Dynamics of picornavirus RNA replication within infected cells

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Replication of many picornaviruses is inhibited by low concentrations of guanidine. Guanidine-resistant mutants are readily isolated and the mutations map to the coding region for the 2C protein. Using in vitro replication assays it has been determined previously that guanidine blocks the initiation of negative-strand synthesis. We have now examined the dynamics of RNA replication, measured by quantitative RT-PCR, within cells infected with either swine vesicular disease virus (an enterovirus) or foot-and-mouth disease virus as regulated by the presence or absence of guanidine. Following the removal of guanidine from the infected cells, RNA replication occurs after a significant lag phase. This restoration of RNA synthesis requires \textit{de novo} protein synthesis. Viral RNA can be maintained for at least 72 h within cells in the absence of apparent replication but guanidine-resistant virus can become predominant. Amino acid substitutions within the 2C protein that confer guanidine resistance to swine vesicular disease virus and foot-and-mouth disease virus have been identified. Even when RNA synthesis is well established, the addition of guanidine has a major impact on the level of RNA replication. Thus, the guanidine-sensitive step in RNA synthesis is important throughout the virus life cycle in cells.

INTRODUCTION

Swine vesicular disease virus (SVDV) and foot-and-mouth disease virus (FMDV) are members of the family Picorniridae, within the genera Enterovirus and Aphythovirus, respectively. These viruses can cause clinically indistinguishable diseases in swine; however, FMDV has a much broader host-range and is responsible for one of the most economically important diseases of farm animals. SVDV is closely related both antigenically and genetically to coxsackie virus B5 (Graves, 1973; Inoue \textit{et al.}, 1989).

Picornaviruses have a positive-sense RNA genome of 7.5–8.5 kb. The RNA is translated after entry into the cellular cytoplasm. Following production of proteins required for RNA replication, the input viral RNA also acts as the template for the synthesis of negative-sense transcripts, which are then used to synthesize positive-sense genomes (reviewed by Paul, 2002). A large excess of positive-strand transcripts is produced compared with negative-sense RNA. Picornivirus RNA replication occurs on membrane-bound replication complexes and involves several different viral proteins in addition to the RNA polymerase (3D\textsuperscript{pol}). The replication of certain picornivirus RNAs is sensitive to guanidine (in the millimolar range) but the level required to achieve inhibition varies. Guanidine-resistant mutants of FMDV (Saunders & King, 1982; Saunders \textit{et al.}, 1985), poliovirus (PV) (Pincus & Wimmer, 1986; Baltera & Tershak, 1989; Tolskaya \textit{et al.}, 1994) and echovirus 9 (Klein \textit{et al.}, 2000) all have amino acid substitutions within the 2C protein. Different mutations have been observed depending on the level of guanidine used to select the mutants. For example, studies on the Mahoney strain of PV showed that substitution of residue 179 [from Asn (N) to Gly (G) or Ala (A)] conferred resistance to 2 mM guanidine, whereas mutants selected in 0.53 mM guanidine had a substitution of Ser (S) 225 to Thr (T) (Pincus \textit{et al.}, 1986).

The specific function of 2C in RNA replication is not clear. The 2C protein interacts with viral RNA (Rodriguez & Carrasco, 1995; Banerjee \textit{et al.}, 1997); it also induces membrane rearrangements within cells (Teterina \textit{et al.}, 1997; Egger \textit{et al.}, 2000) and includes regions which interact with other viral and cellular proteins (Teterina \textit{et al.}, 2006; Tang \textit{et al.}, 2007). It has been demonstrated previously that guanidine blocks the initiation of negative-strand PV RNA synthesis within cell-free replication systems (Barton & Flanagan, 1997) and hence it is inferred that 2C has a role in this process. The block on the initiation of negative-strand RNA synthesis consequently blocks the formation of positive-sense transcripts but guanidine does not block the elongation of initiated chains. It has also been shown that guanidine blocks the uridylylation of VPg (3B), within cell-free replication systems for PV (Lyons \textit{et al.}, 2001); it is noteworthy that the 5' terminal ‘cloverleaf’ is also required for this reaction in this system. In contrast, using purified components in solution, uridylylation of VPg can be achieved with VPg, UTP, 3CD, 3D\textsuperscript{pol} plus an RNA template including cre, thus...
there is no requirement for either 2C or the cloverleaf under those conditions (Paul, 2002). All picornavirus RNA is synthesized with VPg linked to the 5’-terminal nucleotide and it is believed that the uridylylated VPg (VPpgpUpU) acts as the primer for RNA synthesis.

