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Full Paper

Novel heat shock response mechanism mediated by the initiation nucleotide of transcription

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Aya Sato, Misaki Takamatsu, Satona Kobayashi, Michio Ogawa, Yuh Shiwa, Satoru Watanabe, Taku Chibazakura, and Hirofumi Yoshikawa*

Department of Bioscience, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo, 158-8502, Japan

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*Corresponding Author:

Hirofumi Yoshikawa
Department of Bioscience
Tokyo University of Agriculture
1-1-1 Sakuragaoka, Setagaya-ku
Tokyo 156-8502, Japan
Tel: +81-3-5477-2758; Fax: +81-3-5477-2668; E-mail: hiyoshik@nodai.ac.jp
Summary

Among SigA-dependent promoters in *Bacillus subtilis*, we compared the nucleotide sequences of heat shock responding and non-responding promoters. Chimeric promoter experiments revealed that the heat shock response could be ascribed to the initiation nucleotide (iNTP) of the transcription. Our *in vivo* reporter assay results indicated that a full response was achieved using GTP, a reduced response was observed using ATP, and no additional expression was observed using UTP or CTP.

We then investigated the *in vitro* transcription assay in more detail. Enhanced transcription that was dependent upon the iNTP was observed when heat treatment was administered during the pre-initiation period. We next analyzed the efficiency of open complex formation using potassium permanganate footprinting, and our results revealed an increase in the ratio of open complex formation at elevated temperatures. Based on this, we suggest that the overall intensification of transcription at high temperatures was derived from the high efficiency of open complex formation together with the high affinity of RNA polymerase (RNAP) for the initiation nucleotide GTP.

To determine if this mechanism observed in *B. subtilis* RNAP is common among bacterial species, we performed similar experiments using *Escherichia coli* RNAP. Our results indicated that *E. coli* RNAP also exhibited both temperature- and iNTP-dependent enhancement of transcription. Although the temperature ranges and the ratios of enhancement are somewhat different, the overall heat shock response mechanism mediated by the iNTP of transcription appears to be conserved among bacterial RNAP.

**Key Words:** heat shock response, *Bacillus subtilis*, transcription, initiation nucleotide
Introduction

Bacterial heat shock responses have been extensively studied, and two major mechanisms that depend upon either the heat shock sigma factor or the repressor-operator structure (CIRCE element) function to mediate the expression of major heat-responsive genes. Details regarding the heat shock response mechanisms in *Bacillus subtilis* are grouped into six classes that include class I to class VI (Schumann 2003). Briefly, the *grpE-dnaK-dnaJ* operon and the *groES-groEL* operon, both of which are regulated by the HrcA repressor protein, are classified as class I. A specific inverted repeat sequence CIRCE (controlling inverted repeat for chaperone expression) element exists downstream of the transcription start point of each operon. HrcA binds to CIRCE and represses the transcription of both operons. In response to heat shock, the amount of HrcA bound to CIRCE is thought to be decreased, thus resulting in the transcriptional induction of both operons. Genes transcribed by Sigma B that functions as one of the secondary sigma factors to transcribe not only heat shock genes but also genes responding to energy depression or environmental stresses are classified into class II. Class III consists of a series of proteins that are regulated by CtsR repressor containing Clp proteins. ClpC forms Clp ATPase that is a member of the Hsp100 family and is essential in response to high-temperature conditions. ClpP is a peptidase that degrades misfolded proteins that cooperate with ClpC and ClpX (Frees et al., 2004). ClpE, another Hsp100/Clp ATPase, is also regulated by CtsR (Derre et al., 1999). Class IV includes *htpG*, a gene that is induced to nearly 10-fold levels by heat shock at both the transcriptional and translational levels. This gene is suggested to encode a molecular chaperone, and seven nucleotides (GAAAAGG) located downstream of the SigA-dependent promoter of *htpG* are speculated to regulate the heat shock response. Class V includes *htrA* and *htrB*, both of which are positively regulated by the two component-regulatory system CssRS and are suggested to code for membrane-anchored proteins. CssRS recognizes not only heat shock but also secretion stress. All other genes, with the exception of those described above, are classified into class VI as regulation mechanisms-unknown, and these include the *lonA* operon, *clpX*, and *ftsH* as representatives. As the majority of heat shock proteins are included in class VI, the existence of an unknown heat shock response mechanism has been suggested (Hecker et al., 1996, Helmann et al., 2001, Schumann 2003, 2016).

During the course of this study, we observed that the *B. subtilis* chaperone gene lacking the CIRCE element still responded to heat shock. Although it is known that the *B. subtilis* major sigma factor $\sigma^A$-dependent promoters typically require the participation of
transcriptional regulatory factor(s) for the heat shock response, we discovered that the class I
groES/L promoter responds to heat shock without the requirement of a CIRCE element.
Based on this, it is likely that there are certain factor(s) within the promoter sequence that
regulate the heat shock response. We performed detailed analyses of this phenomenon and
verified the novel heat shock response mechanism that is inherent in the transcription
initiation mechanism.

The expression of these heat shock-responsive genes is primarily regulated at the
transcriptional level. Transcription proceeds according to the activity of RNA polymerase
(RNAP) over several steps, and a number of reports have previously reviewed this process
(Murakami and Darst, 2003, Kapanidis et al., 2006, Haugen et al., 2008, Ross and Gourse,
2009, Gaballa et al., 2012, Henderson et. al., 2019). According to the best of our knowledge,
the sigma factor binds to the core enzyme to form the holoenzyme that then recognizes the
promoter sequence upstream of the gene and subsequently initiates RNA synthesis. The
RNAP binds to the DNA strand to form a closed complex (RP$_C$), then turns to an open
complex (RP$_O$) via its intermediate (RP$_I$). The *Escherichia coli* open complex is known to be
stable, while that of *B. subtilis* is unstable (Whipple and Sonenshein, 1992, Prajapati et al.,
2016). In the RP$_C$ state, DNA is double-stranded and does not enter the main channel of the
RNAP. The 1.1 region of the sigma factor begins to leave RNAP in the state of RP$_I$, and
DNA then begins to enter the main channel. At this point, the unwinding of DNA is believed
to begin and then become complete in the RP$_O$ state. The template strand of the DNA enters
the main channel for RNA synthesis. In the state of the scrunched complex, NTP enters from
the second channel, and RNA synthesis begins. The -10 region or its downstream DNA
strand extrudes transiently from the main channel prior to promoter clearance (the
dissociation of the promoter from the RNAP complex). The abortive transcription reaction
that repeats the synthesis of short RNA (from several to several tens of nucleotides) then
begins. Through this reaction, RNAP escapes from the promoter region, forms the elongation
complex (RP$_E$), and synthesizes the complete RNA. In this study, we aimed to determine
which step is responsible for the heat shock response.

