Cloning and sequence of UK bovine rotavirus gene segment 7: marked sequence homology with simian rotavirus gene segment 8

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ABSTRACT

The genome of the UK bovine rotavirus, which consists of eleven segments of dsRNA was polyadenylated and reverse-transcribed into cDNA. Complementary cDNA strands were annealed and the termini of the duplexes completed using DNA polymerase I. Full-length DNA copies of RNA segments 7,8 and 9 were cloned into the Pst I site of pBR322 and a clone containing the entire gene 7 was identified and sequenced. Gene 7 is 1059 nucleotides in length and contains a single long open reading frame capable of coding for a protein of 317 amino-acids. The known gene product of segment 7 is a protein with an estimated molecular weight of 33,000 daltons. When the UK bovine rotavirus gene 7 sequence was compared with the published data for the homologous gene (segment 8) of the simian rotavirus SA11, it was found to be identical to it in size and the arrangement of the proposed coding and non-coding regions, and very similar in nucleotide sequence (88% homology). Most of the base changes are silent and the predicted amino-acid sequences are almost identical (96% homology).

INTRODUCTION

Rotaviruses are important aetiologic agents of diarrhoeal disease in man ,other mammals and birds. They are members of the Reoviridae family of viruses and possess a genome of eleven segments of double-stranded (ds) RNA, enclosed in an isometric double-shelled capsid (for review see ref. 1). All members of the rotavirus group have common inner-shell antigens, whereas up to four neutralization serotypes of human rotavirus have been recognized (2,3). The serotype-specific antigens are present on the outer shell of the capsid (4,5). The level of antigenic variation within serotypes (ie. antigenic drift) is not known.

An RNA-dependent RNA polymerase is present in virus cores and when activated produces single-stranded copies of positive

polarity (ie. mRNA) from each of the eleven dsRNA segments (6,7). In vitro translation of individual dsRNA segments has defined the proteins encoded by the genomes of two different rotaviruses ; the simian rotavirus SA11 (8,9,Kantharidis,P.,Dyall-Smith,M.L. and Holmes,I.H., manuscript in preparation), and the UK bovine rotavirus (10). In both viruses each gene segment appears to specify only a single protein.

For epidemiological purposes the different band patterns produced by electrophoresis of genomic RNA from different rotavirus isolates (electropherotypes,11) allow easier discrimination of strains than conventional serology (12). Although the gene segments encoding the serologically important rotavirus proteins have now been identified (13),the relationship between electropherotype heterogeneity and genetic (and antigenic) variation among rotaviruses has yet to be determined. Unfortunately the RNA segments 7,8 and 9, one of which determines the virus neutralization serotype, are difficult to resolve by polyacrylamide gel electrophoresis and the order of migration of these genes varies from strain to strain (14).

Cross-hybridization studies (15,16) have shown that there is a considerable degree of homology between corresponding genes of different rotaviruses. In an effort to gain more precise information regarding the genetic diversity of this group of viruses we have begun a programme of cloning and sequencing rotavirus genes. In addition to indicating the nature of rotavirus variation, cloned gene segments may be useful for epidemiological or diagnostic purposes, or even for vaccine production In this report we present the sequence of gene segment 7 of UK bovine rotavirus. This gene codes for the protein p33,with an estimated molecular weight of 33,000 daltons (=VP8 of ref. 10). We compare this to the recently published sequence of the homologous gene (segment 8) of SA11 (17).

MATERIALS AND METHODS

Virus Growth and Purification

UK bovine rotavirus (18) was grown in MA104 cells and purified as described (8,9).