The 2C protein contains three conserved ‘Walker’ motifs, which are shared with other NTP-binding proteins including RNA and DNA helicases (Gorbalenya et al., 1990), but no helicase activity has yet been demonstrated for any of the picornavirus 2C proteins. Using recombinant fusion proteins it has been shown that 2C has ATPase activity and that this activity of PV 2C is inhibited by low concentrations of guanidine (Pfister & Wimmer, 1999); however, this was not the case for the echovirus 9 2C protein (Klein et al., 2000). Many of the mutations in 2C that confer resistance to guanidine are in, or close to, the Walker motifs (Pincus et al., 1986; Tolskaya et al., 1994; Klein et al., 2000) but the single amino acid substitution reported in a single guanidine-resistant mutant of FMDV (strain Qc) was not near these motifs but close to the C terminus (Saunders et al., 1985).

The effect of guanidine on picornavirus replication is reversible. Within cell-free replication systems, negative-strand RNA production followed by positive-strand RNA synthesis commences very rapidly after the removal of guanidine (Barton & Flanagan, 1997), but a recent report indicated that within PV-infected cells the resumption of PV RNA replication, as detected by fluorescence in situ hybridization, following guanidine removal occurred after a significant lag phase (Egger & Bienz, 2005). We have now analysed the dynamics of viral RNA replication within cells infected with either SVDV or FMDV in the presence or absence of guanidine using quantitative real-time PCR (qRT-PCR) assays.

METHODS

Samples of SVDV (strains UKG/27/72, Itl 18/92 and Itl 3/97) and FMDV (A22 Iraq 24/64) were obtained from the Institute for Animal Health (Pirbright, UK). The FMDV O-UAE 542/99 isolate was provided by U. Wernery (Central Veterinary Research Laboratory, Dubai). Nucleotide sequences of SVDV UKG and FMDV A22 Iraq 24/64 have been determined previously (GenBank accession nos X54521 and AY593762, respectively). Sequence information for FMDV O-UAE 542/99 (accession no. EU140964), SVDV Itl 18/92 (accession no. EU151448) and SVDV Itl 3/97 (accession no. EU151449) is available at GenBank.

For most experiments, porcine kidney IBRS2 cells (in six-well plates; Costar) were infected with virus, as indicated in the Figure legends, in the absence or presence of guanidine hydrochloride (Sigma), generally 3 mM except where specifically stated. The cells were incubated overnight at 37 °C. Following the indicated treatments, the cells (plus medium, 2 ml) were frozen at −70 °C or, where indicated, the cells were harvested directly in RLT buffer (Qiagen).

RNA was extracted from cell harvests (140 μl) using a viral RNA extraction kit (Qiagen) and eluted in water (50 μl); an aliquot corresponding to 0.1 μl was used to produce cDNA using reverse transcriptase (Tagman RT; Applied Biosystems) with random hexamer primers (Roche). From the cDNA reaction, 1 μl was used in qRT-PCR assays (with AmpliTag; Applied Biosystems) using either a Stratagene MX4000 or MX3005 machine with the primers and probes for SVDEV and FMDV detection as described previously (Reid et al., 2003, 2004). Under these conditions neither cDNA synthesis nor PCR were saturated. Data were analysed using the Stratagene MxPro software. Ct values are the calculated cycle values at which the fluorescence reached a defined threshold during the early exponential phase of the reaction when all reagents are in excess and the products do not compete for primer binding. Each additional cycle required indicates a twofold lower amount of cdNA present in the sample and the difference in Ct values between two samples analysed in parallel is the ΔCt value. All reactions were performed using 50 cycles, the results are presented as a value (N, usually 35) minus the Ct observed, if the Ct was greater than N then N–Ct was set to zero.