**Materials and Methods**

**Bacterial strains, primers, and growth media.** The *B. subtilis* strains used in this study are
listed in Table S1. In general, the *B. subtilis* strains harboring various promoter-*bgaB* fusions
were constructed as follows. Promoter regions were amplified by PCR and cloned into the
**amyE**-integration vector pDLd (Nanamiya et al. 1998) after digestion with EcoRI and BamHI. In these constructs, the SD sequence and the following structural gene of the thermo-tolerant β-galactosidase gene are transcriptionally fused to each promoter region. Amplified plasmids using *E. coli* strain DH5α were used to transform *B. subtilis* 168 to chloramphenicol resistance, and the constructs were confirmed by sequencing amyE loci. The specific procedures used to construct each strain are described in the Methods section of the supplemental material. The primers used in this study are listed in Table S2. All bacterial strains were grown in Luria–Bertani (LB) medium at the indicated temperatures. Antibiotics were used at the following concentrations: erythromycin, 1 μg ml⁻¹; chloramphenicol, 5 μg ml⁻¹; neomycin, 5 μg ml⁻¹; spectinomycin, 100 μg ml⁻¹.

**β-Galactosidase activity assays.** The assay strains were grown in liquid LB medium at 37°C, and during the logarithmic growth phase (OD₆₀₀ = 0.4) each culture was divided into two new cultures, where one was maintained at 37°C and the other was shifted to 49°C. At the indicated intervals, culture aliquots were withdrawn, and β-galactosidase activity was assayed as described previously (Craven et al. 1965, Takada et al., 2018) with the exception that the assays were performed at 60°C.

**Western blotting.** Western blotting was performed as previously described (Takada et al., 2018). SigA was detected using a polyclonal anti-SigA antibody (a gift from K. Asai, Tokyo Univ. Agric).

**Primer extension mapping.** Primer extension analysis was performed as described by Natori et al. (2009). *B. subtilis* cells were collected by centrifugation, resuspended in 10 mM Tris-HCl (pH 8.0), and then subjected to RNA extraction using the QIAGEN RNeasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Thirty micrograms of total RNA and 1 pmol of infrared dye (IRD)-labeled oligonucleotide primer R9 that is complementary to the 5-terminal region of the bgaB gene were mixed and heated at 70°C for 5 min. Samples were cooled to 25°C for 1 h, and then reverse transcription reactions were performed using 200 U of AMV reverse transcriptase (Promega) at 58°C for 60 min. After RNase treatment and ethanol precipitation, the products were separated on 6% polyacrylamide-7 M urea gels along with a sequencing ladder that was generated by PCR cycle sequencing with primer R9, which facilitated the mapping of the 3’ ends of the reverse transcripts (corresponding to the 5’ end of the RNAs). IRD-labeled reverse transcription and
sequencing products were detected using a Li-Cor DNA analyzer (models 4200 and 4300, Aloka).

Preparation of histidine-tagged RNAP. The histidine-tagged RNAP (Fujita and Sadaie, 1998a) was prepared from the culture of the NBS278 strain that lacks the sigB, sigH, and sigW genes (a gift from K. Asai, Tokyo Univ. Agric). An overnight culture of NBS278 was diluted 1:1000 with LB medium and grown at 37°C until the culture was harvested by centrifugation at an OD$_{600}$ of 0.8–1.0. The cell pellet was suspended in suspension buffer (20 mM Tris-HCl [pH 8.0], 15 mM imidazole, and 150 mM NaCl) and centrifuged at 7,000 × g for 10 min. The pellet was resuspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 15 mM imidazole, 10% glycerol, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mg/mL lysozyme) and lysed by sonication after incubation for 30 min at 37°C. A 1/10 (v/v) Ni-NTA agarose slurry was added to the supernatant, and the mixture was incubated by stirring for 1 h at 4°C. The mixture was applied to a Polyprep column (Bio-Rad) for gravity flow chromatography. The resin was drained and washed with 25-fold vol of wash buffer (20 mM Tris-HCl [pH 8.0], 50 mM imidazole, 10% glycerol, and 150 mM NaCl). RNAP was eluted with 5 mL aliquots of elution buffer (20 mM Tris-HCl [pH 8.0], 500 mM imidazole, 10% glycerol and 150 mM NaCl). Individual fractions were subjected to SDS-PAGE. The eluted samples containing the RNAP complex were dialyzed against 30% and 50% glycerol sequentially (20 mM Tris-HCl [pH 8.0], 10 mM NaCl, 30% or 50% glycerol). This enzyme contained a small amount of SigA but was still used as a core enzyme.

Alternatively, histidine-tagged SigA was purified from E. coli Rosetta 2 (Merck Millipore) according to previously described methods (Fujita and Sadaie, 1998b) with slight modifications. When the OD$_{600}$ reached 0.5, IPTG (1 mM) was added to the culture, and the cells were harvested after 1 h of incubation. The cell pellet was suspended in wash buffer (20 mM Tris-HCl [pH 8.0], 5 mM imidazole, and 200 mM NaCl) and centrifuged at 10,000 × g for 3 min. The pellet was resuspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 5 mM imidazole, 10% glycerol, 200 mM NaCl, and 1 mM PMSF) and lysed by sonication. A 1/10 (v/v) Ni-NTA agarose slurry was added to the supernatant, and the mixture was incubated by stirring for 1 h at 4°C. The mixture was applied to a Polyprep column (Bio-Rad) for gravity flow chromatography. SigA was eluted with 5 mL aliquots of elution buffer (20 mM Tris-HCl [pH 8.0], 100 mM imidazole, 10% glycerol, and 2 M NaCl). Individual fractions were subjected to SDS-PAGE. The eluted samples containing SigA were dialyzed against 30% and 50% glycerol sequentially (20 mM Tris-HCl [pH 8.0], 10 mM NaCl, 30% or 50% glycerol).
Holoenzyme RNAP was prepared by incubating core RNAP (final concentration 60 nM) and SigA subunit (500 nM) for 20 min at 37°C.