Synthesis of cDNA from Viral dsRNA

Viral dsRNA was extracted from preparations of purified virus using SDS-phenol-chloroform and precipitated with ethanol as described previously (9). This dsRNA was denatured (5 min. at 100°C) in TE buffer (10mM Tris-Cl,1mM EDTA,pH 8.0), snap chilled and the 3' termini polyadenylated using E.coli poly(A) polymerase (prepared according to Sippel,19). The extent of reaction was followed by monitoring the incorporation of (^{3}H) ATP (Amersham). Polyadenylated RNA (20µg) was denatured (100°C, 3 min.)in 65µl of TE buffer containing 5µg of oligo-dT_{12} (Collaborative Research) and chilled rapidly on ice. This was diluted to give 100µl of reaction buffer containing (final concentrations); 50mM Tris-Cl (pH 8.3),50mM KCl,10mM $MgCl_2$,10mM dithiothreitol,4mM sodium pyrophosphate,0.5mM of each base(dATP, dCTP,dGTP,dTTP),and 10µCi of (α-^{32}P)ATP (2000 Ci/mmol,Amersham). AMV reverse-transcriptase (50U;Life Sciences Inc., USA)was added and the mixture incubated for 2 hr at 42°C. RNA strands were then removed by alkaline hydrolysis,(0.25M NaOH,70°C for 20 min.) neutralized with HCl, extractd with phenol-chloroform and filtered through a Sephadex G-50 column to remove low molecular weight species.

Preparation of ds cDNA and Insertion into pBR322

Since cDNA was produced from both plus and minus RNA strands, ds cDNA was formed simply by heating the cDNA (2 min. at 100°C) in TE buffer and then allowing complementary DNA molecules to anneal as follows:NaCl was added to 0.2M immediately after heating and the solution was incubated at 65°C for 30 min, then at 50°C for 60 min. and finally at 40°C for 30 min., after which it was put on ice. To ensure that any duplexes so formed were flush-ended they were repaired using the Klenow fragment of E.coli DNA poymerase I (Boehringer), the reaction being followed by the incorporation of (α-^{32}P)ATP. The cDNA was then electrophoretically separated on a low-melting agarose gel and the wet gel autoradiographed at room temperature. The band corresponding in molecular weight to RNA segments 7,8 and 9 was excised and the cDNA extracted from the melted gel slice by phenol extraction and concentrated by ethanol precipitation. For insertion into pBR322 the 3' termini of the ds cDNA were extend-

ed with dC residues using terminal deoxynucleotidyl transferase (Ratliff Biochemicals,USA), annealed to Pst I digested and dG tailed pBR322 (New England Nuclear), according to the manufacturers instructions.

E.coli strain MC1016 (20) was transformed by the ds cDNA-pBR322 preparation and transformants containing hybrid plasmids were selected by screening for resistance to tetracycline and sensitivity to ampicillin.

Estimation of Insert Size

The approximate size of inserts in recombinant plasmids was determined by agarose gel electrophoresis of plasmid DNA isolated from transformants (21),in relation to pBR322 and pBR325 DNA. For more precise determinations, inserts were excised with Pst I and then separated on agarose gels along with DNA size markers (Eco RI and Hind III digested phage λ DNA,Boehringer).

Identification of Cloned Gene 7

Transformant bacterial colonies were screened for the presence of gene segment 7 by hybridization to ^{32}P-labelled segment 7 RNA.This segment was isolated by electrophoresis of UK bovine rotavirus dsRNA (50μg) on a 7% polyacrylamide gel (22), staining with ethidium bromide, electroelution of segment 7 from the gel and ethanol precipitation. The RNA was denatured (5 min. at 100°C in TE buffer) and fragmented (50°C,30 min. in 0.1M Na_2CO_3),then the 5' termini were labelled with ^{32}P using polynucleotide kinase (23) and (γ-^{32}P)ATP (BRESA,Australia).

Bacterial colonies were lysed on nitrocellulose filters (24) and then probed with ^{32}P-labelled segment 7 RNA. To confirm the identity of the cloned gene, plasmid DNA was ^{32}P-labelled by nick translation (25) and hybridized to Northern blots (26) of UK bovine rotavirus dsRNA which had been separated on long (38cm) polyacrylamide gels and transferred to APT paper (14,27).

DNA Sequencing

The recombinant plasmid containing UK bovine rotavirus segment 7 was prepared by cleared cell lysis and banding in a CsCl/EtBr gradient. Restriction digests of whole plasmid, or restriction fragments of the insert isolated from acrylamide gels (see Fig. 1) were cloned in various vectors of the M13 mp series (28). Sequences of thes M13-cloned fragments were deter-

mined by the chain termination method (29) using universal primers (30,31) or internal restriction fragments of the plasmid which were isolated from acrylamide gels and treated with exonuclease III (1 unit for 30 min. at 37°C/μg plasmid equivalent).

