

Stress-induced tRNA-derived RNAs: a novel class of small RNAs in the primitive eukaryote *Giardia lamblia*

Yan Li, Jun Luo, Hui Zhou, Jian-You Liao, Li-Ming Ma, Yue-Qin Chen and Liang-Hu Qu*

Key Laboratory of Gene Engineering of the Ministry of Education, State Key Laboratory for Biocontrol, Zhongshan University, Guangzhou 510275, P. R. China

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ABSTRACT

Giardia lamblia is an early diverging and evolutionarily successful protozoan as it can enter into a dormant cyst stage from a vegetative trophozoite. During dormant stage, its metabolic rate decreases dramatically. However, to date, the regulatory molecules participating in the initiation and maintenance of this process have not been fully investigated. In this study, we have identified a class of abundant small RNAs named sitRNAs, which are ~46 nucleotides in length and accumulate in *G. lamblia* encysting cultures. Remarkably, they are derived from the 3' portion of fully matured tRNAs by cleavage of the anticodon left arm, with the 3' terminal CCA triplex still connected. During differentiation, only a limited portion of mature tRNAs is cleaved, but this cleavage occurs almost in the entire tRNA family. sitRNAs begin to accumulate as early as 3 h after initiation of encystation and are maintained at a relatively stable level during the whole process, exhibiting an expression peak at around 24 hr. Our studies further show that sitRNAs can be induced by several other stress factors, and in the case of serum deprivation, both tRNAs and sitRNAs degrade rapidly, with the accumulation of tRNA being halved. Our results may provide new insight into a novel mechanism for stressed *G. lamblia* to regulate gene expression globally.

INTRODUCTION

Transfer RNA (tRNA) holds a central place in protein synthesis by interpreting the genetic information into the amino acid sequence (1). To fulfill this role, an inactive tRNA precursor must be extensively processed into the

standard length, which is usually 73–93 nucleotides (nt), and be post-transcriptionally modified (2,3). In eukaryotes, correctly processed tRNA is aminoacylated in the nucleus to form aminoacyl-tRNA, which functions in a proofreading process and facilitates its nuclear export (4,5). Almost all the maturation steps are accomplished in the nucleus in a precisely coordinated manner, with the exception of some base modifications and certain intron splicing of yeast tRNAs in cytoplasm (1,6).

Besides their adaptor function in translation, some pivotal regulatory roles for tRNAs have been recognized in recent years. In some Gram-positive bacteria, uncharged tRNA can regulate gene expression by interacting with the 5' untranslated region of certain mRNAs (7,8). In nutrient-deficient *Escherichia coli*, the increased proportion between uncharged and aminoacylated tRNA induces the accumulation of ppGpp, a global transcriptional regulator, which ultimately down-regulates the synthesis of tRNA and rRNA while up-regulating the expression of many amino acid biosynthetic genes (9). Also, it has long been held that tRNAs move unidirectionally from nucleus to cytoplasm once they are matured and correctly charged (1). However, in *Saccharomyces cerevisiae*, cytoplasmic tRNAs can retrograde into nucleus for proofreading or for regulating tRNA availability according to amino acid availability (10,11). Later studies further demonstrate that this retrograde movement can also occur in mammalian cells in response to nutrient deprivation, and this may contribute to the post-transcriptional regulation of global gene expression (12).

Under normal growth conditions, compared with mRNAs, tRNAs have a relatively long half-life because of their extensive secondary and tertiary structures and temporal associations with aminoacyl-tRNA synthetases, elongation factors and ribosomes (13). However, under certain physiological stresses or when the tRNA molecules are defective, they can be degraded to some degree (13–15). An increasing number of studies have also

*To whom correspondence should be addressed. Tel: +86 20 84112399; Fax: +86 20 84036551; Email: lssqlh@mail.sysu.edu.cn

Correspondence may also be addressed to: lsszh@mail.sysu.edu.cn

Present address:

Jun Luo, Henan Provincial Key Laboratory of Animal Immunology, Henan Academy of Agricultural Sciences, Zhengzhou 450002, P. R. China.

revealed that both in prokaryotes and in eukaryotes, mature tRNAs tend to be cleaved into 'tRNA halves' under starvation or in a developmentally regulated manner, further demonstrating that tRNAs are not stable in an absolute sense (16–18).

