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*Rep*A protein- and *ori*R-dependent initiation of R1 plasmid replication: identification of a *rho*-dependent transcription terminator required for *cis*-action of *rep*A protein

Hisao Masai and Ken-ichi Arai

Department of Molecular Biology, DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA 94304, USA

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ABSTRACT

Initiation of R1 plasmid replication is dependent on cis-acting repA protein and the 188 base-pair (bp) sequence, oriR. RepA protein synthesized in vitro preferentially activates oriR in cis, regardless of the orientation and location of oriR on the template DNA. RepA protein is not reusable after it activates oriR in the cis-position. Cis-action of repA protein is also dependent on the presence of CIS, a 170 bp sequence, between repA and oriR. CIS contains a rho dependent transcription terminator of the repA transcript, deletion of which results in decrease in transformation efficiency and rapid loss of plasmid in the absence of selection. The significance of transcription termination events in replication was indicated by decreased replication activity in vivo caused by premature termination of the repA transcript between repA and CIS. A model which may account for the role of CIS in mediating the cis-action of the repA protein is presented.

INTRODUCTION

The R1 plasmid, a drug resistance plasmid, belonging to the IncFII incompatibility group, is maintained in Escherichia coli cells with a copy number of one to two per chromosome (1). Three replication functions, copB, copA and repA, are encoded on the basic replicon of the R1 plasmid. The copB and copA products act as inhibitors of expression of repA (2). RepA protein, identified as a 33 kd protein (3), was shown to be required for initiation of R1 plasmid replication in vitro (4,5). Replication origin of the R1 plasmid was localized to a 188 bp segment (oriR) downstream of repA by using an in vitro trans-complementation assay (3).

A unique feature of R1 plasmid replication is the apparent cis-specific action of repA protein in vitro, i.e , newly synthesized repA protein is preferentially utilized by a nearby oriR sequence on the same template from which it was synthesized, and the oriRs on other templates are not activated (3,6).

In vivo, it has also been observed that wild type R100 plasmid, an

IncFII plasmid closely related to R1, is not able to complement defective plasmids carrying a mutation in the repA gene (lack of trans-complementation) (7). We have discovered that repA protein becomes trans-acting in vitro when a nearby oriR is removed (3) and this has been the basis for the in vitro trans-complementation assay by which we mapped oriR. In this communication, we first examine the sequence required for the cis-action of repA protein in vitro. It was shown that the repA protein is cis-acting as long as it is synthesized from a plasmid that carries both repA and oriR on the same template regardless of its location or orientation. We found that a sequence between repA and oriR (designated CIS) is also required for cis-action of repA protein in vitro.

Although CIS is not essential for in vitro replication, plasmids which lack a part of the CIS sequence replicate very poorly in vivo. The sequence within CIS which was essential for in vivo replication was shown to contain rho-dependent transcription terminator activity. Our results indicate that transcription termination or pausing within CIS is important for R1 replication in vivo and that the effect may be through facilitating the interaction of the repA protein with DNA rather than through affecting the repA expression.

MATERIALS AND METHODS

E. coli Strains and Plasmids

E. coli strains MC1061 (araD139, Δ(ara, leu)7697, ΔlacZ74, galU, galK, hsr, strA) C2110 (his, rha, polA1), DH5α (F⁻, endA1, hsdR17, supE44, thi-1, recA1 gyrA96, relA1, Δ(argF-lacZYA) U169, φ80dlacZΔM15, λ) and P3478 (F⁻, polA1, str, thy) are laboratory stock strains. KH6301 (F⁻, trpEam, tyram, thr, ilv, metE, argH, thy) and HD173 (F⁻, trpEam, tyram, thr, ilv, argH, thy, nitA702 [rho-ts702]) (8) were generously provided by K. Shigesada (Institute for Virus Research, Kyoto) P3478 and C2110 were used to prepare fraction I for in vitro reactions and to measure replication activity of pUC-mini R1 hybrid plasmids, respectively. Plasmid p202T2, which contains a rho-independent transcription terminator from rrnB of E. coli, was obtained from K. Moore (DNAX). BamHI digestion of p202T2 releases a 340 bp fragment that contains a terminator which functions in both orientations This BamHI fragment was used for the constructions as a portable rho ndependent terminator fragment. A 28 bp synthetic transcription terminator sequence from trpA was obtained from Pharmacia. Plasmids are described in the text and figure legends.

Reagents and Enzyme Reactions

BAL-31 endonuclease and a Klenow fragment were obtained from Bethesda Research Laboratories and Boehringr Manheim Biochemicals, respectively. All other enzymes were purchased from New England Biolabs. All the enzyme reactions were performed as described (9). Linkers were purchased from Pharmacia. Tetracycline hydrochloride was obtained from Sigma.

In vitro Replication assay

Crude extracts (fraction I) of E. coli cells were prepared from P3478 as described (10). Assay conditions for in vitro replication of R1 plasmid have been described (3).

Two Stage Reaction of in vitro R1 Plasmid Replication

RepA$^+$ plasmid was incubated for 30 min at 30°C in the standard reaction mixture without radioactive deoxynucleotide (the first stage). The first stage reaction mixture of the amount indicated was removed and added to the second reaction mixture that includes 100 μg/ml chloramphenicol and the oriR$^+$ plasmid. The reaction mixture was further incubated for 60 min at 30°C. The reaction was terminated and acid insoluble radioactivity was measured.

Determination of Nucleotide Sequence

The modified procedure of Maxam and Gilbert (11) was employed to determine nucleotide sequence.

Preparation of RNA and S1 Mapping

Preparation of RNA from E. coli cells and S1 mapping of the end of transcripts were performed as described by Nomura et al. (12). Size markers were generated by a Maxam-Gilbert sequencing reaction performed on the same end-labeled fragment that was used for S1 mapping.

RESULTS

RepA Protein Preferentially Activates the cis-Origin in vitro

R1 plasmid replication in vitro, using fraction I, is dependent on ongoing protein synthesis (3,13), because repA protein must be synthesized freshly before each round of replication. If repA protein, synthesized in fraction I from a first template DNA (repA donor), can activate oriR on a second template DNA (repA recipient), replication of the second template should be detected (trans-acting). However, our previous results (3) indicate that the repA$^-$ oriR$^+$ (second template) plasmid does not replicate efficiently in vitro in the presence of a repA$^+$ helper plasmid (1st template) carrying a functional oriR. As shown in Fig. 1A, all the

