Structural features of the priming signal recognized by primase: mutational analysis of the phage G4 origin of complementary DNA strand synthesis

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ABSTRACT

45 mutations (insertion, deletion and base substitution) of the G4 *Ori*$_c$ were tested for their functional activity in M13 and R199 *in vivo*. The critical mutants were also assayed for their ability to synthesize pRNA *in vitro* using SSB and primase. The results demonstrate that the secondary structure and spacing of stem-loops I and III are essential for *Ori*$_c$ activity and that the 5′-CTG-3′ sequence flanking stem-loop I is essential for initiation of pRNA synthesis.

INTRODUCTION

The phage G4 origin of complementary DNA strand synthesis (*Ori*$_c$) determinant sequence is located in the intergenic region between genes F and G (1,2) and directly recognized by primase in the presence of *E. coli* single-strand binding protein (SSB) (3). The deletion analysis of *Ori*$_c$ cloned in a filamentous phage vector has shown that the minimum region for its function is 140-nucleotides (nt), between A_{3868} and A_{4007} (Fig. 1; ref. 4). Many mutations have been introduced into the *Ori*$_c$ determinant sequence and the functional activity of each mutant has been assayed (4−12). Similar analyses have been carried out with the origin of complementary DNA strand synthesis (*ori*$_c$) determinant sequence of a related phage α3 (13−15). These mutational analyses have revealed structural features which appear to be essential for the function of this primase-dependent priming signal. However, the *Ori*$_c$ mutations have been isolated over a number of years and their functional activity assayed by different methods. This makes it difficult to relate the results and reliably describe the functional domains of *Ori*$_c$. It was therefore considered necessary to reassay all of the available mutants (insertion, deletion and base substitution) in a single series by one assay method. The gel method of measuring RF DNA accumulation (9) has proved to be reproducible and correlates well with the results obtained by other methods (conversion of ^{32}P-parental SS DNA to RF DNA, plaque size and burst size). In addition, the most interesting mutants were assayed for *Ori*$_c$ activity in an *in vitro* system using purified primase and SSB protein (16). Together these results build a picture of the structure of *Ori*$_c$ that is necessary for its activity in initiating DNA replication.

MATERIALS AND METHODS

Construction of the mutant *Ori*$_c$'s

Construction of the mutants of *Ori*$_c$ and their structures have been described in (7,8,11 and 12).

Estimation of the RFI accumulation *in vivo*

To monitor the functional activity of each mutant in terms of the DNA synthesis *in vivo*, the accumulation of RFI DNA in *E. coli* cells infected with the recombinant phages was estimated by the method described in (9).

The RNA synthesis *in vitro*

pRNA synthesis by SS phage DNA containing the wt and mutant *Ori*$_c$ was measured *in vitro* according to the method of Stayton and Kornberg (16). A standard reaction mixture of 50 μl contained: 20mM TrisHCl (pH 7.5) 8mM dithiothreitol, 80 μg/ml bovine serum albumin, 8 mM $MgCl_2$, 4%(w/v) sucrose, 100 μM ATP, GTP, CTP, and UTP each at 20 μM (3000−30000 cpm/pmole [α-^{32}P]GTP and/or [α-^{32}P]CTP), 320 ng of ss template DNA, 2.4 μg of SSB (U.S. Biochemical), 0.25 μg (300 replication units) of primase (a gift of Dr. Roger McMacken of Johns Hopkins University), and 4 μg/ml rifampicin. The reaction mixture was incubated at 30°C for 10 min, then the reaction was stopped on a DEAE disc (DE81, Whatman), which was pretreated with 10 mM sodium pyrophosphate, ethanol washed, and dried. Papers were dropped immediately into a wash solution of 0.3 M ammonium formate (pH 7.8), 10mM sodium pyrophosphate, and 0.1% sodium laurylsulfate, stirred for at least 1 hr, washed three additional times for 5 min, twice with 95% ethanol, and counted by liquid scintillation counter.

