tmRNA in *Caulobacter crescentus* Is Cell Cycle Regulated by Temporally Controlled Transcription and RNA Degradation

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SsrA, or tmRNA, is a small RNA found in all bacteria that intervenes in selected translation reactions to target the nascent polypeptide for rapid proteolysis. We have found that the abundance of SsrA RNA in *Caulobacter crescentus* is regulated with respect to the cell cycle. SsrA RNA abundance increases in late G_1 phase, peaks during the G_1-S transition, and declines in early S phase, in keeping with the reported role for SsrA in the timing of DNA replication initiation. Cell cycle regulation of SsrA RNA is accomplished by a combination of temporally controlled transcription and regulated RNA degradation. Transcription from the *ssrA* promoter peaks late in G_1, just before the peak in SsrA RNA abundance. SsrA RNA is stable in G_1-phase cells and late S-phase cells but is degraded with a half-life of 4 to 5 min at the onset of S phase. This degradation is surprising, since SsrA RNA is both highly structured and highly abundant. This is the first observation of a structural RNA that is cell cycle regulated.

Regulation of mRNA and protein levels is fundamental to control of the cell cycle, both in eukaryotes and in bacteria. We now report that a small structural RNA is cell cycle regulated in the bacterium *Caulobacter crescentus*. This RNA, known as SsrA (also known as tmRNA and 10Sa RNA), is a small, highly structured RNA that has been found in every species of bacteria as well as in some chloroplasts and mitochondria of eukaryotes (27). SsrA RNA intervenes in selected translation reactions to release ribosomes from the mRNAs and to target the nascent polypeptides for proteolysis. SsrA provides a quality control function for translation by recognizing stalled ribosomes and targeting the incomplete proteins for degradation before they are released into the cell (8, 14, 29). In addition, findings regarding the phenotypes of SsrA mutations in a number of bacteria (3, 4, 10, 20) and the substrates that have been identified for SsrA (1, 19, 22) suggest that SsrA also has a role in regulating gene expression. Recently, we found that SsrA activity is required for correct timing of initiation of DNA replication in *Caulobacter* (12). The requirement for SsrA activity at a single point in the cell cycle led us to examine the possibility that the SsrA RNA is itself regulated as a function of the cell cycle.

Caulobacter is ideal for cell cycle studies, because it is easy to isolate a population of cells in G_1 phase and this population passes synchronously through the cell cycle. In addition, the cell cycle of *Caulobacter* is coordinated with a program of differentiation which culminates in an asymmetric cell division (see Fig. 4) (24). The progeny swarmer and stalked cells differ with respect to morphology and cell fate. The G_1 phase of the *Caulobacter* cell cycle coincides with the swarmer cell stage, in which cells are motile and have a single polar flagellum. Swarmer cells cannot initiate DNA replication or undergo cell division until they differentiate into stalked cells. This differentiation includes the loss of the polar flagellum, pilus, and chemotaxis apparatus, growth of a new appendage called a stalk at the same pole, and initiation of DNA replication. Thus, the swarmer- to stalked-cell transition is coincident with the G_1-S phase transition. After differentiation, the stalked cell continues to replicate the chromosome, elongates, and eventually forms a division plane, becoming a predivisional cell. The predivisional cell divides asymmetrically to produce a swarmer cell and regenerate the stalked cell.

In *Caulobacter* and related α-proteobacteria, the *ssrA* gene is unusual in that it contains a circular permutation that results in a two-piece SsrA RNA (13). The *ssrA* gene is transcribed as a single transcript with an internal loop connecting the tRNA-like 5′ and 3′ ends. The 30-nucleotide (nt) internal loop of this pre-SsrA RNA is excised by specific ribonucleases, producing the tRNA-like 5′ and 3′ ends and resulting in a mature SsrA composed of two RNA molecules, the coding RNA and the acceptor RNA. Despite this two-piece composition, the *Caulobacter* SsrA RNA is predicted to have a structure very similar to those of the one-piece SsrA RNAs from other bacteria. As in *Escherichia coli*, SsrA RNA in *Caulobacter* tags proteins made from mRNA with no stop codon and the *Caulobacter* SsrA-encoded peptide targets proteins for rapid degradation in vivo (13). We report here that the *Caulobacter* SsrA RNA is transcribed and nucleolytically processed to its mature form at the swarmer- to stalked-cell transition and then subjected to nuclease digestion in the stalked cell. Thus, the abundance of this small structural RNA is under strict cell cycle control. There are many examples of mRNAs that peak in expression during specific stages of the cell cycle (16), but *Caulobacter* SsrA RNA is the first example of a structural RNA that is cell cycle regulated.

