Terminal Heterogeneity at Both Ends of the Satellite Tobacco Necrosis Virus Ribonucleic Acid*

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ABSTRACT: The end groups of the RNA of the satellite of tobacco necrosis virus (STNV) were analyzed by Sanger's methods. The 5′-terminal AGU- was found to be present in both the 5′-diphosphorylated and -triphosphorylated states. The 3′-terminal sequence was found to be -GACUACCC. Since all viral RNA preparations that have been analyzed terminate in -CCC, with or without a terminal A, this sequence is not unusual. However, the finding that about 50% of the 3′-OH group of the terminal C was phosphorylated in STNV-RNA is novel and requires mechanistic explanations. The possible connection between the 3′-phosphorylated RNA chain and the satellite nature of the virus must be considered.

The small size of satellite tobacco necrosis virus (STNV)-RNA,[1] in comparison with other viral RNAs, as well as its dependence on a helper virus (TNV), has made this virus attractive for sequence analysis studies. From sedimentation investigations, an RNA chain length of 1200 nucleotides can be calculated. If all of these nucleotides were used to code for amino acids in a protein, STNV-RNA could serve as a message for 400 amino acids. The molecular weight of STNV coat protein has been estimated at approximately 20,000–22,000 (Lesnaw and Reichmann, 1970; Rees *et al.*, 1970). This implies a protein chain length of about 190 amino acids and leaves room for a second cistron of equal size if all of the STNV-RNA serves as a translatable messenger.

Previous sequence studies of STNV-RNA have provided evidence for the sequences ppApGpUp at the 5′ end (Wimmer *et al.*, 1968), and -G(U,2AC)C as well as -G(U,2AC,C)C at the 3′ end (Wimmer and Reichmann, 1969).[2] As part of a contemplated program of complete sequence elucidation of this RNA by means of the two-dimensional analysis system (Sanger *et al.*, 1965), the terminal oligonucleotides have now been reinvestigated. The 5′ sequence was confirmed, although it was found to be in part triphosphorylated. The 3′-terminal octanucleotide sequence was established. A preliminary report of the results of this work has been presented (Horst *et al.*, 1971).

Materials and Methods

Pancreatic ribonuclease and bacterial alkaline phosphomonoesterase were obtained from Worthington Biochemical Corp. T_1 RNase was obtained from Calbiochem Corp. STNV and TNV were obtained from Dr. M. E. Reichmann as necrotic lesions on mung beans. The viruses were passed twice through single lesions on cowpea leaves [*Vigna sinensis* (*Torner*) *savi*] by R. H. Rice and shown to be a pure strain by investigation of the coat protein (amino acid analysis, polyacrylamide gel electrophoresis) and the RNA (polyacrylamide gel electrophoresis). One hour after infection the leaves were cut with a razor blade near the base of the petiole and placed in Petri dishes. Approximately 50 μl of ^{32}P, neutralized with NaOH, containing 0.2 mCi, was added to the petiole and allowed to be taken up by the leaves (usually 1–3 hr). After that, 3–4 drops of water were applied by the same method to each leaf, and the Petri dishes (with the leaves) were put into a glass container that was closed with parafilm. The leaves were incubated at 21° for 5 days. At the end of the incubation the leaves were placed in a plastic bag, frozen for 1 hr at −70°, and extracted as described (Liu *et al.*, 1969) with the following modifications. The frozen leaves were crushed inside the plastic bag and then loaded, while still frozen, into a blender. Two volumes of buffer (1% KH_2PO_4–0.025 M EDTA, pH 7) were added and the mixture was blended for 3–5 min. Following this, 20 ml of ice-cold 95% ethanol was added for every 100 ml of buffer, the suspension was stirred and then clarified by centrifugation at 7000 rpm in a Servall GSA rotor for 20 min. The supernatant from this was squeezed through four layers of cheesecloth and the virus was precipitated by the addition of 0.3 g of ammonium sulfate/ml. The mixture was allowed to stand at room temperature for 1–2 hr and then it was centrifuged at 7000 rpm for 30 min as before in a GSA rotor. The supernatant was discarded and the pellet was suspended in a small amount of water and dialyzed against water overnight in the cold (4°). The sample was then centrifuged at 9000 rpm in a Servall SS-34 rotor for 15 min and the supernatant from this was centrifuged for 5 hr in a Spinco 40 rotor at 36,000 rpm in half-filled tubes. Further purification of the virus was accomplished, after resuspension of the pellets in water, by sedimentation in a linear sucrose gradient (4–20%, 12-ml tubes) for 3 hr at 33,000 rpm in a SW-36 rotor. The STNV bands appeared approximately in the middle of the sucrose gradient and could be removed with a Pasteur pipet. The amount of virus obtained was estimated from its absorbance at 260 mμ. The virus solution was then dialyzed against distilled water overnight at 4°. For larger scale preparations,