**In vitro transcription assay and detection of abortive transcripts.**

A truncated DNA template harboring the sigA P2 promoter was prepared by PCR using primers R7 and R8. The *in vitro* run off transcription assay was performed according to the method of Fujita and Sadaie (1998a) with some modifications. Template DNA (0.4 pmol) was mixed with the RNAP fraction (3 pmol as a core enzyme) and incubated for 8 min at 37°C or at the indicated temperature in 45 µL transcription buffer (16 mM Tris-HCl [pH 8.0], 8 mM NaCl, 10 mM MgCl₂, 0.1 mM DTT, 0.05% Tween 20, and 0.02 mM EDTA) (pre-initiation step). Transcription was initiated by the addition of 5 µL of a nucleotide/heparin mixture (200 µM each ATP, GTP, CTP, 20 µM UTP with 148 kBq (4 µCi) [α-³²P]UTP, and 1 mg/mL heparin). After 8 min or the indicated time period at each temperature, the reaction was stopped by the addition of an equal volume of solution containing 40 mM EDTA and 400 µg/ml glycogen (initiation to elongation step). Transcripts were precipitated with ethanol, dissolved in 10 µL of gel loading buffer (94% deionized formamide, 9.4 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol), and heated at 95°C for 5 min prior to electrophoresis on a 6% polyacrylamide and 7 M urea denaturing gel.

To detect abortive transcripts, an *in vitro* transcription reaction was performed on a two-fold scale, and the initiation reaction was halted at 2 min. to detect the initial step of transcription. The products were then analyzed using 23% denaturing polyacrylamide gel.

**Potassium permanganate (KMnO₄) footprinting.** Open complex formation and KMnO₄ footprinting were performed according to the methods of Loziński and Wierczkowski (2001) and Chen and Helmann (1997) with the some modifications. The DNA template was amplified from the genome by PCR using a set of primers (D54 and A1), where the A1 primer was 5' terminally labeled with [γ-³²P]ATP and T4 polynucleotide kinase. In this PCR product, the non-template DNA strand of the sigA P2 promoter was labeled. Three pmol RNAP core and 25 pmol SigA were incubated at 37°C for 20 min, mixed with 0.4 pmol labelled DNA template in transcription buffer, and incubated at 37°C for 10 min. To form an open complex, a pre-initiation reaction was performed by further incubation at 37°C or 45°C for 8 min. Then, 10 mM potassium permanganate was added for 3 min at 37°C or 45°C, and the reactions were terminated by the addition of 50 mM EDTA and 4% β-mercaptoethanol. Nucleic acids were deproteinized by phenol-chloroform extraction and recovered by ethanol.
precipitation followed by treatment with 1 M piperidine at 90°C for 30 min prior to heat
denaturation and separation by 7 M urea-6 % PAGE.

**Database analyses.** From currently available databases, *B. subtilis* heat-responsive genes
were extracted and their transcription start sites were examined. To identify differentially
expressed genes under heat shock stress, we used GEO2R
(http://www.ncbi.nlm.nih.gov/geo/geo2r/), which is an online analysis tool of the GEO
database. Firstly, we enter a series accession number GSE27219, which is a publicly
available tiling microarray expression data from Nicolas et al. (2012) which contained 269
samples consisting of *B. subtilis* strain BSB1 transcriptomes measured under a wide range of
different conditions. The group names (control and heat group) were entered after 'Define
groups’ was clicked. BMM_1, BMM_2, and BMM_3 samples (grown in BMM at 37°C until
OD500 nm of 0.4) were assigned to control group and Heat_1, Heat_2, and Heat_3 samples
(10 minutes after shifting from 37 °C to 48 °C) were assigned to heat group. After samples
have been assigned to groups, click ‘Analyze’. Genes with adjusted P-values (adj.P.Val) <
0.05 and logFC (fold change) ≥ 1 were considered at least twofold up-regulated genes under
heat shock stress. A total of 338 CDSs was extracted and each transcriptional profile was
examined using the BSGatlas database (Geissler et al., 2021,
https://rth.dk/resources/bsgatlas/) which integrates and unifies multiple existing annotation
resources including DBTBS (Sierro et al., 2008), BsubCyc (Caspi et al., 2014), SubtiWiki
(Zhu and Stülke, 2018) and so on. Among them, one third was found to be SigB dependent.
By excluding CDSs categorizing class I to V and other sigma factor dependent genes, 170
CDSs seemed to be grouped into class VI. Finally 46 CDSs accompanying reliable
transcription start site with single-nucleotide resolution were collected.

**Results**

**An essential element of the heat shock response in the SigA-dependent promoter**

A heat-resistant β-galactosidase assay was performed to examine the *groES/L*
promoter. In this study, we deleted the CIRCE element to assess its function, and we
compared it to the activity of the wild-type sequence (Fig. 1A). Surprisingly, the *groES/L*
promoter lacking the CIRCE element still responded to heat shock (Fig. 1B). The basal level
of expression at 37°C was increased by approximately 5-fold (from approximately 20 to 100
Miller units), thus indicating that CIRCE repressed the promoter activity. In response to heat shock, the β-galactosidase activity was increased to ca. 500 (CIRCE+) or to ca. 700 (CIRCE-) Miller units within 20 min. Based on this, the fold increase in expression was reduced from 25 (CIRCE+) to 7 (CIRCE-); however, a significant level of heat shock induction was facilitated by the intrinsic promoter sequence.

We then considered the possibility that a heat shock response mechanism may be an underlying modulator of the SigA-dependent promoter. As the groES/L promoter is a composite (Li and Wong, 1992; Völker et al., 1994; Hecker et al., 1996) and the effects of other sigma factor(s) cannot be ruled out, the well-characterized SigA-dependent promoter P2 of the sigA operon (Wang and Doi, 1987) was selected. In fact, we found a SigB-dependent promoter-like sequence in the groES/L promoter region. The promoter region of sigA P2 (-55 to +3, Fig 2A) was fused to the bgaB gene and analyzed. The sigA P2 promoter also exhibited a heat shock response, as indicated in Fig. 2B. Under these conditions, the amount of SigA in the cell did not vary significantly, and the increase in expression was not due to the enhanced amount of sigma factor (Fig. 2C).