To analyze the size of pRNA synthesized, 40μl of reaction mixture was precipitated with 95% ethanol and loaded on a small (10 cm×10 cm) 12% polyacrylamide gel containing 7 M urea. The gels were autoradiographed wet.

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RESULTS AND DISCUSSION

Effect of insertions in *Gori*$_c$ function

The nucleotide sequence of the 278-nt *Eco*RI fragment containing *Gori*$_c$ is shown in Fig. 1. There are three potential stem-loop structures (I, II, and III) in the minimum *Gori*$_c$ region and chemical cleavage studies have confirmed their stable existence in SS DNA (19).

To analyze the relative importance of each stem-loop structure, insertions were made which altered their size, shape and spacing. These were at the unique *Ava*II and *Xho*I endonuclease cleavage sites present in the wt *Gori*$_c$ (5−7,10) and the four newly created *Sma*I/*Xma*I, *Eco*RV, *Hind*III, and *Sma*I/*Xma*I sites of mutants, *Gori*$_c$-IL30, -E, -H, and -X, respectively (11,12). Short nucleotide sequences were inserted at these cleavage sites and the effect on *Gori*$_c$ activity was estimated by measuring accumulation of RFI DNA *in vivo*. RFI DNA accumulation showed good correlation with *Gori*$_c$ activity, as estimated by the clear-plaque forming assay, the phage growth assay, and measurement of parental RF DNA synthesis *in vivo* (17). As summarized in Table 1, the six sites divided into four groups, according to the effect of insertions on *Gori*$_c$ activity. The first group is composed of three sites, the *Sma*I/*Xma*I sites of *Gori*$_c$-IL30 and -X and the *Eco*RV site of *Gori*$_c$-E, where short insertions essentially destroyed *Gori*$_c$ function (average functional activity of all mutations is 18% that of wt *Gori*$_c$). This identifies stem-loop III and the pRNA initiation site as essential for *Gori*$_c$ activity. The second group is the *Ava*II site of wild type *Gori*$_c$, where some insertions slightly depressed *Gori*$_c$ function, while others affected it severely (average for all mutations is 40% that of wt *Gori*$_c$). This identifies the spacer region between stems III and II as fairly important in *Gori*$_c$ function. Insertions at the *Xho*I site of wild type *Gori*$_c$ only slightly depress *Gori*$_c$ activity (average of all mutants is 70% that of wt *Gori*$_c$) and are placed in a third group. This identifies stem-loop II as relatively unimportant in *Gori*$_c$ function. Insertions at the *Hind* III site of *Gori*$_c$-H vary considerably in their effect (none to 70% decrease) and are placed in a fourth group. This indicates that certain structural features at stem-loop I are essential for *Gori*$_c$ function, while others are not. The different insertions at each of the six sites must therefore reflect different functional contributions of each domain of the *Gori*$_c$. These results correlate with those obtained for the *ori*$_c$ of the related single strand phage ϕK, where it was shown that domains equivalent to *Gori*$_c$ stem-loop III are directly involved in primase interaction (18).

