Rotavirus RNA Segments Sized by Electron Microscopy

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SUMMARY

The RNA segments of bovine rotavirus (UK Compton strain) were measured by electron microscopy in mixtures containing φX174 double-stranded DNA as an internal reference molecule. The numbers of base pairs per segment were calculated and compared with data obtained by other procedures (comparative RNA gel electrophoresis; co-electrophoresis of cDNAs in denaturing gels; sequencing).

Rotaviruses, a genus of the Reoviridae family, are the main cause of acute gastroenteritis in infants and young animals (Flewett & Woode, 1978; McNulty, 1978). They possess a genome consisting of 11 segments of double-stranded (ds) RNA. The RNA can be rapidly extracted and the segments separated by electrophoresis on polyacrylamide gels (Newman et al., 1975; Kalica et al., 1976; Verly & Cohen, 1977; Kalica et al., 1978). Differences in the RNA migration patterns have been found among isolates obtained from different animal species (Kalica et al., 1978; Rodger & Holmes, 1979; Todd & McNulty, 1977; Todd et al., 1980) and the relative mobility of corresponding segments may also differ among isolates obtained from a single species (Verly & Cohen, 1977; Kalica et al., 1978; Rodger & Holmes, 1979; Todd et al., 1980; Espejo et al., 1980; Lourenco et al., 1981; Follett & Desselberger, 1983).

The size estimates of corresponding RNA segments vary over a considerable range, and Table 1 gives a comparison of some of the data in the literature (Newman et al., 1975; Rodger et al., 1975; Todd & McNulty, 1976; Verly & Cohen, 1977; Kalica et al., 1978; Todd et al., 1980; results of Rodger & Holmes, 1982, cited by Both et al., 1982). Most of the size estimates were obtained by comparing the relative migration of rotavirus RNA segments with that of reovirus RNA segments. Todd et al. (1980) note that ‘the estimations for [rotavirus] RNA segments 7–11 are questionable since these segments have electrophoretic mobility greater than that of the smallest RNA segment from reovirus’. Shatkin et al. (1968) used sedimentation data obtained for the three size classes of reovirus RNA by Bellamy et al. (1967) to determine the size of individual reovirus segments from gels. Kalica et al. (1978), applying Shatkin’s approach to rotavirus RNAs, obtained estimates (Table 1, column 3) which for RNA segments 7–11 were closer to their real size than the estimates obtained by Newman et al. (1975), Verly & Cohen (1977), Todd et al. (1980) and Rodger & Holmes cited by Both et al. (1982) (Table 1, columns 1, 2, 4 and 5 respectively). However, the size estimates of the large dsRNA segments of rotavirus by Kalica et al. (1978) were considerably smaller than the corresponding values of most other estimates.

Since 1982, procedures have been published which have allowed cloning of the rotavirus genome (McCrae & McCorquodale, 1982; Both et al., 1982, 1983; Imai et al., 1983; Dyall-Smith et al., 1983). DNA complementary to the genomic RNA of rotavirus was obtained in a reaction step using reverse transcriptase and this cDNA has been sized on denaturing gels using plasmid pBR322 DNA fragments of known size as markers. The size values thus obtained by Both et al. (1982) differed considerably from the previous ones (Table 1, compare column 6 with columns 1 to 5), and McCrae & McCorquodale (1982) had results similar to those of Both et al. (1982). The definite sizes obtained from sequence data of segments 7, 8, 9 and 11 of different rotavirus strains (Both et al., 1982, 1983; Imai et al., 1983; Dyall-Smith et al., 1983) are in close agreement with those estimated from the cDNA.
Table 1. Molecular weights of rotavirus RNAs obtained by gel electrophoresis of RNAs and cDNAs, and by electron microscopy