Giardia lamblia is a unicellular intestinal protozoan that commonly causes diarrhea and malnutrition (19). It has a compact genome and represents one of the most primitive eukaryotes examined to date (20). *G. lamblia* is also a successful parasite for its ability to survive in diverse and hostile environments by differentiation from a vegetative trophozoite to a dormant cyst, a process known as encystation. *In vivo*, encystation is triggered when trophozoites travel through the lower part of the intestine where nutrition is limited (19). This simple life cycle can be successfully mimicked *in vitro*, providing an excellent model to study basic cell differentiation. Although encystation has been well studied at morphological, cellular and molecular levels, studies concerning the regulatory molecules involved in the initiation and maintenance of this process are rather limited. Further investigation of the mechanisms concerning encystation of *G. lamblia* may shed light on the complicated differentiation processes that higher eukaryotes undergo.

Small non-coding RNAs (sncRNAs) are important regulators participating in almost every process of gene expression in higher eukaryotes (21). To date, combined with experimental and computational approaches, various kinds of sncRNAs have also been identified in *G. lamblia*, including spliceosomal small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs) and RNase P RNA (22–25). Although no microRNAs have been identified in this unicellular eukaryote, telomeric retroposon-related, small, sense and antisense RNAs do exist in trophozoite (26), demonstrating that in this primitive eukaryote, the sncRNA families are as diverse as those in higher eukaryotes. During encystation, although the whole metabolic rate decreases to 10–20% of that found in normal-growing trophozoites (19), previous studies have revealed up-regulation of several encystation-induced protein and/or mRNA, such as *cwp1* and *cwp2* (27). However, knowledge concerning sncRNAs during this process is rather limited. To further investigate sncRNAs participating in the regulation of the encystation of *G. lamblia*, we have identified and characterized large quantities of stably existing small RNAs derived from the cleavage of the tRNA anticodon left arm. As expected, they could also be induced by several stress factors. Their possible functions are further discussed.

MATERIALS AND METHODS

Cell cultivation and stress treatments

Trophozoites of *G. lamblia* (WB clone C6, ATCC no. 50803) were cultured as described previously (28). Briefly, cells were maintained axenically in freshly filter-sterilized TYI-S-33 medium (pH 7.0–7.2) supplemented with 10% newborn calf serum and 0.5 mg/ml bovine bile (Sigma) in 20-ml glass tubes at 37°C. Trophozoites were grown to confluence (66–72 h) and were then harvested by

centrifugation at 4°C after chilling on ice for 20 min to dislodge adherent cells.

For *in vitro* induction of encystation, we took one of the easiest approaches initially described by Kane *et al.* (29). Firstly, trophozoites were grown to confluent monolayers. Then, the spent growth medium and non-adherent cells were discarded after inverting the tubes several times. Subsequently, the attached trophozoites were re-fed with TYI-S-33 medium with increased pH (7.8) and concentration of bovine bile (10 mg/ml) and were induced to encyst at 37°C for up to 90 h. Total encysting cultures were harvested at different time points and were used for RNA extraction.

In serum-deprivation experiments, monolayer trophozoites were re-fed with TYI-S-33 medium without adding serum. Cultures were then incubated at 37°C at indicated times after this replacement. For serum re-supplementation experiments, after being serum deprived for 15 min, the cultures were re-supplemented with 10% serum for continued growth. For cold or heat stress treatments, monolayer trophozoites were shifted to 4 or 42°C and were harvested at indicated times, respectively.

Oligonucleotides

Oligonucleotides were synthesized and purified by Sangon Co. (Shanghai, China). P47: 5'-CGCCAGGGTTTCC CAGTCACGAC-3'; P48: 5'-AGCGGATAACAATTTC ACACAGGA-3'.

Probes for northern blot analysis were listed in Supplementary Table S1.

RNA detection, construction of cDNA library and sequence analysis

Total RNA was isolated separately from cell cultures under different growing conditions by the acid guanidinium thiocyanate/phenol chloroform method (30). RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the integrity of the RNA was assessed using denaturing agarose gel electrophoresis. Twenty-five micrograms of total RNA from trophozoites and 24-h encysting cultures was separated on a 15% denaturing polyacrylamide gel electrophoresis (PAGE) and directly visualized by ethidium bromide staining.

For nuclease analysis, 25 µg total RNA from 24-h encysting cultures was incubated with (DNase I) or RNase A (TaKaRa), according to the manufacturer's instructions. After phenol extraction and ethanol precipitation, the RNAs were re-dissolved in diethyl pyrocarbonate (DEPC)-treated water, and then separated along with the non-treated RNA on 15% PAGE and ethidium bromide stained.

