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MICROCALORIMETRIC STUDY ON THE TRANSCRIPTION START SITE MUTAGENESIS

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Abstract

The microcalorimetric method and DNA site-directed mutagenesis technique were used together to study the effect of transcription start site mutagenesis on RM07 promoter activity and gene transcription efficiency in *Escherichia coli*. The results revealed that once the putative transcription start site G was mutated to T, the promoter activity of RM07 promoter fragment was decreased and the transcription strength of *cat* reporter gene was weakened. Our work suggests that the nucleotide component of transcription start site is very critical for the promoter strength and the gene transcription efficiency. Our research also provides a new and useful technique for determining the transcription start site using both chemical and biological method.

Keywords: *E. coli*, microcalorimetry, site-directed mutagenesis, transcription efficiency

Introduction

Transcription is the enzymic synthesis of RNA on a DNA template. This is the first step in gene expression and ultimately leads to the synthesis of the protein encoded by a gene. Therefore, transcription is the vital control point in the overall process of gene expression [1].

Transcription process includes three steps: initiation, elongation and termination. Initiation is a very important step. It involves the binding of RNA polymerase to the specific promoter DNA sequence, resulting in local double-strand DNA unwinding. Then the RNA polymerase initiates the synthesis of RNA at the specific nucleotide called transcription start site [2].

The efficiency of transcription initiation influences the rate of transcription, while the efficiency of transcription initiation is determined by the promoter structure and interaction between RNA polymerase and promoter [2]. In this work, we firstly used the microcalorimetric method and site-directed mutagenesis technique together to determine the transcription start site of RM07 promoter fragment [3] in *E. coli*.

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Using the RM07 promoter–chloramphenicol resistance gene (*cat*) fusion product as the model, we also studied the effect of the transcription start site mutation on the promoter strength and gene transcription efficiency. We find that the efficiency of transcription initiation is not only influenced by the promoter structure, but also affected by the nucleotide component of transcription start site.

Experimental

Bacteria and plasmids

The bacterial strains, *E. coli* HB101, *E. coli* HB101/pKK232-8, *E. coli* HB101/pKK232-07-11, *E. coli* HB101/pKK232-07-PMSTART are preserved in the Microbiological Genetics Laboratory in Wuhan University. They were grown in Luria–Bertani (LB) complex medium supplemented with antibiotics when necessary [4].

General molecular biological techniques

General molecular biological techniques (plasmid extraction, DNA fragment isolation, ligation and transformation) were performed as described [4].

Site-directed mutagenesis

The polymerase chain reaction (PCR) was used to perform the site-directed mutagenesis for changing the specific single base pair in RM07 DNA fragment. The experiment was performed according to the method described previously [5].

Calorimeter

A TAM air Isothermal Microcalorimeter, manufactured by Thermometric AB company of Sweden, was used to measure heat output of the metabolism of different *E. coli* strains. This isothermal microcalorimeter is an eight-channel twin instrument. Normally, 20 mL reaction vessels made from glass or stainless steel are used. The thermal power detection limit is stated to be ±2 μW [6]. The microcalorimeter was thermostated at 37ºC. The voltage signal was recorded by a computer. For details of the performance and structure of the instruments, see the Instruction Manual of TAM air Isothermal Microcalorimeter and [6].

Microcalorimetric measurements

The heat flow rate of the system can be measured *vs*. time. The isothermic heat flow–time dependence of bacterial growth was performed. The metabolic thermogenic curves of *E. coli* were determined using the ampoule method. A 20 mL glass ampoule was cleaned and sterilized, 5 mL bacterial suspension was put into the ampoule. The temperatures of the calorimeter system and the isothermal box were controlled at 37ºC. Meanwhile, a computer was used to record the thermogenic curves of *E. coli* growth continuously.

Results

Site-directed mutagenesis of the putative transcription start site

RM07 DNA fragment was isolated from the chromosomal DNA of the halophilic Archaea, *Halobacterium halobium*, and was shown to confer promoter activity in *E. coli* [3]. DNA sequence analysis revealed that RM07 fragment contained the typical –35 and –10 box sequences of bacterial promoters. Using the chloramphenicol resistance gene (*cat*) as the reporter gene, promoter functional research and deletion analysis both confirmed that the putative –35 and –10 box sequences were the accurate functional regions responsible for the promoter activity of RM07 fragment in *E. coli*.

