The Molecular Biology of Rotaviruses. VI. RNA Species-specific Terminal Conservation in Rotaviruses

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SUMMARY

The use of T₁ RNase fingerprinting of terminally labelled genomic double-stranded RNA species from various rotavirus isolates, to analyse the near terminal G-residue positions, has revealed an RNA species-specific fingerprint pattern covering approximately 40 nucleotides at the termini. These RNA species-specific terminal fingerprint patterns were found to be conserved in both rotavirus RNAs isolated from various animal species, and in isolates from a single animal species where gross divergence of internal RNA sequence for a particular RNA species was evident. This conservation of near terminal G-residue positions suggests that, internal to the short regions of absolute terminal sequence conservation that we have previously shown to be present on all rotavirus RNA species, there is a region of conserved sequence which is specific for a particular RNA species.

INTRODUCTION

The rotavirus genome is composed of 11 discrete segments of double-stranded (ds)RNA (Newman et al., 1975; Rodger et al., 1975; Kalica et al., 1976; Todd & McNulty, 1976) each of which appears to be monocistronic, coding for a single primary gene product (McCrae & McCorquodale, 1982a). Detailed structural analysis of the termini of the genomic dsRNAs has shown that they are flush-ended (McCrae & McCorquodale, 1983). Terminal sequencing studies that we have carried out on the U.K. bovine rotavirus and a human rotavirus isolate have revealed two short terminal sequences (< 10 bases), one at each end of the RNA (McCrae & McCorquodale, 1983) that are completely conserved on all the genomic RNAs analysed and which are almost certainly present on the genome RNAs of all rotaviruses carrying the recognized virus group antigen (Woode et al., 1976; McNulty, 1978).

Recently, we have developed a one-dimensional RNA fingerprinting technique (Clarke & McCrae, 1981b) for analysing the structural basis of the widespread variation in electrophoretic mobility of rotavirus genome segments reported by a number of groups (Kalica et al., 1978; Rodger & Holmes, 1979; Clarke & McCrae, 1981a). During the detailed analysis of the fingerprints of individual genomic RNAs from a number of field isolates of bovine rotavirus, it became apparent that there were regions of genome segment-specific sequence conservation lying immediately adjacent to the absolutely conserved terminal sequence. The purpose of this report is to present evidence for these regions of genome segment-specific sequence conservation, and to show that they seem to be present in all rotaviruses carrying the recognized group antigen, irrespective of their animal species of origin.

METHODS

Viruses and cells. The U.K. tissue culture-adapted bovine rotavirus was propagated in BSC-1 cells and virus particles purified as previously described (McCrae & Faulkner-Valle, 1981). Human rotavirus was purified from infected faeces obtained from a 1-year-old child in hospital. The purification procedure used was as for the bovine virus, but with the addition of an extra Arcton extraction step. Field isolates of bovine and porcine rotavirus were
collected as single stools and were supplied to us by Mr Tony Scott of the Central Veterinary Laboratory, Weybridge, U.K.

**Extraction and 3' terminal labelling of viral dsRNA.** In most cases viral genomic dsRNA was extracted directly from the virions present in infected faeces as previously described (Clarke & McCrae, 1981a). When purified virions were available, extraction of viral dsRNA with phenol was as previously described for reovirus (McCrae & Joklik, 1978). Extracted genomic dsRNA was labelled at its 3' termini with $[^{32}\text{P}]\text{PcP}$ using T4 RNA ligase as previously described (McCrae, 1981).

**Isolation of individual genome segments and one-dimensional terminal fingerprint analysis.** Double-stranded RNAs having $^{32}\text{P}$-labelled 3' termini were fractionated and individual genome segments isolated for fingerprinting using the method of Clarke & McCrae (1981b). Following their isolation, individual species of dsRNA were denatured at 50 °C in 90% dimethyl sulphoxide and partially digested with T1 RNase as previously described (Donis-Keller et al., 1977; Clarke & McCrae, 1981b). Random cleavage of isolated species of RNA to produce sequence ‘ladders’ was achieved by partial alkaline hydrolysis of labelled RNA in 50 mM-bicarbonate buffer pH 9-0 at 90 °C for 3 min. $^{32}\text{P}$-labelled DNA restriction fragment size markers were prepared as previously described (Clarke & McCrae, 1981b).

For fingerprint production, partial digestion products were fractionated on thin 16% polyacrylamide gels (Sanger & Coulson, 1978) which were subsequently autoradiographed at -70 °C using DuPont Lightning-plus intensifying screens.

