THE EVALUATION OF HEAT-SHOCK PROMOTER FOR HETEROLOGOUS GENE EXPRESSION IN YEAST

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Ringkasan

Promoter gen kejutan panas (*heat-shock promoter*) *HSP26* yang diisolasi dari khamir telah digunakan untuk mengekspresikan gen heterolog, gen CAT (*Chloramphenicol Acetyl Transferase*), di dalam khamir *Saccharomyces cerevisiae*. Hasil analisis menunjukkan bahwa promoter *HSP26* tersebut mampu mengekspresikan gen CAT dan ekspresinya dapat diinduksi dengan perlakuan kejutan-panas. Diketemukan juga bahwa gen CAT tidak terekspresikan pada waktu sel mencapai fase eksponensial, dan ekspresinya baru terjadi pada waktu sel mencapai fase pertumbuhan stasioner. Aras ekspresi pada saat sel mencapai fase stasioner lebih rendah dibanding dengan aras ekspresi pada waktu diinduksi dengan kejutan-panas. Hasil penelitian ini menunjukkan bahwa promoter gen *HSP26* dapat digunakan untuk mengekspresikan gen heterolog di dalam khamir *S. cerevisiae*.

Abstract

The promoter of the yeast *HSP26* gene has been employed for the expression of CAT gene in *Saccharomyces cerevisiae*. The results showed that the promoter was heat-inducible, as well as induced when the cells reached the stationary phase. Thus, it has been shown that the *HSP26* promoter is potential for the expression of heterologous proteins in yeast.

I. Introduction

The efficient expression of heterologous proteins in yeast relies on yeast promoters. The most commonly used promoters are from the glycolytic enzyme genes. These are attractive because glycolytic enzymes are highly expressed, each can represent from 1 to 5% of total cell protein even though they are generally encoded by a single copy gene. The high-level expression is due to the strong promoters associated with these genes (Goodey *et al.*, 1987; Carter *et al.*, 1987). The glycolytic promoters, however, may present some problems for expression of heterologous genes in that they are most active at the start of fermentation when the sugar concentration is highest. The accumulation of high levels of a foreign protein at the start of

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fermentation maybe inhibitory to the cell and cause the cessation of growth or create a selective pressure for a reduction in plasmid copy number or even loss of plasmid (Goodey *et al.*, 1987). If the heterologous protein expressed is potentially toxic, such as a highly active protease, the growth of yeast may very prematurely cease as soon as the gene is expressed.

In a situation where the heterologous product is potentially toxic to the yeast cell, a promoter system which is switchable during the growth would be preferred. Such a system should consist of a promoter which is completely switched-off during the biomass accumulation, in order to avoid the expression of the product and concomitant cessation of growth, and inducible at the end of growth. By employing this kind of system it may be possible to achieve maximum levels of expression without sacrificing the optimum growth of cells.

A novel regulated system which involves the use of a heat-shock gene promoter has been discovered. It has been shown that when yeast cells are exposed to high temperature and other forms of stress, including stationery growth phase, they respond by producing a small number of proteins called the heat-shock proteins (Hsps).

In yeast, transcriptional regulation plays an important role in the regulation of heat-shock protein. One of the striking feature of heat-shock proteins is that they are induced rapidly and intensely (Lindquist and Craig, 1988). When temperature rises, a specific heat-shock transcription factor (HSF) is rapidly activated through a change in its phosphorylation state, leading to a rapid increase in heat-shock gene transcription (Sorger *et al.*, 1987; Wu *et al.*, 1987). The synthesis of small hsps is also induced at specific stages in development at normal temperatures as well as by heat-shock although the molecular role of these proteins is not clear (Lindquist and Craig, 1988). The yeast hsp26, for example, is not required for cell viability under normal or stressed conditions (Petko and Lindquist, 1986). Deletion mutations of *HSP26* gene have no observable effect on thermotolerance, respiratory or fermentative growth, spore development, germination or viability, dessication and ethanol tolerance, or growth under anaerobic conditions (Lindquist and Craig, 1988). The similar situation was also observed for another yeast small hsp, the hsp12, which is strongly induced by heat-shock and on entry into stationery phase but with no detectable effect on growth at various temperatures, nor on the thermotolerance (Praekelt and Meacock, 1990).

