ISOLATION OF SINDBIS VIRUS 26 S RNA BY cDNA-CELLULOSE CHROMATOGRAPHY

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1. Introduction

Cells infected with Sindbis virus, a member of the alphavirus group, synthesize 42 S viral genome RNA and a subgenomic 26 S viral RNA [1]. The smaller RNA, which has been shown to correspond to the 3′-terminal one-third of the larger species in the related alphavirus, Semliki Forest virus [2,3], codes for the viral structural proteins [4,5]. Both viral RNAs apparently have modified termini, poly(A) at the 3′-terminal [6,7] and a capped 5′-terminal sequence m^7GpppA-U [8]. The mechanism for generating 26 S mRNA in virus-infected cells remains unclear, although it was suggested [9] that its synthesis is initiated at an internal site on 42 S negative strand template RNA. An alternative possibility that Sindbis 26 S RNA is formed from 42 S RNA by removal of internal sequences is raised by recent evidence for a splicing mechanism in the maturation of eukaryotic viral and cellular mRNAs [10]. By this mechanism, the 26 S and 42 S RNAs would have common sequences at the 5′- and the 3′-termini. Rapid nucleic acid sequencing techniques make it possible to compare the terminal sequences of 26 S and 42 S RNA, provided that the RNAs can be obtained in highly purified form. Genome 42 S RNA can be isolated from purified virions. However, 26 S RNA, necessarily obtained from infected cells, is difficult to purify free from contaminating 28 S ribosomal RNA and poly(A)-containing RNAs by gradient centrifugation and oligo(dT)-cellulose chromatography. In order to purify 26 S RNA, we have employed the method in [11] to prepare solid-phase bound cDNA, using Sindbis 42 S RNA as template, AMV reverse transcriptase, and oligo(dT)-cellulose as primer. The following experiments demonstrate the isolation of Sindbis 26 S RNA by hybridization to the cDNA-cellulose.

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2. Materials and methods

Sindbis virus was purified from infected chick embryo fibroblasts, and the genome RNA was extracted and purified by centrifugation in 5–20% sucrose density gradients as detailed in [12]. Sindbis 26 S RNA labeled with [^{3}H]uridine in the presence of 1 μg/ml actinomycin was phenol-extracted from chick cells purified by LiCl precipitation and separated from intracellular 42 S RNA by gradient centrifugation [13]. Because the viral 26 S RNA was contaminated with 28 S ribosomal RNA, its specific activity (660 cpm/ng) was assumed to be the same as that of the [^{3}H]uridine-labeled, co-extracted 42 S RNA sedimented in the same gradient. Vesicular stomatitis virus (VSV) mRNA was synthesized in vitro by virion-associated RNA polymerase with [α-^{32}P]-CTP as radioactive precursor (spec. act. 11 400 cpm/ng) [14].

For the preparation of cDNA-cellulose, a 0.5 ml reaction mixture consisted of 50 mM Tris–HCl (pH 8.3), 4 mM sodium pyrophosphate, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 μg actinomycin D, 1 mM each of dATP, dCTP, dGTP and dTTP, 50 mg alkali-prewashed oligo(dT)-cellulose (T_2 purchased from Collab. Res. Assoc.), 100 μg 42 S Sindbis RNA and

320 units AMV reverse transcriptase (kindly provided by Dr J. W. Beard of Life Sci., St Petersburg, FL, via the Office of Program Resources and Logistics, Natl. Cancer Inst.). After 30 min at 37°C with stirring, an additional 160 units enzyme was added and the incubation continued for another 60 min. The mixture was then packed in a 0.6 × 15 cm water-jacketed column and washed with 15 ml H_2O, 2 ml 0.5 M NaOH and 10 ml H_2O. The resin was finally equilibrated with binding buffer (10 mM Tris–HCl (pH 7.4), 0.5 M NaCl, 1 mM EDTA, 0.1% SDS) containing 30% deionized formamide.

3. Results and discussion

Sindbis virus 26 S ^{3}H-labeled RNA was mixed with ^{32}P-labeled VSV polyadenylated mRNA and loaded onto a column of Sindbis cDNA-cellulose. After extensive washing at 30°C, 37% of the input ^{3}H- and 47% of the ^{32}P-labeled RNAs were retained (fig.1A). When the column temperature was raised to 60°C, all the ^{32}P-labeled VSV RNA was eluted. In contrast, 27% of the input ^{3}H-labeled Sindbis RNA remained bound and was released when the elution temperature was increased to 90°C. When a mixture of a 5-fold weight excess of ^{3}H-labeled chick embryo ribosomal RNA and ^{32}P-labeled 26 S mRNA was analyzed, the elution pattern of the Sindbis RNA was similar to that in fig.1A, while all the rRNA eluted at 30°C (data not shown). These results indicate that the cDNA-cellulose column can separate Sindbis 26 S mRNA from other polyadenylated mRNAs and from chick ribosomal RNA, the major contaminants in viral RNA prepared from infected cells.

