Polyadenylic Acid Sequences in the Virus RNA Species of Cells Infected with Semliki Forest Virus

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SUMMARY

Polyadenylic acid tracts were shown to be present in the 42 S 38 S, 33 S and 26 S single-stranded RNA species found in cells infected with Semliki Forest virus, as well as in the 20 S double-stranded (RF) RNA. The average polyadenylic acid content of the 42 S and 26 S RNAs purified on oligo(dT)-cellulose columns was 100 to 110 and 63 to 67 residues, respectively.

INTRODUCTION

It is now widely accepted that tracts of polyadenylic acid (poly A) are associated with translatable, or potentially translatable, RNA molecules in eukaryotic cells. These RNAs include cellular mRNA and its heterogeneous nuclear precursors (Kates, 1970; Darnell, Wall & Tushinski, 1971; Edmonds, Vaughan & Nakazato, 1971; Lee, Mendecki & Brawerman, 1971), virus particle RNA (Armstrong et al. 1972; Eaton & Faulkner, 1972; Yogo & Wimmer, 1972), and virus-specified RNA (Kates, 1970; Philipson et al. 1971; Eaton, Donaghue & Faulkner, 1972; Bridgen & Kingsbury, 1972; Weinberg, Ben-Ishai & Newbold, 1972; Galet & Prevec, 1973; Soria & Huang, 1973). In this paper we use several different procedures to examine the virus-specific RNA from cells infected with Semliki Forest virus, an alphavirus, for the presence of poly A tracts. Such viruses are of interest because the virus particle contains a molecule of single-stranded RNA which is infectious (Friedman, Levy & Carter, 1966) and which therefore is presumed to act as a messenger for its own RNA replicase. In addition, the infected cell contains three other single-stranded RNAs of the same polarity, two of which have been shown to be associated with polysomes (Kennedy, 1972; Moshowitz, 1973; Rosemond & Sreevalsan, 1973) and are therefore putative messengers. A search for poly A sequences might shed some light on the relationships between these different species and their functional role in infection. We were also interested in examining the feasibility of using any poly A tracts for the purification of virus-specific mRNA.

METHODS

Materials. Naphthalene-1,5-disulphonic acid (di-sodium salt) and sodium dodecyl sulphate (especially pure grade) were obtained from British Drug Houses, Ltd., Poole, Dorset. Deoxyribonuclease (ribonuclease-free) was supplied by Worthington Biochemical Corp., Freehold, N.J., U.S.A. Agarose was obtained from L'Industrie Biologique Francaise S.A., Gennevilliers, France, and oligo(dT)-cellulose from G. D. Searle and Co., Ltd., High Wycombe, Bucks. Actinomycin D was a generous gift from Merck, Sharpe and Dohme...
Acrylamide and N,N'-methylenebisacrylamide were recrystallized from chloroform and acetone, respectively (Loening, 1967). Phenol, ethanol and ether were all redistilled before use. All other chemicals were the best grade obtainable commercially. Lithium dodecyl sulphate (LDS) was prepared by ion exchange chromatography as described by Noll & Stutz (1968). [3H]-Poly U was synthesized from [3H]-uridine diphosphate using polynucleotide phosphorylase as described by Eaton & Hutchinson (1972). The [3H]-poly U had a sp. act. of approx. 60 000 ct/min/μg and was stored in distilled water at −20 °C. Carrier tRNA was prepared by extracting commercial tRNA twice with phenol containing 1% (w/v) SDS, once with chloroform:octanol (24:1 (v/v)) and twice with ether before being precipitated with 2.5 vol. of ethanol at −20 °C.

Virus. Three times plaque-purified wild-type ts+ Semliki Forest virus (SFV) was used throughout (Walters, Burke & Skehel, 1967). Virus inocula were prepared by a single further passage in suspensions of chick embryo cells (Kennedy & Burke, 1972).

Cells and media. Monolayer cultures of BHK cells, clone 13, were grown in sealed 2.5 litre smooth-walled bottles, as described by Morser, Kennedy & Burke (1973). For all biochemical experiments growing cultures were used.