The heat-shock response is also dependent upon *cis*-acting regulatory elements (heat shock element, HSE) in the gene promoter that are characterised by a periodically arranged GAA segments,

repeated at 2 nucleotide intervals in alternating oritentations, and require at least three GAA segment (Amin *et al.*, 1988). It was also observed that translational controls play a very important role in the regulation of heat-shock proteins. Under heat-shock condition, preexisting mRNAs are translationally repressed while the heat-shock mRNAs are translated very efficiently (Lindquist, 1981; Storti *et al.*, 1980).

The regulation of *HSP26* gene, which encodes the small heat-shock protein in *S. cerevisiae*, has also been shown to be induced during stationary phase (Lindquist and Craig, 1988; Praekelt, personal communication). The *HSP26* promoter contains a TATA element (TATAAAT) and a highly conserved 8 bp heat shock element: 5' TTCTAGAA 3' which is also found in a number of other heat shock-induced genes, including *HSP70 (SSA3), HSP84, UB14* and *PGK1* (Tuite *et al.*, 1988; Bossier *et al.*, 1989). This heat shock element is recognised by the heat shock transcription factor to stimulate transcription in response to a sudden increase in temperature. The *HSP26* promoter also contains both repressing and activating elements. It has been shown that an upstream sequences of the *HSP26* gene repress the constitutive expression of a heterologous gene containing a heat shock promoter element. The same sequence, however, also confers heat shock inducibility upon a heterologous gene that does not contain a heat shock promoter element. It has been suggested that transcription of *HSP26* gene is regulated by a mechanism of basal repression during growth and derepression upon heat-shock induction, which is in contrast to other heat-shock genes that are regulated by trancriptional activation (Susek and Lindquist, 1990).

The fact that this gene was strongly induced during stationary growth phase, suggested that its promoter might be useful as a naturally switchable system for the expression of heterologous proteins in yeast. The potential application of the *HSP26* promoter for heterologous proteins expression in yeast was evaluated using the CAT (Chloramphenicol Acetyl Transferase) gene as a reporter gene.

II. Materials and Methods

II.1 Materials

Bacterial strain:

Escherichia coli NM522: (lac-proAB) thi- supE hsd 5 [F' proAB *laq q Z M15]*.

Yeast strain:

Saccharomyces cerevisiae S150-2B: MATa ura3-52 trp1-289 his3-1 leu2-3,12 2 μ m^{+}.

II.2 Methods

All the cloning steps were as described previously (Maniatis *et al.*, 1982).

III. Results

III.1 Cloning of HSP26 *Gene Fragment into pUC19*

The *HSP26* gene had been cloned previously. The genomic clone carrying the *HSP26* gene (kindly provided by U. Praekelt, Leicester Biocentre, University of Leicester) was used as the source of DNA fragments for subsequent cloning experiments. A 4.2 kb *HSP26* gene-containing fragment was isolated by digestion with *EcoR*I and ligated into the *EcoR*I linker in the *lacZ* gene of pUC19 (Figure 1). The identification of the recombinant plasmid in ampicillin resistant transformants of *E. coli* NM522 was confirmed by restriction enzyme analysis. This *HSP26* gene-carrying pUC19 was then used as the source of a promoter fragment for the next cloning step.

