Interference with brome mosaic virus replication by targeting the minus strand promoter

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Sense and antisense strategies for interfering with the replication of brome mosaic virus (BMV) were examined. The effects of 200 nucleotide-long sense and antisense transcripts, corresponding to the viral 3' end (−) strand promoter, on the accumulation of progeny viral RNAs were studied by co-inoculation with wild-type BMV RNAs. Progeny accumulation in barley protoplasts transfected with either sense or antisense transcripts of the (−) strand promoter and BMV RNAs-1 and -2 was decreased by more than 90%, and by 60 to 80% when RNA-3 was also present. This trans interference was concentration-dependent, and reduced both (+) and (−) strand progeny accumulation to a similar extent. The appearance of complementary (−) strands indicated that sense interfering transcripts could serve as templates for (−) strand synthesis, and the use of deletion mutants revealed that the observed interference was in part mediated by this template activity. The reproducibility of the protoplast assay used here allows rapid evaluation of interference strategies and comparisons to be made of alternative approaches to engineered resistance. The results presented here suggest that targeting viral (−) strand promoters with sense and antisense transcripts may be an effective method for engineering plant resistance to viral infection.

Introduction

Molecular genetic studies on brome mosaic virus (BMV) have allowed the identification of cis-acting RNA sequences required for the replication cycle of a diverse range of positive strand RNA plant virus families. Using the concept of pathogen-derived resistance (PDR) (Sanford & Johnston, 1985), these studies have led to the recognition of novel strategies for plant viral resistance which may have broad application (Huntley et al., 1993). Engineered defective interfering RNAs derived from BMV RNA-2 and capable of effectively parasitizing the replication of genomic viral RNAs have been reported (Marsh et al., 1991b, d) and inhibitory aminoacylation-deficient mutants (Rao & Hall, 1991) as well as regulatory elements from the intercistronic region (Huntley & Hall, 1993) have also shown potential for providing engineered resistance to viral pathogens. Other regions of the BMV genome are also likely to provide suitable tools for PDR.

Several groups of RNA plant viruses possess a tRNA-like domain at the 3' terminus of their genomic RNAs (Hall, 1979; Haenni et al., 1982). In BMV, the 3' CCA<sub>OH</sub> terminus is specifically tyrosylated by host-encoded tRNA synthetase (Kohl & Hall, 1974) and can be repaired by interaction with host nucleotidyl transferase, suggesting a telomeric role for this structure (Rao et al., 1989). A novel hybrid-arrested replication approach (Ahlquist et al., 1984) was used to demonstrate in vitro that initiation of (−) strand synthesis occurs opposite the penultimate C of the 3' CCA<sub>OH</sub> (Miller et al., 1986) and deletion mutants specifically deficient in adenylation, tyrosylation and (−) strand promoter activity have been created (Bujarski et al., 1986; Dreher & Hall, 1988a, b; Dreher et al., 1989; Rao et al., 1989). Since the multifunctional tRNA-like structure plays a decisive role in the life-cycle of BMV, we have investigated the possibility that this region could be targeted to inhibit viral replication. We now describe studies on the inhibitory effect that both ‘sense’ and antisense transcripts of the (−) strand promoter have on the replication of genomic BMV RNAs in protoplasts.

Methods

Chemicals and enzymes. Restriction and modifying enzymes were obtained from Boehringer-Mannheim, New England Biolabs (NEB) and Bethesda Research Laboratories (BRL). T7 RNA polymerase and human placental ribonuclease inhibitor (RNAguard) were from Pharmacia. T3 RNA polymerase was obtained from BRL. Ambion T7 and T3 Megascripts were used for non-capped RNA transcription. Cap analogue (m<sup>7</sup>GpppG) and T4 DNA ligase were from NEB. Calf intestinal phosphatase and yeast tRNA were procured from Boehringer-Mannheim.
yielded transcripts with 31 5' and no 3' bases of non-viral origin. Produced from (Loesch-Fries & Hall, 1980) and inoculated (Dreher et al., 1989). In vitro linearization of template plasmids (ptRNA, pAknob, pA5' and pM4) according to the manufacturer's instructions. This yielded transcripts with eight 5' and eight Y nucleotides of non-viral origin. It is known that (-) strand cDNA clones from which infectious full-length wild-type (wt) BMV RNAs-1, -2 and -3 can be transcribed regardless of the presence of an additional 3' non-viral sequence (Miller et al., 1991a). In vitro transcription and protoplast replication assays. Infectious, capped, full-length BMV genomic RNAs were transcribed with T7 RNA polymerase from plasmids linearized with BamHI as described by Dreher et al. (1989) and separated from DNA template by precipitation with 2.6 M-LiCl (Rao & Hall, 1990, 1991) were digested with HindIII and BamHI to release 200 nucleotide (nt) fragments which were ligated into identical sites within the pT7T3 19U (Pharmacia) polylinker. The plasmids thus created are respectively called ptRNA, pAknob, pA5' and pM4 and can be transcribed in vitro to yield both sense and antisense RNAs (Fig. 1).

