Non-replicating deletion mutants of brome mosaic virus RNA-2 interfere with viral replication

Loren E. Marsh,† Gregory P. Pogue, Urzula Szybiak,‡ James P. Connell and Timothy C. Hall*

Department of Biology, Texas A&M University, College Station, Texas 77843-3258, U.S.A.

Naturally occurring defective interfering RNAs (DI-RNAs) and satellite RNAs greatly reduce the accumulation of their helper virus *in vivo*, but often modulate symptom expression in an unpredictable manner. Deletion mutants Nc/S, Na/M and Sa/Nc+M/S, derived from brome mosaic virus (BMV) RNA-2, failed to replicate when co-inoculated with BMV RNAs-1 and -2 to barley protoplasts. However, the inoculum RNA corresponding to these deletion mutants was extremely stable and could have been mistaken for plus-strand progeny had minus-strand progeny analysis been omitted. These results accentuate the need for such tests in evaluating the ability of mutant viral sequences to replicate. One of the mutants, Nc/S, effectively interfered with the accumulation of BMV RNAs-1 and -2 in barley protoplasts. This non-replicating interfering RNA was termed NRI RNA-2 Nc/S. When present with RNAs-1 and -2 at low inoculum amounts (1 μg), NRI RNA-2 Nc/S reduced replication of RNA-2, the parental RNA, by 63% and preferentially interfered with minus-strand RNA accumulation. At higher levels (4 μg), it completely displaced replication of both RNAs-1 and -2. Mutations eliminating translation of a truncated p2a protein from NRI RNA-2 Nc/S did not alleviate the interference effect, demonstrating that a defective replicase protein was not responsible for the decreased accumulation of genomic RNA. At an NRI RNA:genomic RNA inoculum molar ratio of 1:1, NRI RNA-2 Nc/S reduced the accumulation of all helper virus RNAs by 55%. Since this reduction was seen for both wild-type RNA-3 and ΔSGP RNA-3, a deletion mutant of RNA-3 that lacks the subgenomic promoter necessary for coat protein expression, it was evident that the effective interference mediated by NRI RNA-2 Nc/S was not mitigated by encapsidation. The ability of the NRI RNAs to mimic satellite DI RNAs in depressing helper virus replication suggests that their expression in transgenic plants may provide a new and widely applicable approach for inducing resistance to viral infection.

Introduction

Defective interfering RNAs (DI-RNAs) and satellite RNAs are small infectious RNAs that require a helper virus to replicate in infected tissues. DI-RNAs are frequently associated with animal virus infections and are derived from their helper virus by a series of sequence deletions and rearrangements. They have also been identified with the carmo- and tombus- plant virus groups (Morris & Knorr, 1990). Studies on DI-RNAs associated with RNA viruses have been useful in characterizing the sequence elements involved in viral replication and encapsidation (Levis et al., 1986; Weiss et al., 1989; Knorr et al., 1991). In contrast, the genomes of satellite RNAs typically have little sequence homology with their helper virus, but may exhibit profound effects on the severity of pathogenesis and symptom expression in infected plants (Murant & Mayo, 1982; Francki, 1985). Studies elucidating the origin of turnip crinkle virus satellite RNAs (Cascone et al., 1990) have increased our understanding of recombination mechanisms in RNA viruses.

Both DI- and satellite RNAs replicate at the expense of, and compete with, the helper virus genome (Holland, 1990). They often reduce the helper virus to undetectable levels in plants (Hillman et al., 1987) and plant protoplasts (Jones et al., 1990). These interference properties have led many plant virologists to view these subviral RNAs a potential tools for arresting virus infections. Indeed, expression of satellite RNAs in transgenic plants has been shown to confer resistance to tobacco ringspot virus (Gerlach et al., 1987) and cucumber mosaic virus (Harrison et al., 1987). However,
several concerns exist which limit widespread applica-
tion of this approach. Satellite and DI-RNAs are
themselves contagious agents with efficient mechanisms
of packaging and replication. Most importantly, the
symptom-modulating properties of satellite RNAs can
often lead to an aggravation, instead of amelioration, of a
viral infection (Murray & Mayo, 1982; Francki, 1985).
The expression of these differences in pathogenicity is
dependent on both the strain of helper virus and the plant
cultivar infected. In addition, the lack of associated
satellite or DI-RNAs with most plant viruses imposes a
natural limitation on broad implementation of such
protection strategies.