Figure 2 shows the restriction map of the promoter region of *HSP26* gene. The promoter fragment was cloned into plasmid pSG19 by digesting the *HSP26* gene-carrying pUC19 with *Bgl*II and *Sal*I enzymes and ligating the fragments into pSG19 digested with *BamH*I and *Sal*I (Figure 3). The plasmid pSG19 is a centromere-based yeast shuttle vector carrying a CAT reporter gene useful for promoter assay. The *HSP26* fragments were inserted immediately upstream of the CAT gene. As there were two *Bgl*II sites within the promoter region of *HSP26* gene, the small and the larger *Bgl*II - *Sal*I fragments were cloned separately into pSG19. Plasmid pSG19-6 and pSG19-1B contained the small and the larger *Bgl*II-SalI *HSP26* promoter fragments, respectively. Recombinants were screened by restriction enzyme analysis from colonies grown on Luria Agar ampicillin plate.

III.2 Transformation of S. cerevisiae *S150-28 with* HSP26 *Promoter Fragments Cloned into pSG19*

The two recombinant *HSP26* promoters cloned into pSG19 plasmids were used to transform the yeast *S. cerevisiae* S150-2B (*MATa, ura3-52, trp1-289, his3-1, leu2-3,12,* 2μ m+) by the lithium

acetate transformation method. Transformants were selected from colonies grown on selective medium (lacking uracil) and confirmed by the plasmid rescue method. The transformants carrying the recombinant plasmid were then used to assay the capability of *HSP26* promoter fragments in regulating the expression of a reporter gene (CAT gene).

III.3 The Expression of CAT Gene Regulated by Promoter Fragments of HSP26 *Gene*

As the *HSP26* gene is induced by heat shock as well as during growth at stationary phase, it was of interest to see whether a heterologous gene could also be expressed under these conditions if fused to the *HSP26* promoter.

In order to assess the induction by heat shock, the yeast clones carrying recombinant plasmids were grown exponentially at 25°C in 50 ml YEPD medium using overnight culture grown in minimal medium lacking uracil as starters. Heat shock induction was achieved by the addition of fresh YEPD (25 ml), preincubated at 51°C, to 25 ml of cells grown exponentially at 25°C; the cultures were then quickly transferred to 38°C waterbath for 40 minutes. After 40 minutes at 38°C the cells were chilled on ice for 15 minutes and harvested by centrifugation. The cell pellets were then used for CAT assays and RNA preparations.

As a control, the same clones were also subjected to dilution but grown under non heat-shock treatment by adding 25 ml of fresh YEPD, preincubated at 25°C, to 25 ml of exponentially growing cells and continuing the incubation in the 25°C waterbath for 40 minutes. The cells were then harvested as described above.

In Table 1 it is clearly shown here that both *HSP26* promoter fragments were heat-inducible and capable of regulating the expression of CAT gene. The data also show that reducing the size of promoter fragment markedly reduced the level of CAT expression by almost 4 fold. As expected, there was no CAT activity detected when the *HSP26* promoter fragments were used to drive the expression of CAT gene under non heat-shock condition.

Previous work on *HSP26* expression has shown that the gene was induced at stationery phase. It was therefore of interest to see whether these *HSP26* promoter fragments were also able to induce the expression of a heterologous gene at stationary phase. Therefore, yeast cultures carrying the *HSP26* pSG19-6 and pSG19-1B were grown in YPD through exponential to stationary phase. Samples of cells were than removed for CAT assay. Table 2 shows that the *HSP26* pro-

moter was also induced spontaneously at late growth phase without heat shock treatment.

It can be seen here that although the *HSP26* promoter was induced at late growth phase, the level of induction was much lower level than under heat-shock conditions. The results presented both in Table 1 and 2 show that the larger fragment of *HSP26* promoter is more powerful in regulating the expression of CAT gene that the smaller fragment.

III.4 mRNA Analysis

Table 2 shows that the level of CAT expression during stationary growth phase was very low compared to the heat shock induction. In order to examine the induction of *HSP26* promoter fragments during the growth phase in a little more detail, an analysis of mRNA levels was carried out. The mRNAs were probed by using the *HSP26* gene fragment. As a size control, the mRNAs were also probed with the *CYC1* gene fragment.