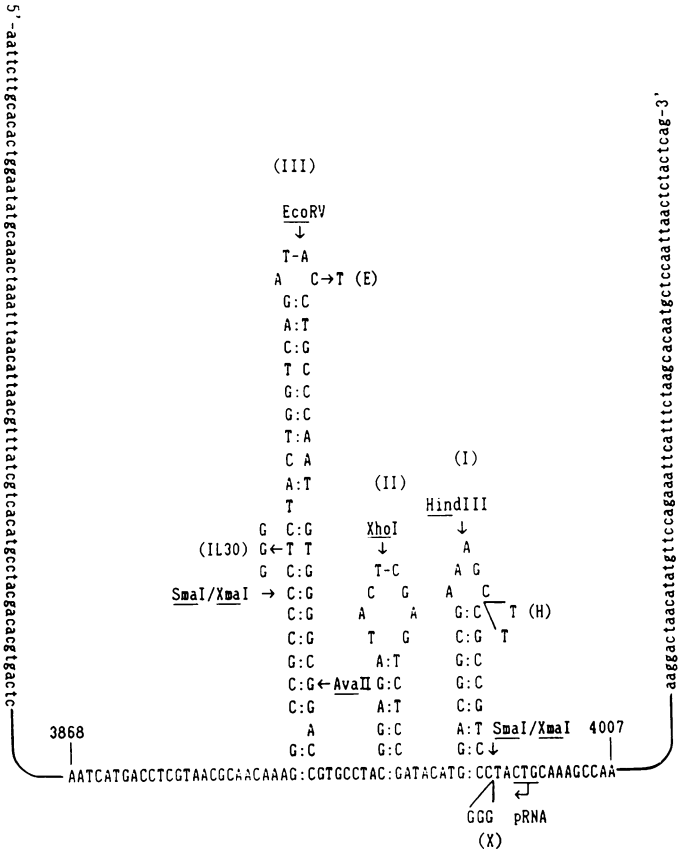

Fig. 1. Nucleotide sequence of *Gori*$_c$. Nucleotide sequence of the 278-nt *Eco*RI fragment, containing *Gori*$_c$ determinant sequence, from R199/G4 (5) is shown. Nucleotide sequence and numbers are according to (1) and the *Gori*$_c$ minimum region (capital letters) is defined in (4). Six restriction endonuclease cleavage sites (see Table 1), including four changed sequences, insertions and substitutions by site-directed mutagenesis, are shown. The origin of pRNA synthesis is marked.

Role of *Gori*$_c$ stem-loop structures

To further identify the functional contribution of each stem-loop structure, deletion and base substitution mutations were made.

Stem-loop I

Lambert *et al.* (9) showed that the stability of stem-loop I is essential for *Gori*$_c$ function. Two point mutations were made in the stem of stem-loop I, which disturbed base-pairing. These mutations considerably reduced the activity of *Gori*$_c$. However, functional activity was recovered when a second mutation was introduced which restored the disturbed base-pairing.

Insertion into the loop of stem-loop I were found to have varying effects on *Gori*$_c$ activity (Table 1). This is probably therefore due to the varying effect of these insertions on the stem of the stem-loop structure. The loop of stem-loop I however contains a sequence 5'-AAGCC-3' which is also present in the loop of one of the stem-loop structures of *sitB*. This is one of the *ssi* signals identified from the *terC* region of the *E. coli* chromosome (20). To examine the importance of this hexanucleotide sequence, base substitutions were made. Two nucleotides, AG, in 5'-AAAGCC-3' were replaced by TT (*Gori*$_c$-IL01) and a 2-nt insertion was made between two C residues in this hexanucleotide sequence (*Gori*$_c$-H). Both mutations had little effect on the function of *Gori*$_c$ (Table 1 and 2). These results indicate that this specific sequence, 5'-AAAGCC-3', is not essential for the function of *Gori*$_c$, and presumably for that of *sitB*. Although the changed stems of stem-loop I of insertion mutants, *Gori*$_c$-HF and -HSH, are more stable than that of wild type (11), the functional activity of these mutations was depressed (Table 1). Surprisingly, no RNA synthesis was observed *in vitro* when the ss DNA templates containing *Gori*$_c$-H, -HF, and -HSH were used (Figure 2), suggesting that the secondary structure of stem-loop I is critical for the RNA synthesis by primase. These results indicate that proper stability and/or size of the stem of stem-loop I are important for the function of *Gori*$_c$.