<table>
<thead>
<tr>
<th>Segment numbers and size groups</th>
<th>Mol. wt. x 10^-6 (and no. of bp in columns 6 and 7 given in parentheses)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>(I–IV)</td>
<td></td>
</tr>
<tr>
<td>I 1</td>
<td>2.2</td>
</tr>
<tr>
<td>II 5</td>
<td>0.9</td>
</tr>
<tr>
<td>III 7–9</td>
<td>0.5</td>
</tr>
<tr>
<td>IV 10</td>
<td>0.3</td>
</tr>
<tr>
<td>11</td>
<td>0.2</td>
</tr>
<tr>
<td>Total mol. wt.</td>
<td>12.88</td>
</tr>
</tbody>
</table>

* Bovine rotavirus, 5% polyacrylamide gel; reovirus RNAs as standard (Martin & Zweerink, 1972).
† Bovine rotavirus, 5% polyacrylamide gel; reovirus RNAs as standard (Wood, 1973).
‡ Human rotavirus, strain D. 0.5% agarose 2.5% polyacrylamide gel using size class molecular weights (Shatkin et al., 1968). The segments whose molecular weights are in bold type co-migrated with corresponding segments of UK calf diarrhoea virus (Fig. 4 and Table 5 in Kalica et al., 1978).
§ Calf rotavirus, 3.8% polyacrylamide gel; reovirus RNAs as standard (Wood, 1973).
¶ Simian rotavirus, 5% polyacrylamide gel; reovirus RNAs as standard. Cited by Both et al. (1982).
†† Simian rotavirus SA11. cDNA was separated on 3% polyacrylamide–7 M-urea gel; marker fragments prepared from pBR322. The mol. wt. were calculated from number of bp (in parentheses) assuming an average mol. wt. of 660 per bp. Segments whose mol. wt. is in bold type co-migrate with corresponding segments of UK calf diarrhoea virus (Kalica et al., 1978: their Table 3).
** Values given are mid-points of the highest columns for each segment (Fig. 2). These values correspond to absolute lengths for the genomic segments of: 1, 1.01 μm; 2, 0.81 μm; 3, 0.79 μm; 4, 0.70 μm; 5, 0.45 μm; 6, 0.40 μm; 7–9, 0.33 μm; 10, 0.21 μm; 11, 0.19 μm.
††† Ratio = (number of bp in column 7)/(number of bp in column 6).
‡‡‡ Both et al. (1983) sequenced segment 9 of SA11 virus which contains 1062 bp. Both et al. (1982) sequenced segment 8 of SA11 and found it to contain 1059 bp; Dyall-Smith et al. (1983) sequenced segment 7 of UK bovine rotavirus and found it to be 1059 bp long. Assuming 660 mol. wt./bp, this would account for 0.70 x 10^6 mol. wt.
§§ Imai et al. (1983) sequenced segment 11 of the human Wa strain and found it to be 663 bp long, i.e. of 0.44 x 10^7 mol. wt. Segment 11 of human Wa strain co-migrates with segment 11 of UK bovine rotavirus (unpublished results).
The size of segments of dsRNA released from reovirus particles has also been determined by electron microscopy (Vasquez & Kleinschmidt, 1968) and has yielded results in agreement with sedimentation coefficients (Bellamy et al., 1967) assuming a molecular mass of $2.1 \times 10^6$ daltons per $\mu$m. Considering the high degree of variation among the different estimates (Table 1) we have redetermined the size of the segments of bovine rotavirus (UK Compton strain) RNA by electron microscopy using circular $\phi$X174 RF DNA as an internal marker and we compare our results with those obtained by other procedures (Table 1).

Bovine rotavirus (UK Compton strain) was propagated in BSC-1 and MA-104 cells, virus was pelleted from the supernatant of infected cells through a 30% sucrose cushion (1 h at 110000 $g$ and 4 °C) and the RNA extracted as described (Follett & Desselberger, 1983).

$\phi$X174 RF DNA (5386 base pairs; Sanger et al., 1978) was obtained from New England Biolabs and mixed as an internal marker with the RNA molecules.

