Characterization of a potato leafroll luteovirus subgenomic RNA: differential expression by internal translation initiation and UAG suppression

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Northern blot analysis of Solanum tuberosum infected with potato leafroll luteovirus revealed the 6 kb genomic RNA and a major 2.3 kb subgenomic RNA. The 5' end of the subgenomic RNA was located at nucleotide 3653 in an intergenic region located at the centre of the viral genome upstream of three open reading frames (ORFs). Transient expression in tobacco and potato protoplasts of the β-glucuronidase reporter gene fused to various putative regulatory sequences present in the subgenomic RNA was used to study their influence on expression levels. We observed a suppression of the amber stop codon separating the coat protein (CP) gene from a downstream ORF (56K protein), to a level of 0.9% to 1.3%. Translation initiation at the AUG of an ORF (17K protein) which is nested within the CP gene, exceeds translation of the CP gene itself by a factor of 7.

Introduction

Luteoviruses are transmitted by aphids in a persistent manner and replicate tissue-specifically in the phloem of infected plants (for a review see Francki et al., 1985). The icosahedral virus particles contain a single-stranded 6 kb RNA of positive polarity, which is not polyadenylated and possesses a genome-linked small protein (VPg) (Mayo et al., 1982; Harrison, 1984). Recently, cloning and nucleotide sequence analysis have been described for the RNAs of three luteoviruses, barley yellow dwarf (BYDV) (Miller et al., 1988), beet western yellows (BWYV) (Veidt et al., 1988) and potato leafroll (PLRV) (Mayo et al., 1989; van der Wilk et al., 1989; Keese et al., 1990).

The 3' halves of the luteovirus genomes contain a cluster of three genes conserved with respect to sequence and arrangement, whereas the 5' halves show greater divergence of sequences. The PLRV genome contains six open reading frames (ORFs) (Fig. 1). Several strategies for their expression have been suggested. ORFs located in the 5' half of the genome are thought to be translated from genomic RNA. For the expression of the putative RNA polymerase (ORF 2b), Mayo et al. (1989) have suggested that translational frameshift occurs during translation of the overlapping ORF 2a. Results of in vitro translation studies with genomic PLRV RNA (Mayo et al., 1982, 1989) suggest that the 3'-located gene cluster is not expressed by internal translation initiation of genomic RNA.

Methods

Preparation of total RNA. Material was harvested from stems and leaves of Solanum tuberosum plants infected with a PLRV field isolate (Sarkar, Hohenheim, F.R.G.). Plant tissue (10 g) was homogenized in 60 ml of a two-phase system consisting of equal volumes of phenol and lysis buffer (1% Triton X-100, 50 mM-MOPS pH 7.0, 50 mM-EDTA, 2 M-urea, 1% 2-mercaptoethanol). After extraction, the aqueous phase was adjusted to 350 mM-NaCl and passed through an ion-exchange column (QIAGEN-pack 100; QIAGEN) equilibrated in buffer A (400 mM-NaCl, 50 mM-MOPS pH 7.0, 15% ethanol). The column was washed with buffer A to remove proteins and polysaccharides. RNA was eluted with a buffer containing 1050 mM-NaCl, 50 mM-MOPS pH 7.0, 15% ethanol, 2 M-urea and precipitated by adding 0.8 volumes of isopropanol.
of 8 tag of total plant RNA or 8 ktg of pCPL1 (Tacke (8 x 10^6 c.p.m, each) were glyoxalated, either alone or after addition (cRNA) was obtained as described above. Identical amounts of cRNA were isolated and ligated in the sense orientation into the transcription vector pSP65. Hybridization was as described above except that 0-2 mg/ml RNase was included in the washing solution. Several stranded riboprobes detecting negative sense (probe 5+) or positive sense (probe 5-) PLRV sequences.