In vitro transcription and protoplast replication assays. Infectious, capped, full-length BMV genomic RNAs were transcribed with T7 RNA polymerase from plasmids linearized with BamHI as described by Dreher et al. (1989) and separated from DNA template by precipitation with 2.6 M-LiCl (Rao & Hall, 1989). Positive strand or 'sense' transcripts (with respect to the polarity of the genomic BMV RNAs) of wt or mutant 3' tRNA-like structures were produced by linearization of template plasmids (ptRNA, pAknob, pA5' and pM4) with BamHI, followed by T7 Megascript transcription according to the manufacturer's instructions. This yielded transcripts with eight 5' and eight 3' nucleotides of non-viral origin. It is known that (-) strand replication commences opposite the appropriate C residue corresponding to the penultimate nucleotide on the wt viral sequence regardless of the presence of an additional 3' non-viral sequence (Miller et al., 1986). Antisense transcripts of the wt tRNA-like structure were produced from HindIII-linearized ptRNA using T3 Megascript. This yielded transcripts with 31 5' and no 3' bases of non-viral origin. Routinely, 30 to 40 µg was obtained from 1 µg of linearized template in a 20 µl reaction.

Barley (Hordeum vulgare cv. Dickson) protoplasts were isolated (Loesch-Fries & Hall, 1980) and inoculated (Dreher et al., 1989) with capped genomic viral RNA transcripts (1 µg of each) and the described molar ratio of 200 nt-long sense or antisense tRNA-like transcripts. To prevent premature hybridization of antisense RNA transcripts to the viral genome, samples of ice-cold transcripts were mixed together on ice just before protoplast inoculation. Following transfection, protoplasts were incubated at room temperature under fluorescent lights for 24 h. Total RNA was extracted with SDS and phenol–chloroform and then ethanol-precipitated (Loesch-Fries & Hall, 1980).

Analysis of progeny viral RNA. Replication of viral RNAs in protoplasts was analysed by Northern blots as described by Dreher et al. (1989). Strand-specific (+)- or (-)-sense RNA probes were 32P-labelled to identical specific radioactivity levels by transcription from plasmid pT73TR, using either T3 or T7 RNA polymerase (Rao et al., 1989). This plasmid contains a cDNA insert corresponding to the tRNA-like structure (3'-terminal 200 bp) which is conserved among all BMV RNAs. The data presented are representative of at least three independent experiments. Northern blots were autoradiographed with pre-flashed film (Laskey & Mills, 1977) and relative band intensities were determined using a Bio-Rad model 620 video densitometer. Where both (+) and (-) progeny are shown, equal amounts were taken from the same protoplast sample. Progeny RNAs are expressed as a percentage relative to replication levels of wt BMV RNAs and the results are the average of at least three independent experiments.

Results

Antisense RNAs to the (-) strand promoter debilitate BMV replication in barley protoplasts

Hybrid arrest studies by Ahlquist et al. (1984) demonstrated that antisense cDNA sequences derived from the tRNA-like structure can successfully block BMV (-) strand promotion during replication in vitro. Co-inoculation of barley protoplasts with wt BMV RNAs-1 and -2 with 15- or 22-fold excess of antisense transcript to the tRNA-like structure reduced the accumulation of the genomic RNAs by 45% and 90%, respectively (Fig. 2a, b; compare lanes 3 and 4 with lanes 1). Input antisense RNAs inoculated in the absence of genomic RNAs were detectable after 24 h of incubation, but the diffuse band near the bottom of the blot resolved for (-) strands (Fig. 2a, lane 5) indicates that some degradation had occurred. The diffuse bands near the bottom of the blot resolved for (+) strands are degradation products derived from (+)-sense progeny genomic RNAs; these RNAs did not give rise to any corresponding (+) strand RNAs (Fig. 2a, lanes 1). However, the faint (+)-sense band detected (Fig. 2a, lane 5) suggests that the supplied antisense transcripts serve as inefficient templates for host-encoded RNA-dependent RNA polymerase activity (Hardy et al., 1979; Huntley & Hall, 1993).

