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Structural Analysis of a Necrogenic Strain of Cucumber Mosaic Cucumovirus Satellite RNA *in Planta*

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Structural studies of plant viral RNA molecules have been based on *in vitro* chemical and enzymatic modification. That approach, along with mutational analysis, has proven valuable in predicting structural models for some plant viruses such as tobacco mosaic tobamovirus and brome mosaic bromovirus. However, *in planta* conditions may be dramatically different from those found *in vitro.* In this study we analyzed the structure of cucumber mosaic cucumovirus satellite RNA (sat RNA) strain D4 *in vivo* and compared it to the structures found *in vitro* and in purified virions. Following a methodology developed to determine the structure of 18S rRNA within intact plant tissues, different patterns of adenosine and cytosine modification were found for D4-sat RNA molecules *in vivo, in vitro,* and in virions. This chemical probing procedure identifies adenosine and cytosine residues located in unpaired regions of the RNA molecules. Methylation data, a genetic algorithm in the STAR RNA folding program, and sequence alignment comparisons of 78 satellite CMV RNA sequences were used to identify several helical regions located at the 5′ and 3′ ends of the RNA molecule. Data from previous mutational and sequence comparison studies between satellite RNA strains inducing necrosis in tomato plants and those strains not inducing necrosis allowed us to identify one helix and two tetraloop regions correlating with the necrogenicity syndrome.

INTRODUCTION

Satellite RNAs (sat RNAs) of cucumber mosaic cucumovirus (CMV) are small (333–405 nt), linear RNA molecules (Roossinck *et al.,* 1992) that multiply using the replication machinery of their helper virus, CMV (Mossop and Francki, 1978). Moreover, CMV sat RNAs use the coat protein of CMV for encapsidation (Kaper *et al.,* 1976), which allows them to be disseminated in nature by numerous aphid species (Palukaitis *et al.,* 1992). The effect of the sat RNAs on helper-induced symptomatology varies with different strains of helper and sat RNAs and with the host plant (Collmer and Howell, 1992; García-Arenal *et al.,* 1987; Sleat and Palukaitis, 1990; Sleat *et al.,* 1994; Zhang *et al.,* 1994) and ranges from attenuation in a majority of hosts to rapid necrosis and death in tomato (reviewed in Roossinck *et al.,* 1992). The CMV sat RNAs can be divided into two major groups, depending on whether or not they induce necrosis in tomato plants (Devic *et al.,* 1990). The necrogenic sat RNAs have resulted in devastating crop losses in southern Europe (Jorda *et al.,* 1992; Kaper and Waterworth, 1977; Kaper *et al.,* 1990). There are no apparent functional open reading frames in CMV sat RNAs (Collmer and Kaper, 1988; Devic *et al.,* 1990; Jaegle *et al.,* 1990; Masuta and Takanami, 1989), indicating that the sat RNA itself interacts with its helper virus and host factors to exert its biological activity (García-Arenal *et al.,* 1987).

A few studies have proposed structural models for six CMV sat RNA strains using *in vitro* enzymatic and chemical probing together with minimum free-energy RNA folding programs (García-Arenal *et al.,* 1987; Gordon and Symons, 1983; Hidaka *et al.,* 1988). The structures proposed for G-, B2-, B3-, and WL1-sat RNA are nearly identical (García-Arenal *et al.,* 1987). However, these sat RNA strains vary in the symptoms induced on tomato plants when coinfected with different CMV helper strains (García-Arenal *et al.,* 1987).

Chimeric and mutational analyses with several CMV sat RNAs have identified regions of the molecule involved in the induction of chlorosis and/or necrosis in solanaceous hosts (Collmer and Howell, 1992; Roossinck *et al.,* 1992; Sleat *et al.,* 1994; Zhang *et al.,* 1994). However, mutations causing functional changes could be affecting the global RNA structure (Bernal and García-Arenal, 1994a,b) rather than having a local effect. Hence the secondary structure of the sat RNA is important, and its solution may lead to a greater understanding of the biological activity of RNA molecules.

In a few systems, RNA and DNA molecules have been chemically modified using an *in vivo* approach (Ares and Igel, 1990; Climie and Friesen, 1988; Ephrussi *et al.,* 1985; Harris *et al.,* 1995; Mayford and Weisblum, 1989; Nick and Gilbert, 1985; Senecoff and Meagher, 1992, 1993; Zaug and Cech, 1995). Such *in vivo* studies should provide information on the structural status of RNA mole-

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cules actively functioning inside the cell and on their interactions with viral and host factors.

Satellite RNAs of CMV have been predicted to have a high degree of secondary structure, with base pairing ranging from 49 to 52% (García-Arenal *et al.,* 1987). In this study, we mapped unpaired adenosine and cytosine residues of a necrogenic CMV sat RNA, D4-sat RNA (Kurath and Palukaitis, 1989), susceptible to modification by dimethyl sulfate (DMS) *in vivo,* inside virions, and *in vitro.* The information obtained was used to generate three working models of the structure of D4-sat RNA, one for each one of the three conditions. Predicted structural motifs were further analyzed by alignment of 78 CMV sat RNA sequences.

MATERIALS AND METHODS

Virus and sat RNA

Fny-CMV was described previously (Owen and Palukaitis, 1988). Virus was purified from infected *Nicotiana tabacum* cv Xanthi nc (tobacco) and viral RNA was extracted following established procedures (Roossinck and White, 1997). The D4-sat is derived from a cDNA clone of D-sat RNA, pDsat4 (Kurath and Palukaitis, 1989). Infectious transcripts were generated from pDsat4 and were coinoculated with Fny-CMV onto tobacco seedlings to obtain gel-purified sat RNA (Palukaitis and Zaitlin, 1984).

Plant inoculations

Seedlings of tobacco at the second-leaf stage were inoculated with Fny-CMV RNA at a concentration of 300 μg/ml and with gel-purified D4-sat RNA at 5 μg/ml. Coinfected plants were kept in a greenhouse at 26° for 12 days. Systemically infected leaves were used for *in planta* methylation and for virus purification.