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MATERIALS AND METHODS

Strains and plasmids. The wild-type *Caulobacter* strain used in this work is CB15N (6). *Caulobacter* strains were grown in M2G or PYE medium at 28°C (5), and growth was monitored by observing the increase in optical density at 660 nm. Using PCR, the *ssrA* promoter transcriptional reporter was constructed by amplifying the 602-bp segment of DNA 5′ of the SsrA transcriptional start site and cloning the fragment into pRKlac290 5′ of the promoterless *lacZ* gene (2). The plasmid was verified by DNA sequencing and mobilized into the *Caulobacter* strains by conjugation from *E. coli* strain S17 (5). The wild-type *E. coli* strain used in this work was MG1655, which was grown in Luria-Bertani broth at 30°C, and culture density was monitored by observing optical density at 600 nm.

Cell cycle studies. Synchronized cultures of *Caulobacter* were obtained by centrifugation in a Ludox density gradient followed by isolation of swarmer cells (6). Swarmer cells were released into M2G medium, and aliquots were removed from synchronized cultures every 15 min for analysis by Northern blotting.

RNA analysis. Total RNA was isolated using the hot phenol method (23). Northern blotting was performed after separating equal quantities of total RNA on polyacrylamide-urea gels. ^{32}P-labeled DNA probes were generated from PCR products by using QuickPrime protocol (Amersham Biosciences), visualized using a PhosphorImager (Molecular Dynamics), and quantified with ImageQuant software. RNA decay experiments were performed by inhibiting transcription in a culture by addition of rifampin to achieve a concentration of 300 μg/ml and removing aliquots into an equal volume of stop buffer (10 mM Tris-HCl [pH 7.5], 5 mM MgCl$_2$, 25 mM sodium azide, 500 μg of chloramphenicol/ml) on ice. RNA was prepared from these samples and analyzed by Northern blotting. The half-life was determined by fitting the data with a single exponential function. To measure the RNA decay in pure populations of swarmer cells, stalked cells, and predivisional cells, synchronous cultures were prepared and analyzed at 15 min for swarmer cells, at 45 min for stalked cells, and at 80 min for predivisional cells.

Promoter activity assays. Cells bearing *lacZ* reporter constructs were synchronized, and at 15-min time points, 1-ml aliquots were removed, labeled with 25 μCi of [^{35}S]methionine for 5 min, and added to 50 μl of 100% trichloroacetic acid on ice. Proteins were collected by centrifugation and resuspended in 50 μl of buffer containing 10 mM Tris-HCl (pH 8.0), 1% sodium dodecyl sulfate (SDS), and 1 mM EDTA. After resuspension, 0.75 ml of RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) was added and the lysates were incubated with agarose conjugated to anti-β-galactosidase antibody (Abcam) for 4 h at 4°C. Immunoprecipitated protein was collected by centrifugation, washed three times with RIPA buffer, and boiled in SDS-polyacrylamide gel electrophoresis sample loading buffer. Proteins were separated on SDS–6% polyacrylamide electrophoresis gels, and the amount of radiolabeled β-galactosidase was quantified using a PhosphorImager. The signal for each synchronous culture was normalized to the level of the 15-min time point, and three normalized experiments were averaged.