**RESULTS**

One-dimensional terminal fingerprinting (Clarke & McCrae, 1981b) was originally developed to study the underlying structural basis of the variation in electrophoretic mobility of genome RNA species seen in field isolates of rotaviruses (Kalica et al., 1978; Rodger & Holmes, 1979; Clarke & McCrae, 1981a). During development of this technique we observed a high level of near terminal conservation of the fingerprint pattern obtained when examining a given RNA species from a number of virus isolates. Thus, Fig. 1 shows the partial T1 RNase digestion fingerprints obtained for the species 5 and 6 RNAs from five independent field isolates of bovine rotavirus. The partial digestion procedure produces a series of overlapping oligonucleotide fragments ending in 3'-terminal G residues; the oligonucleotide fragment running from the cleavage position to the labelled 3' terminus is detected by autoradiography, allowing the positions of G residues within 100 to 200 bases of a 3' terminus to be precisely located. When carried out on rotavirus genomic RNA species, where the two labelled RNA strands had not been separated prior to analysis, the picture obtained represents a composite of G positions in both the plus and minus strand. In Fig. 1 the G-banding pattern expected from the short regions of completely conserved terminal sequence that we have previously reported (McCrae & McCorquodale, 1983) was obtained. However, internal to this, a region of RNA species-specific conservation of T1 RNase banding pattern was evident in all five isolates. This additional region of near terminal conservation ran from nucleotide position 8 to approximately position 40 (Fig. 1). Internal to the sequence of about 40 nucleotides from the terminus of each RNA strand, minor variations in the banding patterns were evident between the different virus isolates. These variations, which were more apparent in the species 5 RNA fingerprints, were of the type that we have previously reported (Clarke & McCrae, 1981b). Having established that these two RNA species carried a region of G position conservation near each terminus, it was obviously of some interest to establish whether a similar phenomenon existed for the other genome segments. We therefore undertook fingerprint analysis of the other RNA species of these isolates, with RNA species 7 to 9 being treated as a single entity for these studies. The results (data not shown) showed that for all the RNAs from these five isolates, there was a region of approximately 35 nucleotides internal to the completely conserved terminal sequences (McCrae & McCorquodale, 1983) whose conserved G-residue banding pattern was specific to a given RNA species.

Despite the absolute conservation of G-banding pattern in the near terminal regions of the virus isolates described above, we were dealing with RNA species whose overall fingerprint patterns only showed evidence of relatively minor sequence changes. Previously, we have reported results on the fingerprinting of the species 11 RNAs from five bovine virus isolates.
Fig. 1. Terminal fingerprint analysis of species 5 and 6 RNAs from five bovine rotavirus isolates. The isolation of terminally labelled individual RNA species and the production of partial T₁ digestion fingerprints was carried out as previously described (Clarke & McCrae, 1981b). M, DNA size markers generated using an HpaII digest of the plasmid pBR322; L, partial alkaline hydrolysis ladder to mark nucleotide positions; A to E, T₁ fingerprints for the five RNAs; N, undigested control to indicate that the bands generated were T₁-specific. The numbers on the left-hand side of the lanes give an indication of nucleotide positions from the termini of RNA molecules.

(Clarke & McCrae, 1982). This analysis showed that these RNAs fell into two distinct groups whose internal regions showed a gross divergence in fingerprint pattern (Clarke & McCrae, 1982). Fig. 2(a) shows the terminal region fingerprint patterns from the species 11 RNAs from these five isolates. The conservation of the G-banding pattern as far as approximately nucleotide position 40 is clearly evident. This was despite the gross internal sequence differences between the two groups which is re-confirmed and emphasized in Fig. 2(b).

Since bovine rotaviruses appear to carry a near terminal region of genome segment-specific sequence conservation in their genomic dsRNA, it was of some interest to establish whether the conservation extended to the genomes of rotaviruses isolated from other animal species. Fig. 3 shows the terminal and internal fingerprint patterns for the species 10 and 11 RNAs of four
human rotavirus isolates. The RNA species-specific conserved region was clearly evident and in the case of the species 11 patterns was the same as that obtained for the bovine isolates (Fig. 2a). Internal to the terminal region there was again considerable divergence of fingerprint pattern. Finally, Fig. 4 shows results of a comparative analysis of RNA species 5 and 6 from further isolates obtained from an infected calf, a piglet and a human infant. The terminal fingerprint analysis showed for both RNA species the characteristic conservation of the G-banding pattern, although in the case of the species 5 RNA of the pig isolate, a single change at about nucleotide position 20 was evident, indicating that the observed conservation may not be absolute. However, internal to about 45 nucleotides from the termini, the species 5 RNA fingerprints
Conserved RNA sequences in rotaviruses