Labelling of virus-specified RNA. Almost confluent monolayers of BHK cells were washed once with 50 ml of maintenance medium (Morser et al. 1973) and infected with 50 p.f.u. SFV/cell in the presence of 1 μg/ml actinomycin D. After adsorption for 1 h at 37 °C the fluids were replaced with 50 ml of pre-warmed maintenance medium containing 1 μg/ml actinomycin D. At the times specified in individual experiments (specified relative to the end of the adsorption period) the fluids were removed and replaced with 10 ml of Earle's solution containing 2% dialysed calf serum, 1 μg/ml actinomycin D and the appropriate radioisotope. The final concentration of these isotopes was: [3H]-uridine 50 μCi/ml; [14C]-uridine 2.5 μCi/ml; [32P]-orthophosphate 100 μCi/ml or 250 μCi/ml. For experiments with the latter isotope 0.1% N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid replaced the phosphate component of the Earle's solution. Under these labelling conditions radioactive precursors were incorporated into virus-specified RNA (Martin & Burke, 1974). However, in experiments where the cells were labelled from 1 h post-infection a small proportion of the [32P]-orthophosphate was incorporated into DNA (S. I. T. Kennedy, unpublished results). At the end of the labelling period the cell monolayers were washed three times with ice-cold phosphate-buffered saline (PBS), once with 50 mm-tris containing 100 mm-NaCl and 1 mm-EDTA (pH 7.5; TNE) and the cells scraped off the bottles with the aid of a specially constructed wiper. The cells were collected by centrifuging at 700 g for 5 min at 4 °C and resuspended in a small vol. of TNE.

Extraction of total nucleic acids. Washed, resuspended BHK cells were lysed by treatment with naphthalene-1,5-disulphonate (added to 0.5% (w/v)) followed by SDS (added to 2% (w/v)). Naphthalene-1,5-disulphonate was included during lysis to inhibit nucleases (Parish & Kirby, 1966) as it was found that this detergent was a much more potent nuclease inhibitor than SDS. The cell lysate was mixed with an equal vol. of phenol saturated with TNE, shaken and centrifuged at 1500 g for 10 min. The aqueous phase was retained and the phenol phase re-extracted with a further quarter vol. of TNE. The aqueous phases were combined and then sequentially extracted twice more with TNE-saturated phenol, once with chloro-
form: octanol (24:1 (v/v)) and finally three times with ether. Residual ether was blown off with nitrogen and nucleic acid precipitated with 2.5 vol. of ethanol at 4 °C overnight. It was recovered by centrifuging at 1500 g for 15 min, washed twice with 70% ethanol containing 50 mM-NaCl and stored at −20 °C.

Preparation of [32P]-labelled cellular RNA. Two cultures of rapidly growing BHK cells were labelled for 24 h with 50 μCi/ml [32P]-orthophosphate added to the growth medium. Cellular RNA was extracted with TNE-saturated phenol in the absence of detergent.

Purification of double-stranded (ds) RNA. Total nucleic acids from infected BHK cells, labelled from 1 to 6 h post-infection with 100 μCi/ml [32P]-orthophosphate, were dissolved in 10 mM-tris containing 100 mM-NaCl, 10 mM-MgCl2, and 1 mM-EDTA (pH 7.2) and incubated at 37 °C for 20 min with 50 μg/ml deoxyribonuclease (ribonuclease-free). The solution was made 1% in SDS and extracted, twice at room temperature with TNE-saturated phenol, once with chloroform:octanol (24:1 (v/v)) and twice with ether, and the RNA precipitated with 2.5 vol. of ethanol at −20 °C overnight. The precipitate was dissolved in 2 ml 50 mM-tris containing 1% SDS and chromatographed through a 20 × 0.9 cm column of Sephadex G-200 in the same buffer in order to remove oligonucleotide fragments. The material eluted in the void vol. was made 2 M in LiCl and kept at 4 °C for 48 h. The bulk of both the single-stranded RNA and the replicative intermediate (RI) was removed by centrifuging at 20000 g for 40 min at 4 °C, and the dsRNA precipitated from the supernatant fluid with 2.5 vol. of ethanol at −20 °C overnight. The dsRNA was further purified by two successive cycles of column chromatography through Whatman CF-11 cellulose using the stepwise gradient elution system described by Franklin (1966). The purified dsRNA was stored at −20 °C under 3 vol. of ethanol. Under these conditions of infection and preparation only one major species of virus-specified dsRNA is present in infected BHK cells.

