An Adenylate-Rich Segment in the Virion RNA of Sindbis Virus

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Summary. When Sindbis virus RNA was digested with RNase at low ionic strength, an adenylate-rich fragment was demonstrated. Zonal sedimentation in sucrose indicated that the fragment was 50-100 nucleotides in length.

Recent studies on the chemical nature of the RNA present in mammalian cells have demonstrated the presence of adenylate-rich segments in both the heterogeneous nuclear RNA (1,2) and cytoplasmic mRNA isolated from polyribosomes (1-6). The A-rich segments from the two cellular RNA species appear to be similar in size (1,2). In the case of mRNA from HeLa cells, the A-rich region may either be contained in or adjacent to that portion of the RNA molecule which hybridizes to the reiterated regions of HeLa cell DNA (1). Several possible functions of the A-rich segment have been suggested. These include transcriptional regulation, selection of specific RNA's for transport to the cytoplasm and translational regulation. Segments rich in adenylate residues have also been found in viral RNAs such as the initial RNA synthesized by the virion associated polymerase of vaccinia virus (7), and the single-stranded portions of reovirus RNA (8,9).

Two considerations have led to the examination of the virion RNA of a single-stranded RNA virus. First, as Sindbis virus does not possess a virion-associated polymerase it is likely that the parental RNA serves as the initial messenger molecule. If an A-rich sequence favors or is necessary for the successful translation of a given message, then an A-rich sequence would be expected in the RNA of a virus such as Sindbis. Second, if an A-rich segment is not present in viral messenger RNA, this might provide a

possible explanation for discrimination against viral messages in interferon-treated cells.

MATERIALS AND METHODS

Growth and Purification of Radioactively Labeled Virus.

A plaque-purified stock of Sindbis virus strain A339 was used. The host cells employed were secondary chicken embryo fibroblast monolayers grown in Eagle's minimal essential medium (10) containing twice the normal concentration of amino acids and vitamins and supplemented with 5% newborn calf serum. The medium was removed from monolayers and replaced with medium containing no phosphate, 3% dialyzed newborn calf serum, and 5 μg/ml of actinomycin D (Sigma). After 2 hours incubation at 37°, the medium was removed and the monolayers were infected with virus at a multiplicity of 50-100. Following 30 minutes for viral adsorption, phosphate-free medium without actinomycin D but containing 5 μCi $H_3{}^{32}PO_4$ (carrier free) and 10 μCi/ml of either ^{3}H-adenine (19 Ci/m mole) or ^{3}H-uridine (28 Ci/m mole) was added. The culture fluids were harvested after an additional 6.5 hours incubation at 37°C and clarified by centrifugation at 5,000 xg for 20 minutes. The labeled virus was concentrated by pelleting through a 15% sucrose (w/w in phosphate-buffered saline) cushion at 153,000 xg in a SW41 rotor for 1 hour. The pellet was resuspended and centrifuged through a 15-30% linear sucrose gradient at 153,000 xg for 2 hours. Fractions containing the cosedimenting peaks of radioactivity and infectivity were collected.

RNA Extraction.

Purified virus was suspended in standard saline citrate (SSC; 0.15 M NaCl, 0.015 M sodium citrate) containing 1 mg/ml unlabeled carrier DNA. The suspension was shaken at room temperature after addition of sodium dodecyl sulfate (SDS) to a final concentration of 0.5% and an equal volume of H_2O-saturated phenol. The aqeous phase was extracted once with ether, precipitated with 4 volumes of 95% ethanol at -20°C for 2 hours, and the precipitate was collected by centrifugation at 10,000 xg for 15 minutes.

RESULTS

RNase Digestion of Virion RNA.

Since bovine pancreatic ribonuclease A hydrolyses only those phosphodiester bonds involving the 3'-OH of pyrimidine nucleosides, RNase-resistant fragments of single-stranded RNA should be enriched in purine oligonucleotides. Figure 1 shows the sedimentation profile of Sindbis viral RNA labeled with ^{3}H-adenine. When each gradient fraction was digested with pancreatic RNase (Worthington, heated to 85°C for 10 minutes to destroy DNase) at a high salt concentration (2 × SSC), an RNase-resistant fragment

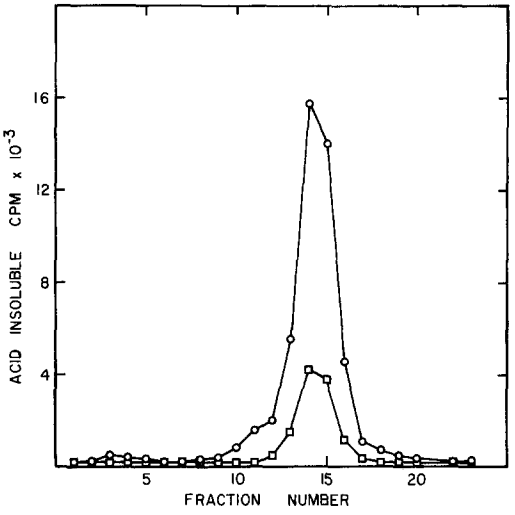

Figure 1. Zonal sedimentation of Sindbis virus RNA. RNA was extracted from purified Sindbis virus radioactively labeled with ^{3}H-adenine. RNA was layered on a 5-20% linear sucrose gradient (w/w in 0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA, 0.05% SDS) and centrifuged for 105 minutes at 200,000 xg in a SW41 rotor. o—o acid-insoluble CPM; □—□ acid-insoluble CPM after 30 minute RNase (1.6 µg/ml) digestion in 2 × SSC at 37°C.