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[1] Abbreviations used are: STNV, satellite tobacco necrosis virus; TNV, tobacco necrosis virus; TEA–HCO_3, triethylamine bicarbonate buffer.

[2] The authors have in all previous publications concerned with terminal RNA sequences used the preferable terminology of 3′-linked and 5′-linked for what are now more commonly called the 5′ and 3′ ends of nucleic acid chains, respectively. They now have regretfully decided to accept these less decriptive terms in deference to the majority of workers in the field.

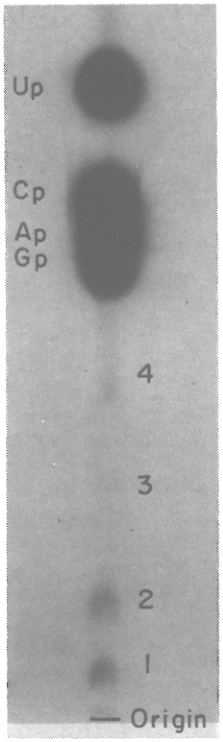

FIGURE 1: Products of KOH hydrolysis of [^{32}P]STNV-RNA. [^{32}P]-STNV-RNA (10^6 cpm) was hydrolyzed in a capillary tube with 0.5 N KOH for 48 hr at 33°. The products were spotted directly onto DEAE paper and an electrophoresis was carried out in 0.5% pyridine–5.0% acetic acid–0.001 M EDTA buffer (pH 3.5). The X-ray plate was exposed for 2–3 days. Material in spots 1 and 2 were resistant to further KOH digestion. Material in spots 3 and 4 could be digested with KOH to mononucleotides after elution.

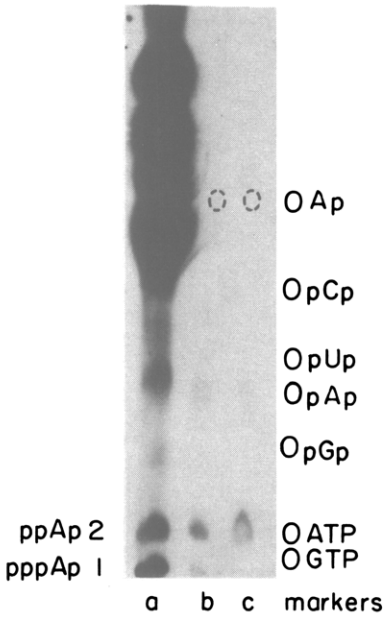

FIGURE 2: Products of HCl hydrolysis of the 5′-terminal nucleotides. Spots 1 and 2 shown in Figure 1 were eluted and treated with 0.1 N HCl for 25 min at 100° and the products were separated by electrophoresis as described in Figure 1. (a) KOH digest of the whole STNV-RNA, (b) after treatment of spot 1 with HCl, and (c) after treatment of spot 2 with HCl. The markers GTP, ATP, and pNp were coelectrophoresed with the samples.

the volume was reduced after the sucrose gradient centrifugations (at 36,000 rpm) by means of ammonium sulfate precipitation (0.3 g/ml).