RESULTS

Levels of SsrA RNA change through the cell cycle. Our observation that SsrA activity is required for proper timing of DNA replication raises the possibility that SsrA RNA is cell cycle regulated. To determine whether the abundance of SsrA RNA changes through the cell cycle, total RNA was isolated from synchronized populations of *Caulobacter* and the amount of SsrA RNA was measured by Northern blotting (Fig. 1). The amount of the SsrA coding RNA (214 bp) and acceptor RNA (83 bp) increased by 2.5-fold in 15 min during the swarmer- to stalked-cell transition and then decreased to near or below the initial levels over 30 min in stalked cells. The amount of the coding and acceptor RNAs then increased slowly over 60 min in predivisional cells. For comparison, the same Northern blots were probed for 5S rRNA and the amount of 5S rRNA did not change significantly over the cell cycle.

A priori, it is possible that the amount of mature SsrA RNA is regulated at several different steps, including transcription of pre-SsrA RNA, processing of pre-SsrA RNA to the mature form, and degradation of mature SsrA RNA. Because there was little accumulation of pre-SsrA at any point during the cell
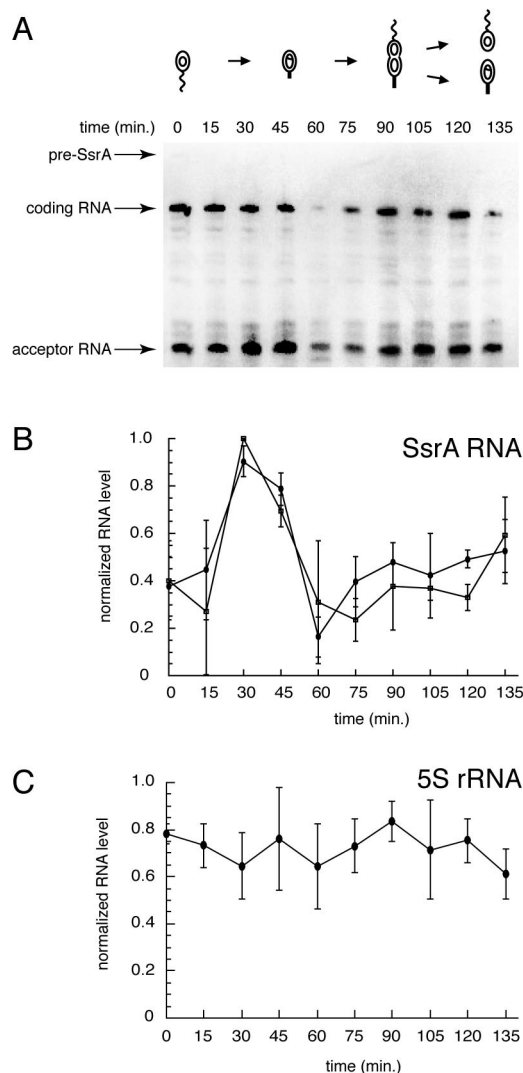

FIG. 1. Cell cycle regulation of SsrA RNA abundance. (A) A representative Northern blot of total RNA from synchronized cultures probed for SsrA RNA. The schematic diagram indicates the stage of the cell cycle for each time point. The bands corresponding to the pre-SsrA RNA, the 214-nt coding RNA, and the 83-nt acceptor RNA are indicated. (B) The amount of SsrA RNA was quantified and normalized to the peak level of SsrA. The averages of the results of four independent experiments are shown, with error bars indicating the standard deviations. Filled squares correspond to the SsrA coding RNA, and filled circles correspond to the SsrA acceptor RNA. (C) The blots were reprobed for 5S rRNA and quantified in the same manner as described for panel B.

cycle (Fig. 1A), processing of pre-SsrA RNA to the mature form occurs at the time of its transcription. Thus, transcription of the *ssrA* gene and degradation of SsrA RNA were examined as possible regulatory pathways for cell cycle control of the abundance of SsrA RNA.