RESULTS AND DISCUSSION

Cloning of Gene Segment 7

The cloning strategy used in this study for rotavirus dsRNA segments followed that described previously for non-polyadenylated RNA species (32,33). The complementary RNA strands were first separated by thermal denaturation and then the free 3' termini were polyadenylated using poly(A) polymerase. Based on the incorporation of (^{3}H)ATP it was calculated that an average of 5 residues were added per 3' end. Oligo-dT_{12} was then used to prime the synthesis of cDNA using AMV reverse-transcriptase. When this cDNA was analysed by agarose gel electrophoresis under denaturing (DMSO-glyoxal) conditions (34), transcripts corresponding in size to all eleven rotavirus RNA segments were produced (data not shown). However the larger RNA segments were transcribed to a lesser extent than the smaller segments, and a considerable number of incomplete cDNA species were present, especially in the 400-900 base range. After annealing the complementary cDNA strands and repairing 3' termini with *E.coli* DNA polymerase I (Klenow fragment), size analysis on non-denaturing agarose gels showed that ds cDNA had been formed; the gel pattern being similar to that described for cDNA.

To avoid cloning incomplete gene segments the ds cDNA preparation was separated on a preparative agarose (low-melting; BioRad) gel and individual bands located by autoradiography, and excised. A prominent ds cDNA species which ran as a sharp band and corresponded in size to segments 7,8 and 9 (*ca.* 1100 bp) was selected for cloning into pBR322. It was extracted from the gel, its 3' termini extended with dC residues using terminal deoxynucleotidyl transferase, annealed with Pst I digested, dG-tailed pBR322, and used to transform *E.coli* strain MC1016.

Screening of Clones for Segment 7

Ninety-eight bacterial colonies harbouring recombinant plasmids were screened for the presence of gene 7 using ^{32}P-

labelled segment 7 RNA. Only one clone hybridized to this probe. The other colonies contained plasmids with inserts of genes 8 and 9. The identity of the gene 7 clone (UK32) was confirmed by Northern blot analysis (see ref. 14). The size of the insert in this clone was estimated to be approximately 1100 base pairs, which is sufficient to account for the entire gene 7 segment.

Gene 7 Sequence

The strategy for determining the sequence of gene 7 is shown in Figure 1. A difficulty was encountered in sequencing through the tailed regions where the fidelity of copying sequences after the G:C/A:T tracts was markedly reduced. Because of this the sequence of one extremity of the insert could only be sequenced in one direction; the result however was clear and unambiguous. The majority of the sequence (91%) was determined in both directions. The nucleotide sequence of the positive strand of UK bovine rotavirus segment 7 is presented in Figure 2. The gene is 1059 nucleotides long and appears to be full-length since it contains the conserved terminal sequences shown to be present on rotavirus gene segments (M.McCrae,personal communic-

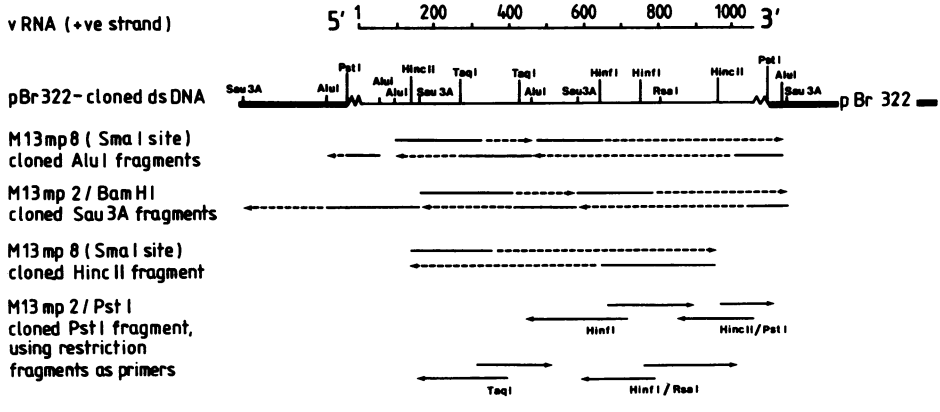

Figure 1. Summary of sequencing strategy.The restriction sites used to determine the nucleotide sequence of a cloned copy of gene segment 7 are indicated.The sense shown is that of the +ve strand (mRNA) of the dsRNA.From this clone Alu I fragments and a Hinc II fragment were subcloned into the Sma I site of M13mp8 (48),Sau 3A fragments into the Bam HI site of M13mp2/Bam HI (49), Pst I into the Pst I site of M13mp2/Pst I (50). Solid lines indicate the extent to which the sequence was determined in each subclone.