[^{32}P]STNV-RNA was obtained by phenol extraction of the virus after addition of ammonium carbonate to 0.1 M. The RNA was precipitated three times with 70% ethanol in the presence of 0.05 M sodium acetate, and washed with 95% ethanol. The highest specific activity obtained in the labeling process was about 0.05 μCi/μg of RNA. This is low compared to the specific activities obtained for R17, Qβ, or rRNA (1 μCi/μg), a fact which limits the extent of possible sequence studies.

The digestion of the STNV-RNA with enzymes or KOH and the separation and analysis of the products were performed essentially as described (Sanger *et al.*, 1965; Brownlee and Sanger, 1967).

Results

Identification of the 5′-Terminal Nucleotide and RNA Chain Length. [^{32}P]STNV-RNA was hydrolyzed with 0.5 N KOH and the products were separated by electrophoresis on DEAE paper as shown in Figure 1. The heavily exposed spots in the upper part of the figure indicate the positions of the mononucleotides. Below are the more acidic components. Each of the slower moving components was cut out, eluted with 2 M triethylamine bicarbonate (TEA–HCO_3) of pH 8.5, and treated with 0.5 N KOH. The products of this second hydrolysis were separated as before and an autoradiogram was made of the positions of the spots. Only the two lower spots (1 and 2) remained unchanged. The upper spots were digested to material that migrated to the positions of the mononucleotides (in some autoradiograms, traces of a third material could be seen in the position of pAp).

When eluates from spots 1 and 2 were treated briefly with 0.1 N HCl and, together with a KOH digest of the whole STNV-RNA, separated by electrophoresis on DEAE paper, a pattern was obtained as shown in Figure 2. It may be seen that spot 1 yielded three other components (Figure 2b): one moving in the position of spot 2, another moving in the position of pAp, and a third in that of Ap. Spot 2 yielded only two other components coinciding with pAp and Ap (Figure 2c). From these results we conclude that spot 2 is ppAp and spot 1 is pppAp.

The ratio of the total radioactivity in the [^{32}P]STNV-RNA to the average radioactivity per phosphate in pppAp and ppAp indicated a chain length of 1350 for STNV-RNA.

Determination of the 5′-Terminal Sequence. [^{32}P]STNV-RNA was digested with pancreatic RNase and the products were separated by the two-dimensional fingerprint method of Sanger *et al.* (1965). An autoradiogram of the pattern obtained is shown in Figure 3. Each of the spots was cut out, eluted with 2 M TEA–HCO_3, and further digested with 0.5 N KOH. The KOH digestion products were separated by electrophoresis on DEAE paper in pyridine–acetate buffer (pH 3.5). Autoradiograms were made and examined for the presence of pppAp- and ppAp-terminal nucleotides. Of all the spots, only 400 yielded pppAp and 401 yielded ppAp. The mononucleotides produced with each of these spots were Gp and Up. This confirms our previous finding of a triphosphorylated terminal nucleotide in addition to the diphosphorylated terminal nucleotide, and further establishes the sequences as pppApGpUp and ppApGpUp. The amount of the diphosphorylated end sequence was approximately equal to that of the triphosphorylated end sequence.