The temperatures used for elution of 26 S RNA from the cDNA-cellulose column were chosen on the basis of another experiment in which the temperature was raised by 10°C increments; from a total of 51 ng RNA retained after washing at 30°C, 10 ng and 7 ng eluted at 40°C and 50°, respectively, <1 ng each at 60°C and 70°C and 25 ng and 7 ng at 80°C and 90°C, respectively. The formamide concentration used for binding was selected from the results shown in table 1. When 26 S RNA was loaded in the absence of denaturing agent, there was very little specific binding (2 ng from an input of 67 ng) as determined by retention at 60°C followed by elution at 90°C. In 30% formamide,

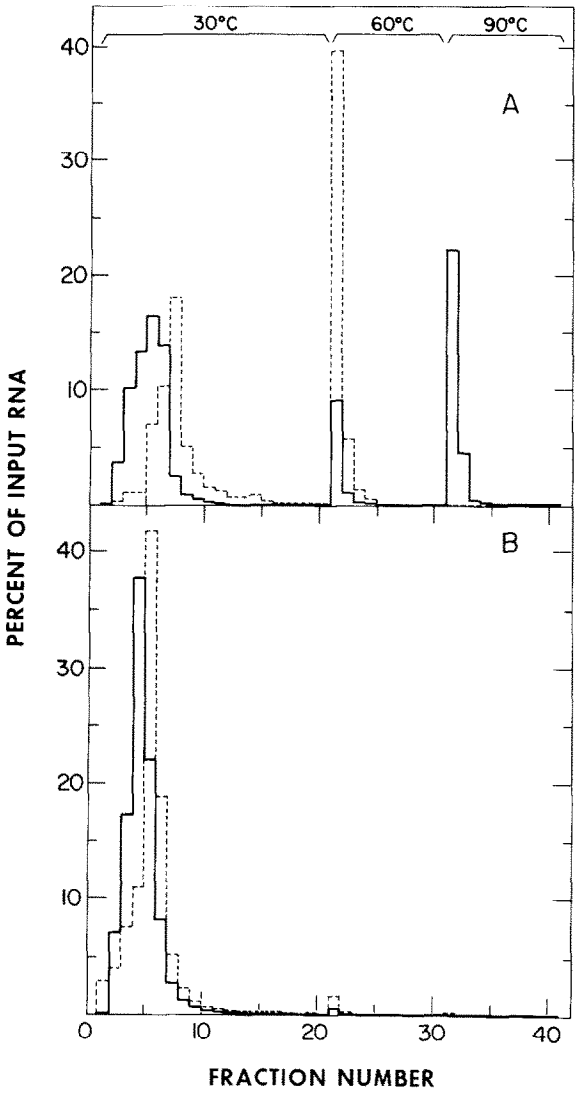

Fig.1. Chromatography of Sindbis 26 S ^{3}H-labeled RNA and VSV ^{32}P-labeled mRNAs on cDNA-cellulose (A) and oligo-(dT)-cellulose (B). (A) A mixture of 116 ng 26 S RNA and 3 ng VSV RNA was applied to the cDNA column at 30°C in 3 ml binding buffer containing 30% formamide at 0.6 ml/h flow rate. The column was then washed with elution buffer (10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.1% SDS) at the same temperature, and 1 ml samples were collected and counted in Aquasol (NEN). The elution was repeated at 60°C and 90°C. Sindbis 26 S ^{3}H-labeled RNA (—); VSV ^{32}P-labeled mRNAs (- - -). (B) Prewashed oligo(dT)-cellulose was directly packed in a 0.6 × 15 cm column and equilibrated with binding buffer and 30% formamide. Sindbis 26 S ^{3}H-labeled RNA (179 ng) and VSV ^{32}P-labeled mRNAs (3.3 ng) were applied and eluted as in panel A.

Table 1
Effect of formamide concentrations on binding of 26 S RNA to cDNA-cellulose

Formamide in sample during loading (%)	Unbound RNA (ng)	RNA (ng) bound and eluted at		
		30°C	60°C	90°C
0	20	42	3	2
30	32	9	15	13
40	56	1	2	8
50	60	<1	<1	7

Sindbis virus 26 S ^{3}H-labeled RNA (67–69 ng) was applied to cDNA-cellulose at 30°C in 3 ml binding buffer containing the indicated concentrations of formamide. The column was washed with 7 ml binding buffer without formamide and the radioactivity in the combined 10 ml sample was counted to obtain the fraction of unbound RNA. Washing was then continued with elution buffer (no formamide) at 30°C (20 ml), 60°C (10 ml) and 90°C (10 ml). The fraction of ^{3}H-labeled RNA eluted at each temperature was determined. The same column was re-used after regeneration by washing with 10 mM NaOH followed by H_2O and equilibration buffer containing the same formamide concentration as in the sample