Total RNA extraction

DMS-treated (see below) and control-treated leaves were directly frozen in liquid nitrogen. Frozen tissue was stored at −20° until use or immediately ground using a chilled mortar and pestle and RNA was extracted (Palmiter, 1974), including a step with 1 *M* LiCl to selectively separate sat RNA (Palukaitis and Roossinck, 1995). After RNA was concentrated it was resuspended in 0.1 m*M* EDTA, pH 8.0, and stored at −20°.

DMS treatments

The DMS (Aldrich, Milwaukee, WI) treatment *in vivo* of infected tobacco leaves was a modification of the procedure described previously by Senecoff and Meagher (1992) for soybean. Leaves were treated in methylation buffer (80 m*M* potassium cacodylate, pH 7.8; 20 m*M* magnesium acetate; 300 m*M* potassium chloride; 0.1% v/v NP-40) with and without DMS. Several concentrations of DMS (0.5 to 2.0%, v/v), several exposure times (10 to 60 min), and two different temperatures (25° or ice) were used to optimize the assay. The conditions selected to carry out the *in vivo* methylation included exposure of tobacco leaves to DMS at 1% (v/v) for 25 min on ice. After DMS treatment leaves were rinsed three times with distilled water. The *in planta* DMS modification experiments were repeated three times.

Gel-purified sat RNA was DMS modified *in vitro* as described previously for 18S rRNA (Senecoff and Meagher, 1992) with some modifications. Five hundred nanograms of sat RNA was DMS-treated in 100 μl of methylation buffer lacking NP-40. Sat RNA was boiled for 1 min and then placed at room temperature for 10 min. RNA molecules were treated with DMS at room temperature for 5 min under native conditions or under semidenaturing conditions using methylation buffer lacking magnesium. Reactions were terminated and DMS was removed with phenol/chloroform (Senecoff and Meagher, 1992). Sat RNA was resuspended in 6 μl of water for the primer extension analysis. The *in vitro* DMS modification experiment was repeated two times.

Purified CMV particles containing genomic viral RNA and D4-sat RNA were treated with DMS following a procedure described for Moloney murine leukemia virus (Alford *et al.,* 1991), with modifications. Five micrograms of virions suspended in 300 μl of CMV buffer C (5 m*M* sodium borate, pH 9.0, 0.5 m*M* EDTA) was mixed with 1 μl of DMS (final concentration 0.3%) or 6 μl of DMS (final concentration 2.0%). DMS was dissolved by vortexing and the reactions were carried out on ice for 5 min. DMS methylation was terminated by addition of 60 μl of DMS stop buffer (1 *M* Tris–HCl, pH 7.5, 1 *M* β-mercaptoethanol, 1.5 *M* sodium acetate, 100 m*M* EDTA, pH 8.0) and incubation at 25° for 10 min. Samples were divided into two microfuge tubes and placed on ice for 10 min. Viral RNA was extracted as described previously (Roossinck and White, 1997) and resuspended in 6 μl of water for the primer extension analysis. The DMS modification experiment carried out in virions was repeated two times.

Primer extension and RNA sequencing

Methylated nucleotides were detected using a primer extension procedure based on a dideoxy RNA sequencing protocol (Mierendorf and Pfeffer, 1987). DMS-treated and untreated sat RNA from each experiment was analyzed by primer extension two to four times. D4-sat RNA sequencing ladders were obtained as previously described (Fichot and Girard, 1990). Three oligonucleotides were used for primer extension (Fig. 1A): primer 343, 5′-CAGAATTCGGGTCCTG; primer 1487, 5′-ATAGACATTCACGGAGATCAGC; and primer 342, 5′-CTGAGCGGGGGCTCA.

Secondary structure and sequence alignments

Prediction models for the secondary structure of D4-sat RNA were obtained using the genetic algorithm of