Amplification of the coding region for the 2C protein from guanidine-resistant SVDV and FMDV was achieved with cDNA preparations used for the qRT-PCR assays in a standard PCR with the primers dTCACGATGACCTCATTAGGG plus dTCGTAATTCTCC-AAGCATIGTG (for SVDV) or dAAGGACCGCTCTTGTTGCC plus dCTCAAGAATTTCAATTGCTGC (for FMDV). The fragments (~1500 and 1240 bp, respectively) were isolated and inserted into the pcR-XL-TOPO vector (Invitrogen) and transformed into competent Escherichia coli (TOP10; Invitrogen). Plasmid DNA was prepared from amplified individual colonies. Inserts were identified by restriction enzyme digestion and gel electrophoresis and sequenced by Agowa (Berlin, Germany).

Virus yield assays were performed using IBRS2 cells, 10-fold dilutions of each sample were assayed in five different wells per dilution in a 96-well plate. Cells were incubated at 37 °C for 3 days and cell death was scored by microscopy. Virus yield was expressed as TCID50 ml−1.

RESULTS

Replication of SVDV RNA is inhibited by guanidine–HCl

Initially, we wanted to determine whether qRT-PCR could be a useful tool for analysing the effect of guanidine hydrochloride (gua–HCl) on picornavirus RNA replication. Porcine cells (IBRS2) were infected with well-characterized field strains of either SVDV (UKG, Itl 18/92 and Itl 3/97) or FMDV (A22 Iraq or O-UAE). Infections were performed in the absence or presence of different concentrations of gua–HCl (1–10 mM). Following overnight incubation and visual inspection for cytopathic effects (CPE), the cells and medium were frozen. After thawing, RNA was extracted from an aliquot of the cell harvest and the level of viral RNA was determined using qRT-PCR as described previously (Reid et al., 2003, 2004). Fig. 1 shows the results obtained using the UKG strain of SVDV. In the absence of gua–HCl, high levels of viral RNA were detected as expected, the presence of 1 mM gua–HCl had no significant effect on RNA production and all the cells still lysed. With 2 mM gua–HCl, the level of RNA accumulated was significantly reduced but using 3–10 mM gua–HCl further depressed the level of RNA detected although similar values were obtained at each of the concentrations tested (3, 5 and 10 mM), which probably corresponded largely to the input virus. There was a
difference of about 10 in the \( C_t \) values observed for the viral RNA in the absence or presence of 3–10 mM gua–HCl, this corresponds to a 2\(^{10} \) = 1024-fold reduction in RNA detected (2\(^{10} \) = 1024).

**Reversibility of guanidine blockade of RNA synthesis**

It is well known that the effect of guanidine on picornavirus RNA replication is reversible (Barton et al., 1995; Barton & Flanegan, 1997). To determine the kinetics of the reversibility of the guanidine-induced blockade of RNA replication we used the minimum concentration of gua–HCl that effectively blocked RNA replication. Cells were infected with SVDV in the presence of 3 mM gua–HCl then, following incubation for 18 h, switched to fresh medium in the presence or absence of 3 mM gua–HCl and incubated for a further 2, 4 or 6 h then harvested. This experiment was performed essentially in duplicate, for one set of samples the viral RNA was extracted from the frozen cell extracts that were also used for virus-yield assays. For the second set, the medium was removed from the cells at the appropriate time and the cells were lysed directly by the addition of RLT buffer (Qiagen), the first step in the RNA extraction procedure. Cells infected with SVDV in the absence of gua–HCl showed extensive CPE by 18 h but no CPE was evident in the presence of this inhibitor. RNA was prepared from both sets of samples and analysed in parallel by qRT-PCR. Results from the samples harvested directly into RLT buffer are shown in Fig. 2 but entirely analogous results were obtained from the frozen cell samples (see below). In the absence of gua–HCl a high level of SVDV RNA was apparent (\( C_t \sim 20 \)) as expected, whereas in the continuous presence of 3 mM gua–HCl a \( C_t \) value of about 32 was observed (i.e. about 2\(^{12} \) or 4096-fold less RNA). The slightly larger difference observed in this experiment in the presence and absence of gua–HCl compared to Fig. 1 probably reflects the removal of much of the input virus when the medium was changed to remove the guanidine for the latter experiment.

