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In vivo and *in vitro* activities of the bacteriophage T4 early promoters

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After infection, bacteriophage T4 redirects transcriptional machinery of *Escherichia coli* to T4 promoters. Because of the sequence properties, T4 early promoters are stronger than *E. coli* promoters. In this work, we studied the deviations from the consensus sequence that exist among T4 early promoters and the factors that determine their activity. We selected putative early promoter sequences from the early regions of T4 genome and tested them for their activities *in vivo* and *in vitro*. Two of the sequences tested were found to be active *in vivo*. Thus, we mapped two new early promoters on the T4 genome. I*n vitro* transcription analysis of the promoter-like sequences that were inactive during phage infection shows that the presence of glucosylated hydroxymethylcytosines in phage DNA may modulate promoter activity *in vivo*.

Key words: bacteriophage T4, early promoters, transcription, consensus sequence

INTRODUCTION

Following infection by bacteriophage T4, *Escherichia coli* RNA polymerase (RNAP) recognizes T4 early promoters and immediately initiates transcription of the early phage genes. The transcription is very efficient and increases after ribosylation of the RNAP by phage ADP-ribosyltransferases. No other phage-encoded factors are required for the early transcription of T4. Efficient recognition of phage early promoters is determined mainly by the properties of phage DNA.

It has long been thought that T4 early promoters are very typical and well conserved. About 40 early phage promoters share features with the consensus sequence, which is characterized by a heptamer GTTTACa/t centered at –35 position relative to the transcriptional start site, a hexamer TAnnnT centered at –10, and the spacer region of 16–17 bp between them [1–3]. Polyadenine tracts located at –52 to –42 positions and the extension of the –10 sequence by a tGTGG motif are also characteristic features of T4 early promoters [1–5]. Only a minor part of T4 early promoters deviate from the consensus sequence, and some of them have the same sequence as the *E. coli* σ^{70} promoters. However, the activities of such deviant promoters have usually been demonstrated using only plasmid or *in vitro* systems and have not been confirmed during phage infection.

Many new middle promoters have been detected on the T4 genome in the recent years [6–8], and their sequences vary significantly. About a half of the presently known middle promoters deviate from the consensus sequence deduced previously. It is indicative that some other features of DNA than simply sequence and spacing constraints may influence the activity of a particular phage promoter.

The aim of this study was to test what deviations from the consensus sequence could be detected among T4 early promoters. Therefore, we analysed the prereplicative regions of phage genome and tested several early promoter-like sequences for their activities *in vivo* and *in vitro*. The preliminary results show that some differences can be detected at –35 position of T4 early promoters, but the presence of glucosylated hydroxymethylcytosines in phage DNA may also modulate promoter activity *in vivo*.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. *Escherichia coli* strain B^E (*sup*^{0}) was kindly provided by Dr L. W. Black. This strain was used for propagation of phage T4, as well as for isolation of the total cell RNA. Bacteriophage T4D wild-type was a gift from Dr W.B. Wood.

Enzymes and isotope. T4 polynucleotide kinase, *Taq* DNA polymerase, RNase inhibitor and rNTPs were obtained from Fermentas AB. AMV reverse transcriptase was obtained from Promega. *E. coli* RNAP and [γ^{32}P]ATP was supplied by Amersham Biosciences.

PCR amplification. The PCR was carried out as described [9]. The DNA templates for the PCR were denatured phage particles or the genomic phage DNA.

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RNA isolation and primer extension analysis of phage mRNAs. Total RNA from T4 phage-infected *E. coli* cells was phenol-extracted and analyzed by primer extension under conditions of primer excess, using avian myeloblastosis virus reverse transcriptase as described previously [8, 10]. The oligonucleotides were 5'-labeled with [γ^{32}P]ATP using T4 polynucleotide kinase.

