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Bacteriophage T7 late promoters: construction and *in vitro* transcription properties of deletion mutants

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ABSTRACT

The construction of plasmids containing T7 class I promoters with deletion mutants was described. Restriction fragments, ending at the Hinf I site located at position -10 in the promoter from 14.8% of the T7 genome, were cloned into pBR322. This produced the deletion of either the left or the right part of the promoter. The in vitro transcription properties of these plasmids were determined. Control plasmids were obtained by cloning wild type class II and class III promoters into pBR322. These plasmids also were used to compare the in vitro transcription properties of the two classes of late promoters.

Much of the leftward part of a T7 late promoter can be deleted without abolishing activity, but deletion of the right part eliminates promoter activity.

Class II, class III, and the mutated promoters have characteristic responses to changes in ionic strength, exogenous glycerol, and temperature.

INTRODUCTION

T7 RNA polymerase is a monomeric protein (105,000 daltons) which is synthesized very early during the infection of E. coli by T7 (1). It is both considerably smaller and less complex than the bacterial RNA polymerase, but nevertheless is responsible for the expression of both the middle (class II) and late (class III) T7 genes (2).

The late promoters have a structure very different from bacterial promoters, consisting of a highly conserved uninterrupted sequence extending from -17 to +2 bps relative to the initiation site. Since the expression of the class II and class III genes is regulated at the level of transcription (3), the late promoters also are divided into class II and class III. Of the 17 late promoters which are mapped and sequenced, 10 are class II, 5 are class III and 2 are near the ends of the genome (4,5,6,7,8). There are two structural differences between class II and class III promoters which probably account for the differences in their expression. Class II promoters are all located in the 14-40% region of the T7 genome while class III promoters are all located to the right of 40% (9). Class III promoters have the conserved

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sequence from -17 to to +2 and extend the region of conservation from -22 to +6.

In order to more fully understand the essential features of the conserved region, a series of T7 late promoters with deletion mutations was constructed. Since these mutant promoters were on plasmids, the isolation of sufficient amounts of templates for a series of quantitative in vitro transcription experiments was facilitated.

MATERIALS AND METHODS

Enzymes

Hinf I was purified by the procedure of Greene et al. (10) from frozen Haemophilus influenzae R_f cells (gift of U. Mueller). Eco RI, Bam HI, and Alu I were generously provided by S. M. Stirdivant, W. Zacharias, and P. F. Lambert, respectively. Micrococcus luteus DNA polymerase (11) was prepared by E. Selsing. T4 DNA ligase was purchased from New England Biolabs.

A partially purified T7 RNA polymerase was prepared from T7 infected E. coli MO cells. A cleared lysate of the infected cells was brought to 0.2M NaCl in P-buffer and run through a phosphocellulose column as described (12). The active fractions were pooled, diluted to 0.2M NaCl with P-buffer (described in 12), and run through the column a second time. This procedure produced a 160 fold purification of the polymerase. A 37% increase in specific activity was observed when the pooled fractions were concentrated by dialysis (vs. P-buffer, 50% glycerol, 50mM NaCl) possibly due to the removal of excess salt. When assayed as described (12), the final pool contained 827 units/ml at a specific activity of 2560 units/mg.

Two methods showed no detectable RNase contamination of the T7 TNA polymerase. In an in vitro transcription experiment, using a 4 fold greater concentration of polymerase than normally used, the incorporation of [^{3}H]-CMP into acid insoluble RNA showed a linear rate of increase which leveled off after about 1 hour and showed no decrease after 4 hours. Also, [^{3}H]-labeled RNA was incubated with an 8 fold greater than normal concentration of polymerase in the transcription buffer at 37° for 1/2 hour; this produced no detectable loss of acid insoluble counts. This preparation also has proved to be stable for at least 6 months when stored at -20°.