Using virus yield assays it was found that the yield of SVDV was reduced from about 10\(^{9.4} \) TCID\(_{50} \) ml\(^{-1} \) in the absence of inhibitor to about 10\(^{4.8} \) TCID\(_{50} \) ml\(^{-1} \) in the presence of 3 mM gua–HCl; these results are consistent with the effects of gua–HCl measured in the qRT-PCR assays.

When gua–HCl was removed from the cells and incubation continued for only 2 h, no significant increase in viral RNA synthesis was observed.

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**Fig. 1.** Determination of SVDV sensitivity to guanidine. IBRS2 cells were infected with SVDV (UKG strain) in the presence of the indicated concentrations of gua–HCl. After overnight incubation, the cells plus medium were frozen and aliquots of the cell extract were used to detect SVDV RNA by qRT-PCR as described in Methods. A negative control (NC) (water alone) was included in the qRT-PCR to ensure the absence of cross-contamination. The same colour codes are used in each panel. (a) Shows the amplification traces generated in the qRT-PCR while (b) displays the data as a bar graph. The \( y \)-axis (30–\( C_t \)) is the value generated by subtracting from 30 (to cover the range of values generated in this experiment) the calculated point (termed the \( C_t \)) at which the fluorescence reached the threshold (T) value. If a high level of RNA is present in the sample then the fluorescence generated by the PCR rapidly exceeds the threshold value (i.e. a low \( C_t \)) and 30–\( C_t \) will be high.

**Fig. 2.** Viral RNA synthesis following removal of gua–HCl from SVDV-infected cells. IBRS2 cells were infected with SVDV (UKG) in the absence (−) or presence (+) of gua–HCl (3 mM) and incubated overnight (o/n). The medium was then replaced by fresh medium with (+) or without (−) gua–HCl and incubated for 2, 4 or 6 h as indicated. In each case, cells were harvested directly into RLT buffer and SVDV RNA was assayed by qRT-PCR. A negative control (NC; water) was also analysed.
was observed; however, after 4 h there was over a 1000-fold ($\Delta C_t = 10$) increase in viral RNA and a further small increase by 6 h (Fig. 2). For comparison, the virus yield assays detected about $10^4$ TCID$_{50}$ ml$^{-1}$ in the virus harvest at 2 h after removal of gua–HCl, this had increased to $10^7$ TCID$_{50}$ ml$^{-1}$ at 4 h and $10^8$ TCID$_{50}$ ml$^{-1}$ at 6 h.