Transcription initiation mapping. Total RNA (100 µg) was annealed at 47 °C for 20 h to 50 ng of a 361 bp PstI–BssHII fragment (co-ordinates 3417 and 3777) which was 5'-labelled at the BssHII site. Annealing conditions were as described by Weaver & Weissmann (1979). Nucleic acids were precipitated with ethanol and dissolved in 500 µl of 30 mM-sodium acetate pH 4.6, 50 mM-NaCl, 1 mM-ZnCl2, 0.1 µg/ml bovine serum albumin (BSA). Unhybridized single-stranded nucleic acids were degraded by digestion with 400 units of mung bean nuclease for 2 h at 30 °C. After ethanol precipitation, the RNA was hydrolysed in 2 N-m NaOH at room temperature for 3 h. The solution was neutralized with acetic acid and precipitated with ethanol. The reaction products were analysed on 8% sequencing gels (Garoff & Ansorge, 1981) and detected by autoradiography.

For determination of the transcriptional start point by a primer extension experiment, 50 ng of the 5'-32P-labelled oligodeoxynucleotide 5' TACACcACCTTGAACATTTCCTTTAACCAGC 3' was annealed with 100 µg of total RNA as described above. The nucleic acids were precipitated, resuspended in 300 µl of 50 mM-Tris-HCl pH 8.3, 50 mM-KCl, 6 mM-MgCl2, 100 µM of each dNTP, 5 mM-DTT, 30 µg/µl actinomycin D and 50 µg/ml BSA and incubated for 1 h at 42 °C with 200 units of murine leukaemia virus reverse transcriptase. After alkaline hydrolysis (see above), the elongation products were analysed on a 15% sequencing gel (Garoff & Ansorge, 1981).

Chimeric PLRV/GUS constructs. Constructs of the β-glucuronidase (GUS) gene from E. coli (Jefferson et al., 1986) translationally fused to the AUG start codons of the CP gene and 17K ORF, respectively, were prepared as follows. A SacI–BstHII fragment (co-ordinates 3503 and 3777) was isolated from the cDNA clone pCPL1 (Tacke et al., 1989). The 270 bp fragment, forming part of the intergenic region and the initial coding sequences for both CP and 17K, was treated with mung bean nuclease or the Klenow fragment of DNA polymerase, and translationally fused to the GUS gene by insertion into the Neo-restricted and mung bean nuclease-treated vector pRT103GUS (Töpfer et al., 1988).

For chimeric constructs of GUS with PLRV sequences surrounding the UAG stop codon between the genes for the CP and the 56K protein, an Alul–BsrNI fragment, containing the CP gene and additional 218 bp upstream and 21 bp downstream sequences, was isolated from the cDNA clone pCPL1 (Tacke et al., 1989; co-ordinates 3500 to 4433). This fragment was cloned into the vector pRT103GUS between the cauliflower mosaic virus (CaMV) 35S promoter and the GUS gene. The polymerase chain reaction (PCR) was used for primer-dependent in vitro mutagenesis. The upstream primer (UP; antisense strand orientation; Fig. 2) of the primer extension experiment (see above) and the downstream primer (DP; sense strand orientation) 5' GCTTGTGAAACCCACAAUAGGUAGAACCUCGGAU 3' were employed with circular plasmid DNA as a template, in a PCR in order to delete the central part of the CP gene. A second downstream primer, in which the UAG stop codon was replaced by the GCC codon for alanine, served in a subsequent PCR mutation experiment to replace the amber stop codon. The integrity of all constructs obtained was verified by sequence analysis. From this analysis, a construct was identified which contained an extra nucleotide (T) at the junction of UP- and DP-initiated cDNA which resulted in a frameshift mutation.

Ribosomal RNA binding assay. The EcoRI inserts of cDNA clone pCPL1 (Tacke et al., 1989; PstI–BssHII co-ordinates 3498 to 4325) of the cDNA clone pCPL1 (Tacke et al., 1989) in the sense (clone 5+) and antisense (clone 5-ø) orientations into the transcription vector pSP65. Hybridization was as described above except that 0-2 µg/ml RNase was included in the washing solution.

