The family Picornaviridae includes important pathogens of humans and farm animals, such as poliovirus (an enterovirus), human rhinoviruses and foot-and-mouth disease virus (FMDV, an aphthovirus). Swine vesicular disease virus (SVDV), another enterovirus, is related closely to the human coxsackievirus B5 (Graves, 1973; Inoue et al., 1989; Zhang et al., 1999) and can cause a disease in pigs that is very similar clinically to foot-and-mouth disease (Dekker, 2000; Lin & Kitching, 2000). However, attenuated strains of SVDV have been isolated from apparently healthy pigs (Kodama et al., 1980). Infectious cDNA clones of SVDV, corresponding to the pathogenic strain J1/73 (termed J1) and the attenuated strain H/3/76 (termed 00) from Japan, have been derived (Inoue et al., 1989, 1990, 1993). By using these plasmids, chimeric genomes were produced and the key determinant of virulence was mapped to the coding region for 1D–2A (Kanno et al., 2001). Within this region, residue 20 of the 2A protease is particularly significant. Inoculation of pigs with mutants of strain 00 containing single amino acid substitutions at this residue leads to the appearance of revertants, often containing an arginine at this position encoded by an AGA codon, one of six codons for this residue. The properties in pigs of two chimeric viruses, each with an arginine residue at this position but encoded by different codons, have been investigated in parallel with the parental pathogenic and attenuated strains. Presence of the arginine residue, but not of the AGA codon, is essential for induction of high viraemia and appearance of significant disease.

Pathogenic and attenuated strains of swine vesicular disease virus (SVDV), an enterovirus, have been characterized previously and, by using chimeric infectious cDNA clones, the key determinants of pathogenicity in pigs have been mapped to the coding region for 1D–2A. Within this region, residue 20 of the 2A protease is particularly significant. Inoculation of pigs with mutant viruses containing single amino acid substitutions at this residue leads to the appearance of revertants, often containing an arginine at this position encoded by an AGA codon, one of six codons for this residue. The properties in pigs of two chimeric viruses, each with an arginine residue at this position but encoded by different codons, have been investigated in parallel with the parental pathogenic and attenuated strains. Presence of the arginine residue, but not of the AGA codon, is essential for induction of high viraemia and appearance of significant disease.

The nature of residue 20 within the 2A protease influences the biochemical activities of this protein (Sakoda et al., 2001; Inoue et al., 2005). Residue 20 is adjacent to His21, one component of the catalytic triad of this protease. In the J1 strain, residue 20 is an arginine (R) specified by an AGA codon, whereas in the 00 strain, this residue is an isoleucine (I, from an AUA codon). Studies using just the 1D–2A coding region showed that the 1D–2A proteins from both viruses were self-cleaved efficiently to produce 1D and 2A. However, the 00 strain 1D–2A was defective, compared with the J1 1D–2A, at inducing cleavage of the translation initiation factor eIF4GI and at stimulating the activity of enterovirus internal ribosome entry site (IRES) elements (Sakoda et al., 2001). The cleavage of eIF4GI, a scaffold component of the cap-binding complex eIF4F (reviewed by Gingras et al., 1999), results in inhibition of cellular cap-dependent protein synthesis. Translation initiation on the picornavirus RNA is dependent on the activity of the IRES (reviewed by Belsham & Jackson, 2000). Modification of I20 to R20 within the 00 1D–2A coding region was sufficient to stimulate both eIF4GI cleavage and IRES activation to levels similar to those observed with the J1 strain (Sakoda et al., 2001). Thus, a key attenuating mutation within the 2A protease has a critical role in determining the pattern of protein synthesis within SVDV-infected cells.

Residue 20 of the SVDV 2A protease (J1 strain) has been changed to each of the other amino acids and the effect of these substitutions on the activities of the 1D–2A was determined (Inoue et al., 2005). The most active mutants were reconstructed into a full-length cDNA. Viable viruses were rescued containing residue 20 of 2A as L, W, R, (AGA codon), I, K, E and V. In cell culture, the mutants R(CGC) and W grew about as well as the wild-type (J1) virus, whereas mutants I, K and V were less fit. In pigs, each of the mutants tested (W, I, K and V) replicated and viraemia was observed in each case (albeit at much lower levels than
with the J1 virus), but the mutants produced little disease, although neutralizing antibodies were produced (Inoue et al., 2005). Unexpectedly, SVDV recovered from the inoculated pigs frequently contained modifications at the codon for residue 20 within the 2A protease to regenerate an AGA codon. As six codons encode an R residue, this seemed like a strong bias operating at the level of the RNA sequence. To test the significance of this observation, we have now compared two viruses each encoding an R at residue 20 but with different codons, either AGA (as in J1) or CGC [as in the R mutant described previously (Inoue et al., 2005)].

