

# Initiating nucleotide identity determines efficiency of RNA synthesis from 6S RNA templates in *Bacillus subtilis* but not *Escherichia coli*

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## ABSTRACT

The 6S RNA is a non-coding small RNA that binds within the active site of housekeeping forms of RNA polymerases (e.g.  $E\sigma^{70}$  in *Escherichia coli*,  $E\sigma^A$  in *Bacillus subtilis*) and regulates transcription. Efficient release of RNA polymerase from 6S RNA regulation during outgrowth from stationary phase is dependent on use of 6S RNA as a template to generate a product RNA (pRNA). Interestingly, *B. subtilis* has two 6S RNAs, 6S-1 and 6S-2, but only 6S-1 RNA appears to be used efficiently as a template for pRNA synthesis during outgrowth. Here, we demonstrate that the identity of the initiating nucleotide is particularly important for the *B. subtilis* RNA polymerase to use RNA templates. Specifically, initiation with guanosine triphosphate (GTP) is required for efficient pRNA synthesis, providing mechanistic insight into why 6S-2 RNA does not support robust pRNA synthesis as it initiates with adenosine triphosphate (ATP). Intriguingly, *E. coli* RNA polymerase does not have a strong preference for initiating nucleotide identity. These observations highlight an important difference in biochemical properties of *B. subtilis* and *E. coli* RNA polymerases, specifically in their ability to use RNA templates efficiently, which also may reflect the differences in GTP and ATP metabolism in these two organisms.

## INTRODUCTION

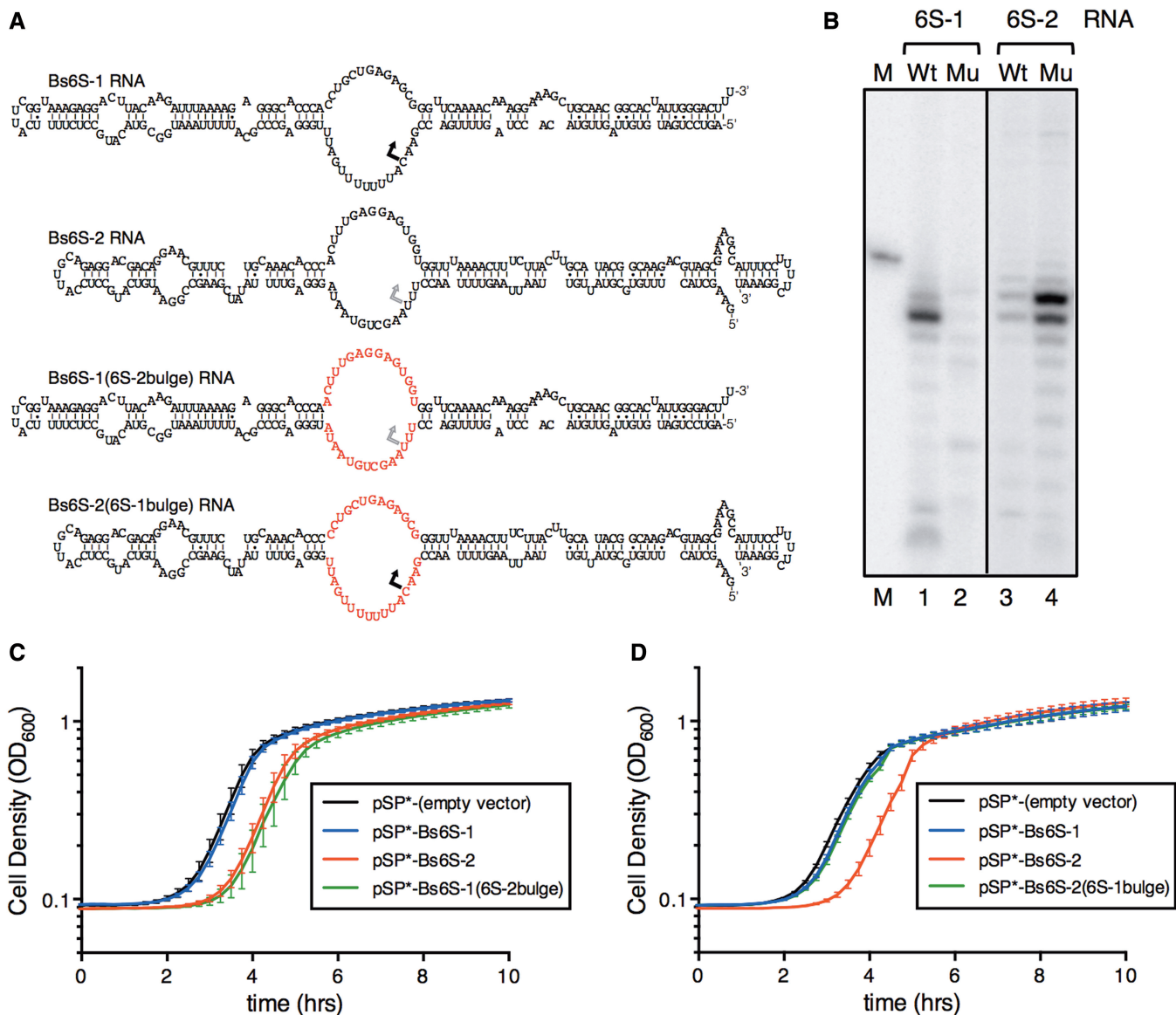
The 6S RNA is a small non-coding RNA that was first identified in *Escherichia coli*, where many of the functional studies have been performed [reviewed in (1,2)]. *E. coli* 6S RNA (Ec6S RNA) binds tightly to the  $\sigma^{70}$ -containing form of RNA polymerase (EcE $\sigma^{70}$ ) and regulates transcription at many  $\sigma^{70}$ -dependent promoters. The Ec6S RNA is largely double stranded with a central single-stranded region that is reminiscent of the conformation of DNA in

the open complex during transcription initiation (3,4). It has been shown that Ec6S RNA binds to EcE $\sigma^{70}$  in the active site in a manner similar to promoter DNA binding to EcE $\sigma^{70}$ . Ec6S RNA interacts with EcE $\sigma^{70}$  similarly enough to DNA templates to be used as a template for the synthesis of a product RNA (pRNA) (5,6). *In vivo*, pRNA synthesis occurs during outgrowth from stationary phase and has been shown to be an important step in release of Ec6S RNA from EcE $\sigma^{70}$  at that time (7). In contrast to the contacts in the active site, other interactions between Ec6S RNA and EcE $\sigma^{70}$  are not the same as those between promoter DNA and EcE $\sigma^{70}$ . For example, the ‘upstream’ region of Ec6S RNA does not mimic promoter DNA, and many residues within  $\sigma^{70}$  region 4.2 contribute differentially to 6S RNA and DNA binding (8).

The 6S RNAs are highly conserved, and genes encoding putative 6S RNAs have been identified from a wide range of bacterial species using biochemical, bioinformatic and sequencing approaches (3,4,9–12). It is the secondary structure that is primarily conserved, in agreement with studies demonstrating the importance of this structure for binding to RNA polymerase (3,13).

