irrespective of whether it is isolated from a heterologous or homologous species.

MATERIALS AND METHODS

Cell cultures. For preparation of rabbit kidney primary cultures, minced kidneys from 3- to 5-week-old New Zealand White rabbits were stirred gently at

room temperature for 1 to 2 hr in a solution of Eagle's minimal essential medium (MEM; Grand Island Biological Co.) containing 0.25% Pronase. The cell suspension resulting from one kidney pair was washed and plated in MEM containing 10% calf serum on a total tissue culture dish surface area of about 1,000 cm^2. With a medium change the next day, confluency was reached in 5 to 7 days after seeding. A line of HeLa cells (HeLa I) from C. Colby, Jr. (University of Connecticut, Storrs) and HeLa S_3 cells (HeLa II) from M. Bishop (University of California, San Francisco) were also grown in MEM plus 10% calf serum. For preparation of RNA, primary or secondary chicken embryo cultures (26) were grown in medium 199 (Grand Island Biological Co.) supplemented with 2% Tryptose phosphate broth and 1% each calf and chicken sera. Periodic inspection of all cell types by electron microscopy showed no evidence of contamination with mycoplasma.

Buffers. Low-salt buffer contained 0.01 M NaCl in addition to 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.4) and 1 mM ethylenediaminetetraacetic acid (EDTA). High-salt buffer was the same except for the NaCl concentration which was 0.3 M.

Preparation of a nuclease-resistant fraction of cellular ^{3}H-RNA. Confluent monolayers were labeled by adding 50 to 100 μCi of ^{3}H-uridine per 100-mm petri dish after 3 to 4 hr of preliminary incubation in 4 ml of the fresh growth medium appropriate for each culture type as indicated above. Actinomycin D was added, where indicated, at 6 μg/ml for 15 min before the addition of radioactive precursor. After 90 to 120 min of labeling, the nuclease-resistant RNA fraction was prepared from purified cellular nucleic acids as described earlier (6). For samples of nucleic acids to be treated with deoxyribonuclease alone, ribonuclease-free pancreatic deoxyribonuclease I (Worthington Biochemical Corp.) was used. Otherwise, the nucleic acids were incubated first with standard grade pancreatic deoxyribonuclease I and then with pancreatic ribonuclease, following published procedures (6).

When the partially digested cellular nucleic acids were chromatographically analyzed on a column of 6% agarose gel, the exclusion volume (determined as in reference 6) routinely contained the first two or three fractions of material labeled with ^{3}H-uridine. These were pooled and extracted twice with phenol before precipitation with ethanol. At this stage, a visible precipitate often formed which made a turbid solution when redissolved in $\leqq$ 1 ml of low-salt buffer (without EDTA). This turbidity was largely removed from solutions without loss of radioactive RNA by centrifugation at 150,000 $\times$ *g* for 10 min, after which the supernatant fraction was stored at -20 C. This fraction is referred to hereafter as the nuclease-resistant RNA or cellular double-stranded RNA.

Standard nuclease treatments and thermal denaturation of RNA. The standard ribonuclease treatment was with 20 μg of pancreatic ribonuclease per ml and 0.5 μg of T_1 ribonuclease per ml (Worthington Biochemical Corp.) in 0.8 ml of high-salt buffer for 30 min at 37 C. The standard ribonuclease plus deoxyribonuclease treatment was the same except that 50 μg of deoxyribonuclease I per ml and 2 mM $MgCl_2$ were also present. To standardize the ratio of enzyme to nucleic acid, yeast soluble RNA (sRNA; CalBiochem) was added (60 μg/ml) to all samples with $\leqq$0.01 absorbancy $(A)_{260}$/ml. Thermal denaturation of ribonuclease-resistant RNA was done in low-salt buffer as reported earlier (6).

Fractionation of RNA with 2 M LiCl solution. Purified total cellular nucleic acids were treated with deoxyribonuclease, extracted with phenol, ethanol-precipitated, and washed with ethanol as described previously for preparation of the nuclease-resistant fraction of cellular RNA. The deoxyribonuclease-treated nucleic acids were dissolved in low-salt buffer at $\leqq 10$ A_{260}/ml, and sufficient LiCl in solution (10 M) was introduced to give a final concentration of 2 M. After 16 hr or more at 0 C, the solution was centrifuged for 15 min at 20,000 $\times$ *g*. Three volumes of low-salt buffer was added to the resulting supernatant fraction and several milliliters was added to the pellet. Then the nucleic acids in each fraction were ethanol-precipitated and washed.

Virus interference assays. Assays were done in duplicate by using 0.1 ml of medium per cell culture in 6-mm wells of microtest tissue culture plates (Falcon Plastic). The assay cultures were either 7- to 14-day-old rabbit kidney cell primary cultures prepared and grown as described above or chicken embryo primary cultures plated at 5×10^4 cells per 6-mm well and incubated for 5 to 6 days in NCI Medium (Schwarz BioResearch) supplemented with 2% Tryptose phosphate broth and 1% each calf and chicken sera. Samples of RNA to be tested for interference with virus replication were precipitated in ethanol with carrier yeast sRNA and dissolved directly in medium 199 with 2% fetal calf serum and 10 μg of diethylaminoethyl-dextran per ml (molecular weight = 2×10^6, Pharmacia) at a concentration of $\leqq$100 μg of RNA per ml. This concentration of sRNA in 0.1 ml failed to induce resistance to viruses and did not affect interference induced by the synthetic polymer poly rI:rC (Miles Laboratories, Inc.).