Stem-loop II

Deletions in the loop of stem-loop II destroy the function of *Gori*$_c$ (8), whereas insertions in the same loop slightly depress but do not abolish its function (5). One of the possible

Table 1 Insertion mutants of $Gori_C$

Group	Structural changes in:	Site of insertion	Mutant[a]	Inserted bases (nt)	Relative functional activity[b] (%)
I	stem III	SmaI/XmaI of IL30	IL30P	4	28
			IL30BA	8	16
			IL30EI	10	17
			IL30HI	8	16
	stem III loop III	EcoRV of E	EBA	8	18
			EEI	10	18
			EHI	8	20
			ESM	8	15
	spacer between CTG pRNA initiation and stem I	SmaI/XmaI	X	3	123
			XF	7	26
			XBA	11	26
			XEI	13	16
			XHI	11	14
			XPT	11	79
			XXB	11	10
II	spacer between stems II and III	wt	Fin10	3	45[c]
		AvaII	Bam4122	13	69[d]
			Eco16	10	22[d]
			Eco1004	13	34[c]
			Hin1010	10	27[c]
III	stem II loop II	wt	fill-in63	4	71[e]
		XhoI	Bam14	14	78[e]
			ϕX54	29	66[e]
			ϕX56	53	66[e]
IV	stem I loop I	HindIII of H	H	2	103
			HF	6	40
			HSH	12	29

[a] Construction of the mutants have been described by Hiasa et al.(11.12).

[b] Relative RFI DNA accumulation is calculated as follows: [(RFI DNA accumulation of mutant at 60 min.)/(RFI DNA accumulation of mutant at 5 min.)]/[(RFI DNA accumulation of wild type at 60 min.)/(RFI DNA accumulation of wild type at 5 min.)]. The method of estimation of the RFI DNA accumulation has been described (11). The relative value of the vector M13ΔE101 is 7%.

[c] Data from Sakai et al.(7).

[d] Data from Sakai et al.(6).

[e] Data from Sakai and Godson (5).

Table 2. Base substitution and deletion mutants of $Gori_c$

Mutant[a]	Structure altered	Substituted bases (nt)	Deleted bases (nt)	Relative functional activity[b] (%)
IL01	loop I	2	0	59
IL20	loop II	2	0	86
IL02	same	3	0	74
IL30	stem I	3	0	60
E	loop III	1	0	59
AX103	spacer between stems II-III	1	0	16[c]
AX39F3	same	5	0	77[c]
AX403	same	4	0	0[c]
AX393	same	0	4	48[c]
ΔX514	loop II	0	4	15[c]
ΔX517	same	0	6	27[c]
ΔX513	same	0	17	0[c]

[a];Described in the footnote[a] of Table 1.
[b]:Described in the footnote[b] of Table 1.
[c]: Data from Sakai et al,(7).

Table 3. Effect of nucleotide sequences at the pRNA synthesis initiation site flanking stem-loop I

Mutant[a]	Nucleotide sequence[b]	Relative functional activity[c] (%)
Gori$_c$ (wild type)	-G:CCTACTGCA-	100
SS	-G:CCggggatcct-	16
SSBA	-G:CCCGGATCCGggggatcct-	13
SSEI	-G:CCCCGAATTCGGggggatcct-	9
SSHI	-G:CCCAAGCTTGggggatcct-	7
SSPT	-G:CCGCTGCAGCggggatcct-	65
SSXB	-G:CCCTCTAGAGggggatcct-	16

[a] Described in the footnote[a] of Table 1.
[b]Nucleotide of the 3′-flanking region of stem-loop I is shown. Small letters indicate the nucleotide sequence of the vector, M13Δ*lac*183, and underlined T residue in the wt *Gori*$_c$ sequence is the initiation site of the pRNA synthesis.
[c]Described in the footnote[b] of Table 1. The relative value of the vector, M13Δ*lac*183, is 15%.