Interestingly, two 6S RNAs have been identified in some species, including *Bacillus subtilis* and *Legionella pneumophila* (3,4,10,12). The *B. subtilis* 6S RNAs were first sequenced as abundant RNAs of unknown function called Bs190 (Bs6S-1 RNA encoded by *bsrA*) and Bs203 (Bs6S-2 RNA encoded by *bsrB*) (14,15) that later were identified as 6S RNAs based on their co-immunoprecipitation with RNA polymerase or based on secondary structure similarity to the Ec6S RNA (see Figure 1) (3,4). Both Bs6S-1 and Bs6S-2 RNAs have been shown to bind the housekeeping form of RNA polymerase (BsE $\sigma^A$  in *B. subtilis*) similarly to Ec6S RNA binding to EcE $\sigma^{70}$  (3). However, these two *B. subtilis* RNAs exhibit some differences in biochemical behavior, expression profiles and phenotypes associated with loss of function. For example, Bs6S-1 RNA is able to be used for pRNA synthesis both *in vitro* and *in vivo* by BsE $\sigma^A$  in a manner similar to the use of Ec6S RNA by EcE $\sigma^{70}$  (7,16,17). In contrast, pRNA synthesis from Bs6S-2 RNA has not been

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**Figure 1.** The efficiency of pRNA synthesis from Bs6S-1 RNA and Bs6S-2 RNA is dependent on sequences in the central bulge. (A) Schematics of *B. subtilis* 6S-1 RNA, 6S-2 RNA and mutant RNAs in secondary structures supported by phylogenetic analyses (4). Nucleotides that have been changed in the mutant RNAs are indicated in red. The template position where pRNA synthesis initiates in Bs6S-1 RNA or Bs6S-2 RNA is indicated by a black or gray arrow, respectively (16). (B) pRNAs generated *in vitro* by BsE $\sigma^A$  from Bs6S-1 RNA (lane 1), Bs6S-1(6S-2bulge) RNA (lane 2), Bs6S-2 RNA (lane 3) or Bs6S-2(6S-1bulge) RNA (lane 4) were visualized on a denaturing gel. Lane M contains a 5' end labeled oligonucleotide 19 nt in length for size comparison. Reactions shown here were done in parallel and run on the same gel, but intervening lanes have been removed, as indicated by a vertical line. (C and D) Growth of *B. subtilis* cells as monitored by optical density at 595 nm (OD<sub>595</sub>) in an absorbance plate reader after stationary phase cells were diluted ~1:500 into 2 $\times$  YT medium. Growth was of *B. subtilis* *AbsrA* $\Delta$ *bsrB* cells (KW590) containing plasmids pSP\*-Bs6S-1 (blue), pSP\*-Bs6S-2 (red), pSP\*-Bs6S-1(6S-2bulge) (green in C), pSP\*-Bs6S-2(6S-1bulge) (green in D), or the empty vector pSP\* (black). Data shown are from one representative experiment with three biological replicates. Similar results were observed in at least three experiments. Error bars correspond to  $\pm$  standard deviations from the averages.

detected *in vivo* and is not as efficient *in vitro*, although pRNAs from Bs6S-2 RNA have been observed under some conditions *in vitro* (7,16,17). Cells expressing Bs6S-2 RNA but lacking Bs6S-1 RNA are delayed in their ability to restart growth when stationary phase cells encounter an upshift in nutrients, whereas cells lacking Bs6S-2 RNA restart growth similarly to wild-type cells (7). In addition, Bs6S-1 RNA and Bs6S-2 RNA are expressed differentially through growth. Bs6S-1 RNA

accumulates during stationary phase to levels at least 10-fold higher than in early exponential phase in a manner similar to Ec6S RNA (3,4,16). However, Bs6S-2 RNA does not accumulate in stationary phase, and its levels may even decrease (3,4,14,16). The difference between Bs6S-1 RNA and Bs6S-2 RNA accumulation patterns might be due to a difference in pRNA synthesis, as it has been shown that pRNA synthesis leads to the release and presumed degradation of Ec6S RNA and

Bs6S-1 RNA (7,18). Other observations suggest differential use for pRNA synthesis is unlikely to be the only difference in behavior between these two RNAs. For example, cells lacking Bs6S-1 initiate sporulation earlier than wild-type cells or cells lacking Bs6S-2 RNA, a time when pRNA synthesis rates are low (19).

Transcription on DNA promoters by RNA polymerase has been extensively studied in both *B. subtilis* and *E. coli*. Initiation involves promoter recognition, several conformational changes at the DNA and protein levels and subsequent initiation of RNA synthesis [reviewed in (20)]. The location where transcription begins, referred to as the +1 position, is often directed by local sequence context. For instance, compilations of *B. subtilis* and *E. coli* promoters indicate a strong preference for initiating transcription with adenosine triphosphate (ATP) or guanosine triphosphate (GTP), although examples of use of uroidine triphosphate (UTP) and cytidine triphosphate (CTP) for initiation sites exist as well (21–23). The nucleotides immediately adjacent to the +1 position also can influence transcription initiation location and efficiency (24,25). For example, pyrimidines on the non-template strand appear to be favored both upstream (–1 position) and downstream (+2 position). In addition to directing location and general efficiency of initiation, the identity of the +1 and sometimes the +2 positions can lead to important regulatory events. For example, rRNA promoters have kinetic properties that make them particularly sensitive to the initiating nucleotide concentration (26–28). For several pyrimidine biosynthetic genes, the initiating sequences are sensitive to the cognate NTP levels such that when concentrations are high, these promoters direct a reiterative transcription cycle that prevents transcription of the structural gene [reviewed in (29,30)].

Here, we investigate the properties of Bs6S-1 and Bs6S-2 RNAs that contribute to differences in efficiency of their use to template pRNA synthesis by  $BsE\sigma^A$ . Sequences of the central bulge are critical and sufficient to direct high or low pRNA synthesis rates.  $BsE\sigma^A$  was found only to carry out high levels of pRNA synthesis when initiating with GTP (iGTP). In contrast to RNA-templated RNA synthesis, this enzyme efficiently uses either ATP or GTP for initiation of transcription on DNA templates and even uses CTP or UTP for initiation on some promoters (21,22). Intriguingly, the requirement for iGTP for high efficiency pRNA synthesis by  $BsE\sigma^A$  is not shared by  $EcE\sigma^{70}$ , which can initiate pRNA synthesis with any of the four nucleotides. In addition to the role of the iNTP identity in pRNA synthesis, we show that the –1 template position contributes to the efficiency of pRNA synthesis for both  $BsE\sigma^A$  and  $EcE\sigma^{70}$ , although  $BsE\sigma^A$  appears to be more sensitive to changes at this position. In contrast, only mild effects of the identity of the +2 template position were observed for either enzyme.