***In vitro* transcription.** *In vitro* transcription experiments were carried out using about 400 bp DNA fragments that were PCR amplified from the genomic T4 DNA and carried a particular promoter sequence. The reaction was performed in a 50 µl reaction mix which contained 1 mg DNA, 10 u of the *E. coli* RNAP, rNTPs (0.5 mM of each) and 40 u of RNase inhibitor in the reaction buffer for the *E. coli* RNAP. The reaction mix was incubated at 37 °C for 30 min. Then EDTA was added up to 0.02 mM concentration, and the mix was extracted with the equal volume of phenol (pH 4.8). The extraction was repeated with phenol:chlorophorm mix and with chloroform. Then 1/10 part of 5 M NaCl and 2.5 parts of 96% ethanol were added to the supernatant, the mix was placed at –20 °C for 2 hours and RNA was collected by centrifugation. RNA was suspended in 10 µl DEPC-treated water and stored at –20 °C.

Oligonucleotide primers. The T4-specific oligonucleotide primers used in this work are listed in Table.

RESULTS AND DISCUSSION

To study what sequence differences can exist among early T4 promoters, we analyzed the prereplicative genomic regions of phage T4 and selected the sequences that potentially could serve as early promoters. The selected sequences had a good match to the –10 consensus sequence, a spacer region of 15 to 19 bp and the –35 regions that were similar to those of the *E. coli* σ^{70} or T4 early promoters (Fig. 1). To test the activities of these sequences *in vivo*, we analyzed transcripts induced after infection by bacteriophage T4. To this end, total RNA was isolated from T4-infected *E. coli* cells and the transcripts were analyzed by primer extension.

Only two of the putative promoter sequences listed in Fig. 1 were found to act as promoters of phage T4 (Fig. 2). We designated these promoters as **$P_E$segD** and **$P_E$pseT1**. Primer extension analysis showed that the