We have recently reported (Marsh et al., 1991b) the
construction of artificial DI-RNAs from brome mosaic
virus (BMV), a tripartite positive-stranded RNA virus
that infects many monocotyledonous species, including
barley, maize and rice. These artificial DI-RNAs, or
parasitic RNAs (pRNAs: Pogue et al., 1990), are
deletion mutants of BMV RNA-2 that replicate in a
dependent manner on BMV genomic RNAs and are
capable of marked interference with the replication of
the co-inoculated helper virus (Marsh et al., 1991b). This
approach overcomes host range limitations associated
with naturally occurring DI-RNAs. We now describe a
series of deletion mutants of BMV genomic RNA-2 that
produce no minus strand progeny. They are incapable of
replication when co-inoculated with BMV RNAs, even
though the plus strand RNA used as the inoculum is very
stable. Additionally, we demonstrate that one of these
mutants, NRI RNA-2 Nc/S (a non-replicating interfer-
ing RNA), effectively interferes with the replication of
genomic BMV RNAs and certain deletion derivatives in
barley protoplasts. The expression of such interfering
RNAs in transgenic plants may be preferable to satellite
RNAs for developing virus-resistant cultivars.

Methods

Chemicals and enzymes. Restriction and modifying enzymes
were obtained from Boehringer-Mannheim, New England Biolabs (NEB)
and Bethesda Research Laboratories (BRL). Cap analogue
(m'GppGpp), T4 DNA ligase and mung bean nuclease were purchased
from NEB. T7 RNA polymerase and human placental ribonuclease
inhibitor (RNAguard) were obtained from Pharmacia. T3 RNA
polymerase and Bal-31 nuclease were obtained from BRL and
International Biotechnologies respectively.

Plasmid constructs and in vitro transcription. The construction of
plasmids pT781, pT782 and pT783, bearing cDNA clones from which
infectious full-length wild-type (wt) BMV RNAs -1, -2 and -3 can be
transcribed in vitro, was described by Dreher et al. (1989), also see Fig.
1. Deletion mutations such as pT782 ΔNcoI/StuI [Nc/S; a deletion of
nucleotides (nt) 883 to 2502, inclusive], pT782 ΔNaeI/MluI (Na/M; a
deletion of nt 220 to 1664) and the double mutant pT782 ΔSalI/NcoI
+ ΔMluI/StuI (Sa/Nc + M/S; a deletion of nt 31 to 886 and nt 1681 to
2502, respectively) were constructed by digestion of pT782 at two
unique restriction sites and subsequent digestion with mung bean
nuclease to blunt single-stranded overhangs prior to ligation. Deletions
were confirmed by restriction enzyme analysis and sequencing.
Construction of deletion mutations pT782 ΔMluI/StuI and pT782
ΔEcoRV/StuI (which yield pRNA-2 M/S and pRNA-2 E/S, respecti-
vely) was described in Pogue et al. (1990) and Marsh et al. (1991b).
Small deletions in the region of the translation initiation codon (nt 104)
were made by linearization of pT782 at the BstBI site (nt 109) and Bal 31
nuclease digestion for 10 s. Recircularization resulted in the constructs
pT782 Δ9@BstBI, a 9 nt deletion (nt 105 to 113) that includes the
initiation codon, and the pT782 Δ23@BstBI deletion (nt 109 to 133),
resulting in a frameshift following the second codon. RNA-2 Δ9
and RNA-2Δ23, respectively, were transcribed from these constructs.
Transfer of these deletions to pT782 ΔNcoI/StuI produced
pT782Δ23@NcoI/StuI + Δ9@BstBI and pT782ΔNcoI/StuI + Δ23@BstBI
which yielded the transcripts NRI RNA-2 Nc/S + Δ9 and NRI RNA-2
Nc/S + Δ23. Capped, infectious, RNAs were transcribed with T7
RNA polymerase as described by Dreher et al. (1989) from plasmids
linearized with BamHI. Transcripts were separated from the DNA
template by precipitation with 2.6 M-LiCl (Rao et al., 1989).