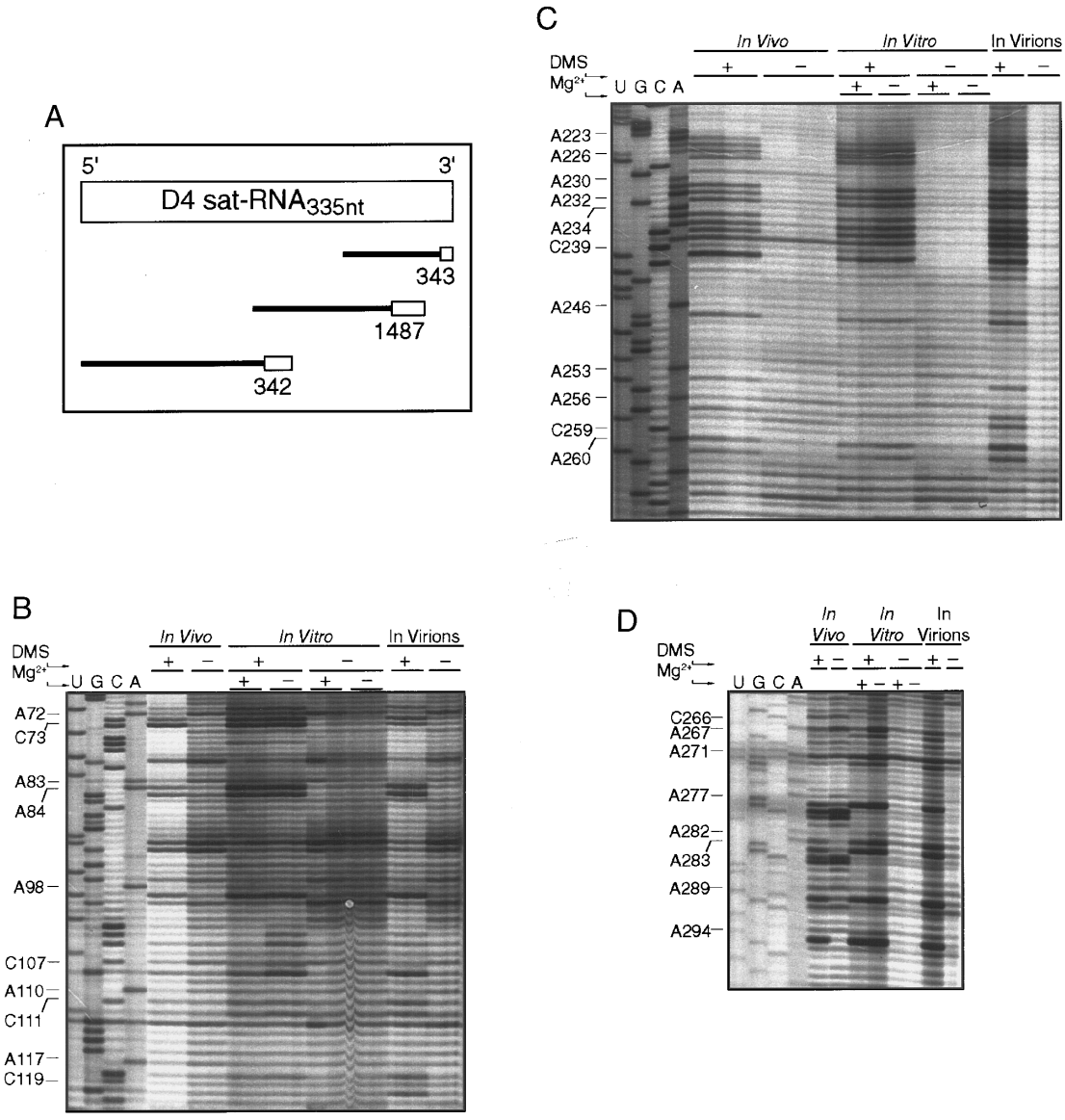

FIG. 1. Primer extension analysis of CMV D4-sat RNA after chemical probing with DMS *in vivo, in vitro,* and in virions. (A) Position of primers on the D4-sat RNA molecule, as indicated with white rectangles, and approximate extension of fragments analyzed. (B, C, and D) Results of primer extension using primers 342, 1487, and 343, respectively. Methylated bases are indicated to the left of each panel. Each methylated base is observed as a band appearing at the nucleotide preceding the modified base in DMS treatment lanes (+) compared to control lanes (−). Semidenaturing conditions in the *in vitro* treatment are indicated as Mg^{2+} (+) and Mg^{2-} (−), referring to the presence or the absence of magnesium in the methylation buffer respectively. Methylation treatments were as described under Materials and Methods. Lanes U, G, C, and A are sequencing lanes.

the program STAR 4.2b (Gultyaev *et al.,* 1995; van Batenburg *et al.,* 1995). Data from experiments (strongly methylated bases) were added for the RNA folding process. Sixty-four CMV sat RNA sequences were obtained from GenBank (Table 1). A sequence alignment was carried out using the program CLUSTALW from the GCG package (Genetics Computer Group sequence analysis software system; Deveraux *et al.,* 1984).

RESULTS

Methylation *in vivo,* in virions, and *in vitro*

Structural studies of RNA molecules and analysis of RNA–protein interactions have been carried out using chemical probing (Ehresmann *et al.,* 1987; Peattie and Gilbert, 1980). Chemical reagents such as DMS modify residues that are not involved in base pairing (Peattie and Gilbert, 1980). DMS modification of adenosine and cytosine residues at positions N_1 and N_3, respectively, blocks subsequent elongation by reverse transcriptase at the nucleotide preceding the modified residue during primer extension (Ehresmann *et al.,* 1987; Inoue and Cech, 1985). A limited reaction with the chemical probe allows fractions of the population of RNA molecules to be modified at one position (Stern *et al.,* 1988). The prematurely terminated cDNAs are then analyzed along with untreated samples in denaturing sequencing gels (Inoue

TABLE 1

Source of sat RNA Sequences and Accession Numbers

CMV sat RNA strains and cDNA clones	Sequence source	Necrogenic in tomato	CMV sat RNA strains and cDNA clones	Sequence source	Necrogenic in tomato
B1	M16586[a]	−	D	M10686	+
B2	M16587	−	pD4	Kurath and Palukaitis (1989)	+
B3	M16588	−	pD5	M30585	+
pB1, 3, 5[b]	M30591	−	pD6	Kurath and Palukaitis (1989)	+
pB2	M30549	−	pD9	M30587	+
pB4	M30593	−	pR1	M18869	−
1	X86409[c]	−	pR2	M18870	−
2	X86410[c]	+	pR3	M18871	−
4	X86415[c]	−	pRa	D00541	−
6	X86414[c]	+	WL1	Garcia-Arenal *et al.* (1987)	−
7	X86411[c]	+	WL2	M16590	−
8	X86420[c]	+	WL3	X51465	−
12	X86416[c]	+	pWL47	M30588	−
16	X86413[c]	+	pWL210	M30589	−
18	X86708[c]	+	pWL324	M30590	−
22	X86412[c]	−	pWLM1	Sleat and Palukaitis (1990)	−
27	X86419[c]	+	pWLM2	Sleat and Palukaitis (1990)	+
57	X86418[c]	+	pWLM3	Sleat and Palukaitis (1990)	−
59	X86423[c]	+	pWLM4	Sleat and Palukaitis (1990)	−
T73	D10037	+	pDW	Sleat *et al.* (1994)	−
77	X86422[c]	+	pWDWLM2	Sleat *et al.* (1994)	+
79	X86417[c]	+	pDWLM2	Sleat *et al.* (1994)	+
80	X86421[c]	+	pWWD	Sleat *et al.* (1994)	+
8A	X86408[c]	−	pWD	Sleat *et al.* (1994)	+
NG71(71)	X86424	−	pC7-2	D42081	?
Ce	X86425[c]	+	pCH20	M20353	+
PG	X86426[c]	+	1	J02061	−
Top stunting	X69136	−	pSq10	M20352	+
Fny-1	X54065	−	E	M20844	−
Fny-2	X54066	−	G	M16585	−
pX7	M20355	+	Q	J02060	−
pX12	M20356	+	S	M14934	−
pX15	M20357	+	IX	M64284	+
pX2c	M20360	−	K8	X53534	−
pX2nT3	M20354	+	F	D00699	?
I17F (D)	M18872	+	pMS1	X57582	?
I17N	A10086	+	YN	Kaper *et al.* (1988)	+
pI17N(1)	M18867	+	X2N	Kaper *et al.* (1988)	+
pI17N(2)	M18868	+	pT73	D10037	−

Note. "p" refers to cDNA clones.

