Synthesis of RepK of Rolling Circle Plasmid pKYM is Regulated by Countertranscript and HU Protein

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Abstract

Replication of rolling circle plasmid pKYM was regulated by RepK, a plasmid-encoded initiator protein, with HU protein and antisense RNA (copRNA) that block the expression of RepK. HU protein bound to the repK promoter in the presence of RepK protein and inhibited the transcription of repK mRNA. The copRNA would hybridize to repK mRNA and induce a stem-loop structure in which the repK Shine-Dalgarno sequence is sequestered by base pairing. Sequence substitution experiments demonstrated that this stem-loop not only inhibits translation but induces premature termination.

Key words: rolling circle; pKYM; HU protein; RepK protein; antisense RNA

1. Introduction

Replication of plasmid DNA is regulated at the initiation step by autogenously encoded negative feedback loops. Antisense RNAs, countertranscripts, serve as the inhibitory component in several plasmids. In all of the systems, the countertranscript is complementary to the 5' end of an essential RNA molecule (the target RNA) such as a mRNA of replication initiator protein. The countertranscript hybridizes to the target RNA and induces a critical change in the folding pattern distal to the region of complementarity and inhibits its normal functions. In the pT181 which is a well studied rolling circle plasmid of Staphylococcus aureus and IncFII plasmid families, the target of the countertranscript is the mRNA of the plasmid-specific initiator protein (RepC and RepA, respectively).1,2 With pT181, the countertranscript-induced conformation contains a strong stem-loop in which the repC Shine-Dalgarno (SD) is sequestered. This stem-loop induces premature termination of transcription of repC mRNA.1 In the ColEI system, binding of the countertranscript causes downstream changes in the secondary structure of the preprimer and prevents formation of an RNA-DNA hybrid at the replication origin processes to form the functional replication primer.3

The rolling circle plasmid pKYM isolated from Gram-negative bacterium Shigella sonnei is a multicopy plasmid of 2083 nucleotides and belongs to the pUB110/pC194 plasmid family of Gram-positive bacteria.4 The replication of pKYM is also regulated at the initiation step by countertranscript, copRNA (our observation). Sequence analysis suggested that repK mRNA would form two stem-loop structures and that the repK SD is sequestered in one of them.

The promoter of repK is located in the replication origin of pKYM. We have reported that HU protein binds to origin only when RepK is bound to the origin.5 Since the HU binding site overlaps to the repK promoter, the transcription of repK would be regulated by RepK itself with HU at the initiation step of transcription. In this paper, we demonstrate that initiation of transcription of repK mRNA was repressed by RepK with HU, and the stem-loop structure not only inhibited translation but induced premature termination of transcription.

2. Materials and Methods

2.1. Bacteria and plasmids

XL1-Blue,6 YK1100 and YK1340 (YK1100 hupA16::Km hupB11::Cm)7 were described before. pOPM3703FT, which expresses RepKY237F, is a derivative of R6K.8 RepKY237F is a site-directed mutant of RepK. Though the mutant protein binds to the pKYM origin, it can not initiate DNA replication.4 The tran-
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Figure 1. Nucleotide sequence around the repK promoter and copRNA promoter. (a) The nucleotide sequence from 391 to 710 is shown. RepK binding site and HU binding sites are indicated. Arrows indicate stem-loop structures. -35 and -10 are promoter sequences. White arrowheads are the initiation sites of repK mRNA and copRNA, and black arrowhead indicates the termination site of copRNA (see text). (b) copRNA-induced conformation of repK mRNA.

2.2. Construction of NM mutant

NM mutant was constructed to prevent formation of SL4. The sequence of 5'-ACAGCCTGCCAGA in SL4 was changed to 5'-TCTAGATGCACTTTT by the method of Kramer.9

2.3. Primer extension to detect repKY237F mRNA

Thirty micrograms of RNA prepared from Escherichia coli YK1100 and YK1340 carrying both dr8CAT and pOPM3703FT were used for the template of primer extension. CAT III primer (5'-AACGGTGGTATATCC) was used as a primer. The reaction conditions and gel electrophoresis were described previously.10

2.4. Primer extension to detect copRNA

Thirty micrograms of RNA extracted from E. coli XL1-Blue carrying pKYM was used as a template. C primer (5'-CATTTCTGAATGTTCACCTC) was used as a primer. Reaction condition and gel electrophoresis were described previously.10

2.5. In vitro transcription of copRNA

Plasmid dr5 was digested with BglII, isolated from agarose gel and purified for use as a template. The experiment was performed as described previously.11 The transcript was electrophoresed through 8% sequencing gel.

2.6. Enzymes and other methods

Restriction enzymes and modification enzymes were purchased from Toyobo Co. (Oska Japan), Takara Shuzo.
3. Results and Discussion

3.1. Repression of the repK promoter by RepK with HU

We have demonstrated that HU binds to two sites in the presence of RepK, and one of the sites overlaps the repK promoter (−35, 5′-TTGACA). To examine whether transcription of repK is repressed by RepK with HU, we assayed the amounts of repK mRNA by primer extension. RepKY237F in which the Tyr237 was mutated to Phe was used instead of native RepK to avoid initiation of DNA replication. RepKY237F binds to pKYM origin but can not initiate DNA replication. As shown in Fig. 2b, the amount of repK mRNA was higher in the absence of RepKY237F and HU. The gel shift assay demonstrated that almost the same amount of RepKY237F which is under control of the lac promoter was produced in both strains (Fig. 2c). Therefore, the repression of repK mRNA in E. coli YK1100 was due to the presence of HU.