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                    SA11 SiRV Segment 8                                    C
                      UK BoRV Segment 7    5'-GGCTTTTAAAGCGTCTCAGTCGCCGTTTGAGCCTTGCGGTGTAGCC 46

                                                                  G           A          GT   A
ATG GCT GAG CTA GCT TGC TTT TGT TAT CCC CAT TTG GAG AAC GAT AGC TAT AAA TTT ATT CCG TTT AAC AAC TTG 121
Met Ala Glu Leu Ala Cys Phe Cys Tyr Pro His Leu Glu Asn Asp Ser Tyr Lys Phe Ile Pro Phe Asn Asn Leu 25
                                                              Arg                          Ser

        G   T          A          G     A          A          T  C      G
GCT ATA AAA TGC ATG TTG ACG GCA AAA GTA GAC AGA AAA GAT CAG GAT AAA TTC TAT AAC TCA ATA ATT TAT GGT 196
Ala Ile Lys Cys Met Leu Thr Ala Lys Val Asp Arg Lys Asp Gln Asp Lys Phe Tyr Asn Ser Ile Ile Tyr Gly 50
                                    Lys

                                  T  C  C  G  G  T                                          A
ATT GCA CCA CCG CCA CAA TTC AAA AAA CGC TAT AAT ACA AAT GAC AAT TCA AGA GGA ATG AAC TAT GAA ACT TCG 271
Ile Ala Pro Pro Pro Gln Phe Lys Lys Arg Tyr Asn Thr Asn Asp Asn Ser Arg Gly Met Asn Tyr Glu Thr Ser 75
                                        Ser

     T              CG T                C                    T                          A  G
ATG TTC AAT AAA GTG GCG GTA CTA ATT TGT GAA GCA TTG AAT TCA ATT AAA GTT ACA CAA TCT GAT GTT GCG AAT 346
Met Phe Asn Lys Val Ala Val Leu Ile Cys Glu Ala Leu Asn Ser Ile Lys Val Thr Gln Ser Asp Val Ala Asn 100
                       Ala                                                              Ser

  A  T     A  A              G    T A  G  C          A  A      G          G           A
GTG CTC TCA AGA GTA GTT TCT GTA AGA CAT CTG GAA AAT TTG GTG CTG AGG AGA GAA AAT CAT CAA GAC GTG CTT 421
Val Leu Ser Arg Val Val Ser Val Arg His Leu Glu Asn Leu Val Leu Arg Arg Glu Asn His Gln Asp Val Leu 125
            Lys Ile

  C  T  A      G T G T  C G          A C                T          C  G  A
TTT CAC TCG AAA GAA CTA CTA TTA AAA TCA GTG TTA ATA GCT ATT GGT CAC TCA AAA GAA ATT GAA ACG ACT GCC 496
Phe His Ser Lys Glu Leu Leu Leu Lys Ser Val Leu Ile Ala Ile Gly His Ser Lys Glu Ile Glu Thr Thr Ala 150

            A  G                      A  T      A                  A  C C G      T
ACT GCT GAA GGG GGA GAA ATT GTT TTT CAA AAT GCT GCG TTT ACG ATG TGG AAA TTG ACG TAT TTA GAA CAC AAA 571
Thr Ala Glu Gly Gly Glu Ile Val Phe Gln Asn Ala Ala Phe Thr Met Trp Lys Leu Thr Tyr Leu Glu His Lys 175

C                              T      A      A  A    C                                  G
TTA ATG CCA ATT TTG GAT CAA AAT TTC ATT GAG TAT AAG ATT ACA GTG AAT GAA GAT AAA CCA ATT TCA GAA TCA 646
Leu Met Pro Ile Leu Asp Gln Asn Phe Ile Glu Tyr Lys Ile Thr Val Asn Glu Asp Lys Pro Ile Ser Glu Ser 200
                                                  Leu