[a] GenBank accession number.

[b] Identical cDNA clones generated from B1-sat RNA.

[c] Isolates described by Grieco *et al.* (1997).

and Cech, 1985), where they are observed as multiple bands which represent the several fractions of RNA molecules modified at each position. These conditions also result in premature termination at nonmethylated bases, but these bands are easily distinguished by comparison with non-DMS-treated samples.

CMV D4-sat RNA molecules exposed to DMS in helper and sat RNA coinfected intact tobacco leaves, in purified CMV particles, and *in vitro* had regions of different patterns of methylation (Table 2). A total of 47 adenosine and cytosine residues were consistently susceptible to methylation by DMS (Table 2), suggesting that these bases are located in unpaired regions of the sat RNA molecule. Twenty-eight bases were susceptible to DMS *in vivo, in vitro,* and in virions, and the remaining 19 bases varied in their susceptibility to DMS under the three conditions (Table 2). Sat RNA molecules inside virions had the highest number of methylated residues with 44. *In vivo* and *in vitro* sat RNA molecules had 37 and 35 modified bases, respectively. The fact that most of the modified bases were adenosine residues was not unexpected, because adenosine is chemically more susceptible to modification by DMS than cytosine (Inoue and Cech, 1985). More than 50% of the adenosine residues

TABLE 2

CMV D4-sat RNA Bases Modified by DMS and Comparison with Enzymatic and Chemical Probing in Four CMV sat RNA Strains

	DMS modification[a]			CMV sat RNAs[b]				
D4 bases	*In vivo*	In virions	*In vitro*	WL1	G	B3	B2	Q
A48	+++	+++	+++					
A72	+++	+++	+++			|A	Å	
C73	+++	+++	+++					
A83	+++	+++	+++	|A		|A	|A	
A84	+++	+++	+++					
A98	+++	+++	+++	|A				
C107		+++	+++					
A110		++			†A		†Å	
C111		++						
A117		++	+		†|A|			
C119		++						
C129	+++	++	++[c]	†C		†C	†C	†C
A131	+++	++	++		|A	†A	†Å	
A164	+++			|A|		|A	|A	
A180	++					|A	|Å	
A201	++	+++	++					
C202	+++	+++	++					
A223	+++	++	+++		†A	†A	†A	†|A|
A224	+++	+++	+++					†A
A225	+++	+++	+++					
A226	+++	+++	+++					
A230	+++	+++	+++	†A	†A	†|A|	†Å|	†A
A231	+++	+++	+++	A	A	A	Å	A
A232	+++	+++	+++	|A	A	A	Å	A
A234	+++	++	+++	†A	† A	A	†A	† A
A235	+++	+++	+++	A	A	A		A
A236	+++	+++	+++	A	A	A		A
C237		+++	+++	C	C	C|	C|	C
A238	+	+		A	A	|A	|Å	A|
C239	+++	+++	+++	C	C			|C
A246	+++	++	+	A	A|	|A	Å	A|
A253	+	+		A		|A	Å	
A256	++				|A	†A	Å	
C259	++	+++						
A260	++	+++	+	†A	†A			
C266		++	+			†C		
A267		++	+			A		
A271		+++	+			|A	Å	
A277	+++	+++	+++				|A|	
A282	+++	+++	++			A		
A283	+++	+++	++			|A		
A289		+++	++	|A|	|A	A	Å	|A
A294	+++	+++	+++			A|		A|
A300	+++	+++	+++		†A			†A
A309	+++	+++		†A			†A	
A310	+++	+++				†A		†A
A316	++	+++		A|	A|	†A		|A

[a] +++, strong methylation; ++, intermediate methylation; + weak methylation.

[b] Cleavage or chemical modification *in vitro* at the analogous positions in other CMV sat RNAs (Gordon and Symons, 1983; Garcia-Arenal *et al.,* 1987). \|M\|, S1 cleavage at 5′ end and/or 3′ end. \|, weak cleavage. M, A or C. †M†, T1 cleavage at 5′ end and/or 3′ end. †, weak cleavage. Å, adenosine residues modified with diethyl pyrocarbamate.

[c] Stronger band under semidenaturing conditions. DMS modification conditions were as described under Materials and Methods.

in D4 sat RNA were susceptible to DMS (37/69; modified adenosine/total adenosine) compared to 12% for cytosine residues (10/77). The 47 residues presented in Table 2 were DMS modified in two or three repetitions of the experiments. However, there was a group of 28 residues (11 A residues and 17 C residues) which were inconsistently methylated in several experiments (data not shown).

The first 47 bases at the 5′ end of the sat RNA molecule were not susceptible to DMS. Higher concentrations of DMS and longer incubation times did not improve the susceptibility of the bases in this region (data not shown). Figures 1B, 1C, and 1D show 36 modified residues from the total of 47 detected. Beginning at residue A_{48} and ending at A_{98}, 6 residues had strong methylation in the 5′ end of the sat RNA molecule under the three conditions tested (Fig. 1B, Table 2). Residue C_{107} is the only clear example of strong methylation both in virions and *in vitro,* but not *in vivo* (Fig. 1B, Table 2). C_{237} (Fig. 1C) had a DMS susceptibility similar to that of C_{107}; however, a strong band appears *in vivo* control lanes, masking the role of methylation in the stop by the enzyme reverse transcriptase.

An adenosine-rich region located between positions G_{222} and U_{240} showed consistent strong chemical modification by DMS (Fig. 1C, Table 2). This region includes an area with high variation in the sequence among all CMV sat RNAs (Kurath and Palukaitis, 1989, 1990; Palukaitis and Roossinck, 1995). In the CMV sat RNA strains WL1, B2, B3, G, and Q, the equivalent region was found to be highly susceptible to chemical and enzymatic probes *in vitro* (Table 2), suggesting that those modified bases were in single-strand regions (García-Arenal *et al.,* 1987; Gordon and Symons, 1983). Further comparisons among D4-sat RNA and those sat RNAs (Table 2) showed that there were few other bases that had the same susceptibility.