Cell cycle-regulated transcription of *ssrA*. The cell cycle dependence of *ssrA* transcription was determined by assaying the production of β-galactosidase from a *lacZ* reporter under the control of the *ssrA* promoter (Fig. 2). This reporter construct contains the P$_{ssrA}$ promoter followed by a Shine-Dalgarno sequence and the *lacZ* gene, such that transcription from
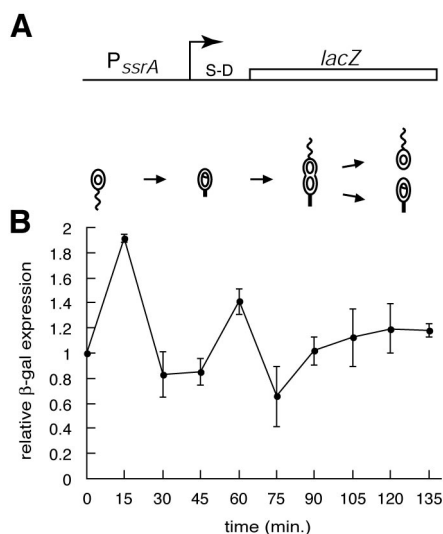
FIG. 2. Regulation of transcription from the *ssrA* promoter. (A) Schematic diagram of the reporter construct used to assay cell cycle regulation of transcription from the *ssrA* promoter. The arrow indicates the transcriptional start site, and the relative location of the Shine-Dalgarno sequence (S-D) is shown. (B) Promoter activity was measured by immunoprecipitation of β-galactosidase from pulse-labeled samples of a synchronized culture. The amount of β-galactosidase was normalized to the initial time point, and the averages of the results of three independent experiments are shown, with error bars indicating the standard deviations.

P*ssrA* results in production of β-galactosidase. In log-phase cultures, the P*ssrA* promoter generated ~6,500 Miller units of activity, making it one of the most active promoters reported for *Caulobacter*. This high level of transcription is consistent with the high abundance of SsrA RNA in the cell. Transcription from P*ssrA* at different points in the cell cycle was compared by pulse-labeling cell samples from a synchronous culture with [35S]methionine and immunoprecipitating the β-galactosidase to determine how much new protein was made from the P*ssrA* reporter construct during the pulse. Transcription increased approximately twofold during the swarmer- to stalked-cell transition and then decreased early in the stalked cell stage (Fig. 2). Transcription from the *ssrA* promoter peaked immediately before accumulation of SsrA RNA, and the increase in transcription during the swarmer- to stalked-cell transition was similar to the increase in SsrA RNA steady-state levels in magnitude. This correlation suggests that the increase in SsrA RNA levels during the swarmer- to stalked-cell transition is the result of increased transcription. There was a smaller peak of transcriptional activity late in the stalked cell stage, immediately before SsrA RNA steady-state levels began to increase.

Degradation of SsrA RNA. Cell cycle-regulated transcription of *ssrA* can account for the increase in SsrA RNA levels during the swarmer- to stalked-cell transition, but the rapid decrease in SsrA RNA levels during the stalked cell stage suggests that SsrA was degraded. To determine whether this was the case, the half-life of SsrA RNA in both log-phase and synchronized cultures was measured by inhibiting transcription with rifampin and monitoring the decay of SsrA RNA by Northern blotting (Fig. 3). In log-phase cultures that contain a mixture of
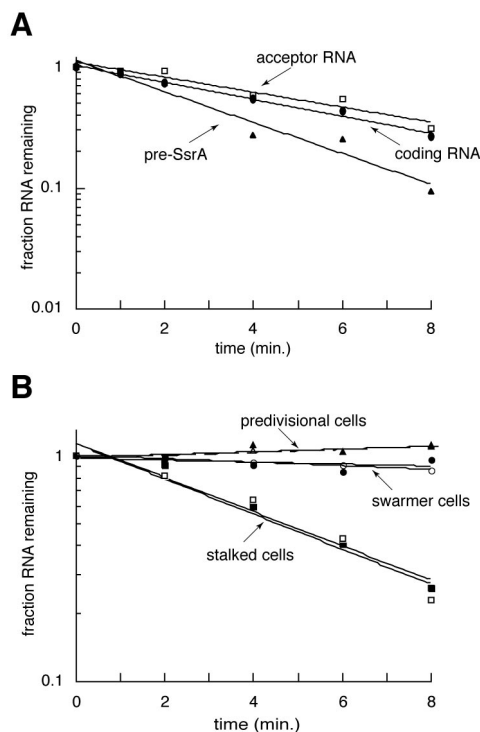

