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Heat-inducible translational coupling in *Bacillus subtilis*

Shinsuke Fujiwara, Nami Tsubokura, Yasurou Kurusu, Kimiyo Minami and Yasuo Kobayashi*
Department of Applied Biochemistry, Faculty of Applied Biological Sciences, Hiroshima University, Higashi Hiroshima 724, Japan

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ABSTRACT

***Bacillus subtilis* plasmid pGR71 is a promoter-probe shuttle vector derived from pUB110 (1). The expression of the *cat* gene on pGR71 in *B. subtilis* requires the insertion of a *Bacillus* promoter and a ribosomal binding site (RBS) into the *Hin*dIII cloning site immediately upstream from the *cat* gene. A recombinant plasmid of pGR71, named pGR71-369, was obtained by a spontaneous deletion of a fragment containing most of the inserted *Hin*dIII fragment and the replication origin necessary for multiplication in *Escherichia coli*. The expression of the *cat* gene in *B. subtilis* cells carrying this plasmid was inducible by heat. Nucleotide sequence analysis of the upstream region of the *cat* gene, deletion analysis, and dot blot hybridization analysis of mRNA in various conditions revealed that the *cat* gene was expressed by heat-inducible translational coupling and that the regulatory region of heat inducibility was present in the upstream region of the *cat* gene.**

INTRODUCTION

In procaryotic systems, translational initiation is strongly influenced by a consensus nucleotide sequence present in 5′ upstream of the initiation codon. The sequence usually contains a polypurine stretch complementary to the 3′ terminus of 16S ribosomal RNA, and is called Shine-Dalgarno (SD) sequence (2) or ribosome binding site (RBS).

Translational coupling has been observed in *Escherichia coli* (3−6) and in *Bacillus subtilis* (7, 8). Recent works indicated that biosynthetic pathways in bacilli were characterized by clustered overlapping genes which were supposed to be regulated by translational coupling (9−11). In the usual case of translational coupling, the expression of a distal gene in an operon is dependent on the translation of a proximal gene which has a translational stop codon close to or overlapping the initiation codon of the distal gene (3).

The exact mechanism of translational coupling is not clearly understood, although two possibilities are considered (3−5, 12). First, the ribosome could terminate and initiate translation without leaving the mRNA or a ribosome could dissociate and rapidly reassociate with the initiation codon of the distal cistron. Second, translational coupling could occur by a mechanism in which the translating ribosome rearranges or exposes an otherwise unknown translation initiation signal of the distal gene. Recently, Sor et al.(13) reported that the nucleotide sequence around the connective region of two cistrons was important for efficient translational coupling.

The plasmid pGR71, constructed by Goldfarb et al.(1), is a promoter-probe shuttle plasmid derived from pUB110, pBR350, and *cat* gene of Tn*9*. In *B. subtilis*, expression of the *cat* gene carried by pGR71 requires the insertion of a *Bacillus* promoter and a ribosomal binding site (RBS) into the *Hin*dIII cloning site located immediately upstream of the *cat* gene. While screening promoters regulated by heat, we obtained a recombinant plasmid pGR71-369 from pGR71 by a spontaneous deletion. *B. subtilis* transformed with pGR71-369 was chloramphenicol resistant (Cm^r) at 42°C, but chloramphenicol sensitive (Cm^s) at 30°C.

Nucleotide sequence and deletion analysis of pGR71-369 and dot blot hybridization analysis of *cat* mRNA synthesized *in vivo* revealed that the *cat* gene was expressed not by an inserted promoter but by translational coupling with the proximal open reading frame (ORF) encoding K1 protein from pUB110, and that the heat inducible expression of the *cat* was regulated at the translational level.

METHODS

Media, phages, bacterial strains, and plasmids

Schaeffer sporulation medium (14) was used for cultivation. In the case of kanamycin resistant (Km^r) strains, 5 μg of kanamycin/ml was added. Defective phage PBSX (15) was induced with mitomycin C from spore forming *B. subtilis* strain 168 Trp (trpC2), concentrated, and purified as described by Yamamoto et al.(16). *B. subtilis* strain MI112 (*leuA8 thr5 argA15 recE4 hsr*$^-$ *hsm*$^-$) (17) was used as a host for promoter-probe plasmid pGR71 (1). Plasmid pGR71 (see Fig.2) was obtained from Dr.T.Kudo with the permission of Dr.R.H.Doi. *E. coli* JM105 (Δ*lacpro thi-1 strA sbcB15 hsdR4 F′ traD36 proAB lacI*^{q}ZΔM15) (18) was used as a host for the sequencing phage vectors M13mp10 and M13mp11 (19) and for plasmid vector pUC18 (20). *E. coli* JM105, M13mp10, M13mp11 and pUC18 were obtained from Amersham Japan Corporation.