In several instances, during the primer extension analysis, bands with the same intensity appeared in DMS-treated and control lanes. Such is the case of residues U_{92}, U_{93}, U_{112} (Fig. 1B), and G_{269} (Fig. 1D). In a few cases, the same blockage to the reverse transcriptase occurred *in vivo* DMS-treated and control sat RNA, but not with virion or *in vitro* samples. Residues C_{237} (Fig. 1C), G_{278}, G_{279}, G_{284}, and G_{285} (Fig. 1D) are examples of such situations.

Cytosine residue C_{129} had stronger methylation under semidenaturing conditions *in vitro* than when the methylation buffer contained magnesium (Table 2), suggesting weak tertiary interactions associated with this residue (Peattie and Gilbert, 1980).

Although DMS methylation of sat RNA was efficient in purified virions, it was not possible to purify virions from DMS-treated infected tissue. Protein–RNA interactions required for the structural integrity of CMV virions (Boatman and Kaper, 1976) may have been disrupted by the methylation (data not shown). DMS methylation under semidenaturing conditions in purified virions was not carried out because variation in salt concentration could disrupt virion stability (Palukaitis *et al.,* 1992). In addition, we considered that DMS methylation under semidenaturing conditions *in planta,* using a methylation buffer lacking magnesium, was unlikely to affect intracellular magnesium concentration.

Structural models for CMV D4-sat RNA

Three models for the structure of D4-sat RNA were obtained using a genetic algorithm of the RNA folding program STAR (Gultyaev *et al.,* 1995; van Batenburg *et al.,* 1995), which included the DMS probing data from *in vivo* (Fig. 2A), *in vitro* (Fig. 2B), and in-virion (Fig. 2C) methylation experiments. Bases that were strongly methylated were not allowed to base pair during the RNA folding and they are indicated in each structure (Figs. 2B, 2C, and 2D). A comparison of structures generated using the three algorithms of the STAR program (Abrahams *et al.,* 1990) and the MFOLD program (Zucker, 1989) run at 25 and 37° was carried out to select the structure showing the highest consistency with the modified bases (Rodriguez-Alvarado and Roossinck, unpublished results).

Comparison of the structures shown in Fig. 2 suggests four major folding regions: (I) the region between position U_8 and position C_{56}; (II) a region which includes bases A_{55} to G_{213} and presents similar and different folding structures among the three models; (III) a region of high susceptibility to DMS modification from G_{222} to U_{240} mentioned above; and (IV) the last 95 nucleotides at the 3′ end. Three of these regions (I, II, and IV) are highly similar among the three models.

Alignment and comparison of sequences involved in structural motifs in CMV sat RNAs

Phylogenetic comparative analysis has been the main approach for the elucidation of higher order structures in homologous RNA sequences (James *et al.,* 1989). Comparative sequence analysis is based on the concept of positional covariance (Gutell *et al.,* 1994). Putative base-pair positions in possible helical regions are indicated by the occurrence of covariance at both positions (James *et al.,* 1989). Although comparative analysis has been a powerful tool to elucidate the structures of a number of RNA molecules (James *et al.,* 1989), its use in identifying structural motifs in CMV sat RNAs is limited for two main reasons. First, no regions of the CMV sat RNA molecule have been associated with specific functions during replication, encapsidation, or pathogenesis. Second, the high similarity of the CMV sat RNA sequences reduces the number of covariant positions to be detected in order to infer a structural motif and show its conservation through evolution (Fraile and García-Arenal, 1991).

Our analysis of secondary structure in sat RNAs is based on a search for canonical and G:U base pairs. Such base pairs constitute a majority in the helical regions of 16S and 23S rRNA structures predicted by comparative sequence analysis (Gutell, 1996). The bases forming the helical regions predicted for the *in vivo* structural model of D4-sat RNA were aligned with 77 other CMV sat RNA sequences and compared for genetic variation (Tables 3 and 4). The search for genetic variation

