In Vivo and in Vitro Activities of Point Mutants of the Bacteriophage T7 RNA Polymerase Promoter†

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ABSTRACT: Two compatible plasmids were recently reported [Ikeda et al. (1992) *Nucleic Acids Res. 20*, 2517–2524] that together can be used to determine whether a mutant T7 RNA polymerase promoter is active or inactive in vivo. The first plasmid, pKGP1-1, carries T7 *gene 1* (the gene encoding T7 RNA polymerase) ligated to a *tac* promoter, while the second plasmid, pCM-X#, carries the gene encoding chloramphenicol acetyltranferase (CAT) ligated to potential T7 promoters. If the pCM-X# plasmid carries a potential T7 promoter that can be utilized by T7 RNA polymerase, then CAT is produced from transcripts generated by T7 RNA polymerase from the potential promoter on the pCM-X# plasmid. To determine whether *Escherichia coli* growth characteristics and chloramphenicol (cam) resistance produced by the plasmids pKGP1-1 and pCM-X# reflect the T7 promoter activity of the possible promoters carried by the pCM-X# plasmids, the in vivo and in vitro strengths of the potential T7 promoters were compared and correlated. In vivo promoter strength was determined by measuring the relative amounts of CAT present in *E. coli* extracts, while relative in vitro promoter strength was measured in transcription assays. The in vivo and in vitro strengths of 22 point mutants of the consensus T7 promoter were shown to correlate with the growth characteristics and cam resistance conferred to *E. coli* harboring the plasmid pKGP1-1 and the respective pCM-X# plasmid. Furthermore, the correlation of T7 promoter activity with the presence and/or absence of specific functional groups in the major grooves of the wild-type and mutant promoters suggest the following are necessary for the efficient utilization of a potential T7 promoter: (1) a hydrogen bond acceptor at the N7 position of the G at −11; (2) either a hydrogen bond acceptor and a hydrogen bond donor at the 4-keto group of the T and 6-amino group of the A at −9 or the 5-methyl group at the T at −9; and (3) either hydrophobic interactions at the 5-hydrogen of the C at −7 or a hydrogen bond acceptor at the N7 of the G at −7.

The highly specific RNA polymerase of bacteriophage T7 is responsible for transcription of the rightmost 85% of the T7 genome. Comparison of the 17 natural T7 RNA polymerase promoters yields a 23 base pair consensus sequence (see Table I for the sequence of the consensus T7 promoter) that spans the site of the initiation of transcription (+1) and extends from −17 to +6 (Moffatt et al., 1984; Dunn & Studier, 1983; Studier & Dunn, 1982; Oakley et al., 1979). Simple footprinting studies (Gunderson et al., 1987; Ikeda & Richardson, 1986; Basu & Maitra, 1986) have shown that T7 RNA polymerase protects the promoter from −21 to −3, while high-resolution footprinting (Muller et al., 1989) has shown that the polymerase protects two regions from −16 to −11 and from −5 to +1.[1]

The importance of both minor and major groove contacts within the T7 promoter has been demonstrated by studies of promoters containing modified nucleotides. These studies showed that specific base modifications within the major and minor grooves of the T7 promoter can prevent binding and utilization of the promoter (Jorgensen et al., 1991; Klement et al., 1990; Joho et al., 1990; Stahl & Chamberlin, 1976, 1978). In addition, studies of mutant T7 promoters have further defined the relationship of structure to function in the T7 promoter. The behavior of specific T7 promoter mutants suggests that the promoter is organized into two domains, an initiation domain from −4 to +5 and a binding domain from −5 to −12 (Chapman et al., 1988; Chapman & Burgess, 1987). Single base changes within the initiation domain reduce the rate of initiation of transcription but have little effect on promoter binding. Conversely, single base changes within the binding domain reduce promoter binding but have little effect on the rate of the initiation of transcription.