  C  C      G            A C   G      A  C  T  A      A      T
CAT GTG AAA GAA CTC ATT GCT GAG TTG CGA TGG CAG TAT AAC AAG TTT GCG GTA ATA ACA CAT GGT AAA GGT CAC 721
His Val Lys Glu Leu Ile Ala Glu Leu Arg Trp Gln Tyr Asn Lys Phe Ala Val Ile Thr His Gly Lys Gly His 225

            A  A                                                C                C
TAC AGA GTT GTC AAG TAT TCA TCA GTT GCG AAT CAT GCA GAT AGA GTT TAT GCT ACT TTC AAG AGT AAT AAT AAA 796
Tyr Arg Val Val Lys Tyr Ser Ser Val Ala Asn His Ala Asp Arg Val Tyr Ala Thr Phe Lys Ser Asn Asn Lys 250

          A G A A           C A  T  T      A      A      G  T                          C
AAT GGA AAT GTA CTG GAA TTT AAT TTG CTA GAC CAA AGG ATA ATT TGG CAA AAC TGG TAT GCG TTT ACG TCT TCA 871
Asn Gly Asn Val Leu Glu Phe Asn Leu Leu Asp Gln Arg Ile Ile Trp Gln Asn Trp Tyr Ala Phe Thr Ser Ser 275
          Met Ile

            C              A              G      G                  G C        C      G
ATG AAA CAA GGT AAT ACT CTT GAC ATA TGT AAG AAA CTA CTC TTC CAG AAG ATG AAA AGA GAA AGT AAT CCA TTT 946
Met Lys Gln Gly Asn Thr Leu Asp Ile Cys Lys Lys Leu Leu Phe Gln Lys Met Lys Arg Glu Ser Asn Pro Phe 300
                        Glu

  G  G                                                                  A    GAA       A
AAA GGA CTG TCA ACT GAT AGA AAG ATG GAT GAA GTT TCT CAA ATA GGA ATT TAATTCGTTATCGGTTTGAAGGTGGGTATGG 1028
Lys Gly Leu Ser Thr Asp Arg Lys Met Asp Glu Val Ser Gln Ile Gly Ile 317

  A  T
CAGAGCAAGAATAGAAAGCGCTTATGTGACC-3' 1059
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Figure 2. Sequence of UK bovine rotavirus gene segment 7. The sequence is that of the +ve strand (mRNA sense). The predicted amino-acid sequence is shown below the gene sequence. Nucleotide differences between this sequence and that of segment 8 of the simian rotavirus SA11 (17) are indicated above the gene sequence, while amino-acid differences are shown beneath the proposed amino-acid sequence.

ation;35),ie.

(+) 5' GGCUUU$^{A}_{U}$AAGC... . ...$_{A}^{U}$UGUGACC 3'

Conserved terminal sequences are similarly found on reovirus (36) and influenza virus (37,38) RNA segments, and are thought to be recognition sites for RNA processing enzymes (39).

The gene 7 sequence contains only one reading frame of significant length. The M13 single-strand containing this sense of the reading frame was shown to be of positive polarity as it did not hybridize to UK bovine rotavirus transcripts (ie. mRNA), whereas the complementary strand did (data not shown). The open reading frame begins at the first AUG from the 5' end (Fig. 2), and is 318 codons in length (including the termination codon). This is sufficient to code for the known gene product,p33, a protein with an estimated molecular weight of 33,000 daltons(see the next section). Since no direct evidence is available for the site of translation initiation we have assumed that initiation occurs at the first AUG codon, a strong initiation site according to the criteria of Kozak (40). The codon usage prior to the next in-phase AUG sequence is consistent with this assumption.

The nucleotide composition of the potential coding region (A 36.7%,T 28.9%,G 19.7%,C 14.7%) is generally reflected in the codon usage at position 3 (A 35%,T 34%,G 20%,C 10%) with C content at this position being slightly low. C content at position 2 is high, yet codons of the type XCC are rarely used (Table 1). G content is quite low at position 2.

