Structural Analysis of Electrophoretic Variation in the Genome Profiles of Rotavirus Field Isolates

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Rotaviruses are the major etiological agents of acute viral gastroenteritis in the young of a wide variety of species, including humans and several species of domestic livestock (4). In human infants, particularly those in underdeveloped countries, rotavirus-induced diarrhea is a major medical problem, thought to be responsible for several million deaths per annum (1). In agriculture their economic importance is shown by the fact that one serological survey found 80% of calf diarrhea outbreaks to be rotavirus associated, and mortality rates in these outbreaks can exceed 30% (4).

Epidemiological surveying of this important virus group has been hampered by difficulties in preparing discriminating serological reagents, and this is due mainly to difficulties encountered in the routine adaptation of these viruses to growth in tissue culture (17). As an alternative to the more traditional seroepidemiological surveying, several groups have proposed using analysis of the genome profile of these viruses by gel electrophoresis as an epidemiological tool (4, 14). Indeed, a number of epidemiological surveys of genome profiles have been undertaken, and collectively these have established that whereas the overall pattern of viral genome segments is highly conserved, considerable variations in the migration of particular segments occur (9, 14). However, to date no detailed structural analysis of this electrophoretic variation has been undertaken to establish whether or not it is the result of nucleotide sequence changes likely to be of serological significance.

The high level of genomic variation observed in field isolates of rotavirus is analogous to the heterogeneity of genome profiles observed in influenza virus and reovirus isolates (7, 13). In influenza, a mechanism involving the exchange of genome segments (gene reassortment) between two viruses upon infection of a single animal has been proposed to explain this variation and to account for the rapid appearance of new pandemic strains of the virus (3). Recently, laboratory-based studies undertaken with temperature-sensitive mutants of a bovine rotavirus and a human virus isolate showed that, when cells were mixedly infected with these two viruses, genetic exchange of segments could occur at high levels to yield recombinant viruses containing genome segments from both parental viruses (5). Obviously, if this type of genetic interaction between rotaviruses from different species occurs to any degree in nature, it will have a profound effect on the strategies adopted for controlling these agents by the use of vaccines.

We have recently developed a relatively rapid and sensitive method for generating diagnostic fingerprints of individual segments of rotavirus double-stranded (ds) RNA (2a). This report describes the application of this method to a number of field isolates of bovine rotavirus, with a view to achieving some understanding of the structural basis for the electrophoretic varia-
tions in genome segments and obtaining evidence for the occurrence of genome segment reassortment between rotaviruses in the field.

MATERIALS AND METHODS

Viruses and cells. The cell culture-adapted strain of U.K. bovine rotavirus was grown in and purified from BSC-1 cells as described previously (11).

Field isolates of bovine rotavirus were extracted from single stools collected from calves on properties in four different areas within the United Kingdom during 1980.

Preparation and terminal labeling of viral dsRNA. Purified tissue culture-adapted bovine rotavirus particles were deproteinized with phenol and sodium dodecyl sulfate as previously described (2). dsRNA was extracted directly from the virus particles in fecal samples by the method of Clarke and McCrae (2).

dsRNA was labeled at its 3' terminus with \(^{32}\)PjPjCp, using T\(_4\) RNA ligase as described previously (2).

Preparation of genome segments. Individual dsRNA segments were isolated by the method of Clarke and McCrae (2a).

Briefly, labeled dsRNA was fractionated by electrophoresis on a 1.5% agarose gel containing 6 M urea, using a Tris-acetate continuous buffer system (6). The RNA segments were located by autoradiography and excised with a scalpel. RNA was recovered from the excised slices by using a "freeze-squeeze" procedure (2a).

The recovered RNA was concentrated by ethanol precipitation, resuspended in 2 to 50 \(\mu\)l of 20 mM Tris (pH 7.4), and stored at -20°C.

Partial nuclease digestion of isolated species with RNase T\(_1\) and partial alkaline hydrolysis. Isolated species of dsRNA were denatured at 50°C in 90% di-methyl sulfoxide as described previously (12). The denatured RNA was partially digested with \(10^{-4}\) units of RNase T\(_1\) per \(\mu\)g of RNA as described previously (2a).

Random cleavage of isolated species of RNA was achieved by partial alkaline hydrolysis carried out in 50 mM bicarbonate buffer (pH 9.0) at 90°C for 3 min.

Fractionation of the digestion products of isolated individual dsRNA species. The oligonucleotide fragments generated by digestion with RNase T\(_1\) were fractionated on thin 14% polyacrylamide gels (10, 15).

After electrophoresis, gels were autoradiographed at -70°C, using Dupont Lightning-Plus image-intensifying screens and Kodak X-Omat H film.

Production of dsDNA size markers. DNA size markers were produced by digestion of 2 \(\mu\)g of the plasmid PBR322 with the restriction endonuclease HpaII. The resulting DNA fragments were labeled with the Klenow fraction of DNA polymerase I and 10 \(\mu\)Ci of dCTP.