FIG. 3. Degradation of SsrA RNA. (A) The decay of SsrA RNAs in a log-phase *Caulobacter* culture was determined by inhibiting transcription and monitoring the loss of SsrA by Northern blotting. Single-exponential functions fit to each data set are shown. (B) The experiment was repeated on pure populations of swarmer cells (circles), stalked cells (squares), and predivisional cells (triangles). Filled symbols correspond to SsrA coding RNA, and open symbols correspond to SsrA acceptor RNA.

swarmer, stalked, and predivisional cells, both the coding and acceptor RNAs decayed with a half-life of 4 to 5 min (Table 1). The pre-SsrA RNA decayed with a half-life of 2.5 min. However, loss of pre-SsrA RNA is not due solely to RNA degradation; it includes processing into mature SsrA, as well as any

TABLE 1. Half-lives of RNA species in *Caulobacter* and *E. coli*

Cell type	RNA species	Half-life (min)
Caulobacter log-phase cultures	SsrA acceptor RNA	5.2 ± 0.2
	SsrA coding RNA	4.4 ± 0.3
	Pre-SsrA RNA	2.5 ± 0.2
	5S rRNA	Stable
	pilA mRNA	<2
	λ repressor mRNA[a]	<2
Caulobacter swarmer cells	SsrA acceptor RNA	51 ± 8
	SsrA coding RNA	56 ± 9
	5S rRNA	Stable
Caulobacter stalked cells	SsrA acceptor RNA	4.5 ± 1.0
	SsrA coding RNA	4.2 ± 0.4
	5S rRNA	Stable
Caulobacter predivisional cells	SsrA acceptor RNA	Stable
	SsrA coding RNA	Stable
	5S rRNA	Stable
E. coli log-phase cultures	SsrA RNA	89 ± 16
	5S rRNA	Stable

[a] λ repressor-M2-H6-*trpAt* variant mRNA measured from *Caulobacter* constitutively expressing a plasmid-borne copy of this gene (13).

complete degradation of the full-length transcript (Fig. 4B). As controls, the Northern blots were stripped and reprobed to measure the decay of two mRNAs, that encoding *pilA* and that encoding a variant of λ repressor, as well as to measure the decay of 5S rRNA (Table 1). For both mRNAs the half-life was 1 to 2 min, consistent with the rapid degradation expected of mRNAs. The amount of 5S rRNA was constant, indicating that there was no general loss of RNA over the course of the experiment. Thus, mature *Caulobacter* SsrA RNA is more stable than many mRNAs but is degraded rapidly enough to be completely turned over during a single cell cycle.

Although it has been assumed that SsrA in *E. coli* is stable because it is highly abundant, a half-life has not been reported. The decay of SsrA in log-phase cultures of *E. coli* was measured using the same protocol as for *Caulobacter*. The half-life of *E. coli* SsrA under these conditions was 89 min (Table 1), considerably longer than in *Caulobacter*. The difference in the half-life durations is even more pronounced when the relative length of the cell cycle is considered; in *Caulobacter* the half-life of SsrA is ~0.03 cell cycles, whereas in *E. coli* the half-life of SsrA is ~3 cell cycles. Thus, with respect to the length of the cell cycle, the degradation of *Caulobacter* SsrA is 100-fold faster than that of SsrA from *E. coli*.

To determine whether the degradation of SsrA RNA is cell cycle regulated, the RNA decay experiments were repeated using pure populations of swarmer, stalked, and predivisional cells (Fig. 3B). In swarmer cells, both forms of SsrA RNA decayed with a half-life of more than 50 min. In stalked cells, however, SsrA RNA was degraded with a half-life of 4 to 5 min. This rate of degradation, coupled to the decrease in transcription from the *ssrA* promoter, is sufficient to account for the fourfold decrease in SsrA RNA levels over 30 min in the stalked-cell stage. In predivisional cells, no degradation of SsrA RNA was detected. These results demonstrate that the amount of SsrA RNA is cell cycle regulated by at least two mechanisms: temporally controlled transcription and temporally controlled degradation (Fig. 4).