Preparation of plasmid DNA

Cleared lysate of *B. subtilis* carrying plasmid was prepared by the pronase-sarcosyl method (21). Purification of the plasmid DNA was carried out by 0.8% agarose gel electrophoresis and

* To whom correspondence should be addressed

DEAE-cellulose chromatography according to the procedure by Smith (22).

Deletion plasmids construction

Deletion plasmids pGR71-369ΔBH, -369ΔBX, -369ΔXH, -369C, and -369D were constructed from pGR71-369 using *Bam*HI, *Xba*I, and *Hin*dIII restriction enzyme sites (see Fig. 2). For pGR71-369ΔBH construction, pGR71-369 DNA was digested with *Bam*HI, treated with Mung bean nuclease, and digested with *Hin*dIII, removing a 633 bp fragment. *Hin*dIII-digested DNA was treated with Klenow polymerase and ligated with T4 DNA ligase. For pGR71-369ΔBX construction, pGR71-369 DNA was digested with *Xba*I and treated with Mung bean nuclease. Then, the DNA was digested with *Bam*HI, removing 325 bp fragment, followed by Klenow polymerase treatment, and ligated with T4 DNA ligase. pGR71-369ΔXH was constructed with the same procedures as pGR71-369ΔBX construction, except that *Hin*dIII was used instead of *Bam*HI. For pGR71-369C construction, pGR71-369 DNA was digested with *Bam*HI and *Hin*dIII, removing a 633 bp fragment, treated with Mung bean nuclease, and ligated with T4 ligase. For pGR71-369D construction, pGR71-369 DNA was digested with *Xba*I and *Hin*dIII, removing a 309 bp fragment, treated with Mung bean nuclease, and ligated with T4 ligase.

Transformation

B. subtilis transformation was performed according to the protoplast method of Chang and Cohen (23). *E. coli* was transformed by a published procedure (24).

DNA sequencing

Specific restriction fragments were cloned into M13mp10 and M13mp11 (19) and sequenced by the dideoxy chain termination method of Sanger and Coulson (25). DNA was labeled with [α-^{35}S]dATP (600 Ci/mmol ; Amersham Japan Corp.).

CAT assay

CAT activity was determined colorimetrically (26) and was expressed as nmol of Cm acetylated per min at 37°C per mg of cellular protein, which was quantitated by the method of Lowry et al.(27) using bovine serum albumin as a standard.

RNA preparation and dot blot hybridization analysis

RNA preparations and dot blot analysis were performed as previously described (28) with slight modification. The DNA fragment used as a probe was labeled with biotin-11-dUTP using a nick translation kit obtained from BRL (Tokyo, Japan). RNA was spotted onto the Hybond-N blotting membrane (Amersham Japan Corp.). Hybridization was performed at a probe concentration of 100 ng /ml. Detection and visualization were performed according to the manual of BRL detection kit.

RESULTS

Derivation of deletion plasmid pGR71-369

To obtain heat-inducible promoter fragments, we digested PBSX DNA from 168Trp strain with the restriction enzyme *Hin*dIII. Digested DNA fragments were inserted into the unique *Hin*dIII site of pGR71. *B. subtilis* MI112 cells transformed with these recombinant plasmids were plated onto DM3 plates (23) containing Km (100 μg/ml) and Km^r were selected. These Km^r transformants were transferred to Schaeffer plates containing Cm