RESULTS

This study was conducted with the U.K. tissue culture-adapted bovine rotavirus and four field isolates of bovine rotavirus collected from geographically distinct areas within the United Kingdom during 1980. A one-dimensional genome profile analysis of 3'-end-labeled RNA extracted from these five isolates is shown in Fig. 1. Comparison of the profiles shows clear electrophoretic variation among these isolates for segments 1, 5, 6, 10, and 11.

Having demonstrated that electrophoretic variation occurred in these isolates, we used our recently developed partial nuclease digestion fingerprinting method to probe the structural basis of this variation. Figure 2 shows the fractionation on a 14% polyacrylamide gel of the terminally labeled partial digestion products of all five species 10 RNAs. Despite the considerable mobility variations seen among these RNA species in one-dimensional analysis (Fig. 1), their partial digestion fingerprints were very similar, with only a few minor changes in the position of G residues being observed. To confirm that these similarities of sequence were not an artifact generated by analyzing near terminal

FIG. 1. Polyacrylamide gel showing a comparative genome analysis of the five bovine isolates used in this study. Viral RNA was 3'-end-labeled as described in the text. Samples were run on a 20-cm 7.5% polyacrylamide gel (2). Although individual species of RNA were isolated from agarose gels, these do not show up the differences in migration apparent from separation on acrylamide gels. The four field isolates were from geographically distinct locations (given in parentheses). Track A, Tissue culture-adapted calf rotavirus (U.K.); track B, isolate 2855 (Carmarthen); track C, isolate 4329 (Shrewsbury); track D, isolate 6597 (V. I. Centre, Weybridge); track E, isolate 117 (Penrith). Migration was from top to bottom; the genome segments of the tissue culture-adapted calf rotavirus are numbered 1 to 11.
regions of the dsRNA, we prepared complete two-dimensional oligonucleotide fingerprints for two of the species 10 RNAs (isolates C and D) that had the clear mobility difference shown in Fig. 1. The results of this analysis (data not shown) confirmed the similarities observed with our terminal analysis procedure, with only one of the five diagnostic size spots undergoing a slight mobility shift. When partial nuclease digestion fingerprinting was performed on three of the other individual dsRNA species showing mobility variation on single-dimension analysis, namely, species 1, 5, and 6, the results were similar to those obtained for species 10, with a high level of overall band pattern conservation and only minor variations in G position being observed. The above results established that detectable variation in electrophoretic mobility can result from minor changes in primary sequence.

We next analyzed those genome segments from the isolates for which no detectable electrophoretic variations existed. Species 2 and 3 and 7, 8, and 9 were analyzed as a combined pair and a triplet, respectively, and species 4 was analyzed as an individual segment. All species 4 RNAs comigrate except that of U.K. tissue culture, which migrates very slightly slower than the others. Autoradiographs of partial nuclease digestion fragments for the 7, 8, and 9 triplet (Fig. 3) and for species 4 (Fig. 4) are shown. From Fig. 3 it is clear that the band patterns are highly conserved, with only one area (indicated by arrows) where clear differences exist. These differences probably represent a minimal estimate of the variation present since in this case a G band at a given position may result from one or more of the six RNA strands under analysis. The variation in band intensities at given G positions which were more evident in these samples may be indicative of minor variation in sequence among the three dsRNA species under test. Partial nuclease fingerprinting of the species 4 RNAs revealed larger sequence differences than those seen for the other species (Fig. 4). On the basis of the partial nuclease digestion patterns of species 4 RNA, the five isolates could be divided into two distinct groups (isolates A, C, and D and isolates B and E); within each group slight variations in banding patterns were evident, but these were less than the differences that existed between the two groups. However, despite these differences in banding patterns between the two groups of species 4 RNAs, there is nevertheless an underlying conservation in the banding pattern of all five of them. These results show that even where there is no detectable variation in mobility of corresponding genome segments, differences in their primary structure can still exist which are as

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FIG. 3. Partial nuclease digestion fingerprints obtained for species 7, 8, and 9 dsRNA "triplets" of the five isolates under study. Track M is the marker track showing radiolabeled DNA size markers. Track L is the partial hydrolysis reference ladder. The partial nuclease digestion products of the five triplet RNAs of isolates A to E are arranged as shown in Fig. 1. Track N contains a control of native ds denatured undigested RNA from the triplet of isolate A. The banding patterns for all five isolates are highly conserved except for a small area indicated by the arrows, where clear differences are visible. This gel (20 by 40 cm) was run at 1.6 kV for 6 h.

FIG. 4. Partial nuclease digestion fingerprints of the five species 4 dsRNAs from the bovine rotavirus isolates. Track M is the marker track containing DNA size markers. Tracks A to E show the partial nuclease digestion fingerprints of the five respective species 4 RNAs, and track N contains undigested denatured species 4 RNA from isolate A. The partial nuclease digestion pattern for species 4 RNAs could be divided into two groups on the basis of these patterns. Group I contains isolates A, C, and D; group II contains isolates B and E. This gel (20 by 40 cm) was run at 1.6 kV for 6 h.
great as, or even greater than, corresponding genome segments that do not comigrate.