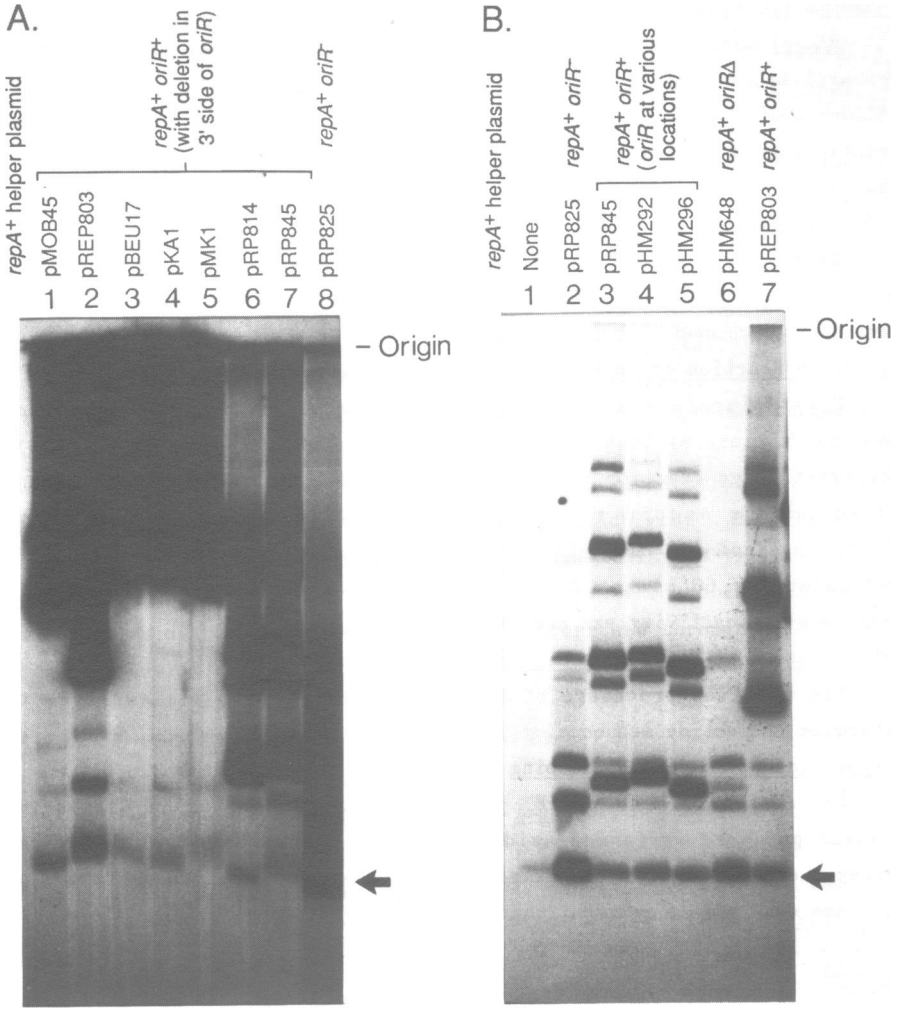


Fig. 1 Cis-action of the repA protein in vitro. Standard reaction mixtures containing 0.2 pmol as circle of pRP814-8 (oriR plasmid, the recipient of the repA protein) were incubated for 60 min at 30°C in the presence of 0.2 pmol as circle of various repA+ plasmids as indicated below. RepA donor plasmids are indicated on top of each lane. A. pMOB45, pBEU17, pKA1 and pMK1 are derivatives of R1 runaway replication plasmids. pREP803, pRP814, pRP845 and pRP825 are described in Fig. 3 and Fig. 2 of ref. 5. B. pHM292 carries two tandemly arranged portable oriR sequences at 3' side of repA with an inverted orientation. pHM296 carries a portable oriR sequence at 5' side of repA. pHM648 carries a 23-bp deletion (position 1548-1570) within oriR. See Fig. 3 for more information on the structures of the plasmids. The arrow indicates the position of form I DNA of the oriR plasmid. Presence of multiple bands is due to the generation of multimers as products.

plasmids carrying both repA and oriR replicated efficiently, whereas coexisting oriR plasmids (pRP814 8) were poorly replicated, indicating that repA protein synthesized from these repA$^+$ oriR$^+$ plasmids is cis-acting in vitro. Addition of 10-fold excess of repA$^-$ oriR$^+$ recipient plasmid did not appreciably affect its replication in the presence of repA$^+$ oriR$^+$ donor plasmid, indicating that repA protein acts stringently in cis in vitro (data not shown).

RepA protein was cis-acting with pRP814, which does not carry any additional sequence to the 3' end of oriR, as well as with pREP803 which carries 2 kb sequence beyond the 3' end of oriR. This indicates that deletion of DNA sequence 3' to oriR does not affect the cis-acting nature of repA protein in vitro. As shown in Fig. 1B, a plasmid carrying oriR at the 3' side of repA in the opposite orientation (lane 4) and one carrying oriR at the 5' side of repA (lane 5) did not support the replication of oriR plasmid in trans. These results indicated that the location and orientation of oriR relative to repA do not affect the cis-action of repA protein in vitro. A plasmid carrying an internal deletion within oriR, which completely lost replicative activity, partially supported the replication of oriR plasmid in trans (lane 7). RepA protein acts fully in trans when oriR is completely removed (lane 2).

The Region Between repA and oriR is Required for cis-Action: Identification of CIS Sequence

RepA and oriR are separated by approximately 170 bp sequence. So far, no function has been assigned to this region. To look for the function of this region a set of plasmids, lacking the 170 bp sequence but carriying repA and oriR, was constructed. One of the deletion derivatives, pHM593, lacks most of the 170 bp sequence while the repA coding region is intact. A portable 206-bp oriR sequence was reinserted upstream or downstream of repA in pHM593. Four different combinations of repA and oriR were recovered, and all but one were shown to replicate in vitro. pHM1971 which carries oriR downstream of repA in the inverted orientation did not replicate in vitro. pHM918 and pHM882 carrying oriR downstream or upstream, respectively, of repA were used as donor plasmids in the trans-complementation assay in vitro. As shown in Fig. 2 (lanes 2-5), both donor and recipient plasmids are replicated to almost the same extent, indicating that the cis-action of repA protein is lost upon removal of the 170 bp sequence. The region between repA and oriR was named CIS, since it is involved in the cis-action of the repA protein. The cis/trans actions of