After 18 to 24 hr of exposure to nucleic acid solutions, chicken or rabbit cells were washed with Tris-buffered saline (TS; reference 8) and challenged, respectively, with either Sindbis virus or vesicular stomatitis virus (VSV, Indiana strain) at a multiplicity of about 5 plaque-forming units (PFU) per cell. Virus was applied in 20 μliters of virus growth medium (NCI plus 6% calf serum for Sindbis, MEM plus 10% calf serum for VSV) which was removed after a 1 hr period of adsorption. Then cells were washed twice with TS before being refed with 0.2 ml of appropriate virus growth medium. After 18 to 24 hr of further incubation, cultures were examined for cytopathological effects (which correlated with viral growth), and the plates were frozen at -70 C until the medium was analyzed by plaque assay on chick embryo secondary monolayers as previously described for Sindbis virus (6).

Control samples of a standard interferon inducer, poly rI:rC, were included in all experiments when interfering activity of cellular nucleic acids was assayed. Poly rI:rC reduced virus growth to titers below

detection at a concentration of 0.1 μg/ml or less when cultures without inducer gave 10^6 to 10^7 PFU/ml for either the chicken or rabbit cell system. Results are reported as the $\log_{10}$ (to the nearest 0.1) of the ratio of virus titer in uninduced control cultures to the average titer in two parallel cultures treated with a given sample to be assayed. This ratio is referred to as $\log_{10}$ virus titer reduction, and in practice duplicate assays gave reductions differing by $\leqq 0.5$ $\log_{10}$. Since, in general, the $\log_{10}$ virus titer reduction was not found to be linear with inducer RNA concentration, no attempt was made to quantify differences in RNA concentration of compared samples when they caused titer reductions which varied outside the range of duplicates. We used this titer-reduction assay as a measure of interferon induction because it has been shown to be more sensitive than assays for extracellular interferon (5, 31), even before adaptation to microassay procedure.

RESULTS

Isolation of a ribonuclease-resistant interference inducer from cellular nucleic acids. To answer the general question of whether the cellular nucleic acids of a given species contain double-stranded RNA able to induce interferon in the same species or other species of cells, the following experiments were carried out. The nuclease-resistant RNA was prepared from purified total cellular nucleic acids of chicken embryo fibroblasts or rabbit kidney cells by treatment with deoxyribonuclease and ribonuclease and chromatographic fractionation of the digest as outlined above. Only that fraction of the ribonuclease-resistant RNA which eluted in the void volume of the 6% agarose column was then analyzed for its ability to interfere with virus replication. In all four tested cell types, about 0.4% of the total ^{3}H-uridine incorporated into RNA during a 90-min pulse was in the ribonuclease-resistant fraction excluded by 6% agarose (Table 1). This amounted to less than 0.1% of the total A_{260} in the digest.

Nuclease-resistant RNA from chicken, rabbit, or HeLa cells depressed virus growth in both rabbit kidney and chicken embryo cell cultures—in all cases to a level lower than 2 $\log_{10}$ below control titers. By contrast, the same amounts of total cell nucleic acids which were used to isolate the interference-inducing, ribonuclease-resistant RNA did not show any interfering activity before digestion with deoxyribonuclease and ribonuclease. After nuclease treatment, however, samples of unfractionated nucleic acids from all cells interfered with virus replication to the same extent (within 0.5 $\log_{10}$ virus titer reduction) as the purified ribonuclease-resistant fraction alone. These findings are summarized in Table 1. Deoxyribonuclease treatment alone also rendered cellular nucleic acids active in inducing interference with virus replication (data not shown). Furthermore, in some experiments, part of the cell suspension from either one chicken embryo or rabbit kidney was cultured for interference assays, whereas the rest of the cells were grown as a source of labeled ribonuclease-resistant RNA. Thus induction of virus interference by the presumed double-stranded RNA fraction of chicken or rabbit cell nucleic acids was demonstrable not only in cells of the same or different species, but also in cells derived from the same piece of tissue as those from which the ribonuclease-resistant RNA was prepared.

Further nuclease treatments of the ^{3}H-RNA purified from degraded cellular RNA and DNA by chromatography on 6% agarose were performed under the standard conditions (*see above*) at high ionic strength. The resistance to ribonuclease (or a mixture of ribonuclease plus deoxyribonuclease) of this fraction of ^{3}H-RNA ranged from 50% to greater than 95% in different preparations from each cell type. However, no significant change ($\leqq 0.5$ $\log_{10}$ virus titer reduction) in the ability of these RNA fractions to interfere with virus replication was observed after standard nuclease treatments of seven of eight RNA samples from chicken and HeLa cells (data not shown). After thermal denaturation (Materials and Methods) of the purified ribonuclease-resistant fraction of cellular RNA at 100 C, less than 5% of the ^{3}H-RNA remained resistant to the standard ribonuclease treatment and all interfering activity was lost (Table 1).