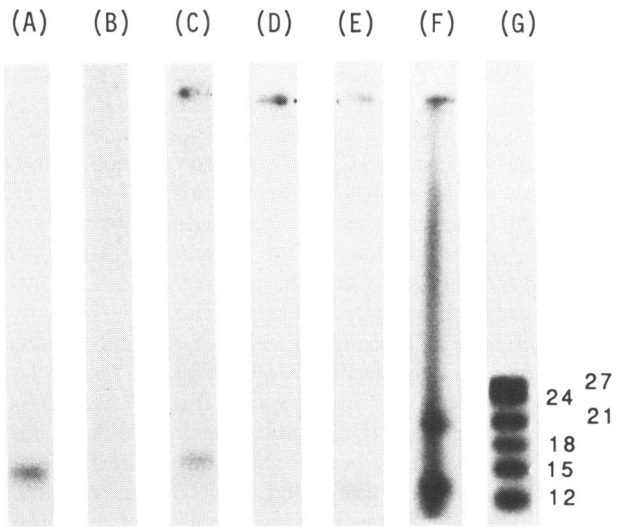

Fig. 2. RNA transcript made on wt and mutant $Gori_c$'s. The ss DNA template used contained the wt $Gori_c$, lane (A); $Gori_c$-HF, lane (B); $Gori_c$-E, lane (C); $Gori_c$-SS, lane (D); $Gori_c$-SSPT, lane (E); $Gori_c$-XPT, lane (G) is oligonucleotides as the size maker (12-, 15-, 18-, 21-, 24-, and 27-nut).

explanations for these observations is that the specific nucleotide sequence in the loop II is essential for $Gori_c$. To examine this hypothesis, base substitutions were made in the loop of stem-loop II. The functional activity of these two mutants ($Gori_c$-IL20 and -IL02) was only slightly affected (Table 2). These results suggest that the shape and/or dimension of the loop II is essential rather than the specific nucleotide sequence. If so, the size of the loop II of wt $Gori_c$ must be the minimum for its function. Alternatively, if stem-loop II is dispensable, the proper distance between stem-loops I and III may be essential for the function of $Gori_c$. This may explain why in the spacer region, between stem-loops I and III, including the stem-loop II domain, base substitutions and insertions can be made without abolishing the function, but deletions cannot. Similar results were obtained from the analysis of the ori_c of α3 (14,15). This explanation is consistent with the tertiary structure model of $Gori_c$ proposed by Sims and Benz (18). However, the differences of the effects on the function both by the mutations in $Gori_c$-AX39F and in $Gori_c$-AX40 and by the 4-nt deletion in $Gori_c$-AX39 and -ΔX514 cannot be explained by the distance between stem-loops I and III nor by this tertiary structure model (Table 2). Even if the secondary structure of stem-loop II is dispensable, there may be the specific nucleotide sequences in the region between stem-loops I and III which are as important for the function of $Gori_c$ as the distance between the two stem-loop structures.

Stem-loop III

Although the location of stem-loop III is the farthest from the primer RNA (pRNA) synthesis initiation site (Fig. 1), it appears to play an essential role in $Gori_c$ function (12). As shown in Table 1, no insertions could be made in stem-loop III, either in the stem or in the loop, without destroying $Gori_c$ function. Base substitutions, however, could be made. A point mutation in the loop III ($Gori_c$-E) and a 3 nt substitution in the middle of stem III ($Gori_c$-IL30) only slightly depressed $Gori_c$ function both *in vivo* and *in vitro*, whereas a point mutation at the bottom of the stem III ($Gori_c$-AX10), replacing C_{3938} by G, abolish its function (8). Another point mutation in the lowermost base pair of the stem III (G_{3893} replaced by C) and a deletion in the stem of

Table 4. RNA synthesis on the wild type and mutant $Gori_c$'s.

Recombinant phage[a]	Reaction condition[b]	Total incorporation[c] (pmole)
M13ΔE101	none	0.07
M13ΔE101	rif	0.07
M13ΔE101/G4	none	0.51
M13ΔE101/G4	rif	0.56
M13ΔE101/G4	primase	0.03
M13ΔE101/G4	SSB	0.06
	ss DNA	0.03
M13ΔE101/G4	UTP	0.04
M13ΔE101/G4	GTP	0.03
M13ΔE101/G4	CTP	0.17
M13Δ*lac*183/G4-SS	none	0.05
M13Δ*lac*183/G4-SSPT	none	0.18