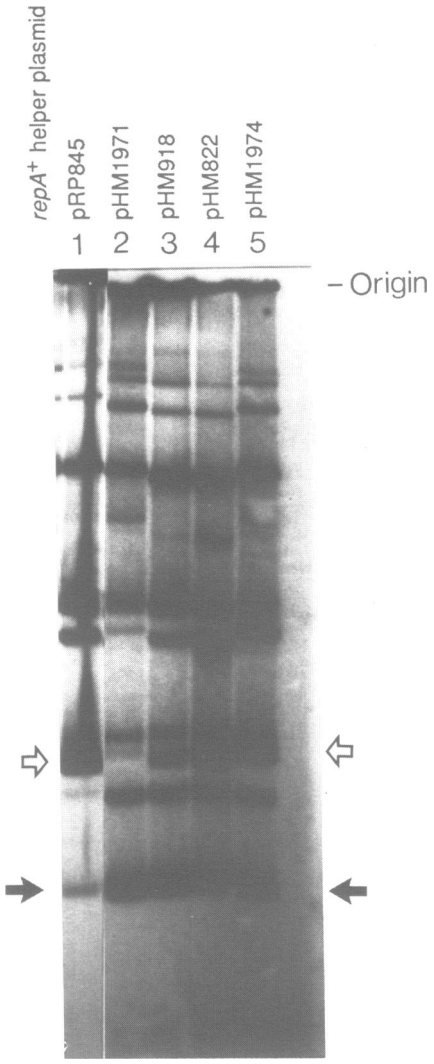


Fig. 2 Effect of deletion of CIS on cis-action of the repA protein. The reactions were performed under the same condition as described in the Fig. 1 legend. The repA donor plasmids are indicated in the figure. pHM1971, 918, 882 and 1974 are CIS$^-$ plasmids with oriR positioned at various locations relative to repA. See Fig. 3 for more information on the structures of the plasmids. The filled and open arrows indicate the positions of form I DNA of the oriR plasmid (the repA recipient) and the repA donor plasmids, respectively. pHM1971, carrying the CIS-oriR DNA downstream of repA in an inverted orientation, was not replicated both in vivo and in vitro, although it carries the functional repA and oriR sequences. It appears that "tail to tail" ligation of repA and oriR inhibits replication. The reason for this inhibition is not clear.

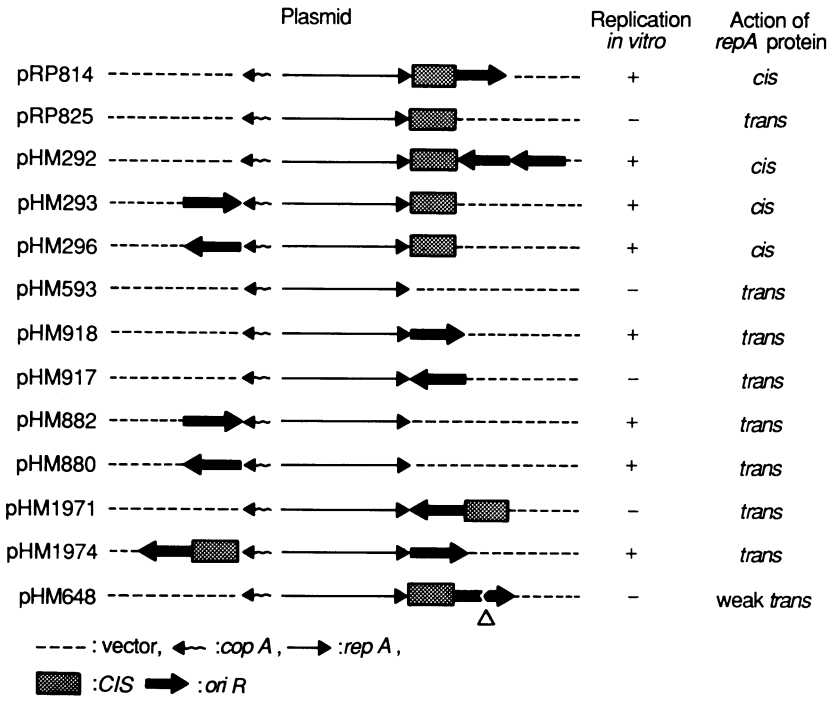


Fig. 3 Schematic representation of repA donor plasmids constructed and their properties.

the repA protein synthesized from various plasmids are summarized in Fig. 3.

RepA Protein is Not Reusable After it Activates oriR in the cis Position

The CIS sequence seems to ensure the cis-action of the repA protein by permitting preferential utilization of de novo synthesized repA protein by the cis-origin. The following results indicate the existence of an additional mechanism to ensure that the repA protein does not participate in the initiation of the next round after it activates the cis-origin. The in vitro replication of R1 plasmid was divided into two stages. The first stage involves protein synthesis on the repA$^+$ plasmid, and the second stage involves DNA synthesis on the oriR$^+$ plasmid in the absence of protein synthesis. When the repA$^+$ oriR$^-$ plasmid was used in the first stage, the oriR plasmid was efficiently replicated in the second stage. However, replication of the oriR plasmid was not observed when the first stage was on the repA$^+$ oriR$^+$ plasmid (Fig. 4), although the analysis of in vitro synthesized proteins indicated the presence of repA protein (See Fig. 3 of

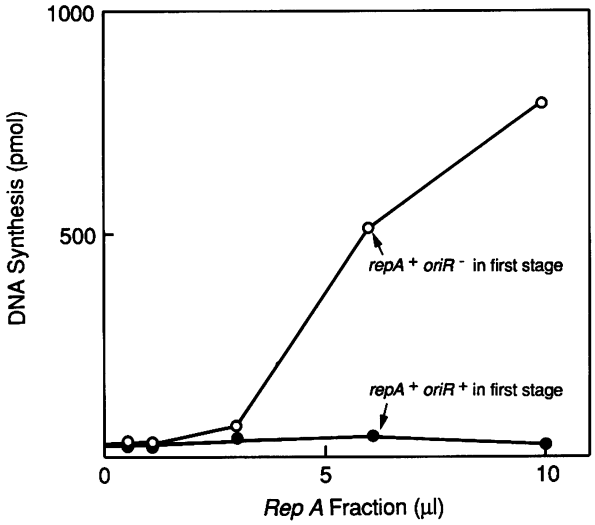


Fig. 4 Two-stage reaction of R1 plasmid replication in vitro: repA protein is not reusable after it interacts with oriR. The first stage reaction with pRP825 (repA⁺oriR⁻, O—O) or pBEU17 (repA⁺oriR⁺, ●—●) was incubated at 30°C. After 30 min, varying amounts of the first stage reaction mixture ("RepA fraction") were removed and added to the second stage reaction mixture containing 100 µg/ml chloramphenicol and 7500 pmol as nucleotide of pMOB45 DNA. The reaction was continued for another 60 min and acid insoluble radioactivity was measured.

ref. 3). Since the synthesis of repA protein is coupled to DNA synthesis during the first stage, repA protein synthesized must interact with the origin. These results indicate that repA protein is not synthesized in excess and that all of the repA protein synthesized de novo is utilized by cis-oriR. Furthermore, after activation of the origin, the repA protein is inactivated or is unavailable for the template in the second stage.

Deletion of CIS Decreases the Transformation Efficiency in vivo

OriR, identified by using the in vitro trans-complementation assay, is sufficient for initiation of DNA replication in vitro, when repA protein is supplied in trans (4,5). OriR, physically separated from repA, can function as a replication origin in vivo as well and neither the location nor orientation of oriR relative to repA affect the replication of R1 plasmid (Fig. 5).