Identification and Determination of the 3′-Terminal

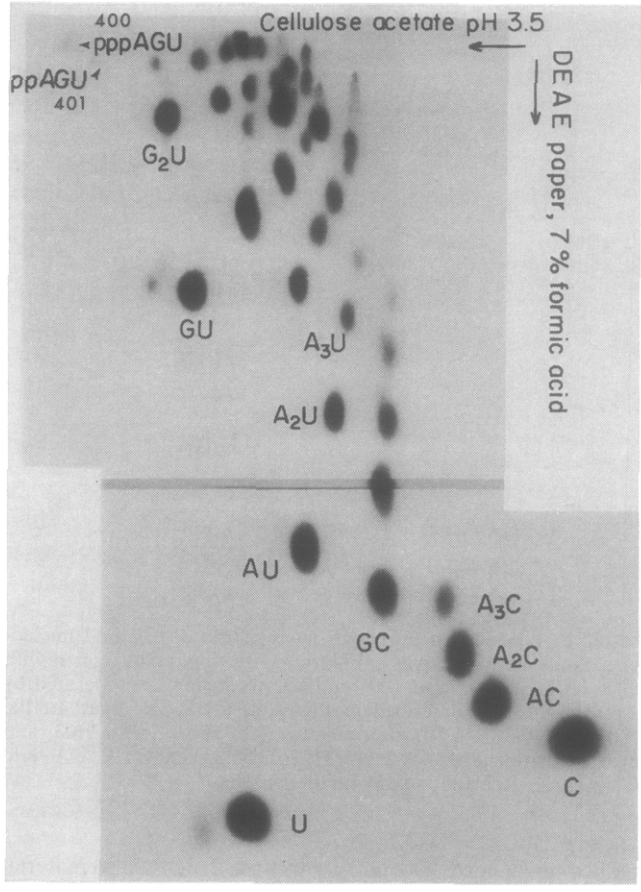

FIGURE 3: Two-dimensional electrophoresis of pancreatic RNase digest of [^{32}P]STNV-RNA. [^{32}P]STNV-RNA (approximately 10^6 cpm) was hydrolyzed with pancreatic RNase (1 U/20 μg of RNA) for 4 hr, 40°. The products were separated first by electrophoresis on cellulose acetate in a 0.5% pyridine–5.0% acetic acid–0.001 M EDTA–7 M urea buffer (pH 3.5 (usually 1.5 hr with 4000 V)). The oligonucleotides were transferred to DEAE paper and the electrophoresis in the second dimension was performed in 7% formic acid (about 14 hr with 1000 V). The fingerprint was dried and exposed to X-ray film for 6 hr.

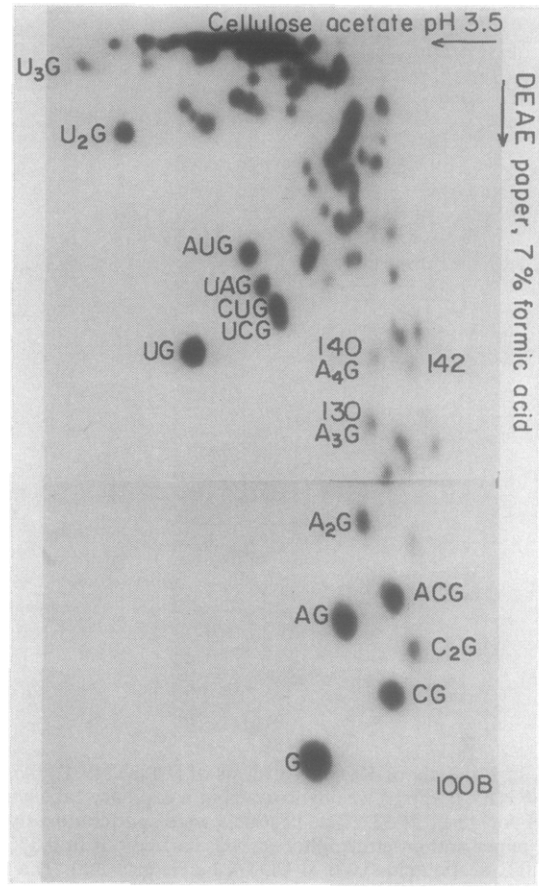

FIGURE 4: Two dimensional electrophoresis of T_1 RNase digest of [^{32}P]STNV-RNA. [^{32}P]STNV-RNA (approximately 2×10^6 cpm) was hydrolyzed with T_1 RNase (1 U/20 μg of RNA) for 4 hr, 40°. The products were separated and visualized as described in Figure 3.

