Studies of genetically defined chimeras of a European type A virus and a South African Territories type 2 virus reveal growth determinants for foot-and-mouth disease virus

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The three South African Territories (SAT) types of foot-and-mouth disease virus (FMDV) display great genetic and antigenic diversity, resulting from the independent evolution of these viruses in different geographical localities. For effective control of the disease in such areas, the use of custom-made vaccines is required. To circumvent the tedious process of vaccine strain selection, an alternative in the control process is being investigated. Specifically, it is proposed to replace the antigenic determinants of an infectious genome-length cDNA copy of a good SAT vaccine strain with those of appropriate field strains, producing custom-made FMDV chimeras for use in vaccine production. Here the construction of an infectious genome-length cDNA copy of the SAT2 vaccine strain, ZIM/7/83, is described, created utilizing an exchange-cassette strategy with an existing A12 genome-length cDNA clone. The virus derived from this cDNA (designated vSAT2) displayed excellent growth properties in cell culture, indicating its potential usefulness in the production of custom-made vaccine strains. Evaluation of the growth of various SAT2/A12 chimeras created during the derivation of SAT2 infectious cDNA suggested incompatibilities between the non-structural proteins of ZIM/7/83 and the 5’ UTR of A12.

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals that affects agricultural economics worldwide. The causative agent (FMDV) is a member of the genus Aphthovirus within the family Picornaviridae and occurs in seven distinct serotypes, of which six [types A, O, C and South African Territories (SAT) types 1–3] have been isolated on the African continent. The three SAT types are located almost exclusively in sub-Saharan Africa and display great genetic (Vosloo et al., 1995; Van Rensburg & Nel, 1999; Bastos et al., 2001, 2003) and antigenic variability (Esterhuysen, 1994). These types are further characterized by their ability to infect multiple wildlife hosts. African buffalo (Syncerus caffer) in southern Africa have been shown to be persistently infected with SAT type viruses (reviewed by Thomson et al., 2003) and are an important source of infection for domestic animals (Dawe et al., 1994; Bastos et al., 1999). The eradication of the disease from the region is therefore unlikely. Control in southern Africa consists mainly of vaccination as well as movement control of animals and animal products (Hunter, 1998). Due to the extensive variability of the SAT types, different lineages establish themselves in different geographical regions (Vosloo et al., 1995; Bastos et al., 2001, 2003). Current inactivated vaccines are often unable to control some lineages arising in these regions, arguing for the development of custom-made vaccines for use in specific geographical localities. However, development of useful cell-culture-adapted vaccine strains from field isolates is time-consuming and expensive, limiting the availability of custom-made vaccine strains.

The FMDV genome consists of a positive-strand RNA molecule of approximately 8.5 kb. This genome encodes a single translational ORF, and the product of the ORF is co- and post-translationally cleaved by viral proteinases (Sobrino et al., 2001). The fact that the viral RNA can be made infectious in the absence of other components of the virion opened the theoretical possibility of genetically engineering new viruses from in vitro-generated RNA...
molecules. Zibert et al. (1990) succeeded in the development of this technology, providing a powerful new tool for the study of FMD and the development of new methods to control the disease. Utilizing this technology, it is possible to engineer recombinant viruses containing manipulated and/or exchanged antigenic determinants (Rieder et al., 1994; Sa-Carvalho et al., 1997; Almeida et al., 1998; Baranowski et al., 1998; Beard & Mason, 2000). Thus, it is possible that chimeric SAT viruses could be designed to retain the desirable biological properties of good vaccine strains, whilst encoding the antigens required for vaccines in different geographical localities.

In a recent study, the usefulness of a type A genome-length clone in the construction of chimeras containing the structural proteins of SAT type 2 was reported (Van Rensburg & Mason, 2002). These studies showed that this chimera, created using a previously constructed type A12 strain, whilst encoding the antigens required for vaccines in different geographical localities.

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METHODS

Cell lines and viruses. Baby hamster kidney (BHK) cells, strain 21, clone 13 [ATCC CCL-10; maintained in Eagle's basal medium (BME; Invitrogen) containing 7% fetal calf serum (FCS; Delta Bioproducts) and 10% trypsin phosphate broth] were used during transfection, virus recovery, plaque assays and one-step growth studies. Plaque assays were also performed using IB-RS-2 [Instituto Biologico renal suino; maintained in RPMI medium (Sigma) containing 10% FCS] and Chinese hamster ovary (CHO) cells strain K1 [ATCC CCL-61; maintained in Ham's F-12 medium (Invitrogen) containing 10% FCS] using a trangacanth overlay method described by Rieder et al. (1993), and stained with 1% (w/v) methylene blue in 10% ethanol and 10% formaldehyde prepared with PBS, pH 7.4.