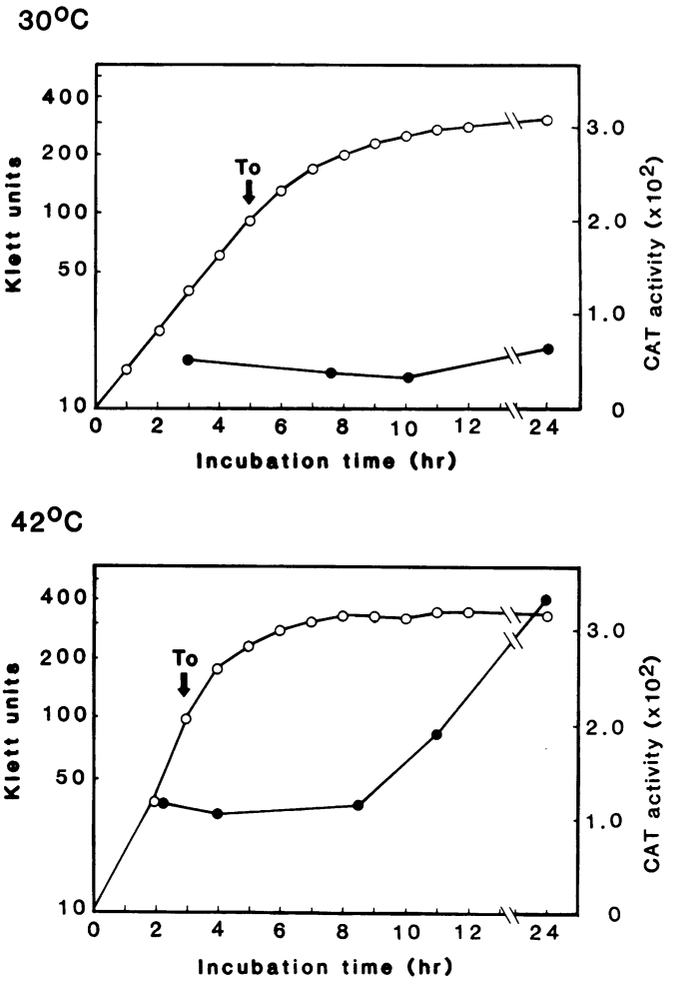

Figure 1. CAT activity as a function of growth and sporulation in *B. subtilis* MI112 harboring plasmid pGR71-369. Symbols : ○ cell growth (Klett unit); ● CAT activity in cells grown in sporulation medium. CAT activity is shown by units of specific activity (nmol DTNB reduced min^{-1} mg^{-1} total soluble protein).

(20 μg/ml) and Km (5 μg/ml). Then, colonies that were chloramphenicol resistant (Cm^r) at 42°C, but chloramphenicol sensitive (Cm^s) at 30°C were selected.

Strain 369 showed the highest CAT activity among obtained transformants. The plasmid from strain 369 was designated as pGR71-369. As shown in Fig. 1, the CAT activity of strain 369 at various stages of growth was low at 30°C, but it was higher at 42°C and stimulated at the late sporulation stage. During sporulation, sets of developmentally regulated genes are turned on, and some of them are known as regulatory factors for sporulation gene expression (29). The CAT activity of *spo0A* and *spo0B* mutants containing pGR71-369 was heat-inducible but not stimulated at the late sporulation stage (data not shown). This result suggests that the stimulation of CAT expression at the late sporulation stage is under the *spo0* gene control.

To analyze the regulatory mechanism of the *cat* gene expression by pGR71-369, the plasmid DNA was purified and used to transform *B. subtilis* MI112. The obtained transformants showed the same phenotype as that of strain 369, suggesting that the cause of the heat-inducible chloramphenicol resistanse is in the plasmid. Agarose gel electrophoresis indicated that the plasmid pGR71-369 was smaller than that of pGR71, suggesting that pGR71-369 had a deletion. To know which region of pGR71 was deleted,