The addition of BMV RNA-3 to RNAs-1 and -2 in protoplast inocula resulted in a 205-fold increase in progeny (+)-sense RNAs (Marsh et al., 1991a), and shifts the ratio of progeny (+):(-) strands from approximately 1:1 to > 100:1 (Rao et al., 1990; Marsh et al., 1991a). Although the details of this dramatic change in replication are not well understood, we have
Interference with BMV replication

Fig. 2. Interference with RNA-1 and -2 replication by co-inoculation of antisense tRNA-like sequences. (a) Autoradiograph showing Northern hybridization to (+) (left-hand panel) and (−) (right-hand panel) strand progeny RNA from barley protoplasts. Samples in lanes 1 to 4 were inoculated with BMV RNAs-1 and -2 (1 μg each). Samples in lanes 2 to 4 were co-inoculated with a 7-, 15- or 22-fold molar excess of 200 nt-long RNA antisense to the tRNA-like structure common to the 3' end of BMV genomic RNAs. In lane 5, protoplast samples were inoculated with antisense RNA alone, equivalent to the amount inoculated in lane 2. Viral and antisense transcripts were mixed on ice directly before co-inoculation. At 24 h post-inoculation, total nucleic acid was extracted and separated in 1% agarose gels following denaturation with glyoxal, and then was electrophoretically transferred to nylon membrane. The blots were probed with 32P-labelled (+)- or (−)-sense probes corresponding to the 3' end of viral RNAs. Positions of RNAs-1 and -2 are shown at the left. D, Position of positive strand degradation products; I, input antisense RNA transcripts. The substantial degradation of RNA reflects the lack of encapsidation in the absence of RNAs-3 and -4. Both (+) and (−) strand panels were exposed for 16 h at −80 °C. Autoradiographs were scanned by densitometry using preflashed film. The replication of RNAs-1 and -2 in lanes 2 to 4 was compared to that of lanes 1 for both (+) and (−) strands. The averaged results of three independent experiments for (+) strands (11) and (−) strands (12) are shown in (b).

suggested that a modified viral replicase with an altered affinity for (+) and (−) strand promoter elements may be involved (Pogue et al., 1993). Given these differences, it was important to compare the inhibition of progeny production by antisense RNAs for protoplasts transfected with RNA-1 and -2 (RNAs 1 + 2) (Fig. 2) with that for protoplasts infected with all three genomic RNAs (Fig. 3). Comparison of Fig. 2 and Fig. 3 reveals that antisense transcripts to the (−) strand promoter reduced progeny accumulation by 60% in protoplasts infected with all three RNAs and by approximately 90% for those transfected with RNAs 1 + 2. However, in both situations, (+) and (−) strand accumulation was equally reduced despite the shift in strand asymmetry that characterizes full genomic infections.

Fig. 3. Interference with full genomic BMV infection by antisense tRNA-like structure. (a) Barley protoplast samples were inoculated with 1 μg each of genomic RNAs-1, -2 and -3 plus no or a 7-, 15- or 22-fold molar excess of antisense tRNA-like structure transcripts (lanes 1 to 4 respectively). RNA blot analysis was performed as described under Fig. 2. The (+) (left) and (−) (right) strand panels were exposed at −80 °C for 1 h and 48 h respectively. (b) Total accumulation of progeny RNAs-1, -2 and -3 (lanes 2 to 4) was compared to that of RNAs-1, -2, and -3 transfected alone (lane 1) for (+) strands (■) and (−) strands (□) and expressed as a percentage value from three independent trials. I, Input antisense RNA transcripts.
Interference with viral replication by sense transcripts