The finding that the nuclease-resistant fraction of cellular RNA is resistant to both deoxyribonuclease and ribonuclease argues against the possibility that this ribonuclease-resistant ^{3}H-RNA which interferes with virus replication is in DNA-RNA hybrids because DNA-RNA hybrids are known to be sensitive to deoxyribonuclease (25) or a combined nuclease treatment (32). However, double-stranded RNA would be expected both to resist ribonuclease at high ionic strength and to be made sensitive by melting at low ionic strength before ribonuclease treatment (14). In addition, only such RNA is known to show a high specific activity of induction of virus interference (4, 5, 15). It can be estimated from the data in Table 1 that less than 0.05 μg (<0.01 A_{260}/ml) of ribonuclease-resistant RNA per ml reduced titers of two different viruses by as much as 5 $\log_{10}$ in two different cell species. Together these observations suggest that the ribonuclease-resistant fraction of cellular RNA which interferes with virus growth is double-stranded RNA.

Interference-inducing RNA appears to be partially single-stranded before ribonuclease treatment. To determine whether the RNA in the

TABLE 1. *Interference with virus growth by cellular nucleic acids before and after nuclease treatments*

Cell species	Nucleic acid fraction	Trichloroacetic acid-precipitable ^{3}H-nucleic acids				Interference assays ($\log_{10}$ virus titer reduction)[d]	
		Nuclease treatment[a]	Counts per min per ml	Per cent of total	A_{260}/ml	Chicken cells	Rabbit cells
Rabbit kidney	Total purified nucleic acids	None	600,000	100	4.0	−0.2	0.0
		Deoxyribonuclease, then ribonuclease					4.0
	Nuclease-resistant fraction excluded by 6% agarose	None	2,700	0.45	<0.001[c]	2.4	4.5
		Melted, then standard ribonuclease treatment	<100[b]			0.0	−0.2
Chicken embryo	Total purified nucleic acids	None	845,000	100	3.5		−0.3
		Deoxyribonuclease, then ribonuclease				3.9	3.6
	Nuclease-resistant fraction excluded by 6% agarose	None	2,600	0.31	<0.001	3.8	3.3
		Melted, then standard ribonuclease treatment	<110			0.2	−0.2
HeLa I	Total purified nucleic acids	None	510,000	100	1.1	0.1	0.0
		Deoxyribonuclease, then ribonuclease					4.3
	Nuclease-resistant fraction excluded by 6% agarose	None	1,170	0.23	<0.001	4.2	4.3
		Melted, then standard ribonuclease treatment	<50			−0.3	0.0
HeLa II	Total purified nucleic acids	None	580,000	100	3.6		
	Nuclease-resistant fraction excluded by 6% agarose	None	2,500	0.43	<0.001	3.4	5.3

[a] Deoxyribonuclease and ribonuclease treatments of the total nucleic acids are described for the preparation of a nuclease-resistant fraction of cellular ^{3}H-RNA. Melting was performed at 100 C in low-salt buffer. The procedure is presented in Materials and Methods with the details of the standard ribonuclease treatment.

[b] Not corrected for background.

[c] Estimated amount in a 10-fold dilution of the original solution which read <0.01 A_{260}/ml.

[d] Defined in Materials and Methods under virus interference assays.

nuclease-resistant fraction of cellular nucleic acids was completely or only partially double-stranded before ribonuclease digestion, its solubility in 2 M LiCl was examined. It is known that double-stranded RNA is soluble but partially double-stranded RNA is insoluble in 2 M LiCl (1). After deoxyribonuclease treatment, total cellular nucleic acids were fractionated with 2 M LiCl according to the procedure described above. Then the supernatant and pelleted nucleic acids were either assayed directly for virus-interfering activity or digested with ribonuclease before isolation of the nuclease-resistant fraction of ^{3}H-RNA by agarose column chromatography (*see above*). Over 90% of the total ribonuclease-resistant ^{3}H-RNA in HeLa I or chicken embryo cell nucleic acids was found in the LiCl-insoluble pellet. Moreover, interference-inducing activity was detectable only in the precipitated fraction of HeLa I or rabbit kidney cell nucleic acids. These results are summarized in Table 2. Under the same conditions, only 12% (195 of 1,560 counts/min) of added influenza virus double-stranded ^{32}P-RNA (10) was precipitated with HeLa I cellular double-stranded RNA. Therefore, precipitation in 2 M LiCl of the interference-inducing activity with the ribonuclease-resistant RNA suggests that, in the total purified nucleic acids of the cell, the nuclease-resistant inducer could be the double-stranded core of an RNA complex that also has

TABLE 2. *Distribution in 2 M LiCl of ribonuclease-resistant ^{3}H-RNA and virus-interfering activity of cellular nucleic acids*[a]

Cellular nucleic acids	2 M LiCl-soluble fraction		2 M LiCl-insouble fraction	
	Ribonuclease-resistant RNA[b] (counts/min)	Virus interference[c] ($\log_{10}$ virus titer reduction)	Ribonuclease-resistant RNA[b] (counts/min)	Virus interference[c] ($\log_{10}$ virus titer reduction)
HeLa I	125 (9)[d]	−0.2	1,240 (91)[d]	3.7
Chicken embryo	77 (10)		785 (90)	
Rabbit kidney		0.1		4.4

[a] Total purified nucleic acids were treated with deoxyribonuclease and then fractionated with 2 M LiCl.