Northern blot analysis. Samples of 10 µg of total RNA were glyoxalated, separated by electrophoresis in a 1.2% agarose gel, transferred to nitrocellulose filters and baked for 2 h at 80 °C (Thomas, 1980). Virus-specific hybridization probes were prepared by isolating DNA fragments from the PLRV cDNA clones pCPL1 and pCPL2 (Tacke et al., 1989) after restriction with appropriate endonucleases (EcoRI, 2173 bp fragment 1, co-ordinates 640 to 2812 according to the published PLRV sequence of Mayo (1989); van Ansorge, 1981) and detected by autoradiography. Additionally, an rRNA-specific probe was prepared as described by Feinberg & Vogelstein (1983, 1984). The DNA was transferred to nitrocellulose filters by vacuum blotting and hybridized at 68 °C in 2 x SSPE, 1% SDS, dried and exposed at -70 °C. Filters with no hybridization signals (Fig. 2b, lane 3) were reused without prior elution and labelled to high specificity as described by Feinberg & Vogelstein (1983, 1984). Single-stranded 32P-labelled RNA probes were obtained after isolation of a 3.6 kb BamHI fragment, clone pTA250.2, carrying the 18S and 26S rDNA sequences of wheat (Appels & Dvorak, 1982).

After hybridization in 3 x SSPE (450 mM-NaCl, 30 mM-Na2HPO4, 3 mM-EDTA), 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone 350, 0.1% SDS at 68 °C for 20 h, the filters were washed twice for 15 min at 68 °C in 2 x SSPE, 1% SDS, dried and exposed at -70 °C. Filters with no hybridization signals (Fig. 2b, lane 3) were reused without prior treatments for prehybridization and hybridization.

Single-stranded RNA probes (riboprobes) were obtained by in vitro transcription of appropriate constructs in the SP6 vector system (Melton et al., 1984) by SP6 RNA polymerase in the presence of [α-32P]CTP. The construction of transcription vectors was performed by cloning an 828 bp SacI–AciI fragment (PLRV co-ordinates 3498 to 4325) of the cDNA clone pCPL1 (Tacke et al., 1989) in the sense (clone 5+) and antisense (clone 5-ø) orientations into the transcription vector pSP65. Hybridization was as described above except that 0-2 µg/ml RNase was included in the washing solution.

Transcript expression assay. Protoplasts were isolated from S. tuberosum (cv. Desireé) and Nicotiana tabacum (SR1), and Ca(NO3)2/polyethylene glycol-mediated DNA transfer was performed as described by Negrutiu et al. (1987), using 3 x 10^6 protoplasts and 10 µg of plasmid DNA per transfection experiment. Activity of GUS was determined by a fluorimetric assay (Jefferson, 1987).
Results

Characterization of viral RNAs

Total nucleic acids were isolated from PLRV-infected potato plants and purified by ion-exchange chromatography. Northern blot analysis was performed after electrophoretic separation of glyoxalated RNAs by hybridization with 32p-labelled cDNA probes specific for different regions of the viral genome (Fig. 2). All probes detected the genomic 6 kb RNA (Fig. 2a, upper arrowhead). Probe 3, covering the non-coding intergenic region, revealed a virus-specific RNA with an apparent size of about 2-6 kb, whereas hybridization with a probe to the more 5' located ORF 2b (probe 2) did not react with RNA of that size. With probe 4, carrying sequences 3' distal to the internal region, the 2-6 kb signal was stronger than that obtained with probe 3. This RNA was also detected when a labelled oligodeoxynucleotide, specific for the 3' end of genomic RNA, was used for hybridization analysis (data not shown). The subgenomic RNA was apparently not polyadenylated as it did not bind to oligo(dT)-cellulose. This large subgenomic RNA, the existence of which has already been mentioned by Mayo et al. (1989), will be referred to in the following as subgenomic RNA 1 (sgRNA 1).

sgRNA 1 was further characterized by hybridization with single-stranded riboprobes (probes 5+ and 5- in Fig. 2b). Only 32P-labelled transcripts of antisense orientation (probe 5-) showed hybridization to RNA from PLRV-infected plants, (lanes 1 and 2) indicating that sgRNA 1 is single-stranded and of positive-sense orientation. The specificity of this probe for PLRV sequences was shown in a Northern blot experiment in which the antisense riboprobe (5-) did not react with total RNA from healthy plants (lanes 3 and 4). The integrity of the plant RNA was proven by rehybridizing the filter with a probe for ribosomal sequences (lane 4, -PLRV).