The mRNA analysis (Figure 4) showed that during growth in exponential phase, there was no transcript detected from either the pSG19-6 or pSG19-1B construction (lane 1 and 4). At early stationary phase, the large *HSP26* promoter fragment resulted in some low levels of transcription (lane 5) while the small promoter fragment did not give any detectable level of transcript (lane 2). Figure 4 also showed that the CAT gene was not transcribed until the cells had reached the late stationery phase (lane 3 and 6).

IV. Discussion

The results presented here have clearly confirmed that the *HSP26* promoter is heat-inducible and able to regulate the expression of a heterologous gene in yeast. The length of the promoter fragment was found to be crucial in determining the level of expression of the heterologous gene. This was indicated by the markedly reduced level of expression of the gene by the shorter promoter fragment.

Since this work was carried out, it has been discovered that the promoter region of *HSP26* gene contains five sequences that match the consensus heat shock element: 5' nTTCnnGAAn 3'(Susek and Lindquist, 1990). The sequences were all mapped between nucleotides -200 to - 456, relative to the translation initiation site, and located upstream of the two *Bgl*II sites. Between these two sites no sequences match very closely to the consensus heat shock element, although part of the motif, TTC, is present. Table 1 and 2 show that

the removal of sequences between the two *Bgl*II sites reduced the level of CAT gene expression. As there were no sequences that match very closely to the heat shock element, it is unlikely that the difference in the levels of expression between the two promoter fragments was due to the removal of a heat shock element. This difference in the levels of expression was more likely due to the removal of the transcription initiation site which has been located between the two *Bgl*II sites at nucleotide -74. The promoter elements that are required for transcription initiation and initiation site selection are located between nucleotide positions -137 and -40, while the two *Bgl*II sites are located at nucleotide positions -131 and -50 (Susek and Lindquist, 1990).

Table 2 and mRNA analysis show that the CAT gene was not expressed during exponential phase when the level of glucose was still very high, suggesting that glucose was the limiting factor for the ability of *HSP26* promoter to regulate the expression of CAT gene. When the level of glucose decreased, at stationery phase, the CAT gene was expressed when the larger *HSP26* fragment was used. These facts are consistent with the suggestion that the expression of the *HSP26* gene is related to the level of cAMP; that is, it is expressed when the cells are in the stress condition.

Another study indicated that a decrease in the level of cAMP-dependent protein phosphorylation resulted in the heat shock-type of response, including elevated synthesis of heat-shock proteins, acquisition of thermotolerance and transient arrest of the cell cycle (Shin *et al.*, 1987). Furthermore, transcriptional regulation via the intracellular cAMP level has been confirmed for a member of other heat shock genes, including *SSA3, HSP12, and UB14* (Boorstein and Craig, 1990; Praekelt and Meacock, 1990; Finley *et al.*, 1987). In the case where this has been investigated, for example the *SSA3* gene, it appears to be due to transcriptional regulatory mechanism independent of the HSE/HSF interaction. Again, the *cis*-acting sequences, defined as UAS_{PDS} for the *SSA3* gene, are located in the 5' region of the promoter (Boorstein and Craig, 1990). Therefore, as before, the differences observed between the small and the larger *HSP26* promoter fragments examined here were due to deletion of the RNA initiation site.

It is possible that the *HSP26* promoter may be more powerful than the alcohol dehydrogenase (ADH) gene promoter in regulating heterologous gene expression in yeast. It was found that in a similar construction the ADH promoter gave CAT activity of only 0.51 μ moles/min/mg protein from a single copy plasmid in *S. cerevisiae* strain S150-2B, and 7.5 μ moles/min/mg protein with 21 copies of plasmid (Hadfield *et al.*, 1986). When the *HSP26* promoter was used

to express the CAT gene on a single copy plasmid in *S. cerevisiae* S150-2B, the CAT activity detected under heat-shock condition was 19.76 μ moles/min/mg (Table 1).