It has been shown that the J1 and 00 viruses grow with similar kinetics in porcine cells in tissue culture (Kanno et al., 1999); furthermore, the R(CGC) mutant virus (Fig. 1) has also been shown to grow with similar kinetics to J1 (Inoue et al., 2005). However it should be noted that the 00 virus produces small plaques, whereas the J1 and R(CGC) viruses display a large-plaque phenotype (Kanno et al., 1999; Inoue et al., 2005). The R(AGA) virus (Fig. 1) was prepared exactly as described previously for other mutants (Inoue et al., 2005). As expected, this virus grew well in porcine cells and displayed a large-plaque phenotype (data not shown).

We have now compared the properties of these viruses in pigs in parallel with the attenuated 00 virus and the virulent J1 virus. Seventeen Landrace cross-bred Large White pigs (between 20 and 30 kg) were housed in biosecure animal accommodation and were divided into five groups in individual boxes as follows: pigs VM36–37, uninfected (control); pigs VM39–41, inoculated with SVDV 00; pigs VM42–45, inoculated with SVDV J1 (wild type); pigs VM46–49, inoculated with SVDV 2A-20 R(CGC); pigs VM50–53, inoculated with SVDV 2A-20 R(AGA).

Pigs were inoculated with 10^7 p.f.u. virus (as measured in SLK cells) subdermally in the heel pad of the left forefoot essentially as described previously (Inoue et al., 2005). The animals were examined for signs of swine vesicular disease and rectal temperatures were recorded daily until 9 days after inoculation (p.i.). The clinical signs were scored as described previously (Inoue et al., 2005); each animal could have a maximum score of 26 points. None of the animals showed a significant fever at any time. All four animals inoculated with the J1 virus showed typical signs of disease, including the generation of severe lesions on most feet from day 2 p.i. (Fig. 2a). In contrast, the three animals inoculated with the 00 strain showed no signs of disease, as expected (Fig. 2d). The animals that received the two chimeric viruses showed a similar picture to each other, which seemed intermediate between those of the 00 and J1 viruses (Fig. 2b, c). In both groups, all four animals showed clinical disease starting from 2 days p.i. The scores for individual animals inoculated with these two viruses are shown in Fig. 2(b, c). The two control animals that received no virus showed no signs of disease. A summary showing mean clinical scores for all groups of animals is presented in Fig. 2(d).

Blood samples, together with nasal and rectal swabs, were taken immediately before inoculation (day 0) and then on days 1, 2, 3, 4, 7 and 9 p.i., as described previously (Inoue et al., 2005). When the animals were killed, at 11 days p.i., samples of epithelium from the feet and tongue were collected into RNAlater (Ambion) and stored at −20 °C. RNA extraction from the samples was performed as described previously (Alexandersen et al., 2003; Zhang & Alexandersen, 2003). Quantitative real-time RT-PCR (qRT-PCR) was used to determine the amount of SVDV RNA in these samples by using the 2B–IR primer/probe set (Reid et al., 2004; Inoue et al., 2005).

The levels of SVDV RNA in serum samples taken during the experiment are shown in Fig. 3(a, b). No viraemia was
detected in uninfected animals or those inoculated with the 00 virus (Fig. 3a). In contrast, all animals (except VM49) inoculated with the J1, R(AGA) or R(CGC) viruses displayed high levels of viral RNA within the serum (Fig. 3a, b). Viraemia was observed only between days 1 and 3 p.i. and generally lasted for 2 days, but in animal VM48, viraemia was observed for three successive days. There was no apparent difference between the levels of viral RNA detected in these three groups. These results indicated that significant replication of these three viruses occurred within the inoculated animals. In contrast, the 00 virus appeared unable to replicate or spread around the animal.

Samples of tongue and foot epithelium were obtained at the termination of the experiment. In addition, nasal- and rectal-swab samples were collected during the course of the experiment. RNA was extracted from these samples and analysed for the presence of SVDV RNA by using qRT-PCR as described above. For clarity, the results are presented in a qualitative manner in Fig. 3(c). No significant level of SVDV RNA was detected in any of the samples taken from pigs inoculated with the 00 virus or from uninfected animals. However, all of the animals inoculated with J1 and the chimeric viruses showed the presence of viral RNA in these samples and no major differences in the distribution of viral RNA were apparent.