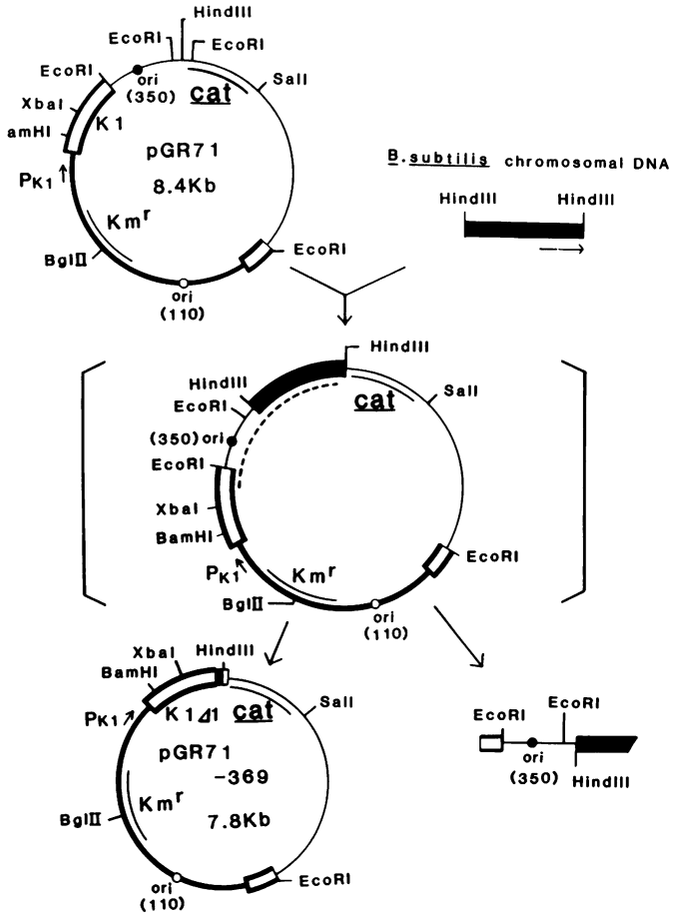

Figure 2. The derivation of plasmid pGR71-369 from plasmid pGR71. The *Hin*dIII DNA fragment from *B. subtilis* chromosome was inserted into plasmid pGR71. Plasmid pGR71-369 is derived by a spontaneous deletion of the region shown by broken line. The open box represents the region derived from pUB110 K1 gene, whereas the solid box represents the *Hin*dIII fragment of *B. subtilis* chromosomal DNA. Km^r and *cat* are genes for the determinant of kanamycin and chloramphenicol resistance, respectively. The replication origins of pBR350 and pUB110 are indicated as ori ● (350), and ori ○ (110), respectively. P_{K1} is the promoter of K1 gene. Thick line indicates the fragment derived from pUB110 and thin line indicates the fragment derived from pBR350 and Tn*9* *cat* gene.

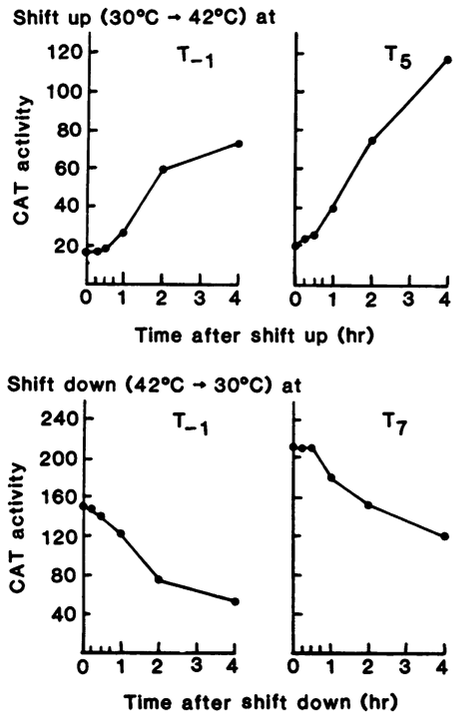

Figure 3. The effect of temperature shift up and shift down on CAT activity from the cells containing pGR71-369. Tn means n hours after the end of logarithmic growth. Units of activity are nmol DTNB reduced min^{-1} mg^{-1} total soluble protein.

pGR71-369 DNA was digested by various restriction enzymes (data not shown). We found that the internal deletion occurred somewhere in a region between the *Bam*HI and *Hin*dIII sites, which contained the inserted DNA fragment and replication origin (ori) functional in *E. coli* as shown in Fig. 2.

The effect of temperature shift up and shift down on CAT activity

It is known that when a variety of organisms are exposed suddenly to higher temperature, a set of proteins, called heat shock proteins are induced (30). Heat shock in *B. subtilis* induces the synthesis of at least 20 proteins (31−33). Synthesis of the majority of heat shock proteins began after about 3 min and returned to a steady state level within 20 to 40 min after heat shock (34, 35). Some heat shock proteins continued to be synthesized at elevated levels for about 60 min after the heat shock in *B. subtilis* and were eliminated by a temperature shift down (31, 32). In present study, CAT activities were increased by temperature shift up and were decreased by temperature shift down (Fig. 3). The induction pattern of CAT would parallel the synthesis of some heat shock proteins during heat shock, but *cat* gene expression from pGR71-369 remained low within 15 min after heat shock and increased gradually to 4 hours. These results suggest that the induction of CAT by pGR71-369 is different from typical heat shock response.