Sequence. [^{32}P]STNV-RNA was digested with T_1 RNase and the products were separated by the two-dimensional fingerprint method. An autoradiogram of the pattern obtained is shown in Figure 4. The spots were cut out and digested further with 0.5 N KOH, and the mononucleotides separated on Whatman 54, located by means of autoradiography, and analyzed by means of a scintillation counter. All of the oligonucleotides from the fingerprint pattern contained Gp except 100B and 142. The base compositions of these oligonucleotides are shown in Table I. Shown also are the results of hydrolysis of each oligonucleotide with venom phosphodiesterase, pancreatic ribonuclease, and ribonuclease U2. A comparison of the KOH and venom phosphodiesterase digests of 100B shows that there are two Ap and three Cp residues in the KOH digest but only one pA and four pC residues in the venom phosphodiesterase digest. This result establishes an A on the 5′ end and a C on the 3′ end of 100B: Ap-(Cp,Cp,Cp,Ap,Up)C. A similar analysis of 142 is precluded because of its resistance to venom phosphodiesterase. This, and the presence of one more Cp, indicates, however, that its right terminus may be blocked with a phosphate in the 3′-OH position.

Partial digestion of 100B with venom phosphodiesterase and separation of the products on DEAE paper at pH 3.5 provided the following M values (Brownlee and Sanger, 1967): 0.68, 0.71, and 0.45. These were the ratios calculated from the mobilities of the oligonucleotides that migrated more slowly than the blue marker, and suggested the presence of three pC residues at the 3′ end: A(pC,pA,pU)pCpCpC.

The products of a pancreatic RNase hydrolysis of 100B established the fact that one Cp precedes Up (Table I). The hydrolysis of the oligonucleotide with U2 RNase produced the products (Table I) that completed the sequence of 100B as: ApCpUpApCpCpC.

TABLE I: Base Compositions of Oligonucleotides 100B and 142 from a T_1 RNase Digest.

	KOH	VPDE[a]	Pan.[a]	U_2[a]
100B	3Cp	4pC	Cp	Ap
	2Ap	pA	Up	(Cp,Up)Ap
	Up	pU	2ApCp	CpCp C
142	4Cp		2Cp	Ap
	2Ap		Up	(Cp,Up)Ap
	Up		2ApCp	CpCpCp

[a] VPDE = venom phosphodiesterase; Pan. and U_2 = pancreatic and U_2 ribonclease, respectively.

TABLE II: Effect of Phosphatase Treatment on Certain T-1 Oligonucleotides.

Spot No.	−PME[a] (cpm)	Ratio to 140	+PME[a] (cpm)	Ratio to 140
100B	3323	0.8	18,065	1.4
142	4111	1.0	Not seen	
130	6860	1.7	18,694	1.4
140	3960	1.0	12,957	1.0

[a] PME = bacterial phosphomonoesterase.

A pancreatic RNase digest and a U2 RNase digest of 142 produced the fragments that are shown in Table I. Because of the similarity of these digestion products with those obtained from 100B, it is reasonable to assume that the sequence of 142 is ApCpUpApCpCpCp. This sequence differs from 100B only in the phosphorylation of the 3′-OH.

To test this further, [^{32}P]STNV-RNA was first treated with bacterial alkaline phosphatase, extracted again with phenol, and precipitated in 70% ethanol. The RNA was then dissolved in buffer and hydrolyzed with T_1 RNase as before. The products were separated by the two-dimensional electrophoresis procedure and an autoradiogram was made of the pattern. As shown in Figure 5, the autoradiogram was identical in every respect with those obtained from untreated STNV-RNA except that spot 142 was missing. In addition, the quantity of ^{32}P oligonucleotide in spot 100B increased almost twofold, relative to other oligonucleotides used for reference (Table II). These results provide further evidence that 142 differs from 100B only by the presence of a phosphate at the terminal 3′-OH position.

