Cloning of complete genome sets of six dsRNA viruses using an improved cloning method for large dsRNA genes

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Introduction

There are six virus families with dsRNA genomes, namely Birnaviridae, Cystoviridae, Hypoviridae, Partitiviridae, Reoviridae and Totiviridae. Several viruses of the family Reoviridae are aetiological agents for disease in humans and animals. Advances in recombinant DNA technology continue to raise expectations of generating a range of new vaccine candidates to combat infectious diseases. For diseases caused by viruses of the family Reoviridae, the key to tapping into this powerful technology is the ability to clone full-length dsRNA genes. In the case of the Rotavirusae and Orbivirinae, the main focus of our research, this requires cloning of dsRNA genes that are classified as large, namely genes of 3–4 kb (Sabara et al., 1991; Crawford et al., 1994; Brussow et al., 1990; McNeal et al., 1992; Madore et al., 1999; Roy et al., 1996, 1990; Martinez-Torrecuadrada et al., 1996; Stone-Marschat et al., 1996; du Plessis et al., 1998; Scanlen et al., 2002). Although dsRNA cloning has progressed steadily, routine cloning of large dsRNA genes remains problematic, especially where dsRNA template is limited, e.g. in cases where viruses have not been cultured, or where no sequence information is available.

Difficulties and limitations of existing dsRNA cloning methods are as follows: the first dsRNA cloning methods based on polyadenylation of genomic dsRNA, oligo(dT)-primed reverse transcription, followed by blunt-ended cloning or dC-tailing and cloning into dG-tailed pBR322 (Cashdollar et al., 1982, 1984) were generally technically complicated, the various steps were very inefficient and required relatively large amounts (> 1 µg) of dsRNA starting material. The homopolymeric tails of cloned genes presented difficulties for subsequent sequencing and expression. The second generation cloning procedures used PCR amplification of cDNA, which made it possible to clone from much smaller amounts of starting material. Initially, PCR-based procedures depended on the

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prior availability of flanking sequence information of the gene of interest. The conservation of termini within the different Reoviridae species allowed amplification of specific full-length cDNA of dsRNA genes using segment termini-specific primers (Kowalik et al., 1990; Cooke et al., 1991). A milestone in dsRNA cloning was the development of a single-primer amplification sequence-independent method for rotaviruses that cannot be cultured (Lambden et al., 1992). However, only dsRNA genes smaller than 1–5 kb could initially be cloned intact (Lambden et al., 1992; Bigot et al., 1995; James et al., 1999). We reported progress in cloning large dsRNA genes when we cloned full-length 3–4 kb African horsesickness virus (AHSV) genome segments by extending the primer ligating to dsRNA with a poly(A) tail to facilitate oligo(dT) priming of poly(dA)-oligonucleotide-ligated dsRNA and by enriching oligo-ligated dsRNA for larger genome segments (Vreede et al., 1998). However, this method, as well as the additional modifications to the single-primer amplification sequence-independent method and other procedures reported recently (Attoui et al., 2000a; Zhang & Rowhani, 2000; Chen et al., 2002), are still not suitable for routine cloning of large, full-length dsRNA genes and complete genome sets, since they require sequence data for segment-specific primers, need large amounts of dsRNA (at least 20 ng) per segment or hybridization screening of a library with segment-specific probes. Currently none of these methods can achieve the ultimate goal of amplifying a complete set of dsRNA genome segments of a dsRNA virus using one-tube reactions.

The objective of this investigation was to further develop and optimize the single-primer amplification sequence-independent dsRNA technique described by Lambden et al. (1992) for routine cloning of large dsRNA genes. We report here the use of our optimized technique in cloning full-length genes of complete dsRNA genome sets of six different viruses, each amplified in a one-tube PCR. We further demonstrate the use of the cloned genes in obtaining sequence information and recombinant protein expression. The ability to clone large dsRNA genes with ease is expected to speed up recombinant vaccine development initiatives and help to unravel the importance of terminal sequences for replication and gene expression of dsRNA viruses.

Methods

- **Viruses.** AHSV serotypes 1 and 2, bluetongue virus (BTV) serotype 2, equine encephalitis virus (EEV) serotypes Bryanston and Kylami (reference strains) and reovirus Dearing strain were obtained from the OIE Reference Laboratory at the Onderstepoort Veterinary Institute (Onderstepoort, South Africa). Bacteriophage Φ12 and its host (LM 2333) were obtained from Dr Paul Gottlieb (Sophie Davis School of Biomedical Education, New York, USA) and propagated as described by Mindich et al. (1999).

