Presence of Genomic Polyadenylate and Absence of Detectable Virion Transcriptase in Human Coronavirus OC-43

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SUMMARY

Human coronavirus RNA, prepared by extraction of purified virions with phenol-chloroform, consists of a major 15 to 55S class and a minor 4S class of RNA fragments. Polyadenylic acid [poly (A)] sequences are present in 15 to 55S but not in 4S RNA, suggesting different functions for each class. A stretch of poly (A) of approximately 19 adenosine monophosphate residues was obtained in sizing experiments after digesting OC-43 RNA with pancreatic and T1 ribonucleases. An OC-43 virion RNA transcriptase could not be detected with systems optimal for detecting the transcriptases of influenza and Newcastle disease virus.

INTRODUCTION

Polyadenylate [poly (A)] sequences are present in many virus RNAs having the same polarity as their intracellular messengers (Johnston & Bose, 1972). Where these polarities differ, a transcriptase (usually present within the virion) must synthesize a complementary intracellular copy of the genome as the functional messenger (Baltimore, 1971). Thus tests for genomic poly (A) or a virion transcriptase allow a classification of RNA viruses based upon early events in their replication.

Other more direct tests of messenger function are not practical for human coronaviruses because of the properties of the two model viruses available. Of these, human coronavirus strain OC-43 is relatively stable and can be grown to high titre in suckling mouse brain but not in cell cultures (McIntosh et al. 1967), while human coronavirus strain 229E is extremely labile and can be grown to workable titres for biochemical analyses only with considerable difficulty (Hierholzer, 1976).

Because of these difficulties, human coronavirus RNA was characterized using OC-43 virus propagated in suckling mouse brain in the presence of 32P-orthophosphate (Tannock & Hierholzer, 1977). When isolated from purified virions by sodium dodecyl sulphate (SDS) lysis, a single RNA species is obtained which has a sedimentation coefficient of 70S, a mol. wt. of $6.1 \times 10^6$ and is heat-labile (Tannock & Hierholzer, 1977). Phenol extraction isolates a range of RNAs with sedimentation coefficients of 15 to 55S, and some 4S RNA is present as a minor component (Tannock & Hierholzer, 1977). In the present paper, human coronavirus OC-43 RNA was examined for the presence of poly (A) by affinity column chromatography, and attempts were made to detect a virion transcriptase with systems optimal for the transcriptases of myxov- and paramyxoviruses.

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Solutions and reagents. Buffers and reagents for RNA analysis by centrifugation in sucrose gradients and polyacrylamide gel electrophoresis (PAGE) were those described by Tannock & Hierholzer (1977). For affinity column chromatography, they included elution buffer (EB): 9 parts formamide and 1 part NET (Tannock & Hierholzer, 1977) and equilibrium buffer (EQB): 0.7 M-NaCl, 0.001 M-EDTA, 25% formamide, 0.5% SDS, 0.005 M-Tris, pH 7.5. Other reagents included poly (U)-Sepharose 4B (Pharmacia), T<sub>1</sub> and pancreatic ribonucleases (Worthington), adenosine-, guanosine-, cytidine- and uridine-5'-triphosphates (ATP, GTP, CTP and UTP, Calbiochem), Nonidet P-40 (NP-40, Shell).

Isotopes. Carrier-free ³²P-orthophosphate, ³H-uridine-5'-triphosphate (41.5 Ci/mmol) and 5,6-³H-uridine (40 to 50 Ci/mmol) were obtained from New England Nuclear Corporation.

Virus and RNA markers. ³H-uridine ribosomal RNA and ³H-uridine poliovirus type 1 were prepared by the method of Tannock et al. (1970). Preparations of purified ³H-uridine vesicular stomatitis virus (VSV) and ³H-uridine Rous sarcoma virus (RSV) were provided by Dr J. F. Obijeski, Center for Disease Control (CDC), Atlanta, Ga., and Dr P. H. Duesberg, University of California, Berkeley, Ca., respectively. RNA was extracted from each and from purified poliovirus by the phenol-chloroform (PC) method (Tannock & Hierholzer, 1977).

