Fusion of the promoter region of rRNA operon *rrnB* to *lac Z* gene

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ABSTRACT

A Lambda phage was constructed in which the structural gene for β galactosidase is fused to a DNA segment carrying the ribosomal promoter *rrnB* of *E. coli*. In this hybrid operon β galactosidase synthesis in vitro is repressed by ppGpp. Repression of β galactosidase synthesis by cAMP is reported.

INTRODUCTION

The molecular mechanisms involved in the regulation of rRNA synthesis in bacteria are only partially understood. The ribosomal RNA genes are known to respond to environmental conditions of the cell. When cells are starved for amino acids rRNA synthesis is repressed. Repression of rRNA synthesis coincides with the accumulation of Guanosine 5' diphosphate 3' diphosphate (ppGpp) (1). However in vitro analyses led to contradictory results (2,3,4). Further experiments are necessary for determining the factors involved in ppGpp repression of rRNA promoters.

We have used recombinant DNA techniques in order to construct a λ phage in which the ribosomal RNA promoter *rrnB* directs the synthesis of β galactosidase. In this communication we describe the construction and in vitro use of this phage. As expected, β galactosidase synthesis in vitro is subject to regulation by ppGpp. However we find that in this hybrid operon β galactosidase synthesis is also repressed by cAMP.

METHODS

a. Bacteria phages and plasmids. The *E. coli* strains M72 (*lac* ⁻ *am* *trp* ⁻ *am* *Su* ⁻) (5) were used in growing phage stocks. The phage λ W205 (6,7) and λ R167 were used in recombinant DNA construction (8). Plasmid pGG1 carries the *rrnB* promoters (2).

b. Media: Thryptone broth (TB) (1% Bacto-tryptone, 0.5% NaCl) TB bottom

agar (TB + 1% Bacto agar) and TB top agar (TB + 0.6% Bacto agar) were used for growth of bacteria and phages. MacConkey-lactose plates and X-gal plates were used for identification of λ lac⁺ phages.

c. β galactosidase assays. Assays were performed as described by Miller (10).

d. Restriction enzymes and restriction conditions. Restriction enzymes were from New England Biolabs. All restriction reactions were performed at 37°C for 1 hr in buffer mixtures recommended by the manufacturers.

RESULTS

Construction of recombinant phage. The construction of an hybrid operon in which lac Z gene expression is directed by the rRNA promoter is shown in Fig. 1. Three DNA fragments were joined in vitro. This experimental approach is a modification of the method used before (8,9,11). One fragment was the left half of λ W205 which bears a trp-lac fusion (6,7) and is missing the lac and trp promoters. This phage carries the λ immunity region and the sus S7 mutation. The fragment was generated by cleavage with Hind III which cuts in the trp B gene. Another fragment was the right half of λ RP167 cII⁻ that bears a single EcoRI site to the left of the phage attachment site. These two fragments

Fig. 1. Construction scheme of λ 913. Details of construction are described in the text.

were joined by a DNA fragment derived from the plasmid pGG1 by cleavage with EcoRI and HindIII. Following ligation for 48 hours at 4°C the assembled phage DNA was packaged in vitro with extracts containing λ head and tail proteins (11). The recombinant phages were plated with M72 Su⁻ on MacConkey lactose plates. This procedure selects against the λ W205 donor. The lac⁺ phages yielded deep red plaques on MacConkey lactose and deep blue plaques on X-gal plates. The putative recombinants were purified and checked to contain the λ imm21 cII⁻ immunity region.

Heteroduplex analysis

In order to prove the structure of λ 913 we performed heteroduplex analysis. The plasmid pGG1 carrying the rRNA promoter region and the att int exo region of phage λ was partially cleaved by HindIII and allowed to hybridize to λ 913 DNA. Fig. 2 shows one heteroduplex in which pGG1 hybridizes to the expected 5 kb DNA fragment carrying rrnB promoters. The direction of the rRNA promoters with respect to the lac Z gene can be deduced from the location of the two unhybridized tails with respect to the unhybridized λ arms. The long (6kb) tail extends near the short arm of phage λ and is colE1 DNA. The short (0.5 kb) tail extends near the long arm of the λ phage. This piece is from the 16S rRNA and is not present in λ 913.