Cloning Methods

DNA fragments for cloning were isolated from polyacrylamide gels (13). If the sticky ends of the fragment and vector were incompatible, they were filled in with Micrococcus luteus DNA polymerase before ligating (14). Ligation reactions (15) and transformations (16) were essentially as

described. Several restriction sites in the tet^r region of pBR322 were used for the cloning of the fragments. Clones of the desired fragments were found by screening the $amp^r tet^s$ colonies by toothpick assays (17), followed by micro-scale plasmid DNA preparations (18) and restriction mapping.

Plasmid DNA samples were prepared as described (19). The E. coli strain MO (14) was used for purification of plasmid DNA and for the preparation of the T7 RNA polymerase.

Construction of Promoters with Deletion Mutations

Three DNA fragments, containing either a late promoter or a part of a late promoter, were obtained from pRW351 (Fig. 1). Deletions of either the left or the right part of the promoter were produced by cloning the 107 bp or the 241 bp fragment into pBR322. In these clones, the base-pairs missing from the promoter are replaced by the vector DNA adjacent to the cloned fragment.

Construction of Plasmids with Wild Type Promoters

The 122 bp Eco RI fragment, which contains a class II promoter (Fig. 1), was cloned into the Eco RI site of pBR322 in both possible orientations. A 98 bp Hae III fragment, containing the class III promoter from 46.5% of the T7 genome (5), was isolated from T7 DNA and cloned into the Bam HI site of pBR322.

In Vitro Transcription

Unless otherwise stated, all in vitro transcription reactions were done at 37° in 50 mM Tris-HCl, pH 7.6, 0.1mM dithiothreitol, 10mM $MgCl_2$, 2.5mM spermidine, and ATP, GTP, UTP, and CTP (0.2mM each) (plus 20 μCi/ml [^{3}H]-CTP).

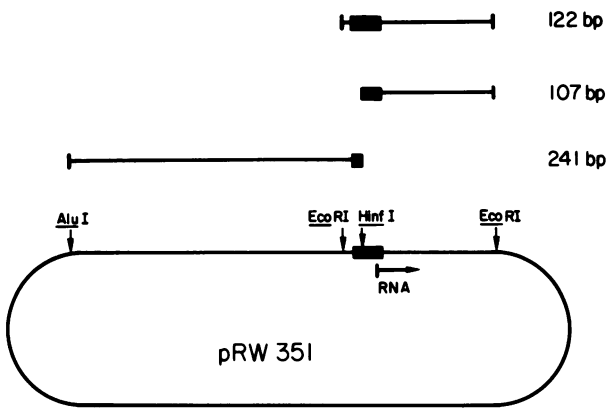

Figure 1. DNAs used in this study. pRW351 was constructed by cloning a 116 bp Hpa II - Taq I fragment, which was shown by N. Panayotatos to contain the class II promoter from 14.8% of the T7 genome (4), into the Eco RI site of pVH51 (21). The thick line represents promoter sequences.

33 units/ml of the partially purified T7 RNA polymerase were added to each reaction. The template was at 0.1mM in terms of nucleotide monomer. This concentration of DNA was saturating for the amount of polymerase used, the plasmids with the wild type promoters saturated the polymerase at about 10nM whereas the mutants did not reach saturating concentration until about 80nM (data not shown). The incorporation of [^{3}H]-CMP into acid insoluble material was assayed as described (20).

RESULTS

Plasmids Containing Wild Type and Mutant Promoters

Table 1 describes the plasmids used in these studies. pRW370 - pRW376 contain promoters with deletion mutations, pRW362 and pRW363 contain a class II promoter and pRW380 contains a class III promoter. The sequences of the promoters with deletion mutations were determined by the method of Maxam and Gilbert (13).

The promoters in pRW362, pRW370, pRW371 and pRW380 all are located in the first quarter of the tetracycline resistance operon and all are oriented in the same direction, with transcription proceeding into the ampicillin resistance operon.