To confirm that the viruses replicating within the inoculated pigs corresponded to the input virus and to check for signs of selection for mutant viruses, cDNA from tissue or serum samples (as used in the qRT-PCR) was used in a conventional PCR to amplify the SVDV 2A coding region, which was then sequenced. From each animal, the codon for residue 20 of the 2A protease in the SVDV RNA matched the expected sequence for the inoculated virus. It is apparent that the 00 virus is unable to replicate significantly within pigs, even though it grows well within porcine cells in tissue culture, albeit producing small plaques (Kanno et al., 1999). In previous studies, it was shown that this virus did not cause clinical disease, but no analysis of virus replication within the inoculated animals was performed. The failure to produce viraemia (Fig. 3a) suggests a severe block on virus replication in vivo, which presumably underlies the lack of clinical disease. This result is consistent with the low production of anti-SVDV antibodies in pigs inoculated with this virus (Kanno et al., 1999). It is not clear how such a virus is maintained within

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**Fig. 2.** SVDV pathogenicity in experimentally infected animals. Pigs were inoculated with the indicated viruses (10^7 p.f.u.) and monitored during the following 9 days for signs of disease. (a–c) Clinical scores for individual animals inoculated with the J1 virus (a) or the chimeric viruses R(CGC) (b) and R(AGA) (c) are shown. In (d), the mean clinical scores for each of these groups are shown [▲, J1; △, R(CGC); ●, R(AGA)], together with the results from the uninfected animals (×) and those inoculated with the 00 virus (□). The drop in scores observed towards the end of the experimental period is due to healing of the lesions.
a population of animals. Potentially, it may co-exist with other pathogenic viruses. If the reduced ability of the 2A protease from the 00 strain to induce cleavage of eIF4GI results in failure to block production of interferon, then this may normally inhibit virus replication. However, if infection occurred concurrently with another virus that did block interferon synthesis (or function), then perhaps the 00 strain could also replicate within the same cells.

As observed previously (Kanno et al., 1999; Inoue et al., 2005), the J1 virus induced extensive disease; the mean clinical score reached about 20 in this study. The viruses R(AGA) and R(CGC) also induced significant clinical disease; however, it appeared less severe than that observed with the J1 virus, with mean clinical scores reaching about 12 in each case, but whether this difference is truly significant or merely reflects biological variation is uncertain. The distribution of virus in animals inoculated with the J1 and chimeric viruses was very similar. The clinical scores observed with R(AGA) and R(CGC) are much higher than those observed using viruses in which residue 20 of the 2A protease had been changed to V, K, I or W (Inoue et al., 2005); these each had peak clinical scores of <5. Thus, the R residue is critical for efficient replication in pigs.

In earlier studies, there had been reversion of residue 20 from mutant viruses back to R encoded by an AGA codon (Inoue et al., 2005), as in the J1 and other pathogenic isolates of SVDV (plus other enterovirus B isolates). However, we have not detected any difference between the presence of an AGA or a CGC codon, which each encode an R residue. Altogether, six codons specify an R residue; some of these other codons may be less well tolerated. Four of the six codons, including CGC and AGA, are used about 20% of the time (in humans), whereas CGU and CGA are only used about 10% of the time. It is noteworthy that two recent studies have shown that large-scale codon deoptimization in poliovirus causes severe attenuation (Burns et al., 2006; Mueller et al., 2006). The presence of important RNA structures, as exemplified by the cis-acting replication element (cre) (reviewed by Paul, 2002), within the coding sequence of the virus can clearly induce a strong

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**Fig. 3.** SVDV distribution within infected pigs. (a, b) Blood samples were collected on days 0, 1, 2, 3, 4, 7 and 9 p.i.; levels of SVDV RNA within the serum were determined and are presented as log_{10}(genome copies ml^{-1}). For clarity, when no significant viral RNA was detected (Ct > 45), then the concentration of RNA was set at 1 copy ml^{-1} (log_{10} 1 = 0). (c) Tissue samples from the tongue (T) and foot epithelium (E), collected at the termination of the experiment (11 days p.i.), plus nasal (NS) and rectal (RS) swabs, collected during the course of the experiment, were analysed for SVDV RNA by qRT-PCR. When significant levels of SVDV RNA (Ct < 45 in each case) were detected in each sample type, a score of 1 was awarded; hence, for each animal, a maximum score of 4 was achievable.
bias for conservation of RNA sequence in addition to requirements for conservation of amino acid sequence.

It remains unclear why the presence of an R residue at position 20 of the 2A protease has such a strong influence on the pathogenicity of SVDV. Alternative residues, e.g. tryptophan (W), can produce a 2A protease with very similar biochemical properties and a virus that grows about as well as the J1 virus in tissue culture (Inoue et al., 2005). However, this virus was much less fit within infected animals. It is quite common for viruses to be maintained within their host without causing apparent disease. The 00 strain was isolated from apparently healthy animals, but the complete inability of the 00 strain of SVDV to induce viraemia and the absence of detectable virus excretion point to the complexity of virus maintenance within the animal population.

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