In constructing the recombinant phage the plasmid pGG1 donates a DNA fragment originated from λ rifd 18 (12). This fragment carries att int xis exo region. A similar segment carrying att int xis exo is also donated by the λ imm²¹ donor. This segment carries a mutated EcoRI site in exo and a small b region (containing EcoRI and HindIII sites) (9). The heteroduplex analysis suggests that the λ 913 has lost a 4 kb segment from the short arm. It is feasible that this deletion occurs by att to att recombination which might eliminate the duplicated region in the hybrid phage.

Restriction analysis of the structure of λ 913.

Restriction analysis of λ 913 DNA revealed the presence of a single EcoRI site in the lac Z gene. This restriction analysis confirms the conclusion drawn from the heteroduplex analysis that the tandem repeat of the att int exo region is deleted by recombination since the second EcoRI site (expected to be present in exo) is missing.

Restriction by BamHI shows BamHI sites that are in agreement with the expected sites in phage λ (8). The BamHI site in the b region is removed

Fig. 2. Electron micrograph of heteroduplex between λ 913 and HindIII restricted pGG1. DNAs were denatured by alkali for thirty minutes, then neutralized with Tris buffer pH 7.6 and HCl 1.8N. DNAs were then mixed and allowed to heteroduplex in the presence of 50% deionized formamide. SV40 DNA was used as a size standard. Magnification 30000 X. The molecules were measured relating them to the SV40 DNA. For detailed description see text. S represents short λ tail, C - colE1, H - heteroduplex, R - ribosomal RNA tail and L stands for long tail of λ .

by the Trp A - lac Z substitution. The BamHI site in the right end of the λ map is eliminated by the nin₅ deletion present in λ 167 from which the right arm of λ 913 was constructed (8).

Using the Southern blotting technique (13) we could demonstrate that the largest BamHI fragment that ends at the BamHI site in int, hybridizes to a nicked translated two kilobase pstI fragment (see fig. 1) originated

Fig. 3. Schematic map of λ913. All details of the map are described in the text, P₁ and P₂ are included in the blackened block. B represents BamHI site, R - EcoRI and H - HindIII. The numbers stand for size in kilobases.

from pGG1 used as labeled probe. Since this fragment carries the *rrnB* promoter region and does not carry any λ sequences, this result clearly demonstrates the presence of the *rrnB* promoter region in λ913. Restriction by HindIII suggests that the HindIII site at the λW205 and pGG1 junction was modified, probably during the cloning procedure.

β galactosidase synthesis coded by λ913.

The results presented in table 1 clearly demonstrate that the hybrid operon carrying the *rrnB* - *lacZ* fusion can direct expression of β galactosidase. Expression of the *lacZ* gene is independent of the presence of the inducer IPTG that induces the *lac* operon (Table 1). Cells lysogenic for λ913 are immune to λimm21 (see fig. 1). The fact that *lacZ* is expres-

STRAIN	<u>β galactosidase units</u>	
	-IPTG	+IPTG
M72 <i>lac</i> ⁻	8	9
M72 <i>lac</i> ⁺	16	1716
M72 <i>lac</i> ⁻ (λ913)	499	512

Table 1. Expression of the *lacZ* gene. Cells were grown to stationary phase in LB media (1% Difco Tryptone, 0.5% NaCl, 0.5% Difco Yeast extract). IPTG, an inducer of the *lac* operon was added at a concentration of 1mM. Enzyme assays were performed as described by Miller (10). Cells lysogenic for λ913 were prepared following infection of non lysogenic cells. A λimm21cI⁻b₂ phage was used to complement the cII function missing in λ913.

sed by these lysogens in the presence of the λ imm21 repressor (Table 1) strongly indicates that phage promoters are not involved in β galactosidase synthesis by the hybrid operon.

The extent of expression of rRNA genes depends on the rate of cellular growth (14,15). We therefore compared the level of β galactosidase in cells growing in rich and poor media. As was previously shown for rRNA (14), the specific activity of β galactosidase was higher in fast growing cells. Expression of lac Z from a related prophage carrying the lac promoter was unaffected by growth conditions. These results suggest that the lac Z gene is under control of the ribosomal RNA promoters.

In vitro synthesis of β galactosidase directed by λ 913 DNA.