Figure 5 shows the result of partial nuclease digestion of the species 11 RNAs from the five isolates. The pattern obtained could again be divided into two groups (isolates A, C, and E and isolates B and D) within which slight variation in banding patterns could be distinguished. However, on comparison of the banding patterns of the two groups it was clear that they were generated by RNA species having grossly different nucleotide sequences. The almost total difference in the sequence between the two groups was again confirmed for two of the isolates, using classical two-dimensional T₁ oligonucleotide fingerprinting, which showed a complete divergence in migration of the four unique T₁ oligonucleotides obtained (data not shown).

DISCUSSION

The difficulties encountered in the routine in vitro cultivation of rotaviruses (17) have severely curtailed seroepidemiological studies on this important virus group. Analysis of the electrophoretic banding of dsRNA genome segments among a number of rotavirus isolates (14) has revealed variations in the migration of each segment. The ease and sensitivity (2) with which the genome profile of field isolates of these viruses can be analyzed have therefore provided an attractive alternative to seroepidemiological surveying. However, for genome segment mobility studies to be used as a measure of the similarities or differences between two independent isolates, some understanding of the structural basis of this variability is clearly essential.

The results of analyzing species 1, 5, 6, and 10, all of which showed considerable electrophoretic variation in the five isolates studied, have revealed that despite these electrophoretic variations the sequence of each segment was highly conserved. A few minor changes in sequence of the type that would be characteristic of "antigenic drift" were seen. The overall conclusion reached from these results was that the observed variations in electrophoretic mobility of the segments did not result from gross variations in the sequence of the genome segments concerned. Similar observations have been made previously with reovirus, in which a temperature-sensitive mutation which was presumably the result of a single base change within the RNA segment concerned was able to induce an easily detectable change in the electrophoretic migration of the RNA species in question (8).

Partial nuclease digestion of corresponding genome segments which showed no migrational variation on one-dimensional analysis showed, particularly in the case of species 4, that despite

FIG. 5. Partial nuclease digestion obtained for the species 11 dsRNAs from the five isolates. Track M is the marker track showing the DNA size markers. Tracks A to E contain the partial nuclease digestion fingerprints of the five respective species 11 dsRNAs, and track N shows the ds denatured undigested species 11 RNA of isolate A. On the basis of these digestion patterns, the five species 11 RNAs can be divided into two distinct groups. Group I contains isolates B and D; group II contains isolates A, C, and E. This gel (20 by 40 cm) was run at 1.6 kV for 6 h.
their unchanged mobility, they contained variations in sequence at least as large as those in segments showing variations in electrophoretic mobility. This type of situation has been reported previously for the orbiviruses Wallal and Mudjinbarry, in which corresponding segments with identical electrophoretic mobility had grossly dissimilar oligonucleotide fingerprints (16). This leads to the conclusion that substantial nucleotide sequence heterogeneity is not restricted solely to those genome segments with different electrophoretic mobilities.

The overall conclusion that can be drawn from these analyses is that the use of simple single-dimension genome profile analysis as an epidemiological tool for surveying and grouping rotavirus isolates could be very misleading. Thus, whereas it is clear that changes in genome segment mobility are associated with detectable changes in the nucleotide sequence of the genome segment under study, it is also true that sequence changes at least as great as those causing a mobility shift can occur in a genome segment without having any effect on its overall electrophoretic mobility. Consequently, on the basis of genome profile analysis alone it is not possible to conclude that two rotaviruses with identical genome profiles are necessarily genetically identical, and further structural characterization of the genome segments must be done for such a conclusion to be reached.

Analysis of the five species 11 genomic segments revealed that they fall into two distinct classes. The electrophoretic variation of these segments on one-dimensional analysis showed no greater mobility variation than any of the other genome segments. However, the two classes of segment 11 had gross differences in their sequences of the type that would be predicted from an antigenic shift type of variation induced by gene reassortment between two viruses simultaneously infecting a single host.

During genome profile analysis of a large collection of bovine rotavirus isolates, we have, on several occasions, obtained genome profiles indicating that a single animal was simultaneously infected with two viruses having different electrophoretic profiles (Clarke and McCrae, unpublished data). This is a necessary prerequisite for gene reassortment to occur. Further studies will be required to ascertain whether the gene reassortment for which we have obtained evidence occurred between parental viruses from the same or different animal species. Gene reassortment has been shown to occur under strong selective pressure in laboratory-based experiments (5); if it also occurs among rotaviruses in the wild and if it can occur across species barriers, as has been shown for influenza, then this will have a profound bearing on the strategies to be used for producing effective vaccines against rotaviruses.

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