Sucrose gradient sedimentation. Samples were dissolved in TLE with, except where noted, 0.5% LDS, layered on a preformed 60 ml 6 to 30% (w/v) linear sucrose gradient in the same buffer and centrifuged at 70000 g for 18 to 20 h at 4 °C in a 3 × 70 ml MSE swing-out rotor (Measuring and Scientific Equipment, Ltd., Crawley, Sussex). On occasion 4.8 ml gradients centrifuged at 135000 g for 2.5 to 3 h at 4 °C in a 3 × 5 ml MSE swing-out rotor were used. Gradients were unloaded by aspiration using an Auto-densiflow unloader (Buchler Instruments, Fort Lee, N.J., U.S.A.).

Assay of trichloroacetic acid (TCA)-insoluble RNA. Samples containing 50 μg carrier tRNA were made 5% in TCA + 0.1 M-sodium pyrophosphate and the precipitated material was collected and washed on glass fibre discs as described by Skehel & Burke (1968). Double-stranded RNA was assayed by its TCA precipitability after treatment with ribonucleases. The RNA sample, in TLE, was brought to 500 mM-NaCl + 10 mM-magnesium acetate by addition of concentrated salt solutions and incubated at 37 °C for 20 min with 50 μg/ml pancreatic ribonuclease and 200 units/ml ribonuclease T1. TCA-insoluble material was analysed as described.

Nitrocellulose filter binding of RNA. This was carried out as described by Lee et al. (1971).

Binding of [3H]-poly U to RNA in solution. One bottle of BHK cells was infected with SFV and a second was mock-infected using maintenance medium. Eight h post-infection both cultures were harvested and total nucleic acids prepared. These preparations were treated with deoxyribonuclease and separately chromatographed through Sephadex G-200 as described above. Binding of [3H]-poly U to these samples was performed using a modification of the technique of Fraser & Loening (1973). Specifically the RNA samples were dissolved in TNE containing 0.5% LDS and 74 μg of each preparation were mixed with
 Binding of RNA to oligo(dT)-cellulose. The procedure was adapted from that described by Edmonds (1971). For preparative purposes, the RNA solution in TLE containing 0.5% LDS (TLEL; up to 18 ml of sample and containing up to 30% (w/v) sucrose) was passed at 0.6 ml/min through a 30 x 6 mm column of oligo(dT)-cellulose maintained at 0 °C by circulation of ice-water around a cooling jacket. After the sample had passed through the column temperature was raised to 20 °C, and the non-binding material washed off with 20 ml of TLEL at a flow rate of 1.5 ml/min. Material containing poly A was recovered by raising the column temperature to 30 °C, and eluting with 10 ml 10 mM-tris (pH 7.5) containing 0.1% SDS, also at a rate of 1.5 ml/min. Fractions of 2 ml were collected from the column, counted and concentrated by precipitation at −20 °C with 100 μg carrier tRNA and 2.5 vol. of ethanol, after addition of NaCl to 100 mM where necessary. For analytical purposes the column height was reduced to 10 mm, and both washing with TLEL and elution with 10 mM-tris (pH 7.5) containing 0.1% SDS carried out at 30 °C. Fractions of 1 ml were collected and assayed for radioactivity by appropriate methods.