- **dsRNA preparation.** dsRNA of the AHSV, BTV, EEV and reovirus was extracted from one 75 cm² flask of infected BHK cells. The dsRNA of bacteriophage Φ12 was extracted from phage purified from a 50 ml sloppy agar culture. The phage was purified from the agar suspension first by removal of the agarose and bacterial host by centrifugation at 8000 g. Phage was then purified from the supernatant by centrifugation at 25 000 r.p.m. (Beckmann JS-21) over a 40 % sucrose cushion. dsRNA from all these viruses was extracted using the commercial TRI-Reagent (Molecular Research Centre), according to the manufacturer’s instructions, followed by precipitation with 2 M LiCl to remove ssRNA. The dsRNA was purified from the supernatant using a column from the Qiagen Gel Extraction kit. The human rotavirus dsRNA from the group A and C viruses was extracted directly from 600 µl of 10 % stool samples using TRI-Reagent.

- **Oligo-igation and cDNA preparation.** Primer PC3 (5’ PO3-GGATCCCGGGAATTCGG(A)3’-NH2 3’) was ligated to a total of 1–700 ng dsRNA as described by Vreede et al. (1998), with the exception that the reaction was incubated at 17 °C for 16 h. For amplification of specific individual genome segments, ligated dsRNA segments were separated by electrophoresis on either 1% TBE or TAE agarose gels. Individual segments were excised and purified using the Gel Extraction kit. cDNA of the individual segments was prepared as described by Vreede et al. (1998), without the incorporation of radioactive nucleotides. Removal of excess RNA, cDNA annealing and filling in of partial duplexes was carried out as described by Lambden et al. (1992), except that cDNA hybridization was done for only 1 h at 65 °C. Amplification of dsRNA was performed as described by Vreede et al. (1998) using primer PC2 (5’ PO3-GGATCCCGGGAATTCGGATCC-OH 3’), with the exception that Takara Ex Taq (Takara Shimadzu) was used as the DNA polymerase and only 22–30 cycles of PCR were performed, depending on the amount of starting material. The protocol for amplification of the whole genome in one PCR was similar except that the oligo-igation reactions of the complete genomes were cleaned up using the Gel Extraction kit: cDNA was prepared from all the genome segments in one cDNA reaction and the whole genomes were amplified in one tube, as described for single segments. For the amplification of the bacteriophage Φ12 genome, the incubation time at 72 °C was extended to 7 min.

- **Cloning and sequencing of amplified dsRNA products.** Amplified cDNA products were separated on a 1% TAE agarose gel and individual segments were purified using the Gel Extraction kit. Restriction enzyme analyses of PCR products were performed to distinguish between segments that co-migrated on agarose gels. Purified PCR products were cloned into T/A cloning vectors pGEM-4 (Promega) or pT-NOT (Franz & Dear, 1998). Positive clones were identified on the basis of the size of the inserts. The clones with inserts from co-migrating cDNA segments were separated on the basis of their restriction enzyme profiles. The terminal ends of cDNA inserts were all sequenced with M13 forward and reverse primers using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit, version 2.0, on an ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems). Terminal sequences of the recombinants representing the different cloned genome sets were aligned using DNASIS (Lynnon Biosoft). Terminal sequences of AHSV-1, AHSV-2, BTV and rotavirus genes were compared with conserved terminal sequences from the same virus species from computerized databases using the BLAST software program (NCBI) and those from bacteriophage Φ12 with sequences from partial and full-length clones obtained by Gottlieb et al. (2002a).

- **Baculovirus expression.** The genes encoding the EEV-Kylami proteins were cloned into either a quadruple baculovirus expression vector pBACgus4x-s (Novagen) or a dual expression vector pFastBacDual (Life Technologies). The rotavirus VP6 gene was cloned into pFastBacHTb (Life Technologies). Recombinant viruses were prepared following the manufacturer’s specifications. Proteins of cells...
infected with recombinant viruses were labelled with $^{35}$S-methionine for 1 h at 72 h after infection. After labelling, the cells were harvested, washed twice in PBS and their total protein content was separated by SDS–PAGE and analysed by autoradiography. The dried radioactive gels were exposed overnight to Hyperfilm MP X-Ray film (Amersham Life Sciences). Core-like particles (CLPs) from cells infected with the dual baculovirus recombinant expressing EEV-Kyalami VP3 and VP7 were purified as described by Maree et al. (1998). CLPs were stained with uranyl acetate prior to electron microscopy and photography at a magnification of 45,000.