## MATERIALS AND METHODS

### Strains

*B. subtilis* strains were wild-type 168 (KW586, 1A1 Bacillus Genetic Stock Center and *ΔbsrA::tet*

*ΔbsrB::spec* [KW590, (7)]. *B. subtilis* was grown in 2× YT (Yeast Extract Tryptone) medium (31); 25 μg/ml kanamycin was included in medium for growth of cells containing pSP\* plasmid derivatives.

### Plasmids

pT3-Bs6S-1 (7), pT3-Bs6S-2 (7), pT3-6S (3) and variants were used for synthesis of RNAs for *in vitro* experiments. Various mutations were introduced by Quikchange II site-directed mutagenesis according to manufacturer protocols (Agilent). See Supplementary Table S1 for oligonucleotide sequences. All relevant regions of plasmids were confirmed by sequencing.

pSP\*-Bs6S-1, pSP\*-Bs6S-2, pSP\*-Ec6S and derivatives were used for expression of Bs6S-1 RNA, Bs6S-2 RNA, Ec6S RNA or variants in *B. subtilis* cells and contained *bsrA*, *bsrB*, *ssrS* (from *E. coli*) or variants under control of the Pspac promoter in pDG148-stu (32). All experiments here were done using uninduced levels of RNA, which were previously shown to result in levels similar to endogenous Bs6S-1 and Bs6S-2 RNAs (7). Cloning was essentially as described by Joseph *et al.* (32); 6S RNA sequences were introduced into pDG148-stu after PCR amplification from the pT3 derivatives. See Supplementary Methods for further details of cloning and oligonucleotide sequences. All relevant regions of plasmids were confirmed by sequencing.

### *In vitro* pRNA synthesis reactions

pRNA synthesis reactions were essentially as described previously (5). Briefly, unlabeled *in vitro* transcribed and gel purified *E. coli*, *B. subtilis* or mutant 6S RNAs (80 nM) were incubated with  $BsE\sigma^A$  or  $EcE\sigma^{70}$  (40 nM active) in 20 mM Hepes (pH 7.5), 120 mM KCl, 0.5 mM  $MgCl_2$ , 5% glycerol and 1 mM dithiothreitol for 5 min at room temperature. Five minutes was sufficient for full binding of RNA to RNA polymerase at these concentrations (8). pRNA synthesis was initiated by addition of nucleotides (0.05 mM final concentration including 1 μCi of  $^{32}P$  γ-GTP,  $^{32}P$  γ-ATP, α- $^{32}P$ -CTP or α- $^{32}P$ -UTP) and  $MgCl_2$  (2.5 mM final concentration). After incubation for 15–20 min at 37°C, reactions were stopped by addition of loading dye (10 M Urea, 45 mM Tris, 45 mM Boric Acid, 1 mM Ethylenediaminetetraacetic acid, pH 8.3, 0.1% Bromophenol Blue, 0.1% Xylene Cyanol). RNAs were separated on denaturing gels (20% polyacrylamide, 8 M Urea, 89 mM Tris, 89 mM Boric Acid, 2 mM Ethylenediaminetetraacetic acid, pH 8.3) and visualized on a Typhoon phosphorimager (GE Life Sciences). For experiments comparing pRNA levels with differential labeling ( $^{32}P$  γ-GTP,  $^{32}P$  γ-ATP or α- $^{32}P$ -CTP), reactions with internally labeled pRNA (i.e. with α- $^{32}P$ -CTP) were diluted 1:5 before gel electrophoresis to normalize levels of the 20 nt pRNA<sub>Ec6S</sub> that would contain 5 C residues per transcript.

His-tagged *B. subtilis* RNA polymerase was purified using Ni-NTA agarose affinity chromatography from KW591 cells as previously described (33), followed by additional purification on HiTrap Heparin HP (GE Healthcare). *B. subtilis*  $\sigma^A$  was purified from inclusion

bodies after overproduction from pET-Bs $\sigma^A$  in *E. coli* BL21 Star cells (Invitrogen), followed by refolding as previously described for *E. coli*  $\sigma^{70}$  (34,35). BsE $\sigma^A$  was reconstituted by incubation of His-tagged RNA polymerase with 10-fold molar excess of  $\sigma^A$  in storage buffer [50 mM Tris (pH 8), 0.5 mM Ethylenediaminetetraacetic acid, 0.1 M NaCl, 50% glycerol, 0.1 mM dithiothreitol] for 30 min at room temperature. The active concentration of BsE $\sigma^A$  or EcE $\sigma^{70}$  (Epicentre) was estimated from the minimum protein concentration required to obtain maximum binding to 10 nM DNA with a consensus extended -10 promoter for *E. coli*, or to 10 nM 6S-2 RNA for *B. subtilis*, as described in Roe *et al.* (36).

### Outgrowth

Outgrowth experiments were as previously described (7). Briefly, 2 $\times$  YT medium was inoculated with diluted stationary phase cells in a 96-well microtiter plate (flat bottomed, polystyrene, Corning). The final dilution represents ~1:500 dilution of the original stationary phase culture. Each experiment examined at least three independent stationary phase cultures, and outgrowth from each culture was tested in two independent wells of the microtiter plate. Growth at 37°C was monitored as OD<sub>595</sub> in an absorbance microplate reader (Biotek Instruments, ELx808); readings were taken every 15 min.