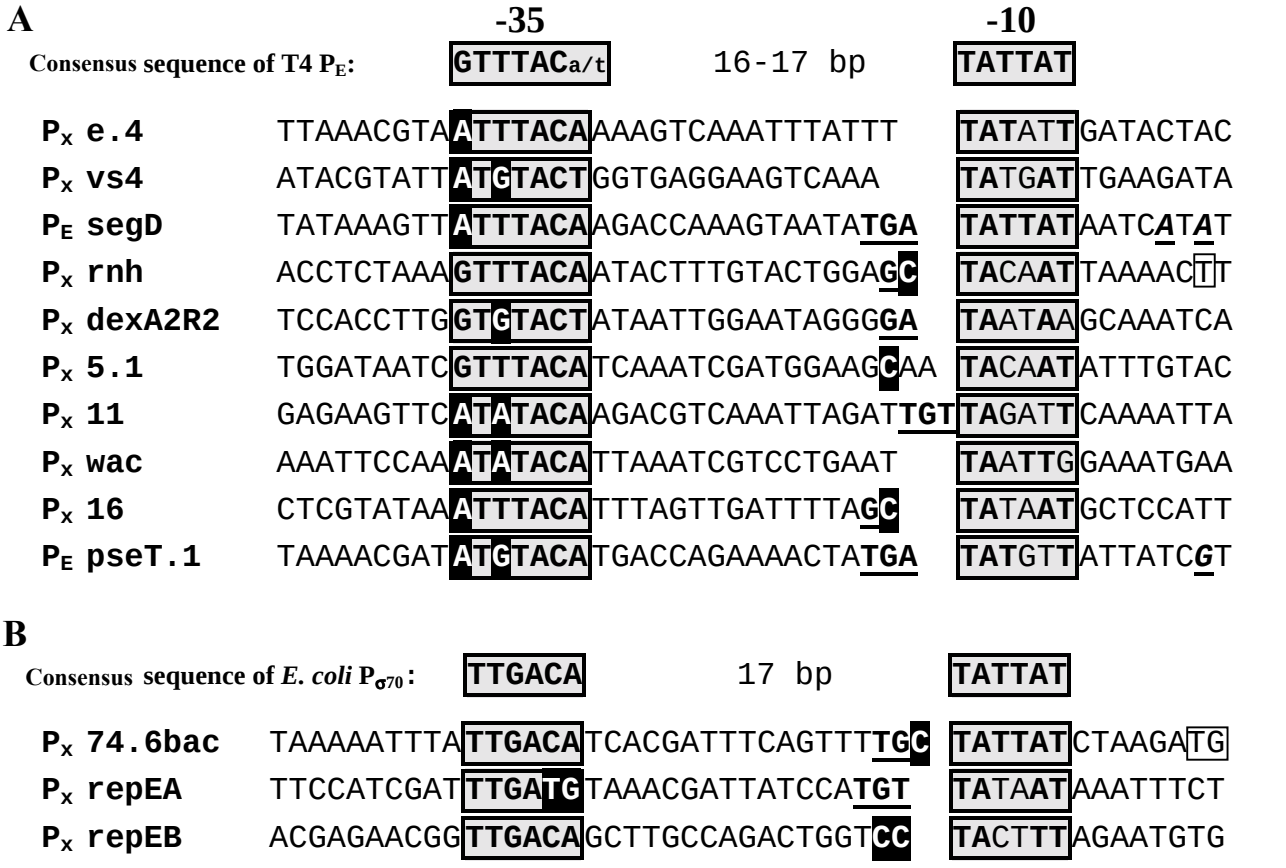

Fig. 1. Early promoter-like sequences selected from prereplicative regions of bacteriophage T4 genome. (A) Sequences matching the consensus sequence of T4 early promoters. (B) Sequences matching the consensus sequence of *E. coli* σ^{70} promoters. The *in vivo* active promoters are designated as P_E. Conserved promoter elements are shown in grey boxes. Differences from the consensus sequence are shown in black boxes. Extensions of –10 sequences are shown in bold and underlined. The initiating nucleotides for the transcripts directed from *in vivo* active promoters are shown in bold, italised and underlined. The initiating nucleotides for the transcripts directed *in vitro* are given in white boxes

Table. **Primers used to get PCR fragments from T4 DNA (A) and primers used to prime AMV reverse transcriptase (B)**

A

Primer	Oligonucleotide sequence
T4P74.6bacD1	5'-CCACTACGCTTTACACCGCTTAA
T4P74.6bacR1	5'-ACCTGTTACTGATACCGTGACTG
T4rnhD1	5'-GTGCAGCAGAAGTAAATCTACG
T4rnhR2	5'-GTACCGATGCTAACTGGATTACG

B

Primer	Oligonucleotide sequence
T4pseT.1R	5'-GTTGGAGGTAAAGATTCTAACCGCT
T4segDR	5'-GTCAAGAGTAGCATGAGCTCCGATG
T4e.4R	5'-GGAAGGTATAGTGATGGCTTTGAC
T4vs.4R	5'-GTCCTAAACGAACTTCAGCG
T4rnhPeR	5'-GCAGCTGGAAATGACATCACG
T4dexA2R2	5'-GGAGCATTACTGCGTTTAACTTCTG
T4g5.1PeR	5'-GTAATGTCAGCATTACCTTCAAC
T4g11PeR	5'-GAACCTCAAGAGAGTTAGGA
T4wacPeR	5'-GCCGTCAACGAATGGTAAGTC
T4g16PeR	5'-GGAGATGGATTATACTGCG
T4P74.6bacR	5'-GTCGTGGATAGGAATGTATGAATGG
T4repEAR	5'-GAAGAAGTATCACCATCAGGAAG
T4repEBR	5'-GAAGCAGCCAGTTCGTGATATG

transcripts coming from these promoters appeared immediately after infection and their amount increased up to 4 min post-infection. Moreover, analysis of the transcripts isolated in the presence of chloramphenicol showed that transcription did not depend on phage protein synthesis (Fig. 2). These transcription features are characteristic of the transcription initiated from early T4 promoters.

Both promoters have differences in their –35 regions, but their –10 sequences and the spacer regions match the consensus very well. Promoter **$P_E$segD** has the sequence ATTTACA in its –35 region and promoter **$P_E$pseT1** has ATGTACA. These results indicate that some differences can exist in the –35 region of T4 early promoters. The first and the third base positions of –35 region are usually well-conserved among early T4 promoters, but apparently are not absolutely required for the recognition.