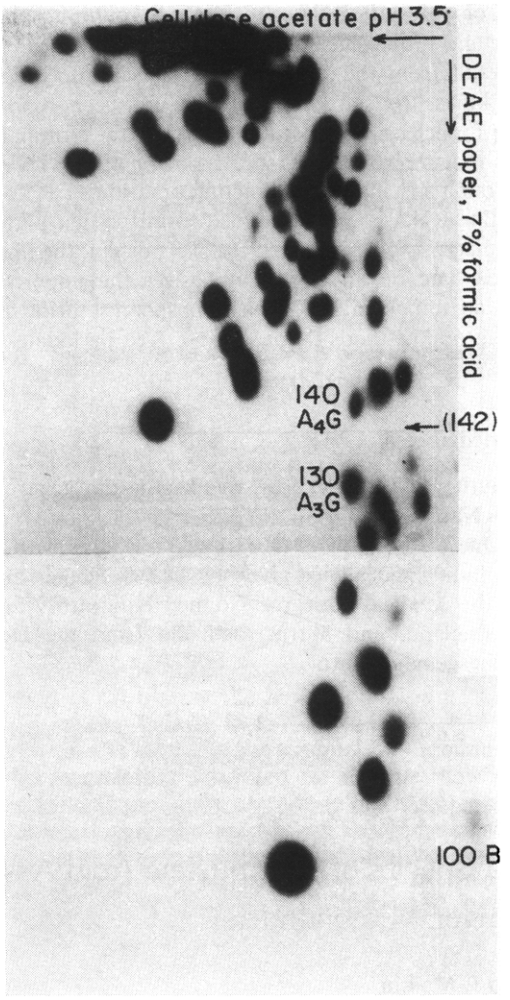

FIGURE 5: T_1 RNase digest of phosphomonoesterase-treated [^{32}P]-STNV-RNA. [^{32}P]STNV-RNA was treated with bacterial alkaline phosphomonoesterase to remove any terminal phosphate groups. After removal of the phosphomonoesterase, the RNA was digested with T_1 RNase and the products were separated and visualized as described in Figure 3. The arrow marks the normal position of spot 142 that is missing in this case.

Discussion

The results of our sequence studies on STNV-RNA differ from those of Wimmer *et al.* (1968, 1969) in two respects. We have found a triphosphorylated 5′-terminal sequence pppApGpUp... in addition to the diphosphorylated end sequence ppApGpUp..., whereas they reported only the diphosphorylated terminal sequence. Furthermore we have presented evidence for the existence of two 3′-terminal sequences, namely, ...(Gp)ApCpUpApCpCpC and ...(Gp)ApCpUpApCpCpCp in roughly equal amounts. The first sequence is compatible with one of the previously reported compositions (Wimmer and Reichmann, 1969), G(U,2AC,C)C, but neither is compatible with G(U,2AC)C.

The presence of both pppApGpUp... and ppApGpUp... as 5′ termini resembles the situation with reovirus, since the multiple double strands of the RNA of this virus were found to carry both pppG and ppG termini (Levin *et al.*, 1970). The possible role of the nucleoside triphosphate hydrolases of that virus in producing the pp-G termini was discussed by Kapuler *et al.* (1970), and similar enzymes might be sought in STNV-infected plants. The extent of the dephosphorylation of newly synthesized nucleic acids possibly reflects the length of time that the nucleic acid is exposed to endogenous phosphohydrolases and phosphomonoesterases. As a result, newly synthesized nucleic acids that are rapidly assembled into virus particles may suffer the least dephosphorylation. Others, such as STNV-RNA may become partially dephosphorylated to ppAp. The extreme example of this may be TMV-RNA which is completely unphosphorylated and indeed shows some nucleotide heterogeneity at the 5′ end (unpublished observations).

