Mutation of the GKS motif of the RNA-dependent RNA polymerase from potato virus X disables or eliminates virus replication

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The RNA-dependent RNA polymerase (RdRp) of potato virus X (PVX) contains a glycine-lysine-serine (GKS) motif. This motif is present in the replication enzyme of many RNA viruses and is thought to be required for nucleoside triphosphate-binding. Three single amino acid changes, glycine to alanine (AKS), lysine to asparagine (GNS) and lysine to glutamate (GES) within the GKS motif of the PVX RdRp were tested for their effect on PVX accumulation. The GNS and GES mutations rendered the virus unable to accumulate in either tobacco plants or protoplasts, whereas substitution of glycine with alanine had only a minor effect on accumulation of PVX. The glycine to alanine mutation reverted to wild-type after passage on Nicotiana clevelandii plants. These findings suggest that the GKS motif is required for PVX replication and that strong selection pressures are active to maintain necessary sequences of the viral RdRp.

The RNA-dependent RNA polymerase (RdRp) of potato virus X (PVX) has several conserved sequence motifs characteristic of RdRp enzymes (Koonin & Dolja, 1993) from animal, plant and bacterial viruses. One such sequence, a glycine-aspartate-aspartate (GDD) motif, is the likely active site of the RdRp and is essential for virus replication (Inokuchi & Hirashima, 1987; Kroner et al., 1989). The RdRp is an RNA-dependent RNA polymerase from potato virus X (PVX) contains a glycine-lysine-serine (GKS) motif. This motif resembles the ‘A’ site of the putative nucleoside triphosphate (NTP)-binding motif (NTBM) (Walker et al., 1982). Cowpea mosaic virus (CPMV) and poliovirus mutant genomes encoding modified forms of the GKS motif are unable to accumulate in isolated cells (Mirzayan & Wimmer, 1992; Teterina et al., 1992; Peters et al., 1994), suggesting that the motif is essential for the replication of these viruses. In some viruses this GKS motif may be required for RNA helicase activity, which is necessary to unwind duplex RNA formed during virus replication (Gorbalenya et al., 1988; Hodgman, 1988).

To determine whether the GKS motif is essential for PVX replication three amino acid substitutions were introduced into the GKS motif of the PVX RdRp. The glycine to alanine change would be unlikely to disturb the tertiary structure of the RdRp (Bordo & Argos, 1991) but would disable replication of PVX if the glycine residue is important for NTP-binding. Lysine to asparagine or glutamate substitutions, which replace a basic residue for either an uncharged residue or an acidic residue, could have more profound effects on the structure of the RdRp. If the GKS motif is a nucleotide-binding site, substitution of the lysine residue would interfere with the interaction between the protein and the negatively charged phosphate group of an NTP (Logan & Knight, 1993; Story et al., 1993; Konola et al., 1994).

Single point mutations, encoding one of the three amino acid substitutions described above, were introduced into the RdRp open reading frame of the PVX cDNA. These mutations were a guanine to cytosine substitution at nucleotide position 2303 (glycine to alanine, AKS), an adenine to cytosine at position 2307 (lysine to asparagine, GNS) and an adenine to guanine at position 2305 (lysine to glutamate, GES). The mutations were introduced by PCR mutagenesis (Yao et al., 1992), using two primers for each mutation, into plasmid pXHA which is a 540 bp HindIII–Asp718I restriction fragment derived from the PVX cDNA clone (pTXX, positions 2247 to 2787) (Kavanagh et al., 1992) cloned into Bluescript KS(−) (Stratagene). One of the primers used had identity to the sense strand of the plasmid from nucleotide position 2291 to 2319 within the viral sequence, whereas the other primer had identity to the complementary strand from position 2290 to 2318. The primer sequence was identical to the plasmid target sequence but contained one of the three point mutations described above. The sequences of the primers were G740A + (5′ cttggagttctgaaagctatgctc 3′) and G740A − (5′ atgctatgctttttgcgacactc 3′) for the AKS mutation, K741N + (5′ cttggagttctgaaagctatgctc 3′) and K741N − (5′ atgctgatgctttttgcgacactc 3′) for the GNS mutation.
To analyse the effects of the GKS mutations on PVX accumulation in single cells, transcripts of pTXS, pTXS-AKS, pTXS-GNS and pTXS-GES were inoculated to protoplasts of N. tabacum cv. Samsun NN. RNA blot analysis confirmed that PVX-GNS and PVX-GES did not accumulate and revealed that PVX-AKS accumulated more slowly than the wild-type PVX (Fig. 2). The failure of PVX-GES and PVX-GNS to accumulate in protoplasts or intact plants shows that mutation of the lysine residue of the GKS motif inactivated the PVX RdRp. This effect, also reported for mutation of the GKS motif lysine residue in the CPMV 5kDa protein (Peters et al., 1994) and the poliovirus 2C protein (Mirzayan & Wimmer, 1992; Teterina et al., 1992), is consistent with the proposed function of this amino acid in NTP-binding (Bradley et al., 1987; Story & Steitz, 1992; Story et al., 1993).