On the other hand, primer extension analyses failed to detect any 5' ends coming from the rest of the putative promoters listed in Fig. 1. This was not very surprising, since these sequences had various deviances from the early promoter consensus. Interestingly, the sequences $P_x$rnh and P_x5.1, which had a good match to the consensus sequence of T4 early promoters, as well as the sequences P_E74.6bac and $P_E$repEB which had a good match to the *E. coli* σ^{70} promoters appeared to be inactive *in vivo*. Promoters P_E74.6bac [1, 11] and $P_E$repEB [12] have been already mapped on the T4 genome previously based on their activities in the plasmid systems. However, here we have found that they do not function during phage infection. These facts suggest that the sequence context of the genomic

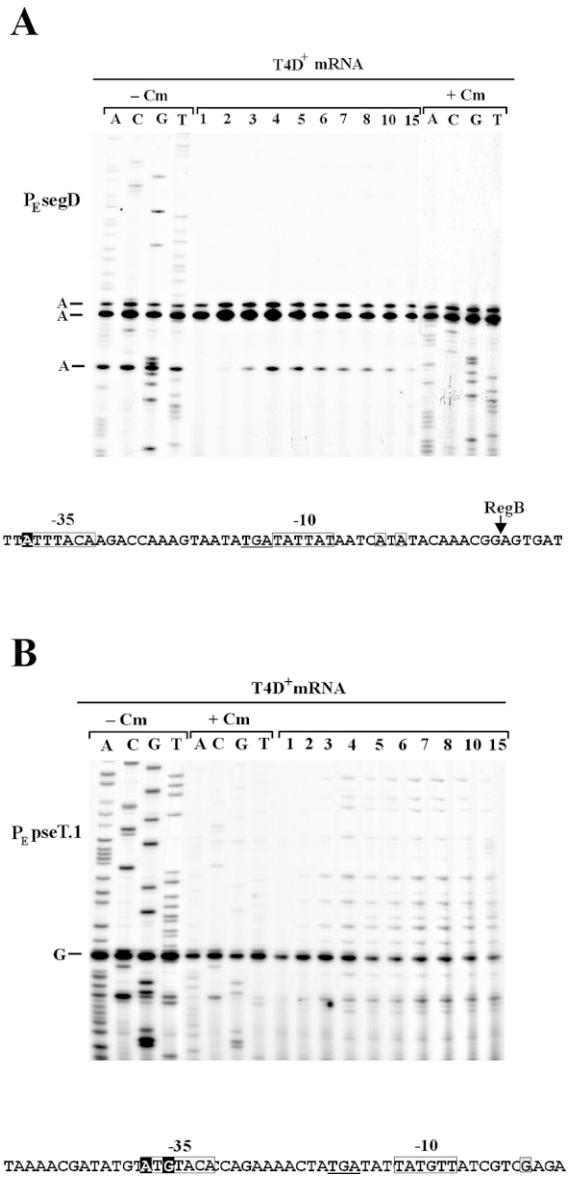

Fig. 2. Primer extension analysis of transcripts for genes *segD* (A) and *pseT.1* (B) of bacteriophage T4. Primer extension sequencing was done on RNA isolated from *E. coli* B^E cells at 3 min post-infection with bacteriophage T4 in the absence or presence of chloramphenicol at 30 °C. Primer extension reactions of RNA isolated at 1 to 15 min post-infection from the cells that were infected with phage T4 at 30 °C are shown. Primers T4segDR and T4pseT.1R were used to prime AMV reverse transcriptase. The sequencing lanes are labelled with the dideoxynucleotides used in the sequencing reactions. The post-infection time (minutes) at which each RNA was isolated is noted on the top of the autoradiograph. The initiating nucleotides of transcripts and the 5′-end nucleotide of the endoribonuclease RegB-truncated transcript are noted. Nucleotide sequences of the 5′ flanking regions of genes *segD* and *pseT.1* of phage T4 are shown at the bottom of the autoradiographs. The –35 and –10 regions of early promoters and the initiating nucleotides for transcripts are shown on grey background. The base that differs from the consensus is on black background. Vertical arrow denotes the position of RegB cleavage