Results

dsRNA cloning

Previously, we reported improved cloning of large (4–2.8 kb) AHSV dsRNA segments 1, 2 and 3 using sucrose-gradient centrifugation to separate them from the smaller genes prior to oligo-ligation (Vreede et al., 1998). However, sucrose gradients could not be used for the enrichment of large genes from small amounts of starting material (100–700 ng of total RNA per genome) as used in this study. Therefore, agarose gel electrophoresis was investigated for separating genome segments. When cDNA was prepared from a pool of EEV-Bryanston dsRNA of segments 1, 2 and 3 and amplified by PCR (Fig. 1), the yield of amplicons from segments 1 and 2 was low compared to that of segment 3 (Fig. 1, lane 5). Both the yield of full-length cDNA and PCR amplicons of the largest genome segments increased significantly when individual agarose gel-purified dsRNA genome segments were used. These PCR products could readily be cloned into T/A cloning vectors such as pGEM-t to yield full-length complete cloned virus genomes sets, such as that of AHSV-1 (Fig. 2).

Surprisingly, it was found subsequently that the yield of PCR amplicons from agarose gel-purified dsRNA genome segments, either before or after oligo-ligation, was significantly lower than when oligo-ligation and cDNA synthesis was performed in one-tube reactions without fractionation of dsRNA or cDNA. Whether it was as a result of contaminating agarose inhibiting the enzyme reactions is not known. The yield of full-length PCR amplicons of the whole genome in a single tube improved the PCR amplicon yield of all segments to such an extent that only 22 cycles of PCR were needed for the amplification of the full-length genes of complete genomes of AHSV, BTV, EEV, reovirus and rotavirus (Fig. 3A). When the number of PCR cycles was increased to more than 28, however, there was a bias favouring the yield of amplicons from medium and smaller segments compared to the yield of the large segments, as is evident for our human group C rotavirus cDNA (Fig. 3B, lane 2).

Two further aspects were investigated to obtain an indication of the efficiency of the method. The first was to determine how efficient it would be for cloning from very small amounts of starting dsRNA and the second was to get an indication of the upper size limit of dsRNA that can be cloned.

Amplification of an uncultured group C rotavirus from less than 1 ng of starting material in 30 amplification cycles yielded enough full-length amplicons of all the genes for cloning (Fig. 3B). All three dsRNA genome segments of bacteriophage Φ12, family Cystoviridae, are large: 2·3, 4·1 and 6·8 kb, respectively (Mindich et al., 1999; Gottlieb et al., 2002b). Amplification of the complete genome of bacteriophage Φ12 could be performed in a one-tube reaction in 25 cycles (Fig. 4A). This was used to clone the entire genome (Fig. 4B).

The molecular mass of all the various PCR products correlated either with that of the genes of published sequences (AHSV, BTV, reovirus and rotavirus) or with the molecular mass of their corresponding dsRNAs (EEV and Φ12). The amplified PCR products could be cloned into T/A cloning vectors such as pGEM-t or pT-NOT (Figs 2C and 4B). Most cloned inserts could be gene-assigned based on their size. However, co-migrating segments, namely segments 7, 8 and 9 from AHSV, segments 7 and 8 from EEV and segments 3, 4 and 7, 8 and 9 of rotavirus were easily differentiated based on differences in their restriction enzyme profiles.