These finding raise the possibility of exploiting the *HSP26* gene promoter for the expression of foreign protein which may be toxic to the cell if expressed at the early stages of growth. On the one hand, this may be achieved by growing the cells carrying the recombinant plasmid with the gene of interest to exponential phase and then subjecting them to a heat-shock condition. Alternatively, if the cells are allowed to grow until reaching the stationary phase the protein could be produced spontaneously.

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Table 1. **The expression of CAT gene regulated by *HSP26* promoter fragments in *S. cerevisiae* after heat-shock treatment**

Plasmid	**CAT units (μ moles/min/mg protein)**	
	Heat-shock	**Non heat-shock**
pSG19-6[a]	5.0	0
pSG19-1B[b]	19.76	0

a pSG19 carrying [*Bgl*II - *Sal*I] small fragment of *HSP26* promoter

b pSG19 carrying [*Bgl*II - *Sal*I] large fragment of *HSP26* promoter.

Table 2. **The Expression of CAT gene regulated by *HSP26* promoter at different growth phases**

Plasmid	**CAT units (μ moles/min/mg protein)**		
	Exponential	**Early stationary**	**Late stationary**
pSG19.6[a]	0	0	0
pSG19.1B[b]	0	0	2.68

[a] pSG19 carrying [*Bgl*II - *Sal*I] small fragment of *HSP26* promoter

[b] pSG19 carrying [*Bgl*II - *Sal*I] large fragment of *HSP26* promoter

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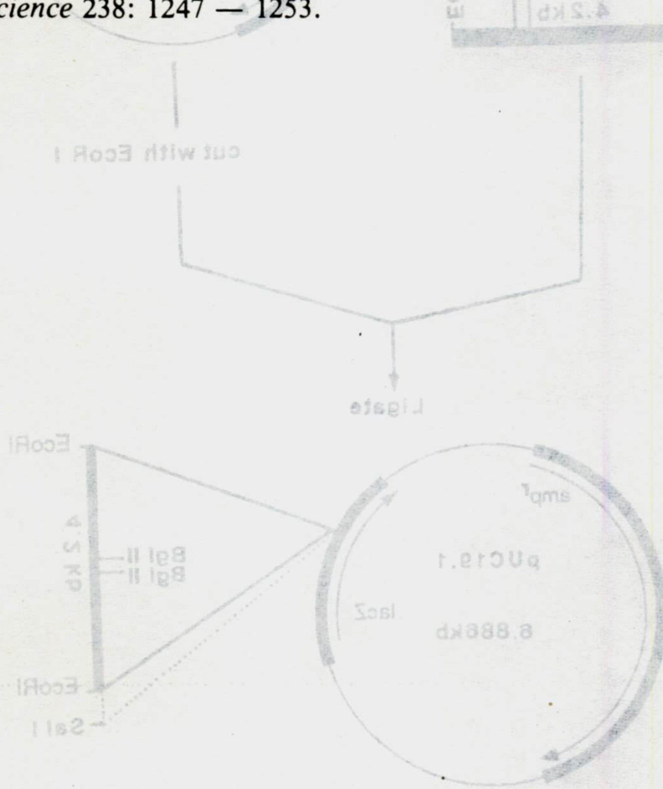