OC-43 growth and purification. The procedure described by Tannock & Hierholzer (1977) was used to grow and purify ³²P-labelled virus. The labelling step was omitted in transcriptase experiments.

Influenza and Newcastle disease virus growth and purification. Influenza strain B/Lee/40 and Newcastle disease virus (NDV) strain LaSota were obtained from Dr A. P. Kendal, CDC. Each virus was diluted 1000-fold and inoculated into the allantoic cavities of thirty 10-day-old chick embryos. After 48 h, the embryos were chilled and the fluids harvested and clarified by centrifuging at 3000g for 20 min. Calf serum was added to the supernate to 5% concentration followed by an equal volume of neutral saturated ammonium sulphate and the suspension was stirred at 0°C for 30 min to precipitate the virus. The precipitate was collected by centrifuging at 3000g for 30 min and suspending in NET to one-tenth the initial volume of allantoic fluid. The precipitate was further concentrated by centrifuging through a 15% sucrose interface to a 3 ml cushion of 65% sucrose in NET at 60000g for 30 min in a Spinco SW 25.1 rotor.

The concentrates were pooled, diluted with NET and centrifuged to equilibrium for 15 h at 97100g through a 10 ml, 25 to 65% sucrose gradient in NET at 7°C in a Spinco SW 36 rotor. Fractions were collected at the peak of haemagglutinin activity in the density range of 1.16 to 1.20 g/ml, diluted with NET, and centrifuged in a velocity gradient in the SW 36 rotor for 1 h at 43200g in a 90 ml 15 to 65% sucrose gradient in NET. Fractions collected from the upper haemagglutinin-rich band were pooled for immediate use in transcriptase experiments.

Protein determinations. The method of Lowry et al. (1951) was used with BSA standards and spectrophotometric readings at 750 nm.

Preparation of poly (U)-Sepharose 4B columns. A suspension containing 1 g of poly (U)-Sepharose 4B in NET was poured on to a glass filter and washed with 100 ml NET, 100 ml of EB and 100 ml of EQB. A final suspension was made in EB. Short columns were prepared using Pasteur pipettes with a small pledget of glass wool placed at the beginning of the constriction. Poly (U)-Sepharose 4B suspension was added to a column height of 2.5 cm (total bed volume = 0.25 ml).
Poly (A) but no transcriptase in OC-43

Fig. 1. Elution profile of PC-extracted OC-43 RNA from poly (U)-Sepharose 4B columns. Mixtures consisting of OC-43 RNA and (a) 3H-uridine poliovirus RNA, (b) 3H-uridine VSV RNA, or (c) 3H-uridine RSV RNA, each in a total volume of 0.5 ml, were applied to poly (U)-Sepharose 4B columns. The initial filtrate was collected and non-adherent RNAs were removed with three 0.5 ml washes with EQB. Poly (A)-containing RNAs were then eluted in four 0.5 ml steps with EB. Radioactivity was determined for all samples and for an aliquot of each RNA mixture before they were applied to the column. The RNA in the filtrate, washes, and eluates is expressed as a percentage of the total RNA present. (a) \(\Box\), Poliovirus RNA, total recovery 83\%; \(\square\), OC-43 RNA, total recovery 92\%. (b) \(\Box\), VSV RNA, total recovery 94\%; \(\square\), OC-43 RNA, total recovery 94\%. (c) \(\Box\), RSV RNA, total recovery 84\%; \(\square\), OC-43 RNA, total recovery 94\%.

RNA analysis. Centrifugation studies were carried out with 4.4 ml, 15 to 30% sucrose gradients in NET, as described by Tannock & Hierholzer (1977). Similar conditions for PAGE were also used, except that the gels were prepared with 5% acrylamide and 0.34% bisacrylamide.