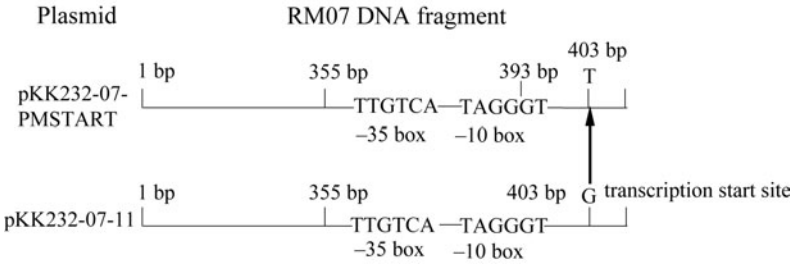

Fig. 1 Site-directed mutagenesis of the RM07 DNA fragment. In pKK232-07-PMSTART, the 403 bp G (putative transcription start site) was changed to T

According to above research results and known DNA sequence, we deduced that the 403 bp G (relative to the first base in RM07 fragment) in the 492 bp RM07 fragment was the transcription start site of RM07 promoter in *E. coli* (as shown in Fig. 1). To confirm it, we used the site-directed mutagenesis technique to change the 403 bp G to T and then checked the effect of this mutation on reporter gene transcription. The mutated RM07 fragment (1 bp-404 bp) was inserted upstream of the promoter–less *cat* gene in the promoter probe vector pKK232-8 [7], generating plasmid pKK232-07-PMSTART. The unmodified RM07 fragment (1 bp-404 bp) was also inserted upstream of the promoter–less *cat* gene of pKK232-8, generating plasmid pKK232-07-11 (as the control, shown in Fig. 1). Above two plasmids plus pKK232-8 were transformed into *E. coli* HB101. Because the chloramphenicol resistance gene (*cat*) was used as the reporter gene, the level of chloramphenicol resistance corresponded to the promoter activity and the *cat* gene transcription efficiency.

Microcalorimetric study on the effect of mutation on the promoter strength and gene transcription efficiency

The microcalorimetric method is one of the important techniques for thermodynamic studies.

By monitoring the heat effect with a sensitive calorimeter, microcalorimetry can directly determine the biological activity of a living system and provide a continuous measurement of heat production, thereby giving much information in both qualitative

and quantitative ways. For these advantages, we further used the microcalorimetry technique to study the effect of the transcription start site mutation on the promoter strength and gene transcription efficiency.

In the logarithmic growth phase, the heat output of cell growth is exponential [8],

$$P_t=P_0\exp(k_m t)$$

or

$$\ln P_t=\ln P_0+k_m t \quad (1)$$

where k_m is the metabolic rate constant. The growth thermogenic curves of the logarithmic growth phase correspond to Eq. (1). Therefore, using the data $\ln P_t$ and t taken from the curves to fit a linear equation, one can obtain the metabolic rate constant (k_m). The rate constants (k_m) are shown in Tables 1 and 2. All of the experimental results have a very good reproducibility and correlationship.

High concentrations of antibiotics will inhibit the growth of *E. coli*, and the metabolic rate constant will decrease. Therefore, the inhibitory ratio (I/%) can be defined as:

$$I=100[(k_0-k_c)/k_0]$$

where k_0 is the rate constant of the control, k_c is the rate constant for *E. coli* growth inhibited by the antibiotic with a concentration of C. When the inhibitory ratio (I) is 50%, the corresponding half-inhibitory concentration of the antibiotic can be represented as IC_{50}, which can be regarded as the inhibiting concentration causing 50% decrease of the *E. coli* growth rate constant. Data for I and IC_{50} are shown in Table 1.