Although CIS, a 170 bp sequence between repA and oriR, is dispensable for in vitro replication of R1 plasmid, the repA⁺ oriR⁺ plasmid, consisting of the minimum repA (78-1254) and the minimum oriR (1406-1611), transformed

Plasmid	*in vitro* replication activity (pmol)	*in vivo* replication activity
1	450	100
2	350	100
3	400	63
4	400	80

----: vector, ←: *cop A*, →: *rep A*, ▩: *CIS*, ➡: *ori R*

Fig. 5 Replication activities in vivo and in vitro of repA$^+$ oriR$^+$ plasmids with varied location and orientation of oriR. In vitro replication activity was measured in the standard reaction mixture using 2.5 μg (7500 pmol as nucleotide) of each plasmid DNA as template. In vivo replication activity was measured by transforming C2110 (polA1) with each plasmid DNA. 2 μg of each supercoiled plasmid DNA was mixed with 50 μl of calcium-treated cells ($3X10^8$ cells). The tubes were kept on ice for 30 min, heat-shock was given by placing the tube at 42°C for 90 sec, 3 ml of L-broth was added to each tube and the cultures were incubated at 37°C for 30 min with vigorous shaking. Twenty-five μl of culture was plated on an L-plate containing 50 μg ampicillin per ml. After 16 hr incubation at 37°C, the number of transformants was counted and presented in the table as a normalized value relative to plasmid 1.

the C2110 (polA1) strain with low efficiency, which suggested that CIS is involved in plasmid replication in vivo.

In order to localize the sequence in CIS which is important for efficient transformation, an internal deletion was introduced (Fig. 6). While no significant effect on replication activity in vitro was observed by deleting CIS, the transformation efficiency of some of the deletion derivatives dropped to one to two percent of that achieved by the parent plasmid. These results suggest that replication activity in vivo is profoundly affected by a deletion in CIS. Nucleotide sequence analysis of deletion ends indicated that the sequence from position 1253 to 1335 was important for efficient transformation (Figs. 6 and 8). The plasmid copy number is also decreased by these deletions (data not shown).

Reintroduction of CIS Sequence into repA$^+$ CIS$^-$ oriR$^+$ Plasmid Restores Transformation Efficiency

The decreased transformation efficiency is due to the lack of the CIS sequence and not to the shortened distance between repA and oriR, since the transformation efficiency is restored only when the CIS segment (1256-1433) is reintroduced into the repA$^+$ CIS$^-$ oriR$^+$ plasmid in one orientation (Fig. 7). The CIS fragment inserted in an inverted orientation did not restore the transformation efficienty. 330 bp and 550 bp fragments derived from

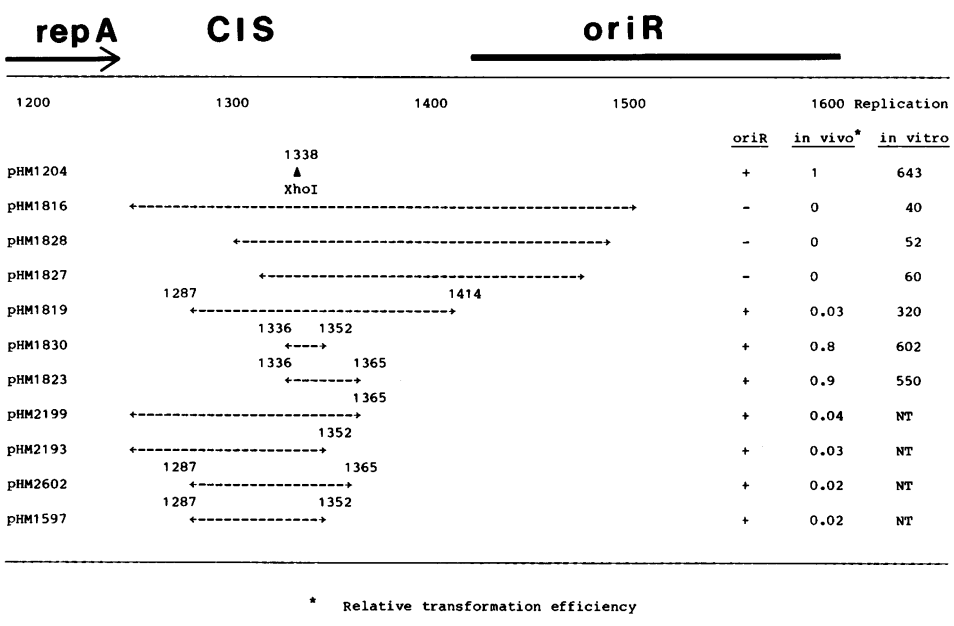
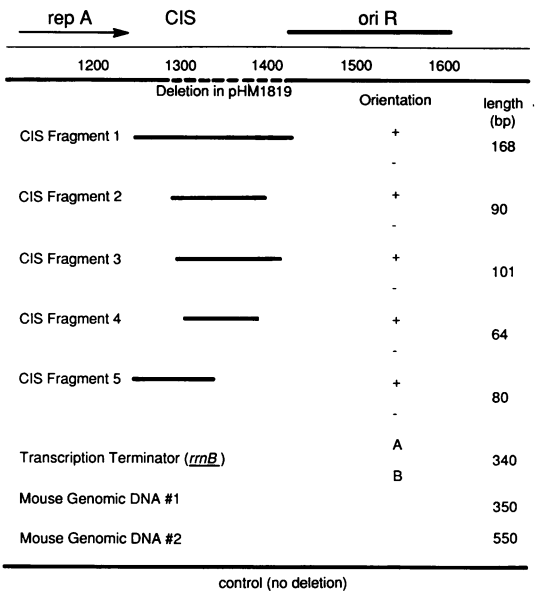


Fig. 6 Replication activities in vivo and in vitro of pHM1204, that carries a unique XhoI site in the CIS region, and its CIS deletion derivatives. Replication activity in vivo and in vitro was measured as described in the legend to Fig. 5. The dotted lines with two arrows indicate the extent of deletion carried by each plasmid DNA. The numbers written at the ends of the arrows show the nucleotide numbers at the deletion endpoints. pHM1816, 1828, and 1827 were judged to be oriR$^-$ since they were not replicated in vitro even in the presence of repA$^+$ oriR$^-$ helper plasmid (data not shown). No transformants were obtained with these three plasmids. pHM2199, 2193, 2602, and 1597 were constructed with the combination of pHM1816 and 1823, 1816 and 1830, 1819 and 1823, and 1819 and 1830, respectively. NT, not tested.

mouse genomic DNA, used as a control, had very little or no stimulation activity (Fig. 7). The recovery of replication activity to some extent by the 550 bp fragment is due to the presence of transcription terminator activity, as will be discussed later.