In order to test whether β galactosidase in the constructed phage is under the control of ribosomal promoters we used the in vitro transcription - translation system developed by Zubay (16).

We could show that λ 913 DNA dependent β galactosidase synthesis was inhibited by the addition of 0.2mM ppGpp to 30% of the control value (Table 3). Surprisingly, the addition of 1mM cAMP had also caused 60% inhibition of β galactosidase synthesis directed by the phage DNA. The addition of a combination of ppGpp and cAMP completely abolished enzyme synthesis (Table 3). The same results were obtained using different isolates of the constructed phage (λ 917, λ 918).

In agreement with previously published results (17) it can be seen that λ lacPs DNA directed β galactosidase synthesis was stimulated by both cAMP

STRAIN	<u>β galactosidase units</u>	
	Rich Medium	Poor Medium
M72 <u>lac</u> ⁻	8	11
M72 <u>lac</u> ⁻ (λ lacP5)	40	36
M72 <u>lac</u> ⁻ (λ 913)	545	226

Table 2. Effect of growth medium on lac Z gene expression. An experiment was performed essentially as described in Table 1 except that cells were grown in LB (rich medium) or M9 medium supplemented 40 μ g/ml Tryptophan and 0.25% glycerol. λ lacP5 carries authentic lac operator and promoter.

DNA	cAMP 1mM	ppGpp 0.2mM	β galactosidase units	
			exp. 1	exp. 2
λ 913	-	-	100	112
"	+	-	40	50
"	-	+	30	34
"	+	+	0	10
λ lacPs	-	-	18	15
"	+	-	110	140
"	-	+	50	60
"	+	+	183	190

Table 3. In vitro expression of Lac Z gene. β galactosidase synthesis was performed in an S-30 in vitro assay according to Zubay (16). Reaction mixtures (0.1 ml) contained 50-60 μ g/ml of the various DNAs. Strain CF 546 (obtained from M. Cashel) ($gal^- bio^- uvr^S B$) $\Delta argA^- trp$ Lac $\Delta RelA^+$ str^R Rec B - was used for the preparation of the S-30. At the end of two hours of incubation at 29°C, 50 μ l were taken from each reaction mixture for enzyme assay. β galactosidase was assayed according to Miller (10).

and ppGpp in the S-30 Zubay system.

DISCUSSION

The control of stable RNA synthesis in bacteria plays a central role in the regulation of cell growth rate (14,15,18). Substantial increases in the relative synthesis of the ribosomes and related components can be shown to accompany rapid growth (14). Thus far there is no satisfactory molecular explanation for this "growth control" phenomenon. A second well-known control of the expression of stable RNA genes is the stringent response (1). Here again the lack of regulatory mutants and the fact that we could not demonstrate a direct effect of ppGpp on the transcription of ribosomal promoters by RNA polymerase (2), has hindered the understanding of the molecular mechanism underlying this phenomenon. These facts prompted us to perform the experiments described here, the main goal being the construction of a recombi-

nant phage that will express efficiently the *rrnB* promoters through an easily assayed gene product. From the results presented here it is likely that this goal has been achieved. It seems that the β galactosidase gene in λ 913 is being transcribed from the fused *rrnB* promoters, originated in the plasmid pGG1. The expression of β galactosidase in λ 913 is shown to be subject to growth control (Table 2). It is not induced by IPTG and does not respond to λ repressor control. The *in vitro* experiments in the coupled transcription-translation system show that enzyme synthesis is inhibited by ppGpp. This result together with our previous observation in a purified transcription system, where we could not demonstrate any effect of ppGpp (2), favors the conclusion that the effect of ppGpp on ribosomal RNA transcription may involve other still undiscovered factors.

Both cAMP and ppGpp exert either a positive or negative effect on different promoters (19,20). In the present study both were found to have a negative effect on the synthesis of β galactosidase in λ 913.

The presence of two tandem promoters for all studied ribosomal RNA cistrons (2,21,22,23) raises the possibility of different control elements governing each of the two promoters. The change in the intracellular level of these two molecules, cAMP and ppGpp, under different growth conditions may determine the rate of synthesis of rRNA.

The biological system, the construction of which is described in the present communication, provides a convenient tool for further investigation of the control mechanisms involved in stable RNA synthesis.

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