**Maintenance of replication competent RNA within SVDV-infected cells in the presence of guanidine**

From the results described above (Fig. 2) it was apparent that viral RNA was maintained within the infected cells incubated overnight in the presence of gua–HCl. We then wished to determine how long this block on RNA replication could be continued while maintaining the ability to allow virus replication to occur when gua–HCl was removed. Cells were infected with SVDV on day 0 in the presence of 3 mM gua–HCl and on days 1, 2 and 3 the cell medium was removed and either fresh medium with gua–HCl (3 mM) was added and incubation continued or medium lacking gua–HCl was added and the incubation continued for 5 h prior to harvesting by freezing. In all cases RNA was extracted from the cells and measured by qRT-PCR, the results are shown in Fig. 3. On day 1, the removal of gua–HCl for 5 h resulted in a large increase ($\Delta C_t \approx 12$) in SVDV RNA synthesis; these results are entirely consistent with those obtained for intracellular viral RNA shown in Fig. 2. On day 2, a much smaller increase [$\Delta C_t \approx 4$, (16-fold)] in the amount of SVDV RNA was observed following removal of gua–HCl, furthermore the amount of viral RNA detected was much lower than observed on day 1 in the absence of gua–HCl but was higher, both in the presence and absence of gua–HCl, than observed in the continued presence of gua–HCl on day 1 (Fig. 3). On day 3, very similar high levels of SVDV RNA were detected both in the presence or absence of gua–HCl, comparable to that observed in the absence of gua–HCl on day 1 (Fig. 3). It appeared that maintaining the presence of gua–HCl for 3 days on SVDV-infected cells resulted in guanidine-resistant virus becoming predominant.

**Characterization of guanidine-resistant SVDV**

Using cDNA prepared from SVDV-infected cells harvested on day 3 in the presence of gua–HCl (3 mM), a conventional PCR was performed using primers that flanked the 2C coding region. The fragment generated (~1500 bp) was inserted into the pcR-XL-TOPO vector and transfected into *E. coli*. Plasmid DNA from individual colonies was isolated and the presence of the SVDV cDNA insert was determined by restriction enzyme analysis. The sequences of 15 different fragments were determined. The sequence changes that conferred amino acid substitutions compared with the parental UKG virus within the 2C protein are listed in Table 1. It was found that nine of 15 sequences encoded the substitution of Ala133 by Thr (A133T), however, in only one of these fragments did this change occur alone (Mut 17), within the other fragments encoding the A133T substitution eight different site changes were observed. The A133T substitution is within the Walker A motif (Gorbalevna et al., 1990). The second most common change was a D160A modification, five of 15 sequences had this substitution and, in contrast to most of the A133T mutants, it was the sole change in each of these fragments (this residue lies between the Walker A and B motifs). One other fragment (Mut 6) contained neither of these changes but had two other substitutions, R119H and Q256R, interestingly the R119H change was also found in two of the fragments containing the A133T modification (Mut 8 and Mut 15). An N228S change was also found in two fragments (Mut 8 and Mut 11), thus the Mut 8 fragment had three different modifications. In studies on another enterovirus, echovirus 9, it has been found that the substitution A133T conferred resistance to gua–HCl (Klein et al., 2000) and this substitution has been detected in some guanidine-resistant mutants of PV as well (Tolskaya et al., 1994). It seems that the A133T and D160A changes in the SVDV 2C protein are each able to confer resistance to guanidine alone. It is not possible to say whether the substitutions R119H and Q256R are able to confer resistance individually or possibly only in conjunction with another change. It is apparent that all of the fragments amplified by RT-PCR encoded amino acid substitutions within the SVDV 2C protein, which is consistent with the fact that between days 1 and 3 the level of virus replication in the presence of guanidine increased by a factor in excess of 1000.

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**Fig. 3.** Generation of guanidine-resistant SVDV. IBRS2 cells were mock (M) or SVDV (+) infected in the presence of gua–HCl (3 mM) as indicated and incubated overnight. The medium was then changed each day to fresh medium with gua–HCl, except for two dishes that were incubated either with (+) or without (−) gua–HCl for just a further 5 h prior to freezing. Residual cells were incubated until day 2 when the medium was again changed to fresh medium with gua–HCl, except for two dishes that were incubated with (+) or without (−) gua–HCl for a further 5 h prior to freezing as before. The remaining cells were incubated for a third day with gua–HCl and then shifted to fresh medium with (+) or without (−) gua–HCl and incubated for 5 h prior to freezing. RNA was extracted from all samples and SVDV RNA was detected by qRT-PCR.
Table 1. Amino acid substitutions within the 2C protein of guanidine-resistant SVDV

The amino acid substitutions A133T and D160A are capable of conferring resistance to gua–HCl alone.