A deletion was introduced into the portable CIS segment from both ends and inserted into the repA$^+$ CIS$^-$ oriR$^+$ plasmid. When the deletion extended from 1295 to 1306 (CIS-Fragment 3) or 1310 (CIS-Fragment 4), transformation efficiency was decreased to 20% or 8%, respectively, indicating that the sequence 1295-1309 is essential (Table 1). Sequences 1336-1365 and the 3' side of 1385 are dispensable, since they can be deleted without affecting transformation efficiency (pHM1823 and pHM1819 carrying CIS-Fragment 2).



	Orientation	length (bp)	Transformation Efficiency Ampicillin (μg / ml) 50	100	200	300
			3	0	0	0
CIS Fragment 1	+	168	77	41	10	0
	-		11	2	0	0
CIS Fragment 2	+	90	125	50	13	2
	-		19	4	0	0
CIS Fragment 3	+	101	19	12	2	0
	-		6	4	0	0
CIS Fragment 4	+	64	8	2	0	0
	-		3	0	0	0
CIS Fragment 5	+	80	20	NT	NT	NT
	-		10	NT	NT	NT
Transcription Terminator (*rrnB*)	A	340	6	1	0	0
	B		27	15	0	0
Mouse Genomic DNA #1		350	5	0	0	0
Mouse Genomic DNA #2		550	51	7	1	0
			100	80	42	20

Fig. 7 In vivo replication activities of the plasmids that carry a CIS fragment at BamHI site of pHM1819. Solid bars represent the CIS-derived regions that were inserted into pHM1819. Transformation efficiency was measured as described in the legend to Fig. 5. The values are normalized to the number of transformants obtained with control DNA (repA$^+$ CIS$^+$ oriR$^+$) on an L+ampicillin (50 μg/ml) plate. Orientation (+) indicate that the CIS fragment is in a native orientation, while (-) refers to the opposite orientation. A DNA fragment carrying a transcription terminator from the rrnB operon was inserted with two orientations (A and B). Mouse genomic DNA 1 and 2 are derived from the mast cell growth factor gene cloned by Miyatake et al. (29). NT, not tested.

Sequences 1310-1335 and 1366-1384 may be important. The latter is missing in CIS-Fragment 5 (1256-1335) which only partially recovered transformation efficiency. These results suggest that the sequences 1295-1335 and 1366-1384 are important for efficient replication activity in vivo (Fig. 8).

S1 Mapping of 3' ends of the repA Transcripts within CIS

Since the fate of the repA transcripts, initiated from either of the two promoters (PrepA or PcopB), is unknown, its 3' ends were determined by S1 mapping (Fig. 9). The major 3' end within CIS was mapped at position 1299, 48 bp downstream of TGA codon of repA. Another strong 3' end was mapped near position 1600, at the 3' edge of oriR. Minor 3' ends were mapped at positions 1319, 1384 and 1423. Densitometric scanning of the

Table 1

Correlation between replication stimulation activity and transcription terminator activity conveyed by fragments derived from the CIS region

DNA Fragment Inserted	Size (bp)	Position	Relative Transformation Efficiency	Efficiency of Transcription Termination
CIS Fragment 1	168	1256-1433	0.80	81%
CIS Fragment 2	90	1295-1384	1.00	72%
CIS Fragment 3	101	1306-1406	0.20	26%
CIS Fragment 4	64	1310-1373	0.08	0%
CIS Fragment 5	80	1256-1335	0.20	80%
rho-Independent Transcription Terminator				
From *rrnB*	~340		0.14 (pHM2714)	81%
From *trpA*	~80		0.10 (pHM2948)	80%
rho-Dependent Transcription Terminator				
From λt_{L1}	763		0.30 (pHM3100)	98%
	450		0.20	76%
	313		0.15	85%

For CIS fragments 1-5, only values with the plasmid carrying them in the native orientation are presented. Transformation efficiency is expressed as the relative value of the number of transformants with each plasmid compared to that for the parent *repA*$^+$ CIS$^+$ *oriR*$^+$ plasmid. Efficiency of transcription termination was calculated as follows. First, a "growth value", calculated as $Gx=OD_{t=60}/OD_{t=0}$, was determined for each plasmid (designated X) where $OD_{t=0}$ and $OD_{t=60}$ stand for the values of OD_{600} when Tc was added and sixty min later, respectively. Then, $(G_{tcv}-G_x)/G_{tcv}x100$ was calculated and presented as the "Efficiency of transcription termination", where G_{tcv} represents the "growth value" of the terminator cloning vector. A synthetic 28 bp *trpA* teminator, obtained from Pharmacia, was first cloned at SmaI site of pUC8. The terminator sequence, isolated as an EcoRI-HindIII fragment, was filled in with Klenow fragment, BamHI linker was attached to both ends and the resulting 80 bp long DNA fragment, carrying additional sequences derived from the pUC8 linker segment on both ends, was inserted into pHM1819 or teminator cloning vector. The *rho*-dependent transcription terminator sequences (a gift from K. Shigesada and M. Imai) are derived from λt_{L1}. 763, 450, and 313 bp λt_{L1} DNA fragments are derived from pMS100, pMS108, and pMS109, respectively (constructed by M. Hirano). The values for termination efficiency for λt_{L1} fragments are those obtained by M. Hirano.

intensity of each band showed that 41%, 2%, 2%, 2%, and 53% of the *repA* transcript is terminated at positions 1299, 1319, 1384, 1423 and ~1600, respectively. When RNA from HD173 (*rho*-ts702) harboring pMOB45 was analyzed in the same way, the band at position 1299 decreased and the one at 1423 increased, suggesting that termination at position 1299 is *rho*-dependent (data not shown). On the other hand, terminations at other positions are likely to be *rho*-independent, since stem-loop structures followed by T-stretches (14) can be drawn (Fig. 8). A highly stable stem-

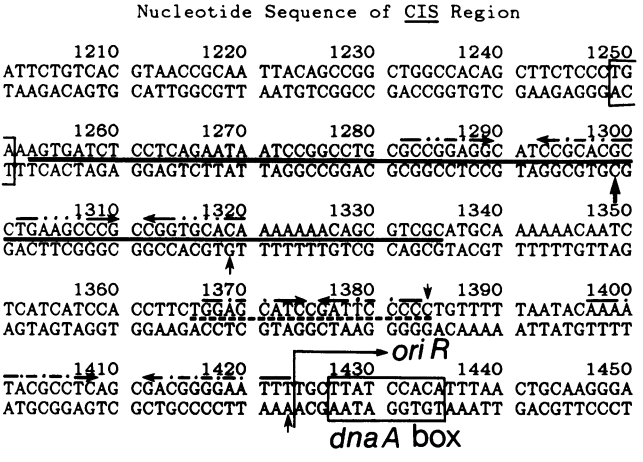


Fig. 8 Nucleotide sequence of CIS region of R1 plasmid. Nucleotide number is according to those in the reference (30). The coding frame for repA terminates at TGA (1249-1251). The minimum oriR starts from the residue at position 1424. Sequences with solid lines were shown to be important for efficient replication of R1 plasmid in vivo and those with a dashed line are also likely to be important. The big arrow indicates the position of the major rho-dependent transcription termination site of the repA transcript and the smaller arrows indicate other minor rho-independent terminations. Possible stem-loop structures are indicated above the sequence with two arrows pointing to each other. dnaA box (position 1427 to 1435), a recognition sequence for host dnaA protein, is also indicated.

loop structure can be formed near the 3' end mapped at ~1600 as well (position 1593 to 1611).