Transcriptase reaction mixtures. Purified viruses for transcriptase experiments were concentrated immediately before use by centrifuging in a Spinco SW 36 rotor at 97100g for 2 h. Each virus pellet was taken up in 0.3 ml distilled water and samples were held for protein determinations.

(i) Influenza-type system. Standard reaction conditions were those described by
Table I. Relative percentages of total RNA recovered in Fig. 1

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<th>Fig. 1(a)</th>
<th>Fig. 1(b)</th>
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<td>Filtrate and washes [Non-poly (A)]</td>
<td>OC-43 Poliovirus</td>
<td>OC-43 VSV</td>
<td>OC-43 RSV</td>
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<td></td>
<td>54</td>
<td>40</td>
<td>61</td>
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<tr>
<td>Eluates [Poly (A)]</td>
<td>46</td>
<td>60</td>
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Oxford & Perrin (1974) for influenza B/Lee/40. Mixtures (total volume 100 µl) contained 50 mM-tris-HCl, pH 8.0, 5 mM-MgCl₂, 2 mM-MnCl₂, 0.5 mM each of GTP, ATP and CTP, 0.005 mM-³H-UTP (7.5 µCi), 0.2 % NP-40, and 30 to 50 µg virus protein. The incubation temperature was 37 °C, and 20 µl samples were taken immediately after ³H-UTP was added (time 0) and at intervals up to 60 min later. The samples were spotted on to 2 × 3 cm filter-paper strips, which were immediately dried and washed successively with 6 % trichloroacetic acid containing 0.1 M-sodium pyrophosphate, 6 % trichloroacetic acid, distilled water, and ethanol-ether (1:1). Acid insoluble radioactivity was determined on the dried samples by counting in a toluene-based scintillation fluid.

(2) NDV-type system. Reaction conditions similar to those described by Huang et al. (1971) for NDV were used. Mixtures (total volume 100 µl) contained 50 mM-tris-HCl, pH 7.3, 4 mM-magnesium acetate, 3 mM-dithiothreitol, 100 mM-NaCl, 0.5 mM each of GTP, ATP and CTP, 0.005 mM-³H-UTP (7.5 µCi), 5 µl 2-mercaptoethanol, 0.2 % NP-40, and 30 to 50 µg virus protein. Incubation was at 32 °C for 1 h and radioactivity was determined as described for the influenza system.

RESULTS

Poly (U)-Sepharose 4B chromatography of OC-43 RNA prepared by phenol-chloroform (PC) extraction

³²P-OC-43 RNA was extracted from purified virions by the PC method, precipitated with ethanol in the presence of carrier RNA and dissolved in NET, as described by Tannock & Hierholzer (1977). Mixtures consisting of OC-43 RNA and (A) ³H-uridine poliovirus RNA, (B) ³H-uridine VSV RNA, or (C) ³H-uridine RSV RNA were applied to a poly (U)-Sepharose 4B column prepared as described in Methods, and the filtrates were collected. Non-adherent RNA was removed with EQB washes, and poly (A)-containing RNAs were eluted with EB. The profiles for total radioactivity in the initial filtrate and in each wash and eluate are shown in Fig. 1. Clearly, for each mixture a significant proportion of OC-43 RNA can only be removed with EB, indicating that poly (A) sequences are present. Significant amounts of poliovirus and RSV RNA [each containing poly (A)] in mixtures A and C were also eluted with EB, but very little VSV RNA [containing no poly (A)] remained after the washing steps. The relative percentages of total RNA recovered from (a) the combined filtrate and washes and (b) the combined eluates in each mixture are shown in Table 1. Values for the proportion of OC-43 RNA in the poly (A)-containing eluates are 46 %, 39 % and 33 %. With poliovirus and RSV RNA these figures are 60 % and 69 %, respectively, compared with only 0.5 % for VSV RNA. Similar values for the poly (A) content of poliovirus, RSV, and VSV RNAs have been obtained by others (Johnston & Bose, 1972; Lai & Duesberg, 1972; Newman & Brown, 1976).