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Generation of guanidine-resistant FMDV

In similar experiments to those described above for SVDV, it was found that the replication of FMDV strains O-UAE and A22 Iraq was also inhibited by 3 mM gua–HCl (data not shown) but the sensitivity of these viruses to gua–HCl was less than for the SVDV UKG. We essentially then repeated the experiment described in Fig. 3 for SVDV using these FMD viruses. Cells were infected with either FMDV type O-UAE or A22 Iraq and were maintained for 1–3 days in the presence of 3 mM gua–HCl. When FMDV O-UAE was used to infect cells, it was clear that on day 1 post-infection removal of the guanidine lead to a large increase in viral RNA replication; however, on day 3 there was little change in viral RNA synthesis when the gua–HCl was removed (Fig. 4a). These observations are analogous to the results obtained using SVDV as described above (Fig. 3). In contrast, no FMDV RNA was detectable, under these assay conditions, after 3 days in cells infected with the A22 Iraq strain of FMDV when maintained in 3 mM gua–HCl (Fig. 4b). However, on days 1, 2 and 3 a large increase in viral RNA was detected following incubation for 5 h in the absence of gua–HCl (Fig. 4b). Thus, A22 Iraq remained guanidine-sensitive throughout the experiment. The level of FMDV A22 RNA detected declined on each day, whereas the level of O-UAE RNA was maintained as it acquired the ability to replicate in the presence of gua–HCl.

The 2C coding region from the O-UAE virus generated by treatment of infected cells with 3 mM gua–HCl for 3 days was amplified by RT-PCR, yielding a fragment of about 1200 bp, which was inserted into pCR-XL-TOPO and plasmid DNAs from individual colonies were sequenced, as described above for the guanidine-resistant SVDV. The amino acid substitutions detected within the FMDV O-UAE 2C coding region are listed in Table 2. All 19 inserts examined encoded the substitution M158L (n.b. the FMDV 2C is 317 residues long compared with the SVDV 2C of 329 residues). This substitution lies within the conserved Walker B motif (Gorbalenya et al., 1990). Seven of these

![Fig. 4](http://vir.sgmjournals.org)
fragments contained additional substitutions but these were each different. These changes were also distinct from the single amino acid substitution in a gua–HCl-resistant mutant of FMDV (O6) reported by Saunders et al. (1984).

Characteristics of renewed RNA synthesis following release of guanidine block

As shown previously (e.g. Barton et al., 1995; Barton & Flanagan, 1997) and above, the effect of gua–HCl on picornavirus RNA synthesis is reversible. Within an in vitro replication system the removal of gua–HCl resulted in almost immediate resumption of negative- and then positive-strand PV RNA synthesis (Barton & Flanagan, 1997). However, within PV- or SVDV-infected cells there is a significant lag phase between the removal of gua–HCl and the detection of RNA replication (see Fig. 2 and Egger & Bienz, 2005). We wished to explore how long the absence of gua–HCl was required to permit the generation of significant new RNA synthesis. Cells were infected with SVDV and maintained in the presence of gua–HCl overnight prior to removal of the inhibitor, then at later times, gua–HCl was added back and the incubations continued until 6 h after removal of the gua–HCl. Cells were then harvested and the SVDV RNA was quantified (Fig. 5). As in previous experiments (Figs 2 and 3), the release of the gua–HCl block resulted in a great increase in the amount of viral RNA was observed. Thus, consistent with the results shown in Fig. 2, a 2 h window without guanidine is not sufficient to allow RNA synthesis to proceed at a significant rate even though gua–HCl does not block the elongation phase of RNA synthesis or the initiation of positive-strand RNA synthesis in vitro.

Table 2. Amino acid substitutions within the 2C protein of guanidine-resistant FMDV O-UAE

The amino acid substitution M158L is capable of conferring resistance to gua–HCl alone.