For cDNA library construction, 60 µg total RNA from 24-h encysting cultures was separated on 15% PAGE. RNAs ~46 nt in length were excised and eluted in 0.3 M NaCl at 4°C overnight, followed by ethanol precipitation. Then, a specialized cDNA library was constructed using TaKaRa's Small RNA Cloning Kit (Code: DRR065) with minor modifications. Briefly, after ligation of 5' and 3' adaptors, reverse transcription was carried out, followed

by PCR amplification. Half of the PCR product was separated on 15% denaturing PAGE gel. Then, fragments around 90 nt were excised from the gel after ethidium bromide staining and eluted in 0.3 M NaCl at 4°C overnight, followed by ethanol precipitation. Finally, the purified cDNA was directly cloned into pMD18-T vector (TaKaRa). The cDNA library was screened by colony PCR with P47 and P48 universal primer pair. Only the insert-containing clones were sequenced with an automatic DNA sequencer (ABI Prism 377) using the BigDye terminator cycle sequencing kit (Applied Biosystems). Valid sequences were blasted against the NCBI databases (<http://www.ncbi.nlm.nih.gov/BLAST/>), as well as the *G. lamblia* genomic database (<http://www.giardadb.org/giardadb/>). tRNA secondary structures were folded by tRNAscan-SE Search Server (<http://lowelab.ucsc.edu/tRNAscan-SE/>) (31). Similar experimental and computational procedures were used to analyze the tRNA halves from serum-deprivation cultures.

Northern blot analysis

Northern blot analysis was performed as described previously, with some modifications (32,33). Briefly, 20 µg total RNAs from cell cultures under different growing conditions was fractionated on denaturing 15% PAGE and then electrophoretically transferred onto Hybond-N⁺ membranes (Amersham Biosciences) using the semi-dry blotting apparatus (BioRad), followed by UV light irradiation for 4 min. DNA oligonucleotides complementary to the 3' part of tRNA isoacceptors were 5'-end labeled with [γ -³²P]ATP (Yahui Co.) using T4 Polynucleotide Kinase (TaKaRa). The membranes were pre-hybridized for at least 1 h in hybridization buffer [300 mM NaCl, 50 mM Na₂HPO₄·12H₂O, 7% sodium dodecyl sulfate (SDS), pH 7.0] and then were hybridized overnight at 42°C. After being washed three times with 2× SSPE/0.1% SDS at room temperature, the membranes were exposed to a phosphor screen and visualized by Typhoon 8600 variable mode imager (Amersham Biosciences).

RESULTS

Detection of an abundant encystation-induced ~46 nt RNA species

As a first step to identify sncRNAs specifically expressed in encysting *G. lamblia*, we examined total RNA from trophozoites and encysting cultures by ethidium bromide staining of denaturing gels. Compared with normal-grown trophozoites, a distinct encystation-specific band, ~50 nt relative to the DNA marker, was detected in 24-h encysting cultures (Figure 1a). To confirm if this band was composed of RNA, we used DNase I and RNase A to digest total RNAs from 24-h encysting cultures. The DNase I (RNase free) treatment had no effect on this band, while the RNase A effectively degraded total RNA and this band (Figure 1b), indicating that this band was indeed comprised of RNA. Because DNA migrated slightly faster than RNA samples in our gel, these small RNAs were actually ~46 nt. They differed in length from 20 to 29 nt telomeric retroposon-related small RNAs of *G.*

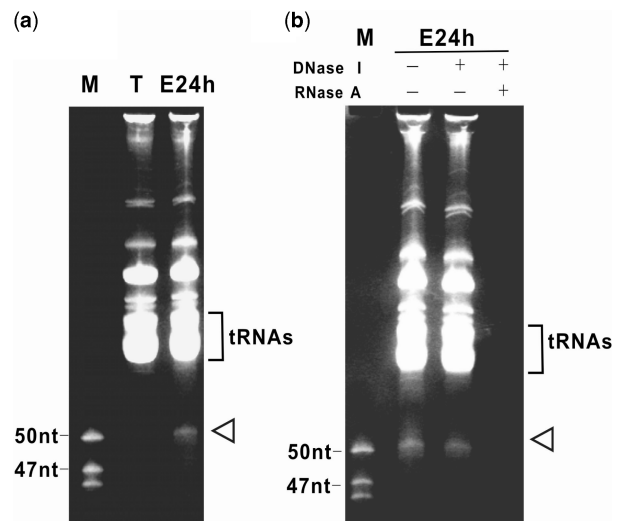


Figure 1. (a) Detection of an encystation-induced ~46 nt RNA species. Equal amounts of total RNA from trophozoites (T) and 24-h encysting cultures (E24h) were separated on 15% PAGE and ethidium bromide stained. A distinct band indicated by an open arrowhead was detected and represents encystation-induced RNA. DNA oligomers were used as markers (M). (b) Nuclease analysis of total RNA from 24-h encysting cultures. Equal amounts of total RNA treated with DNase I and RNase A were separated along with non-treated samples on 15% PAGE and stained with ethidium bromide.

lamblia (26), and any other small RNA families identified to date, including the recently identified tRNA halves that are 30–35 nt (16–18), and therefore represent a totally novel class of small RNAs. They could be directly visualized by ethidium bromide staining, indicating their relative high abundance during differentiation of *G. lamblia*.