DISCUSSION

The data presented here show that SsrA RNA abundance is cell cycle regulated, with peak expression during the G_1-S transition, and that at least two mechanisms are employed for this regulation. The regulation of SsrA RNA is not a response to changes in the amount of translation, because the amount of translation in *Caulobacter* increases at an even rate through most of the cell cycle and does not increase sharply during the G_1-S transition (7). If the only function of SsrA RNA is quality control for translation and the total amount of translation does not increase during the G_1-S transition, then there must be a dramatic increase in the rate of ribosome stalling during this transition. Alternatively, it is possible that SsrA activity plays a regulatory role in addition to its translational quality control function and that the increase in SsrA RNA levels generates or facilitates a change in gene expression during the G_1-S transition. In fact, the increase in the abundance of SsrA RNA during the G_1-S transition is correlated with the requirement for SsrA activity for proper timing of DNA replication initiation (12).

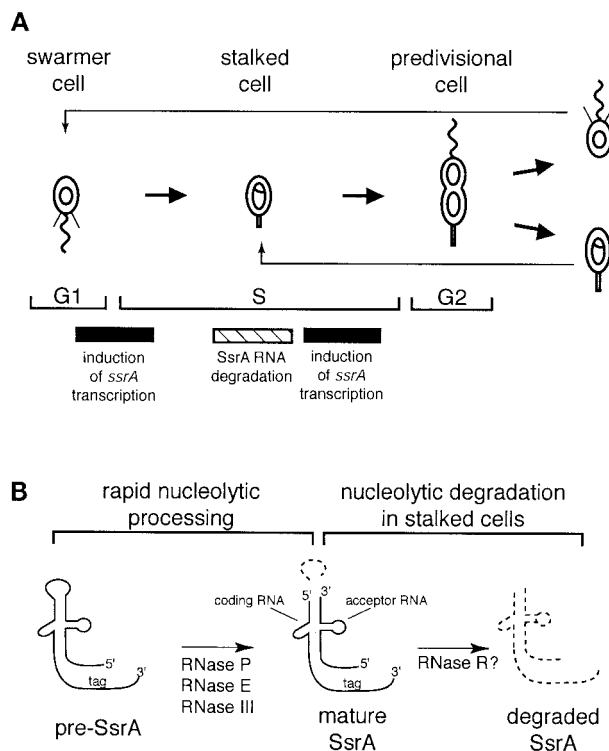The control of both transcription and degradation provides

FIG. 4. Mechanisms of regulation of the abundance of SsrA RNA through the *Caulobacter* cell cycle. (A) Schematic diagram of the *Caulobacter* cell cycle showing the time of induction of *ssrA* transcription and degradation of SsrA RNA. The circles and theta structures within the cells represent nonreplicating and replicating chromosomes, respectively, and the G_1, S, and G_2 phases of the cell cycle are indicated. The single polar flagellum (squiggly line) and polar pili (thin lines) are lost during the swarmer- to stalked-cell transition and are replaced at the same pole by a stalk (thick line). (B) Schematic diagram of nucleolytic processing and degradation of SsrA RNA. A 30-nt loop is excised from pre-SsrA RNA by specific nucleases to produce the two-piece mature SsrA. The mature SsrA RNA is then completely degraded by one or more nucleases in stalked cells. Degraded RNA is indicated by dashed lines. Candidates for these different nucleases, based on results from *E. coli* experiments, are indicated.

redundant mechanisms to ensure that there is sufficient SsrA RNA during the G_1-S transition and that SsrA is cleared from the cell at a critical time after the initiation of DNA replication. Early in the G_1-S transition, transcription of *ssrA* increases and there is no degradation of SsrA RNA; in early S phase, transcription decreases and SsrA RNA degradation is induced (Fig. 4A). Late in S phase, transcription of *ssrA* increases again and degradation of SsrA RNA is turned off (Fig. 4A). This redundant control by transcription and degradation is analogous to the regulation of CtrA, in which both proteolysis and dephosphorylation are used to ensure that CtrA is not active during the G_1-S transition. It is possible that there are also additional mechanisms to regulate SsrA activity in *Caulobacter*, including control of the abundance and activity of the SsrA RNA-binding protein SmpB, which is required for SsrA activity (12), charging of SsrA RNA with alanine, interaction of SsrA RNA with target ribosomes, and cell cycle regulation of SsrA substrates.