The interpretation of the effects of the glycine to alanine mutation in PVX-AKS is less clear cut. From the data in Fig. 2 and a replicate experiment it is estimated that the accumulation rate of PVX-AKS RNA was 30 to 50% of that of wild-type PVX RNA. This slower accumulation of viral RNA could indicate that the AKS mutation leaves the PVX RdRp with 30 to 50% of the activity of the wild-type enzyme. However, it is also possible that the mutation disables the RdRp to a much greater extent but that the mutation reverts to the wild-type in the accumulated viral RNA. To distinguish between these alternative explanations the sequence of the progeny RNA of PVX-AKS was determined in samples taken from an inoculated leaf at 7 days p.i. The RNA of the progeny virus was amplified by RT–PCR using primers TXS2828 (5′ tcggaggctggagaagatcatgccatc 3′) and TXS2014 (5′ atggcatgttctcagactctacg 3′), representing nucleotides 2828 to 2852 on the negative strand. The PCR products were cloned from the mutant derivatives of pXHA (pXHA1–3) containing the AKS, GNS and GES mutations were inserted into pTXS to produce plasmids pTXS-AKS, pTXS-GNS and pTXS-GES, respectively.
confirmed that the sequence was that of the progeny RNA and not that of the residual inoculum or of plasmid DNA in the inoculum. In this experiment four *N. clevelandii* plants were inoculated with equal amounts (5 µg or 0.05 µg) of pTXS-AKS transcripts and non-infectious pTXS-GES transcripts. Only the AKS mutation, not the GES mutation, was detected by sequence analysis in the RT–PCR products from either inoculated or systemically infected leaves of these plants. Thus, the accumulation of PVX-AKS indicates that glycine in the GKS motif was not essential for the activity of the PVX RdRp.

Mutations in the glycine residue have not been reported in the RdRp GKS motifs of other RNA viruses. However, mutation of the glycine residue to valine in a similar (GKT) motif abolished the cytotoxicity of the B19 parvovirus with a DNA genome (Momoda et al., 1994). Furthermore, the *E. coli* RecA protein was completely inactivated by mutation of the glycine residue to either aspartate or valine in the GKT motif (Logan & Knight, 1993; Konola et al., 1994). These findings, together with the fact that glycine is highly conserved in NTBM-containing proteins (Saraste et al., 1990; Koonin & Dolja, 1993), suggest that glycine is important for the function of the motif. The finding that there was a substantial residue of RdRp activity in PVX-AKS can be explained if the glycine residue has only a structural role and does not participate directly in the nucleotide interaction. An alanine mutation would perturb the protein secondary structure to a relatively minor extent (Bordo & Argos, 1991).

A second experiment was designed to determine whether the AKS mutation in PVX-AKS would revert to the wild-type GKS under strong selection pressure for efficient replication due to the use of dilute inocula. *N. clevelandii* plants were inoculated with transcripts (10 µg) of pTXS-AKS diluted in a series of 10-fold dilutions up to 100,000-fold. A sap inoculum for secondary passage was prepared at 7 days p.i. from the plant that became infected with the most dilute inoculum (1000-fold). This inoculum was applied to *N. clevelandii* plants at 10-fold dilutions up to 100,000-fold and a total of three passages was carried out in the same way. The inoculum at each passage was taken from the plant infected with the most dilute inoculum. RNA was extracted after each passage from inoculated and systemically infected leaves and cDNA of the accumulated PVX RNA was produced by RT–PCR. Direct sequence analysis of the PCR products (Fig. 3) revealed that PVX-AKS was the predominant PVX RNA in the inoculated leaves of the transcript-inoculated plants but that in the systemically infected leaves and plants infected with the passaged inoculum there were PVX genomes with wild-type identity at residue 2303 (guanine residue) mixed with AKS (cytosine residue) and PVX mutants with adenosine (PVX-EKS) or uridine (PVX-VKS) at this position. The wild-type PVX was the only form detected in the third passage of the inoculum. There were no other sequence changes between nucleotide positions 2236 and 2535 of the PVX cDNA. In a similar experiment using high titre inocula the revertant wild-type virus (PVX-GKS) was detected after three passages together with the mutant PVX-AKS. The greater stability of PVX-AKS in this experiment suggested that the selection pressure was less than in plants inoculated with dilute virus.
This reversion of the mutation in PVX-AKS illustrates the power of selection as a means of eliminating genetic drift in viral genomes. A practical corollary to this observation concerns the identification of neutral mutations in viral genomes. Clearly, a test of site-directed virus mutants at high titre and after a single passage is not sufficient to rule out an effect of a mutation in essential virus functions.

We are grateful to Julie Gilbert and Cinza Dedi for the preparation and electroformation of tobacco protoplasts and to Patrick Bovill for operating the ABI PRISM 377 sequencer and the Expedite oligonucleotide synthesizer (PerSeptive Biosystems). The Sainsbury Laboratory is supported by the Gatsby Charitable Foundation.

References


Received 20 December 1996; Accepted 3 March 1997