tRNA-dependent cleavage of the ColE1 plasmid-encoded RNA I

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Effects of tRNA^{Ala}(UGC) and its derivative devoid of the 3'-ACCA motif [tRNA^{Ala}(UGC)ΔACCA] on the cleavage of the ColE1-like plasmid-derived RNA I were analysed *in vivo* and *in vitro*. In an amino-acid-starved *relA* mutant, in which uncharged tRNAs occur in large amounts, three products of specific cleavage of RNA I were observed, in contrast to an otherwise isogenic *relA*⁺ host. Overexpression of tRNA^{Ala}(UGC), which under such conditions occurs in *Escherichia coli* mostly in an uncharged form, induced RNA I cleavage and resulted in an increase in ColE1-like plasmid DNA copy number. Such effects were not observed during overexpression of the 3'-ACCA-truncated tRNA^{Ala}(UGC). Moreover, tRNA^{Ala}(UGC), but not tRNA^{Ala}(UGC)ΔACCA, caused RNA I cleavage *in vitro* in the presence of MgCl₂. These results strongly suggest that tRNA-dependent RNA I cleavage occurs in ColE1-like plasmid-bearing *E. coli*, and demonstrate that tRNA^{Ala}(UGC) participates in specific degradation of RNA I *in vivo* and *in vitro*. This reaction is dependent on the presence of the 3'-ACCA motif of tRNA^{Ala}(UGC).

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INTRODUCTION

ColE1-like plasmids use only enzymes encoded by the host, *Escherichia coli*, for their replication (Cesareni *et al.*, 1991; Sharpe *et al.*, 1999; Chatwin & Summers, 2001; Kim *et al.*, 2005a). This replication is initiated by a transcript, called RNA II, which forms a persistent hybrid with its template DNA and acts as a pre-primer RNA. This process is negatively regulated by RNA I, an antisense RNA, which is complementary to the 5'-end of RNA II. RNA I can bind to the RNA II to prevent the formation of the persistent hybrid of RNA II and template DNA (Tomizawa, 1990; Kues & Stahl, 1989). Therefore, RNA I level is a key element in the control of ColE1 plasmid copy number (Wong & Polisky, 1985; Polisky, 1988; Wang *et al.*, 2004). It appears that regulation of RNA I decay during bacterial growth is important for the control of ColE1 plasmid DNA replication (Wagner & Simons, 1994).

Four cellular factors that regulate RNA I decay or cleavage through endonucleolytic or exonucleolytic activity have been reported: (1) RNase E was found to have an endonucleolytic activity in RNA I decay (Lin-Chao & Cohen, 1991); (2) polynucleotide phosphorylase has been identified as one of the exonucleases implicated in RNA I

decay (Xu & Cohen, 1995); (3) a role of RNase III in RNA I decay was discovered (Binnie *et al.*, 1999); and (4) poly(A) polymerase I has been demonstrated to have a role in the regulation of ColE1-like plasmid DNA copy number and RNA I decay (Lopilato *et al.*, 1986; He *et al.*, 1993; Xu *et al.*, 1993; Jasiecki & Węgrzyn, 2003, 2006).

It has been suggested that uncharged tRNA can interact with RNA I to regulate ColE1 plasmid replication (Wróbel & Węgrzyn, 1998; Węgrzyn, 1999; Wang *et al.*, 2002, 2004). It was speculated that tRNA–RNA I interactions (and possibly also tRNA–RNA II interactions) may prevent RNA I–RNA II hybridization, thus allowing more efficient formation of the pre-primer RNA and initiation of plasmid DNA replication (see Węgrzyn, 1999, for a discussion). This regulation may be of special importance under conditions of amino acid starvation, when large amounts of uncharged tRNAs appear in bacterial cells.

In amino-acid-starved wild-type bacteria, the appearance of large amounts of specific nucleotides, guanosine pentaphosphate (pppGpp) and guanosine tetraphosphate (ppGpp) causes a strong inhibition of synthesis of stable RNAs, i.e. rRNA and tRNA (Cashel *et al.*, 1996). However, in relaxed (*relA*) mutants, (p)ppGpp synthesis is impaired during

amino acid starvation, which leads, among other things, to accumulation of uncharged tRNAs. Various tRNAs have different levels of homology to RNA I and RNA II; thus it was proposed (and supported experimentally) that starvation of *relA* mutants for different amino acids results in amplification of ColE1-like plasmids to various extents (Wróbel & Węgrzyn, 1997, 1998). The tRNA interference model in the control of replication of ColE1-like plasmids, based on the interaction of the 3'-CCA sequence of uncharged tRNAs with RNA I, has also been proposed (Wang *et al.*, 2002, 2004). Nevertheless, the mechanism(s) of interaction between uncharged tRNAs and RNA I remain(s) largely unknown.