In some Northern blot experiments additional hybridization signals smaller than 2 kb were detected e.g. by probe 5- (Fig. 2). As the genomic 6 kb RNA was clearly visible in this experiment, we can rule out degradation of the RNA sample prior to electrophoretic separation. In order to test whether these signals were artefacts due to comigration of PLRV RNA with excess cellular RNA, a 32P-labelled single-stranded sense RNA (sRNA), representing 2-5 kb of the 3' half of the viral genome, was synthesized in vitro. After addition of total RNA from healthy plants or E. coli tRNA, these mixtures, as well as pure sRNA, were glyoxalated and electrophoresed in an agarose gel. In all three lanes, a band corresponding to sRNA is visible (Fig. 2c, arrow), with additional smaller RNA of undefined size at the bottom, presumably due to incomplete RNA synthesis or partial degradation. In lane 1, additional bands were detected that are comparable in size to ribosomal RNAs (visualized by a toluidine-stained gel of total plant RNA; Fig. 2c, lane T). This indicates that some of the PLRV sRNA comigrates with...
ribosomal RNAs under the conditions employed. Such comigration of viral RNAs with excess ribosomal RNA was also observed during Northern blot analysis of total RNA extracts from tobacco mosaic virus (TMV)-infected plants (Zaitlin et al., 1983).

Transcriptional start analysis for sgRNA 1

To determine the position of the transcriptional start of sgRNA 1, total RNA from PLRV-infected potato plants was hybridized to a 32P-labelled 365 bp BssHII/PstI fragment representing the intergenic region of the viral genome. The resulting RNA/DNA hybrids were treated with mung bean nuclease and the protected labelled DNA was analysed on sequencing gels with appropriate size markers (Fig. 3). Fragments of 125, 126 and 130 nucleotides in size were apparent after autoradiography. Similar heterogeneity in protection experiments has been reported for other systems (see e.g. Sommer & Saedler, 1986).

To characterize further the start of transcription, a primer extension experiment was carried out using a 32P-labelled primer complementary to sequences in the 5' terminus of ORF 3. This was hybridized to total RNA and the template/primer complex was incubated with reverse transcriptase and deoxynucleoside triphosphates. Gel electrophoresis revealed an elongation product of 82 nucleotides in length. This corresponds to the 125 nucleotide fragment detected in the mung bean nuclease protection experiment. This would place the start of transcription at a guanosine residue 40 nucleotides upstream of the AUG translational start codon of ORF 3 (capsid protein), corresponding to residue 3653 in the PLRV sequence (Mayo et al., 1989). The additional two bands visible in the nuclease experiment may represent incomplete digestions of the DNA/RNA hybrids in the presence of the vast excess of cellular RNA, as the primer extension experiment does not show this ambiguity. From this analysis, the size of sgRNA 1 would be only 2334 bases, thus being overestimated in the Northern blot experiment (total length of PLRV genomic RNA 5987 nucleotides, according to Mayo et al., 1989).

The PLRV sequence encompassing the putative transcriptional start site and the untranslated leader of the sgRNA 1 was compared with the corresponding regions upstream of the CP gene AUG start codon of the luteoviruses BWYV and BYDV. Two conserved sequence blocks were evident (Fig. 4). One is located immediately upstream of the AUG start codon whereas the second is composed of a sequence stretch of some 27 nucleotides, rich in uridine residues as noted before by Mayo et al. (1989). This conserved sequence is centred
Expression of a PLRV subgenomic RNA

The sequence UUAUAUU, located 5' to the transcription start site, is very similar to the sequence UUAUUAUU, which is part of the subgenomic promoter of brome mosaic virus (BMV) and is present in the intergenic region of other RNA plant viruses (March et al., 1988). Additionally, the 5' untranslated end of sgRNA 1 is rich in adenosine and uracil residues, as observed for the 5' ends of subgenomic RNAs of other RNA viruses (TMV, Goelet et al., 1982; BMV, Ahlquist et al., 1981; alfalfa mosaic virus, Koper-Zwarthoff et al., 1980).