A novel tRNA cleavage phenomenon during encystation

To further characterize this novel RNA species, size-fractionated small RNAs were ligated to 3' and 5' adaptors sequentially, and then a specialized cDNA library was constructed and analyzed. We randomly sequenced 188 valid clones. Sequences were blasted against the NCBI databases and the *G. lamblia* genomic database (Materials and Methods). Among these sequences, 11 did not match the genome, suggesting that they may represent unsequenced genomic regions, 4 were antisense to mRNAs of potential proteins and 18 might represent the degradation products of rRNAs, mRNAs and a snoRNA (data not shown). Remarkably, 155 sequences were derived from tRNAs, of which, 150 were from the 3' part (Supplementary Table S2). Because they were induced by encystation, a kind of stress factor, we named them sitRNAs (stress-induced tRNA-derived RNAs).

Sequences analysis indicated that they were between 44 and 49 nt, with a predominant size of 46–47 nt (Figure 2a), coinciding well with the gel-staining result (Figure 1a). They were derived from 18 kinds of tRNA isoacceptors, which were mapped to the *Giardia's* genome. The most frequently cloned sitRNAs were from tRNA^{Asp}, followed by tRNA^{Gly}. Obviously, the cloned sitRNAs do not reflect the relative codon usage in *G. lamblia* (34). Approximately

97% of sitRNAs bore 3' terminal CCA nucleotides. A few exceptions (3%) had only -CC or -C or none of these, which might arise from cleavage during the RNA isolation process due to technique limits (Supplementary Table S2). Nevertheless, it is difficult to rule out the possibility that they might be lost when the tRNA is not re-charged shortly after leaving the ribosome when completing protein synthesis. In addition, some cDNA sequences mismatched with the genomic sequences, possibly because of sequencing errors and/or polymerization errors when reverse transcriptase passed through the modified bases in the process of library construction. Furthermore, none of the sitRNAs contained 5' leader, 3' trailer or intron sequences. All these characteristics demonstrated that sitRNAs were derived from fully matured tRNAs.

Although the cloned sitRNAs from the same tRNA isoacceptor showed a slight heterogeneity at their 5' ends, the majority of the 5' ends (145 clones) were located at various positions of the anticodon left arm in the cloverleaf secondary structure (Figure 2b). The only exception is tRNA^{Leu}_{AAG} (five clones). Unlike other tRNA isoacceptors, it bears an extended variable loop 10 nt in length, so the cleavage sites fell in the anticodon right arm

(data not shown). Accordingly, as reported in *Tetrahymena thermophila*, in contrast to the most cloned tRNA halves, tRNA^{Leu}_{UAA} was also an exception, with the cleavage occurring in the loop of the extended variable stem (16). Our results combined with the work of Lee and Collins (16) indicate that the endonucleases responsible for these cleavages might recognize the length counting from the 3' terminal instead of the secondary structure of the tRNAs.

The global cleavage and stable expression profile of sitRNAs during encystation

It is well known that eukaryotic tRNAs are rich in post-transcriptional modification, which may interfere with the process of reverse transcription *in vitro*. To overcome the potential bias of cloning procedures, we chose one isoacceptor from each of the 20 tRNAs to examine whether this cleavage exhibits specificity for certain kinds of tRNAs. Compared with trophozoites, all sitRNAs tested showed signals around 46 nt in 24-h encysting cultures, irrespective of whether they were cloned (Figure 3, marked by asterisks) or not (Figure 3, without asterisks). This clearly indicates that the cleavage is a global phenomenon for the entire tRNA family. As in the case of tRNA^{Leu}_{AAG}, tRNA^{Ser}_{AGA} also bears an extended variable loop, but its corresponding sitRNAs accumulated at a lower level and were below the detection limit of northern analysis.