Here, we aimed to investigate tRNA–RNA I interactions in more detail, and demonstrated tRNA-dependent RNA I cleavage. We selected tRNA^{Ala}(UGC) as a model tRNA molecule, and analysed effects of this tRNA on RNA I cleavage *in vivo* and *in vitro*. The results presented show that uncharged tRNA^{Ala}(UGC) can induce RNA I decay both *in vivo* and *in vitro* and that this reaction can play an important regulatory role in the control of ColE1 plasmid DNA replication in *E. coli* cells, especially during amino acid starvation.

METHODS

Construction of plasmids pCW, pCW1 and pCW2. ColE1 plasmid DNA (GenBank no. NC_001371) was used for plasmid

construction as depicted in Fig. 1. Primers P1F (5'-GCA ATC CAA ATG GGA TTG CTA GGA-3') and P1R (5'-CAT CGG TAT CAT TAC CCC ATG AAC-3') were used to amplify the replication origin of ColE1 by PCR with AccuPrime Pfx DNA polymerase (Invitrogen). Another PCR reaction was used to amplify an ampicillin-resistance gene (*bla*) from pUC18 (Invitrogen) with the forward primer 5'-GAG TAA ACT TGG TCT GAC AGT-3' and reverse primer 5'-GGT TAA TGT CAT GAT AAT AAT-3'. Then, the PCR product of the ColE1 replication origin was ligated with the 5'-phosphorylated, blunt-ended ampicillin-resistance gene (*bla*) to construct plasmid pOri1 (this construct was verified by DNA sequencing). To amplify the whole pOri1 plasmid by PCR, primers P2F (5'-TAA TTA AGC TCA TAA ATT AAA CCT CGC CAT ATA-3') and P2R (5'-GAT ACT ACA TAA AAA ATA TTT TAT TTG GCC-3') (which includes the TAA stop codon) were employed. The PCR product was ligated with a 5'-phosphorylated DNA fragment, encompassing the ϕ 10 promoter of bacteriophage T7 (further referred to as the T7 promoter, and obtained by hybridization of two deoxyoligonucleotides: 5'-TA ATA CGA CTC ACT ATA GGG AGA and 5'-TCT CCC TAT AGT GAG TCG TAT TA-3'; the bold G corresponds to the start of the RNA), to construct plasmid pCW.

To construct plasmids pCW1 [bearing a gene encoding tRNA^{Ala}(UGC)] and pCW2 [bearing a gene encoding tRNA^{Ala}(UGC) Δ ACCA] the T7 promoter-fused DNA fragments encoding appropriate tRNAs were phosphorylated at the 5' terminus and ligated with the PCR product of pOri1 DNA. In order to produce the tRNA^{Ala}(UGC) with and without the 3'-ACCA sequence, DNA regions encoding tRNA^{Ala}(UGC) and tRNA^{Ala}(UGC) Δ ACCA were fused in-frame with a truncated T7 promoter 5'-TA ATA CGA CTC ACT ATA-3', to construct plasmids pCW1 and pCW2, respectively. These plasmids were verified by DNA sequencing.

Fig. 1. Construction of tRNA^{Ala}(UGC)-producing plasmids. See Methods for detailed description. Open arrows indicate primers used for PCR reactions performed for cloning purposes.