Nucleotide sequence analysis of pGR71-369

When the *Bam*HI-*Hin*dIII fragment of pGR71-369 was recloned in a fresh pGR71 vector and strain MI112 was transformed with the reconstructed plasmid, the phenotype (Cm^r at 42°C, Cm^s at 30°C) of the transformants was identical to that of the strain 369. This result indicates that the *Bam*HI-*Hin*dIII fragment causes the temperature-dependent expression of the *cat* gene. However, we found that this fragment does not have a promoter, because when it was inserted into another promoter-probe plasmid pSB (36) by use of a linker, no activity was observed at 30°C and 42°C (data not shown). To analyze the regulatory region, we determined the nucleotide sequence of this fragment. The nucleotide sequence is shown in Fig. 4. The sequence of the upstream region of the *Bam*HI site was referred from pUB110 sequence (37) and that of the downstream region of the *Hin*dIII site was from Tn*9* sequence (38). The nucleotide sequence of the *Bam*HI-*Hin*dIII fragment revealed that most of this fragment (No.351−977) was derived from pUB110.

The insertion of Bacillus promoter and RBS into the *Hin*dIII cloning site of pGR71 results in a production of a fusion CAT protein. However, in case of pGR71-369, no fusion protein could be synthesized, since an in-frame translation termination codon (No.982) is present at the upstream of the initiation codon of the *cat* gene (No.1021). Previously, Goldfarb et al.(1) indicated that the RBS of the *cat* gene derived from Tn*9* could not be used efficiently by the *B. subtilis* translational system and native size CAT was not produced. But Zaghloul et al.(8) found that the

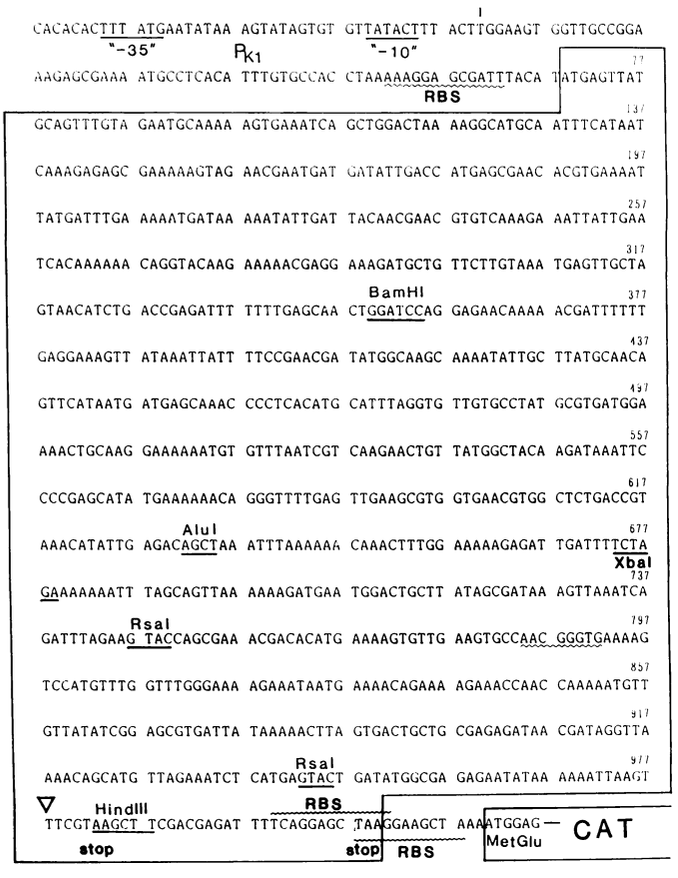

Figure 4. Nucleotide sequence of the 5′ upstream region of the *cat* gene. The upstream region from *Bam*HI site is referred from pUB110 sequence (37) and the downstream region from *Hin*dIII site is referred from Tn*9* sequence (38). Solid line box indicates K1Δ1. Open triangle (▽) indicates an end point of pUB110-derived region. P_{K1} is the promoter of K1 gene. RBS indicates the ribosome binding site. A position of +1 is assigned as a site of P_{K1} transcription initiation determined by Zyprian et al.(40). K1Δ1 coding region starts at number 69.