In the encystation of *G. lamblia*, the majority of mature tRNAs remained intact, with only a limited portion of them being cleaved into sitRNAs. Noticeably, some sitRNAs showed a strong signal compared with their corresponding mature tRNAs, such as sitRNAs from tRNA^{Gly}_{UCC} and tRNA^{His}_{GUG}, indicating that the cleavage efficiency may vary among different tRNA isoacceptors. It is also plausible that the cleavage products might be more stable than the other sitRNAs.

Encystation is a gradual process and can be divided into three stages: (i) reception of the stimulus and activation of encystation-specific genes, (ii) biogenesis of secretory organelles and preparing cyst wall materials and (iii) assembly

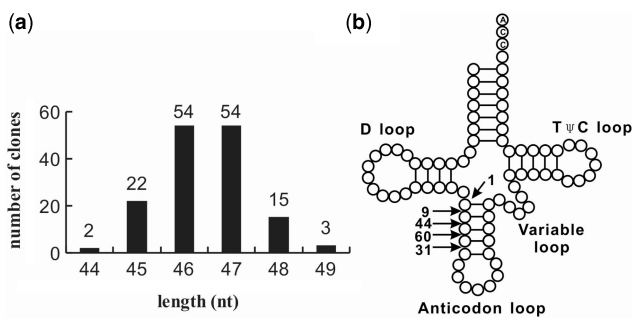


Figure 2. Sequence analysis of sitRNAs. (a) Length distributions of cloned sitRNAs. (b) The 5' cleavage sites of a total of 145 sitRNAs, and number of times cloned are denoted by arrows and numbers, respectively. SitRNAs from tRNA^{Leu}_{AAG} (five clones) were not included, with the cleavage sites located in the anticodon right arm.

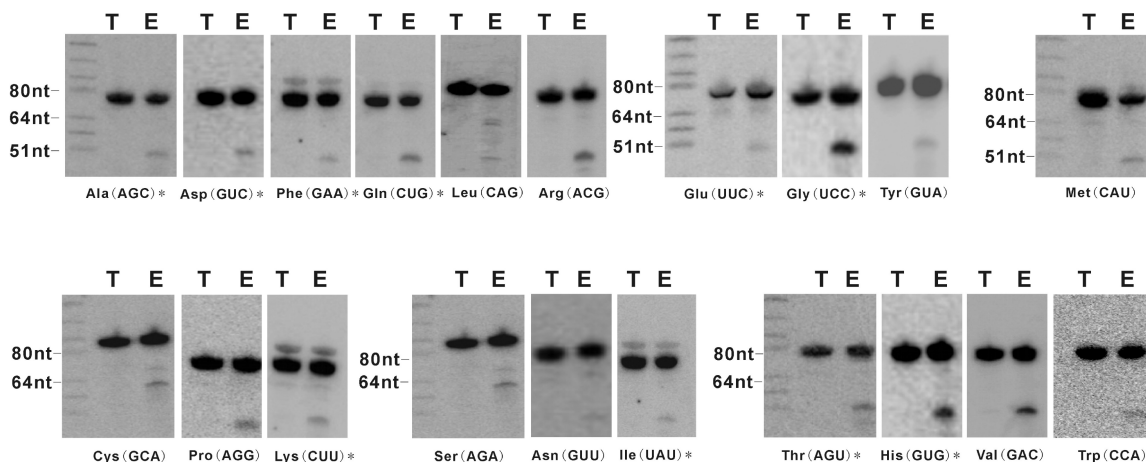


Figure 3. Northern blot analysis of individual sitRNAs. Equal amounts of total RNA from trophozoites (T) and 24-h encysting cultures (E) were used to detect individual sitRNAs cloned in our cDNA library (with asterisks) or not represented in the library (without asterisks). Probes were designed complementary to the 3' part of corresponding tRNAs and are listed in Supplementary data. 5.8S rRNA served as a loading control.

of the extracellular cyst wall (35). Therefore, we examined the sitRNA expression profile at various stages of encystation, ranging from 3 to 90 h after initiation of differentiation, with cyst numbers increasing gradually (Figure 4a). Surprisingly, sitRNAs began to accumulate even at an early stage of differentiation (3 h), when there were no discernible cysts in the culture, and were maintained at a relatively stable level till 90 h, when the cysts almost achieved maximum number, with an expression peak around 24 h (Figure 4b). It is evident that sitRNAs were induced not at a specific encystation period but are universal during the whole differentiation course.

The effects of serum on the accumulation of sitRNAs

Encystation is a kind of stress response when normal-grown trophozoites are exposed to adversity. To investigate whether this cleavage phenomenon is specific to encystation or represents a more universal response to other stresses, we changed the normal growing conditions, which might lower the metabolic rate of trophozoites.