Bacterial strains and growth conditions. To analyse the effects of amino acid starvation, *E. coli* K-12 strain CP78 (*leu arg thr his thi*), referred to as *relA*⁺, and its otherwise isogenic *relA2* derivative, CP79 (Fiil & Friesen, 1968), were employed. Cultivations were performed in M9-glucose minimal medium supplemented with necessary amino acids [the medium composition was as follows: 12.8 g Na₂HPO₄·7H₂O l⁻¹; 3.1 g KH₂PO₄ l⁻¹; 0.5 g NaCl l⁻¹; 1.0 g NH₄Cl l⁻¹; 0.5 g MgSO₄·7H₂O l⁻¹; 4.0 g glucose l⁻¹ plus required amino acids (L-leucine, L-histidine, L-arginine, and L-threonine, 50 mg l⁻¹ each) and 50 µg ampicillin ml⁻¹]. Amino acid starvation was achieved by removal of all amino acids from the medium. Briefly, the bacterial culture was centrifuged, and the pellet was washed twice with an equal volume of 0.9% NaCl and resuspended in M9-glucose minimal medium lacking amino acids, and cultured for another 2 h. All cultivations were performed in a 5 l BIOSTAT B-DCU fermenter (Sartorius BBI Systems).

In other *in vivo* experiments, the *E. coli* strain BL21(DE3) (Invitrogen), bearing the T7 RNA polymerase gene under control of an IPTG-inducible promoter, was used. A 1 ml inoculum (2.5 × 10⁸ cells) from a single colony harbouring a plasmid was added to 100 ml LB medium supplemented with ampicillin in a flask and incubated at 37 °C for 24 h. The resulting culture was inoculated into 2 l LB containing 50 µg ampicillin ml⁻¹ in a fermenter. The cultivation was performed at controlled temperature (37 °C), pH (7.0), and dissolved oxygen tension (30 %). *E. coli* cultures were induced with 1 mM IPTG at OD₆₀₀ 0.4, and samples were withdrawn at the indicated times.

Determination of RNA I level and charging ratio of tRNA^{Ala}(UGC) and tRNA^{Ala}(UGC)ΔACCA *in vivo*. RNA I concentration was determined by a Northern blotting method. *E. coli* cells bearing pCW, pCW1 or pCW2, and grown in a fermenter, were poured into 10% (w/v) trichloroacetic acid at 37 °C. Total RNA was separated electrophoretically in 15% polyacrylamide gels containing 7 M urea and transferred to Hybond-N nylon membranes (Amersham). A ³²P-labelled complementary deoxyoligonucleotide, corresponding to the full-length RNA I, was used to detect RNA I by Northern blotting, performed as described by Sambrook *et al.* (1989).

The charging ratio of tRNA^{Ala}(UGC) and tRNA^{Ala}(UGC)ΔACCA was determined as described by Sorensen (2001). Briefly, tRNAs were purified as described previously (Cayama *et al.*, 2000; Sorensen *et al.*, 2005) and separated electrophoretically in 15% polyacrylamide gels containing 7 M urea. Following RNA transfer to Hybond-N nylon membranes (Amersham), the charging level of tRNA^{Ala}(UGC) or tRNA^{Ala}(UGC)ΔACCA was analysed by hybridization with the ³²P-labelled probe 5'-GCG TGC AAA GCA GGC GCT CTC CCA GCT-3'. Membranes used for detection were washed for 30 min at 60 °C to remove unspecific cross-hybridization. The radioactivity present in specific bands was estimated using a phosphorimager scanner.

Real-time PCR analysis of plasmid DNA copy number. *E. coli* cells (10⁹) were harvested and resuspended in 250 µl TGE buffer (1 M Tris/HCl, pH 8.0, 50 mM glucose, 0.5 M EDTA). Bacteria were lysed with 250 µl SDS-NaOH lysis buffer (0.2 M NaOH, 1% SDS), and then 350 µl 3 M potassium acetate solution (adjusted to pH 4.8 with acetic acid) was added to the lysates. Following incubation on ice for 10 min, the suspensions were centrifuged at 12 000 g for 10 min. Supernatants were used for real-time PCR analysis with the QuantiTect SYBR Green PCR kit (Qiagen). Primers 5'-ATG AGT ATT CAA CAT TTC CGT GTC-3' and 5'-CTT CCG GCT GGC TGG TTT ATT GCT-3' were used to amplify the ampicillin-resistance gene of pCW, pCW1 or pCW2. The PCRs were performed with the iQ5 real-time PCR detection system (Bio-Rad). Serial dilutions of pOri1 plasmid DNA solutions (from 1 mg ml⁻¹ to 10⁻⁵ mg ml⁻¹) were used as standards for determination of plasmid DNA concentration in cellular lysates. Plasmid copy number

(PCN) was determined using the following equation: $PCN = 6.02 \times 10^{23} \times (C \times V) / (N \times M \times 2 \times BP)$, where *C* is the plasmid DNA concentration in the lysate, *V* is the volume of cleared lysate, *N* is total number of *E. coli* cells used, *M* is the formula 'molecular' weight of plasmid DNA (determined as described by Wang *et al.*, 2001) and *BP* is the number of base pairs in plasmid DNA.