Protoplast isolation and inoculation. Barley (Hordeum vulgare cv.
Dickson) protoplasts were isolated (Loesch-Fries & Hall, 1980) and
inoculated (Dreher et al., 1989) with capped viral RNA transcripts
(1 μg of each unless otherwise noted). Following transfection,
protoplasts were incubated at room temperature under fluorescent
lights for 24 h. RNA was extracted with SDS and phenol–chloroform,
followed by precipitation with ethanol (Loesch-Fries & Hall, 1980).

Analysis of progeny viral RNA. Replication of viral RNAs in
protoplasts was analysed by electrophoretic separation in 1% agarose
and Northern blotting as described by Dreher et al. (1989). Specific
plus- or minus-sense RNA probes were 32P-labelled to identical
specific radioactivity by transcription from plasmid pT772TR, using
either T3 or T7 RNA polymerase (Rao et al., 1989). This plasmid

Fig. 1. BMV RNA-2 deletion mutants used in this study. Map of the
RNA-2 cDNA insert in pT782 (Dreher et al., 1989) showing relevant
restriction sites, T7 promoter (cross-hatched). AUG start site and the
polymerase (Kamer & Argos, 1984) CDD Motif (*) of the p2a reading
frame (open box). Large deletions, such as ΔMluI/StuI, ΔEcoRV/StuI,
and frameshift mutations in the region of the p2a AUG, S9@BstBI and
A23@BstBI, are indicated by solid bars. Plasmids containing the
indicated internal deletions within the viral cDNA were used as
transcription templates to derive the various pRNAs and non-
replicating (NR) RNAs.
contains a cDNA insert corresponding to the tRNA-like structure (3'-terminal 200 bp) which is conserved among all BMV RNAs. The use of these probes allowed the densitometric data to be readily converted into molar ratios and facilitated direct comparison of plus- and minus-strand progeny RNA levels. The data presented are representative of at least two independent experiments. Northern blots were autoradiographed with pre-flashed film (Laskey & Mills, 1977) and relative RNA levels were measured from appropriately exposed autoradiographs using a Bio-Rad model 620 video densitometer.

Results

Interference of NRI RNA-2 with the replication of genomic RNAs-1 and -2

RNA transcripts of five deletion mutants of BMV RNA-2 were screened for their ability to replicate in the presence of genomic RNAs-1 and -2. As reported previously (Pogue et al., 1990; Marsh et al., 1991b), pRNA-2 M/S and pRNA-2 E/S replicated efficiently, with both plus- and minus-strand progeny accumulating to levels similar to those of wt RNAs-1 and -2 (Fig. 2a, b). In contrast, RNA blot analysis of RNA-2 deletion mutants Na/M, Sa/Nc + M/S and Nc/S revealed the presence of only plus-strand RNA (compare lanes 4 to 6 of Fig. 2a, b). In addition, the hybridization signals obtained for these mutants when co-inoculated with RNAs-1 and -2 were equal or lower than those obtained from inoculations containing the mutant RNAs alone (compare Fig. 2a and c). In such inoculations, no replication occurred and the signal represented undegraded residual inoculum RNA. Consequently, the failure to detect minus-strand accumulation taken together with the lower signals for plus-strands revealed that the RNA-2 deletion mutants Na/M, Sa/Nc + M/S and Nc/S did not replicate. In spite of the inability of RNA-2 Nc/S to replicate, the presence of this mutant significantly reduced the accumulation of both co-inoculated BMV RNAs (Fig. 2a and b, lanes 6), showing that the Nc/S mutant possesses interfering properties.