3.2. Identification of countertranscript

Sequence analysis suggests that promoter of countertranscript (copRNA) is located upstream of the repK SD (Fig. 1). As shown in Fig. 3a, copRNA was de-
Figure 3. Identification of copRNA. (a) Northern hybridization of RNA extracted from XL1-Blue carrying pKYM. Oligonucleotide (C2 primer: 5’-TGTACTGGCTGCTGAATGCACAGGT) was labeled and used as a probe. Lane 1, RNA was extracted from E. coli XL1-Blue which carried pKYM. Lane 2, RNA was extracted from E. coli XL1-Blue. (b) In vitro transcription of copRNA. Bgl II-digested dr5 which carries a 741-bp fragment of pKYM was used as a template to transcribe RNA. Three transcripts were detected. Arrow indicates copRNA. Black arrowhead and white arrowhead indicate truncated repK mRNA and premature terminated repK mRNA, respectively. (c) Primer extension assay. RNA was prepared from XL1-Blue carrying pKYM. End-labeled oligonucleotide (C primer: 5’-CATTTCGAATGTGTCACCTC) was used as a primer. Lane 1, RNA was extracted from E. coli XL1-Blue which carried pKYM. Lane 2, RNA was extracted from E. coli XL1-Blue. (d) Location of oligonucleotides. C primer for the primer extension assay and C2 primer for the northern analyses are indicated. The length of truncated repK mRNA is 200 bases. PR indicates the repK promoter and PL indicates the copRNA promoter.

3.3. Countertranscript induced premature termination of repK mRNA

As shown in Fig. 1b, RepK mRNA would form a stem-loop, SL4, in the presence of the countertranscript, copRNA. In pT181, which is a well studied rolling circle plasmid of Staphylococcus aureus, the stem-loop structure induced by the countertranscript functions as a transcription terminator. To examine whether SL4 would act...
as a transcription terminator, we constructed plasmids in which the cat gene with its own SD was joined under the repK promoter, PR, and assayed for CAT activity. As shown in Fig. 4, CAT activity was reduced by 50% when SL4 was inserted between the promoter and the cat gene. However, SL1 and NM1 which can not form SL4 did not affect CAT activities. These results indicate that about 50% of the transcript did not reach the cat gene when SL4 was inserted. The premature transcript should be slightly longer than 156 bases in size since the distance between the transcription initiation site and the 3' end of SL4 is 156 bases. As shown in Fig. 3b, the RNA of 170 bases was detected as same intensity as 200 bases of run-off product. This result also indicates that about 50% of transcript is terminated by SL4.

3.4. Translation was also repressed by copRNA
The SD of repK mRNA would be sequestered in SL4 when copRNA is hybridized to mRNA (Fig. 1b). Therefore translation of repK mRNA would be repressed by copRNA via the formation of SL4. We constructed plasmids and assayed CAT activity to confirm this hypothesis. When the cat gene was joined downstream of SL4 without its own SD, 80% of CAT activity was repressed. Since SL4 terminates about 50% of transcription, the other 50% of mRNA would be repressed to 20% at the translation step. NM1 did not affect translation of repK mRNA (Fig. 4). Therefore, the inhibition of translation was due to the formation of SL4.

3.5. Discussion
We demonstrated that SL4 induced both transcription termination and inhibition of translation. We assume that the formation of SL4 depends on the presence of copRNA, and in the absence of copRNA, a different structure is formed to release SD to facilitate the translation. Sequence analysis of repK mRNA suggests that a large stem-loop (L-SL) could be formed in the absence of copRNA (Fig. 5). Since L-SL contains half of SL4, SD would be released. The regulatory circuit of the synthesis of RepK is predicted from these results and is shown in Fig. 5.

SL1 is important for the control of copy number and incompatibility since the plasmid cop3, which has a base change at C575 to T in SL1, shows a high copy number and weak incompatibility (our observation). repK mRNA and copRNA would initiate interaction at the loop of SL1. Without interaction of copRNA, mRNA will form L-SL and release SD to facilitate translation. When copRNA hybridizes to mRNA, SL4 will be induced and sequester SD, and the sequesteration of SD would inhibit translation.

SL4 also functions as a terminator, however, the mechanism by which SL4 terminates transcription is unclear. In the rho-independent transcription terminator, the stem-loop is followed by an AUU sequence. It has been reported that RNA polymerase pauses at the AUU sequence and detaches from DNA. Since pKYM replicates normally in rho null strain, SL4 would be a rho-independent terminator (our observation). SL4 is followed by an AU-rich sequence as shown in Fig. 1. RNA polymerase would be released from DNA at this region.

We showed in this paper that the countertranscript of repK mRNA induced both termination of transcription and inhibition of translation and that the synthesis of RepK was additionally regulated by RepK itself with HU. Once RepK was synthesized, it would bind to the replication origin and HU would cover the repK promoter to prevent the production of excess amount of RepK. Since an excess of RepK is deleterious to the host cell, repression of RepK expression is important for the maintenance of pKYM.

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Figure 5. Schematic model of negative feedback circuit to repress expression of RepK. The conformations of repK mRNA with or without interaction of copRNA are shown. Without the interaction of copRNA, RepK protein is expressed and binds to the origin region. HU then binds to the repK promoter to repress the initiation of transcription. The repK SD is sequestered by base pairing in SL4 when mRNA interacts with copRNA. SL4 not only inhibits translation but terminates transcription (see text). The altered nucleotide which is identified in SL1 of cop3 is shown. PR indicates the repK promoter and PL indicates the copRNA promoter.

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