[b] The 2 M LiCl-soluble and -insoluble nucleic acids were treated with ribonuclease, and the ribonuclease-resistant fraction of RNA was purified by agarose gel exclusion chromatography as described for preparation of the nuclease-resistant fraction of total cellular nucleic acids.

[c] After precipitation with ethanol, the nucleic acids in the LiCl-soluble and -insoluble fractions were assayed for interference with the replication of vesicular stomatitis virus on rabbit kidney cells.

[d] Numbers in parentheses are expressed as per cent.

single-stranded regions which cause precipitation at high ionic strength.

Melting profiles of nuclease-resistant RNA and interference inducer. As expected for an RNA duplex (20), melting profiles with sharp transitions (at 86 C in 0.01 M NaCl) from ribonuclease resistance to ribonuclease sensitivity were obtained for the purified nuclease-resistant fractions of the different cellular RNA species (Fig. 1A, B and C). In the case of HeLa I nuclease-resistant RNA, the ability to induce virus interference in rabbit kidney cultures was completely lost when the ^{3}H-RNA was completely melted (Fig. 1C). Measurement of the T_m of cellular double-stranded RNA on the basis of its interfering activity in the virus titer reduction assay would require both the measurement of a twofold (0.3 $\log_{10}$) virus titer reduction and the assumption of some precise relationship between the concentration of interference-inducing double-stranded RNA and titer reduction. Our experiments were not designed to determine either such a small change in virus titer or the exact nature of the relation between inducing RNA concentration and titer reduction (see above for the limits of the interference assay).

To test whether the T_m of the material which interferes with virus growth is increased with increasing ionic strength as has been shown for double-stranded RNA (2), HeLa I ribonuclease-resistant ^{3}H-RNA was heated at 100 C in high-salt buffer with 0.3 M NaCl (*see above*). It was found that 21% (95 counts/min) of the ^{3}H-RNA and enough virus-interfering activity to give 1.5 $\log_{10}$ virus titer reduction remained resistant to ribonuclease after heating under these conditions. By contrast, less than 3% of the ^{3}H-RNA and no measurable interfering activity (-0.1 $\log_{10}$ virus titer reduction) survived the standard ribonuclease treatment after heating to 100 C at low ionic strength (Fig. 1C). This indicates that the T_m for ribonuclease resistance of the interference-inducing ^{3}H-RNA was increased with increasing ionic strength.

Buoyant density in Cs_2SO_4 of ribonuclease-resistant RNA and inducer of virus interference before and after melting. To examine further the suggested identity between the purified ribonuclease-resistant interference inducer and double-stranded RNA, the density distributions of ^{3}H-RNA and inducer were determined in gradients of Cs_2SO_4. In all cases, the ribonuclease-resistant ^{3}H-RNA banded in a sharp peak at a density of 1.61 to 1.63 g/ml. Single-stranded tobacco mosaic virus (TMV) RNA marker had a density of 1.68 g/ml, and salmon sperm DNA had a density of less than 1.50 g/ml under the same conditions. Interference assays on rabbit kidney cells showed higher levels of antiviral activity only in the gradient fractions containing the peak of the nuclease-resistant ^{3}H-RNA (Fig. 2A, B, and C). The density of the biologically active RNA fraction is in good agreement with that of known double-stranded RNA (29) and is much greater than those reported for DNA-RNA hybrids (1.49 to 1.54 g/ml; references 9, 29). When a sample of ribonuclease-resistant ^{3}H-RNA from each cell type was melted at 100 C before analysis, the ^{3}H-RNA and TMV RNA marker subsequently banded together at 1.68 g/ml (Fig. 2D, E, and F), and interference-inducing capacity was lost. Similar density shifts and loss of activity were noted after treatment of ribonuclease-resistant RNA with 97% dimethylsulfoxide (data not shown). The temperature-dependent density transition of the ribonuclease-resistant ^{3}H-RNA is consistent with the melting of an RNA duplex (29), as is the loss of ability to induce virus interference (6).

Sedimentation properties of the interference-inducing nuclease-resistant RNA before and after standard nuclease treatments. The ribonuclease-resistant ^{3}H-RNA separated from cellular nucleic acid digests by agarose gel chromatography sedimented through sucrose gradients in a broad band