containing adenine was demonstrated. In Figure 2, the time course of digestion is illustrated. RNA from Sindbis virus which had been doubly-labeled with either ^{3}H-adenine and ^{32}P or ^{3}H-uridine and ^{32}P was treated with RNase in salt solutions of different ionic strengths. It may be seen that at the lower ionic strengths (0.1 and 0.5 × SSC), the uridine label was digested at approximately the same rate as the uniform ^{32}P label. The adenine label, however, remained as a residual nuclease-resistant fragment. At the higher

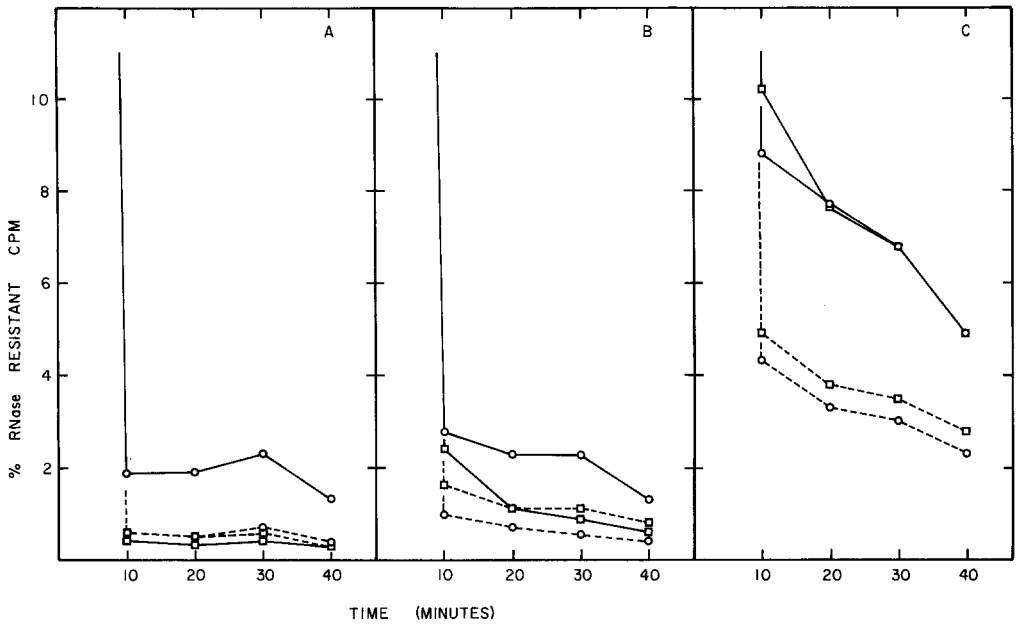

Figure 2. RNase digestion of Sindbis virus RNA at different salt concentrations. RNA was extracted from purified virus labeled with either ^{3}H-adenine and ^{32}P or ^{3}H-uridine and ^{32}P. Percent RNase (1.6 µg/ml) resistance of the ^{3}H and ^{32}P labels at different salt concentrations was determined for each preparation. ^{3}H-adenine and ^{32}P labeled RNA: o—o , ^{3}H-adenine; o---o , ^{32}P. ^{3}H-uridine and ^{32}P labeled RNA: □—□ , ^{3}H-uridine; □---□ , ^{32}P. A. 0.1 × SSC; B. 0.5 × SSC; C. 2.0 × SSC.

ionic strength, the uridine label was also present in the nuclease-resistant fraction suggesting the formation of a partially double-stranded region at high salt concentration or the protection of a previously existing double-stranded region by the high ionic strength buffer. In Table 1, the data are presented as the ratio of % RNase resistant ^{3}H/% RNase resistant ^{32}P such that a ratio above 1.0 indicates an enrichment of the RNase-resistant fraction for the ^{3}H-base indicated. Again, it can be seen that the enrichment for adenine and uridine at high ionic strength was equivalent, whereas at lower ionic strengths, enrichment for adenine alone was observed.

Sedimentation Analysis of the A-Rich Fraction.

Sindbis virus RNA labeled with ^{3}H-adenine was digested with RNase for 30 minutes in 0.5 × SSC. The reaction was stopped by re-extraction of

Table 1. Enrichment of the RNase resistant fragment for adenylate. RNA was digested with RNase for 40 minutes in different salt concentrations as in figure 2.