The amino-acid composition of the predicted protein is given in Table 2. There is a preponderance of basic residues (42 Lys+Arg,10 His) over acidic residues (34 Glu+Asp), indicating a positively charged protein at neutral pH. In this respect it resembles the non-structural protein σNS' of reovirus, which is also positively charged(41). This reovirus protein has recently been shown to be involved in RNA synthesis (41). However there is as yet no published data on the functions of any of the non-structural (or inner-shell) proteins of rotavirus.

Comparison of the Sequences of UK Bovine Rotavirus Gene 7 and SA11 Rotavirus Gene 8.

Gene 7 of UK bovine rotavirus and gene 8 of the simian rotavirus SA11 have been shown to be homologous by low string-

TABLE 1. Codon usage of the predicted coding region of UK bovine rotavirus.

First Position	Second Position U	Second Position C	Second Position A	Second Position G	
U	10,UUU	4,UCU	12,UAU	3,UGU	
	6,UUC	0,UCC	1,UAC	2,UGC	69[a]
	4,UUA	12,UCA	1,UAA	0,UGA	
	9,UUG	2,UCG	0,UAG	4,UGG	
C	2,CUU	0,CCU	6,CAU	0,CGU	
	3,CUC	1,CCC	4,CAC	1,CGC	47
	5,CUA	6,CCA	9,CAA	1,CGA	
	4,CUG	2,CCG	3,CAG	0,CGG	
A	15,AUU	6,ACU	19,AAU	2,AGU	
	0,AUC	0,ACC	7,AAC	1,AGC	115
	7,AUA	4,ACA	22,AAA	9,AGA	
	9,AUG	5,ACG	7,AAG	2,AGG	
G	8,GUU	8,GCU	9,GAU	5,GGU	
	1,GUC	1,GCC	5,GAC	0,GGC	86
	6,GUA	4,GCA	16,GAA	5,GGA	
	7,GUG	6,GCG	4,GAG	1,GGG	
	96	61	124	36[b]	

(a),First position totals; (b), Second position totals. The third position totals are;U 109,C 33,A 110,G 65.

ency hybridization (14). This homology is now confirmed by comparison of our sequence data with that recently published for SAll segment 8 (17). As shown in Figure 2 the two genes are identical in size and in the disposition of the potential coding

TABLE 2. Amino-acid composition of the predicted coding region* of UK bovine rotavirus gene 7.

Amino-acid	Total Number	Amino-acid	Total Number
Ala	19	Leu	28
Arg	13	Lys	29
Asn	26	Met	9
Asp	14	Phe	16
Cys	5	Pro	8
Gln	12	Ser	21
Glu	20	Thr	15
Gly	11	Trp	4
His	10	Tyr	13
Ile	22	Val	22

*Calculated molecular weight= 36,700 daltons.

and non-coding regions. The base sequence homology is 88%, with most of the differences being silent mutations (106/122). Thus the predicted protein sequences are almost identical (96% homology) and the twelve amino-acid changes which do occur are all conservative.

The proteins produced by both of these genes have recently been identified by in vitro translation of isolated dsRNA segments (10, Kantharidis,P.,Dyall-Smith,M.L. and Holmes,I.H., manuscript in preparation). As determined by SDS-polyacrylamide gel electrophoresis the two proteins have identical molecular weights of 33,000 daltons (data not shown), which is consistent with the sequence information. While McCrae and McCorquodale (10) considered the gene 7 product of the UK bovine rotavirus to be a structural protein (VP8 in their study), it had previously been identified as a non-structural protein (NS1) by Thouless (42), and all studies of p33 of SA11 rotavirus have agreed that it is non-structural (9,43,44).

SA11 and UK bovine rotaviruses were isolated 16 years apart at different geographical locations (South Africa and UK, respectively) and from different animal species. They also appear to be serologically distinct (45), yet the gene and predicted amino-acid sequences of these two RNA segments are highly conserved. This lack of variation is consistent with a gene coding for a non-structural protein, which would not be expected to be directly influenced by the host immune system (46). For example, the gene segments encoding the non-structural proteins of two influenza A viruses (A/PR8/34, and A/Udorn/72) show 91% nucleotide sequence homology and have identical lengths although the strains were isolated 38 years apart (47).

It will be even more interesting to compare the extent of sequence variation of rotavirus segments which code for major antigens.

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