Fig. 5. Translational fusion of the amber termination codon to a GUS reporter gene. Synthesis of chimeric PLRV/GUS constructs starting from an AluI/BstNI fragment of cDNA clone pCPL1 (Tacke et al., 1989) and the wild-type GUS gene in pRT103GUS (Töpfer et al., 1988). Pr and T are CaMV promoter (35S) and terminator sequences, respectively. UP and DP are oligodeoxynucleotide primers used for PCR-mediated mutagenesis.

Transient expression of chimeric PLRV/GUS constructs

To determine the frequency of suppression of the UAG stop codon separating ORF 3 (CP) and ORF 5 (56K protein), the CP gene and the first 21 bases of ORF 5 were translationally fused to the GUS gene (Jefferson, 1987), with the CaMV 35S promoter and terminator as control sequences (Fig. 5). As GUS activity may decrease in fusion proteins with increasing length of the foreign protein, most of the CP gene (encoding a 208 amino acid coat protein) was internally deleted in a PCR using 5' UP and 3' DP terminal primers (Fig. 5). Readthrough of this chimeric construct would result in a GUS fusion protein containing 28 foreign N-terminal amino acids (20 amino acids of CP, one corresponding to the suppressed UAG codon and seven amino acids of the 56K protein). In the final construct, the amber stop codon remained located within the original sequence of 18 bp to the 5' side and 21 bp downstream (Tacke et al., 1989), which is considered to be sufficient for suppressor tRNA interaction (Bossi, 1983). By an additional PCR mutation, the UAG codon of the construct was changed into the GCC codon for the uncharged amino acid alanine. This GCC construct served as a positive control for GUS expression. A third GUS construct, carrying an out-of-frame mutation in the PLRV sequence preceding the GUS coding sequence, was used as a negative control in order to account for any putative GUS activity resulting from internal translation initiation.

These constructs were tested for transient expression of GUS activity in potato and tobacco protoplasts. The data summarized in Table 1 were obtained with different protoplast preparations and each value represents the mean of four experiments. Both solanaceous plant species allowed in vivo suppression of the amber stop codon leading to the synthesis of GUS enzyme at a frequency of 0.9 to 1.3%, normalized with respect to the two control constructs. Although tobacco is not a natural host for PLRV (Rich, 1983), isolated tobacco mesophyll protoplasts support PLRV multiplication (Takanami & Kubo, 1979; Mayo et al., 1982). Our results also showed no significant difference between host species.

The second approach to the study of translational signals present on the sgRNA 1 was the determination of the relative expression of the CP gene and ORF4 (17K...
Fig. 6. Translational fusion of the CP ORF and the 17K protein ORF with the GUS reporter gene. A SacI/BssHII pcPL1 cDNA fragment, containing part of the intergenic region and the 5' region of the genes for the CP (ORF 3) and 17K protein (ORF 4), was treated with mung bean nuclease or the Klenow fragment of DNA polymerase and translationally fused to pRT103GUS to yield the CP-GUS (left) or the 17K-GUS (right) construct. Homologies of the different translational initiation codons to the consensus sequence in plants (Liitcke et al., 1987) are indicated by dots. Pr, CaMV promoter sequence; T, CaMV terminator sequence.

Table 2. GUS activity of 17K/CP constructs in protoplasts

<table>
<thead>
<tr>
<th>Protoplasts</th>
<th>Construct</th>
<th>pmol MU/mg protein/min</th>
<th>Ratio CP:17K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>17K-GUS</td>
<td>388630</td>
<td>1:6-8</td>
</tr>
<tr>
<td></td>
<td>CP-GUS</td>
<td>57127</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17K-GUS</td>
<td>237023</td>
<td>1:6</td>
</tr>
<tr>
<td></td>
<td>CP-GUS</td>
<td>33653</td>
<td>1:7-9</td>
</tr>
<tr>
<td>Tobacco</td>
<td>17K-GUS</td>
<td>51466</td>
<td>1:6-7</td>
</tr>
<tr>
<td></td>
<td>CP-GUS</td>
<td>7734</td>
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</tr>
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<tr>
<td></td>
<td>CP-GUS</td>
<td>15653</td>
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</table>

* Results obtained as in Table 1.