In Vitro Transcription Using Wild Type and Mutant Promoters

Figure 2 shows the results of an in vitro transcription experiment using various supercoiled plasmids as templates. Under these conditions, the

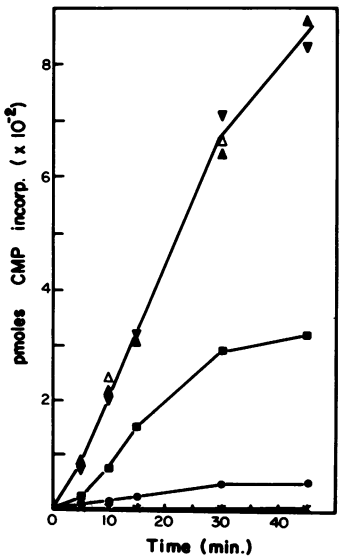

Figure 2. In vitro transcription using wild type and mutant promoters. Templates are supercoiled plasmids. The symbols are: ▲, pRW362, ▼, pRW363, Δ, pRW380, ■, pRW371, ●, pRW370, ×, pBR322. Other details are described in MATERIALS AND METHODS.

Table 1. Plasmids used for promoter studies.

Plasmid	Fragment Cloned (bp)[a]	Cloning Site[c]	Promoter Sequence[e]
			-20 -10 +1 +10
pRW370	107	Hind III	ataaactaccgcattaaagctACTCACTATAGGAGAACCTTAAGGTTTA
pRW371	107	Hind III	gataaactaccgcattaaagcACTCACTATAGGAGAACCTTAAGGTTTA
pRW373	107	Bam HI	cacgatgcgtccggcgtagaggatCACTATAGGAGAACCTTAAGGTTTA
pRW375	241	Eco RI	GCATAGAATTCGGTTAATACGACTgaccaaacaaagtaaattcttgaag
pRW376	241	Eco RI	GCATAGAATTCGGTTAATACGACTaattctcatgtttgacagcttatca
pRW362	122	Eco RI	catgagAATTCGGTTAATACGACTCACTATAGGAGAACCTTAAGGTTTA
pRW363	122	Eco RI[d]	ttcaagAATTCGGTTAATACGACTCACTATAGGAGAACCTTAAGGTTTA
pRW380	98[b]	Bam HI	CAAGGTCCCTAAATTAATACGACTCACTATAGGGAGATAGGGGgatcca

a Unless otherwise noted, all fragments are from pRW351 (Fig. 1).
b A Hae III fragment isolated from T7 DNA which contains the class III promoter from 46.5%.
c All of these plasmids were constructed by cloning a fragment of T7 DNA into pBR322.
d pRW362 and pRW363 differ in the orientation of the cloned fragment.
e Capitals indicate fragment sequences, lower case indicates vector sequences, the underlined base pairs show homology with the class III consensus sequence, the box delineates the highly conserved region of class II promoters (4,5,22).

plasmids bearing wild type promoters shown indistinguishable rates of transcription, even though the class II promoters in pRW362 and pRW363 are oriented in opposite directions and pRW380 has a class III promoter. When pBR322 is used as the template, the activity observed is about 100 fold less than that seen for the plasmids bearing wild type promoters. pRW373, pRW375, and pRW376 produce no discernable transcription (data not shown), presumably because the promoters in these plasmids have essential features deleted. However, two promoters with deletion mutations, in pRW370 and pRW371, are active. When pRW371 is used as the template, the rate of transcription is about 1/3 that of wild type, whereas pRW370 produces a rate of about 1/20 wild type.

Further in vitro experiments then were performed to determine if altered conditions would affect differently the class II, class III, and mutant promoters.

Glycerol Effect on Transcription

Figure 3 shows that addition of glycerol to standard in vitro transcription reactions reduces the rate of transcription observed for the plasmid bearing a wild type promoter, but enhances the rate for plasmids with mutant promoters. The net result is that pRW371 is nearly as active as pRW362 in 15-20% glycerol. The addition of glycerol has no effect when using pBR322, pRW373, pRW375, or pRW376 as the template.