Preparation of tRNA^{Ala}(UGC) and tRNA^{Ala}(UGC)ΔACCA.

Genomic DNA was prepared from *E. coli* K-12 (GenBank no. NC_000913) using the Qiagen genomic DNA preparation kit. The DNA fragment encoding tRNA^{Ala}(UGC) was amplified by PCR with the following primers: 5'-GGG GCT ATA GCT CAG CTG GGA GAG-3' and 5'-TGG TGG AGC TAT GCG GGA TCG AAC-3'. The PCR product was cloned into pCR2.1 vector (Invitrogen), using the TOPO TA cloning kit (Invitrogen), to construct plasmid pCR2.1tRNA. One of the clones was selected and sequenced. Then pCR2.1tRNA was digested with *Eco*RI, and the DNA fragment encoding tRNA^{Ala}(UGC) was purified with the Qiagen gel purification kit. The T7 promoter was fused with the DNA fragment encoding tRNA^{Ala}(UGC) in a PCR with forward primer 5'-TA ATA CGA CTC ACT ATA GGG GCT ATA GCT CAG CTG GGA GAG-3' (containing the sequence of this promoter; the bold G corresponds to the start of the RNA) and reverse primer 5'-TGG TGG AGC TAT GCG GGA TCG AAC-3'. Analogously, the T7 promoter was fused to the PCR product to construct the DNA fragment encoding tRNA^{Ala}(UGC)ΔACCA (primers 5'-TA ATA CGA CTC ACT ATA GGG GCT ATA GCT CAG CTG GGA GAG-3' and 5'-GG AGC TAT GCG GGA TCG AAC-3' were used for PCR). T7 promoter-fused DNA fragments encoding tRNA^{Ala}(UGC) and tRNA^{Ala}(UGC)ΔACCA were transcribed using the Ambion MEGAscript T7 kit. The final tRNA^{Ala}(UGC) and tRNA^{Ala}(UGC)ΔACCA products were purified with the Ambion MEGAclear kit.

***In vitro* preparation of ³²P-labelled RNA I.** The T7 promoter was fused with a DNA fragment encoding RNA I using PCR with primers 5'-TA ATA CGA CTC ACT ATA ACA GTA TTT GGT ATC TGC GCT CTG-3' and 5'-ACA AAA AAA CCA CCG CTA CCA-3' and ColE1 plasmid DNA as a template. The PCR product was used for preparation of RNA I *in vitro* by a transcription reaction with the Ambion MEGAscript T7 kit and incorporating [³²P]UTP. The RNA I product was purified using the Ambion MEGAclear kit.

Cleavage of RNA I *in vitro*. To analyse the cleavage of RNA I, 2 µg tRNA^{Ala}(UGC) or tRNA^{Ala}(UGC)ΔACCA was mixed with 2 µg ³²P-labelled RNA I in 20 µl of the PBS buffer system containing 50 mM MgCl₂. Following incubation at 37 °C for the indicated times and polyacrylamide gel electrophoresis with 7 M urea, RNA I decay was analysed by densitometric scanning of the exposed, ³²P-labelled RNA I bands on the autoradiograms.

Mapping of the cleavage sites. Following incubation of RNA I with tRNA^{Ala}(UGC) or tRNA^{Ala}(UGC)ΔACCA, the RNA I fragments were separated electrophoretically in 15% polyacrylamide gels containing 7 M urea. RNA I fragments were extracted from the gels with GeBAflex-tube (Gene Bio-Application). The purified RNA I fragments were reverse transcribed with either reverse transcription primer Seq1R (5'-ACC AAC GGT GGT TTG TTT GCC-3') or Seq2R (5'-ACA AAA AAA CCA CCG-3'). Then RNA fragments were removed from the cDNA with RNase H, and the 3' end of the cDNA was tailed using dGTP and terminal transferase (Biolab Inc.). Primer Seq2F, (C)₁₂, was used to synthesize the second strand of cDNA with *Taq* DNA polymerase. The double-stranded cDNA was amplified by PCR using primers Seq2R and Seq2F with AccuPrime *Taq* DNA polymerase, and the PCR products were cloned into pCR2.1 TOPO vector (Invitrogen) and sequenced.