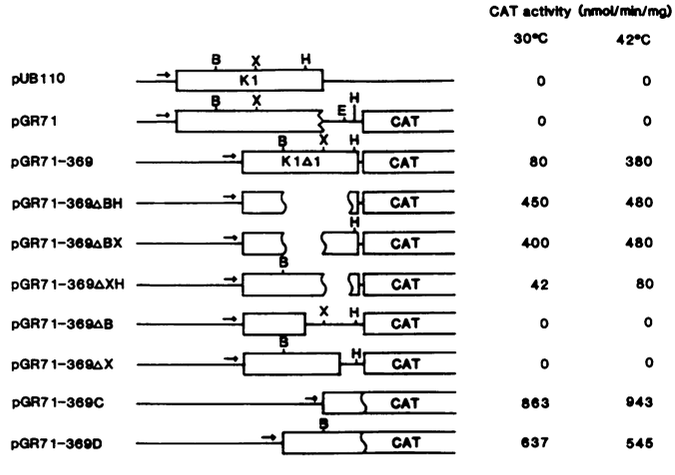

Figure 5. Effects of various deletions of K1 gene on the CAT activity at 30°C and 42°C. Cell-free extracts obtained from overnight culture at 30°C or 42°C were used for CAT assay. E; *Eco*RI, H; *Hin*dIII, B; *Bam*HI, X; *Xba*I. Arrow is the promoter of K1 gene (P_{K1}).

Tn*9* RBS could be used much more efficiently in *B. subtilis* by translational coupling in pGR71-derived plasmids. They reported that if translation of a *B. subtilis* − *E. coli* fusion mRNA was initiated at the *B. subtilis* RBS and if the translation of the proximal *B. subtilis* gene terminated within the Tn*9* RBS of the distal *E. coli* gene, translational coupling occurred. This mechanism is similar, but not identical, to the translational coupling phenomenon observed in other native and synthetic operons (3−5, 7).

Since these facts suggest the possibility of translational coupling in expression of the *cat* gene of pGR71-369, we searched for an ORF which terminates at the translational termination codon TAA(No.1008). As shown in Fig. 4, there was an ORF in the upstream region of the *cat* gene. The ORF started at ATG(No.69) which coincided with the initiation codon of K1 gene of pUB110. Five pUB110-specified proteins, designated as K1 to K5 in decreasing order of molecular weight, were identified by Shivakumar et al.(39) in *B. subtilis* minicells harboring pUB110. We named this K1-derived ORF as K1Δ1. The promoter of K1 gene was determined by Zyprian and Matura (40) and it was indicated as P_{K1} in Fig. 4.

The translation of K1Δ1 is necessary for the CAT expression

To confirm that the translation of the cat gene was coupled with that of K1Δ1, we constructed two plasmids pGR71-369ΔB and pGR71-369ΔX (Fig. 5). The pGR71-369 DNA was digested either with *Bam*HI or with *Xba*I, treated with Mung bean nuclease, and ligated with T4 DNA ligase. The reading frame downstream of *Bam*HI or *Xba*I site of K1Δ1 was changed by this treatment, and a new termination codon for K1Δ1 was created at 549 bp or 333 bp upstream of the *cat* initiation codon, respectively. The translation of the *cat* gene cannot couple with that of the newly created ORFs because of the distance. As shown in Fig. 5, CAT activity of cells containing pGR71-369ΔB or pGR71-369ΔX was not observed at 30°C, nor at 42°C. These results strongly suggested that translation of K1 1 was necessary for the efficient expression of the *cat* gene.

Effects of in-frame deletions in K1Δ1 on CAT expression

To know whether K1Δ1-*cat* translational coupling depends on the upstream region of the *cat* gene, we constructed a series of in-frame deletions in K1Δ1. As shown in Fig. 5, deletion plasmids pGR71-369ΔBH, -369ΔBX, and -369ΔXH were constructed by deletion of the *Bam*HI-*Hin*dIII, *Bam*HI-*Xba*I, and *Xba*I-*Hin*dIII regions of K1Δ1 without changing the reading frame, respectively. Details of deletion plasmids construction were described in Methods. *B. subtilis* strain MI112 was transformed with these deletion plasmids and CAT activities were measured.