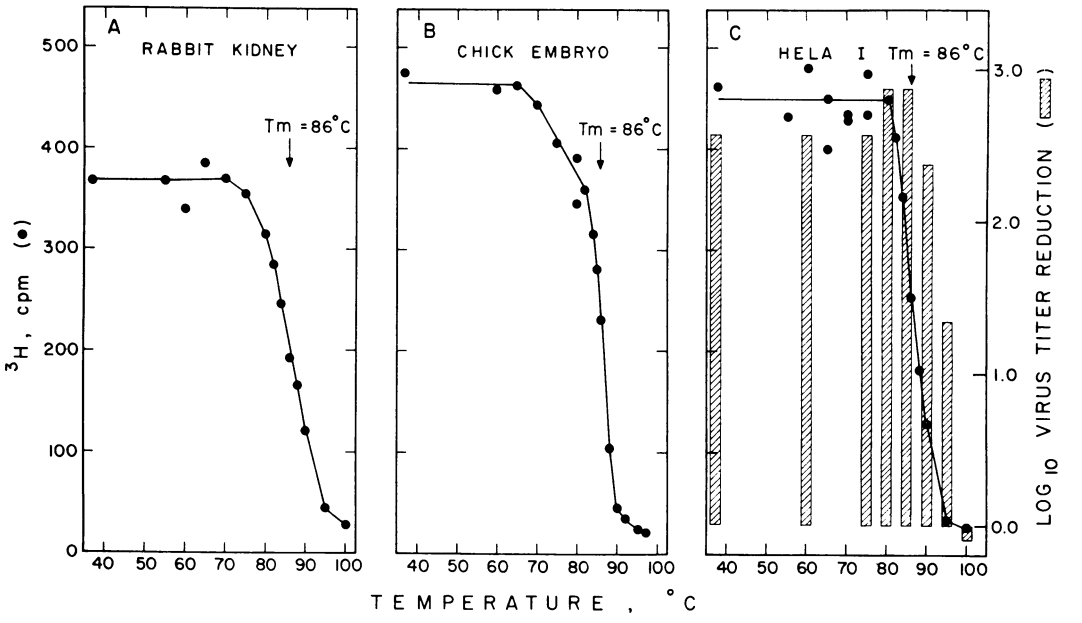

FIG. 1. *Melting profiles of nuclease-resistant RNA and virus interference inducer purified from nucleic acid digests by exclusion chromatography on 6% agarose. Samples of RNA were heated at the indicated temperatures and assayed for trichloroacetic acid-precipitable radioactivity after the standard ribonuclease treatment. For part C, samples of nuclease-resistant HeLa cell RNA were also analyzed for interference inducer. After thermal denaturation and treatment with ribonuclease under the standard conditions, the RNA was phenol extracted three times before precipitation with carrier sRNA by addition of two volumes of ethanol. The RNA was then dissolved directly in the medium used for virus interference assays with rabbit kidney cell cultures.*

with a major peak occurring at about 7*S* for rabbit kidney, 9*S* for chicken embryo, and 11*S* for HeLa I cells when compared with 4*S* yeast sRNA marker (Fig. 3). Sedimentation coefficients ranged from 6 to 8*S* for rabbit cell ribonuclease-resistant RNA (seven gradients of four independent preparations), 7 to 9*S* for chicken cell RNA (six gradients of four preparations), and from 10 to 11*S* for HeLa I cell ribonuclease-resistant RNA (four gradients of two preparations). For a given preparation, little change in sedimentation behavior of the ribonuclease-resistant ^{3}H-RNA was observed after the standard nuclease treatments, although still more ribonuclease digestion reduced the average *S* value by more than 15% (data not shown). Similar results were reported for dependence of sedimentation on ribonuclease treatment in the case of MS2 double-stranded RNA (2).

The lowest sedimentation coefficient obtained in our experiments after the standard deoxyribonuclease plus ribonuclease treatment of purified ribonuclease-resistant RNA is presented for each cell species in Fig. 4. A shoulder (Fig. 3B) or distinct second component (Fig. 4B) with a lower sedimentation coefficient than the main peak was observed in most preparations of chicken cell RNA. We did not examine the possibility that the physiological state of the cells in culture determines the sedimentation characteristics of different isolates of cellular double-stranded RNA. Nor was it shown whether the variations reflect differences in the original size or in the ribonuclease sensitivity of the base-paired portion of the undigested molecules. In all cases, however, it was found that the purified ribonuclease-resistant RNA which induced virus interference was distributed in sucrose gradients from over 7*S* to at least 20*S*. Most of the active fractions (15 to 40% of the total) were sedimented faster than the main peaks of cellular nuclease-resistant ^{3}H-RNA (Fig. 4).

Using the formula of Franklin for the molecular weight of double-stranded RNA (molecular weight = $2.42S^{5.16}$; reference 13), one can estimate average molecular weights for cellular double-stranded RNA of 5×10^4 daltons for 7*S* rabbit kidney cell RNA, 2×10^5 for 9*S* chicken cell RNA, and 6×10^5 for 11*S* HeLa I RNA. The interference-inducing double-stranded RNA, with sedimentation coefficients from 8 to 20*S*, can be estimated to possess a minimum molecular weight of 10^5 daltons and a maximum of several millions. A minimum molecular weight of 1.5×10^5 to