## RESULTS

### The central bulged regions of 6S-1 RNA and 6S-2 RNA are responsible for directing efficiency of pRNA synthesis

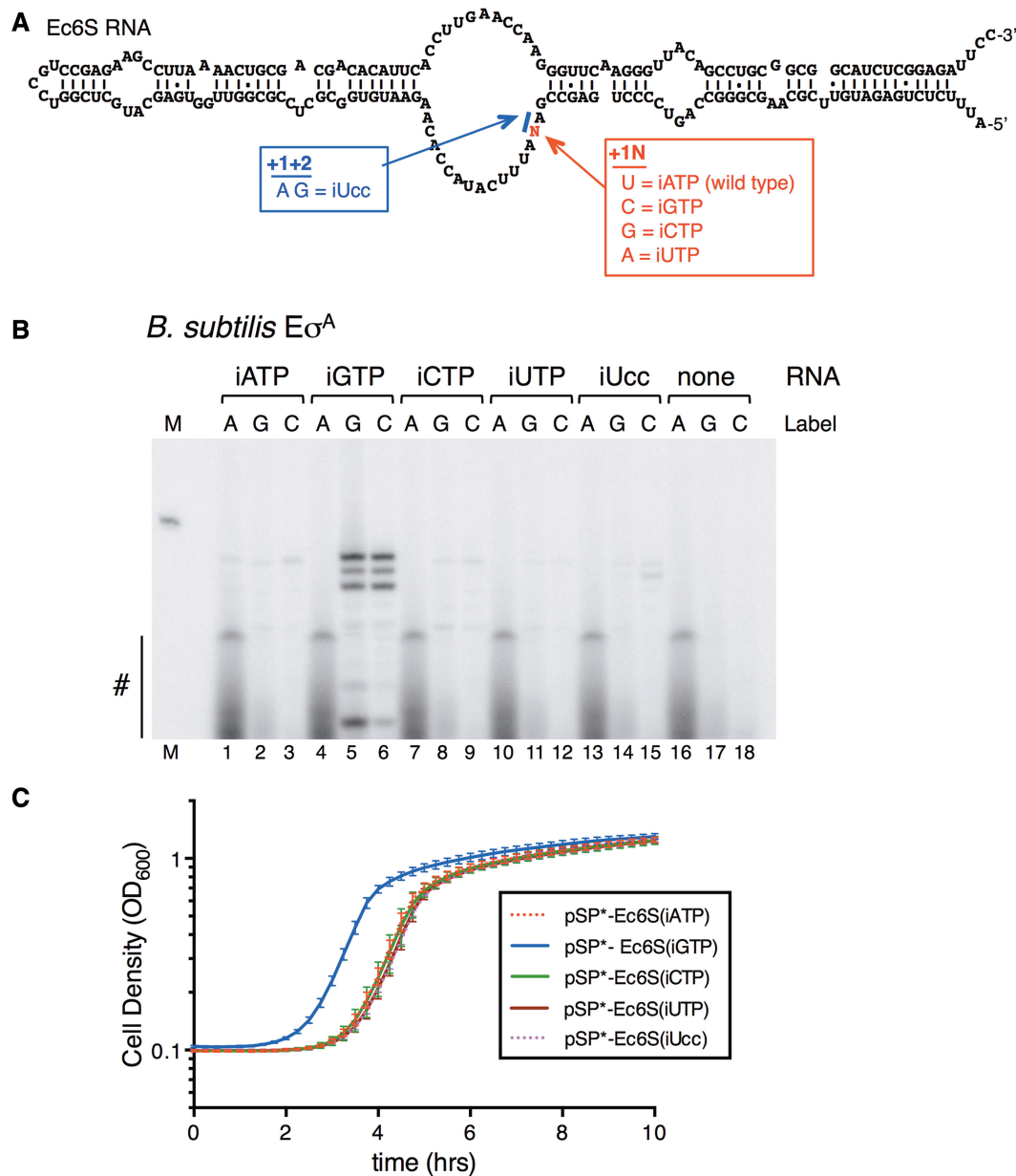
Previous work has suggested that 6S-2 RNA does not support efficient pRNA synthesis *in vivo*, in contrast to 6S-1 RNA, which does support robust pRNA synthesis during outgrowth from stationary phase (7,16,17). In agreement with *in vivo* observations, *in vitro* pRNA synthesis from 6S-2 RNA is lower than from 6S-1 RNA, although pRNA synthesis from 6S-2 RNA has been observed at different levels under different assay conditions (7,16) (see 'Discussion' section). In an effort to explain the biologically significant differences in behavior of 6S-1 and 6S-2 RNAs for pRNA synthesis *in vivo*, we sought to understand what features in RNA templates could determine the efficiency of pRNA synthesis in general. We hypothesized that sequence surrounding the start site of pRNA synthesis might be important. To test whether sequences within the central bulge were sufficient to direct efficient pRNA synthesis or whether differences in sequences between 6S-1 RNA and 6S-2 RNA outside of the central region were critical, mutant 6S-1 and 6S-2 RNAs were generated in which the central bulge of each were swapped (Figure 1A). pRNA synthesis with BsE $\sigma^A$  on *in vitro* generated RNAs was examined using <sup>32</sup>P  $\alpha$ -UTP so that all full-length RNA products would be labeled, regardless of iNTP (Figure 1B). We observed that the presence of Bs6S-1(6S-2bulge) RNA resulted in reduced pRNA levels compared with Bs6S-1 RNA (compare lanes 2 and 1), whereas the presence of Bs6S-2(6S-1bulge) RNA resulted in increased pRNA relative to Bs6S-2 (compare lanes 4 and 3). These results

suggest that the sequence in the central bulge region is most important for efficiency of pRNA synthesis. All RNAs and mutants described here retained the ability to bind BsE $\sigma^A$  (and EcE $\sigma^{70}$ ) efficiently under conditions tested as measured by native gel electrophoresis (Supplementary Figure S1), and all pRNA synthesis reactions were done with excess RNA to minimize any small binding differences. Therefore, changes in levels of pRNA generated reflect changes in efficiency of pRNA synthesis.

Next, we tested whether the *in vitro* behavior of these RNAs represented their behavior *in vivo*. Prior work demonstrated that *B. subtilis* cells that only expressed Bs6S-2 RNA (i.e. cells lacking Bs6S-1 RNA) were delayed in outgrowth from stationary phase [(7), see Figure 1C]. Therefore, we tested whether expression of Bs6S-1(6S-2bulge) RNA, which had a low pRNA synthesis efficiency *in vitro*, would similarly delay outgrowth. Indeed, expression of Bs6S-1(6S-2bulge) RNA in *B. subtilis* cells lacking both endogenous RNAs (*ΔbsrAΔbsrB*) led to a delay in outgrowth similar to expression of Bs6S-2 RNA (Figure 1C). In contrast, expression of Bs6S-2(6S-1bulge) RNA did not delay outgrowth, similar to observations with Bs6S-1 RNA (Figure 1D). Furthermore, we found that expression of Bs6S-2(6S-1bulge) RNA was able to rescue normal outgrowth timing when expressed in *B. subtilis* cells that only express 6S-2 RNA (*ΔbsrA*), but that expression of Bs6S-1(6S-2bulge) RNA had no effect (data not shown). These results suggest that the *in vitro* assay conditions examined here were able to recapitulate the *in vivo* observations for pRNA synthesis.

### The use of RNA templates for pRNA synthesis by BsE $\sigma^A$ is strongly influenced by iNTP identity

pRNA generated from Bs6S-1 RNA (pRNA<sub>Bs6S-1</sub>) initiates with GTP, whereas pRNA generated from Bs6S-2 RNA (pRNA<sub>Bs6S-2</sub>) initiates with ATP (16), leading us to hypothesize that initiating nucleotide identity might be important for efficiency of pRNA synthesis. Therefore, we next made mutant RNAs with changes in the template +1 position to direct Bs6S-1 RNA to initiate with ATP [Bs6S-1(iATP) RNA] or Bs6S-2 RNA to initiate with GTP [Bs6S-2(iGTP) RNA]. Previously, a similar RNA to Bs6S-1(iATP) RNA [i.e. 6S-1(C40U)] was shown to direct reduced pRNA synthesis *in vivo* (16). However, under *in vitro* conditions examined here, the behavior of both the Bs6S-1(iATP) and Bs6S-2(iGTP) mutants was complex with substantial changes in the level of abortive relative to full-length products making analysis of the specific effect(s) of iNTP identity difficult. Therefore, we chose to move to a heterologous system and examine the behavior of Ec6S RNA and mutant Ec6S RNAs to investigate the potential role of individual nucleotide positions one at a time (see Figure 2A). pRNA generated from wild-type Ec6S RNA (pRNA<sub>Ec6S</sub>) initiates with ATP by EcE $\sigma^{70}$  [(5,6) see Figure 3]. However, we observed that Ec6S RNA does not serve as an efficient template for pRNA synthesis by BsE $\sigma^A$  (Figure 2B, lane 3), although this RNA binds to BsE $\sigma^A$  efficiently under conditions tested (Supplementary Figure S1).