Temperature Effect on Transcription

Figure 4 shows the temperature dependence of the in vitro transcription reactions. The wild type promoter bearing plasmids, pRW380 and pRW362, produce indistinguishable temperature dependences and are represented as a single line. The effect of increasing the temperature is qualitatively

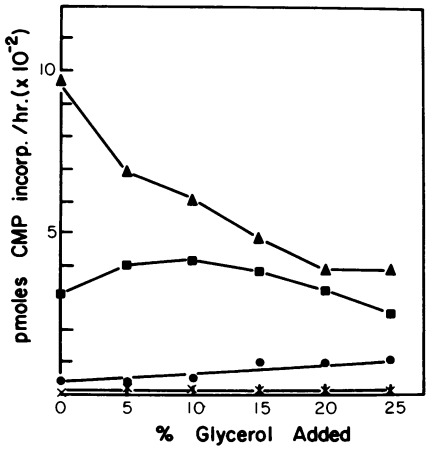

Figure 3. Effect of glycerol on the rate of transcription using wild type or mutant promoters. Conditions are described in MATERIALS AND METHODS except for the addition of glycerol. Rates were determined by linear regression analysis of time course assays, as shown in Fig. 2, using at least 3 time points between 0 and 30 minutes. Symbols are as in Fig. 2.

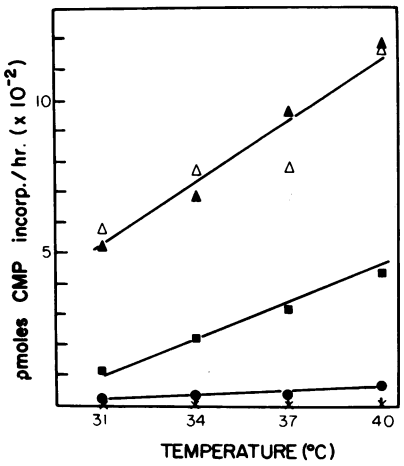

Figure 4. Effect of temperature on the rate of transcription using wild type or mutant promoters. Conditions are described in MATERIALS AND METHODS. Rates were determined as in Fig. 3. Symbols are as in Fig. 2.

similar to that of adding glycerol, since an increase is found in the ratio of the rate of transcription using a mutant promoter compared to the wild type rate. For pRW371, this ratio increases from 15% at 30°to 42% at 40°; for pRW370, this ratio changes very little, from 4% at 30° to 6% at 40°.

Ionic Effect on Transcription

Figure 5 shows the effect of KCl on in vitro transcription reactions. The interaction between T7 RNA polymerase and the mutant promoters in pRW370 and pRW371, is very sensitive to increased ionic strength. The rate of transcription from pRW370 and pRW371 is reduced to 1/2 of its initial value by the addition of approximately 20mM KCl. This is in contrast to transcripton from pRW362 which is not reduced to 1/2 of its initial value until the addition of about 140mM KCl. The resistance to increased ionic strength of

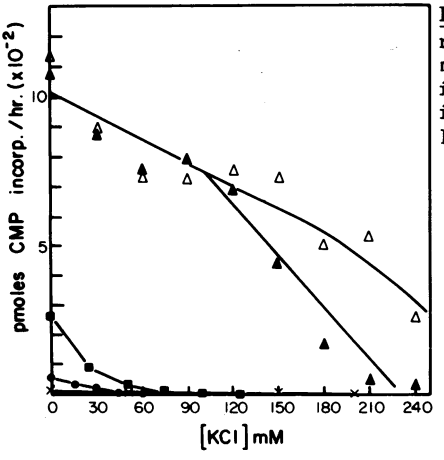

Figure 5. Effect of ionic strength on the rate of transcription with wild type and mutant promoters. Conditions are described in MATERIALS AND METHODS. KCl was added as indicated. Rates were determined as in Fig. 3. Symbols are the same as in Fig. 2.

the cloned class III promoter in pRW380 is even greater than that of the class II promoter in pRW362.