The column affinity of 70S OC-43 RNA, isolated by SDS lysis and gradient centrifugation (Tannock & Hierholzer, 1977), was examined in parallel with RSV RNA. Only 23 % of OC-43 compared with 49 % of RSV RNA could be detected in the eluates.
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Fig. 2. Size distribution of OC-43 RNAs with and without poly (A). A sample of 0.5 ml of \(^{32}\)P-OC-43 RNA was passed through a poly (U)-Sepharose 4B column as described (see Fig. 1 legend), and pools were made of the filtrate and washes (a) and the eluates (b). Each pool and a control sample (c) containing 1.0 ml of OC-43 RNA were mixed with marker \(^{3}H\)-uridine RSV RNA and precipitated with ethanol in the presence of carrier RNA. The precipitates were resuspended in NET and analysed in sucrose-density gradients. The profiles for acid-insoluble \(^{32}\)P-OC-43 (●—●) and \(^{3}H\)-RSV (○—○) RNAs are shown.
Fig. 3. Analysis of OC-43 RNA for the presence of poly (A) in low mol. wt. species by PAGE. A sample of 0.5 ml 32P-OC-43 RNA was passed through a poly (U)-Sepharose 4B column as described in Fig. 1, and pools were made of (a) the filtrate and washes and (b) the eluates. Each pool and a third control sample (c) containing 1.0 ml of OC-43 RNA and 0.5 ml of 3H-uridine ribosomal RNA were precipitated with ethanol in the presence of carrier RNA. The precipitates were resuspended in 50 μl NET and electrophoresed in 5 % gels for 2 h and 7 mA/gel, as described in Methods. The profiles for 32P-OC-43 (●—●) and 3H-ribosomal (○—○) RNAs are shown.
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Fig. 4. Sizing of poly (A) sequences in OC-43 RNA. One ml of 32P-OC-43 RNA prepared by PC extraction in NET (0.1 M-NaCl, 0.01 M-EDTA, 0.01 M-tris, pH 7.4) was incubated with 50 units of ribonuclease T1 and 10 μg of pancreatic ribonuclease for 60 min at 34 °C. The mixture was extracted three times by the PC method and precipitated with ethanol in the presence of carrier RNA. The precipitate was taken up with NET and passed through a poly (U)-Sepharose 4B column as described in Fig. 1. The eluates were further precipitated with ethanol in the presence of carrier and 3H-ribosomal RNAs and analysed in a sucrose-density gradient. The distributions of acid-insoluble 32P-OC-43 poly (A) (●—●) and 3H-ribosomal RNAs (○—○) are shown.

Size distribution of OC-43 RNAs with and without poly (A)

A sample of 32P-OC-43 RNA was passed through a poly (U)-Sepharose 4B column as above, and pools were made of (a) the filtrate and washes and (b) the eluates. Each pool and a third, control sample (c) were mixed with marker 3H-uridine RSV RNA and precipitated in the presence of carrier RNA. The RNAs were then analysed in sucrose-density gradients (Fig. 2). The profiles for acid-insoluble radioactivity in the control mixture (Fig. 2 c) suggest that the major OC-43 RNAs are distributed in the 15 to 55S region of the gradient with a minor low-mol. wt. component sedimenting just ahead of 4S RNA, as we have shown previously (Tannock & Hierholzer, 1977). This minor component is present in the filtrate and wash samples (Fig. 2 a), but absent from the poly (A)-containing eluates (Fig. 2 b).

Better resolution of the minor RNA from the major 15 to 55S species was obtained by PAGE of three similarly prepared 32P-OC-43 RNA samples in 5% acrylamide gels (Fig. 3). The control sample was co-electrophoresed with 3H-uridine ribosomal marker RNA and the radiolabelled profiles suggest that a number of minor RNA species of similar size to ribosomal 4S RNA are present. These are also present in the filtrate and washes (a), but absent in the poly (A)-containing eluates (b), thus confirming the results in Fig. 2.
Fig. 5. Tests for the presence of OC-43 virion transcriptase. Transcriptase activity, according to the extent of acid-insoluble $^3$H-UMP incorporation, was measured for (a) influenza and (b) OC-43, with an influenza-type system, and for NDV (c) and OC-43 (d), with an NDV-type system. Standard reaction conditions are described in Methods. For the influenza-type system, besides the standard mixture ('S') additional mixtures were prepared in which (i) 2 μl of 10% 2-mercaptoethanol was added, and (ii) manganous chloride and (iii) magnesium chloride was omitted. The influenza-type system was incubated at 37 °C and the NDV-type system at 32 °C.