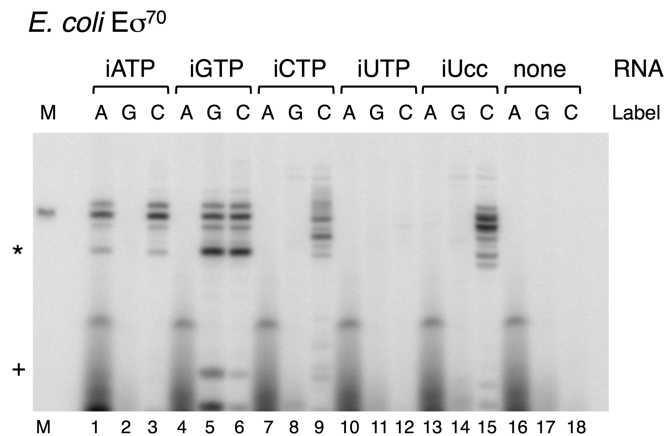


**Figure 2.** Efficient pRNA synthesis by BsE $\sigma^A$  requires initiation with GTP. (A) Schematic of Ec6S RNA in a secondary structure supported by phylogenetic and experimental analyses (3,4). The +1 position is indicated by red N in the sequence, with changes and names of RNA mutants in the red box. For Ec6S(iUCC), the changes in position +1 and +2 are shown in the blue box. (B) pRNAs generated *in vitro* by BsE $\sigma^A$  from various RNAs or no RNA (indicated at top) when containing  $^{32}\text{P}$   $\gamma$ -GTP,  $^{32}\text{P}$   $\gamma$ -ATP or  $^{32}\text{P}$   $\alpha$ -CTP (indicated by A, G or C label at top) were visualized on a denaturing gel. Lane M contains a 5' end labeled oligonucleotide 19 nt in length for size comparison. Hash mark indicates a smear present in the  $^{32}\text{P}$   $\gamma$ -ATP preparation, as it is present in the absence of RNA in lane 16. (C) Growth of *B. subtilis* cells as monitored by optical density at 595 nm (OD<sub>595</sub>) in an absorbance plate reader after stationary phase cells were diluted  $\sim$ 1:500 into 2 $\times$  YT medium. Growth was of *B. subtilis* *AbsrA**AbsrB* cells (KW590) containing plasmids pSP\*-Ec6S(iATP) (dotted red), pSP\*-Ec6S(iGTP) (blue), pSP\*-Ec6S(iCTP) (green), pSP\*-Ec6S(iUTP) (brown) or pSP\*-Ec6S(iUcc) (dotted pink). Data shown are from one representative experiment with three biological replicates. Similar results were observed in at least three experiments. Error bars correspond to  $\pm$  standard deviations from the averages.

Examination of a mutant EcRNA that changed the template position to direct initiation with GTP [Ec6S(iGTP)] revealed that this RNA does support efficient pRNA synthesis by BsE $\sigma^A$  (Figure 2B, lane 6).

To test which iNTP was actually being used for pRNA synthesis, reactions were done containing  $^{32}\text{P}$   $\gamma$ -GTP or  $^{32}\text{P}$   $\gamma$ -ATP in which only products that initiate with GTP or ATP, respectively, would be labeled. In

addition, reactions containing  $^{32}\text{P}$   $\alpha$ -CTP were done to allow visualization of pRNA products, regardless of iNTP. There was substantial contamination of a smeary small product in the  $^{32}\text{P}$   $\gamma$ -ATP label (marked by hash in Figure 2B) that was not from pRNA synthesis, as it was present in reactions with no added RNA (Figure 2B, see lane 16) and was observed at varying levels in different lots of radiolabeled nucleotide. As predicted, pRNA synthesis



**Figure 3.** EcE $\sigma^{70}$  can use any NTP for initiation of efficient pRNA synthesis. pRNAs generated *in vitro* by EcE $\sigma^{70}$  from various RNAs or no RNA (indicated at top) when containing  $^{32}\text{P}$   $\gamma$ -GTP,  $^{32}\text{P}$   $\gamma$ -ATP or  $^{32}\text{P}$   $\alpha$ -CTP (indicated by A, G or C label at top) were visualized on a denaturing gel. Lane M contains a 5' end labeled oligonucleotide 19nt in length for size comparison. These reactions were done at the same time, as the experiment shown in Figure 2B and therefore also have the smear from the  $^{32}\text{P}$   $\gamma$ -ATP preparation. The shorter product and larger abortive RNA that are enhanced from 6S(iGTP) RNA are indicated by asterisk and plus sign, respectively.

from Ec6S(iGTP) RNA with BsE $\sigma^A$  initiated with GTP as indicated by the presence of labeled products when  $^{32}\text{P}$   $\gamma$ -GTP was included in the reaction (see lane 5). As has been previously observed for pRNA synthesis with both BsE $\sigma^A$  and EcE $\sigma^{70}$ , several bands are present that represent 3' end heterogeneity of the pRNA products as demonstrated by sequencing (5,16). Ec6S(iGTP) RNA did not initiate pRNA with ATP as indicated by the lack of products when  $^{32}\text{P}$   $\gamma$ -ATP was included (see lane 4). In addition, pRNA products observed were similar when labeled internally with  $^{32}\text{P}$   $\alpha$ -CTP, indicating that most, if not all, of the pRNAs initiated with GTP. In contrast, little pRNA synthesis was observed from Ec6S(iATP) RNA with any of the labeled nucleotides (see lanes 1–3). We also observed that BsE $\sigma^A$  did not efficiently initiate pRNA synthesis from templates initiating with CTP or UTP (see lanes 9 and 12). We have found that the presence of the dinucleotide UU in the +1+2 or +2+3 positions can lead to low pRNA synthesis and sometimes a high abortive ratio with EcE $\sigma^{70}$  (KMW unpublished results). Therefore, we also tested the potential for initiating with UTP on Ec6S(iUcc) RNA in which the +2 position of the template also was altered to prevent a UU dinucleotide start, but pRNA synthesis remained extremely low (see lane 15).