(a) Species 10          Species 11
L  A  B  C  D  M  A  B  C  D  L

(b) Species 10          Species 11
L  A  B  C  D  M  A  B  C  D  L

Fig. 3. Terminal and near terminal fingerprint analysis of RNA species 10 and 11 from four human rotavirus isolates. The lane designations are as for Fig. 1 except that four human rotaviruses, A to D, were analysed. (a) Fingerprint patterns for the terminal 30 to 40 nucleotides. (b) Fingerprint patterns for the 30 to 80 nucleotide region. The arrow on the left-hand side of each panel is to aid continuation from (a) to (b), and is at the same nucleotide position on each panel. The numbers on each side of the lanes give an indication of nucleotide positions from the termini of RNA molecules.

completely diverged; this is consistent with there being a radical difference in the internal sequence of the three RNAs. Unlike species 5, the fingerprint patterns for species 6 RNAs showed only relatively minor variations between the three viruses. This observation would be consistent with VP6, the major polypeptide of the inner capsid shell which is coded for by species 6 RNA (McCrae & McCorquodale, 1982a) representing the group antigen of rotaviruses detectable using a number of techniques in a majority of the virus isolates analysed to date (Woode et al., 1976; McNulty, 1978).

DISCUSSION

Our initial studies using the one-dimensional partial nuclease digestion fingerprinting technique enabled us to demonstrate that changes in the electrophoretic mobility of a particular genome segment are always associated with a detectable change in primary sequence (Clarke & McCrae, 1982). However, in those studies the changes in primary sequence, which varied from relatively minor changes of the type expected from antigenic drift to the gross sequence changes expected from antigenic shift, were confined to regions internal to the terminal approximately 45 nucleotides at either end of the molecule. The present study has concentrated on analysing
Fig. 4. Terminal and near terminal fingerprint analysis of the RNA species 5 and 6 from a bovine, a porcine and a human virus isolate. L, partial alkaline hydrolysis ladder to mark nucleotide positions; A, T₁ fingerprint of the bovine virus RNA; B, T₁ fingerprint of the porcine RNA; C, T₁ fingerprint of the human virus RNA. (a) Fingerprint patterns for the terminal 30 to 40 nucleotides. The majority of the species 6 RNA from the porcine virus was lost during preparation and so the fingerprint pattern is only very faintly evident. (b) Fingerprint patterns for the 30 to 80 nucleotide region. The arrow on each side of each panel is to aid continuation from (a) to (b), and is at the same nucleotide position on each panel. The numbers on each side of the lanes give an indication of nucleotide positions from the termini of RNA molecules.

This near terminal region of the genome segments. The striking feature immediately apparent on fingerprinting this terminal region from a particular genome segment was a region of 40 to 45 nucleotides that was completely conserved and whose fingerprint pattern was diagnostic of the genome segment being analysed. This RNA species-specific terminal conservation was maintained both in viruses that showed gross internal RNA sequence changes and had been isolated from the same animal species (Fig. 2) and in those viruses, isolated from different animal species, where a complete divergence of internal sequence was apparent. Complete nucleotide sequence analysis of these terminal regions of each genome segment will obviously be required to establish conclusively that no sequence changes occur in them; the absolute conservation of G residue positions strongly suggests this is the case. The requirement of current RNA sequencing strategies for relatively large amounts of virus for synthesis of the mRNA used
in displacement hybridization (Darzynkiewicz & Shatkin, 1980; Li et al., 1980; McCrae, 1981) coupled with the difficulties in adapting rotavirus isolates to high-yield growth in tissue culture (Estes et al., 1979; Wyatt et al., 1980), has precluded such sequence studies on the isolates described in this report. However, using the recently developed cloning strategy for rotavirus genome RNA species (McCrae & McCorquodale, 1982b) this sequence analysis should be possible on the cloned DNA copies and work towards this end is currently in progress.

Concerning the functional role played by these RNA species-specific conserved regions, the fact that they lie immediately adjacent to the absolutely conserved terminal sequences which we have previously reported to be present on all rotavirus genome RNA species (McCrae & McCorquodale, 1983) suggests some role in delineating quantitative control. Thus, modulation of RNA transcription and/or replication, operation of quantitative control in translation and an involvement in genome segment selection for virus particle assembly all suggest themselves as possibilities. However, any defensible speculation concerning these or other possible functional roles for the RNA species-specific terminal conserved sequences will require both their complete definition in terms of sequence and the much more difficult task of developing assays to investigate their functional role in virus replication. Experiments designed to approach both these goals are currently in progress.

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REFERENCES


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