Heat-inducibility of the *cat* gene expression was observed only in pGR71-369ΔXH (Fig. 5). We checked the effect of temperature shift up and down on *cat* gene expression in pGR71-369ΔXH. CAT activity was increased by temperature shift up and decreased by shift down (data not shown). The induction and reduction patterns of pGR71-369ΔXH were similar to those of pGR71-369. Such patterns were not observed in pGR71-369ΔBH nor pGR71-369ΔBX. These results indicated that the *Bam*HI-*Xba*I region of pGR71-369 affects the heat inducibility of the *cat* gene expression.

However, the CAT activities of cells containing pGR71-369ΔXH were low compared with those of pGR71-369. We supposed that the declined *cat* gene expression in pGR71-369ΔXH might be due to a composed stem structure around the junctional region of K1-derived ORF and *cat* region. In fact, by using an NEC PC-9801 computer (Nippon Electric

Co., Tokyo, Japan) and the GENETYX system (Software Development Co., Tokyo, Japan), we found a composed nucleotide sequence, 5′-TTAGCTTCGACGAGATTTTCA-GGAGCTTAA-3′, around the junctional region of *cat* gene in pGR71-369ΔXH. This sequence could form stem and loop structure (ΔG = −9.60 kcal/mol) and the RBS sequence of *cat* gene was situated in the stem region. Das et al.(12) reported that the RBS sequence is necessary for efficient expression of the distal gene of translationally coupled gene pair. Therefore, the declined *cat* gene expression in pGR71-369ΔXH might be due to the stem structure comformed around the junctional region.

The *cat* gene expressions in pGR71-369ΔBH or in pGR71-369ΔBX were higher than that in pGR71-369. These results agree with that of Zaughloul and Doi (6) who suggested that the shorter translational distance preceding the *cat* RBS allowed more efficient expression of the *cat* gene. However, heat inducibility of the *cat* gene expression was lost in pGR71-369ΔBX and pGR71-369ΔBX (Fig. 5). These results indicated that the *Bam*HI-*Xba*I region of pGR71-369 affects strongly the temperature-dependent *cat* gene expression.

The translation of K1 gene is not heat-inducible

It is of interest to investigate whether the frequency of translation of the upper cistron, K1Δ1, changes markedly with temperature. Recently, Kubo et al.(46) reported that translational efficiency was changed by mRNA structure in stacking region in *B. subtilis*. As shown in Fig. 4, there were nucleotide sequences AAGCAAAATATTGCTT (No.414−No.429) and AAAAACAAACTTTGGAAAAAGAGATTGATTTT (No.643−No.674) which could be a weak stacking region. Therefore, at 42°C K1 gene might be translated more frequently than at 30°C because of the mRNA conformation transcribed from *Bam*HI-*Xba*I region.

To examine this possibility, we constructed two translational fusions between K1 gene and *cat* gene. Construction schemes were described in Methods. In pGR71-369C and pGR71-369D, K1 gene could be fused with *cat* gene at *Bam*HI site and at *Xba*I site, respectively. As shown in Fig. 5, temperature-dependent *cat* gene expression was not observed from cells containing pGR71-369C nor pGR71-369D. We also found that the expression of K1 was not affected by temperature by using K1-*lacZ* gene fusion (data not shown). These results suggested that K1 gene translation was not heat-inducible, but *Bam*HI-*Xba*I region of pGR71-369 affects the temperature-dependent translational coupling of the *cat* gene expression.

How does the *Bam*HI-*Xba*I region affect *cat* gene expression? We found sequences in *Bam*HI-*Xba*I region which could make base pairing with the RBS sequence of *cat* gene (No. 556-TCCCCGAGCATATGAAA---TTTCAGGAGCTAAGGAA-No.1014). This structure might affect the efficiency of RBS utilization of *cat* gene. However to reach a final conclusion, more detailed analysis should be done.