A SAT2 vaccine strain, originating from western Zimbabwe (ZIM/7/83; bovine passage 1, BHK passage 5, bovine passage 1), was selected for the construction of a genome-length backbone. This virus was previously obtained from the Botswana Vaccine Institute, Gabarone. To distinguish the parental virus from its cDNA derivative, we will refer to the natural isolate by its accession code (ZIM/7/83). The virus used for plaque assays and growth studies was further passaged three times in BHK cells. All genetically engineered viruses utilized for plaque assays and growth studies were passaged five times in BHK cells (see also ‘In vitro RNA synthesis, transfection and virus recovery’ below). A previously constructed genome-length cDNA copy of type A12 (pRMC35; Rieder et al., 1993) was used in the construction of the SAT2 genome-length cDNA copy. The derived virus, vRMC35, as well as another genetically engineered virus, vCRM4 (Sa-Carvalho et al., 1997), were included in this study as reference strains, using passage 5 on BHK cells. pCRM4 was previously constructed by exchanging the four capsid-coding proteins of type A12 in pRMC35 with the corresponding regions of type O.Campos.

RNA extraction and cDNA synthesis. RNA was extracted from the tissue culture sample of ZIM/7/83 using TRizol (Life Technologies) according to the manufacturer’s specifications and used as a template for cDNA synthesis. Superscript Moloney murine leukaemia virus reverse transcriptase (Life Technologies) and random hexamers as primers were used for the reverse transcription reactions, which were carried out for 1 h at 42 °C.

PCR amplification, construction of genome-length cDNAs and sequencing. A cassette-exchange strategy, based on an existing genome length cDNA of serotype A12 (pRMC35; Rieder et al., 1993) was used in the step-wise construction of an infectious SAT2 cDNA, similar to the strategy employed by Beard & Mason (2000) (Fig. 1). Regions of the SAT2 cDNA were obtained through PCR amplification from cDNA using AdvanTaq DNA Polymerase (Clontech). The reaction was carried out in the presence of oligonucleotides specifically designed to contain restriction enzyme sites that would facilitate insertion into the A12 genome (Fig. 1). The nucleotide sequence of pSAT2 was subsequently determined using selected oligonucleotides and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.0 (Applied Biosystems), followed by resolution on an ABI PRISM 310 Genetic Analyser (Applied Biosystems). To confirm successful insertion into the A12 genome, the cloning sites of all the intermediate SAT2/A12 constructs were also verified through nucleotide sequencing.

In vitro RNA synthesis, transfection and virus recovery. Following successful selection of plasmids containing the desired cDNA constructs, these plasmid DNAs were linearized at a restriction endonuclease site following the poly(A) tract (NotI) and RNA was synthesized with the MEGAscript T7 kit (Ambion) according to the manufacturer’s specifications. BHK cells were transfected with the in vitro-generated RNA by means of electroporation as described by Mason et al. (2002) using an Electrocell Manipulator 600 (BTX). The electroporated cells were then diluted into cell growth medium and allowed to attach to 35 mm diameter tissue culture plates. Dead cells and medium were removed after 3–5 h and replaced with BME containing 1% fetal bovine serum (HyClone) and incubated at 37 °C for up to 24 h. Tissue culture plates containing the monolayers of electroporated cells were frozen at −70 °C. Following thawing, 10% of the lysate was used to infect fresh BHK monolayers and incubated for up to 48 h at 37 °C prior to freezing. This process was repeated four times and aliquots of the lysate obtained during each step were frozen at −70 °C. Unless otherwise stated, passage 5 was used for analyses.