LABELED RNA	% RESISTANT ^{3}H/% RESISTANT ^{32}P			
	UNDIGESTED	DIGESTION BUFFER STRENGTH		
		2.0X	0.5X	0.1X
^{3}H-ADENINE + ^{32}P	1.0	2.1	3.3	3.3
^{3}H-URIDINE + ^{32}P	1.0	1.8	0.8	1.0

the RNA with phenol. Following ethanol precipitation, the residual A-rich RNA fragment was mixed with ^{32}P-labeled marker RNA obtained from uninfected cells and analyzed by zonal sedimentation in sucrose. Figure 3 shows that the A-rich fraction consists of a heterogeneous population sedimenting in the 2-4S region of the gradient.

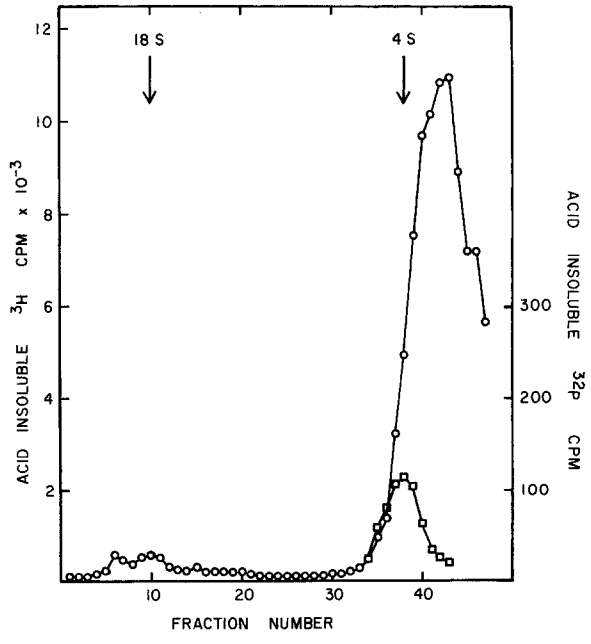

Figure 3. Sedimentation of the adenylate-rich fragment. RNA was extracted from purified virus labeled with ^{3}H-adenine and digested with RNase (1.6 µg/ml) in 0.5 × SSC for 30 minutes at 37°C. The residual fragment was centrifuged on a 5-20% linear sucrose gradient (w/w in 0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA, 0.05% SDS) at 234,000 xg for 4 hours in a SW50.1 rotor. o—o ^{3}H-adenine; □—□ ^{32}P marker RNA extracted from uninfected cells.

DISCUSSION

The evidence presented in this report suggests the presence of an adenylate-rich region in the virion RNA of Sindbis virus. This is based on the finding that an RNase-resistant oligonucleotide rich in adenine remains after RNase digestion of Sindbis RNA at low ionic strength. The size of the fragment has yet to be determined accurately, but based on the sedimentation data, its length is 50-100 nucleotides. If the RNase-resistant fraction is assumed to contain a single fragment composed of 100% adenylate from each genome, then an approximate length of 60 nucleotides may be calculated from the percentage of RNase-resistant ^{32}P and the molecular weights of AMP and of undigested Sindbis RNA. These estimates are in general agreement with the determinations made by others (1,4,5) regarding the lengths of A-rich segments in various RNA species.

The function of the A-rich sequence of Sindbis RNA is unknown. One possibility is that the presence or absence of A-rich sequences provides the basis for discrimination between viral and cellular messages in interferon-treated cells. This now seems remote as such sequences have been reported in cellular mRNA and in three animal virus groups all of which are sensitive to interferon. A second possible function is involvement of the A-rich segment in translational regulation or messenger stability. If the presence of an A-rich segment enhances successful translation of a messenger RNA, a great deal of selective pressure at each round of viral replication would encourage the evolution and subsequent conservation of an A-rich segment in viral RNA. If this is the case, then A-rich segments should be present in single-stranded RNA viruses whose genomes serve as messenger RNA. A third possibility is suggested by the finding that at high ionic strength, the RNase-resistant fragment is enriched in uridine as well as adenine. If uridine residues were randomly distributed throughout the molecule, then the behavior of ^{3}H-uridine in these experiments should have been comparable to that of ^{32}P at all ionic strengths. The data suggest that in addition to the A-rich region, there is also a uridine-rich segment. A partially double-

stranded region may be produced artificially by the action of the high ionic strength buffer which renders the segment resistant to RNase. Alternatively, a double-stranded region may have existed previously within the molecule and was protected from RNase only at high ionic strength. The virion RNA of Sindbis as well as other group A arboviruses can be converted from 40-45S to 26S by heating (11,12), or dialysis against low ionic strength buffer (11). A small double-stranded region (3-4% of the genome) linking two 26S strands together as a 40S molecule could not be detected as an RNase-resistant fraction at the ionic strengths and with the radioactive labels routinely employed. Additional studies are presently in progress in our laboratory to characterize the A-rich segment in Sindbis virion RNA further and to elucidate its function in the replicative cycle of the virus and its contribution to the secondary structure of the genome.

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