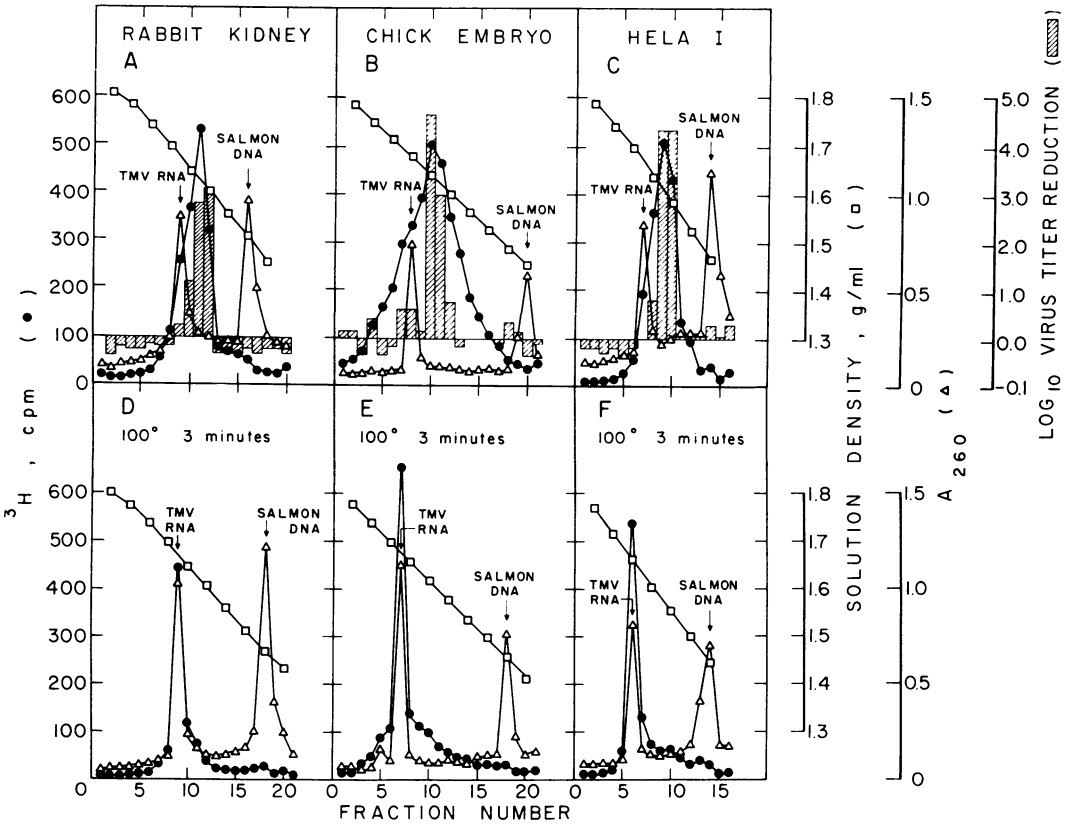

FIG. 2. *Density distribution of cellular nuclease-resistant ^{3}H-RNA and virus interference inducer in Cs_2SO_4 gradients before and after thermal denaturation. For parts A, B, and C, samples of nuclease-resistant RNA fractions were analyzed after a further standard nuclease treatment, followed by three phenol extractions and ethanol precipitation in the presence of one A_{260} unit each of tobacco mosaic virus RNA and salmon DNA. For D, E, and F, samples were heated in low-salt buffer at 100 C for 3 min, quenched in melting ice, and precipitated with marker nucleic acids as above. Each sample was then dissolved in 3.8 ml of Tris-hydrochloride buffer (0.05 M, pH 7.4) containing sufficient Cs_2SO_4 to give a final average density of about 1.58 g/ml. Centrifugation was at 20 C and 31,500 rev/min for 3 days in a Spinco SW 50.1 rotor. Gradients were fractionated by collecting drops from a pinhole in the bottom of the centrifuge tubes. Samples of gradient fractions were taken up immediately in 0.100-ml tared micropipettes which were weighed to measure solution density. To each fraction an equal volume of water was added before reading A_{250}, and samples were assayed either for trichloroacetic acid-insoluble radioactivity or, after ethanol precipitation, for interference inducer in rabbit cells. The latter assays were not performed for parts D, E, and F because melting destroys the inducer. The slight interference induction observed under the tobacco mosaic virus RNA marker for the gradient in part B probably indicates nuclease-resistant RNA co-precipitating with single-stranded RNA due to incomplete removal of single-stranded regions from the nuclease-resistant core of the RNA complex.*

1.6×10^5 daltons for interferon induction has been determined similarly for the synthetic polymer duplex rI:rC (12).

Effects of actinomycin D on the biosynthesis of cellular double-stranded RNA. Incorporation of ^{3}H-uridine into the double-stranded RNA fraction of cellular nucleic acids was inhibited by actinomycin D, as reported previously (24). Inhibitation was 90% or more in the two species of cells grown in primary cultures, whereas the synthesis of ribonuclease-resistant ^{3}H-RNA in the two HeLa cell lines was reduced by 78 to 85% (Table 3). Total incorporation of ^{3}H-uridine into cellular RNA was inhibited by over 98% under the same conditions (data not shown). The inhibition of incorporation into cellular double-stranded RNA by actinomycin D suggests that its synthesis may be dependent on DNA template.