Concerning the 3′ terminus, the finding of a substantial amount of a 3′-phosphorylated sequence is unexpected and is not easy to explain. If the terminal sequences of other viral RNAs, as reported by various laboratories, are compared with that of STNV-RNA, a certain homology can be seen

STNV-RNA-1	-GACUACCC$_P$
STNV-RNA-2	-GACUACCC
TMV-RNA	-GCCCA
R17 RNA, MS2-RNA, f2	-GUUACCACCCA
Qβ-RNA	-GCCCUCCUCUCUCCCCA

It appears, by analogy, that the terminal sequences of STNV-RNA have lost, respectively, one adenosine and one 5′-adenylic acid. The loss of the adenylic acid may be due to an exonuclease activity similar to that of venom phosphodies-

terase, or of an RNA.....CpCpA pyrophosphorylase (Preiss *et al.*, 1961). The 3′-phosphorylated sequence, in turn, could result from the action of a 3′-specific polynucleotide phosphokinase (an enzyme that has not been reported to date), or a nucleosidase that removes only the terminal nucleoside. It is also possible that the 3′-phosphate in STNV-RNA arises from a specific nuclease acting on a longer RNA. Thus the satellite RNA could be derived from the first part of the tobacco necrosis virus. Regardless of its origin, the finding of a 3′-phosphate on an RNA emphasizes the importance of treating an unknown RNA with phosphatase prior to using any end group methods that require a free periodate-susceptible glycol group.

Acknowledgments

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Minor Species of Ribonucleic Acid Associated with Rat Liver Mitochondria*

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ABSTRACT: Species of RNA sedimenting at approximately 20 and 22 S in sucrose–sodium dodecyl sulfate gradients are associated with both rat liver mitochondria and purified cytoplasmic ribosomes. Mitochondria are normally grossly contaminated with cytoplasmic ribosomes and this in itself could explain the presence of these RNAs in mitochrondria preparations. However, a dual localization of these RNAs cannot be excluded. We show here that treatment of mitochondria with low levels of digitonin removes contaminating cytoplasmic ribosomes without affecting mitochondrial integrity. Such treatment also removes the 20S and 22S RNAs, lending strong support to the argument that these RNAs are nonmitochondrial and arise from contamination of mitochondria by microsomal membrane-bound cytoplasmic ribosomes or ribosomal subunits.

Various size classes of RNA are observed in preparations from mitochondria of several animal species. Among these are the mitochondria-specific RNAs, presumably from mitochondrial ribosomes, which, in rat liver preparations, sediment at 13 and 16 S in sucrose gradients (Borst and Grivell, 1971; Bartoov *et al.*, 1970). In addition, there are the 18S and 28S RNAs which arise from microsomal membrane-bound cytoplasmic ribosomes that contaminate mitochondria preparations (Malkin, 1971). Attardi and Attardi (1971) have described two other species of RNA from HeLa cell mitochondria which sediment at approximately 21 and 23 S and whose labeling is insensitive to ethidium bromide. In earlier work, these RNA peaks were poorly resolved but radioactive RNA from the 21S to 23S region of sucrose gradients was shown not to hybridize with mitochondrial DNA (Attardi and Attardi, 1969). Thus it appears likely that these RNAs are not transcribed on mitochondrial DNA. However, this says nothing about their location in the cell since conceivably they could be made elsewhere and then transported to the mitochondria. Recent work (Swanson, 1971) has demonstrated that isolated intact mitochondria can incorporate high molecular weight polyribonucleotides from the surrounding medium into the mitochondrial inner compartment. We have recently shown that the 20S and 22S RNAs found in rat liver mitochondria preparations can be demonstrated in cytoplasmic ribosomes free of mitochondria (Malkin, 1971). These results indicated that these RNAs are nonmitochondrial and arise from contamination of mitochondria by cytoplasmic ribosomes. However, a dual localization of these RNAs cannot be ruled out, *i.e.*, they may be

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