The data about the growth of *E. coli* HB101, *E. coli* HB101/pKK232-8, *E. coli* HB101/pKK232-07-11 and *E. coli* HB101/pKK232-07-PMSTART in LB medium containing chloramphenicol and ampicillin were shown in Tables 1 and 2. The metabolic power–time curves of *E. coli* HB101/pKK232-07-11 and *E. coli* HB101/pKK232-07-PMSTART growing in LB medium containing different concentrations of chloramphenicol were obtained by microcalorimetric measurement and shown in Fig. 2. The following results were acquired from these data: (*i*) the host cell *E. coli* HB101 was sensitive to ampicillin (amp), when the concentration of ampicillin reached 100 μg mL^{-1}, *E. coli* HB101 couldn't grow (k_m=0) (as shown in Table 2). *E. coli* HB101 was also sensitive to chloramphenicol (cm), IC_{50} for chloramphenicol was 1.91 μg mL^{-1} (Table 1). (*ii*) *E. coli* HB101/pKK232-8 was resistant to ampicillin. But it was still sensitive to chloramphenicol, IC_{50} for chloramphenicol was 1.79 μg mL^{-1}. When the concentration of chloramphenicol reached 2 μg mL^{-1}, it couldn't grow. (*iii*) *E. coli* HB101/pKK232-07-11 was not only resistant to ampicillin, but also resistant to chloramphenicol. IC_{50} for chloramphenicol was 73.0 μg mL^{-1} (Table 1). When the concentration of chloramphenicol reached 300 μg mL^{-1}, *E. coli* HB101/pKK232-07-11 could still grow. This result suggests that RM07 fragment confers promoter activity in *E. coli.* (*iv*) The level of resistance to chloramphenicol of *E. coli* HB101/pKK232-07-PMSTART was decreased compared with *E. coli* HB101/pKK232-07-11. IC_{50} for chloramphenicol was 40.0 μg mL^{-1} (Table 1). When the concentration of chloramphenicol reached

Table 1 Data about the growth of *E. coli* in LB medium containing chloramphenicol[a]

E. coli	C_{cm}/ μg mL^{-1}	k_m/ min^{-1}	P_m/ μW	t_p/ min	I/ %	IC_{50}/ μg mL^{-1}
HB101	0	0.01556	206.4	173	–	1.91
	0.5	0.01264	179.5	197	18.8	
	1	0.00978	129.4	238	37.1	
	2	0.00749	100.5	264	51.9	
	4	0	–	–	100	
HB101/ pKK232-8	0	0.01397	212.5	211	–	1.79
	0.5	0.01169	175.2	228	16.3	
	1	0.00986	151	266	29.4	
	2	0	–	–	100	
	4	0	–	–	100	
HB101/ pKK232-0 7-11	0	0.01433	213.1	220	–	73.0
	10	0.01035	210	223	27.8	
	30	0.01027	167.7	291	28.3	
	50	0.00908	107.3	482	36.6	
	100	0.00499	73.0	811	65.2	
	300	0.00316	35.2	2048	77.9	
	400	0	–	–	100	
HB101/ pKK232-07-PMSTART	0	0.01522	196	196	–	40.0
	10	0.01190	181.1	220	21.8	
	30	0.00972	105.1	443	36.1	
	50	0.00537	72.5	784	64.7	
	100	0.00278	45.0	1417	81.7	
	200	0	–	–	100	

[a]cm: chloramphenicol

Table 2 Data about the growth of *E. coli* in LB medium containing ampicillin[a]

E. coli	C_{amp}/μg mL^{-1}	k_m/min^{-1}	P_m/μW	t_p/min
HB101	100	0	–	–
HB101/pKK232-8	100	0.01421	194.8	236
HB101/pKK232-07-11	100	0.01362	198.5	241
HB101/pKK232-07-PMSTART	100	0.01373	197.5	251