Morch et al. (1987) noted that short transcripts of the turnip yellow mosaic virus (−) strand promoter could compete with genomic viral RNAs as templates for (−) strand synthesis in an in vitro replicase assay. To test the efficacy of this concept in vivo, RNA transcripts corresponding to the 3′ 200 nt that include the (−) strand promoter were co-inoculated with wt BMV genomic RNAs-1, -2 and -3 into barley protoplasts. A concentration-dependent interference with the accumulation of both (+) and (−) strand progeny RNAs was observed (Fig. 4a, lanes 1 to 4). At the greatest molar excess tested (22-fold; Fig. 4a, lanes 4), genomic RNA accumulation was reduced by approximately 85% from that obtained using wt RNAs 1 + 2 + 3 (Fig. 4a, lanes 1). Residual supplied (+)-sense RNAs and their (−) strand progeny can be seen at the bottom of lanes 2 to 4 of Fig. 4(a); in the absence of viral genomic RNAs, no (−) strand progeny were generated (Fig. 4a, lanes 6).

Evidence that the decrease in viral RNA accumulation in the presence of added (+)-sense RNAs does not result from reduced uptake of inoculated viral RNAs by the barley protoplasts at the higher inoculum levels is provided by the results shown in Fig. 4(a), lanes 5, where a 45-fold molar excess of yeast tRNA was coinoculated without significant effect on BMV RNA accumulation. These results are similar to those of Rao & Hall (1991), Marsh et al. (1991d) and Huntley & Hall (1993), who all showed that co-inoculation of barley protoplasts with BMV genomic RNAs and excess non-specific RNA (up to 12 μg) had no appreciable effect on viral replication.

Effect of deletions on interfering properties of (+)-sense transcripts

To examine further the interfering properties of sense promoter fragments, three well characterized mutants with lesions located within the tRNA-like structure (Fig. 1) were tested for their ability to serve as templates for complementary (−) strand synthesis in vivo. Mutants M4 and Δ5' have stem–loop deletions of arms D and E that are respectively deficient in adenylation and tyrosylation by host enzymes, but show at least wt (−) strand promoter activity in vitro (Marsh et al., 1991c). The Δknob mutation removes the four unpaired bases that form a small bulge near the base of stem–loop C, a conserved feature in the RNAs of bromo- and cucumoviruses; it yields only 6% of wt (−) strand promoter activity in vitro (Dreher & Hall, 1988a). At a 22-fold molar ratio, the replication of RNAs-1 and -2 was inhibited > 80% by the wt, M4 and Δ5' (+)-sense transcripts and by 50% by the Δknob (+)-sense transcripts (Fig. 5). In the blot probed for detection of (−) strand progeny (Fig. 5a), novel high mobility replication products are evident in samples co-inoculated with wt, M4 and Δ5'. No such products were detectable from the reaction that included the Δknob transcript. Together, these findings suggest that interference with genomic replication by the sense promoter fragments is
Interference with BMV replication

Sanford & Johnston (1985) suggested that engineered resistance to insect, fungal and viral parasites of plants could be achieved by utilizing portions of the pathogen’s own genome. We have attempted two distinct strategies to interfere with the replication of BMV. Both are designed to block (−) strand synthesis, the first step in the viral replication cycle. This represents a vulnerable point at which infection can be prevented because relatively few viral molecules are present. As new (−) strand genomes become available, they serve as templates for a 100-fold increase in (+)-sense progeny (Marsh et al., 1991a), making interference with replication proportionally more difficult.

Our first interference strategy involved the use of antisense RNA to produce an RNA–RNA hybrid which physically obstructs the function of the targeted complementary RNA strand. Regulation of gene expression by this mechanism has been recognized in nature and, as discussed by Inouye (1988), has been shown to work at several different levels including translational and transcriptional inhibition, and interference with DNA replication. Ahlquist et al. (1984) made use of a hybrid arrest strategy to localize the site of BMV replicase recognition by use of cDNAs complementary to the 3' ends of viral RNAs. Recently there have been a number of attempts to apply the antisense strategy both to gene expression in plants (Delauney et al., 1988; Oeller et al., 1991) and to interfere with virus replication (Rezaian et al., 1988; Cuozzo et al., 1988; Hemenway et al., 1988; Powell et al., 1989).