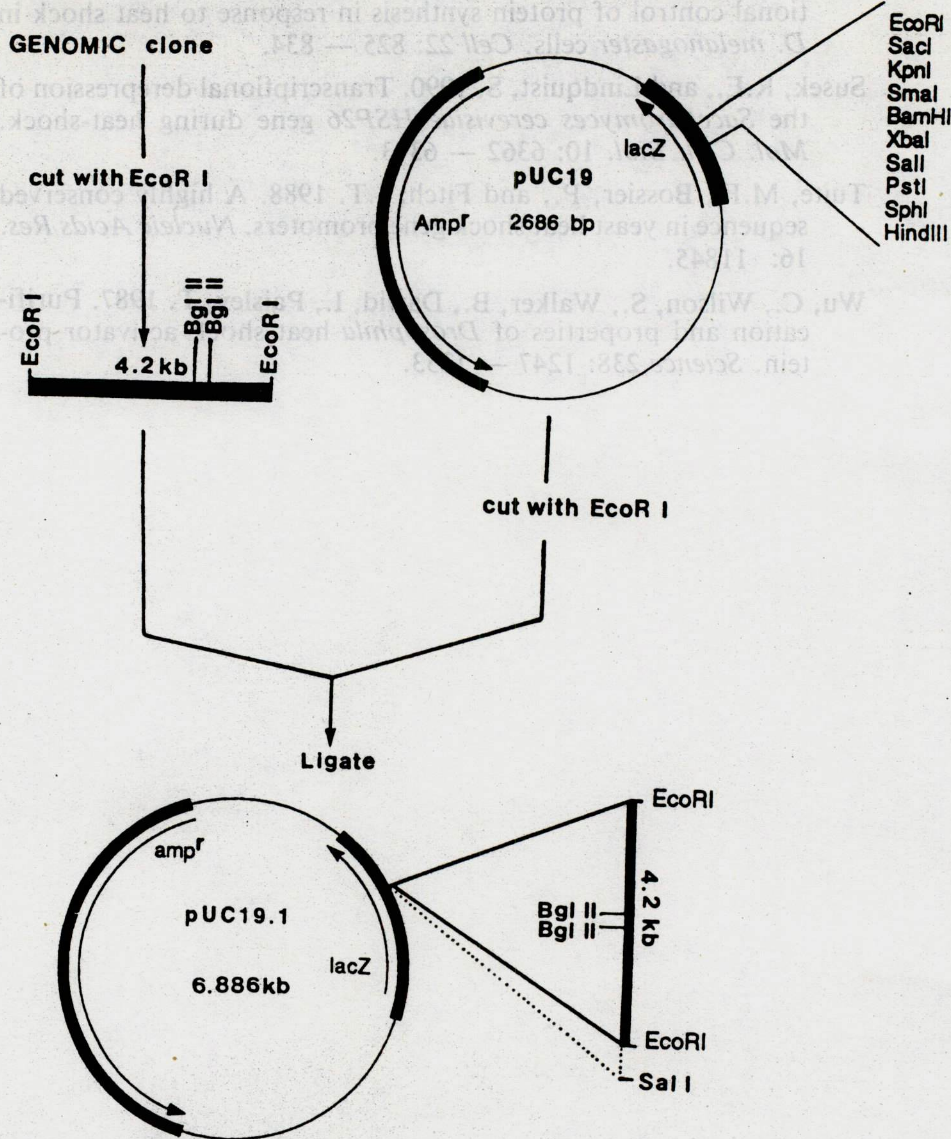

Figure 1. **Diagram of Cloning of the *HSP26* Gene into pUC19 Vector.**

The *EcorR*I 4.2 kb DNA fragment carrying the *HSP26* gene was inserted into *EcoR*I site wihtin the *lacZ* gene of pUC19, thus disrupting the *lacZ* gene. Therefore, the transformants carrying the recombinant plasmid can be distinguished from the non-recombinant as white colonies on the LUA-IPTG-Xgal-Ampicillin plate. A^r: ampicillin resistance gene. Black region: the 4.2 kb DNA fragment carrying the *HSP26* gene.