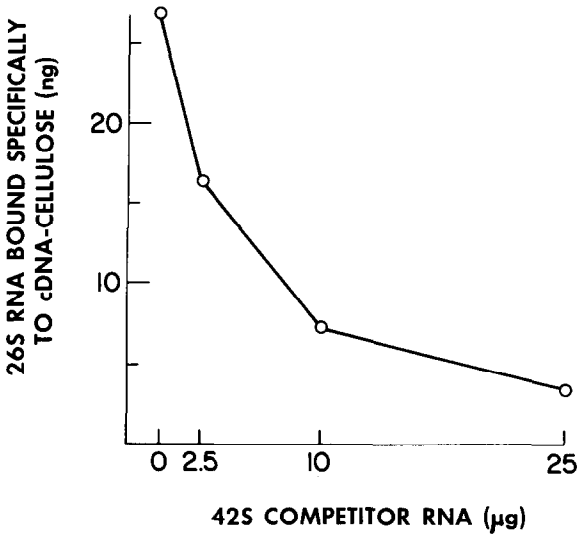

Fig.2. Inhibition of 26 S RNA binding to cDNA-cellulose by Sindbis 42 S RNA. The indicated amounts of virion 42 S RNA were mixed with 67 ng ^{3}H-labeled 26 S RNA in 3 ml binding buffer with 30% formamide and analyzed as in fig.1A. The same column was used after regeneration and equilibration with binding buffer containing 30% formamide.

the specific binding increased 6-fold to 13 ng from 69 ng total input. The more efficient binding in the presence of denaturant presumably reflects melting of short intrastrand base-paired regions in the input RNA and/or the cDNA, allowing hybrid formation. At higher formamide concentrations specific binding was decreased, probably due to loss of molecules that hybridized with the shorter cDNA chains on the column. The capacity of the cDNA column was tested by measuring the specific binding (i.e., elution at 90°C) of 26 S RNA at different input levels. When the input was increased from 109–934 ng, the specific binding of 26 S RNA increased from 27–38 ng, demonstrating that binding of cDNA-specific sequences was approaching saturation.

The specificity of the cDNA column was also shown. VSV and Sindbis RNAs were mixed and loaded onto oligo(dT)-cellulose lacking cDNA. Under the same conditions in fig.1A, only 1–2% of the viral RNAs were retained after washing at 30°C. This small amount of retained RNA eluted at 60°C (fig.1B). Thus, specific binding depends upon the presence of cDNA bound to the oligo(dT)-cellulose. As another test of the specificity of the resin for binding Sindbis RNA, chromatography of ^{3}H-labeled 26 S RNA was done in the presence of 42 S viral genome RNA. Binding was decreased by increasing quantities of the unlabeled 42 S viral RNA (fig.2). A 1000-fold excess (~300-fold molar excess) of 42 S RNA was required to compete out 90% of the specifically-bound 26 S RNA. The requirement for a high level of 42 S RNA to inhibit 26 S RNA binding suggests that only a small fraction of the 42 S molecules can act as competitors under these experimental conditions. Consistent with the possibility that 42 S RNA has secondary structure constraints, it was found that its template efficiency for reverse transcriptase also is low (2 μg 42 S template yielded a maximum of 0.032 μg cDNA). Sindbis genome RNA has been reported [15,16] capable of forming a circular configuration. This and other types of intrastrand structures may limit 42 S RNA interactions with other molecules.

Passage over the cDNA column and elution at 90°C did not result in degradation of the RNA. As shown in fig.3, the sedimentation of 26 S RNA after elution from the column was essentially unchanged. The Sindbis cDNA column should allow selection of 26 S RNA and other subgenomic viral RNA species for

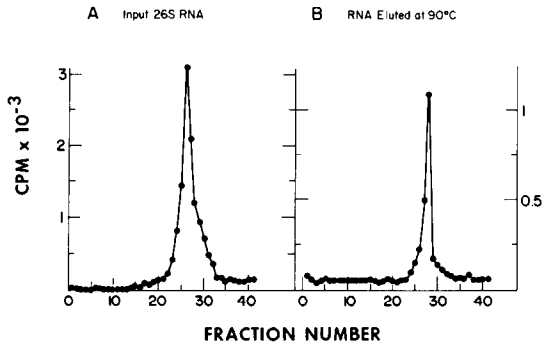

Fig.3. Sedimentation profile of 26 S RNA. (A) A sample of ^{3}H-labeled 26 S RNA was sedimented in a 5–20% sucrose gradient in 50 mM Tris–HCl (pH 7.4), 0.1 M NaCl, 1 mM EDTA and 0.5% SDS (SW-40 rotor, 3 h, 39 000 rev./min). (B) Another portion of the 26 S RNA was purified by cDNA-cellulose chromatography as in fig.1A and an aliquot analyzed from the material eluted at 90°C. Sedimentation is from right to left.

further studies on their structure and mechanism of replication.

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