Conversely, similar experiments examining the rpoB promoter (-65 to +3, Fig. 3A), another SigA-dependent promoter (Boor et al., 1995), did not reveal a heat shock response (Fig. 3B), thus suggesting that there may be heat shock-responsive elements other than the SigA recognition sequences. We then constructed four chimeric promoters for sigA P2 and rpoB (Fig. 3A), and we analyzed their heat shock responses. As presented in Fig. 3C, D, chimeras 1 and 4 responded to heat shock, thus indicating the importance of the initiation site (+1) of the sense strand and two downstream nucleotides (+2 and +3). We performed similar analyses of the constructs in which +2 or +3 nucleotides were replaced, and we observed that altering of those nucleotides had no effect on heat shock responses (data not shown). Therefore, we concluded that the critical element of the heat shock response was the initiation nucleotide (iNTP) of transcription.

We decided to further focus on the +1 nucleotide, and we performed a similar reporter assay where we replaced the promoter region from +1G of sigA P2 with the other three nucleotides. When +1G was replaced with A, the level of the heat shock response was reduced to approximately 1.7-fold (Fig. 4A, B). In other scenarios, when the +1 nucleotide was replaced with T or C, the basal level of promoter activity was markedly diminished, and no heat shock response was observed (Fig. 4A, B).

*The transcription start site was not altered upon heat shock*
To verify that the enhanced level of expression in response to heat shock was due to promoter activity, the transcription start site was determined using a primer extension experiment. The pattern of primer extension at 49°C for the sigA P2 promoter was similar to that at 37°C (Fig. 5). Alternatively, the transcription start sites of promoters containing substituted +1 nucleotides were analyzed (Fig. 5). In the case of the +1A promoter, the same start site as that of the original sigA P2 promoter was only weakly detected, and an additional start site of A at 3 nucleotides upstream was observed. This band pattern was similar at both 37°C and 49°C. The intensity of both bands was very weak in accordance with the reduced level of β-galactosidase activity (Fig. 4A, B). In the case of the +1T or +1C promoters, no detectable band was observed at either 37°C or 49°C. The primer extension experiments demonstrated the correspondence between β-galactosidase activity and transcription levels. Consequently, +1G was determined to be the most important site in this promoter in regard to the response to heat shock.

**Significance of the length of the discriminator sequence**

The results of the experiments analyzing the start site shift in +1A promoter led us to examine the significance of the distance between the -10 region and the initiation site (the length of the discriminator sequence). We then constructed sigA P2 promoter variants where +1G was shifted from 1 base downstream (D1) to 2 bases upstream (U2), as presented in Fig. 6A. It should be noted that the U1 promoter where +1G was shifted to 1 base upstream exhibited a comparable level of activity in the basal condition (37°C) to that of the sigA P2 promoter, and a higher 4.2-fold expression was obtained upon heat shock (Fig. 6B, C). Interestingly, the primer extension assay indicated that transcription began from 1 base upstream in the U1 promoter (Fig. 7). When the new +1G in the U1 promoter was replaced by A (U1A in Fig. 6A), the promoter still exhibited a heat shock response; however, this response occurred at a reduced level (Fig. 6B, C). Transcription also began from the same site as that in U1, and an additional start site was observed (Fig. 7). This additional site was the same as that of the +1A promoter, indicating that if intrinsic G was missing, most downstream nucleotide of the A stretch could function as a second initiation site. In the D1 and U2 constructs, promoter activity was greatly decreased, and no heat shock response was exhibited (Fig. 6B, C). Additionally, no band was detected in the primer extension experiment (Fig. 7), and this was similar to the results of the +1T or +1C experiments (Fig. 6C).
These results suggested that transcription began preferably with guanine nucleotides when the discriminator sequence was 7 or 8 nucleotides in length.

**In vitro transcription experiments reflected in vivo results**

To analyze the mechanism underlying the heat shock response in the context of the SigA-dependent promoter, we performed *in vitro* transcription analyses examining purified *B. subtilis* RNAP and sigma A factor using the *sigA* P2 promoter as a template. In addition to the original +1G promoter, three other variant promoters (+1A, +1T, and +1C) were also used as templates. The initiation of transcription is largely divided into two steps that include pre-initiation (the step until an open complex is formed) and initiation (the step where ribonucleotide polymerization is initiated). We first determined which step resulted in an increase in transcript levels upon heat shock. The data presented in Fig. 8 reveal that the amount of full-length transcript was increased by approximately 2.3-fold when the pre-initiation reaction was maintained at 45°C compared to that of the entire reaction at 37°C. A higher reaction temperature more than 45°C during pre-initiation reduced the products compared to the levels at 45°C, and this was likely due to inactivation the enzyme itself in response to this *in vitro* condition (data not shown). Incubation at higher temperatures during the initiation step did not enhance the transcript, as presented in Fig. 8A and B.

*In vitro* transcription using the +1A promoter resulted in reduced amounts of full-length products compared to those using +1G; however, similar to the observations from the +1G experiment, the effects of high temperature treatment resulted in increased amounts of full-length products, particularly during the pre-initiation period (Fig. 8A, B). Coincidently with *in vivo* experiments, *in vitro* transcription using +1T and +1C templates resulted in the production of only trace amounts of transcripts (Fig. 8A).

**The open complex was more efficiently formed at higher temperatures during pre-initiation**

As the overall stimulation of transcription upon heat shock was ascribed to the pre-initiation step, we next examined open complex formation at both high and normal temperatures using potassium permanganate footprinting. In this procedure, piperidine cleaves specifically thymine nucleotide in the single stranded DNA, therefore, the amount of open complex is quantified by the total T-bands intensity. In the experiments involving the use of the +1G promoter, bands were detected at T loci in the region of -13 to -2, as indicated by the square bracket in Fig. 9A. This indicated that the open complex was formed in this
area. An extra band that was observed in the +1A promoter experiment (indicated by arrowhead) is considered to be due to an additional A at +1 locus that extends the region for single-strand scission. The relative amount of open complex formed in the *sigA* P2 promoter (+1G) at 45°C was 1.5-fold higher than that at 37°C (Fig. 9B). The level of the open complex was also increased by 1.3-fold in the +1A promoter experiment, and a notably similar level of enhancement was observed in the +1C promoter experiment. Thus, a relatively higher level of open complex was formed when the pre-initiation reaction was performed at high temperature, and this occurred regardless of the +1 nucleotide.