[a]amp: ampicillin

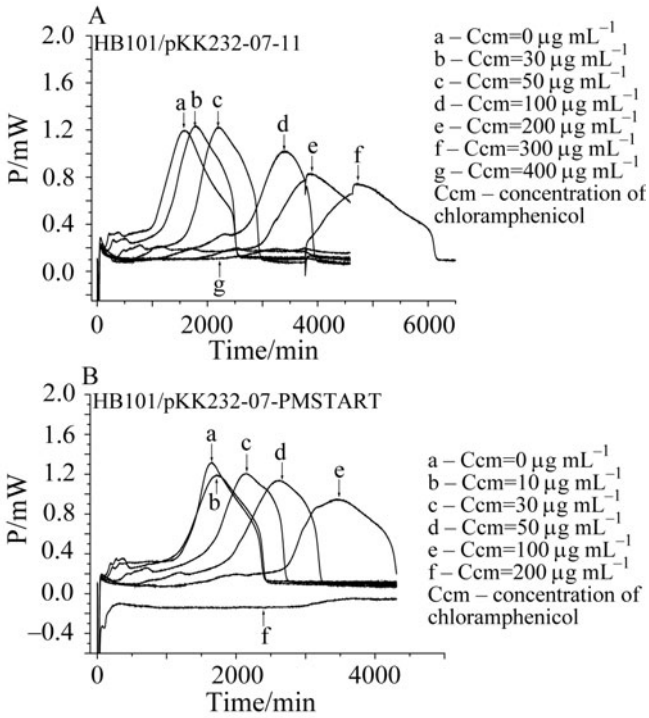

Fig. 2 The metabolic power–time curves of *E. coli* transformants growing in LB medium containing various concentrations of chloramphenicol. A: *E. coli* HB101/pKK232-07-11 (the concentration of chloramphenicol is 0, 30, 50, 100, 200, 300 and 400 μg mL^{-1}); B: *E. coli* HB101/pKK232-07-PMSTART(the concentration of chloramphenicol is 0, 10, 30, 50, 100 and 200 μg mL^{-1}). C_{cm}: concentration of chloramphenicol

200 μg mL^{-1}, *E. coli* HB101/pKK232-07-PMSTART couldn't grow. The putative transcription start site G was mutated to T in pKK232-07-PMSTART, which resulted in the decrease of the RM07 promoter activity and the transcription efficiency of *cat* gene, so the level of resistance to chloramphenicol of *E. coli* HB101/pKK232-07-PMSTART was decreased.

Relationship between P_m and the concentration of chloramphenicol(C)

When the concentration of chloramphenicol(*C*) increased, the growth of *E. coli* was inhibited, the maximum heat power (P_m) of logarithmic growth phase decreased (shown in Table 1). If we make a linear regression of P_m *vs. C,* we can obtain the linear relationship between P_m and *C*.

HB101: P_m=201.42–54.25*C*, *R*=–0.9685 (*C*: 0–2 μg mL^{-1})

HB101/pKK232-8: P_m=210.32–61.5*C*, *R*=–0.9925 (*C*: 0–2 μg mL^{-1})

HB101/pKK232-07-11:

$$P_m=223.51–2.17C,\ R=–0.9778\ (C\text{: }0–100\ \mu g\ mL^{-1})$$

$$P_m=110.83–0.26C,\ R=–0.9537\ (C\text{:}100–300\ \mu g\ mL^{-1})$$

HB101/pKK232-07-PMSTART:

$$P_m=202.6–3.14C,\ R=–0.9838\ (C\text{: }0–30\ \mu g\ mL^{-1})$$

$$P_m=122.15–0.80C,\ R=–0.9578\ (C\text{: }30–100\ \mu g\ mL^{-1})$$

Discussion

Our research suggest that besides the –10 and –35 box sequences of the promoter, the nucleotide component of transcription start site is also critical for the promoter strength and the efficiency of gene transcription. In this study, once the transcription start site G is mutated to T, the efficiency of transcription initiation is decreased and the transcription strength of *cat* gene is weakened. Previous research have revealed that the transcription start site is a purine in 90% of all *E. coli* genes. G is more common than A, but T is rare [9]. Our research results further confirm it. In summary, the conserved sequences of –10 and –35 box of the promoter and the transcription start site nucleotide are all very important for the transcription initiation and transcription efficiency.

Our research also provides a new method for determining the transcription start site by means of chemical and molecular biological methods. Accurate mapping of the transcription start site is important for the success of subsequent promoter analysis. The traditional biological methods for transcription start site mapping include primer extension and S1 nuclease analysis. But all of these methods are involved with mRNA isolation and analysis, so it is time-consuming and cumbersome [10]. In this study, according to the information obtained from the DNA sequence analysis, we firstly used the microcalorimetry analysis (chemical method) and DNA site-directed mutagenesis technique (biological method) together to determine the transcription start site. Microcalorimetry method is a very useful tool for studying microbiological genetics because it is accurate, sensitive, simple to perform and reproducible [11, 12]. By monitoring the heat effect with a sensitive calorimeter, microcalorimetry can directly determine the biological activity of a living system and do not disturb the normal activity of biosystems. Site-directed mutagenesis is a very powerful tool to change specific nucleotide in a DNA sequence and then check the biological effect of the specific mutation, which can provide much information about the transcription regulation and gene expression. Therefore, we believe that the combination of the microcalorimetry analysis and DNA site-directed mutagenesis technique has the promising application value in biological research fields.

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