Unfortunately, this antiviral strategy has, thus far, proven to be of limited value in transgenic plants since it provides protection only when low levels of inoculum are supplied. Experiments by Powell et al. (1989) using antisense RNAs to inhibit the replication of tobacco mosaic virus revealed that these RNAs were effective only when sequences complementary to the 3' tRNA-like structure were present. Rezaian et al. (1988) obtained only weak protection against cucumber mosaic virus (CMV) using sequences complementary to the 5' translational start site of CMV RNA-1, and antisense transcripts directed against the 3' non-coding region that did not include the terminal 135 bases were ineffective. The above studies illustrate that, for effective interference with viral replication, antisense RNA sequences must block initiation of (−) strand synthesis.

due in part to competition for viral replicase and the synthesis of short non-replicating (−) strand fragments.

Fig. 5. Interference with RNA-1 and -2 replication by co-inoculation of wt and mutant tRNA-like structures. (a) The ability of deletion mutants M4, ΔS' and Δknob (see Fig. 1) to debilitate the replication of BMV RNAs-1 and -2 was compared to that of the wt tRNA-like structure in co-inoculation experiments. Inhibition of viral replication by the addition of the indicated molar excess of (+)-sense RNAs is shown in lanes 2 to 13 and was quantified by comparison with the accumulation of RNAs-1 and -2 inoculated alone to protoplasts (lane 1). Positive strand degradation products (D) and input BMV 3' ends (I) are shown at the left-hand border for lanes 1 to 10, and at the right-hand border for lanes 11 to 13. The position of progeny (−)-sense strands derived from (+)-sense 3' fragments is shown (P). (b) Average accumulation of (+) strand progeny RNAs resulting from three independent trials.

Discussion

Sanford & Johnston (1985) suggested that engineered resistance to insect, fungal and viral parasites of plants could be achieved by utilizing portions of the pathogen’s own genome. We have attempted two distinct strategies to interfere with the replication of BMV. Both are designed to block (−) strand synthesis, the first step in the viral replication cycle. This represents a vulnerable point at which infection can be prevented because relatively few viral molecules are present. As new (−) strand genomes become available, they serve as templates for a 100-fold increase in (+)-sense progeny (Marsh et al., 1991a), making interference with replication proportionally more difficult.

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it will also have to compete with an increasingly large number of viral RNA templates.

The results shown in Fig. 2 and 3 confirm that a decrease in progeny virus accumulation can occur in the presence of antisense RNA. However, this is concentration-dependent: over 90% inhibition of both (+) and (−) strand accumulation was observed in the presence of a 22-fold molar excess of antisense transcripts directed against the 3’ end of RNAs-1 and -2 (Fig. 2) and 60% in the presence of RNA-3 (Fig. 3); at lower molar ratios the effect was substantially decreased. These data are consistent with the formation of RNA:RNA hybrids, thereby reducing the number of functional infecting RNAs available to the viral replicase, attenuating (−) strand synthesis and delaying the infection.

A second interference strategy used in these studies employed a sense approach to debilitate genomic viral (−) strand promoter activity similar to that used by Morch et al. (1987) for in vitro replicase experiments with turnip yellow mosaic virus. Their experiments showed that 3’ (+)-sense fragments competed with genomic (+)-sense RNAs for (−) strand replicase, resulting in non-productive transcription of complementary (−) strand fragments. Our results, using 3’ (+)-sense fragments of BMV RNA, confirm in vivo the value of the positive-sense approach to interference. The results shown in Fig. 4 reveal that the presence of (+)-sense 3’ transcripts has an inhibitory effect on virus accumulation, and that the effect is dependent on the concentration of RNA supplied. Additionally, the substantial amounts of (−)-sense products that corresponded in size to input (+) strand tRNA-like structures confirmed that the RNAs supplied served as templates for (−) strand initiation and transcription.

Several possibilities exist for interference with (−) strand promoter function by the (+)-sense RNA fragments. These include an ability to form inhibitory RNA:RNA hybrids with (−) strand RNAs and to compete effectively with viral genomic RNAs both for host factors that interact with the virus genomic tRNA-like structure and, especially, for the limited pool of viral replicase.