Size of poly (A) RNA sequences in OC-43 RNA

The size of the poly (A) residues in OC-43 RNA was determined by sucrose-density gradient centrifugation after ribonuclease digestion. $^{32}$P-OC-43 RNA prepared by PC extraction was incubated with ribonucleases, extracted by the PC method to remove enzyme activity, and precipitated with ethanol in the presence of carrier RNA. The precipitate was solubilized in NET and passed through a poly (U)-Sepharose 4B column. Poly (A)-containing eluates were further precipitated with ethanol in the presence of carrier and $^3$H-uridine ribosomal RNA and then analysed in sucrose-density gradients (Fig. 4). The profiles for acid-insoluble radioactivity indicate that the poly (A) is present as a single peak with a sedimentation coefficient of 2S located near the top of the gradient. The peak is well resolved from what appears to be some incompletely digested RNA fragments too large to contain poly (A) alone. With the formula $M = 1550 \times S^{-2.1}$ (Spirin, 1962), the mol. wt. of the 2S poly (A) is 6645, which corresponds to a stretch of approximately 19 adenylate residues. Spirin's formula, however, takes no account of differing amounts of secondary structure between RNAs with similar sedimentation coefficients and this value must be regarded as only approximate.

Similar profiles were obtained in two further experiments. When pancreatic ribonuclease
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was omitted from the RNA-enzyme mixture, a larger peak with a sedimentation coefficient of 4S was obtained, indicating the presence in small amounts of nucleotides other than A. The polyadenylate 2S fragment represents approx. 0.3% of the total RNA before digestion. The latter figure is a minimum value, however, because larger, incompletely digested, poly (A)-containing fragments are also found (see Fig. 4), which could be due to regions of base pairing within the RNA genome; these may or may not be related to the 2S fragment.

Tests for the presence of a virion transcriptase

Transcriptase experiments were carried out on (a) influenza virus and (b) OC-43 with an influenza-type system, and on NDV (c) and OC-43 (d) with an NDV-type system. For the influenza-type system, transcriptase activity was also measured in mixtures in which (i) 2-mercaptoethanol was added, (ii) manganous chloride was omitted and (iii) magnesium chloride was omitted. The incorporation obtained for each set of conditions is shown in Fig. 5.

For influenza (Fig. 5a), maximum incorporation of 77 pmol UMP/mg virus protein was obtained after 60 min at 37 °C in the standard mixture and in mixtures (i) and (ii). When magnesium chloride was omitted (iii), no incorporation occurred above background. In the same system for OC-43 (Fig. 5b), no incorporation above background occurred under any conditions. With NDV in an NDV-type system (Fig. 5c), incorporation occurred to a maximum of 7 pmol UMP/mg virus protein after 60 min at 32 °C, but no incorporation above background was noted for OC-43 (Fig. 5d).

Discussion

Poly (A) can be detected in 33 to 46% of PC-extracted OC-43 RNA (Fig. 1) but in only 23% of 70S RNA released from virions by SDS lysis (G. A. Tannock & J. C. Hierholzer, unpublished observations). The lower column affinity for 70S RNA may indicate that poly (A) sequences are masked from the poly (U)-Sepharose 4B acceptor groups because of secondary structures which are absent in RNAs prepared by PC extraction (Tannock & Hierholzer, 1977). Apparent variation in the poly (A) content of OC-43 RNA may be related to the great variation in fragment length (Fig. 2c). Heterogeneity has been reported for the subunit RNAs of RSV; all intact subunits have poly (A), and the absence of poly (A) is attributed to ribonuclease activity (King & Wells, 1976). Great variation in 15 to 55S OC-43 RNA fragment size may be due to internal ribonuclease activity. However, poly (A)-containing fragments appear to have a similar size distribution to those without poly (A) (Fig. 2a and b) which is consistent with the presence of a subunit structure more complex than for retroviruses. Accurate measurements of the RNA(s) of newly-matured coronavirions, grown in a more strictly defined system than the suckling mouse brain, are required to resolve this question.