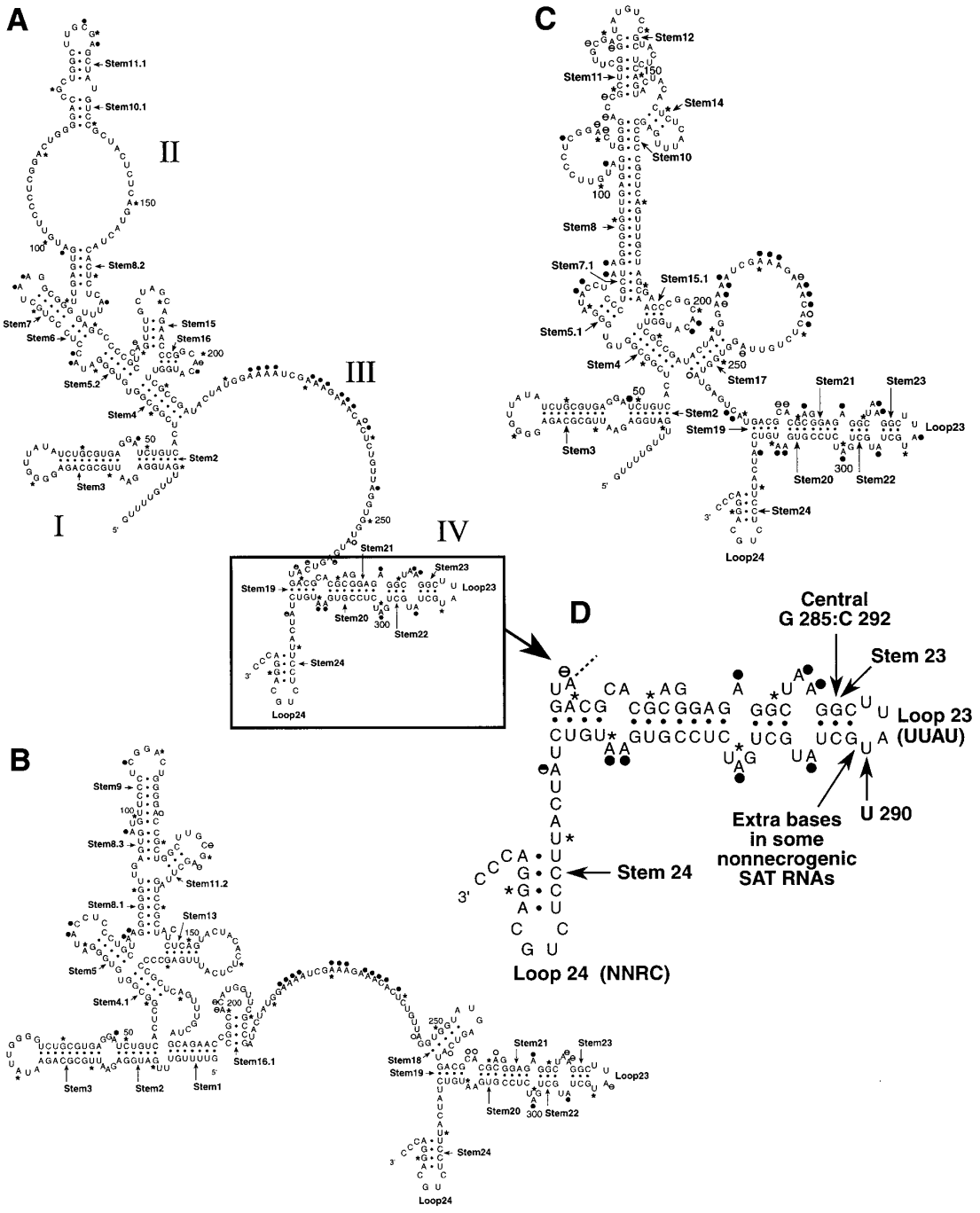

FIG. 2. Structural models for D4-sat RNA *in vivo* (A), *in vitro* (B), and in virions (C). Residues susceptible to modification by DMS are indicated as follows: ●, strongly methylated bases; ø, intermediate methylated bases; ○, weakly methylated bases. Numerals I, II, III, and IV in A indicate regions of the structure described in the text. Inset in D includes the necrogenic domain (Devic *et al.,* 1990; Kurath and Palukaitis, 1989). An asterisk indicates a 10-nt interval.

in the helical regions followed the arrangement of the motifs into the four regions described above. Except as noted below most stems contain noncovariant changes compared to other sat RNA sequences.

Region I presents two helices, Stems 2 and 3, which are present in all three models (Figs. 2A, 2B,and 2C). Comparison of sequences among sat RNAs showed that Stem 2 had one example of sat RNA sequences with covariant changes (data not shown), and Stem 3 had 31 sat RNA sequences with covariant changes at either one or two of three base-pair positions (Table 3).

Region II presents nine helical regions in the *in vivo* model (Fig. 2A). Alignment of sequences among the sat RNAs showed one example of a covariant change in Stem 11.1 in this region only (data not shown). Stem 5.2 (Fig. 2A) was the only helical region conserved in all sat

TABLE 3

Sequence Alignment Comparison of Bases Forming Stem 3 in the Structure Model of CMV D4-sat RNA with 77 CMV sat RNA Sequences

Sat RNA sequences	Necrogenic in tomato	Stem 3	Stem 3′	D4 base pair	Other strains base pair	Canonical or G:U base pair	Covariant base pair
D4	+	UUGCGCAGA[a]	UCUGCGUGA				
21 sequences	+	UUGCGCAGA	UCUGCGUGA				
IX	+	UcGCGCAGA	UCUGCGUGA	U-G	c-G	+	
WLM2	+	UUGCGuAGA	UCUaCGUGA	C-G	u-a	+	+
WD	+	UUGCGuAGA	UCUaCGUGA	C-G	u-a	+	+
WDWLM2	+	UUGCGuAGA	UCUaCGUGA	C-G	u-a	+	+
WWD	+	UUGCGuAGA	UCUaCGUGA	C-G	u-a	+	+
4 sequences	+	UUGCGugGA	UCUGCGUGA	A-U	g-U	+	
				C-G	u-G	+	
T59	+	UUGCGugGA	UCUcgGUGA	A-U	g-U	+	
				C-G	u-c		
				G-C	G-g		
To80	+	UUGaGagGA	UCUGCGUGA	C-G	a-G		
				C-G	a-G		
				A-U	g-U	+	
2 sequences	+	UUGCGuAGA	UCUGCGUGA	C-G	u-G	+	
T57	+	UUGCGCCAGA	UUGCGCCAGA	C-G	C-a		
2 sequences	+	UUGCGCCgGA	UCUGCGUGA	A-U	g-U	+	
3 sequences	−	UUGCGCAGA	UCUGCGUGA				
20 sequences	−	UUGCGuAGA	UCUaCGUGA	C-G	u-a	+	+
E	−	cUGCGugGA	UCUGCGUGg	U-A	c-g	+	+
				C-G	u-G	+	
				A-U	g-U	+	
2 sequences	−	UUGCGCgGA	UCUaCGUGA	A-U	g-U	+	
				C-G	C-a		
2 sequences	−	UUGCGuAGA	UucaCGUGA	C-G	u-a	+	+
				G-C	G-u	+	
				A-U	A-C		
F	−	UUaCGCgGA	UCUGCGUGA	G-U	a-U	+	
				A-U	g-U	+	
Top stunting	−	UUGCGugGA	UucaCGUGA	C-G	u-a	+	+
				A-U	g-c	+	+
				G-C	G-u	+	
S	−	UUGCGuAGA	UCUaCGU_A	C-G	u-a	+	+
2 sequences	−	UUGCGuAGA	UCUauguga	C-G	u-a	+	+
				G-C	G-u	+	
FNY1	−	UUGCGuAGA	UCU_CGUGA				
B2	−	UUGgGuAGA	UCUaCGUGA	C-G	u-a	+	+
				C-G	g-G		
2 sequences	−	UUGCGCCAGA	UCUGCGUaA	U-G	U-a	+	
MS1	−	UUGCGCCgGA	UCUGCGUGA	A-U	g-U	+	
T1	−	UUGCGCCAGA	UUGCGCCAGA	C-G	C-a		

[a] Uppercase letters are residues as in D4-sat RNA. Lowercase letters are different from those in D4-sat RNA.

RNA sequences, and Stem 16 (Fig. 2A) was conserved in all sat RNA necrogenic sequences (data not shown).

Region III contains a large number of adenosine residues which were susceptible to methylation. These modified residues forced the RNA folding program to establish a long single-stranded region (Fig. 2A).