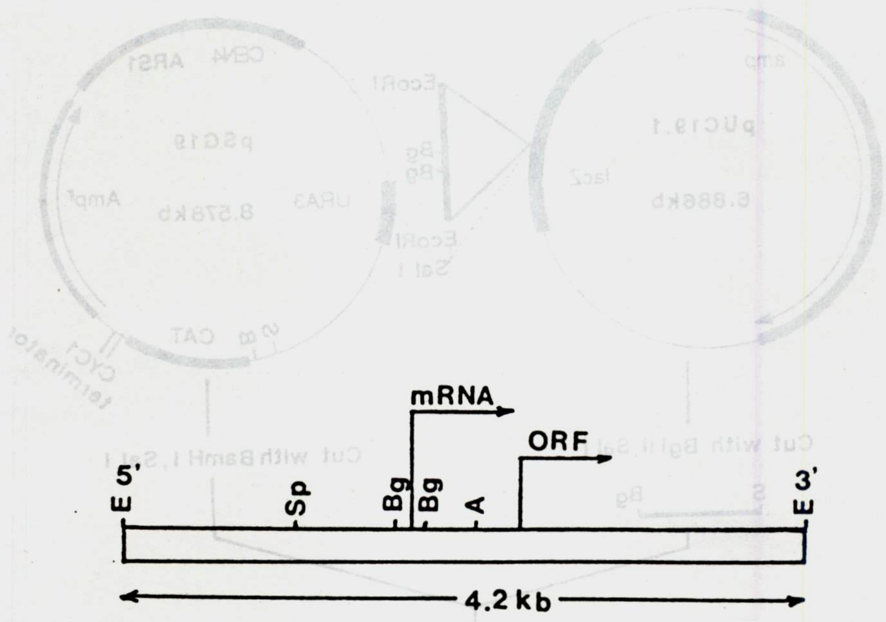

Figure 2. **Restriction Map of the *HSP26* Gene**
A: *Afl/III* Bg: *Bgl*II, E: *EcoR*I Sp: *Sph*I, X: *XbaI,* ORF: open reading frame.