Sequencing of cloned genes

Sequencing of the terminal ends of the cloned genes was performed to establish whether these genes were full-length cDNA copies of the template dsRNA. The genes of AHSV-2 and rotavirus that we cloned here have not been previously cloned or sequenced. We determined that they have the same conserved terminal sequences as other viruses of their respective species (Table 1). The sequences of the terminal ends of our bacteriophage Φ12 genes (data not shown) were
**Fig. 2.** Cloning of a complete AHSV-1 genome set from individually purified genome segments. (A) Agarose gel analysis of purified oligo-ligated dsRNA of AHSV-1. Lanes: 1, PstI-digested λ DNA size marker; 2, segment 1 dsRNA; 3, segment 2 dsRNA; 4, segment 3 dsRNA; 5, segment 4 dsRNA; 6, segment 5 dsRNA; 7, segment 6 dsRNA; 8, segments 7, 8 and 9 dsRNA (segments 7, 8 and 9 of AHSV-1 co-migrate on 1% agarose gels); 9, segment 10 dsRNA. (B) Agarose gel analysis of PCR products from cDNA prepared from individually purified segments of oligo-ligated AHSV-1 RNA. Lanes: 1 and 10, PstI-digested λ DNA size marker; 2, segment 1 PCR; 3, segment 2 PCR; 4, segment 3 PCR; 5, segment 4 PCR; 6, segment 5 PCR; 7, segment 6 PCR; 8, AHSV-1 segments 7, 8 and 9 PCR (segments 7, 8 and 9 of AHSV-1 co-migrate on 1% agarose gels); 9, segment 10 PCR. (C) Agarose gel analysis of the complete genome of AHSV-1 cloned into pGEM-T and excised with Smal. Lanes: 1, PstI-digested λ DNA size marker; 2, segment 1; 3, segment 2; 4, segment 3 (pGEM-T and segment 3 have the same size and co-migrate); 5, segment 4; 6, segment 5; 7, segment 6; 8, segment 7; 9, segment 8; 10, segment 9; 11, segment 10.

**Fig. 3.** One-tube oligo-ligation and PCRs of five different dsRNA viruses. (A) Agarose gel analysis of purified oligo-ligated dsRNA of the five different viruses and the PCR amplicons from their corresponding cDNAs prepared in one-tube reactions. Lanes: 1 and 12, PstI-digested λ DNA size marker; 2, total dsRNA of AHSV-2; 3, PCR amplicons of the complete AHSV-2 genome after 22 cycles of amplification; 4, total dsRNA of BTV-2; 5, PCR amplicons of the complete BTV-2 genome after 22 cycles of amplification; 6, total dsRNA of EEV-Bryanston; 7, PCR amplicons of the complete EEV-Bryanston genome after 21 cycles of amplification; 8, total dsRNA of a human group A rotavirus; 9, PCR amplicons of the complete rotavirus group A genome after 25 cycles of amplification; 10, total dsRNA of reovirus Dearing; 11, PCR amplicons of the complete reovirus genome after 22 cycles of amplification. The amount of PCR amplicons of each virus represents the yield of PCR products from cDNA prepared from one-fifth of its respective dsRNA. (B) Agarose gel analysis of PCR products of cDNA prepared from 1 ng of total oligo-ligated dsRNA of a human group C rotavirus strain prepared in a one-tube reaction. The amplification was done in 30 PCR cycles. The dsRNA was not enough to be visible on an ethidium bromide-stained agarose gel. Lanes 1, PstI-digested λ DNA size marker; 2, rotavirus C amplification products.
Complete genome set cloning for dsRNA viruses

Fig. 4. Cloning of the complete genome of bacteriophage Φ12. (A) Agarose gel analysis of purified bacteriophage Φ12 dsRNA and amplicons of cDNA prepared in a one-tube reaction. Lanes: 1, bacteriophage Φ12 dsRNA; 2, full-length amplification products of cDNA prepared from oligoligated bacteriophage Φ12 dsRNA in 22 cycles of PCR amplification using the one-tube procedure; 3, PstI-digested λ DNA size marker. (B) Agarose gel analysis of the complete genome of bacteriophage Φ12 cloned into pGEM-t and excised with SmaI. Lanes: 1, segment L; 2 and 6, PstI-digested λ DNA size marker; 3 and 4, segment M; 5, a negative miniprep result; 7, segment S.