RESULTS

Construction of the genome-length cDNA copy of SAT2 FMDV

Through step-wise replacement of regions in the previously constructed genome-length A12 cDNA copy (pRMC35; Rieder et al., 1993) with cDNAs encoding the corresponding regions of the genome of ZIM/7/83, several chimeric constructs were created, including a genome-length copy of ZIM/7/83 (pSAT2) (Fig. 1). A single amino acid change [Val (GTC) to Leu (TTG)], was introduced in the 1B region of ZIM/7/83 to facilitate the insertion of the external capsid-coding region of the SAT2 strain into the A12 genetic backbone, resulting in pSAT2/A12 (step 1; Fig. 1).
Substitution of the P2, P3 and 3′ untranslated region into this cDNA clone was used to create pP1-AnSAT2 (step 2; Fig. 1). pCn-AnSAT2 was created by replacement of the poly(C)–1A region of A12 with the corresponding region of ZIM/7/83 in pP1-AnSAT2, producing a cDNA that contained almost the entire SAT2 genome, lacking only the S-fragment of this serotype (step 3; Fig. 1). To facilitate the assembly of a full-length SAT2 cDNA, the S-fragment was amplified using an oligonucleotide containing the first 18 nt of a different SAT2 strain, TAN/2/75 (Harris, 1980), and substituted for the A12 S-fragment in pCn-AnSAT2 (step 4; Fig. 1). An additional clone (pA12/SAT2) was created by substituting the external capsid-coding region of A12 into pSAT2, using the same restriction sites identified in Fig. 1.

Recovery of viable chimeras in BHK cells

These five genome-length cDNA clones, pSAT2/A12, pP1-AnSAT2, pCn-AnSAT2, pSAT2 and pA12/SAT2, together with pRMC35 and pCRM4 (A12 genetic background containing the four capsid proteins of a heparan-sulfate-binding O1 Campos virus; Sa-Carvalho et al., 1997), were used as templates for the in vitro synthesis of RNAs which were used to transfect BHK cells. Viable viruses were recovered from RNAs obtained from all five cDNAs. To verify that the resulting viruses had the expected genetic composition, RNAs extracted from each recovered virus were sequenced using RT-PCR. Analyses of the sequence data obtained from the junction sites of these viral genomes revealed that all of the recovered viruses had the expected genotype (results not shown). Despite our ability to recover viruses from all of these constructs, side-by-side transfections revealed that it was more difficult to recover viruses from some of the synthetic genomes. In particular, RNA synthesized from one construct, pP1-AnSAT2, failed to produce viable virus on several occasions, although when virus was recovered it had the expected sequence.

Comparisons of the ease with which viruses were recovered from all the constructs (except for pP1-AnSAT2) are shown in Table 1. These data showed that RNA derived from pSAT2 produced virus as early as that seen with the RNA derived from the highly tissue-culture-adapted virus (pCRM4; Sa-Carvalho et al., 1997). The CPE observed during the sequential passaging of viruses vSAT2/A12, vCn-AnSAT2, vA12/SAT2 and vRMC35 lagged behind vCRM4 and vSAT2, but by BHK passage 4, all viruses shown in Table 1 displayed similar ability to cause CPE.

To further characterize the properties of these newly created viruses, the titre that they achieved upon overnight growth was determined in three different cell lines (Fig. 2). These studies employed the five viruses described above along with ZIM/7/83 and the two viruses known to display different receptor specificity (vRMC35 and vCRM4). High titres were observed for all viruses on BHK and IB-RS-2 cells. All viruses containing ZIM/7/83 capsids yielded titres on CHO cells, similar to vCRM4, indicating that viruses with the ZIM/7/83 capsid could utilize heparan sulfate as a receptor.
Plaque morphologies in BHK, CHO and IB-RS-2 cells

Comparisons of the plaque morphologies of the viruses on different cell lines reflected the ability of their capsids to interact differently with these cells (Fig. 3). In BHK cells the A12 virus (derived from pRMC35) and vA12/SAT2 displayed plaques with turbid morphology. All of the other viruses tested produced plaques in BHK cells that had a clear morphology, with vCRM4 producing plaques that were smaller than all the ZIM/7/83 external capsid-containing viruses (Fig. 3). Both viruses with the A12 capsid region failed to produce plaques on CHO cells (Fig. 3), consistent with studies showing that these cells do not make integrins suitable for FMDV infection (Mason et al., 1993). Among the remaining viruses the plaques produced by vCRM4 were extremely small (less than 0.5 mm diameter) on CHO cells (as reported by Sa-Carvalho et al., 1997), whereas all of the viruses with ZIM/7/83-derived capsids were small. Fewer differences in morphologies were readily distinguishable on IB-RS-2 cells (Fig. 3), suggesting that the turbid morphology produced by A12 capsid-containing viruses on BHK cells could be due to a limitation in the number of appropriate receptors for these viruses in BHK cells that were present in higher numbers on the IB-RS-2 cells.