Polyacrylamide-agarose gel electrophoresis. Twenty cm gels containing 1.7% (w/v) acrylamide, 0.085% bis-acrylamide and 0.05% (w/v) agarose were used as described by Levin & Friedman (1971). Gels were run at 100 V fixed potential for 8 h at room temperature with buffer recirculation. After electrophoresis gels were extruded onto aluminium troughs and either frozen prior to slicing or placed on Whatman 3MM filter paper and dried down in toto under vacuum with the aid of an infrared lamp for autoradiography. Gels were sliced into 1 mm discs, solubilized and counted as previously described (Kennedy & Burke, 1972).

Isolation and nucleotide composition analysis of oligo(dT)-cellulose-binding fragments. Total nucleic acids from 10 infected cultures of BHK cells labelled with [32P]-orthophosphate (250 μCi/ml) from 1 to 6 h post-infection were prepared and fractionated on six 60 ml sucrose gradients. The peak fractions encompassing the 42 S and 26 S regions of the gradients were collected and chromatographed through oligo(dT)-cellulose. The material which bound to oligo(dT)-cellulose was then applied to a cellulose CF-11 column to remove trace amounts of multi-stranded and dsRNA and the single-stranded RNA was ethanol-precipitated in the presence of 250 μg of carrier tRNA. The material was recovered, dried, and dissolved in 20 mM-tris containing 200 mM-NaCl and 1 mM-EDTA (pH 7.2; 1.5 ml) and incubated with 10 μg/ml pancreatic ribonuclease and 50 units/ml ribonuclease T1 for 30 min at 37 °C. The samples were then made 1% in LDS and rechromatographed through oligo(dT)-cellulose. The material which bound to the column was recovered, made 0.1 M in NaCl and ethanol-precipitated in the presence of 500 μg carrier tRNA at −20 °C. The precipitates were recovered by centrifuging, dissolved in 500 μl distilled water and lyophilized. Each sample was then dissolved in 50 μl of 0.3 M-KOH and hydrolysed at 37 °C for 16 h, as were two samples of [32P]-labelled BHK cellular RNA. After neutralization with perchloric acid the supernatant solutions were spotted on to 45 cm x 2.5 cm strips of Whatman 3MM paper and electrophoresis performed at 1.8 kV for 2.5 h using the apparatus and conditions described by Sanger & Brownlee (1967). Following electrophoresis the strips were thoroughly dried and the position of the markers noted before autoradiography. Finally, the appropriate regions of the strips were cut out and counted in scintillation fluid.

Radioactivity measurements and autoradiography. Radioactivity due to [32P] was usually measured by Čerenkov radiation. Other radioisotopes in solution were counted in a Triton X-100:toluene scintillator as previously described (Kennedy & Burke, 1972). Radioactivity
Fig. 1. Nitrocellulose filter binding of virus-specific RNA. Total RNA from SFV-infected cells, labelled with [\(^{3}H\)]-uridine from 2 to 6 h post-infection, was mixed with \([^{14}C]\)uridine-labelled RNA from mock-infected cells and fractionated on 6 to 30 % (w/v) sucrose gradients. Samples of each fraction were tested for: (a) TCA-insoluble \([^{3}H]\)-labelled (O—○) and \([^{14}C]\)-labelled (△—△) RNA, \([^{3}H]\)-labelled dsRNA (□—□) and (b) nitrocellulose filter binding \([^{3}H]\)-labelled (○—○) and \([^{14}C]\)-labelled (△—△) RNA.

on glass fibre or nitrocellulose filters was measured in a scintillator consisting of 0.475 % diphenyloxazole in toluene. Autoradiography was performed using Kodirex X-ray film.