As we were using a heterologous RNA (e.g. Ec6S and variants), we next tested whether the behavior observed under the *in vitro* conditions tested here with BsE $\sigma^A$  also would be reflected in *B. subtilis* cells. Consistent with the observed low pRNA synthesis *in vitro*, expression of Ec6S(iATP), Ec6S(iCTP), Ec6S(iUTP) or Ec6S(iUcc) in *B. subtilis* cells lacking both endogenous RNAs (*AbsrA* $\Delta$ *AbsrB*) led to a delay in outgrowth similar to expression of Bs6S-2 RNA (Figure 2C). In contrast,

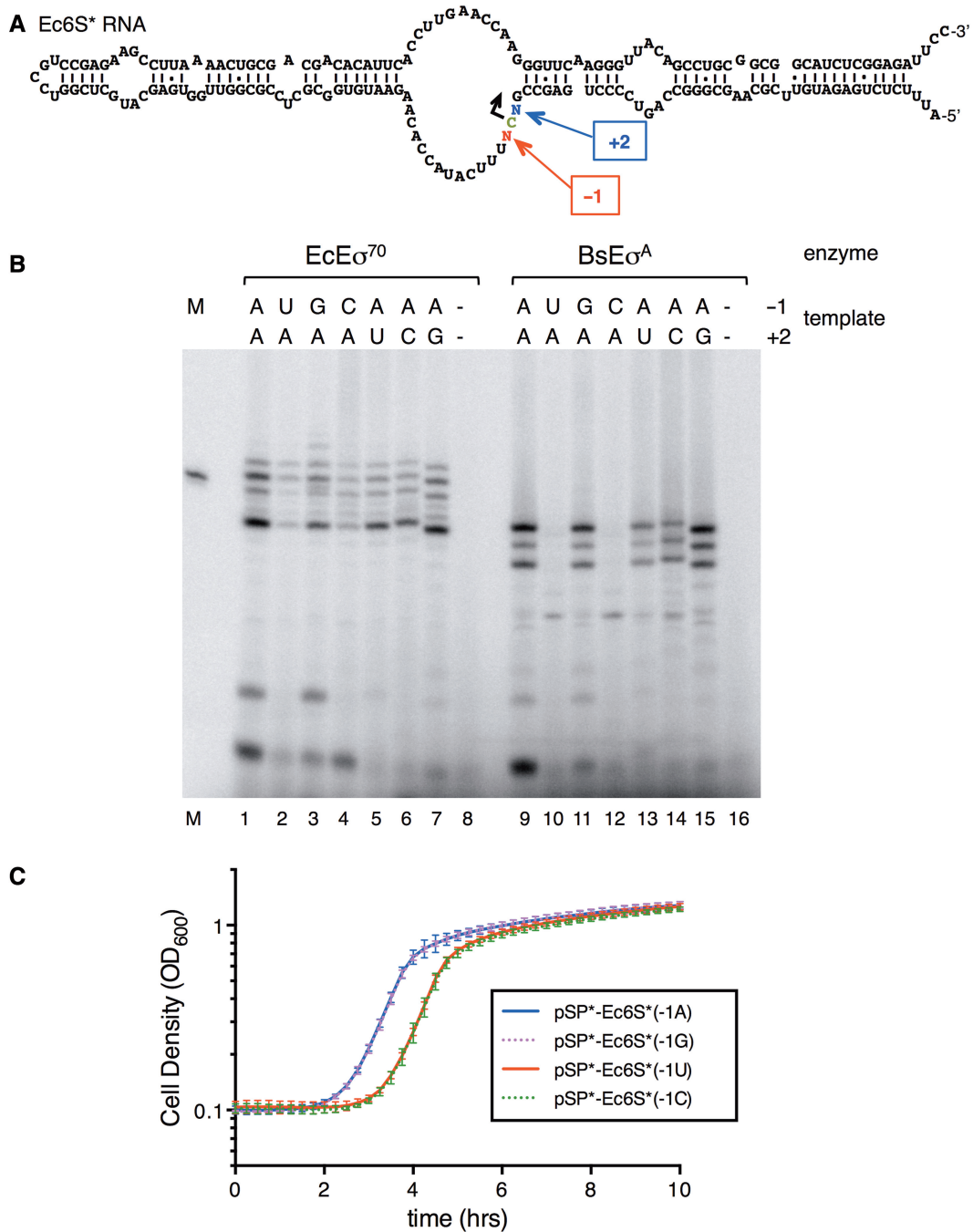
expression of Ec6S(iGTP) RNA, which did support efficient pRNA synthesis, did not delay outgrowth. Again consistent with *in vitro* behavior, expression of Ec6S(iGTP) RNA, but not the other mutant RNAs, restored normal outgrowth timing in *B. subtilis* cells that only express 6S-2 RNA (*AbsrA*), similar to observations when Bs6S-1 RNA is expressed (data not shown).

#### In contrast to BsE $\sigma^A$ , EcE $\sigma^{70}$ does not have a strong preference for iNTP identity for pRNA synthesis on RNA templates

We were surprised that the wild-type Ec6S RNA [Ec6S(iATP) RNA] does not serve as a template for BsE $\sigma^A$ , as it clearly does for EcE $\sigma^{70}$  (5,6). To examine any potential effects of iNTP on EcE $\sigma^{70}$ -dependent pRNA synthesis, we next examined pRNA synthesis from the Ec6S RNA variants. Again, reactions contained  $^{32}\text{P}$   $\gamma$ -GTP,  $^{32}\text{P}$   $\gamma$ -ATP or  $^{32}\text{P}$   $\alpha$ -CTP to distinguish initiation with GTP, ATP or another NTP (i.e. UTP or CTP). The wild-type Ec6S RNA (i.e. iATP) does serve as an efficient template for EcE $\sigma^{70}$  and does initiate with ATP, as indicated by labeling of products when  $^{32}\text{P}$   $\gamma$ -ATP is present, and in agreement with previous work (5,6) (Figure 3, lane 1). Once again, a ladder of products was observed that represent heterogeneous 3' ends as has been previously reported (5). EcE $\sigma^{70}$  also uses Ec6S(iGTP) for pRNA synthesis with a GTP start (Figure 3, lane 5), as indicated by the presence of labeled products when  $^{32}\text{P}$   $\gamma$ -GTP is present. However, the pattern of products observed was altered slightly in two ways. First, there was an enhancement of a slightly shorter product (marked by asterisk in Figure 3), which may be similar to the 13 nt pRNA observed for pRNA synthesis on Ec6S RNA by EcE $\sigma^{70}$  in the presence of heparin (37). This shorter product observed with EcE $\sigma^{70}$  on the Ec6S(iGTP) RNA is the same size as the predominant full-length pRNA generated by BsE $\sigma^A$  on Ec6S(iGTP), which may represent a connection between the iNTP identity and length of pRNA generated, although release, and thus pRNA length, is a complex process (18,37). The second change in pattern of products is the appearance of a band (marked by plus sign in Figure 3) presumed to be an abortive product based on size. EcE $\sigma^{70}$  also is able to initiate with CTP and UTP on Ec6S(iCTP) and Ec6S(iUcc) as well (lanes 9 and 15). EcE $\sigma^{70}$  is not able to generate pRNA products from the Ec6S(iUTP) RNA (lane 12), which we presume is due to a disfavored UU start. EcE $\sigma^{70}$ -dependent pRNA synthesis from these RNA variants all generate multiple product bands and varying levels of shorter presumed abortive products. Therefore, it is difficult to precisely quantitate the relative utilization of the different RNA templates to each other. Nevertheless, it is clear that all four iNTPs are fairly well tolerated by EcE $\sigma^{70}$ , which is in stark contrast to BsE $\sigma^A$ .