Growth kinetics of genetically engineered viruses in BHK cells

One-step growth studies in BHK cells indicated that the release of virus particles from cells infected with vSAT2 and the parental ZIM/7/83 virus were indistinguishable from one another (Fig. 4a). The chimeras in the SAT2 construction series performed poorly compared to vSAT2 and ZIM/7/83: vCn-AnSAT2 > vSAT2/A12 > vP1-AnSAT2. In the first experiment an approximately threefold lower titre for vCn-AnSAT2 (4 × 10^4 p.f.u. ml^-1) was obtained at 10 h post-infection in comparison to vSAT2 (1 × 10^5 p.f.u. ml^-1) and ZIM/7/83 (1 × 10^5 p.f.u. ml^-1). In contrast, the titre obtained for vSAT2/A12 (2 × 10^4 p.f.u. ml^-1) was five- to sixfold lower than that observed for vSAT2 and ZIM/7/83, whilst vP1-AnSAT2 produced a titre of only 7 × 10^3 p.f.u. ml^-1 (20-fold lower than the titre produced by vSAT2) 10 h post-infection. The poorer performance of vSAT2/A12 in comparison to the parental virus has been observed previously (Van Rensburg & Mason, 2002), but the experiments were performed in the absence of vRMC35 (the genetic backbone used for this construct). However, in Fig. 4(b) similar growth was observed for vSAT2/A12 and vRMC35, reaching a titre of 5 × 10^4 p.f.u. ml^-1 at 10 h post-infection, almost 10-fold lower than the titre obtained for vSAT2.
Growth determinants for FMDV

Fig. 3. Schematic representation of the chimeric FMD constructs described in this study together with constructs vRMC35 (Rieder et al., 1993) and vCRM4 (Sa-Carvalho et al., 1997), described previously. Grey bars, A12; white bars, SAT-2, ZIM/7/83; hatched bars, heparan-sulfate-binding O1Campos. Plaque morphologies of the derived genetically engineered viruses and parental ZIM/7/83 (passage history described in Methods) as obtained on BHK, CHO and IB-RS-2 cells are shown on the right.

(4·3 × 10^5 p.f.u. ml^-1), vA12/SAT2 (A12 external capsids in SAT2 genetic backbone) exhibited a higher rate with which virus particles were released from infected cells compared to vRMC35, especially during initial stages of infection. The rate observed for vA12/SAT2 was comparable to vCRM4 10 h post-infection. Slightly lower titres of
Fig. 4. One-step growth studies as determined on BHK cells. To compare the relative release of virus particles by the genetically engineered viruses from infected cells, two independent experiments were performed. (a) vSAT2/A12 (diamonds), vP1-AnSAT2 (squares), vCN-AnSAT2 (circles), vSAT2 (triangles) and the parental ZIM/7/83 strain (crosses). (b) vRMC35 (filled circles), vA12/SAT2 (open circles), vSAT/A12 (diamonds), vCRM4 (crosses) and vSAT2 (triangles). Virus passages used are described in Methods. Cell monolayers were infected for 1 h at 37°C at an m.o.i. of 0.5 p.f.u. per cell and rinsed with 25 mM MES/145 mM NaCl, pH 5.5 (to eliminate residual extra-cellular virus). Following the addition of BME containing 1% FCS, incubation at 37°C was continued. At the times indicated, 2% of the supernatant was removed, diluted ten times into BME containing 1% FCS and frozen at -80°C. Virus titres were performed on the thawed samples by plaque assay on BHK cells. Error bars indicate values obtained from duplicate wells prepared from samples harvested from the same experiment.

1·1 x 10^5 p.f.u. ml^-1 for vA12/SAT2 and 1 x 10^5 p.f.u. ml^-1 for vCRM4 were observed compared to vSAT2.