Localization of the Transcription Terminator Function on CIS and its Correlation with Replication Stimulation Activity

The presence of a transcription terminator in CIS, which was suggested by S1 mapping, was demonstrated by using a terminator cloning vector which carries a unique restriction site between the promoter and the coding region of the tetracycline (Tc)-resistance gene. The fragment, encompassing the entire CIS sequence, inserted into the terminator cloning vector, severely inhibited the expression of Tc. The CIS fragment decreased the Tc expression only in one orientation i.e. the same orientation as in the original plasmid (Fig. 10A). The terminator in CIS did not function in the rho-ts strain HD173 (pHM2730) (Fig. 10B), demonstrating that it is rho-dependent. A typical rho-independent terminator derived from the E. coli rrnB functioned in the same strain

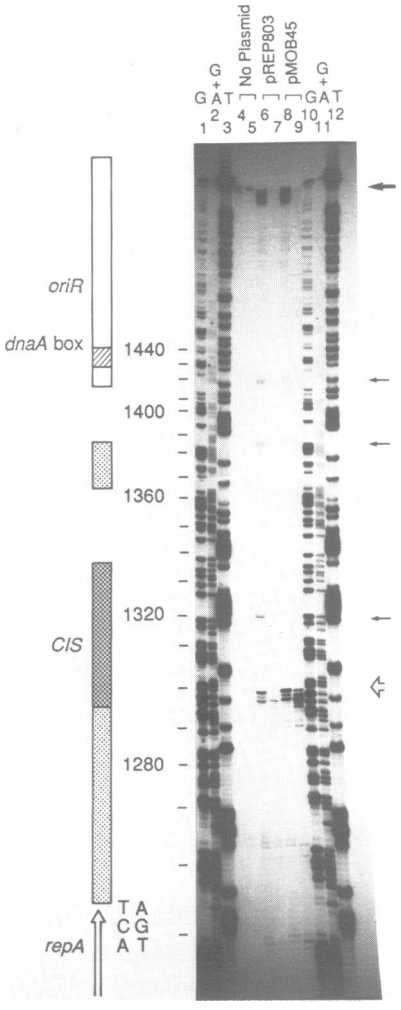


Fig. 9 S1 mapping of 3' ends of the repA transcript. Total RNA was isolated from E. coli MC1061 strain without plasmid (lanes 4 and 5), harboring pREP803, a pUC8-mini R1 hybrid plasmid containing from position 1 to ~ 3600 (lanes 6 and 7), and pMOB45, a derivative of a run-away replication mutant of the R1 plasmid (lanes 8 and 9) and hybridized with 3' end-labeled MluI-EcoRI fragment (position 1118 to 1623, labeled at MluI). RNA/DNA hybrids were digested with 50 units (lanes 4, 6, and 8) or 200 units (lanes 5, 7 and 9) of S1 nuclease. Products were analyzed on 6% polyacrylamide gel containing 7M urea. Lanes 1 to 3, 10 to 12 are Maxam-Gilbert sequencing ladders generated on the same fragment as size markers. The open arrow and the filled arrows indicate the positions of rho-dependent and -independent termination sites, respectively. Those sequences within CIS which are important for the plasmid replication in vivo are shown by the hatched boxes on the left of the gel.

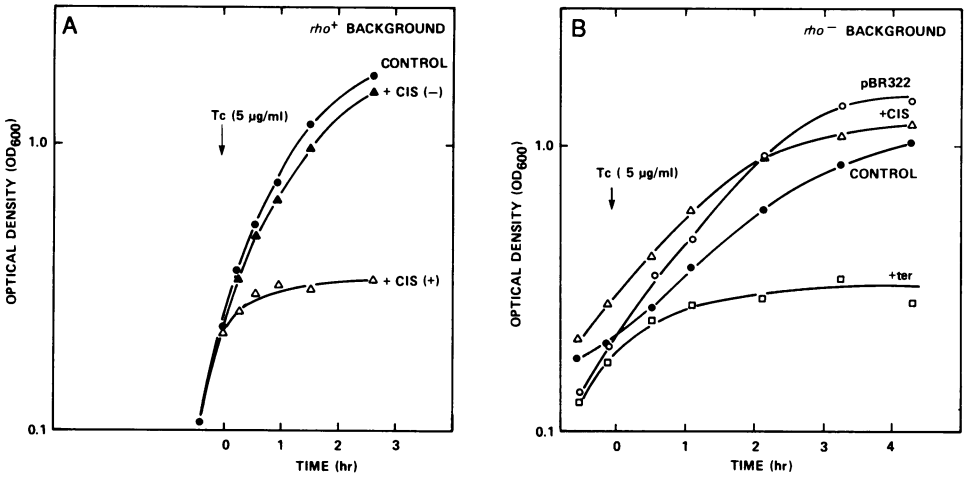


Fig. 10 A. Transcription termination activity in the CIS region. The CIS fragment 1 (described in Fig. 7) was inserted into the terminator cloning vector in both orientations, resulting in pHM2730 (△—△) with the original orientation and pHM2739 (▲—▲) with the opposite orientation. Each plasmid was introduced into MC1061 and the strains were grown in L-broth at 37°C with vigorous shaking, and growth of the cells was monitored by measuring OD_{600}. At an early log phase (OD_{600}=0.2-0.3), tetracycline was added to the culture to a final concentration of 5 μg/ml and growth was further monitored. Severe growth inhibition with pHM2730 indicated the presence of a transcription terminator in CIS. The plasmid carrying the CIS fragment in the opposite orientation exhibited little or no termination activity. ●—●, MC1061 harboring terminator cloning vector. B. Transcription termination activity of CIS in rho⁻ background. pHM2730 (carrying CIS), pHM2734 (carrying the rrnB terminator), and terminator cloning vector were introduced into rho-ts702 strain HD173. The strain harboring each plasmid was tested for transcription termination activity as described above except that cultures were grown at 30°C. ●—●, HD173 (terminator cloning vector); △—△, HD173 (pHM2730); □—□, HD173 (pHM2734); ○—○, HD173 (pBR322).

(pHM2734). Both terminators were functional in the parent rho⁺ strain KH6301 (data not shown).