RESULTS

Nitrocellulose binding of virus RNA species

After infection with SFV, BHK cells contain three types of virus-specified RNA resolvable on sucrose gradients (Friedman et al. 1966; Sonnabend, Martin & Mécs, 1967; Cartwright & Burke, 1970). These are the 42S and 26S single-stranded RNAs and a largely double-stranded RNA sedimenting at about 18 to 22 S. In order to examine the distribution of
poly A sequences among these species, and to compare their behaviour with that of the cellular (largely ribosomal) RNA from normal cells, RNA from SFV-infected BHK cells labelled with $[^{3}H]$-uridine was mixed with RNA from mock-infected cells, labelled with $[^{14}C]$-uridine, and fractionated on two 60 ml 6 to 30 % sucrose gradients run simultaneously as described in Methods. Samples from the fractions of the first gradient, which contained 0·5 % LDS, were assayed for TCA-precipitable RNA and for nitrocellulose filter binding RNA, while those from the fractions of the second gradient, which was free of LDS, were assayed for ribonuclease-resistant TCA-precipitable RNA, i.e. dsRNA. Fig. 1 (a) shows that the expected three virus RNA species are well resolved in this gradient system, and that dsRNA is almost entirely confined to the peak sedimenting about 20 S. From the sedimentation profile of the RNA which binds to nitrocellulose filters, shown in Fig. 1 (b), it is clear that major portions of both the 42 S and the 26 S RNA are able to bind, but that the dsRNA is completely incapable of binding. The specificity of the nitrocellulose binding is indicated by the fact that no binding of the $[^{14}C]$-uridine-labelled ribosomal RNA (rRNA) from the mock-infected cells takes place.

In separate experiments with unfractionated $[^{32}P]$-labelled RNA from infected cells, the prior binding of excess synthetic poly A to nitrocellulose filters reduced binding from 79 % to 1·4 % of the total radioactivity present, whereas similar treatment with poly U failed to alter it at all. Conversely, incubation of the infected cell RNA with poly U at 0 °C for 5 min reduced the radioactivity binding to the filter to 13 % of the total. We conclude that the poly A tracts account for the ability of the 42 S and 26 S virus RNAs to bind to nitrocellulose filters.

In Fig. 2 is shown the result of annealing $[^{3}H]$-poly U with RNA extracted from SFV-infected and mock-infected cells. Although no measure of the proportion of the molecules binding $[^{3}H]$-poly U is obtainable from this experiment, at least some 42 S and 26 S virus RNA

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Fig. 2. Annealing of virus-specific RNA with $[^{3}H]$-poly U. Seventy-four µg of unlabelled RNA from SFV-infected or mock-infected cells was annealed in separate experiments with 1·92 µg $[^{3}H]$-poly U and fractionated on 6 to 30 % (w/v) sucrose gradients. Radioactivity due to $[^{3}H]$-poly U associated with virus-specific (○—○) and normal cellular (△—△) RNA was measured. Arrows indicate the position of cellular $[^{32}P]$-labelled rRNA added as markers.
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Fig. 3. Chromatography of partially purified 26S RNA on an oligo(dT)-cellulose column. [\(^{32}P\)]-labelled 26S RNA from infected cells, labelled 1 to 6 h post-infection, was partially purified by sucrose gradient centrifuging and applied to a column of oligo(dT)-cellulose. The column was washed and eluted as described in Methods. Whole fractions were counted by Cerenkov radiation. The inset shows the proportion of the total radioactivity which binds to oligo(dT)-cellulose in preparations of rRNA, 42S, 26S or dsRNA.

molecules carry the [\(^{3}H\)]-label, and are therefore likely to contain poly A tracts. Any possible interaction of the [\(^{3}H\)]-poly U with the dsRNA is obscured by the large quantity of un-annealed [\(^{3}H\)]-poly U at the top of the gradient.

**Binding of virus RNA species to oligo(dT)-cellulose**

The nitrocellulose binding technique for the detection of poly A sequences does not, at least in our hands, lend itself to the preparation of reasonably large quantities of material containing poly A, because of the low capacity of the filters and difficulty in eluting bound material. For this reason the use of small columns of oligo(dT)-cellulose was investigated. A procedure based on that of Edmonds (1971) was adopted, and preliminary experiments showed that synthetic poly A could be bound and recovered in \(\sim 100\%\) yield using the procedure described in Methods.