Table 2. Inverted terminal repeats of the cloned EEV-Bryanston gene encoding VP6 and EEV-Kyalami gene encoding VP7

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size (bp)</th>
<th>Inverted repeat</th>
<th>Position (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP6</td>
<td>1080</td>
<td>AAATAACGTTC</td>
<td>4 and 1065</td>
</tr>
<tr>
<td>VP7</td>
<td>1175</td>
<td>TTTGCC</td>
<td>7 and 1161</td>
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Baculovirus expression

To establish whether the open reading frames of the cloned genes could be translated to full-length functional proteins, the four newly cloned EEV-Kyalami genes (cloning data not shown) equivalent to the major structural proteins of BTV, namely the EEV VP2, VP3, VP5 and VP7 genes, were expressed using a baculovirus expression system. Co-expression of various combinations of recombinant EEV proteins as well as the single expression of rotavirus VP6 yielded proteins with the expected molecular mass (Fig. 5). Co-expression of the EEV-Kyalami VP3 and VP7 proteins resulted in the intracellular accumulation of particles (Fig. 6), which we concluded are EEV CLPs, since their size and morphology resembles that of native EEV cores and they have capsomeres typical of orbiviruses.

Table 1. Consensus terminal sequences of segments of the cloned genomes of AHSV-1, AHSV-2, EEV-Bryanston, EEV-Kyalami and a human group A rotavirus and known consensus sequences of viruses from the same species (where available)

Published sequences refer to sequences from the ICTV Virus Taxonomy Report (1997).

<table>
<thead>
<tr>
<th>Virus</th>
<th>RNA terminal sequences (positive strand)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHSV (published)</td>
<td>5′ GUUWA......ACWUAC 3′</td>
</tr>
<tr>
<td>AHSV-1 and -2</td>
<td>5′ GUUUWW......YTHUAC 3′</td>
</tr>
<tr>
<td>EEV-Bryanston and -Kyalami</td>
<td>5′ GUUWAD......HSIUAC 3′</td>
</tr>
<tr>
<td>BTV (published)</td>
<td>5′ GUUAAA......RCUUAC 3′</td>
</tr>
<tr>
<td>Rotavirus (published)</td>
<td>5′ GGWWU......KKRCC 3′</td>
</tr>
<tr>
<td>Rotavirus group A</td>
<td>5′ GCCUW......RUGACC 3′</td>
</tr>
</tbody>
</table>

analysis, which made it possible to assign all the genes, including the novel sequences of EEV, to their corresponding proteins. The coding assignment of the EEV genes correspond to that of AHSV and BTV (data not shown) except for the fact that the NS2 gene (segment 7) is slightly larger than the VP7 gene (segment 8). This could be seen after gel electrophoresis of the cDNAs of these two segments on a large agarose gel for an extended time period and full-length sequencing of the VP7 gene (1177 bp) (data not shown).
**Fig. 5.** Baculovirus expression of cloned EEV-Kyalami genes encoding the four major structural proteins and a human rotavirus group C gene encoding VP6. (A) Autoradiograph of $^{35}$S-methionine-labelled proteins from Sf9 cells infected with recombinant EEV baculoviruses. The proteins expressed are indicated above each lane. All proteins were expressed from the quadruple baculovirus expression vector pBACgus4x-1 carrying genes as indicated. (B) Autoradiograph of $^{35}$S-methionine-labelled proteins from Sf9 cells infected with the dual recombinant of EEV VP3 and VP7 that was used for the production of CLPs (lanes marked 1 and 2) and with the rotavirus VP6 baculovirus recombinant (lane marked VP6). Lanes: M, 14C-labelled molecular mass marker (AEC Amersham CFA626); C, negative control (mock infection). Arrows point at the relevant proteins.

**Fig. 6.** Electron micrographic images of two fields of negatively stained EEV-Kyalami CLPs. The insert is a computer enlargement of the particle directly above it in the picture. The typical orbivirus capsomere structure is indicated with an arrow.