The replication of deletion mutants pRNA-2 M/S and pRNA-2 E/S was previously shown to be dependent upon (Pogue et al., 1990) and at the expense of (Marsh et al., 1991b) the genomic BMV RNAs, leading to their designation as parasitic RNAs (pRNAs). Since interference with viral replication by the pRNAs was most marked at elevated inoculum levels (Marsh et al., 1991b), the effect of increased concentrations of the non-replicating RNA-2 mutants on genomic RNA accumulation was evaluated. The presence of 4 μg of RNA-2 Nc/S reduced replication of genomic RNAs-1 and -2 to less than 10% of the control (Fig. 3a, b; compare lanes 1 and 5); at lower inoculum amounts (2 μg), marked interference with RNA-2 replication was evident. At 1 μg, although reduction in RNA-2 accumulation is most readily seen for minus-strand progeny (Fig. 3b, lane 3), quantitative analysis revealed a 60% decrease in RNA-2 plus-strand progeny relative to RNA-1 (Fig. 3a, lane 3). Indeed, this preferential decrease in accumulation of
parental genomic RNA progeny is characteristic of these interfering RNAs. Under these conditions, the presence of pRNA-2 M/S (1 µg) decreased plus-strand RNA-2 progeny by 40% (Fig. 3a, lane 2). Regardless of inoculum concentration, no minus-strand progeny were detected for RNA-2 Nc/S (Fig. 3b). Consequently, these data show that an RNA incapable of replication can interfere with viral RNA accumulation and the term non-replicating, interfering (NRI) RNA-2 Nc/S is used subsequently for this mutant. The other non-replicating (NR) deletion mutants (Na/M and Sa/Nc + M/S) showed no ability to interfere with genomic RNA replication (Fig. 2).

Evidence that the observed interference is not due to decreased uptake of the viral RNAs by protoplasts at the higher inoculum levels is provided by the lack of a significant decrease in BMV replication at inoculum levels exceeding 12 µg of wt RNA-2 (Rao & Hall, 1991). Additionally, inocula containing yeast tRNA at an approximately 50-fold molar excess over that of the BMV genome produced no inhibition of viral replication (data not shown).

**Effect of blocking translation of NRI RNA-2 on its interference properties**

Very small amounts of protein p2a, the putative BMV core polymerase, translated from the non-replicating mutant of RNA-2 support replication of RNAs-1 and -3 in trans (Rao & Hall, 1990). Defective polymerases encoded by mutants of Qβ, a plus-stranded RNA phage of *Escherichia coli*, have been shown to interfere with its replication (Inokuchi & Hirashima, 1987). Since the p2a encoded by NRI RNA-2 Nc/S is truncated at the C terminus, the possibility existed that it generated a peptide that was primarily responsible for replication interference. To examine this possibility, we tested the ability of two NRI RNA-2 Nc/S derivates (Nc/S + A9 and Nc/S + A23) to interfere with viral replication. The deletions in these mutants block translation of p2a; indeed, RNA-2 bearing either of these deletions did not support replication of viral RNAs (Marsh *et al.*, 1991b; data not shown).

At an inoculum level of 1 µg (Fig. 4, lane 2), NRI RNA-2 Nc/S reduced the RNA-2 plus-strand progeny by 63% and total RNA-1 and -2 progeny by 41%. Inoculations of a low amount (1 µg) of NRI RNA-2 Nc/S + A9 or NRI RNA-2 Nc/S + A23 did not show the preferential reduction in RNA-2 accumulation seen for NRI RNA-2 Nc/S (Fig. 4). At 4 µg, NRI RNA-2 Nc/S + A9 reduced the replication of genomic RNAs-1 and -2 by 69% (Fig. 4; compare lanes 7 and 3). This confirmed that interference did not primarily depend on the production of a defective replicase. Although NRI
RNA-2 Nc/S + Δ23 caused a lower (41%) reduction in genomic RNA accumulation, taken together, these results indicate that the modulation of interference effects caused by the NRI RNA-2 derivatives results from the introduced sequence deletion rather than the elimination of a translation product. The present data do not provide insight to the mechanism by which certain sequence modifications modulate interference and further studies are required to determine whether RNA-RNA interactions (e.g. in the template being replicated) are involved.

**NRI RNA-2 interference in the presence of wt and mutant RNA-3**

Genomic RNA-3 is essential *in planta* for both systemic spread and local lesion formation because it encodes both the putative transport protein and the viral coat protein. Although RNA-3 is not required for replication of RNAs-1 and -2 in protoplasts, its presence markedly affects the ratio (symmetry) of plus : minus strand RNA accumulation (Marsh *et al.*, 1991a). Whereas progeny plus : minus ratios are close to unity in the absence of RNA-3 or in the presence of a mutant (ΔSGP RNA-3) lacking the subgenomic promoter core, they are approximately 100:1 in the presence of wt RNA-3. Because it seemed possible that the interference effects of NRI RNA-2 Nc/S may be diminished by this ability of RNA-3 to enhance plus-strand synthesis relative to minus-strand synthesis, assays were carried out in the presence of wt RNA-3 or ΔSGP RNA-3.