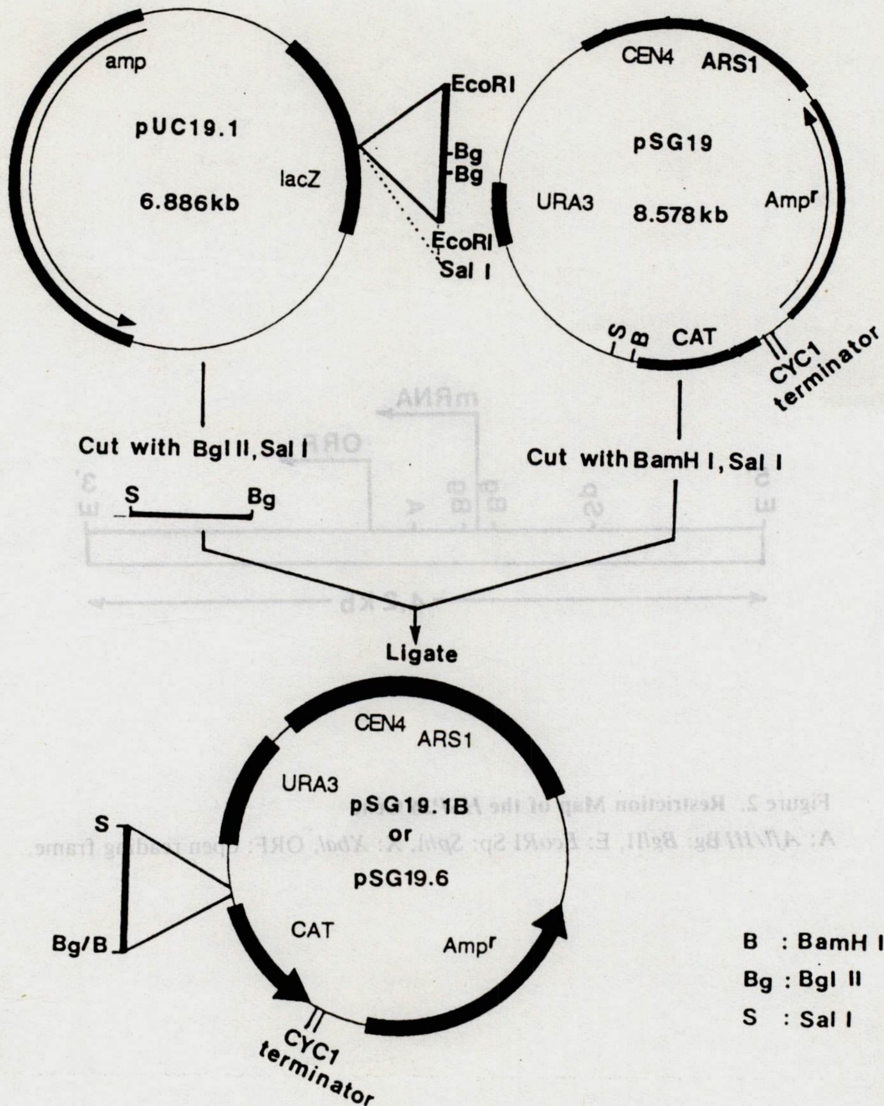

Figure 3. **Diagram of Cloning of the *HSP26* Promoter Fragments into pSG 19 Vector**

The promoter fragment were cut from pUC19.1 with *BglII* and *Sal*I. As there were two *Bgl*II sites within the promoter, two promoter fragment of different sizes were obtained, both were ligated independently with pSG19 into *BamH*I and *Sal*I sites. The ligation products were used to transform *E. coli*NM522 and plated onto ampicillin plate. The recombinants were verified by restriction enzyme analysis.
B: *BamH*I, S: *Sal*I. CAT: chloramphenicol acetyl transferase gene. A^r: ampicillin resistance gene. ORI: origin of replication. *lacZ:* gene encoding β-galactosidase (in this construction, the gene was disrupted by the *HSP26* gene).

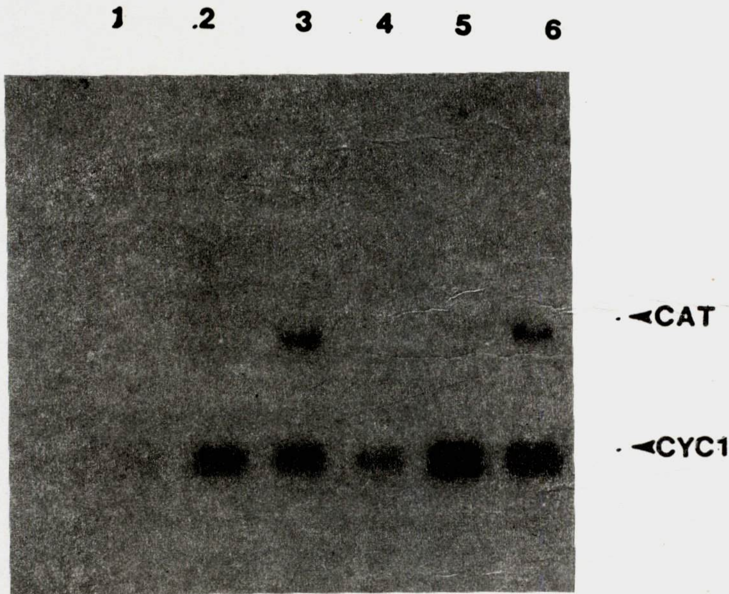

Figure 4. Northern-blot of RNA from CAT Gene Expression Regulated by the *HSP26* Promoter

RNAs were extracted from samples of cells taken at specific growth stages. The RNAs from CAT gene expression are shown by the arrow. The RNA bands underneath the CAT transcripts are *CYC*1 transcripts used as a size control.

1 : RNA from cells carrying pSG19-6, exponential phase
2 : RNA from cells carrying pSG19-6, early stationery phase
3 : RNA from cells carrying pSG19-6, late stationery phase
4 : RNA from cells carrying pSG19-1B, exponential phase
5 : RNA from cells carrying pSG19-1B, early stationery phase
6 : RNA from cells carrying pSG19-1B, late stationery phase.