#### The –1 position has a greater influence on BsE $\sigma^A$ than EcE $\sigma^{70}$ for pRNA synthesis

We next tested the potential role of the –1 and +2 positions on efficiency of pRNA synthesis by BsE $\sigma^A$  and



**Figure 4.** The  $-1$  position contributes to efficiency of pRNA synthesis for BsE $\sigma^A$  and EcE $\sigma^{70}$ , but the  $+2$  position has minimal impact. (A) Schematic of Ec6S(iGTP) RNA (indicated as Ec6S\* here). The  $-1$  and  $+2$  positions where changes were made for various mutants are indicated in red and blue, respectively. The  $+1C$  position, which directs initiation of pRNA synthesis with GTP, is indicated in green. (B) pRNAs generated *in vitro* by BsE $\sigma^A$  (right) and EcE $\sigma^{70}$  (left) from various RNAs (indicated at top) when labeled with  $^{32}P$   $\gamma$ -GTP were visualized on a denaturing gel. Lane M contains a  $5'$  end labeled oligonucleotide 19 nt in length for size comparison. There are slight changes in apparent size of pRNA resulting from changes in sequence at the  $+2$  position consistent with the change in molecular weight of each NTP (i.e. GTP > ATP > UTP > CTP). (C) Growth of *B. subtilis* cells as monitored by optical density at 595 nm (OD<sub>595</sub>) in an absorbance plate reader after stationary phase cells were diluted  $\sim 1:500$  into  $2\times$  YT medium. Growth was of *B. subtilis*  $\Delta$ bsrA $\Delta$ bsrB cells (KW590) containing plasmids pSP\*-Ec6S(-1A) (blue), pSP\*-Ec6S(-1G) (dotted pink), pSP\*-Ec6S(-1U) (red) or pSP\*-Ec6S(-1C) (dotted green). Data shown are from one representative experiment with three biological replicates. Similar results were observed in at least three experiments. Error bars correspond to  $\pm$  standard deviations from the averages.

EcE $\sigma^{70}$  by examining additional Ec6S RNA variants (see Figure 4). For these experiments, all RNA templates were based on Ec6S(iGTP) RNA; therefore, pRNA synthesis could be directly compared between BsE $\sigma^A$  and EcE $\sigma^{70}$ ,

and all reactions were labeled with  $^{32}P$   $\gamma$ -GTP. RNA names indicate the template position identity at the relevant position. For BsE $\sigma^A$ , there was a strong preference for A or G in the  $-1$  position on the template RNA,

as minimal pRNA products were observed for Ec6S\*(-1U) and Ec6S\*(-1C) RNAs compared with Ec6S\*(-1A) and Ec6S\*(-1G) RNAs (compare lanes 10 and 12 with 9 and 11). In addition, expression of Ec6S\*(-1U) RNA or Ec6S\*(-1C) RNA in *B. subtilis* lacking both endogenous RNAs resulted in an outgrowth delay, but expression of Ec6S\*(-1A) RNA or Ec6S\*(-1G) RNA did not (Figure 4C), consistent with *in vitro* observations of efficiency of pRNA synthesis.

Although EcE $\sigma^{70}$  also shows some preference for A or G in the -1 template position, the preference is not as strong, as there was still significant pRNA observed for Ec6S\*(-1U) and Ec6S\*(-1C) RNAs (Figure 4B, compare lanes 1 and 3 with 2 and 4). Once again, there are some subtle changes in patterns of pRNAs from the different templates making exact quantitation difficult, but it is the trends and differences between BsE $\sigma^A$  and EcE $\sigma^{70}$  that we are focused on here.

In contrast to the -1 and +1 positions, we found that any preferences observed at the +2 position were modest and similar between BsE $\sigma^A$  and EcE $\sigma^{70}$ . Specifically, there was a small, but reproducible, preference for A or G over U or C at the +2 template position (Figure 4B, compare lanes 1 and 7 with 5 and 6 or lanes 9 and 15 with 13 and 14).

## DISCUSSION

We set out to understand properties of RNA templates that contribute to their use for pRNA synthesis. We found that sequences within Bs6S-1 or Bs6S-2 central bulge regions are sufficient to determine efficiency of pRNA synthesis by BsE $\sigma^A$  (Figure 1). It appears that the identity of the initiating nucleotide is particularly important, as BsE $\sigma^A$  generated pRNA much more efficiently when initiating with GTP compared with ATP, CTP or UTP (Figure 2). In contrast, EcE $\sigma^{70}$  efficiently initiates RNA synthesis from RNA templates with any of the four nucleotides (Figure 3) including from Bs6S-2 RNA (data not shown). These observations clearly demonstrate a difference in behavior of these two enzymes and may have direct bearing on the lack of detectable pRNA from Bs6S-2 RNA in *B. subtilis* cells, as pRNA<sub>Bs6S-2</sub> initiates with ATP. In addition to the contribution of the iNTP identity to pRNA synthesis, the -1 position also influences efficiency of pRNA synthesis. Both BsE $\sigma^A$  and EcE $\sigma^{70}$  prefer an A or G at the -1 position in the template, but BsE $\sigma^A$  appears to have a stronger preference, as there is little pRNA synthesis if the -1 position is U or C (Figure 4). In contrast, there may only be a slight preference for A or G at the +2 position for both BsE $\sigma^A$  and EcE $\sigma^{70}$  (Figure 4). Our results provide a direct mechanism by which Bs6S-2 RNA behaves differently than Bs6S-1 RNA. They also highlight important differences in requirements for RNA synthesis by BsE $\sigma^A$  and EcE $\sigma^{70}$  and raise questions about what differences between these enzymes are responsible.

The discovery of two 6S-like RNAs in *B. subtilis*, both of which bind to BsE $\sigma^A$ , initially raised questions about whether these two RNAs were redundant or whether they

might provide independent functionality to the cell (3,4). Phenotypic analyses of cells lacking one or the other RNA demonstrated that these two RNAs do not serve the same purpose, as they are not interchangeable. For instance, cells lacking 6S-1 RNA but expressing 6S-2 RNA are delayed in their ability to restart growth when stationary phase cells encounter fresh nutrients (7). Given that 6S-2 RNA does not appear to serve as an efficient template for pRNA synthesis during outgrowth, we suggest that it remains bound to BsE $\sigma^A$ , resulting in a delay in growth presumed to be due to inappropriate regulation of transcription at this time. However, the mechanism underlying this change in behavior of 6S-2 RNA had not been uncovered previously. Here, we demonstrate that the sequence in the central bulge region, specifically the sequence surrounding the region where pRNA synthesis initiates, is responsible for the efficiency of pRNA synthesis by BsE $\sigma^A$ . Interestingly, Bs6S-2 RNA not only initiates with ATP, which is highly disfavored for BsE $\sigma^A$ , but also has an A at the +2 position, which is sub-optimal. In contrast, Bs6S-1 has the optimal sequence at -1, +1 and +2 positions, which probably contributes to its high usage. Although EcE $\sigma^{70}$  is much more promiscuous in that it can use any iNTP and is less sensitive to changes at position -1, the wild-type Ec6S RNA also has a strong initiating sequence with -1A +1U and +2A.