The class III promoter also shows more resistance than the class II promoter to increased ionic strength when the DNA is not supercoiled, as shown in Figure 6. This confirms the observations made by McAllister and Carter (6). For this experiment, pRW380 and pRW363 were linearized with restriction enzymes such that the run off transcript size would be 389 and 474 bases respectively.

DISCUSSION

pRW370 and pRW371 contain active promoters, whereas pRW373 does not. Thus, we conclude that the upstream end of the minimum promoter sequence required *in vitro* lies between -10 and -8. Restriction fragments bearing T7 promoters with similar deletions were constructed by Osterman and Coleman and were shown qualitatively to be active *in vitro* (22). These promoters, and those in pRW370 and pRW371, all have some base pairs, in the region from -22 to -11, which are homologous to the wild type consensus sequence. There may be functional DNA-protein contact points in this region, but since all of these promoters are different, it can be concluded there are no base pairs upstream from -10 which are essential.

The difference in the activities of the promoters in pRW370 and pRW371 may be due to the conservation of different bps in the region from -22 to -12, but it is possible that the change of position -11 from CG to TA accounts for the reduced activity of pRW370. In the wild type promoters at

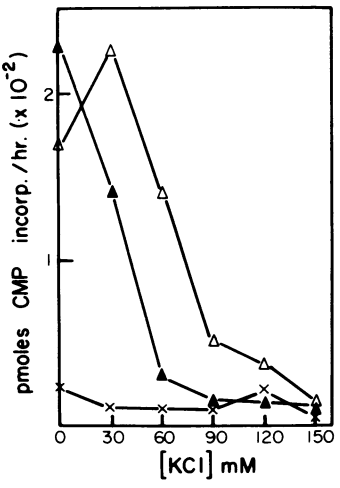

Figure 6. Effect of ionic strength on the rate of transcription using linearized plasmids containing late promoters. Symbols: Δ, pRW380 cut with Eco RI, ▲, pRW363 cut with Bam HI, X, pBR322 cut with Bam HI. Conditions were as in Fig. 5 and rates were determined as in Fig. 3.

1.0% and 28.0%, position -11 is an AT bp rather than the GC bp found in the other promoters. Thus position -11 can be occupied by any of the four possible base pairs.

The high degree of conservation of the -17 to -8 region is indicative of the involvement of this region in promoter utilization. However, the inactivation of the promoter by the deletion of the region to the right of the Hinf I site demonstrates that the -17 to -8 region by itself is not sufficient for promoter activity. This has been confirmed by the inactivity of pRW375 and pRW376 even under those conditions (the presence of supercoiling or the addition of glycerol) that cause increased activity of the other mutant promoters.

The greater resistance to increased ionic strength of class III promoters relative to class II promoters, observed by McAllister and Carter (6), also occurs with supercoiled DNA. This may be important since even a linear DNA molecule, such as the T7 genome, could be supercoiled in vivo if it is anchored at both ends; DNA gyrase is involved in both T7 transcription and replication (23,24).

The promoters in pRW371 and pRW370 are extremely sensitive to salt. This is indicative of either a less stable closed complex or a reduced rate of open complex formation. It may be the former possibility since the region which has been shown to be melted in the open complex is unchanged (25). Other studies on promoters of this type were done at low salt (22), making it possible that salt sensitivity is a general feature of such mutated promoters. This hypothesis would explain the failure to detect in vivo utilization of the weak promoters at 37.9, 40.8, and 44.4% of the T7 genome (26), and would predict that the promoters pRW371 and pRW370 would be inactive in vivo. In vitro studies with highly purified polymerase and mutant promoters should reveal these details.

It has not been possible to identify specific transcripts in standard in vitro reactions using supercoiled pRW362, pRW370, pRW371, or pRW380 as the templates, as analyzed by urea-polyacrylamide gel electrophoresis (data not shown). However, we believe that the average transcript lengths should be approximately the same since there is no strong terminator for transcription by T7 RNA polymerase in pBR322 in this orientation (27). Thus, any differences in the rates of transcription probably reflect differences in the rates of initiation.

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