The data presented in Fig. 5 reveal strong interference by NRI RNA-2 Nc/S, when present in the inoculum at elevated levels, of genomic RNA replication in the...
presence of either ∆SGP RNA-3 or wt RNA-3. The nearly equal production of plus- and minus-strand progeny in the presence of ∆SGP RNA-3, which produces no subgenomic RNA-4, is evident from the identical exposure times used for the autoradiographs in panels (a) and (b) of Fig. 5. A preferential decrease in minus-strand synthesis is readily visible in the presence of 8 μg of NRI RNA-2 Nc/S (Fig. 5a, b; compare lanes 5), although this effect is detectable at all concentrations used. The greatly increased production of plus-strand progeny when all three wt genomic RNAs are present in the inoculum is reflected in lane 1 of Fig. 5(c), which was exposed for approximately one-fifth the time of the corresponding panel (Fig. 5d) showing minus-strand progeny. Under these conditions, RNAs-3 and -4 constitute 80% of the total plus-strand progeny (Marsh et al., 1991a). In the inoculations containing NRI RNA-2 Nc/S, both the preferential debilitation of minus-strand synthesis and the characteristic reduction in accumulation of the parental genomic RNA-2 are evident in the presence of wt RNA-3 or ∆SGP RNA-3 (Fig. 5).

Quantitative densitometry of progeny accumulation showed that in assays containing 1 μg of NRI RNA-2 Nc/S, plus-strand RNA-2 decreased by 37% and 28% in the presence of wt RNA and ∆SGP RNA-3, respectively; more substantial decreases of 68% and 37% were observed for minus-strand RNA-2. Total plus-strand RNA was reduced by 55% and total minus-strand RNA by 67% in the assays containing wt RNA-3 and 2 μg of NRI RNA-2 Nc/S (Fig. 5c and d). When 8 μg of NRI RNA-2 Nc/S was included in the inoculum, a dramatic (>90%) reduction in total minus-strand RNA accumulation was seen in assays containing either wt RNA-3 or ∆SGP RNA-3. Smaller decreases in plus-strand RNA were observed when the amount of NRI RNA-2 Nc/S in the inoculum increased from 2 to 8 μg.

Discussion

Substantial evidence now exists for a complex series of motifs within the BMV genome that regulate plus-strand replication (Pogue et al., 1990), minus-strand (Dreher & Hall, 1988; Dreher et al., 1989) and subgenomic RNA synthesis (French & Ahlquist, 1988; Marsh et al., 1988). Although certain internal deletions in BMV RNA-3 dramatically reduce its replication (French & Ahlquist, 1987), extensive deletions in BMV RNA-2, represented by E/S and M/S, do not deplete replication of the sequence bearing them (Fig. 2), indicating that essential promoter elements are not present in the deleted region. As shown here, other large deletions (Na/M, Sa/Nc+M/S, and Nc/S) extending 5′ of the EcoRV site on RNA-2 cDNA (Fig. 1) eliminate replication of the viral RNA bearing them. The absence of detectable minus-strand RNA corresponding to the non-replicating (NR) RNAs (Fig. 2b) indicates that minus-strand synthesis in these mutants has been greatly reduced, although reductions in plus-strand synthesis may also occur.

Although it might be assumed that the extensive deletions involved in creating the NR RNAs could result in instability of the viral RNA (thereby contributing to poor replication), it is evident from Fig. 2(c) that, when inoculated alone, NR RNA Na/M, Sa/Nc+M/S and NRI RNA-2 Nc/S are much more stable than the efficiently replicating pRNA-2 M/S. Previous studies have shown that when RNA-1 and -2 are inoculated separately, residual inoculum RNA is scarcely detectable 24 h post-inoculation (Rao et al., 1990). However, when both RNAs-1 and -2 were supplied as inoculum, the signals corresponding to their progeny at 24 h post-inoculation (Fig. 2, lane 1) were similar to those for the NR RNAs (Fig. 2c, lanes 3 to 5) inoculated alone. The input NR RNAs can, therefore, readily be mistaken for progeny RNA. Consequently, it is essential to evaluate minus-as well as plus-strand accumulation when characterizing the properties of mutant RNAs. This important control has been omitted in several studies which assessed the replication competence of mutant virus sequences.