**Discussion**

This paper describes the cloning of complete genome sets of a range of dsRNA viruses of the family *Reoviridae*, representing three different species, namely *Reovirinae*, *Orbivirusinae*, *Rotavirusinae* and one member of the family *Cystoviridae*, bacteriophage φ12, from as little as 1 ng and for genes up to 6.8 kb. Our successful cloning of large (> 3 kb) dsRNA genes finally perfects the original single-primer amplification sequence-independent cloning method and reaches the two ultimate goals for dsRNA cloning formulated by Lambden *et al.* (1992). The first goal, that of being able to clone non-cultivatable human rotavirus genomes directly from small clinical samples, was met by our cloning of two complete rotavirus genomes directly from stool samples, one from an uncultured human group A rotavirus and another from 1 ng starting material of a group C rotavirus. The second goal, namely that the method should be applicable without prior knowledge of any sequence information and generate full-length cDNA clones of each genome segment, allowing unequivocal delineation of the 5′- and 3′-terminal gene sequences, was achieved by our cloning of two complete EEV genome sets. Agarose gel electrophoretic sizing and nucleic acid sequencing confirmed that the cloned genes were full-length. The finding that proteins of the correct molecular mass were produced by baculovirus expression of five of the cloned genes, those of EEV Kyalami encoding the four major structural proteins, VP2, VP3, VP5 and VP7 and the human rotavirus group C gene encoding VP6 indicates that these genes have functional open reading frames. This report is the first on cloning of complete genome sets using one-tube amplification reactions. It is also the first report of cloning of full-length EEV genome sets and identification of their terminal sequences. The result that complete genome sets can now routinely be cloned from small amounts of starting material together with the recent progress in sequencing entire dsRNA genomes (Attoui *et al.*, 2000a, b) means that dsRNA cloning and molecular characterization and analysis is now coming of age. It should be possible to begin delivering on many of the expectations that were envisaged for dsRNA viruses since the advent of molecular cloning.
There is no one single aspect that is the key to our success with cloning of large dsRNA genes. The progress is the result of a series of optimization and mixing and matching of some of the original procedures and incorporation of some additional and/or newer technologies and reagents. The important parameters for cloning large dsRNA genes include further optimization of the ligation reaction and the use of methyl-mercuric hydroxide for denaturing dsRNA instead of the more commonly used combination of heat and DMSO. The finding that it is important to avoid a PCR amplification bias for smaller products and that agarose gel electrophoresis was more efficient for fractionating oligo-ligated dsRNA genome segments than sucrose-gradient centrifugation, described by Vreede et al. (1998), confirms recent results of Attoui et al. (2000a). Other modifications included using a long distance, high fidelity polymerase and commercial ion-exchange columns. The most significant contribution our method for cloning of large dsRNA segments is that it is the first and only one with which complete genome sets can be cloned using a series of one-tube reactions. Eliminating the need for purification of individual dsRNA genome segments and cDNAs simplifies, speeds up and improves the efficiency of the cloning procedure significantly by avoiding the inevitable losses associated with purification and recovery of the various stages of cloning genes.

The genomes of all the viruses in this investigation could be amplified in one-tube PCRs (Figs 3 and 4A) as opposed to the need of other methods for individual purification of large (> 3 kb) dsRNA genes (Attoui et al., 2000a). The genome of EEV-Bryanston was cloned from amplicons generated by 21 PCR cycles (Fig. 3A, lane 7) and that of bacteriophage Φ12, of which all three genome segments are > 3 kb (Mindich et al., 1999), was cloned from 25 PCR cycles (Fig. 4A). The best other methods for cloning of large genome segments need at least 20 ng dsRNA per segment and 35 PCR cycles for amplification of full-length large segments; these segments have to be purified individually and need to be 5′-methylated (Attoui et al., 2000a). Lowering the number of PCR cycles significantly reduces the risk for PCR-induced mutations, especially for the larger genes and for cloning genes destined for expression, such as in recombinant vaccine initiatives and investigations on structure and function. We have not fully investigated the upper size limit of dsRNA genome segments that can be cloned. In a pilot experiment, we did not manage to clone a 13 kb chimaeric bacteriophage Φ6 genome (data not shown). We speculate that the range of the reverse transcriptase (5 kb is indicated by the manufacturer Promega) used could be the limiting factor.

Our main motivation for developing this cloning method was to facilitate recombinant vaccine development for rotaviruses and the orbiviruses, AHSV and BTV. The major protective antigens of these viruses and the genes that encode them have been identified, and proof of concept and the feasibility of developing recombinant subunit vaccines that induce protective, (sero)type-specific humoral immunity has been established (Sabara et al., 1991; Crawford et al., 1994, 1999; Brussow et al., 1990; McNeal et al., 1992; Madore et al., 1999; Roy et al., 1990, 1996; Martinez-Torrecuadrada et al., 1996; Stone-Marschat et al., 1996; du Plessis et al., 1998; Scanlen et al., 2002). The genes are all outer capsid proteins that determine (sero)type and induce neutralizing antibodies, namely rotavirus glycoprotein VP7 and spike protein VP4 and outer capsid protein VP2 of AHSV and BTV. Unfortunately, apart from rotavirus VP7 genes, all the genes fall in the 2–4 kb size range, which has thus far been difficult to clone. For rotavirus vaccine development, the progress reported here is particularly timely in view of the setback that the long awaited, new tetravalent rotavirus vaccine (Rotashield) introduced in 1998, a rhesus rotavirus background with three human VP7 genes incorporated by reassortment, had to be withdrawn after infants developed intussusception (McCarthy, 1999). This setback resulted in the need for new rotavirus vaccine initiatives to explore various alternative options for developing vaccines.