Region IV contains positions G_{262} to the 3′ end of the molecule and includes six predicted helices (Stems 19–24) which are present in all three proposed structures for D4-sat RNA (Figs. 2A, 2B, and 2C). Covariation was observed with one sat RNA sequence for Stem 19 (data not shown) and with most nonnecrogenic sequences for Stem 23 (Table 4). The comparison of sat RNA sequences for Stem 23 (Table 4) indicated that covariation occurred at the central G:C base pair (G:C ↔ A:U). However, a large number of sat RNAs had the G:U pair changed to A:C, which is a noncanonical pair. Stems 22 (data not shown) and 23 (Table 4) were the only stems conserved in all necrogenic sequences. Furthermore, Stems 23 and 24 showed four-base loops similar to tetraloops that have been found capping helices in rRNA and are highly conserved in many organisms (Woese *et al.,* 1990). It has

TABLE 4

Sequence Alignment Comparison for Bases Forming Stem 23 and Loop 23 in the Structure Model of CMV D4-sat RNA with 77 sat RNA Sequences

Sat RNA sequences	Necrogenic in tomato	Stem 23	Loop 23	Extra bases	Stem 23′	D4 base pair	Other strains base pair	Canonical or G:U base pair	Covariant base pair
D4	+	GGC[a]	UUAU		GCU				
37 sequences	+	GGC	UUAU		GCU				
4 sequences	−	GaC	UUAg		GuU	G-C	a-u	+	+
2 sequences	−	GaC	UUAU		GuU	G-C	a-u	+	+
7 sequences	−	aaC	cUAU	AUG	Guc	G-U	a-c		
						G-C	a-u	+	+
5 sequences	−	aaC	cUAU	ACG	Guc	G-U	a-c		
						G-C	a-u	+	+
10 sequences	−	aaC	cUAU	AAG	Guc	G-U	a-c		
						G-C	a-u	+	+
F	−	aaC	UcAU	AAGA	Guc	G-U	a-c		
						G-C	a-u	+	+
3 sequences	−	GGC	UUAU		GCU				
X2c	−	aaC	cU__	AAG	GuU	G-U	a-U	+	
						G-C	a-u	+	+
MS1	−	GGC	UUAg		GCU				
WLM1	−	GaC	UUAU		GCU	G-C	a-C		
WLM3	−	GGC	UUAU		GuU	G-C	G-u	+	
WLM4	−	GGC	UUAg		GCU				
WL1	−	GaC	UUAg		GuU	G-C	a-u	+	+
DW	−	GaC	UUAg		GuU	G-C	a-u	+	+

[a] Uppercase letters are residues as in D4-sat RNA. Lowercase letters are different from those in D4-sat RNA.

been suggested that such structures are involved in tertiary interactions and nucleate folding of rRNA (Gutell, 1996).

Table 4 presents a further analysis of Stem 23 which shows residues important for necrogenicity (Devic *et al.,* 1990; Sleat and Palukaitis, 1990). Stem and Loop 23 include positions G_{284}–U_{293}. In many nonnecrogenic sat RNAs there is a region of three to four extra bases located between U_{290} and G_{291} (Devic *et al.,* 1990). In our model this region of extra bases would disrupt the UUAU tetraloop structure of Loop 23, conserved in most necrogenic sequences and in some nonnecrogenic sequences (Table 4).

DISCUSSION

Previous structural models for CMV sat RNAs have been based on analysis of the RNA structure in solution (García-Arenal *et al.,* 1987; Gordon and Symons, 1983). The functional sat RNA structure(s) *in planta* may have similarities with structures formed *in vitro,* but the conditions inside the plant cell could make these molecules acquire different or transient configurations. The three proposed structures for D4-sat RNA presented here (Figs. 2A, 2B, and 2C) were generated using data from DMS probing experiments *in vivo, in vitro,* and in virions and using an RNA folding program. Only those residues that were consistently methylated were used to generate the proposed structures. The fact that several adenosine and cytosine residues showed inconsistent modification by DMS (data not shown) indicates that further optimization of this assay is necessary to map all A and C residues with more precision. In addition, there was a lack of susceptibility to DMS modification in A and C bases located in putative single-stranded regions (Figs. 2A, 2B, and 2C), and there is no information on the status of G and U residues for D4-sat RNA. These points are an indication that these proposed structures for D4-sat RNA are working models which may need further refinement.

The structures proposed for several nonnecrogenic CMV sat RNAs are identical (García-Arenal *et al.,* 1987; Gordon and Symons, 1983). Assuming that these structures are correct, the nucleotide differences at the primary structure level would be responsible for the variation in pathogenicity observed (Palukaitis *et al.,* 1992). It seems more likely that structural motifs specific for each strain are responsible for the observed biological characteristics. Indirect evidence for the latter is given by the variation in patterns of enzymatic cleavage observed for B2-sat and B3-sat in regions with identical sequence (García-Arenal *et al.,* 1987). This suggests that accessibility of phosphodiester bonds in those regions is different in the two strains. B2-sat RNA induces mild deformation on tomato plants in coinfection with LS- or WL-CMV strains, but B3-sat RNA induces severe leaf deformation and stunting (García-Arenal *et al.,* 1987).

The structural models for D4-sat RNA proposed here present few similarities with structures proposed for several nonnecrogenic CMV sat RNAs (García-Arenal *et al.,* 1987; Gordon and Symons, 1983). The hairpin-loop in region I at the 5′ end in the nonnecrogenic sat RNAs structures is identical to Stem 3 (Fig. 2) in the D4-sat RNA structure. The single-stranded motif located between regions IV and VI in nonnecrogenic sat RNAs is similar to the long single-stranded region III shown in Fig. 2A for D4-sat RNA.

The structural model proposed for CMV Y-sat RNA (Hidaka *et al.,* 1988) shows some similar motifs to the models proposed here for D4-sat RNA. The 5′ end for both models shows two similar hairpin loops (Stems 2 and 3, Fig. 2). The 3′ end is highly structured in both sat RNAs models and they present structural motifs different from one another. Sequences at the 3′ end in Y-sat RNA are involved in inducing necrosis in tomato plants (Devic *et al.,* 1990). Comparison of structural motifs in the center of the molecule is more difficult because Y-sat RNA contains an insertion of 34 nt between nucleotides 131 and 166 (Hidaka *et al.,* 1984). Although there are differences in the Y- and D4-sat RNA models at the 3′ end, these two models are more similar than the models for Q- and D4-sat RNAs.