Samples were prepared for electron microscopy using the spreading technique described by Dunnebacke & Kleinschmidt (1967) and by Kleinschmidt (1968). The grids were observed at a primary magnification of 6100 $\times$ using a Jeol 100S electron microscope which was calibrated against a standard grating, and molecules were measured from photographs at a final magnification of 42500 $\times$.

The lengths of RNA segments were measured from the photographs using a Summagraphics ID digitizing tablet controlled by a PDP-11 minicomputer. This digitizing tablet has a resolution of 0.2 mm, which corresponds to approximately 14 base pairs of the dsDNA marker at the magnification used. A computer program was written to calculate the length of each curve by circular interpolation which assumes that successive triplets of sample points lie on the arc of a circle. It was found in practice that usually the first two measurements of each molecule deviated from their mean value by less than 1%, and this mean value was stored for subsequent analysis. When, however, the deviation exceeded 1%, the program automatically requested further
measurements of the same molecule, until it was able to select two measurements within the deviation of 1%.

Fig. 1 shows an electron micrograph of a mixture of rotavirus RNAs and φX174 RF DNA marker molecules. The length of 24 relaxed, circular φX174 marker molecules was measured and the arithmetic mean calculated; the standard deviation was ± 1.7%. This length was defined as containing 5386 base pairs (bp) and used to calculate the number of bp for RNA molecules from their length measurements. Assuming the B form for the dsDNA marker (10 residues per turn, 0.346 nm translation per residue) and an intermediate between the A and B form for the dsRNA rotavirus molecules (RNA 10 helix: 10 residues per turn, 0.30 nm translation per residue; both sets of values from Cantor & Schimmel, 1980) it was calculated that dsRNA contains 1.15 times more bp per unit length than does dsDNA. Therefore, the length measurements of rotavirus RNA molecules were converted into numbers of bp by direct proportionality calculations from the average length of the dsDNA reference molecule and then multiplied by a factor of 1.15. A total of 415 molecules from two complete visual fields of the same grid was measured. Care was taken to measure every distinguishable molecule per field in order to obtain a representative sample of the whole grid.

Subsequently, the length distribution histogram was produced (Fig. 2). For this purpose, the computer was programmed to print out the number of molecules for x ± 21 bp (x ranging from 448 to 13708 in 42 bp intervals). The length distribution histogram (Fig. 2) shows from left to right seven major peaks comprising segments 11 + 10, 9 + 8 + 7, 6, 5, 4, 3 + 2 and 1. Peaks comprising segments 11 + 10 and 3 + 2 clearly show a bimodal structure. A small number of measurements was found between the segmental peaks and beyond segment 1. These measurements most probably relate to aggregates of segments of varying lengths, and the observation is in close agreement with findings reported by Vasquez & Kleinschmidt (1968; their Fig. 6) for reovirus RNA. As the molecules larger than those forming the segment 1 peak represented only 4.6% (19/415) of the total number of molecules measured (Fig. 2), we believe that possible aggregate formation did not affect the determination of segment lengths.

The numbers of molecules contained in major peaks of Fig. 2 were compared with one another. In the two cases of adjoining segments, 11 + 10 and 3 + 2, one half of the molecules in the lowest column between the peaks was assigned to each of the flanking segments. When the frequency of the number of molecules in the smallest peak (segment 11, 29 molecules) was

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Fig. 2. Length distribution histogram of molecules of dsRNA segments of bovine rotavirus (UK Compton strain). Total number of molecules = 415. Peaks corresponding to individual segments (no. 1 to 11) are indicated by arrows on the top. A small number of molecules were found to be outside the size range shown. They had the following sizes (in number of bp): 301, 345; 4155, 4237, 4259, 4266, 4920, 5196, 6076, 6342, 7855, 8368, 8468, 10293, 11115, 11957, 13607.
defined as 1.00, the following frequencies were found for the other segments (in parentheses): 0.80 (segment 10); 3.33 (segments 7, 8 and 9 in one peak); 0.50 (segment 6); 0.97 (segment 5); 0.83 (segment 4); 1.43 (segment 3); 0.90 (segment 2); 1.46 (segment 1). In view of the statistically small size of the individual segment samples, the frequency distribution thus obtained by measuring molecules by electron microscopy is in moderate agreement with the equimolar occurrence of segments obtained by densitometric analysis of bovine rotavirus RNAs in silver-stained gels (Whitton et al., 1983).