Poly (A) is present in 15 to 55S OC-43 RNA obtained by PC extraction but absent from the minor 4S RNA (Fig. 2, 3), suggesting a different function for each RNA class. A stretch of approx. 19 AMP residues is obtained by T1 and pancreatic ribonuclease digestion. The function of coronavirus 4S RNA in PC extracts is unknown. It may be that the same 4S RNA which is freed from 70S OC-43 RNA complex by heating (Tannock & Hierholzer, 1977) functions as a specific t-RNA. A 4S RNA with t-RNA function is released from 70S retrovirus RNA by heating (Erikson & Erikson, 1971; Dahlberg et al. 1974).

Our data provides no evidence as to whether poly (A) in OC-43 RNA is internal or located at the 3' terminus. An appropriate test for its location which has been used for
RSV RNA (Wang & Duesberg, 1974) might be to compare the size distribution profiles of poly (A)-containing RNAs after treatment with ribonuclease H in the presence of poly (dT). However, the great heterogeneity of OC-43 RNA when prepared by PC extraction [Fig. 2; Tannock & Hierholzer (1977)], suggests that it would be extremely difficult to detect size differences between treated and untreated RNA, irrespective of whether internal or external poly (A) is present.

Poly (A) tracts of 30 to 200 AMP residues have been reported for avian retroviruses and are located at the 3' end of each RNA subunit (Lai & Duesberg, 1972; Stephenson et al. 1973; Wang & Duesberg, 1974). The function of poly (A) is unknown, but it may have a role in regulating protein translation. However, the functional messenger for retrovirus protein synthesis is not virion RNA, but an m-RNA of the same polarity, which is transcribed from a DNA provirus (Temin & Baltimore, 1972). If a 3' terminal location for coronavirus RNA is assumed, its presence also suggests that these RNAs have the same polarity as the m-RNAs which specify coronavirus proteins. However, experiments with inhibitors (Akers, 1963; Hierholzer, 1976) reveal that there is no DNA-dependent stage in coronavirus replication, which indicates that the virion RNAs are themselves capable of acting as messengers.

The above evidence supplies strong theoretical reasons for supposing that a virion RNA transcriptase is absent from coronaviruses. Despite two preliminary reports to the contrary for other coronaviruses (Bingham, 1975; Kennedy & Johnson-Lussenburg, 1976), we were unable to detect a transcriptase in OC-43 virions with systems optimal for influenza and NDV. Additionally, no activity could be detected in human coronavirus 229E with a system optimal for VSV (J. C. Hierholzer & J. F. Obijeski, unpublished observations). While these findings do not entirely rule out the presence of a virion transcriptase specific for yet undefined reaction conditions, they are consistent with earlier evidence for the presence of poly (A).

A recent report by Schochetman et al. (1977) also describes the presence of genomic polyadenylate and the absence of detectable RNA transcriptase in the coronavirus, avian infectious bronchitis virus (IBV). Furthermore, they reported that IBV RNA was infectious for chicken fibroblasts. However, one difficulty in regarding this as corroborative evidence arises from the other major finding in the same paper that the IBV genome can be isolated from purified virions as a heat-stable, single RNA species. This is at variance with other findings for IBV RNA (Tannock, 1973; Watkins et al. 1975), porcine coronavirus RNA (Garwes et al. 1975), and human coronavirus OC-43 RNA (Tannock & Hierholzer, 1977) for reasons which are not clear.

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