DISCUSSION

Experiments described in this paper indicate that animal cells contain a fraction of RNA that

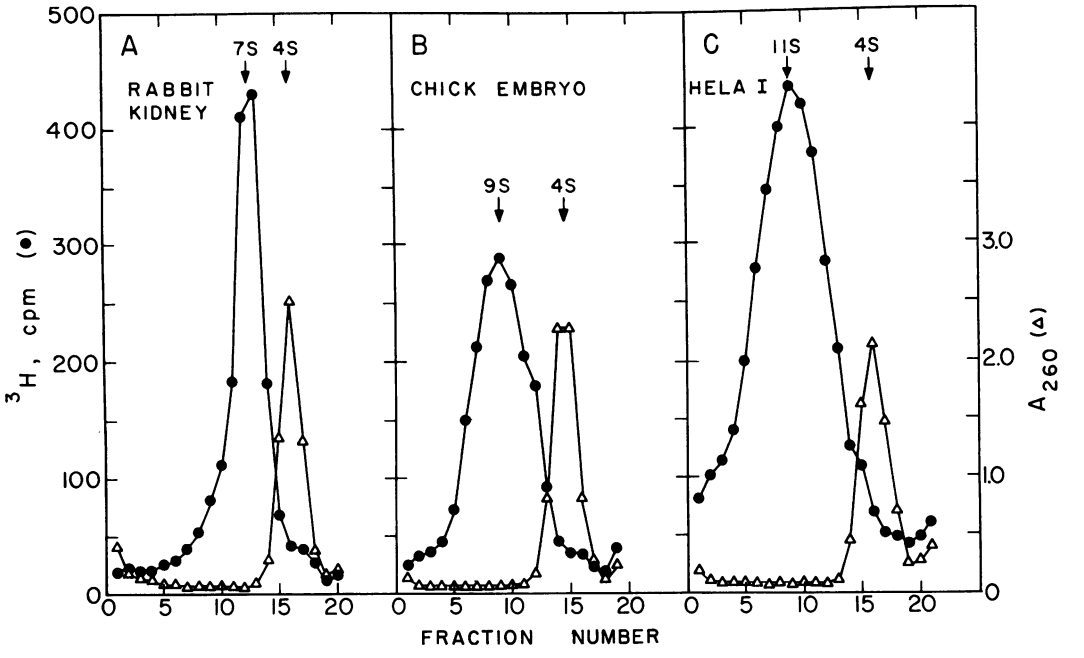

FIG. 3. *Sedimentation analysis of purified cellular ribonuclease-resistant RNA. Samples were precipitated with two volumes of ethanol in the presence of 50 μg of 4S yeast sRNA marker. Pellets were then dissolved in 0.15 ml of buffer containing 0.01 M Tris-hydrochloride (pH 7.4), 0.1 M LiCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate and layered on 5-ml linear gradients of sucrose (10 to 25%) in the same buffer. A 0.3-ml cushion of 65% sucrose in D_2O was included at the bottom of each gradient. Centrifugation was for 315 min at 20 C and 65,000 rev/min in a Spinco SW 65 rotor. Gradients were fractionated as in Fig. 2 and were similarly assayed for A_{260} and trichloroacetic acid-precipitable radioactivity.*

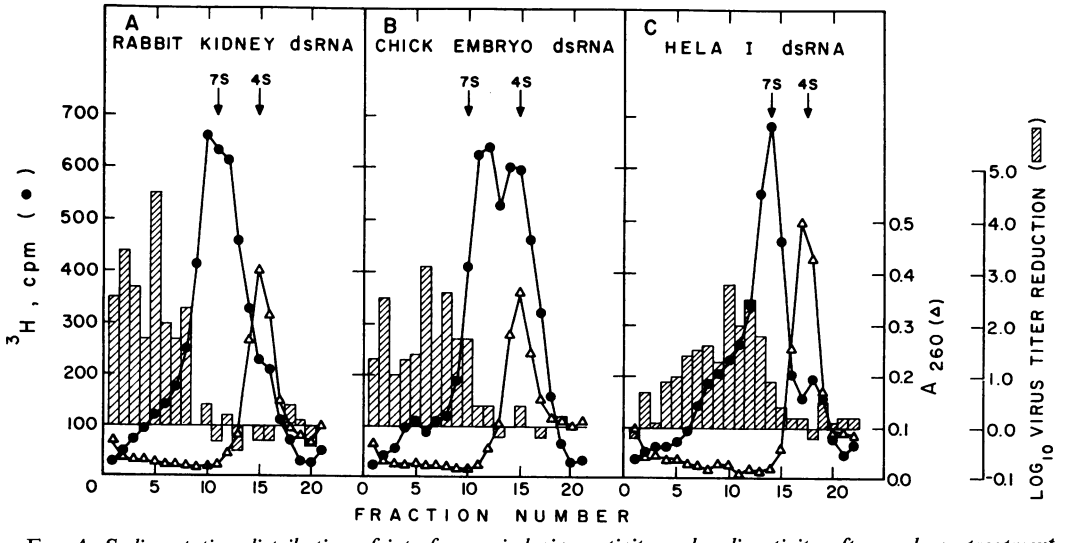

FIG. 4. *Sedimentation distribution of interference-inducing activity and radioactivity after nuclease treatment of cellular double-stranded RNA labeled with ^{3}H-uridine. After the ribonuclease-resistant RNA fraction was given the standard ribonuclease plus deoxyribonuclease treatment, it was phenol-extracted and precipitated with ethanol. Velocity sedimentation analysis on sucrose gradients is described in Fig. 3. In this case, after A_{260} readings, part of each fraction was assayed for trichloroacetic acid-insoluble radioactivity, and the remainder was ethanol-precipitated for virus interference assays with rabbit kidney cultures.*