**Abortive initiation was decreased at higher temperatures during pre-initiation**

Abortive initiation of transcription has been extensively studied, and short RNAs (2 to 20 nt in length) are synthesized apparently prior to promoter clearance where RNAP exits the promoter region and shifts to the elongation complex. Considering the possibility that the level of abortive initiation affects the amount of full-length transcripts, we examined the effect of temperature on the production of abortive transcripts. We clearly observed 15- to 18-nucleotide-long abortive transcripts in the *sigA* P2 promoter (Fig. 10A). At 45°C, full-length transcripts were produced more efficiently than they were at 37°C, and only 60%–80% of abortive transcripts were produced (Fig. 10B). Interestingly, similar levels of abortive transcripts were observed for the alternate promoters with +1 nucleotides from the original G to the other three nucleotides (Fig. 10A). Additionally, a smaller amount of each of the short 15 to 18 nucleotide RNAs was detected at a higher temperature of 45°C (Fig. 10B). These results suggested that the amount of full-length RNA, and thus the level of elongation complex, was independent of the level of abortive transcripts.

**Kinetics of in vitro transcription during different periods of elongation**

To analyze the effect of high temperature on the reaction stage of transcription, we performed *in vitro* transcription experiments over a finer time period of the elongation reaction. A typical result regarding the amount of full-length transcript is shown in Fig. 11A, and the average levels of three independent experiments are shown in Fig. 11B. Only a slight difference in the slope angle of the line during the first 60 s was observed, and the products of full-length transcripts at both 37°C and 45°C were not significantly increased after 60 s. This suggested that the difference in the level of full-length product between 37°C and 45°C was largely due to the amount of open complex formed and not due to the rate of nucleotide incorporation into the transcription machinery.
E. coli RNAP also exhibited heat and nucleotide dependent increases in transcriptional activity

To determine if iNTP-dependent heat shock induction of B. subtilis RNAP is a universal phenomenon among bacterial RNAP, we performed the same in vitro transcription experiments using the E. coli RNAP and sigA P2 promoters. Considering the heat sensitivity of the E. coli enzyme, we performed experiments at either 30°C or 42°C (Fig. 12). The sigA P2 promoter (+1G) possessed the highest relative value of ca. 1.5, and alternate promoters exhibited more reduced levels. iNTP-dependent heat induction was also observed in E. coli RNAP predominantly with +1G. The pattern of the basal level of transcription at 30°C was slightly different compared to that of B. subtilis. In particular, the enzyme activity was quite high in the +1C promoter. This may reflect the different nature of the affinity of the polymerase for nucleotides. A clear heat-dependent enhancement of transcription was evident in the +1G promoter experiments.

Discussion

Several reviews have described bacterial heat shock response mechanisms and commented on the existence of an unknown mechanism (Hecker et al., 1996, Helmann et al., 2001, Schumann 2003, 2016). Our serendipitous finding of heat shock response in the context of a CIRCE-deleted SigA-dependend groES/L promoter may provide a clue regarding this unknown mechanism. Based on this, we analyzed in detail the mechanism underlying this phenomenon. We demonstrated that transcription was increased in response to heat shock even in the case of SigA-dependent promoters without any binding site for transcriptional regulators, thus revealing that the heat shock response mechanism was dependent upon the core promoter sequence (Fig.1, 2). In agreement with Arnosti et al. (1986), the level of SigA did not increase and instead decreased after heat shock (Fig. 2C). Therefore, the overall promoter activity of sigA did not increase significantly, and the heat induction of the sigA P2 promoter was not due to an increase in SigA factor. Conversely, almost no induction occurred in response to heat shock in the context of the rpoB promoter, and chimeric promoter analyses revealed that the nucleotide at the transcription start site (+1) was responsible for this response (Fig. 3). The reporter assay using promoters with various substitutions of the +1 nucleotide indicated that promoters possessing a +1 purine responded strongly to heat shock, while those with a +1 pyrimidine did not (Fig. 4). The transcription
initiated 3 bp upstream from the initial site when the original +1 nucleotide G was replaced with A (Fig. 5), thus suggesting that iNTP affects the global transcription behavior of RNAP.

Based on the results described above, we constructed promoter sequences possessing +1G that was shifted to various locations within its discriminator sequence, and we performed in vivo reporter assays (Fig. 6). We observed that only the 1 base shift to the upstream region from the original position (the case of U1 or U1A promoter) was effective, and in regard to the sigA P2 promoter, its promoter activity was enhanced by locating the +1G or +1A bases at a position that was 8 to 9 bases apart from the -10 consensus sequence. Furthermore, the actual start site (+1) became the moved G or A, and U1A also caused transcription to begin from the upstream A base at a similar rate (Fig. 7). These results demonstrated that the promoter does not function unless an adequate base (+1) exists within a limited region in proximity to the consensus sequence.

We demonstrated that within the promoter recognition mechanism of RNAP, the effect of temperature upshift led to an increase in transcripts if the pre-initiation step (the transition step from the closed complex to the open complex) occurred under high temperature (Fig. 8). We used 45°C for this experiment instead of 49°C, as we could not obtain a substantial amount of transcripts at 49°C. This was likely due to the thermo-sensitivity of RNAP itself under this in vitro condition. Quantitative analysis of the open complex revealed that the amount of open complex was enhanced at higher temperatures in the sigA P2 promoter, and a similar enhancement was also observed in the +1 nucleotide-substituted promoter (Fig. 9). Therefore, the increase in the open complex at high temperature may explain the higher transcript yield; however, it cannot account for the purine-specific heat shock induction that was more pronounced in the +1G promoter. As substantial amounts of the total band intensities were obtained in the +1A promoter and even in the +1C promoter experiments (which greatly reduced the promoter activity) and they exhibited similar levels of increase upon temperature upshift, this suggested that the open complex forms in a manner that is dependent upon the temperature and independent of the +1 nucleotide. We considered that RNAP may not be able to efficiently bind to promoters that possess +1C or +1T, and this may result in reduced promoter activity. However, from the open complex formation experiments, it was demonstrated transcription was initiated less efficiently in these +1 pyrimidine promoters, despite the ability of RNAP to bind to the promoter region.