Appropriately pooled fractions from 60 ml sucrose gradients enriched for 42S RNA, 26S RNA or dsRNA, labelled with [\(^{32}P\)]-orthophosphate, were applied to oligo(dT)-cellulose columns and the distribution of material between non-binding and binding fractions determined. A typical elution profile from a column containing 26S RNA is shown in Fig. 3, together with an inset giving the percentage of the total radioactivity which bound to the oligo(dT)-cellulose for each of the three classes of virus RNA, and for rRNA prepared.
Fig. 4. For legend see facing page.
separately from uninfected cells. It is apparent that a high proportion of all three species of virus RNA tested are capable of binding to oligo(dT)-cellulose whereas rRNA did not bind. When samples of the binding fractions were passed again through an oligo(dT)-cellulose column, nearly 100% of the input RNA was bound, as might be expected. The non-binding material in the 26S and dsRNA preparations does not bind on a second passage through the column, but a significant fraction (about 30%) of the non-binding fraction of the 42S preparation does bind on a second passage. The reason for this anomaly is not yet clear, but investigation to clarify this point is in progress (see Discussion).

While all the experiments so far described consistently indicate the presence of poly A tracts on the 42S and 26S RNA species, there is a contradiction between the non-binding of the dsRNA to nitrocellulose and its binding to oligo(dT)-cellulose. In order to make sure that the binding of dsRNA to the oligo(dT)-cellulose was due to the presence of poly A tracts, we compared the effect on the binding of pre-incubation of poly U and poly C with dsRNA, rigorously purified as described under Methods, with their effect on the binding of 26S RNA, purified by sucrose gradient sedimentation and oligo(dT)-cellulose chromatography. We show in Table 1 that pre-incubation with poly U completely inhibits the oligo(dT)-cellulose binding capability of both 26S RNA and dsRNA, a finding consistent with masking of the poly A tracts by the complementary poly U. Poly C, in a similar experiment, had only a small effect on the binding, presumably because it is unable to complex with poly A. In a further control experiment [3H]-uridine-labelled dsRNA from reovirus (kindly given by L. Garcia of this laboratory) which does not contain any covalently linked poly A (Bellamy & Hole, 1970) completely failed to bind to oligo(dT)-cellulose. It is concluded, therefore, that the binding ability of dsRNA from SFV-infected cells is not due to its double-stranded nature, but is almost certainly a consequence of the presence of a tract of poly A. No explanation of its inability to bind to nitrocellulose filters is forthcoming at present.

The nature of the binding species in the 26S peak from sucrose gradients of RNA from SFV-infected cells labelled from 1 to 6 h post-infection with 100 µCi/ml [32P]-orthophosphate was examined by acrylamide-agarose gel electrophoresis (Fig. 4). The radioactive species on the gels were identified by reference to gels of [32P]-labelled rRNA and purified dsRNA run at the same time. As well as 26S RNA, the material taken direct from the gradient contains small quantities of dsRNA and DNA, and what is probably RI which is unable to enter the gel (Levin & Friedman, 1971; Martin & Burke, 1974). Binding to oligo(dT)-cellulose removes the DNA, the bulk of the RI, and the low levels of heterogeneous material around the 26S RNA band, while leaving the 26S and dsRNA, confirming the data already presented. Treatment with deoxyribonuclease does not alter the appearance of the binding material, thus confirming the identity of the band co-migrating with marker dsRNA as RNA, rather than another fragment of DNA.

Oligo(dT)-cellulose binding of minor species of RNA

Sucrose gradients do not permit the resolution of the minor species of single-stranded RNA discovered by gel electrophoretic analysis of the RNA from SFV-infected cells (Levin

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Fig. 4. Electrophoretic analysis of 26S RNA preparations. [32P]-labelled RNA preparations were electrophoresed on polyacrylamide gel and an autoradiograph prepared as described in Methods. Migration is from top to bottom. (A), rRNA; (B), material from the 26S region of sucrose gradients; (C), oligo(dT)-cellulose-bound material; (D), material bound to oligo(dT)-cellulose after treatment with deoxyribonuclease; (E), purified dsRNA.
Fig. 5. Acrylamide gel electrophoresis of oligo(dT)-cellulose binding, virus-specific RNA. Total [\( ^{3}H \)]-uridine-labelled RNA from SFV-infected cells, and that fraction which binds to oligo (dT)-cellulose were analysed by gel electrophoresis, using [\( ^{32}P \)]-labelled normal cellular RNA as marker. Migration is from left to right. (a) total RNA; (b) oligo(dT)-cellulose-binding RNA. [\( ^{3}H \)]-labelled RNA, (○—○); [\( ^{32}P \)]-labelled RNA, (△—△).