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| Mut 20 |     |      |      |       |      |      |       |       |       |+
| Mut 21 |     |      |      |       |      |      |       |       |       |+

Fig. 5. Guanidine sensitivity of RNA replication is maintained throughout the virus life cycle. IBRS2 cells were infected with SVDV (UKG) in the presence of gua–HCl (3 mM) and incubated overnight. The medium was then changed (at t=0) and the incubation was continued for a further 6 h prior to freezing. During this incubation gua–HCl was added again to cells at t=0, t=1 h, t=2 h, t=3 h, t=4 h or t=5 h as indicated. (+) Indicates that gua–HCl was present for only part of the incubation time. RNA was extracted from all samples and SVDV RNA was detected by qRT-PCR. A negative control (NC; water) was assayed in parallel.
**Protein synthesis is required for the resumption of RNA synthesis following removal of guanidine from infected cells**

To determine if *de novo* protein synthesis is required for the increase in RNA synthesis following the removal of guanidine from SVDV-infected cells, the influence of two different protein synthesis inhibitors, cycloheximide and puromycin, on this process was determined. These agents function in different ways and have distinct consequences, cycloheximide treatment locks ribosomes onto the RNA, while puromycin treatment results in the release of ribosomes from the RNA (Barton *et al.*, 1999). The increase in RNA production following removal of gua–HCl was totally blocked by the presence of either cycloheximide (Fig. 6a) or puromycin (Fig. 6b). Thus, pre-existing viral proteins present within the infected cells were insufficient to generate significant new RNA synthesis.

**DISCUSSION**

Guanidine is a useful tool for analysing the replication of picornaviruses since it can be used to separate the process of translation from RNA replication, although the amount of translation that proceeds in the absence of RNA replication is necessarily limited by the low level of viral RNA present within cells. Guanidine has also been central to identifying a role for the 2C protein within RNA replication since all mutations identified as conferring resistance to gua–HCl have been mapped to the 2C protein (see also below). However, the precise function of 2C in this process remains obscure.

The use of qRT-PCR has proved to be a rapid, simple and sensitive means of following both RNA replication and the appearance of guanidine-resistant mutants (Figs 3 and 4). Presumably this would also apply to the development of other drug-resistant variants too. The great change in sensitivity of SVDV to guanidine between days 1 and 3, demonstrated by the >1000-fold increase in virus RNA detected in the presence of this agent, made it quite straightforward to identify sequence changes within the 2C coding region. The changes detected within the SVDV genome predominantly encoded the substitutions A133T or D160A, the former change exactly corresponds to the substitution detected in guanidine-resistant mutants of echovirus 9 (Klein *et al.*, 2000) and has been detected in some guanidine-resistant mutants of PV (Tolskaya *et al.*, 1994). This substitution is within the Walker A motif (Gorbalenya *et al.*, 1990). It should be noted that residue 179 of the SVDV 2C protein (adjacent to the Walker B motif) is a cysteine (C) and thus is already different from the wild-type N residue in PV 2C, which is substituted by G or A in many guanidine-resistant mutants. In similar analyses using FMDV O-UAE, it was found that all 19 cDNA fragments corresponding to the FMDV 2C sequence encoded the amino acid substitution, M158L, which is within the Walker B sequence and adjacent to the highly conserved DDL motif. Thus, the sequence changes detected explain the changes in virus properties. It is not known if any of the additional amino acid substitutions (Tables 1 and 2) contribute to guanidine resistance. In the absence of a 3D structure for any of the picornavirus 2C proteins, the spatial relationship between the Walker motifs is not known and the relative location of other residues, e.g. D130 within the SVDV 2C protein, which also determine resistance to gua–HCl is undefined.

During the early stages of these studies, it was found that two closely related strains of SVDV, Itl 18/92 and Itl 3/97, differed significantly in their sensitivity to gua–HCl. The Itl 3/97 isolate produced significant CPE in the presence of 3 mM gua–HCl (albeit that the virus yield was inhibited), whereas the replication of the Itl 18/92 strain was...
completely blocked under these conditions. Sequence analysis of these virus strains has been undertaken (P. Normann & Soren Alexandersen, unpublished results) and only a single amino acid difference was found within the 2C coding regions, changing residue A96 to T. This substitution, which apparently confers partial resistance to gua–HCl, is not within a Walker motif.