RESULTS

Cleavage of RNA I in amino-acid-starved *E. coli* cells

It has been reported that in relaxed (*relA*) *E. coli* mutants, during amino acid starvation, the concentration of uncharged tRNAs is significantly increased; however, in stringent (wild-type, *relA*⁺) cells the level of uncharged tRNAs is tightly controlled (Sorensen, 2001). We found that in amino-acid-starved *relA2* bacteria bearing a ColE1-like plasmid (CP79/pOri1), the RNA I molecules were cleaved efficiently (Fig. 2a, c), in contrast to the otherwise isogenic *relA*⁺ cells (Fig. 2b, d). A possible hypothesis to explain these results was that uncharged tRNAs might have a role in the RNA I cleavage. Therefore, in subsequent experiments, we aimed to determine whether overexpression of uncharged tRNA can induce RNA I decay, and – if so – which tRNA motif can induce the RNA I cleavage *in vivo* and *in vitro*.

Overproduction of tRNA^{Ala}(UGC) and tRNA^{Ala}(UGC)ΔACCA in *E. coli*

E. coli cells bearing plasmid pCW (vector), pCW1 [over-expressing a gene for tRNA^{Ala}(UGC)] or pCW2 [over-expressing a gene for 3'-truncated tRNA^{Ala}(UGC)ΔACCA] were cultured in a fermenter in LB medium. Total RNA was isolated, tRNAs were purified and tRNA^{Ala}(UGC) was analysed quantitatively. No accumulation of tRNA^{Ala}(UGC) was found in cells harbouring the pCW vector (Fig. 3a, d). However, tRNA^{Ala}(UGC) was produced at a high rate after IPTG induction of pCW1-bearing bacteria (Fig. 3b, e). Under these conditions, most of the tRNA^{Ala}(UGC) molecules were uncharged (Fig. 3b, e). Similarly, tRNA^{Ala}(UGC)ΔACCA was produced at a high rate after cells bearing pCW2 were induced with IPTG (Fig. 3c, f) and, as expected, the overproduced tRNA^{Ala}(UGC)ΔACCA molecules were uncharged (Fig. 3c, f).

Effects of tRNA^{Ala}(UGC) and tRNA^{Ala}(UGC)ΔACCA accumulation on RNA I decay

We examined effects of tRNA^{Ala}(UGC) and tRNA^{Ala}(UGC)ΔACCA overproduction on the RNA I decay *in vivo*. RNA I was rapidly degraded in cells overproducing tRNA^{Ala}(UGC) (Fig. 4b, e). However, such a rapid decay was not observed in the tRNA^{Ala}(UGC)ΔACCA-overproducing strain (Fig. 4c, f). In control experiments with the pCW vector, no increase in the rate of RNA I decay was found after IPTG induction (Fig. 4a, d). These results identified tRNA^{Ala}(UGC) as a cellular factor involved in RNA I degradation, and revealed that the 3'-terminal ACCA motif of tRNA is necessary for this reaction.

Fig. 2. Cleavage of RNA I in amino-acid-starved bacteria. *E. coli relA2* (a, c) and *relA*⁺ (b, d) cells bearing a ColE1-like plasmid (pOri1) were investigated. Amino acid starvation was initiated at time 0 as described in Methods. The RNA I level was analysed by Northern blotting. The data in (a) and (b) represent means $\pm$ SD from three experiments. A relative RNA I level of 1 corresponds to the value measured at time 0. (c, d) Examples of Northern blots. RNA I degradation products are marked as I, II and III.

Fig. 3. Charging levels of overproduced tRNAs. Northern-blot analysis of charged and uncharged tRNA^{Ala}(UGC) concentrations during cultivation of *E. coli* bearing plasmid pCW (vector; a), pCW1 [bearing a gene encoding tRNA^{Ala}(UGC); b] or pCW2 [bearing a gene encoding tRNA^{Ala}(UGC) Δ ACCA; c]. Cultures were induced with IPTG at OD₆₀₀ 0.4. Samples were withdrawn at the indicated times to determine concentrations of charged (black columns) and uncharged (open columns) tRNA. Results are expressed as the ratios at the indicated times of uncharged tRNA levels after and before IPTG induction, and the relative ratios of charged tRNA levels after and before IPTG induction. The data in (a–c) are means $\pm$ SD from three experiments. (d–f) Representative results of Northern blotting experiments with probes designed to detect charged and uncharged tRNA^{Ala}(UGC) in cells bearing pCW (d), pCW1 (e) and pCW2 (f).