& Friedman, 1971; Martin & Burke, 1974). To determine whether or not these minor species contained poly A sequences, unfractionated [\( ^{3}H \)]-labelled RNA from cells infected with SFV was passed through an oligo(dT)-cellulose column and the electrophoretic profile of the bound material compared with that of the total RNA (Fig. 5). The profile clearly distinguishes the 42S, 38S, 33S, 26S single-stranded RNAs, and the dsRNA. The dsRNA and all the single-stranded RNA species are represented in the profile of the oligo(dT)-cellulose binding material and therefore all contain poly A sequences.
Fig. 6. Nucleotide composition analysis. The [\textsuperscript{32}P]-labelled material from the 42 \textit{S} and 26 \textit{S} RNAs which was able to bind to oligo(dT)-cellulose after treatment with ribonucleases was hydrolysed with alkali and the resultant nucleoside monophosphates, together with internal markers, were separated by high voltage electrophoresis. Samples of normal [\textsuperscript{32}P]-labelled RNA were also hydrolysed in the same way. \(\times\) denotes the respective origin. (1) and (4) total cellular RNA. (2) material from the 42 \textit{S} RNA. (3), material from the 26 \textit{S} RNA. The inset shows the nucleotide composition of the material from the 42 \textit{S} and 26 \textit{S} RNAs determined by scintillation counting of the separated spots. A letter identifies the base of each nucleoside-2'-(3')-monophosphate.
Table 1. Oligo(dT)-cellulose binding of 26S and dsRNA in
the presence of homopolyribonucleotides

<table>
<thead>
<tr>
<th>Pre-incubation</th>
<th>26S (%)</th>
<th>dsRNA (%)</th>
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<tbody>
<tr>
<td>None</td>
<td>88</td>
<td>80</td>
</tr>
<tr>
<td>With poly U</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>With poly C</td>
<td>78</td>
<td>76</td>
</tr>
</tbody>
</table>

Samples of [32P]-labelled 26S and dsRNA in TLE containing 0.1% SDS were incubated with 50 µg of either poly U or poly C, and the fraction of the radioactivity binding to oligo(dT)-cellulose was determined.

Isolation and nucleotide composition analysis of oligo(dT)-cellulose binding material

As further confirmation of the presence of poly A sequences in 42S and 26S RNAs, the ribonuclease-resistant portions of these molecules were isolated. The oligo(dT)-cellulose binding material from appropriate regions of sucrose gradients of [32P]-labelled RNA from infected cells was freed of any traces of multi-stranded RNA, which would also be largely resistant to ribonuclease, by passage down CF-11 cellulose columns (Franklin, 1966; Martin & Burke, 1974). After nuclease digestion of the single-stranded material under conditions which permit the survival of poly A tracts (Beers, 1960), oligo(dT)-cellulose columns were used to recover the binding material. The fraction of the total quantity of RNA digestion products which bound to the column was 0.85% in the case of the 42S RNA and 1.32% in the case of the 26S RNA. Analysis of the nucleotide composition of the alkaline hydrolysate of the material bound to the columns of oligo(dT)-cellulose showed it to be almost entirely composed of adenylic acid in both cases (Fig. 6). This is confirmation that poly A tracts are present in the 42S and 26S RNA species of SFV-infected cells.