Dot blot hybridization analysis of the cat mRNA synthesized in vivo

To clarify that the heat-inducible *cat* expression was not controlled at the level of transcription, we measured the amounts of the *cat* mRNA in cells carrying pGR71-369 by dot blot analysis using a 538 bp *Rsa*I fragment obtained from the *cat* structure gene as a probe. Fig. 6 showed that the level of the *cat* mRNA was almost constant in vegetative, T_6- and T_{20}-cells cultivated at 30°C and 42°C. We also measured the amounts of the *cat* mRNA in cells

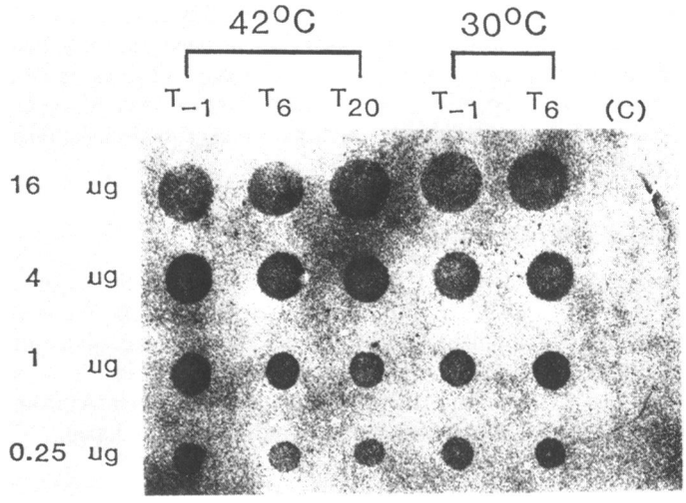

Figure 6. Dot blot analysis of *cat* mRNA synthesized *in vivo*. Two μl of diluted RNA samples (16 μg, 4 μg, 1 μg, 0.25 μg) were spotted horizontally across the blotting membrane (Hybond-N membrane, Amersham Japan), which was then processed for hybridization and visualization. *Rsa*I fragment (538 bp) in *cat* gene was used as a *cat* gene probe. mRNAs from cells carrying pGR71-369 were isolated from vegetative (T_{-1}: 1 hour before the end of logarithmic growth) and sporulation (T_6: 6 hours after the end of logarithmic growth, T_{20}: 20 hours after the end of logarithmic growth) cells incubated at 30°C and 42°C in Schaeffer sporulation medium. As a control (c), RNA purified from vegetative cells of MI112 strain grown at 42°C was used.

carrying pGR71-369ΔA or pGR71-369ΔB and confirmed that there were not any significant differences in *cat* mRNA levels in various conditions (data not shown). These results indicated that the *cat* expression was not controlled at the level of transcription.

DISCUSSION

In *B. subtilis*, translational coupling has not been directly demonstrated in natural chromosomal operons (41), although there are some data suggesting the occurence of the translational coupling in *B. subtilis* chromosomal operon, such as pyrimidines (42), arginine (43) and histidine (44) operons. In this paper, we reported a temperature-dependent translational coupling in *B. subtilis* plasmid. The expression of *cat* gene in *B. subtilis* cells carrying pGR71-369 was inducible by heat. Nucleotide sequence analysis, deletion analysis, and dot blot hybridization analysis of mRNA revealed that the *cat* gene was expressed by translationl coupling and inducible by heat. We also found that the regulatory region of heat induction was present in the upstream region of the *cat* gene.

Since the translational coupling is modulated at the post-transcriptional level, structure of mRNA might play an important role in the regulation. In attenuation of the tryptophan operon in *E. coli* (44), or the *cat* gene expression in *B. subtilis* (45), mRNA structure played a major role. Schottel et al.(45) reported that if the RBS sequence was situated in a region of mRNA that was capable of base pairing, the efficiency of translation was decreased. Kubo and Imanaka reported that translational efficiency could be changed by alteration of mRNA structure in an open reading frame in *B. subtilis* (46).

In general, the stability of secondary structure of nucleic acids is related with AT(AU) contents (47, 48). High AT contents make secondary structure unstable at high temperature (47). These facts suggest that the *cat* gene expression of pGR71-369 might be

affected by its mRNA secondary structure. The analysis of AT contents of the upstream region of the *cat* gene indicated that the upstream region was occupied with many ATs. There is a possiblity that the mRNA conformation transcribed from the *Bam*HI-*Xba*I region at high temperature makes the translational coupling more easily than at 30°C.

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