The 3’ tRNA-like structures of BMV and other plant virus RNAs have been shown to interact with several host proteins, including aminoacyl-tRNA synthetase (Kohl & Hall, 1974; Dreher & Hall, 1988b), nucleotidyl transferase (Bujarski et al., 1986; Dreher & Hall, 1988b; Rao et al., 1989) and elongation factors (Bastin & Hall, 1976). However, co-inoculation of yeast tRNAs with BMV RNAs at a molar ratio of 45:1 had no detectable effect on the production of virus progeny (Fig. 4, lane 5). This detracts from the possibility that binding to host proteins involved in tRNA functions could result in their depletion to limiting amounts that reduced genomic RNA accumulation to the levels seen in Fig. 4, lanes 2 to 4.

If inhibition of replication by the 3’ (+)-sense construct functions by competition with the genomic RNAs for replicase, 3’ fragments corresponding to sequences known to be defective in (−) strand promoter activity should also be defective in competition. Several such sequences have been characterized. These include the Δknob mutation, in which a structural motif conserved in bromo- and cucumovirus is eliminated (Fig. 1), dramatically reducing (−) strand promoter activity (Dreher & Hall, 1988a; Rao et al., 1990). The data shown in Fig. 5(a), lanes 11 to 13, confirm that the 3’ fragment bearing the Δknob mutation was inactive as a template for generation of complementary (−) strands in protoplasts and was 50% less effective than the wt sequence for inhibiting progeny accumulation (Fig. 5b).

The observation that the Δknob sequence retained a measurable level of interference suggests that competition for replicase initiation represents only part of the interaction and tight binding to viral replicase or hybridization to (−) strand progeny RNA may also participate in the overall effect. Additional evidence that several mechanisms participate in competition comes from the experiments using two 3’-terminal deletion mutants of BMV RNA-2, M4 and Δ5’. These RNAs are respectively deficient in their ability to be adenylated and tyrosylated in vitro (Bujarski et al., 1985, 1986). Since M4 exhibits twice wt template activity for (−) strand initiation in vitro (Bujarski et al., 1985), it might be expected to be substantially more effective than the wt 3’ fragment in reducing genomic RNA accumulation. In fact, its activity was not significantly different from wt (Fig. 5b), suggesting that its debilitated interaction with host nucleotidyl transferase may have countered its high template activity.

When present on full-length RNA-2, both the M4 and Δ5’ mutations interfere in trans with the replication of other genomic RNAs (Rao & Hall, 1991). Consequently, it was possible that either of these mutations could enhance the interference of the wt 3’ sequence. However, when present on 3’ fragments, the Δ5’ and M4 mutations produced substantial amounts of the complementary (−) strand product but their interference with genomic RNA accumulation was only similar to that arising from the wt 3’ RNAs (Fig. 5). These observations support our previous finding that the ability of the M4 and Δ5’ mutations to interfere in trans is modulated by the context of RNA-2.

Attempts to provide engineered resistance to plant viral infections have been made with a variety of viral proteins (Beachy et al., 1990; Golemboski et al., 1990) and bioactive RNAs (Marsh et al., 1991b; Hemenway et al., 1988). However, the extreme variation between
individual plants and the frequent lack of correlation between gene expression levels and the degree of resistance (Kawchuck et al., 1991; Lindbo & Dougherty, 1992) has made it difficult to compare directly the efficacy of each strategy. We have attempted to circumvent this problem by using protoplasts for replication assays that mimic the constitutive expression of a viral inhibitor, and by ensuring that the molar ratio of the interfering construct to the viral genome is strictly defined at the outset of inoculation. We have previously shown (Loesch-Fries & Hall, 1980) that > 90% of protoplasts are infected in these experiments. Consequently, each sample inoculation represents thousands of individual interference trials. The reproducibility of this assay system allows repeated trials to be conducted with a minimum of variation which is not possible with transformed plants, and it also allows the relative comparison of antiviral strategies prior to the labour-intensive application to transgenic plants. We recently reported that a sixfold molar excess of (−)-sense transcripts from the intercistronic region from BMV RNA-3 decreased viral replication by > 90% (Huntley & Hall, 1993). This represents a three- to fourfold increase in efficiency when compared with the sense and antisense approaches described here. On the basis of these considerations, we anticipate that the (−) RNA-3 construct will prove a more effective approach for achieving engineered resistance and have embarked on testing this prediction.

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