Effects of increased levels of tRNA^{Ala}(UGC) and tRNA^{Ala}(UGC) Δ ACCA on ColE1-like plasmid DNA copy number

Since RNA I negatively regulates replication of ColE1-like plasmids, we investigated effects of overproduction of tRNA^{Ala}(UGC) or tRNA^{Ala}(UGC) Δ ACCA on pCW, pCW1 and pCW2 plasmid copy number in *E. coli*. We found that the copy number of pCW1 [bearing a tRNA^{Ala}(UGC) gene] was significantly increased after IPTG induction, in contrast to pCW2 [bearing a gene for tRNA^{Ala}(UGC) Δ ACCA] and pCW vector (Fig. 5). These results are compatible with measurement of RNA I decay during overproduction of tRNA^{Ala}(UGC) and tRNA^{Ala}(UGC) Δ ACCA.

Effects of tRNA^{Ala}(UGC) and tRNA^{Ala}(UGC) Δ ACCA on the RNA I decay *in vitro*

In order to analyse whether the effect of tRNA^{Ala}(UGC) on RNA I degradation is direct, we investigated RNA I decay *in vitro* in the presence of this tRNA and its 3'-truncated Δ ACCA derivative. tRNA^{Ala}(UGC) or tRNA^{Ala}(UGC) Δ ACCA was mixed with RNA I in PBS buffer containing 50 mM MgCl₂ or devoid of this salt. RNA I was degraded rapidly ($t_{1/2}$ = 5 min) in the presence of tRNA^{Ala}(UGC) in PBS buffer containing 50 mM MgCl₂, and degradation products could be observed (Fig. 6a, c). However, in the presence of tRNA^{Ala}(UGC) Δ ACCA, RNA I was as stable as it was with no additional transcripts (Fig. 6b, e, f). The efficiency of tRNA^{Ala}(UGC)-dependent RNA I decay was decreased dramatically in the absence of MgCl₂ (Fig. 6a, d). Moreover, RNA I was stable in the presence of MgCl₂ and absence of tRNA (Fig. 6g).

As shown in Fig. 6(c), in the presence of tRNA^{Ala}(UGC), RNA I was cleaved to form three different fragments, suggesting the presence of at least two cleavage sites in the RNA I molecule. The RNA I fragments were collected from the gel after electrophoresis (performed as shown in Fig. 6c), reverse transcribed into cDNA, and converted into double-stranded DNA. DNAs were amplified by PCR reactions, and PCR products were cloned into the pCR2.1 vector and sequenced. The results of DNA sequencing revealed that the first cleavage site was located between U(60) and A(61), and the second cleavage site was located between U(93) and A(94) (Fig. 7a, b). The location of the cleavage sites on the secondary structure of tRNA^{Ala}(UGC) is presented in Fig. 7(c).

DISCUSSION

In bacterial cells, tRNA represents up to 20% of the total RNA (Dittmar *et al.*, 2004). Although the basic features of tRNA were discovered almost 50 years ago (Hoagland *et al.*, 1958), unexpected functions of this RNA were later demonstrated, including those connected with DNA replication. For example, tRNA may act as a primer in DNA synthesis (Marquet *et al.*, 1995), and a role for tRNA in the regulation of ColE1 plasmid DNA replication in amino-acid-starved *E. coli* has been proposed (Wróbel & Wegrzyn, 1997, 1998; Wegrzyn, 1999; Wang *et al.*, 2002). Recently, it was also demonstrated that tRNA can be used as a kind of T helper 1 immune adjuvant (Wang *et al.*, 2006).