[a]Recombinant phage used as the ss template DNA in the reaction mixture.
[b]Component omitted from the reaction mixture.
[c]The total ^{32}P-incorporation measured by a liquid scintillation counter.

stem-loop III, between A_{3916} and G_{3934}, could not be made (unpublished results). With the ori_c of a related phage α3, Kodaira *et al.* have reported the similar observations (13). All mutations which change the secondary structure of $Gori_c$ stem-loop III severely affect or abolish $Gori_c$ function. These results indicate that the secondary structure of stem-loop III is essential for $Gori_c$. Although there is no experimental evidence, it might be involved in the direct recognition of $Gori_c$ by primase in the presence of SSB.

Specific nucleotide sequence required at the pRNA synthesis initiation site

The *in vitro* studies by Kornberg and his coworkers suggest that in the presence of SSB two molecules of primase form a complex with $Gori_c$ (16). pRNA synthesis starts at T_{3997}, 5-nt downstream from stem-loop I. Though 26- to 29-nt RNA transcripts are synthesized in the absence of deoxyribonucleotide triphosphates (22), pRNA made by primase is much shorter in the presence of deoxyribonucleotide triphosphates (23). pRNA synthesis starts at T residue in the trinucleotide sequence, 5′-CTG-3′, on the template DNA, so that the first sequence of pRNA is always pppAG-. This is the case not only at the ori_c of G4 but also at those of related phages (24,25). The T residue is always located at 5-nt downstream from the secondary structure, which is equivalent to stem-loop I of $Gori_c$, in all of these ori_c sequences. These conserved structural features appear to be essential for the function of ori_c's of these phages.

A deletion mutant, $Gori_c$-SS, which lacks the 3′-flanking region of stem-loop I of $Gori_c$, was constructed on a recombinant phage M13Δ*lac*183/G4 (11). A 14-nt deletion, between T_{3994} and A_{4007}, at the 3′-end of the $Gori_c$ minimum region (Fig. 1), destroyed $Gori_c$ function completely when measured both *in vivo* (Table 3) and *in vitro* (Table 4 and Fig. 2). To examine the effects of different nucleotide sequences in this domain where the pRNA synthesis starts, linkers were inserted into the unique *Sma*I/*Xma*I site at the 3′-end of the $Gori_c$-SS determinant sequence (11). As shown in Table 3, the functional activity was restored only when the *Pst*I linker is inserted ($Gori_c$-SSPT). This is best explained by the fortuitous addition of a CTG sequence from the *Pst*I linker (G*CTG*CAGC). This result indicates that the conserved trinucleotide sequence,

5′-CTG-3′, is an essential motif for $Gori_c$ function, probably functioning as a priming signal to start the pRNA synthesis by primase. The same requirement for 5′-CTG-3′ was observed *in vitro* (Table 4). No pRNA synthesis was observed when the deletion mutant $Gori_c$-SS (M13Δ*lac*183/G4-SS) was used as a template for purified primase (Fig. 2). pRNA synthesis however was partially restored and a full length RNA transcript was synthesized when M13Δ*lac*183/G4-SSPT, which had a CTG sequence from the inserted *Pst*I linker, was used as a template (Fig. 2). These results strongly support the hypothesis that the trinucleotide sequence, 5′-CTG-3′, functions as the priming signal recognized by primase to start the RNA synthesis.