In vivo, a high concentration of nutrients is required for the survival and proliferation of trophozoites, especially those unable to be synthesized themselves. *In vitro*, trophozoites are normally cultured in a rich medium containing serum as a source of nutrients and some vital growth factors, which are indispensable for them to multiply. To examine the effects of serum on the metabolism of tRNAs, we performed serum deprivation and re-supplement experiments.

To our surprise, when confluent monolayer trophozoites were re-fed with TYI-S-33 medium lacking serum, a strong sitRNA band and a faint ~37 nt band representing tRNA half-molecules were immediately visualized by northern blot even 15 min after serum deprivation (Figure 5a). However, when the serum-deprived cultures were re-supplemented with serum and allowed to recover for another 15 and 35 h, sitRNAs and tRNA halves were still visible at 15 h, but disappeared at 35 h (Figure 5b), indicating that the emergence of sitRNAs was reversible.

It is known that trophozoites cannot survive under serum-deprived condition. In our experiments, as

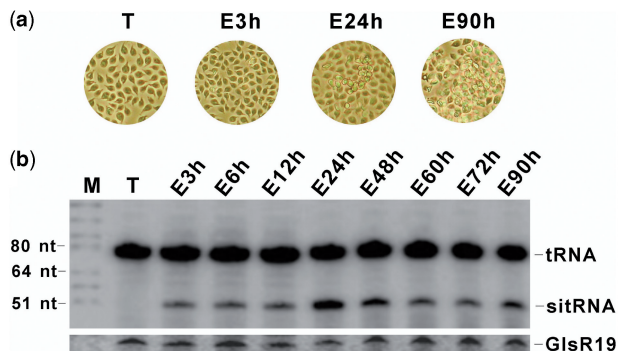


Figure 4. Expression profile of sitRNAs during the whole encystation process. (a) Microscopic observations of trophozoites and encysting cells at indicated times during encystation. Trophozoites are pear shaped, while cysts are oval and refractive. (b) Equal amounts of total RNA from encysting cultures at different time points indicated were blotted and probed with sit-Arg(ACG), sit-Arg(CCU) and sit-Arg(UCU) mixed probes. GlsR19 snoRNA served as a loading control (24).

starvation time prolonged to 1, 2 and 3 h, trophozoites gradually died. Most protein synthesis machineries were shut off, and accordingly, mature tRNAs were no longer needed for mRNA translation. They and sitRNAs began to degrade rapidly (Figure 5c). A cDNA library representing tRNA halves from 3-h non-serum cultures (LS3h) was constructed and analyzed. Careful sequence analysis indicated that all cloned tRNA halves bore no intact 3' CCA ends, and all the cleavage sites were in the anticodon loop region (Figure 5d, Supplementary Table S3). However, all halves were from the 3' part of tRNAs, while no 5' halves were cloned, indicating that the 5' halves may be resistant to the cloning procedures used, or possibly may be unstable in serum-deprived *G. lamblia*. Northern blot analysis using probes specific for the 5' halves did not show any bands corresponding to the 5' halves of tRNAs. Instead, some smaller bands around 30 nt, which might derive from the 5' halves, did exist (data not shown), thus favoring the second possibility.

The effects of non-permissible temperature on the accumulation of sitRNAs

Compared with cysts that are insensitive to temperature changes and can survive at 4°C for several days, trophozoites are relatively sensitive to low temperature. To date, although no studies concerning the changes of metabolic level have been reported, cell activity is decreased