Here, we demonstrate that tRNA can be involved in the cleavage of the ColE1 plasmid-encoded regulatory

Fig. 4. Stability of RNA I in tRNA-overproducing cells. Relative RNA I levels in *E. coli* bearing plasmid pCW (vector; a), pCW1 [bearing a gene encoding tRNA^{Ala}(UGC); b] or pCW2 [bearing a gene encoding tRNA^{Ala}(UGC) Δ ACCA; c]. Relative RNA I levels were determined as the ratio of RNA I amount after and before IPTG induction. Samples of the cultures were withdrawn at the indicated times and relative RNA I levels were determined by Northern blotting. The data in (a–c) are means $\pm$ SD from three experiments. (d–f) Representative results of Northern blotting experiments for cells bearing pCW (d), pCW1 (e), and pCW2 (f).

transcript, RNA I, both *in vivo* and *in vitro*. Physiological significance of this process has been demonstrated by showing effects of increased levels of uncharged tRNA^{Ala}(UGC), a model tRNA used in this study, on the regulation of ColE1-like plasmid copy number in *E. coli*

(Fig. 4). It is worth noting that greatly increased levels of uncharged tRNAs occur under some physiological conditions, particularly during amino acid starvation, and especially during the relaxed response to this kind of starvation (Chatterji & Ojha, 2001).

Fig. 5. ColE1-like plasmid copy number in tRNA-overproducing cells. Copy number of plasmids pCW (vector; black columns), pCW1 [bearing a gene encoding tRNA^{Ala}(UGC); open columns] or pCW2 [bearing a gene encoding tRNA^{Ala}(UGC) Δ ACCA; hatched columns] after IPTG induction of *E. coli* cultures. Cultures were induced with IPTG at OD₆₀₀ 0.4. Samples were withdrawn at the indicated times to determine plasmid copy number. Plasmid DNA was digested with *Sca*I, then analysed by electrophoresis in 0.8% agarose gel. (a) Means $\pm$ SD from three experiments. (b–d) Typical results for pCW, pCW1 and pCW2, respectively.

Fig. 6. tRNA-dependent RNA I cleavage *in vitro*. RNA I cleavage *in vitro* in the presence of tRNA^{Ala}(UGC) (a, c, d) or tRNA^{Ala}(UGC)ΔACCA (b, e, f) was tested. Samples were withdrawn at indicated times after addition of either tRNA^{Ala}(UGC) or tRNA^{Ala}(UGC)ΔACCA to RNA I, and relative RNA I levels were determined. Mg²⁺ ions (final concentration 50 mM) were either present (■) or absent (○) in the reaction mixture. Results in (a–c) are means ± SD from three experiments. (c–g) Representative Northern-blotting results: (c) RNA I, tRNA^{Ala}(UGC) and Mg²⁺; (d) RNA I, tRNA^{Ala}(UGC) and no Mg²⁺; (e) RNA I, tRNA^{Ala}(UGC)ΔACCA and Mg²⁺; (f) RNA I, tRNA^{Ala}(UGC)ΔACCA and no Mg²⁺; (g) RNA I and Mg²⁺.

Specific sites of the RNA I cleavage were determined (Fig. 7c). Since the cleavage sites are located in RNA I loops I and II, and these loops are involved in formation of the kissing complex at the first stage of RNA I–RNA II interactions (Kues & Stahl, 1989; Tomizawa, 1990), it is likely that disruption of these loops prevents the antisense activity of RNA I. This should result in an increased frequency of ColE1 plasmid replication initiation, and an increased plasmid copy number, which is what was actually observed in tRNA^{Ala}(UGC)-overproducing cells (Fig. 5).

tRNA^{Ala}(UGC)-mediated cleavage of RNA I requires the 3'-terminal ACCA sequence and Mg²⁺ ions. The former factor is discussed in more detail below. Regarding Mg²⁺ ions, it was found previously that they have a special role in stabilizing the native tertiary structure and favouring the folding reaction of many RNAs (Fedor, 2002; Misra & Draper, 2002; Fedor & Williamson, 2005). Therefore, a similar function of Mg²⁺ ions may be necessary for stabilization of either tRNA^{Ala}(UGC) or a complex of this molecule with RNA I.

The question remains what the mechanism of tRNA^{Ala}(UGC)-dependent cleavage of RNA I is. Since this reaction occurs in a mixture composed only of a buffer (including Mg²⁺ ions) and RNAs, it is clear that an RNA must be the catalytic agent. The original demonstration that RNA can act as an enzyme came from studies in which it was demonstrated that an intron can excise itself from pre-RNA and the flanking exons are joined to the mature RNA independent of proteins and additional energy (Kruger *et al.*, 1982). Subsequent experiments showed that the RNA component of RNase P from *E. coli* is able to process its substrate, a pre-tRNA, in the absence of its protein subunit (Guerrier-Takada *et al.*, 1983).