The distance between the pRNA initiation site, 5′-CTG-3′, and stem-loop I (or the equivalent secondary structure in ori_c of related phages) may be important for ori_c function. The T residue, where pRNA synthesis starts, is located 5-nt downstream from the secondary structure in all the ori_c sequences of G4 and its related phages. To change this distance, insertions were made between C_{3993} and T_{3994} (11) and the functional activity of each mutant was estimated *in vivo* (Table 1). All the insertion mutants which have a distance longer than 12-nt lost their function. This result is coincident with the observation that the distance between the CTG sequence at the 5′-end of nascent RNA molecule and the transition site from RNA synthesis to DNA synthesis is 1- to 11-nt (29). The only exception is $Gori_c$-XPT, which has two CTG sequences and that two T residues in the CTG sequence are located at 4-nt and 16-nt downstream from stem-loop I. *In vitro* $Gori_c$-XPT templates appeared to synthesize two pRNA molecules corresponding to the lengths predicted by the two CTG sequences (Figure 2). It indicates that any CTG within a certain distance of stem-loop I can serve as a pRNA initiation site. However, all the results of the *in vivo* and *in vitro* assays suggest that the distance from the pRNA initiation site to stem-loop I may be essential for the transition from the pRNA synthesis to DNA synthesis.

The differences of the functional activity among the priming site mutants (Table 1 and 3), indicate however that not only the CTG sequence but also nucleotide sequences around T_{3997} affect the function of $Gori_c$.

Model of $Gori_c$ functional domains

The tertiary structure model (18) cannot explain all of the results described above and it is clear that further *in vivo* and *in vitro* studies are needed to identify the $Gori_c$ features that are recognized by primase. However, as a working hypothesis, it is proposed that one primase molecule may recognize and interact with stem-loop III, and another molecule may interact with stem-loop I and its 3′-flanking region, where the priming event takes place. This model explains the results of the mutational analyses and the observation (16) that two primase molecules form a complex with $Gori_c$. It is of interest to note that the 19-nt repeated sequence (21) exists in $Gori_c$, but whether this repeated sequence is involved in the interaction with primase molecules has not been tested. Both *in vivo* and *in vitro* studies are in progress to examine this hypothesis.

Generality of G4 types of *ssi* signal

Recently, *ssi* signals have been isolated from plasmids, F, R100, R6K, and ColE2. Three of these were found to be 'G4-type' *ssi* signals, which required only primase and SSB for the priming (26). In all of these signals, as well as the *ssi* from R1 (the priming signal for the leading strand synthesis (27)), pRNA synthesis starts at the T residue in the sequence, 5′-CTG-3′ (28). An *ssi* signal from plasmid ColIb also seemed to be 'G4-type' (Tanaka, K., Sakai, T., Honda, Y., Hiasa, H., Sakai, H. and Komano, T., unpublished results). Only limited sequence homologies were observed between $Gori_c$ and the 'G-4-type' *ssi* signals. The trinucleotide sequence, 5′-CTG-3′, is also commonly observed in the 5′-end of nascent RNA molecules mapped on phage λ *in vivo* (29). All these results indicate that the trinucleotide sequence, 5′-CTG-3′, may function as the priming signal which is recognized by primase to start the pRNA synthesis. However, in the in *vitro* replication system using primosome proteins and DNA polymerase III holoenzyme, it is not yet clear whether the Okazaki fragment synthesis always starts at the sequence, 5′-CTG-3′, or not (30). It is possible that the association of primase with the helicase (DnaB protein) and/or DNA polymerase III affects the specificity both for the start point of the active pRNA synthesis and the transition from RNA synthesis to DNA synthesis. The interaction of primase with helicase (probably DnaB protein) and/or DNA polymerase III holoenzyme may occur *in vivo*, even in the cases of G4 and of 'G4-type' *ssi*'s.

CONCLUSIONS

1. The trinucleotide sequence, 5′-CTG-3′, is an essential motif for the function of $Gori_c$, and probably the primase-dependent *ssi*'s. It may be the priming signal to start the pRNA synthesis.
2. The distance between the pRNA start point, T_{3997}, and stem-loop I of $Gori_c$ is very important.
3. Loop structures, not the specific sequence but the dimension, are important. And, each loop structure might make a distinct functional contribution to the function of $Gori_c$.
4. The secondary structure of stem-loop III of $Gori_c$ is essential for its function.
5. The nucleotide sequences at the bottom of stem III as well as 3′-flanking region of stem I, which are equivalent to the domains in the φK ori_c sequence protected by primase, are very important for its function of ori_c's.

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