The sequence data of rotavirus RNAs available so far are of the smaller segments [663 bp for segment 11 of human Wa strain (Imai et al., 1983); 1059 bp for segment 8 of simian SA11 virus (Both et al., 1982); 1059 bp for segment 7 of UK bovine rotavirus (Dyall-Smith et al., 1983); 1062 bp for segment 9 of SA11 virus (Both et al., 1983)]. Our data are in good agreement with these sequences (3.7% higher for the triplet of segments 7, 8 and 9; 3.9% lower for segment 11 of bovine rotavirus assuming its real number of bp to be very close to that of segment 11 of the human Wa strain). Priess et al. (1980) found a linear relationship between length and number of bp of dsDNA in the range of 400 to 10000 bp and there is no reason that this should not be the case for the size range of dsRNAs investigated (Vasquez & Kleinschmidt, 1968). Our measurements agree, with a difference of ≈ 6% (Table 1, column 8), with the cDNA size estimates of size classes II (segments 5 and 6), III (segments 7 to 9) and IV (segments 10 and 11) (Both et al., 1982), whereas they are 8 to 17% lower for molecules of size class I (segments 1 to 4). Segments 1, 2 and 3 of SA11 virus from which the values of Both et al. (1982) are obtained and the corresponding segments of the UK bovine rotavirus migrate very similarly (Pereira et al., 1983) whereas segment 4 of the UK bovine rotavirus migrates slightly more slowly, i.e. is apparently larger on gels than is segment 4 of SA11 rotavirus (Pereira et al., 1983). It is true that cDNA size measurements using dsDNA standards in denaturing gels proved to be superior to comparative RNA size measurements in gels even under denaturing conditions as was observed for the influenza virus genome (see Table II of Brownlee, 1981) and this statement is certainly also correct to a large extent for the measurements of the rotavirus RNA genome (Table 1; compare columns 1, 2, 4, 5 with column 6). But it is possible that single-stranded cDNAs > 2500 bases long do not migrate only according to their size. This question should be decided when sequences of size class I segments are available. The molecular weight of the total rotavirus genome obtained by us is between the corresponding value obtained by Both et al. (1982) and those obtained by comparative RNA gel electrophoresis using reovirus RNA as standard (Table 1, columns 4 to 7).

For reoviruses, good agreement of molecular masses derived from sedimentation data with electron microscopy was obtained (Vasquez & Kleinschmidt, 1968) by assuming 2.1 × 10^6 daltons per µm of dsRNA (Bellamy et al., 1967). The sequence of the S2 gene of reovirus (Cashdollar et al., 1982) confirms the corresponding part of these data. From measurements of sequenced dsDNA standards by electron microscopy (Priess et al., 1980) a number of 1.91 × 10^6 mol. wt. of dsDNA per unit length (µm) was calculated, which is 10% below the assumed value for dsRNA (Bellamy et al., 1967). Priess et al. (1980) found that the lengths of DNA–mRNA hybrids of Escherichia coli phage T7 are shorter than the corresponding dsDNAs and had to be multiplied by a factor of 1.15 in order to calculate the correct number of bps present. These differences obtained from measurements by electron microscopy are in the same size range as those obtained from physicochemical studies (Cantor & Schimmel, 1980) which we have taken as a basis for calculations comparing lengths of dsDNAs and dsRNAs measured in one preparation. It is clear from our results that the use of dsDNA molecules of known size as the internal standard has allowed accurate measurement of the dsRNA segments of bovine rotavirus to within 4% of the known sizes of individual sequenced segments. Thus, measurement of nucleic acid molecules by electron microscopy seems to be a reliable means to determine molecular weights of molecules whose primary sequences are not available.

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REFERENCES


Short communication


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