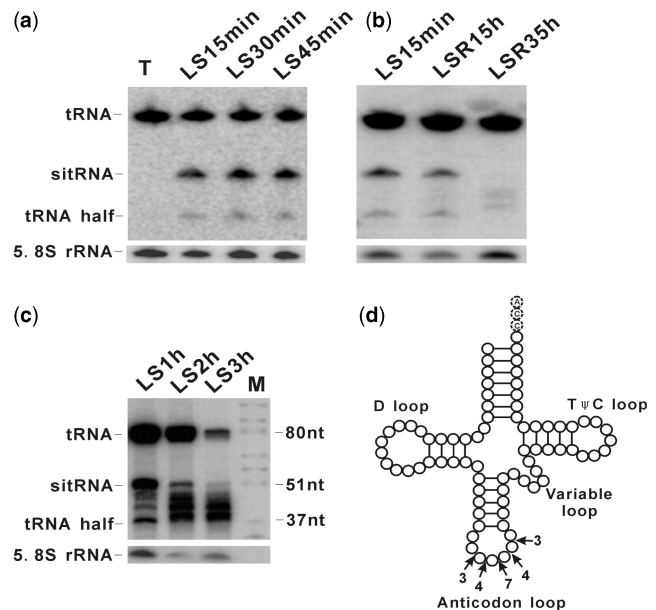


Figure 5. Serum deprivation and re-supplementation on the accumulation of sitRNAs. (a) TYI-S-33 medium for monolayer trophozoites was replaced by non-serum medium and growth continued for another 15 min (LS15min), 30 min (LS30min) and 45 min (LS45min). (b) After deprivation of serum for 15 min (LS15min), the cultures were re-supplemented with 10% serum for another 15-h (LSR15h) and 35-h (LSR35h) growth. (c) The medium for monolayer trophozoites was replaced by non-serum medium and was cultivated for 1 h (LS1h), 2 h (LS2h), and 3 h (LS3h), respectively. Total RNAs under each growing condition were isolated and probed with sit-Arg(ACG) and sit-Arg(CCU) and sit-Arg(UCU) mixed probes. 5.8S rRNA served as a loading control in (a–c) (d) The 5' cleavage sites of tRNA halves, and number of times cloned are denoted by arrows and numbers, respectively.

dramatically under non-permissible temperature. This is reflected that when confluent monolayer trophozoites were cultivated at 4°C (cold stress), the cells originally attaching to the surface of the tube became detached, and this attachment depend on active metabolism (19). We, therefore, speculated that under cold stress, the metabolic rate decreased. Accordingly, sitRNAs were clearly induced over time (Figure 6a). After returning to 37°C for 6-h recovery, this band disappeared, indicating that the cold response was reversible (Figure 6b). sitRNAs could also be induced during continued cultivation at 42°C (data not shown). As protein synthesis is not completely stopped and a low metabolic rate still exists under these adversities, sitRNAs only occupy a limited portion of mature tRNAs, as observed in encysting cultures.

DISCUSSION

tRNAs, representing 15% of cellular total RNAs, have long been known as a class of stable RNAs. However, this concept has been constantly challenged. Recent studies revealed a commonly existing phenomenon that in both prokaryotes and eukaryotes, mature tRNA could be cleaved into tRNA halves under suboptimal growing conditions (Table 1). In *E. coli*, plasmid-encoded colicins such as colicin D and colicin E5 target tRNAs for cleavage in the anticodon loop, and this cleavage exhibits specificity for only a limited number of tRNA isoacceptors (36,37). In essential amino acid-starved *T. thermophila*, similar

loop cleavage occurs in the entire tRNA family (16). Quite recently, tRNA halves have also been identified in bacterium *Streptomyces coelicolor* and fungus *Aspergillus fumigatus* during their differentiation into relative resting states (17,18). However, different organisms may adopt slightly diverse mechanisms to cleave tRNA at different sites/region, namely, between positions 34 and 35 for colicin E5, between positions 38 and 39 for colicin D, at a position 3' adjacent to the anticodon in *A. fumigatus* or in the anticodon loop region in *T. thermophila* and *S. coelicolor* (16–18,36,37), revealing some species-specific characteristics of tRNA cleavage. The endonucleases involved might be different from each other. Apparently, these cleavages are not meaningless random degradation phenomenon, and might participate in the regulation of gene expression, especially in response to stress conditions (Table 1).

Taking advantage of the excellent differentiation model of *G. lamblia*, we identified a novel global tRNA cleavage phenomenon as *G. lamblia* encysted into dormant cysts. In contrast to the well-studied tRNA halves, the cleavage occurred predominantly in the anticodon left arm of mature tRNAs to produce ~46 nt sitRNAs. This cleavage could also be induced by some other adverse growing conditions, thus adding to the complexity of tRNA internal cleavage when confronting suboptimal growing conditions. However, in our experiments, elevated concentrations of oxygen as performed by Morrison *et al.* (38) could not induce the accumulation of sitRNAs (data not shown). It is reported that *G. lamblia* is a microaerophilic protozoan and can use different metabolic pathways under various concentrations of oxygen (19), so oxidative pressure is not stressful enough to induce tRNA cleavage. Some unidentified endonucleases may be activated by these stresses and are responsible for the cleavage of mature tRNAs around ~46 nt counting from the 3' end. Further specifying the endonucleases participating in these different cleavage activities will provide clues to the biogenesis of the various tRNA fragments.