Our cloned EEV genome represents the first complete set of intact, full-length genes and the terminal sequences the first nucleic acid sequence data generated for this virus species. The VP2 genes of AHSV-1 and -2 were not cloned previously and are important for recombinant vaccine development. The AHSV-1 and -2 and EEV-Bryanston and -Kyalami VP2 genes generated here will be slotted into recombinant subunit vaccine initiatives and new diagnostic methods for typing for the respective virus species, as described recently for AHSV and BTV (du Plessis et al., 1998; Scanlen et al., 2002; Koekemoer et al., 2000; Sailleau et al., 2000; Bremer et al., 1994; Cloete et al., 1994). Our progress with routine cloning of large dsRNA genome segments will finally make it possible to develop complete recombinant VP2-based vaccine repertoires against all nine AHSV (van Dijk, 1999) and 24 BTV (van Dijk, 1993) serotypes, as well as for other orbiviruses, such as EEV, Palyam virus and Kemerovo virus. The prospect that it might be possible to generate recombinant vaccines directly from fully virulent isolates from field cases or directly from clinical samples that have not been cultured is particularly exciting and can now be investigated. If successful, it will create the opportunity to readily incorporate local strains and isolates in recombinant vaccines. This will constitute a workable alternative, especially for those viruses that have not been attenuated.

Partial nucleic acid sequence data obtained for each of the EEV genes revealed that, in line with what has been observed with other members of the family Reoviridae (Mertens et al., 2000), all 10 segments of the EEV-Bryanston and -Kyalami genomes have conserved terminal sequences. We established that the EEV conserved terminal bases (5′ GUU and UAC 3′) are identical to those of AHSV and BTV. The coding assignments of the EEV genes could be deduced from the partial 5′- and 3′-terminal coding nucleic acid sequences obtained in the process of characterizing the terminal ends and
compared to orbivirus sequences in the databases (data not shown). EEV has the typical protein complement and coding assignment of AHSV and BTV except for segments 7 and 8, which are reversed and encode NS2 and VP7, respectively. The coding assignment confirmed deductions made by earlier hybridization experiments that showed that partial clones of EEV genome segments 3, 8 and 10 were highly conserved and a partial genome segment 2-specific probe did not hybridize with dsRNA from any of the other EEV serotypes, suggesting that this segment encodes the serotype-specific antigen of EEV (Viljoen & Huismans, 1989). Segment-specific inverted terminal repeats were identified for EEV genome segments encoding VP7 and VP6 (Table 2). These conserved motifs have been postulated to act as sorting signals during replication for packaging plus-sense ssRNA of each genome segment into the progeny viral capsids (Anzola et al., 1987; Xu et al., 1989) and to interact by homologous base pairing, holding RNA transcripts in a panhandle structure that might guide the RNA-dependent RNA polymerase (Attoui et al., 1997, 1998). Baculovirus expression of a few selected genes cloned in this study demonstrated that these cloned genes have functional open reading frames. The baculovirus-expressed rotavirus VP6 had the expected molecular mass and EEV-KyalaMi VP3 and VP7 assembled into CLPs. The assembled EEV CLPs are envisaged to serve as a scaffold for attaching outer capsid proteins in vaccine development approaches similar to what has been done for BTV (Roy et al., 1994) and rotavirus (Madore et al., 1999) and for morphogenesis studies.

The progress reported in this paper for cloning large dsRNA genes, together with recent advances in full genome set sequencing for dsRNA genomes (Attoui et al., 2000a), is expected to introduce a powerful new era in Reoviridae research and advance application of molecular knowledge in vaccine development, diagnostics, treatment and epidemiology. Extensive cloning and sequencing investigations can now all be initiated to unravel the importance of the terminal ends of dsRNA genome segments for replication and expression of genes of viruses of the family Reoviridae and form a basis for the development of reverse genetic systems for many of these viruses.

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