The DNA sequence which contains the terminator activity was determined by measuring transcription terminator activity of the same CIS-fragments used in the experiment shown in Fig. 7. Deletion of the sequence 1295-1309 (CIS-fragments 2, 3, and 4), which includes the major 3' end of the repA transcripts, dramatically reduced transcription terminator activity, in parallel with the loss of replication stimulation activity (Table 1). Transcription terminator activity is contained within the 80 bp CIS-

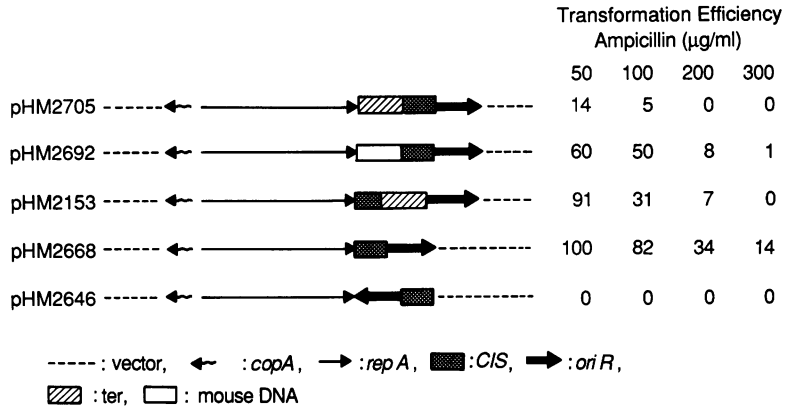


Fig. 11 Effect of a rho-independent transcription terminator inserted at the 5' side or the 3' side of CIS on replication activity in vivo. pHM2668 (repA$^+$ CIS$^+$ oriR$^+$) is a derivative of pRP845 and carries a unique BglII site between repA and CIS. pHM2705 carries the rrnB terminator, inserted at BglII site of pHM2668. pHM2692 carries a control fragment, MCGF fragment 1, at the same site. pHM2153 carries the rrnB terminator, inserted at BamHI site of pHM1823 (located between CIS and oriR). Transformation efficiency with each plasmid DNA was measured as described in the legend to Fig. 5. The number of transformants on plates containing different concentrations of ampicillin was counted and the values normalized to that with repA$^+$ CIS$^+$ oriR$^+$ plasmid (pHM2668) on a plate with 50 μg ampicillin per ml are presented. Figures are not drawn to scale. pHM2646, carrying CIS and oriR downstream to repA in the opposite orientation, did not give any transformants and the replication of this plasmid in vitro was also very poor (data not shown, see pHM917 and pHM1971 in Fig. 3).

fragment 5 (1256-1335), although this fragment cannot fully recover transformation efficiency when inserted into the CIS$^-$ plasmid.

Transcription Termination Is Not Sufficient to Stimulate in vivo Replication

In order to examine whether other transcription terminators can replace the function of CIS which is required for efficient replication in vivo, a rho-independent terminator derived from E. coli rrnB was introduced into the repA$^+$ CIS$^-$ oriR$^+$ plasmid. The terminator, inserted in both orientations, increased the transformation efficiency up to 14% of that of the parent plasmid at ampicillin concentration 50 μg/ml (Fig. 7 and Table 1). However, with ampicillin concentration above 200 μg/ml, transformants were not obtained, while the parent plasmid, carrying CIS, gave a substantial number of transformants. Mouse genomic fragment 2 increased the transformation efficiency to some extent when inserted into the CIS$^-$ plasmid (Fig. 7), presumably because this fragment carries

terminator activity as measured by the terminator cloning vector (data not shown). pHM2948, carrying the trpA terminator in place of CIS, transformed the polA1 strain with efficiency similar to pHM2714 which carries the rrnB terminator (Table 1). CIS$^-$ plasmids are extremely unstable in the absence of selection; only 1~2% of the population maintained CIS$^-$ plasmids after growth in a non-selective media for six generations, whereas CIS$^+$ plasmids are stably maintained under the same conditions. Insertion of the rrnB or trpA terminator improved the stability only to 9% or 8%, respectively (data not shown). These results suggest that rho-independent terminators cannot functionally replace CIS although they restore replication efficiency of CIS$^-$ plasmid to some extent.

A rho-dependent transcription terminator from λt_{L1} was also examined (Table 1) Although the transformation efficiency of CIS$^-$ plasmids carrying the λt_{L1} terminator was improved to 15~30% of the control at 50 μg/ml ampicillin and plsmid DNA was more stably maintained than CIS$^-$ plasmids (data not shown), a recovery was still not complete. These results suggest that rho-dependent transcription terminators, which work slightly better than rho-independent ones, still cannot fully replace the CIS function.

Transcription Terminator Placed at the 5' Side of CIS Affects Replication Activity in vivo

Close correlation found between transcription terminator activity and replication stimulation activity in CIS suggests that the repA transcript terminated within the CIS region is involved in maintaining efficient replication in vivo. If this is the case, premature termination of the repA transcription upstream to CIS should affect replication activity. This possibility was tested by inserting the rrnB transcription terminator at either the 5' or 3' side of CIS. Measurements of transformation efficiency with each plasmid DNA revealed that a transcription terminator inserted between repA and CIS significantly reduced transformation efficiency (pHM2705), while one inserted between the CIS and oriR (pHM2153) did not affect it appreciably except that the copy number was somewhat reduced (Fig. 11). A DNA fragment carrying no terminator activity, inserted between repA and CIS, did not significantly reduce transformation efficiency (pHM2692). This indicates that premature termination of repA transcription before reaching CIS causes reduced transformation efficiency. Plasmid maintenance also dropped to a level similar to pHM2714 or pHM3100 (CIS$^-$ plasmids carrying other terminators) when a transcription terminator

was inserted between repA and CIS (data not shown). These results support the idea that efficient in vivo replication of R1 plasmid is dependent on proper transcription termination of the repA transcript within CIS.

DISCUSSION

Initiation of R1 plasmid replication is dependent on two plasmid functions, repA and oriR, both in vivo and in vitro (3-5). A DNA fragment containing the replication origin of a plasmid such as F (15) and R6K (16) can be replicated in vivo in the presence of a helper plasmid carrying a DNA segment encoding a trans-acting factor. In contrast, IncFII plasmids are unique in that trans-complementation has never been observed (7), which led us to believe that the repA protein is cis-acting. With the aid of the in vitro system for R1 plasmid replication, this was demonstrated in vitro (3). As has been observed in vivo, the repA protein acts preferentially in cis in vitro (3,6, and Fig. 1). To our knowledge, this is the first example where cis-specific action of a protein was observed in vitro. Using this in vitro system, nucleotide sequences involved in the cis-action were examined. Deletion of the region downstream of oriR did not significantly affect the cis-action of repA protein (Fig 1). Deletion of oriR completely abolished the cis-action; the repA protein synthesized from $repA^+$ $oriR^-$ plasmid is efficiently utilized by oriR in trans (3). Position and orientation of oriR relative to repA did not affect the cis-action of the repA protein. An internal deletion of oriR (pHM648, positions 1548 to 1570), which completely blocks the origin function, partially impairs the cis-action. Since the deletion carried by pHM648 does not affect binding of repA and dnaA proteins to oriR in vitro (our unpublished data), it is expectd that the newly synthesized repA protein will still preferentially bind to oriR in the cis-position. However, because the subsequent reaction may not take place due to the deletion, a portion of the repA protein may leak from the repA-oriR complex and become available for the oriR in trans. When CIS, the sequence between repA and oriR, is deleted, the repA protein is capable of activating oriR in the trans position (Fig. 2). This suggests that there is a signal in the CIS region that confers cis-specificity on the repA protein.