Unlike tRNA halves, one of the most prominent characteristics of sitRNAs is that with only a few exceptions, they unanimously have 3' terminal CCA nucleotides still connected. This template-independent 3' terminal sequence is of critical importance during protein synthesis for interaction with amino acid. It is reported that aminoacylation protects the 3' termini of tRNAs from

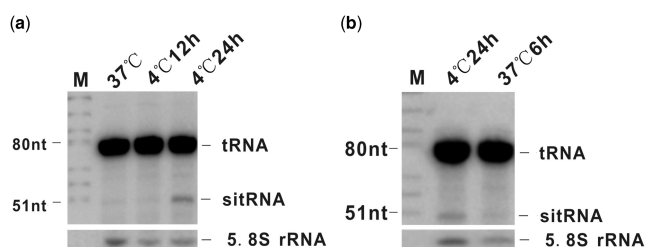


Figure 6. Temperature stress on the accumulation of sitRNAs. (a) Monolayer trophozoites grown at 37°C were transferred to 4°C for another 12 h (4°C12h) and 24 h (4°C24h) cultivation, respectively. Then, cells were allowed to recover at 37°C for 6 hr (37°C6h). (b) Total RNA was probed with sit-Arg(ACG), sit-Arg(CCU) and sit-Arg(UCU) mixed probes. 5.8S rRNA served as a loading control.

Table 1. tRNA cleavage phenomenon studied in various organisms

Species	Conditions inducing tRNA cleavage	Cleavage specificity	Cleavage position	Possible endonuclease	Proposed function
<i>E. coli</i> (36,37)	Stress-induced signals	tRNA ^{Tyr, His, Asn, Asp} tRNA ^{Arg} isoacceptors	Positions 34 and 35 Positions 38 and 39	Colicin E5 Colicin D	Impair protein synthesis and cause cell death
<i>T. thermophila</i> (16)	Essential amino acids starvation	Global tRNA cleavage	Anticodon loop region	Unknown	Eliminate uncharged tRNA or signaling molecule
<i>S. coelicolor</i> (17)	Cell differentiation	Global tRNA cleavage	Anticodon loop region	Unknown	A metabolic cue to switch developmental programs
<i>A. fumigatus</i> (18)	Cell differentiation	Not shown	3' Adjacent to the anticodon	Unknown	Downregulate protein synthesis
<i>G. lamblia</i>	Encystation and other stresses	Global tRNA cleavage	Anticodon left arm	Unknown	Downregulate global gene expression

ribonucleases (13). In *E. coli* and yeast, uncharged tRNAs are more susceptible to be attacked by exonucleases, resulting in the loss of 3' residues (39–41). In our study, however, sitRNAs are presumably cleaved from tRNAs, which are still aminoacylated; therefore, they are also likely to be connected with amino acids, and the aminoacylation may maintain the intactness of their 3' CCA termini.

All organisms have developed elaborate mechanisms to cope with various abrupt physiological or environmental changes, which may hinder their normal growth. *G. lamblia* is frequently exposed to environmental pressures. In order to continue its life cycle, it must develop adaptive responses that allow them to survive under adverse conditions. Although low/high temperature and serum deprivation could not induce encystation, one of the most important characteristics shared by these suboptimal conditions including encystation is that they ultimately result in the depression of the whole metabolic level, so that some protein synthesis is also down-regulated. We, therefore, conclude that sitRNAs may be induced by environmental stresses, which influence the normal metabolic rate of trophozoites. Clearly, this cleavage cannot stop protein synthesis completely for the majority of the mature tRNAs remain intact, with only a limited portion being targeted for cleavage. More importantly, compared with the subsequent degradation of the identified tRNA halves, sitRNAs maintain a rather abundant and stable level during the stress course, and their appearance is reversible while the stress is removed. Thus, we propose that this novel cleavage phenomenon might be coupled to down-regulation of global gene expression required for cells to maintain a relatively low metabolic level.

tRNAs can directly contribute to the efficiency of protein synthesis, which makes them excellent regulators in gene expression under stress or during development. In one way, the slightly increased proportion of uncharged tRNAs can initiate the stringent response of bacteria, and this process is reversible by favorable growing conditions (9). Likewise, tRNAs retrograded into nucleus in response to nutrient availability can be released into cytoplasm once nutrients are re-supplemented (42). However, the cleavage of tRNAs into sitRNAs influences the whole tRNA level directly and therefore represent a straightforward regulation. To the best of our knowledge, this cleavage represents a novel phenomenon during the stress response of *G. lamblia* and will shed light on the diversity of tRNA regulation pathways. Whether the similar cleavage of tRNAs occurs in other phylogenetically related organisms remains to be explored.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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