DISCUSSION

The evidence presented in this paper suggests that poly A sequences are associated with all four of the single-stranded RNA species found in BHK cells infected with SFV, as well as with the 20S double-stranded replicative form (RF). It seems likely that all the 26S RNA and RF molecules contain poly A judging from the high percentage of each species which is able to bind to oligo(dT)-cellulose. Although no quantitative data on the 38S and 33S minor species are available, comparison of the electrophoretic profile of total RNA from infected cells with that which binds to oligo(dT)-cellulose indicates that certainly a very large proportion of these molecules contain poly A tracts. Taken in conjunction with the findings of Kennedy (1972) that 26S and 33S RNAs are found in the polysomes of SFV-infected cells, the presence of poly A tracts in these molecules strongly suggests that these RNA species act as messenger RNAs.

The data on the binding of 42S RNA to oligo(dT)-cellulose seem quantitatively somewhat different from those on the 26S and dsRNA binding. A lower proportion of the 42S RNA from sucrose gradients binds to oligo(dT)-cellulose, and although one might suggest that this was due to a greater quantity of contaminating material, particularly DNA, in the lower part of the gradients, there is also a loss of 42S RNA relative to the other binding species when the electrophoretic profiles of total infected cell RNA are compared before and after oligo(dT)-cellulose binding (see Fig. 5). This behaviour might suggest some heterogeneity in the poly A content of 42S RNA similar to that reported by Eaton & Faulkner.
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(1972) in Sindbis virus RNA. However, in one of the tests used by these authors, namely the nitrocellulose filter binding assay, we find that the SFV 42 S RNA binds almost quantitatively. The interpretation of the results is complicated by the fact that on a second passage through the oligo(dT)-cellulose column some of the 42 S RNA which did not bind on the first passage is bound. One might speculate that some conformational equilibrium obtains in solutions of 42 S RNA, and that the poly A tract responsible for binding is masked in one state but not the other. Experiments are in progress to try and clarify this point.

The observation that the 26 S RF RNA of SFV-infected cells contains poly A parallels work on poliovirus RF, in which Yogo & Wimmer (1973) showed a long poly A tract at the 3'-terminus of the positive strand. One of their most intriguing findings was the presence of a tract of poly U at the 3'-terminus of the negative strand. Since this has a number of interesting implications for the way in which RNA replication takes place, we are currently analysing SFV RF for similar poly U tracts.

The apparent inability of RI to bind to oligo(dT)-cellulose columns is rather puzzling in view of the efficient binding of RF. Since RF can be generated from RI by treatment with ribonucleases (Martin & Burke, 1974) we consider it likely that RI does contain poly A tracts, but that they are not available for base pairing with oligo(dT)-cellulose because they are masked by the secondary or tertiary structure of the assembly. However, this should be regarded as a tentative conclusion since positive identification of a species which does not enter the acrylamide-agarose gel cannot be made.

From the fraction of the total radioactivity in the 42 S and 26 S RNA molecules which retained its ability to bind to oligo(dT)-cellulose after treatment with ribonucleases A and T1, the average total length of the poly A tracts associated with each of these molecules may be calculated. Taking values of 4·0 to 4·3 x 10^6 and 1·6 to 1·8 x 10^6 for the mol. wt. of the 42 S and 26 S RNAs, respectively (Levin & Friedman, 1971; Kennedy, 1972; Simmons & Strauss, 1972; Martin & Burke, 1974), the poly A tracts can be calculated to contain 100 to 110 residues in the 42 S RNA, and 63 to 73 residues in the 26 S RNA. Since the actual lengths of these poly A sequences have not yet been determined directly, it is not possible to say whether the 42 S RNA contains a single tract of average length greater than that of the 26 S RNA, or whether in fact it contains two separate tracts of size similar to that of the 26 S RNA. This question, together with the location of the tracts within the molecules, is currently under investigation.

Since the RNA species which have been identified as possible messengers in SFV-infected cells (the 33 S and 26 S RNAs, Kennedy, 1972) contain poly A tracts, it is expected that the high binding capacity of oligo(dT)-cellulose columns will allow the purification of these species on a sufficiently large scale for studies of their function in in vitro protein-synthesizing systems to be made.

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Poly A in SFV-specific RNA


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