DISCUSSION

Several genome-length cDNA clones useful for producing genetically engineered type A (Rieder et al., 1993) and O (Zibert et al., 1990; Beard & Mason, 2000) FMDV have been reported. Here we describe the first such clone for an SAT-type virus. Evaluation of the virus derived from our SAT2 genome-length clone revealed that it has maintained the high-titre growth of its parental virus, ZIM/7/83, in cell cultures. Furthermore, this virus, as well as a panel of chimeric viruses containing the capsid region of ZIM/7/83, all grew well in CHO cells, suggesting these viruses were able to use heparan sulfate as a receptor. Since ZIM/7/83 is a tissue-culture-adapted virus, this finding adds to those of others that tissue-culture-adapted viruses are capable of binding heparin and using heparan sulfate proteoglycans as cellular receptors (Sa-Carvalho et al., 1997; Neff et al., 1998; Fry et al., 1999; Jackson et al., 2003). These properties of vSAT2 suggest that the two viruses (vSAT2 and ZIM/7/83) are indistinguishable phenotypically. However, due to the fact that the complete genome sequence of the recovered vSAT2 virus was not compared to that of the parental strain, it is possible that the two viruses differ genotypically.

We previously reported that vSAT2/A12, a chimeric virus with the SAT2 capsid in an A12 genome, performed poorly in BHK cells when compared to the parental ZIM/7/83 (Van Rensburg & Mason, 2002). This finding, and studies showing that 3C^pro (which cleaves the capsid proteins of SAT2) differs significantly from type A 3C^pro (Van Rensburg et al., 2002), lead us to propose that the poor growth properties of vSAT2/A12 were due to suboptimal processing of the SAT2 external capsid proteins by the A12 3C^pro (Van Rensburg & Mason, 2002). However, side-by-side growth comparisons of this chimera with an infectious clone derived from a type A12 virus (vRMC35) and our genetically engineered SAT2 virus (vSAT2) indicated that the chimera grows just as well as the type A12 virus. This result suggests that, rather than being compromised by an incompatibility of various portions of its genome, the inferior growth of vSAT2/A12 relative to ZIM/7/83 may reflect the superior properties of the latter virus in cell culture.

Evaluations of the growth properties of other chimeras suggest that there is an incompatibility between different portions of the A12 and SAT2 genomes. The clearest case of this type of genetic incompatibility is revealed by the properties of the RNA derived from pP1-AnSAT2. The RNA derived from this chimeric genome did not reproducibly produce virus when transfected into cells. The resulting virus also produced plaques smaller than any of the other SAT2 capsid-expressing virus on all the cell lines investigated. This genome contains the 5' UTR, L^pro and protein 1A of type A12, with the remainder of the genome derived from ZIM/7/83. Since all of the fragments utilized in this clone produced a highly viable virus with excellent growth properties, we concluded that there were incompatibilities between the 5' UTR of A12 and the SAT2 polypeptide and 3' UTR. One of the components in this region, L^pro, was of particular interest since it was shown to differ substantially between A12 and SAT2 (Van Rensburg et al., 2002). L^pro is, however, followed by its own cleavage site and it therefore...
seems unlikely that \( L^{pr} \) incompatibility accounts for the poor growth properties observed for vP1-AnSAT2.

Investigation of the predicted RNA structural folding of the 5’ UTR of SAT2, and that of the \( A_{12} \) genome (Mason et al., 2003) revealed a strong similarity in the structure, with only a few differences observed between the two genomes in the cre and previously mapped protein–RNA interaction sites for the FMDV IRES (results not shown), despite a low overall nucleotide identity (73% for the pseudoknots and 77% for the cre and IRES structures). However, it is unclear whether these differences could result in incompatible interactions between the 5’ UTR and the non-structural proteins suggested by the poor growth of vP1-AnSAT2. Analyses of the predicted structure of the S fragment of SAT2 revealed a 37 nt deletion relative to that predicted for type \( A_{12} \) (results not shown). However, comparisons of vCn-AnSAT2 with vSAT2 (which differ only in their S fragments) failed to reveal any striking differences, providing further evidence of the difficulty in reaching a conclusion regarding functional interactions using predicted RNA structures.

Despite some slight differences observed with plaques on IB-RS-2 cells, reflected by small differences in growth kinetics in these cells (results not shown), the genetically derived virus, vSAT2, appears to grow as well as its parent, ZIM/7/83, in BHK cells. Since BHK is the cell line of choice for production of inactivated FMD vaccines, it appears that the cDNA used to derive vSAT2 is a good candidate for a genetic background upon which useful genetically engineered vaccine strains of FMDV can be built.

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