Currently it is generally accepted that RNA-mediated catalytic processes are widespread in nature, although this does not concern tRNAs. In many cases, RNA-mediated RNA cleavage also requires the assistance of specific proteins. Examples are the activities of siRNA and microRNAs (Zhang *et al.*, 2002; Doi *et al.*, 2003; Zeng *et al.*, 2003; Dorsett & Tuschli, 2004; Lee *et al.*, 2004; Shen &

Fig. 7. Mapping of the RNA I cleavage sites. cDNAs obtained after reverse transcription of products of RNA I cleavage and addition of poly(G) were cloned into the pCR2.1 vector, and sequenced with M13 forward sequence primer (Invitrogen) as described in Methods. Primers used for reverse transcription and cloning are indicated by arrows, and cloned sequences are shown by bars. In (a), the last nucleotide (T) before the poly(G) sequence (added during the cloning of DNA sequences corresponding to RNA fragments into the vector) is complementary to the A(94) residue in RNA I, which allows the first cleavage site between U(93) and A(94) to be determined. In (b), the last nucleotide (T) before the poly(G) sequence (added during the cloning of RNA fragments into the vector) is complementary to the A(61) residue in RNA I, which allows determination of the second cleavage site between U(60) and A(61). In (c), the secondary structure of tRNA^{Ala}(UGC), with the cleavage sites (arrows) is shown.

Goodman, 2004; Yekta *et al.*, 2004; Kim *et al.*, 2005b; Behlke, 2006; Ronemus *et al.*, 2006; Valencia-Sanchez *et al.*, 2006). Nevertheless, other reports have described reactions of direct RNA-mediated cleavage of RNA (Tanner, 1999; Lilley, 1999; Doudna & Cech, 2002; Sago *et al.*, 2004). These reactions include self-cleaving of RNA [for example:

hammerhead motif ribozyme (Birikh *et al.*, 1997); hairpin motif ribozyme (Fedor, 2000); hepatitis delta virus ribozyme (Been & Wickham, 1997); varkud satellite (VS) ribozyme (Collins, 2002)], and self-splicing RNAs [for example: group I introns (Schmidt *et al.*, 1992) and group II introns (Jacquier, 1996)].

Fig. 8. A putative mechanism for tRNA-mediated RNA I cleavage, assuming the crucial role of the 3'-ACCA motif. See text for details.

There are two possible mechanisms of tRNA^{Ala}(UGC)-dependent RNA I cleavage. First, tRNA^{Ala}(UGC) might act as a ribozyme, directly cleaving RNA I molecules. Second, RNA I might be a self-cleaving nuclease, and the tRNA would be required as a cofactor to activate the RNA I nuclease activity. Nevertheless, considering the requirement of the 3'-terminal ACCA sequence of tRNA^{Ala}(UGC), as well as a previously published model for tRNA interference, in which direct interactions between 3' termini of tRNAs with RNA I were proposed (Wang *et al.*, 2002), the tRNA-mediated direct cleavage of RNA I seems to be more likely than RNA I autocleavage.

Assuming this more likely option, a possible mechanism for tRNA^{Ala}(UGC)-induced RNA I cleavage may be suggested (Fig. 8). Interaction between 3'-ACCA of tRNA and the UGGU sequences in the regions of RNA I loops might be capable of forming a catalytic structure, requiring an involvement of Mg²⁺ ions. Note that the UGGU motifs occur close to both cleavage sites in loop I and loop II of RNA I (Fig. 7c), while they are absent in the non-cleavable loop III and in other single-stranded RNA I regions. Both 2'-OH and 3'-OH groups of the 3'-ACCA motif of tRNA and the 2'-OH group adjacent to the phosphodiester bond of RNA I could be critical for the cleavage reaction, and these groups might be involved in Mg²⁺ binding (Fig. 8). The 3'-ACCA structure of tRNA could then play a key role in the catalysis of the reaction, in addition to binding to RNA I. The UGGU sequences at the loop regions of RNA I might also contribute to the optimal catalytic activity.

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