The replication of the pRNAs is dependent upon (Pogue et al., 1990) and at the expense of the genomic RNAs, leading to substantial interference with BMV accumulation (Marsh et al., 1991b; Fig. 2). However, one of the non-replicating RNAs, NRI RNA-2 Nc/S, maintains the ability to interfere with replication of the genomic RNAs (Fig. 2 and 4). The accumulation of the parental RNA-2 is particularly reduced by the presence of NRI RNA-2 Nc/S, and both pRNAs, when present at low inoculum levels (Fig. 2 and 4). Such specific interference events imply that the limited sequence differences between the 3′ non-translated regions of the three genomic BMV RNAs may mediate RNA-specific interactions by the replicase or other proteins. Indeed, deletion of bases constituting the translational start site of p2a of NRI RNA-2 Nc/S eliminated its specific interference with RNA-2, suggesting that cis-specific interactions between 5′ and 3′ sequences are responsible for this phenomenon. Higher proportions of the interfering RNAs in the inoculum strongly inhibited the replication of all BMV RNAs, thereby concealing any RNA-specific interactions.

Although marked decreases in viral plus-strand accumulation were observed (Fig. 3, and 5), NRI RNA-2 Nc/S showed preferential interference with minus-strand accumulation, and the mechanism by which the NRIs interfere with genomic RNA replication is unclear. One explanation could be an ability to sequester...
replicase in unproductive complexes, with minus-strand elongation being blocked in the region of the deletion. Morch et al. (1987) suggested a similar mechanism for the ability of short, positive-sense 3' fragments to debilitate turnip yellow mosaic virus RNA replication \textit{in vitro}. Because we were unable to detect the incomplete minus-strands expected to be present if blocking of elongation were the only effect, it appears that they are rapidly degraded, or that no initiation of minus-strand synthesis occurs. Discrimination between these and other possible mechanisms will be the focus of future studies. Comparisons \textit{in vitro} of minus-strand promoter activities of non-replicating deletion mutants with those which replicate efficiently (e.g. NRI RNA-2 Nc/S compared to pRNA-2 M/S) may provide important insight to the processes involved.

The ability of NRI RNA-2 Nc/S to inhibit BMV genomic RNA accumulation by over 50\% at an inoculum molar ratio of approximately 1:1 demonstrates its effectiveness in reducing viral replication. This level of interference is similar to the 65\% reduction in tomato bushy stunt virus replication by a naturally occurring DI-RNA when inoculated at a 1:1 molar ratio (Jones et al., 1990). An over 60\% reduction in BMV RNA accumulation was observed when pRNA-2 E/S was inoculated at a 1.5:1 molar ratio (Marsh et al., 1991b). Although inhibition of BMV replication by NRI RNA-2 Nc/S has not been demonstrated in plants, the large reductions in BMV accumulation in protoplasts (Fig. 4) indicates that such constructs may be useful tools for controlling viral infection when highly expressed in transgenic plants. Since previous studies have shown that BMV is unable to establish a systemic infection in barley when the replication of RNA-3 is reduced by over 60\% (Dreher et al., 1989), complete inhibition of viral replication may not be necessary to provide significant levels of protection.

The expression of satellite RNAs in transgenic plants can confer resistance to virus infection (Gerlach et al., 1987; Harrison et al., 1987). However, the limited number of satellites associated with plant viruses presents a major barrier to wide application of this approach. Additionally, the possibility exists that naturally occurring satellites can mutate or spread to other plants where their presence will aggravate rather than ameliorate viral pathogenesis. Controlling viral diseases by NRI RNAs, which can be constructed from the cDNAs of a wide range of viruses, has certain important advantages. The enhanced stability of the NRI RNAs will aid in the accumulation of sufficient levels of interfering RNAs within the transformed plant cells to establish and maintain protection. The range of viruses inhibited by NRI RNAs will probably reflect the commonality in sequence elements recognized by host- and virus-encoded protein factors. The non-replicating nature of NRI RNAs would prevent their amplification in the presence of a helper virus, as seen with naturally occurring DI-RNAs and satellites, and therefore prevent acquisition of mutations or additional properties, such as encapsidation signals, which would allow their spread from the resistant plant to a more sensitive cultivar. The present data substantiate the functionality of NRI RNAs in protoplasts and provide a rationale for future tests of their efficacy in transgenic plants.

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