We speculated that in case of +1C or +1T promoter, there was a little uptake of the iNTP and/or promoter clearance after the formation of the open complex. Our analysis performed to detect the abortive transcripts clearly demonstrated that similar levels of short
RNAs were produced regardless of the +1 nucleotide (Fig. 10). These results indicated that the amounts of the full-length transcripts were independently regulated at the level of abortive transcripts, where the reduced abortive products did not cause a higher level of promoter clearance that resulted in greater amounts of full-length transcripts. Although it remains unknown if the reduction of abortive products at higher temperatures yielded more full-length transcripts, this is unlikely due to the observation that the results presented in Fig.10A indicate that +1T or +1C did not enhance the full-length transcripts at higher temperatures.

Shimamoto (2013) presented a very fine comparison of the two models concerning the production of abortive transcripts. According to the conventional sequential mechanism, RNAP yields abortive transcripts to escape from the promoter region, proceed to promoter clearance, and complete production of full-length transcripts. In contrast, according to the updated branched mechanism, RNAP initially yields two different complexes (productive and moribund complexes). The moribund complexes result in dead-end yielding abortive transcripts, while productive complexes accomplish promoter clearance to yield full-length transcripts. The moribund complex is a by-product that occurs when the holoenzyme binds to DNA. These two complexes exhibit different modes of sigma factor association and function through independent pathways, with the exception of the participation of certain factors such as GreA/B in *E. coli*. The ratio of the amounts of these two complexes that are produced depends upon the nature of the promoter. In favor of this branched mechanism, the full-length transcripts were derived from the productive complex, and the abortive transcripts were derived from the moribund complex. This indicates that these two reactions were independent. Therefore, the amount of abortive transcripts is unrelated to the level of the full-length transcript. In agreement with the assertion of this review, our results support the branched mechanism. Moreover, the abortive initiation mechanism was independent of iNTP and was not related to the heat shock mechanism.

As we demonstrated that high temperatures during the pre-initiation period resulted in an increase in full-length transcripts, more detailed time course analyses incorporating the various periods of the pre-initiation step were performed in our *in vitro* run-off assay. The inclination of both curves during the initial 60 s (Fig. 11B) was not very different, thus indicating that the elongation rate at 45°C was only slightly improved compared to that at 37°C. Both reactions reached a plateau within 120 s., and the maximum level therefore reflected the amount of open complexes. These results indicated that the increase in transcript at high temperature was not due to a more efficient elongation rate and instead was due to the
presence of a greater number of open complexes. Based on these results and the results presented in Figs. 8 and 9, it is postulated that a similar level of open complex was formed in the \textit{sigA} P2 promoter depending on the temperature; however, the incorporation of the iNTP into RNAP differed, where GTP was the most highly incorporated, ATP was the next highest, and CTP and UTP were incorporated the least. The affinity of RNAP to GTP or ATP at higher temperature is probably more predominant than that to CTP or UTP. These promoter-associated mechanisms in combination with the iNTP function represent the heat shock response.

To examine if this novel heat shock response mechanism in the \textit{sigA} P2 promoter could be applied to other bacteria, we performed an \textit{in vitro} transcriptional analysis using the RNAP of \textit{E. coli} as a model organism of Gram-negative bacteria. We observed that while there was a difference in the amount of transcriptional products at 30°C (the basic level), this model organism also exhibited the highest heat shock response in the +1G experiments and a smaller response in the +1A experiments, and this was similar to that observed in the \textit{B. subtilis} RNAP. Based on these results, it is likely that a similar heat shock response mechanism is conserved among the other bacteria (Fig. 12).

We speculate that the heat shock response mechanism discovered in this report may explain the mechanism underlying the function of a number of heat-responsive genes. We then performed database analyses and extracted 46 SigA dependent and at least twofold heat-responsive genes that categorized into class VI as well as having reliable transcription start site data with single-nucleotide resolution (see Materials and Methods). The distribution of their iNTP is G (37.0%), A (56.5%), T (2.2%), and C (4.3%), while that of known 315 SigA dependent promoter having single iNTP site (DBTBS: Sierro et al., 2008) is G (30.2%), A (53.7%), T (9.8%), and C (6.3%). These data demonstrated the predominancy of purine nucleotide, especially G, as iNTP of SigA dependent heat-responsive genes.

It is likely that regulation by the nucleotide sequence itself without the need for transcriptional regulators enables a more rapid response to heat shock. For example, transcriptional regulation that is dependent upon iNTPs has been widely established in the context of \textit{B. subtilis} stringent control. The transcriptional initiation efficiency of several genes strongly depends upon the intracellular concentration of nucleotide triphosphates that are used as iNTPs (Turnbough 2008). In response to amino acid starvation, the alarmone guanosine (penta)tetra-phosphate [(p)ppGpp] facilitates a decrease in GTP and a reciprocal increase in ATP. These alterations cause transcriptional alterations that occur in an iNTP-dependent manner. For example, when intracellular GTP concentrations are decreased, the
transcription of *rrn* operons that possess a promoter containing +1G are decreased (Krásný and Guarse, 2004). In contrast, the genes involved in branched-chain amino acid (BCAA) biosynthesis, such as *ilvBHC-leuABCD* whose promoter possesses +1A, increase in response to reciprocal changes in GTP and ATP concentrations (Krásný et al., 2008, Tojo et al., 2008, Sojka et al., 2011, Fujita et al., 2012, Osaka et al., 2020). Thus, the mechanism underlying the nature of RNAP has been recognized as an inherent cellular function, and it has provided a new perspective that has been reviewed from the aspect of heat shock phenomena. In fact, extensive massive analysis of the transcriptomes of *B. subtilis* using tiled microarrays to examine 269 RNA samples from 104 different conditions allowed for the quantification of the contribution of each sigma factor to transcriptome plasticity. The proportion of transcriptome variation attributed to SigA was exceptionally low among sigma factors, thus suggesting that condition-specific regulation of SigA-dependent promoters relies primarily upon other transcription factors (Nicolas et al., 2012). The iNTP-dependent mechanism described in this report may explain at least a portion of these unknown factors.