Only one guanidine-resistant mutant of FMDV (strain O6) has been sequenced previously (Saunders et al., 1985). The mutation was identified as a U to C change within an oligonucleotide that corresponds to nt 4749–4769 within the FMDV O1K sequence (GenBank accession no. X00871). This encodes an amino acid substitution of Y238 to H rather than an M to T change that was misstated by Saunders et al. (1985). This substitution is clearly distinct from the change identified in FMDV O-UAE and is also outside of the Walker motifs.

The large increase in RNA replication that occurs following gua–HCl administration at 18–24 h post-infection demonstrated that the viral RNA is maintained in cells for at least 24 h. At later times, the appearance of guanidine-resistant viruses complicated the picture, in some cases, since a much reduced stimulation of viral RNA production was observed when gua–HCl was removed. The appearance of guanidine-resistant virus could occur by two different mechanisms, it could be de novo-generated mutants implying that a low level of RNA replication is occurring even in the presence of gua–HCl or else, more likely, the gua–HCl-resistant virus observed may reflect the multiplication of a small population of pre-existing guanidine-resistant virus variants present within the input virus.

There was a significant time delay (~3 h) between the removal of gua–HCl and the detection of new viral RNA synthesis (Figs 2 and 5). These results are consistent with the observations of Egger & Bienz (2005) within PV-infected cells, as detected by fluorescence in situ hybridization, but contrast with the very rapid initiation of RNA synthesis that occurs in cell-free replication systems following gua–HCl removal (Barton et al., 1995; Barton & Flanegan, 1997). It has been proposed that following removal of gua–HCl from infected cells the viral RNA has to be translated again before RNA synthesis can commence (Egger & Bienz, 2005). In the cell-free replication systems, guanidine is present during an active translation phase and RNA replication is only allowed to proceed when the guanidine is removed. By this time relatively high levels of viral proteins (including precursors) have accumulated since translation has been driven by a large input of viral RNA.

We explored the effects of cycloheximide and puromycin, inhibitors of protein synthesis, on the restoration of RNA synthesis following gua–HCl removal within infected cells (Fig. 6). Cycloheximide completely blocked the regeneration of RNA synthesis (Fig. 6a), in principle this could result from a blockade of RNA replication as well as inhibition of protein synthesis since ribosomes remain associated with the RNA in the presence of this drug (Barton et al., 1999), although Gamarnik & Andino (1998) showed that inhibition of protein synthesis with cycloheximide strongly stimulated RNA replication in their cell-free translation/replication system. However, puromycin, which causes the release of ribosomes from the RNA, also completely abolished the increase in RNA following removal of gua–HCl (Fig. 6b). Thus, there is a clear need for the synthesis of viral proteins before RNA replication can recommence within cells after removal of guanidine. This contrasts with the lack of requirement for protein synthesis, once replication complexes are formed, for RNA synthesis that is observed in the HeLa cell-lysate-based in vitro virus replication system (Barton et al., 1995, 1999). The requirement for de novo viral protein synthesis within cells may result from a need for certain precursor proteins, e.g. 3CD. This precursor (and possibly others) have unique roles in RNA synthesis (Paul, 2002) but might be expected to decay over time to the mature products within infected cells, which only contain fairly low levels of viral proteins (in the presence of gua–HCl). It is interesting to note that even after RNA synthesis is well established (i.e. 3 h after removal of gua–HCl) the readddition of gua–HCl still resulted in a strong reduction (at least 64-fold, Fig. 5) in the amount of viral RNA production achieved after 6 h. Thus, the gua–HCl-sensitive step in viral RNA replication is clearly still required at this stage of the virus life cycle within cells. These observations raise the issue of the mechanism by which the viral RNA is programmed to re-enter a translation phase following the removal of guanidine.

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REFERENCES