The mechanisms for control of the *ssrA* promoter are not

evident from the DNA sequence. The temporal transcription pattern is similar to that of *dnaA*, but the sequence elements that have been identified in the *dnaA* promoter (11, 21, 28) are not found in the *ssrA* promoter. There are also no consensus CtrA binding sites or binding sites for other characterized transcription factors in *Caulobacter*. Therefore, understanding the cell cycle regulation of transcription of the *ssrA* gene requires mutagenesis of the promoter and identification of the transcription factors that regulate its activity.

The regulation of SsrA by ribonucleases includes both the processing of pre-SsrA RNA to the mature form and complete degradation of mature SsrA RNA (Fig. 4B). The processing of pre-SsrA RNA to the mature form is fast enough to prevent accumulation of pre-SsrA RNA throughout the cell cycle. Several ribonucleases, including RNase P, RNase E, and RNase III, have been implicated in the processing of SsrA RNA in *E. coli* (15, 17, 18, 25, 26). *Caulobacter* contains homologues of each of these RNases, and it has been proposed that they are responsible for excising the 30-nt loop from *Caulobacter* pre-SsrA RNA (13). Because each of these nucleases is also involved in the processing of tRNAs and other RNA molecules, it is likely that they are constitutively active in *Caulobacter*, accounting for the rapid processing of pre-SsrA RNA.

How is degradation of SsrA RNA controlled through the cell cycle? Two possibilities are that SsrA RNA is specifically stabilized in swarmer and predivisional cells and that SsrA RNA is specifically degraded in stalked cells. It is possible that SsrA RNA is stabilized by RNA-binding proteins such as SmpB. Notably, the level of SsrA RNA in log-phase cultures is reduced by 90% in the absence of SmpB (12), although it is not known whether the lower steady-state level is due to decreased transcription or increased degradation. If SmpB is expressed only in swarmer and predivisional cells, then its absence in stalked cells might cause degradation of SsrA RNA. It is also possible that the regulated degradation of SsrA RNA is due to the presence of a specific endo- or exoribonuclease. One-piece SsrAs are likely to be resistant to exoribonucleases, since the 5′ and 3′ ends of SsrA are folded in a tRNA-like conformation, but the two-piece *Caulobacter* SsrA has additional 5′ and 3′ ends that are possibly available to an exoribonuclease. RNase activity taking place exclusively in the stalked cell would result in the observed regulation of SsrA RNA degradation. One candidate for the nuclease that degrades SsrA RNA in stalked cells is the 3′-5′ exoribonuclease RNase R. RNase R has been found in association with SsrA RNA in *E. coli* (9), and *Caulobacter* also has an RNase R homologue. Identification and characterization of the RNase or RNases that degrade SsrA RNA are required to understand this mode of regulation.

Are SsrA RNAs in other organisms also degraded? It has been assumed that SsrA RNA is stable in *E. coli* and other species because of its abundance and structure, and SsrA RNA in *E. coli* is in fact very stable (Table 1). However, SsrA RNA in *Caulobacter* is also highly abundant and structured (13) but is degraded. If the two-piece composition of *Caulobacter* SsrA is important for its degradation, then two-piece SsrAs in other species, including all α-proteobacteria and some cyanobacteria (13), should be degraded as well. In addition, RNase R is conserved throughout bacteria, so if RNase R is responsible for the degradation of SsrA RNA in *Caulobacter* strains, it is possible that other bacteria also use RNase R to degrade SsrA

RNA. One intriguing possibility is that the regulation of SsrA is correlated with its role in cellular physiology. In *Caulobacter* strains, SsrA activity is required for correct timing of replication initiation during the G_1-S transition (12) and SsrA RNA is degraded once per cell cycle. In *E. coli* strains, SsrA activity is not required for log-phase growth and SsrA is very stable. It would be instructive to examine the regulation of SsrA RNA in other bacteria that have a requirement for SsrA activity.

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