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**Legends to figures.**

Fig. 1. Promoter activity of the *groES/L* gene in response to heat shock. (A) Promoter sequence of the *groES/L* gene with (+) or without (-) the CIRCE element. Consensus promoter sequences of the -10 and -35 regions are highlighted with pale gray, and the inverted repeat sequences of CIRCE element are highlighted with dark gray. The locus of the initiation site of transcription is indicated as +1 in the sense strand. (B) The NBS742 (CIRCE+) and NBS699 (CIRCE-) strains were grown in LB medium, and the thermo-tolerant β-galactosidase activity (Miller unit ± standard deviation) at 49°C (solid line) or at 37°C (dotted line) is presented.
Fig. 2. Promoter activity of the sigA gene in response to heat shock. (A) Sequence of the sigA gene P2 promoter (from -55 to +3). The locus of the transcription initiation site guanine (G) is indicated as +1 in the sense strand. Consensus sequences are highlighted with pale gray. (B) The NBS1074 strain was assayed for β-galactosidase activity (Miller unit) as described in Fig. 1B. (C) Western blotting of SigA protein with anti-SigA antiserum at various time points during incubation (top). The relative intensity of each band is plotted, and the band at 37°C and 0 min is set to 1 (bottom).

Fig. 3. Heat shock responses by rpoB and rpoB/sigA P2 chimeric promoters. (A) Sequence alignment of the sigA P2 (from -55 to +3), rpoB (from -65 to +3), and rpoB/sigA P2 chimeric promoters. Capital letters indicate sequences derived from sigA P2, and small letters indicate those derived from rpoB. Consensus sequences are highlighted with pale gray. The locus of the transcription initiation sites that include guanine (G) of sigA P2 and thymine (t) of rpoB are indicated as +1. Heat shock responses by rpoB (B) and rpoB/sigA P2 chimeric promoters (C). The NBS746 (rpoB) and four chimeric strains (NBS695 to 698, chimera 1 to 4, respectively) were assayed for β-galactosidase activities as described in Fig. 1B. (D) Relative amounts of β-galactosidase activities at 49°C and 20 min vs that at 37°C. P2; The data for sigA P2 were obtained from Fig. 2B.

Fig. 4. Heat shock response by sigA P2 promoters containing +1 nucleotide substitutions. (A) The NBS692 (+1A), NBS693 (+1T), and NBS694 (+1C) strains were assayed for β-galactosidase activities as described in Fig. 1B. (B) Relative amounts were determined as described in Fig. 3D.

Fig. 5. Determination of transcription start site of the sigA P2 promoter and its +1 variants using primer extension. The NBS1074 (sigA P2) and three +1 variant strains (NBS692 to 694) were grown in LB medium, and RNAs were extracted from cells at 20 min after temperature shift. Each transcription start site of bgaB gene was then determined. M; Sequence ladder of the sigA P2 promoter region. The transcription start site (+1) and -10 nucleotide of the sigA P2 promoter are indicated.

Fig. 6. Effects due to the length of the discriminator sequence of the sigA P2 promoter. (A) Discriminator sequences of various +1 shift mutants. D1 (NBS656); +1G is shifted 1 base
downstream. U1 (NBS657); +1G is shifted 1 base upstream. U1A (NBS659); +1G is shifted 1 base upstream and replaced with A. U2 (NBS658); +1G is shifted 2 bases upstream. (B) The β-galactosidase activity of various +1 shift mutants was determined as described in Fig. 2B. (C) Relative amounts were determined as described in Fig. 3D.

Fig. 7. Determination of the transcription start site of various +1 shift mutants using primer extension. The NBS1074 (sigA P2), NBS692 (+1A), and four +1 shift mutants (NBS656 to 658) were grown in LB medium, and RNAs were extracted from cells at 20 min after temperature shift. Each transcription start site of the bgaB gene was then determined. M; Sequence ladder of the sigA P2 promoter region. The transcription start site (+1) and -10 nucleotide of the sigA P2 promoter are indicated.

Fig. 8. In vitro run-off transcription assay of the sigA P2 promoter and the +1 nucleotide mutants. (A) Templates were prepared from the strains NBS1074 (sigA P2), NBS692 (+1A), NBS693 (+1T), and NBS694 (+1C). Autoradiograph of run-off transcription assays for each promoter. Five (1 to 5) temperature combinations during pre-initiation and initiation period were examined. (B) The relative intensity of each band is plotted, and the result without heat treatment (case 1) is set to 1. Only the data for the sigA P2 promoter (dark gray) and the +1A mutant (pale gray) are plotted.

Fig. 9. Potassium permanganate footprinting analysis of sigA P2 (NBS1074) and two +1 nucleotide mutant promoters that include +1A (NBS692) and +1C (NBS694). (A) Autoradiograph of potassium permanganate footprinting alongside of sequencing ladder (left). Pre-initiation temperature is indicated as 37°C or 45°C. Bands derived from single strand scission are indicated by a parenthesis. Additional bands that appeared in the +1A promoter are indicated by an arrowhead. (B) Total band intensities derived from single strand scission indicated by a parenthesis in A are considered to as a ratio of open complex formation, and those at 37°C are set to 1. The relative intensities at 45°C vs those at 37°C are plotted.

Fig. 10. Detection of abortive transcripts of sigA P2 (NBS1074) and of +1 nucleotide mutant promoters, including +1A (NBS692), +1T (NBS693) and +1C (NBS694). (A) Autoradiograph of the in vitro transcription assay, where two portions for full-length transcripts and abortive transcripts are enlarged. The numbers 37 and 45 indicate the pre-
initiation temperatures. Nucleotide numbers of abortive transcripts were obtained from the autoradiograph generated under a longer exposure (data not shown). (B) The relative intensities at 45°C vs those at 37°C of each transcript (full-length and short RNAs) are plotted. P2, A, T, and C; sigA P2, +1A, +1T, and +1C promoter, respectively.

Fig. 11. Time-course kinetics of *in vitro* transcription assays. (A) Autoradiograph of *in vitro* run-off transcription assay of the *sigA* P2 promoter after various time periods of elongation. Reactions were performed at either 37°C or 45°C. (B) Band intensities ± standard deviation at 45°C (solid line) or at 37°C (dotted line) are plotted.