Negative and/or positive effects of the N-terminal GUS modification present in both chimeric constructs could have contributed to the 7:1 ratio in favour of 17K protein synthesis. However, another possible factor is that the second putative AUG start codon of the 17K protein (Fig. 6) is in a sequence that more closely resembles the consensus sequence for optimal translation initiation in plants (Lütcke et al., 1987) than that of the CP gene AUG.

**Discussion**

Sequence analysis of the RNA of two luteoviruses, BWYV (Veidt et al., 1988) and PLRV (Mayo et al., 1989; van der Wilk et al., 1989), has identified a conserved cluster of three genes, ORF 3 (CP gene), ORF 4 (17K protein) and ORF 5 (56K protein), in the 3' half of the genomic RNA (Fig. 1). Earlier attempts to translate the CP gene from genomic PLRV RNA in vitro have failed (Mayo et al., 1982), suggesting that the CP gene is translated from a subgenomic RNA, similarly to those of several other RNA plant viruses (Davies, 1985). Luteoviral RNAs smaller than unit length have been reported for BYDV (3-0 kb; Gerlach et al., 1987) and BWYV (2.9 kb; Falk et al., 1989). This communication characterizes a subgenomic RNA detected in PLRV-infected potato plants of 2.3 kb in length (sgRNA 1). The subgenomic PLRV RNA is 3' coterminal with PLRV genomic RNA and is transcribed from the intergenic region. Its transcriptional start maps at the guanosine residue within the uridine-rich sequence UAUUUU-GUUUAAC, a region which is highly conserved with respect to nucleotide sequence (Fig. 4) as well as distance (34 nucleotides) from the CP gene initiation codon in the RNAs of PLRV, BWYV and BYDV. By analogy to PLRV, this region may therefore represent part of the subgenomic promoter for all luteoviruses, with A presumably being the start of transcription for the subgenomic RNAs of BWYV and BYDV. As pointed
expression by UAG readthrough, translation initiation and the 17K protein may be subject to high turnover.

The 17K protein is very efficient and exceeds by sevenfold on purified virus particles by monospecific antisera. This reflects the actual accumulation of the PLRV subgenomic promoter.

Using constructs containing parts of the PLRV genome fused to the GUS gene, we observed suppression of the amber stop codon separating the CP gene and ORF 5 (Fig. 1) in both tobacco and potato protoplasts. GUS activity was from 0.9% to 1.3% of the level measured for a construct in which the UAG stop codon had been replaced by the alanine codon GCC. These and other data (see below) provide molecular evidence that protoplasts of the non-host tomato can support PLRV replication by expression of a suppressor tRNA capable of recognizing the PLRV UAG codon, and possibly interacting with flanking sequences. Tobacco tRNAs promoting suppression of UAG stop codons of TMV RNA have been identified and characterized by sequence analysis (Beier et al., 1984a, b). It will be interesting to see whether these tobacco suppressor tRNAs will promote readthrough of PLRV subgenomic RNA in vitro.

The low amount of CP/ORF 5 readthrough product measured in protoplasts (Fig. 5) may reflect a possible regulatory role of the fusion protein, or the released ORF 5 product, during virus replication, either in the infected host plant or in the aphid vector that transmits the virus. Such regulatory effects of readthrough products are known for E. coli phage Qβ replication. The Qβ CP gene terminates in a leaky stop codon and readthrough occurs at a frequency of about 2% (Weiner et al., 1973). In the absence of readthrough, infectious phage particles are not formed (Hofstetter et al., 1974). In this context it should be noted that the PLRV 56K protein is detected on purified virus particles by monospecific antisera directed against this protein (E. Tacke, unpublished results). This protein could possibly play an important role in transmission by the insect vector Myzus persicae.

Our data suggest that, in contrast to low level ORF 5 expression by UAG readthrough, translation initiation at an internal AUG of the sgRNA 1 to produce the ORF 4 17K protein is very efficient and exceeds by sevenfold the synthesis of CP in both potato and tobacco protoplasts. These values, however, may not necessarily reflect the actual accumulation in vivo, as the CP is sequestered for morphogenesis of progeny virus particles and the 17K protein may be subject to high turnover rates. Experiments are under way to study the synthesis and distribution of this non-structural protein in transgenic as well as virus-infected plants by the use of monospecific antisera.

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References


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