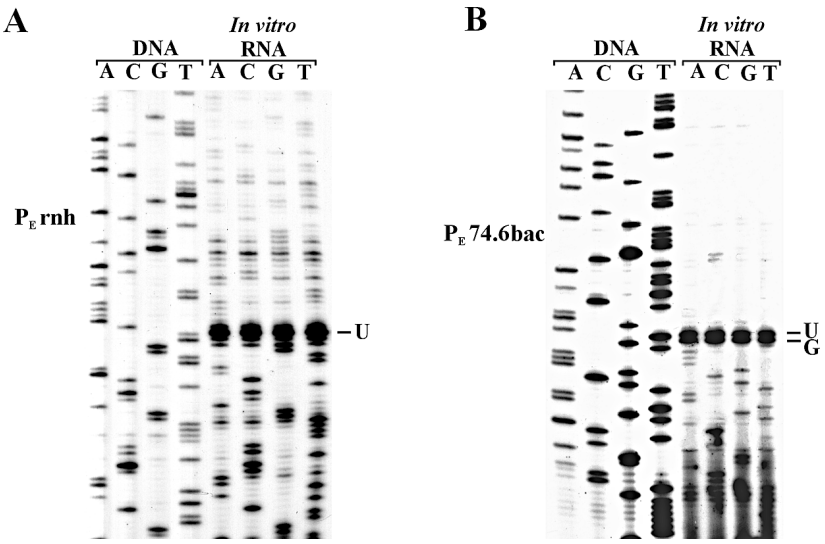

Fig. 3. Primer extension sequencing of the transcripts directed *in vitro* from the putative T4 early promoters $P_x$rnh (A) and P_x74.6bac (B). Sequencing reactions were done on RNA obtained by *in vitro* transcription from PCR fragments carrying a sequence of putative promoters. Primer extension reactions were performed using primers T4rnhPeR (A) and T4P74.6bacR (B). Sequencing of phage genomic DNA is also presented. The sequencing lanes are labelled with the dideoxynucleotides used in sequencing reactions. The initiating nucleotides for the transcripts are noted and given in white boxes in Fig. 1

phage DNA may have an important contribution to the function of T4 promoters.

It has been observed previously that both T4 early [2] and middle [7] promoters do not have cytosines at the positions –14 and –13 relative to the transcriptional start site. This fact has induced the idea that glucosylated hydroxymethylcytosines of phage DNA can impair the binding of *E. coli* RNAP to the promoter sequence. To test this idea, we made *in vitro* transcription experiments with the PCR-generated DNA fragments carrying either the T4 early promoter-like sequence $P_x$rnh or the *E. coli* σ^{70} promoter-like sequence P_X74.6bac. These sequences were selected for the experiment, since they had cytosines at the –13 position relative to the transcriptional start site. Such sequences should be recognized by the *E. coli* RNAP *in vitro*, since PCR-generated DNA has no cytosine modifications.

As we expected, primer extension sequencing of *in vitro* generated transcripts showed initiation of transcription from both $P_x$rnh and P_E74.6bac sequences (Fig. 3). The transcription started at expected positions indicating that *E. coli* RNAP recognized these sequences correctly. Therefore, our results show that cytosine modification of the T4 DNA can modulate activity of a particular promoter during phage infection.

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T4 BAKTERIOFAGO ANKSTYVŲJŲ PROMOTORIŲ AKTYVUMAS *IN VIVO* IR *IN VITRO*

S a n t r a u k a

T4 bakteriofago infekcijos pradžioje fagas nukreipia *E. coli* RNR polimerazę savo genų transkripcijai. Tai pasiekiama stiprių ankstyvųjų promotorių dėka, kurių stiprumą lemia DNR sekos elementai. Šiame darbe mes tyrėme, kokie sekos skirtumai nuo tipinės sekos gali būti aptinkami tarp ankstyvųjų T4 promotorių. Tam tikslui iš ankstyvųjų fago genomo sričių buvo atrinktos sekos, kurios potencialiai galėtų funkcionuoti kaip ankstyvieji promotoriai, ir buvo tiriamas jų aktyvumas *in vivo* ir *in vitro*. Dvi tirtos sekos buvo patvirtintos kaip ankstyvieji T4 promotoriai. *In vitro* transkripcija nustatyta, kad fago infekcijos metu neaktyvios sekos gali būti atpažįstamos *E. coli* RNR polimerazės *in vitro*, jeigu jos atitinka tipinę seką. Tirtų sekų analizė rodo, kad modifikuota fago DNR gali nulemti ankstyvųjų fago promotorių aktyvumą *in vivo*.