Fig. 12. *In vitro* run-off transcription assay of the *sigA* P2 promoter and the +1 nucleotide mutants using *E. coli* RNAP. (A) Templates were prepared as described in Fig. 8, and autoradiographs of the run-off transcription assay at either 30°C or 42°C are presented. (B) Relative intensity of transcripts at 42°C vs that at 30°C.
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**A**

**groES/L promoter**

| CIRCE (+) | -35 | -10 | +1 |
|-----------|-----|-----|----|
| AATTTTTTATCTTATCACCAATGGAAGGAGATCTTTATATTATAAGATTG | GGTTAGCACTCTTTAGTCGCTGAGTCTAAGAATTAAATTA |

| CIRCE (-) | -35 | -10 | +1 |
|-----------|-----|-----|----|
| AATTTTTTATCTTATCACCAATGGAAGGAGATCTTTATATTATAAGATTG | GGTAGCACTCTTTAGTCGCTGAGTCTAAGAATTAAATTA |

**B**

| CIRCE (+) | **CIRCE (-)** |
|-----------|--------------|
| ![Graph](Image) | ![Graph](Image) |

**Fig. 1.**
**A**

**sigA P2**

GGGAATAATGAAAAACAATAGCATCTTTGTGAAGTTTGTATTATAATAAAAAATT

**B**

\[ \beta \text{-galactosidase activity (Miller units)} \]

| Time (min) | 0 | 10 | 20 |
|------------|---|----|----|
| 37°C       | 50| 50 | 50 |
| 49°C       | 0 | 0  | 0  |

**C**

| Relative value | 0 min | 10 min | 20 min |
|----------------|-------|--------|--------|
| 37°C           | 1.5   | 1.5    | 1.5    |
| 49°C           | 0.5   | 0.5    | 0.5    |

*Fig. 2.*
**A**

\[ \text{sigA P2} \]

\[
\begin{array}{cccc}
\text{-35} & \text{-10} & \text{+1} \\
\text{GGGAATAATGAAAAACAATAGCATCTTTTGTGAAAGTTTGTATTATAATAAAAATTTG} \\
\text{rpoB} \\
\text{-35} & \text{-10} & \text{+1} \\
\text{ggctattatcatcaaaagcaaaaaaaaagtttgactcggtatatttaactatgttaatattgtaaaaatg} \\
\end{array}
\]

**Chimera 1**

\[
\text{GGGAATAATGAAAAACAATAGCATCTTTTGTGAAAGTTTGTATTATAATt} \\
\text{ggctattatcatcaaaagcaaaaaaaaagtttgactcggtatatttaactatgttaatattatTTg} \\
\]

**Chimera 2**

\[
\text{GGGAATAATGAAAAACAATAGCATCTTTTGTGAAAGTTTGTATTATAATAAAAATTTg} \\
\text{ggctattatcatcaaaagcaaaaaaaaagtttgactcggtatatttaactatgttaatattatTTg} \\
\]

**Chimera 3**

\[
\text{GGGAATAATGAAAAACAATAGCATCTTTTGTGAAAGTTTGTATTATAATAAAAATTTg} \\
\text{ggctattatcatcaaaagcaaaaaaaaagtttgactcggtatatttaactatgttaatattatTTg} \\
\]

**Chimera 4**

\[
\text{GGGAATAATGAAAAACAATAGCATCTTTTGTGAAAGTTTGTATTATAATAAAAATTTg} \\
\text{ggctattatcatcaaaagcaaaaaaaaagtttgactcggtatatttaactatgttaatattatTTg} \\
\]

*Fig. 3.*
**Fig. 3.**

**B**

- Graph showing β-galactosidase activity (Miller units) over time (min) for the rpoB gene.

**C**

- Graphs showing β-galactosidase activity (Miller units) over time (min) for Chimera 1, Chimera 2, Chimera 3, and Chimera 4.

**D**

- Bar graph comparing β-galactosidase activity (Miller units) at 49°C/37°C for different chimeras (P2, rpoB, 1, 2, 3, 4).
|          | M | sigA P2 | +1A | +1T | +1C |
|----------|---|---------|-----|-----|-----|
| A        | 37| 37      | 37  | 37  | 37  |
| C        | 49| 49      | 49  | 49  | 49  |
| T        |   |         |     |     |     |
| G        |   |         |     |     |     |

**Fig. 5.**
A

sigA P2
+1 shift D1
+1 shift U1
+1 shift U1A
+1 shift U2

TATAATAAAAAATTTGG
TATAATAAAAAATGTTG
TATAATAAAAAATATTG
TATAATAAAAAAGTTTG

B

+1 shift D1

+1 shift U1

+1 shift U2

+1 shift U1A

C

49°C/37°C

0
10
20

P2 D1 U1 U1A U2
Fig. 7.
**A**

|          | 1   | 2   | 3   | 4   | 5   |
|----------|-----|-----|-----|-----|-----|
| pre-initiation | 37°C | 42°C | 45°C | 37°C | 37°C |
| initiation    | 37°C | 37°C | 37°C | 42°C | 45°C |

**sigA P2**

**+1A**

**+1T**

**+1C**

**B**

**Fold increase**

| 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|
| 1 | 2 | 3 | 0.5 |

*Fig. 8.*
Fig. 9.

A

|   | T | C | A | G |
|---|---|---|---|---|
| sigA P2 | 37 | 45 | 37 | 45 |
| +1A      | 37 | 45 | 37 | 45 |
| +1C      | 37 | 45 | 37 | 45 |

B

![Bar chart showing relative value of whole bands for different conditions](chart.png)

- SigA P2: 1.5 ± 0.1
- +1A: 1.3 ± 0.2
- +1C: 1.4 ± 0.1
Fig. 10.

Free-RI

| sigA P2 | +1A | +1T | +1C |
|---------|-----|-----|-----|
| 37      | 37  | 37  | 37  |
| 45      | 45  | 45  | 45  |

Full-length

18 nt
17 nt
16 nt
15 nt
Fig. 10.
Fig. 11.

**A**

| Time (s) | 0  | 30 | 45 | 60 | 90 | 120 | 480 |
|----------|----|----|----|----|----|-----|-----|

- 37°C
- 45°C

**B**

- Amount of transcript (PSL)
- Time (s)
Fig. 12.

(A) Table showing relative values (42°C/30°C) for different conditions:

|        | sigA P2 | +1A   | +1T   | +1C   |
|--------|---------|-------|-------|-------|
| 30°C   | 0       | 0.5   | 1     | 1.5   |
| 42°C   | 1       | 2     | 1.5   | 1     |

(B) Bar graph showing relative values (42°C/30°C) for different conditions:

- sigA P2: 1.5
- +1A: 1
- +1T: 1
- +1C: 1

Fig. 12.