Others (16) have observed higher levels of *in vitro* pRNA synthesis from Bs6S-2 RNA by BsE $\sigma^A$  than observed by us [(7) and see above]. There are several differences in assay conditions between these studies, but we suggest of note is a difference in NTP concentration (200  $\mu$ M versus 50  $\mu$ M). We have observed that the relative utilization of Bs6S-1 and Bs6S-2 RNAs *in vitro* by BsE $\sigma^A$  is strongly influenced by NTP concentration (Supplementary Figure S2). Given that conditions used here correlate well with *in vivo* observations (e.g. low Bs6S-2 directed pRNA synthesis *in vitro*, undetectable pRNA *in vivo*), we suggest they are most relevant to expand our understanding of the roles and behavior of Bs6S-1 and Bs6S-2 RNAs *in vivo*.

For example, understanding the mechanism underlying the difference in Bs6S-1 and Bs6S-2 to support efficient pRNA synthesis gives good insight into why cells lacking Bs6S-1 RNA are delayed in outgrowth. However, this difference in behavior is probably not the only difference in activity between these two RNAs. Cells lacking 6S-1 RNA also exhibit an early sporulation phenotype, which has been postulated to result from a more rapid depletion of nutrients by these cells compared with wild-type cells (19). However, cells lacking 6S-2 RNA sporulate with normal timing, and it is difficult to imagine how changes in pRNA synthesis rates would alter cell behavior after entry into stationary phase when pRNA synthesis of 6S-1 RNA already is low (7,16). Future experiments are needed to continue to dissect the role of both Bs6S-1 and Bs6S-2 RNAs at various times in growth.

One question raised by these experiments is what determines the difference in behavior of BsE $\sigma^A$  and EcE $\sigma^{70}$  on RNA templates. Bacterial RNA polymerases are highly conserved, and it is often assumed that housekeeping enzymes will behave similarly in regards to their



promoter recognition and basic polymerization activities. However, there are several examples illustrating that these assumptions are not always appropriate. For instance, many *E. coli* promoters are not active in *B. subtilis* (38,39), indicating there are distinctions in promoter recognition and/or utilization, although the details about the underlying mechanisms remain unclear. Differences also have been observed in the frequency of abortive transcription, pausing at intrinsic pause sequences and even the general transcription rates (40). In addition, differences in open complex stability have been shown (41,42), but 6S RNAs examined here bind both BsE $\sigma^A$  and EcE $\sigma^{70}$  similarly and tightly (7). Whether the differences in efficiency of pRNA synthesis are due to the same underlying mechanisms that determine differences in transcription remains to be seen.

Perhaps surprisingly, the reported differences in the behavior of BsE $\sigma^A$  and EcE $\sigma^{70}$  do not include differences in preference for iNTP in transcription from DNA templates, suggesting it may be a special feature of RNA-templated RNA synthesis. Both BsE $\sigma^A$  and EcE $\sigma^{70}$  appear to prefer to initiate transcription with ATP or GTP, with a slight preference for ATP in compiled promoter sequences (21–23), although frequency of use may not necessarily represent a biochemical preference. Examples of initiation of transcription with UTP and CTP at some genes also are known for both organisms. Therefore, the strong preference for GTP here by BsE $\sigma^A$  may be unique to the use of an RNA template. Given that we have observed the strong preference for iGTP on both *B. subtilis* and *E. coli* RNAs by BsE $\sigma^A$ , it seems unlikely that this preference is driven by *B. subtilis*-specific RNA elements outside the initiating region in Bs6S-1 or Bs6S-2 RNA. However, it is possible that there are differences in the general manner in which BsE $\sigma^A$  interacts with these RNA templates that make it more sensitive to iNTP. Both BsE $\sigma^A$  and EcE $\sigma^{70}$  bind all the RNAs discussed here tightly under conditions tested, indicating differences are unlikely to be due to differential affinities between different RNA:protein pairs, and any such differences would have to be entirely dependent on the +1 template position, as changes of a single nucleotide were sufficient to alter activity. Further study elucidating the details of the RNA:protein interaction in both species will be needed to fully understand the similarities and differences between these two enzymes.

Although there are some differences in extent of preference, both BsE $\sigma^A$  and EcE $\sigma^{70}$  exhibit similar preferences for an A or G at the –1 position. A similar preference is observed for both enzymes on DNA promoters as well (e.g. pyrimidine at –1 in the non-template strand, which is equivalent to a purine on the template strand seen here) (23). Similarly, in some cases, the identity of the +2 position has been implicated as well on DNA templates, which might be consistent with the mild effects observed here for RNA templates by both BsE $\sigma^A$  and EcE $\sigma^{70}$ .

Several well-studied promoters are known to be sensitive to concentration of iNTP for regulation. In these cases, the identity of the iNTP is important to determine which NTP the promoter is sensitive to, but the identity per se is not important to allow regulation. For instance,

both *B. subtilis* and *E. coli* rRNA promoters are sensitive to iNTP concentrations (28,43). However, changing the iNTP identity (e.g. A to G in *E. coli* or G to A in *B. subtilis*) does not change the ability to be regulated but only the identity of which NTP they are sensitive to. Mechanistic studies have demonstrated it is the kinetics of transcription initiation events that are important to allow iNTP sensing (44). It is interesting to postulate that the kinetics of initiation events in pRNA synthesis could be different between *B. subtilis* and *E. coli* leading to altered iNTP requirements, although further experiments are needed for a full understanding.

pRNA synthesis has been shown to relieve RNA polymerase from 6S RNA regulation (5,7,18,45), and that this release is important to allow efficient outgrowth from stationary phase in both *B. subtilis* and *E. coli* (7). The switch from 6S RNA inhibition to release of RNA polymerase has been hypothesized to result from changes in NTP concentrations. It is possible that the concentration of the iNTP may be of primary importance, similar to observations at several DNA promoters. If so, it is notable that *B. subtilis* uses GTP rather than ATP for initiation, given that changes in concentrations of GTP and ATP are not always coordinated in this organism. Although both ATP and GTP increase during outgrowth, during the transition into stationary phase GTP levels decrease while ATP levels increase (46). These changes are important in *B. subtilis* for regulation of iNTP-sensitive promoters that decrease (with iGTP) or increase (with iATP) during stringent response (22,47,48). Therefore, it would appear that GTP concentration may be a better signal of overall nutritional status generally in *B. subtilis*. In contrast, *E. coli* GTP and ATP levels appear to both follow nutrient levels coordinately. Although these observations do not explain the mechanism of why BsE $\sigma^A$  does not prefer to use iATP in pRNA synthesis, it suggests that pRNA synthesis, and therefore 6S RNA regulation, might be inappropriate in *B. subtilis* if it did.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1, Supplementary Figures 1 and 2 and Supplementary Methods.

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