Several examples of cis-acting proteins have been described (17). The gene A protein, which initiates a rolling circle mode of replication of the replicative form of ΦX174 phage DNA by introducing a nick at a specific site on the genome (18), is cis-acting. Mixed infection of wild-type and

gene A amber mutant phages resulted in selective replication of wild-type phage and no complementation was observed (19). λQ anti-terminator protein acts preferentially on the nearby qut site (20). A mutation in dsdC, a gene encoding an activator for D-serine deaminase, cannot be complemented by the wild type gene on a multi-copy plasmid (21). Transposase of Tn5 acts more efficiently on the direct repeats present near the transposase gene (22). Cis-specific action would permit the efficient utilization of proteins which are expressed only in a limited amount. Although the numbers of known cis-acting proteins are limited, they may in fact be more common. Classical cis/trans tests generally assume that proteins are diffusible in the cytoplasm and can act in trans. However, it is still possible that the target site in cis-position is more efficiently recognized than ones in trans.

Although only repA and oriR are required for in vitro replication of R1 plasmid, CIS must be present downstream of repA for efficient replication of the plasmid in vivo (Fig. 6). In the absence of CIS, transformation efficiency, average copy number and plasmid stability decreased. CIS contains a rho-dependent transcription terminator, which terminates repA transcription at position 1299 (Figs. 9 and 10). Deletion analysis indicates that transcription terminator activity is necessary but not sufficient for efficient replication (Table 1).

How does the CIS sequence affect replication with its rho-dependent transcription terminator activity? A possible model for cis-action of repA protein is postulated below (Fig. 12). The precise termination in CIS may stabilize the repA transcript and thereby increase the repA expression, as was the case for some genes whose expression is regulated at the level of transcription termination (23). However, S1 mapping of the 3' end of the repA transcript in rho-ts702 strain indicated that the transcript is stable in the absence of the rho-dependent termination at position 1299. Failure of other rho-dependent or independent transcription terminators to replace CIS (Fig. 7) also suggests that control by mRNA stability seems to be unlikely. It is more likely that CIS somehow facilitates interaction of the newly synthesized repA protein with oriR. Generally, rho-dependent termination is mediated by two sequential steps; a rho-independent stalling or pausing of RNA polymerase, and a rho-mediated release of the RNA transcript (24). Pausing at or near position 1299 may occur at positions between 1282 and 1300 which can form a weak secondary structure (Fig. 8). The boxA sequence, which may play an important role in the action of host

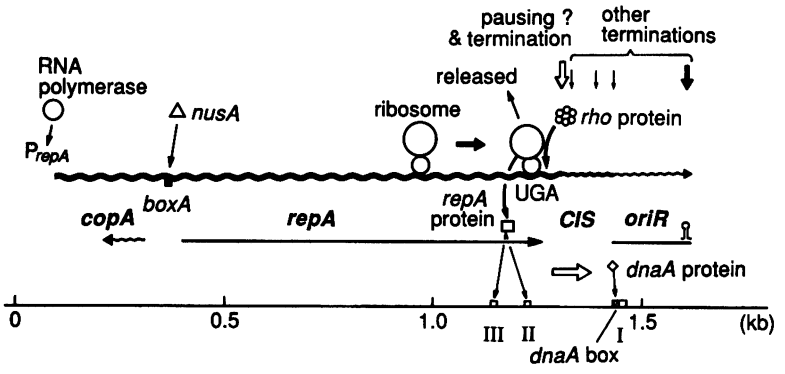


Fig. 12 Transcriptional pattern in the basic replicon of R1 plasmid and a model for the role of CIS in efficient plasmid replication in vivo and in cis-action of repA protein in vitro. Wavy lines represent RNA transcripts and their thickness indicates relative amount of the transcript. The repA coding region and oriR are indicated by an arrow and a bar above the scale. An open vertical arrow indicates the major rho-dependent termination site, while the filled vertical arrows show the positions of other rho-independent terminations. I, II and III represent the binding sites of the purified repA protein. I is the primary binding site within oriR and II and III are secondary binding sites for the loading of repA protein on the template DNA. The open horizontal arrow above CIS indicates the possible translocation of repA protein on DNA (see the texts for details).

nusA protein (25), has been discovered in the leader region of the repA trascript (26). NusA protein may enhance pausing at this site, as it does so in vitro (27). The pausing of RNA polymerase will enable ribosome to complete the translation of the repA transcript which still forms an RNA/DNA hybrid at its 3' end. Freshly synthesized repA protein may bind immediately to the DNA template and thus be protected from inactivation. Using purified repA protein, we have recently identified secondary repA binding sites in the C-terminus of the repA coding frame (position 1141-1150 and 1211-1220) (28). Considering their physical proximity to the site of repA synthesis, it is tempting to speculate that the newly synthesized repA protein, loaded onto DNA through secondary binding sites, translocates on DNA until it locates oriR, with which it forms a nucleoprotein-like structure (4,28) to initiate replication. In this model, oriR can be placed anywhere on the template for efficient replication, as long as the repA protein can be loaded onto the template DNA through CIS and the secondary repA protein binding sites. The repA protein may not be loaded onto the template DNA efficiently if the repA transcript is released from the template DNA without sufficient pausing, as

may be the case for other transcription terminators which only partially replace CIS function. Alternatively, the CIS sequence may contain information to maintain a stable RNA/DNA hybrid, so that the newly synthesized repA protein on the ribosome is kept close to the template DNA. In the absence of CIS, newly synthesized "free" repA protein, which is not trapped at the loading sites, can activate oriR in trans. In vivo, CIS$^-$ plasmids do not replicate efficiently, because "free" repA protein, which diffuses into the cytoplasm, may be unstable and cannot be utilized for the initiation. RepA protein seems to be no longer available for reutilization and protected from being reused by a second template once it binds to oriR and initiates DNA replication (Fig. 4). At present, we do not know whether this is achieved by physical sequestering of repA protein through its tight association with DNA even after replication is completed, or by functional inactivation by some kind of modification. Availability of an in vitro system, in which cis-action of a protein is observed, would help to understand the molecular mechanism of cis-action.

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