TABLE 3. *Effects of actinomycin D on labeling of ribonuclease-resistant ^{3}H-RNA*[a]

Cell species	Trichloroacetic-acid-precipitable ^{3}H-RNA (counts/min)		Per cent inhibition of labeling
	Without actinomycin D	With actinomycin D	
Chicken embryo...	11,200	557	95
Rabbit kidney.....	927	95	90
HeLa I.....	5,018	1,095	78
HeLa II.....	1,639	242	85

[a] Nuclease-resistant fraction of cellular ^{3}H-nucleic acids was prepared from parallel cultures with and without actinomycin D.

is double-stranded and induces interference with virus replication. In this study, it has not been demonstrated directly that interference with the growth of Sindbis virus or VSV by cellular ribonuclease-resistant RNA is mediated by interferon. But experiments carried out under similar conditions in this (5, 6) and other (12, 13) laboratories have suggested that induction of interferon by double-stranded RNA is the basis of such interference.

Cellular double-stranded RNA and interference-inducing activity purified from cellular nucleic acids share these properties: they are both resistant to ribonuclease and deoxyribonuclease, they have the same distinctive solubilities in salt solutions of different concentrations, and the T_m values for their thermal denaturation are coordinately affected by changes in ionic environment. Also they have the same buoyant density in Cs_2SO_4. However, the distributions of ribonuclease-resistant interference-inducing activity and RNA were only partially coincident after fractionation by velocity sedimentation: inducing RNA sedimented faster than the average-sized molecule of cellular double-stranded RNA.

Therefore, it may be concluded that the high-molecular-weight double-stranded RNA in cells is the most efficient component of cellular nucleic acids reported to induce interferon.

Earlier reports on ribonuclease-resistant cellular RNA did not show induction of virus interference (6, 24), but presumably the negative result (6) was due to lower sensitivity of the assay system which employed cultures 100 times larger than those used here. The observation in this work that total cellular nucleic acids had no interference-inducing action but were rendered potent after deoxyribonuclease treatment cannot be explained from our experiments.

This work shows that cells of a given animal produce double-stranded RNA which induces virus interference in other cells of the same animal, as well as in those of another species of animal. This evidence indicates that double-strandedness may be the only essential property for interferon induction by natural RNA, rather than the source of the RNA as postulated previously (18). Such a conclusion raises the question of why resident cellular double-stranded RNA, as it exists in the cell, does not induce detectable interference with virus growth. Perhaps the intracellular concentration of double-stranded RNA is too low for interferon induction. From long-term labeling experiments with ^{32}P-phosphate, it is possible to estimate that of the total RNA (10^{-5} μg per chicken cell) only about 0.01% ($\sim 10^{-9}$ μg) is double-stranded (*our unpublished data*), in agreement with immunochemical measurements (28). The minimum level of poly rI:rC for interference induction was found to be 10^{-8} μg per chicken cell (5). Assuming that poly rI:rC and cellular double-stranded RNA have similar specific activities of interferon induction, then it may be estimated that about 10 times more extracellular double-stranded RNA than is present in the cell is necessary for induction of virus growth interference.

It is possible, however, that enough double-stranded RNA is present in the cell to cause interference with viruses, but the cellular double-stranded RNA in situ may not be able to interact with components necessary for interferon induction. It is also possible that in the cell less RNA exists in the double-stranded form before deproteinization which occurs during isolation.

The biological role and origin of the cellular double-stranded RNA are still open to speculation: this RNA may be generated by a latent virus infection or it could be a true cellular component. The general occurrence of double-stranded RNA in different primary cells (chicken, rabbit, mouse) and cell lines (human, hamster; compare this report, 24, 28, and our unpublished observations) argues against a viral origin of this RNA. Moreover, the finding that the biosynthesis of cellular double-stranded RNA is inhibited by actinomycin D further dispels the suspicion that this RNA may represent the replicative intermediate of a latent RNA virus, because viral RNA-dependent RNA synthesis is generally known to be resistant to actinomycin D. However, we cannot exclude the possibility that the double-stranded RNA might derive from infection by a DNA virus which, like vaccinia virus (6), is capable of synthesizing double-stranded RNA.

It seems more likely, however, that the interference-inducing, double-stranded RNA described here is transcribed from complementary

regions of cellular DNA. This idea is compatible with the ubiquity of this RNA species in animal cells and with the sensitivity of its biosynthesis to actinomycin D. It is also conceivable that some cellular RNA-dependent RNA synthesis exists, since we found that the biosynthesis of cellular double-stranded RNA appears to be less sensitive to inhibition by actinomycin D than total RNA synthesis (also mentioned in 24).

Further characterization of the cellular double-stranded RNA will be necessary to speculate on its biological role.

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ADDENDUM IN PROOF

Results similar to those reported by us have been published by E. De Maeyer, J. De Maeyer-Guignard, and L. Montagnier [Nature New Biology (London) **229**:109–110, 1971].

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