The D4-sat RNA induces rapid, systemic necrosis and death in tomato and attenuation of viral-induced symptoms in tobacco. DMS probing of CMV D4-sat RNA *in vivo, in vitro,* and in virions showed that some bases display variation in accessibility depending on the environment in which the molecule was tested. RNA structural studies on other organisms have also shown differences when *in vivo* and *in vitro* conditions have been compared. In a recent study, *in vivo* analysis of the spliced leader RNA from *Trypanosoma brucei* and *Leptomonas collosoma* (trypanosomatid protozoa) indicated that Form 2 was the predominant structure, while *in vitro* studies showed that Form 1 was the dominant structure (Harris *et al.,* 1995). In addition, structural analysis of *Tetrahymena thermophila* nuclear RNAs *in vivo* showed that the DMS methylation patterns of telomerase RNA and U2 snRNA fit the structural models determined using comparative sequence analysis better than the data obtained from *in vitro* analysis (Zaug and Cech, 1995).

The DMS probing experiments and analysis of covariant changes in CMV sat RNA sequences that were carried out, together with previous chemical and enzymatic probing analyses of other CMV sat RNA strains (García-Arenal *et al.,* 1987; Gordon and Symons, 1983) and mutational studies (Devic *et al.,* 1990; Sleat and Palukaitis, 1990), indicate the presence of structural motifs in regions I, III, and IV (Fig. 2A).

Region I contains Stem 3 which is closed by a loop of nine residues including two A residues (A_{28} and A_{30}; Fig 2A). Although DMS modification did not occur on adenosine residues in any of the three situations in the loop of Stem 3, other evidence strongly suggests that Stem 3 occurs in CMV sat RNAs. The covariance observed at three base pairs (Table 3) and the enzymatic cleavage of phosphodiester bonds in residues located in the loop of Stem 3 (Stem I in Gordon and Symons, 1983) of several sat RNA strains suggest that these residues are indeed in a single-stranded region in those strains. The lack of DMS modification in this region may indicate that those residues are somehow modified as are several tRNA residues (Rich and Rajbhandary, 1976). Stem-loop structures at the 5′ end of several viral RNAs have been shown to be involved in replication *in planta* (Andino *et al.,* 1990; Gilmer *et al.,* 1992, 1993; Pogue and Hall, 1992). Interestingly, structures at the 5′ end predicted for Q-CMV RNAs 1 and 2 (Pogue and Hall, 1992) present a strong similarity with Stems 2 and 3 of the model proposed for D4-sat RNA (Fig. 2A). The role of the sequences at the 5′ end of D4-sat RNA in replication is still unknown.

Region III presents a highly conserved adenosine-rich area with susceptibility to methylation in D4-sat RNA and to enzymatic cleavage in nonnecrogenic sat RNA strains (García-Arenal *et al.,* 1987). These results suggest that this single-stranded region is a common structural motif among sat RNAs that induce diverse symptomatology in various hosts, and hence this region may not be involved in the specificity of symptom development.

Region IV in D4-sat RNA (Fig. 2A) contains sequences responsible for necrogenicity in tomato plants (Devic *et al.,* 1990; Kurath and Palukaitis, 1989; Sleat and Palukaitis, 1990; Sleat *et al.,* 1994). Sleat and Palukaitis (1990) used a nonnecrogenic sat RNA strain (WL47) to identify three residues involved in necrogenicity in tomato. When positions A_{285}, G_{290}, and U_{292} of WL47 (numbering is A_{293}, G_{299}, and U_{304} in García-Arenal *et al.,* 1987) were changed to G_{285}, U_{290}, and C_{292}, the mutant sat RNA became necrotic in tomato in coinfection with a subgroup II CMV strain but not with a subgroup I CMV strain (Sleat and Palukaitis, 1990; Sleat *et al.,* 1994). In our structural model for D4-sat RNA, residues G_{285} and C_{292} form a base pair in the center of Stem 23 (Fig. 2D), while nonnecrogenic sequences have residues A_{285} and U_{292}. These results suggest that the G:C base pair in Stem 23 cannot be replaced by a thermodynamically weaker A:U base pair (Cantor and Schimmel, 1980) without losing some structural features involved in necrogenicity.

Loop 23 is present in all necrogenic strains and in some nonnecrogenic strains as a tetraloop $_{287}UUAU_{290}$. Many nonnecrogenic strains contain a different tetraloop sequence, and three or four extra bases between U_{290} and G_{291} (Fig. 2D). WL47-sat RNA contains the sequence UUAG in Loop 23, and as mentioned before, a triple mutation including one in Loop 23 (changed to UUAU) changed WL47-sat RNA into a partially necrogenic mutant. This result indicates that a conserved UUAU tetraloop is required for necrogenicity in tomato plants.

Methylation data in the region of Stem and Loop 24

for D4-sat RNA (Fig. 2D) was unavailable because of the positioning of the primer. Enzymatic and chemical probing information for this region in other sat RNAs is also inconclusive (García-Arenal *et al.,* 1987; Gordon and Symons, 1983). Sequence alignment comparison showed that two C:G base pairs in the center part of Stem 24 are conserved in all sat RNA sequences (data not shown). Moreover, a comparison of sequences located in Loop 24 showed that all necrogenic sequences and 26 nonnecrogenic sequences have a consensus sequence NNRC (N represents any nucleotide; R represents G or A).

The structural model presented here for the D4-sat RNA is consistent with methylation data, covariance analysis, and previous mutational analysis of the necrosis domain (Devic *et al.,* 1990; Sleat and Palukaitis, 1990). The model strongly suggests that it is the secondary structure, rather than the primary sequence, that is responsible for the necrogenic phenotype. Further understanding of the mechanism of necrosis in tomato is required before we can fully understand precisely how this small parasitic RNA molecule interacts with viral and host factors to overwhelmingly disrupt the life of its host.

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