Recently, we described two compatible plasmids that together can be used to determine whether a mutant T7 promoter is active or inactive in vivo (Ikeda et al., 1992). The first plasmid, pKGP1-1, is a pACYC177 (Chang & Cohen, 1978) derivative that carries T7 *gene 1* (the gene encoding T7 RNA polymerase) ligated to a *tac* promoter (deBoer et al., 1982; 1983), while the second plasmid, pCM-X#,[2] is a pKK232-8 (Brosius & Lupski, 1987; Brosius & Holy, 1984) derivative that carries the gene encoding CAT[3] ligated to a potential T7 promoter. If the pCM-X# plasmid carries a potential T7 promoter that can be utilized by T7 RNA polymerase, then CAT is produced from transcripts generated by T7 RNA polymerase from the potential promoter on the pCM-X# plasmid; consequently, *Escherichia coli* harboring the two plasmids pKGP1-1 and pCM-X# is cam resistant if the

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[1] The boundaries of the protected regions are the average of the boundary positions reported for the two DNA strands of the T7 promoter.

[2] pCM-X# is the general designation for this family of plasmids derived from pKK232-8. A specific plasmid within this family is designated with a letter and a number in place of X#.

[3] Abbreviations: A_x, absorbance at the designated wavelength (x) in nanometers; amp, ampicillin; bla, β-lactamase; CAT, chloramphenicol acetyltransferase; cam, chloramphenicol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CoA, coenzyme A; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; kan, kanamycin; LB, Luria–Bertani (medium); NTP, nucleoside triphosphate; Tris, tris(hydroxymethyl)aminomethane.

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pCM-X# plasmid carries an active T7 promoter and is cam sensitive if the pCM-X# plasmid carries an inactive T7 promoter.

Point mutations that were found to inactivate the T7 promoter are a C to A or G substitution at −7, a T to A substitution at −8, a C to A, T, or G substitution at −9, and a G to T substitution at −11 (Ikeda et al., 1992), but it was also observed that pKGP1-1 in combination with different pCM-X# isolates could produce *E. coli* that was cam resistant but did not behave like *E. coli* that harbored pKGP1-1 and a pCM-X# containing a wild-type T7 promoter (pCAT10-1) (Ikeda et al., 1992). One possible explanation of these observations is that some T7 promoter mutations reduce promoter activity but do not inactivate the promoter. Reduced promoter activity, however, is not the only possible mechanism for producing the observed results. Changes in plasmid copy number of changes in cellular translation efficiency could affect the amount of CAT produced by the cells and could produce the observed intermediate growth and resistance characteristics. To determine whether *E. coli* growth characteristics and cam resistance produced by the plasmids pKGP1-1 and pCM-X# reflect the T7 promoter activity of the possible promoters carried by the pCM-X# plasmids, the amount of CAT produced by 22 point mutants of the consensus T7 promoter was measured in cell extracts, and the relative in vivo promoter strength of the point mutants was determined. Transcription assays were then used to measure the relative in vitro strength of the same 22 T7 promoter point mutants, and the in vitro and in vivo strengths of these promoters are shown to correlate with the growth and resistance characteristics conferred to *E. coli* harboring the plasmid pKGP1-1 and the respective pCM-X# plasmids.

EXPERIMENTAL PROCEDURES

Materials

Cell Strains. *E. coli* JM101 was obtained from Stratagene.

Enzymes. T7 RNA polymerase was purified according to standard methods from *E. coli* HMS12 harboring plasmids pGP1-1 and pGP1-5 (Tabor & Richardson, 1985; Ikeda & Richardson, 1987). The T7 RNA polymerase was greater than 98% pure and was estimated to have a specific activity of 483 000 units/mg (Chamberlin et al., 1970).

Chemicals. Buffers and DTT were of electrophoresis grade. IPTG was of molecular biology grade. Media were from Difco. Antibiotics were from Sigma. DTNB and CENTA β-lactamase substrate were from Calbiochem. All other chemicals were of reagent grade.

Media. LB media consists of 10.0 g of tryptone, 5.0 g of yeast extract, and 10.0 g of NaCl per liter of media; the pH of the media was adjusted to 7.5 with NaOH. SOC media consists of 20 g/L tryptone, 5.0 g/L yeast extract, 0.6 g/L NaCl, 0.5 g/L KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose; the pH of the media was adjusted to 7.5 with NaOH.

Nucleoside 5′-Triphosphates. [2,8-^{3}H]ATP (25–40 Ci/mmol) was purchased from Du Pont/New England Nuclear Research Products. Ribonucleoside triphosphates were obtained from Pharmacia/LKB.

Plasmids. The construction of plasmids pKGP1-1 and pCAT10-1 and the plasmids listed in Table I has been previously described by Ikeda et al. (1992). All plasmids were prepared according to standard methods (Sambrook et al., 1989). The identities of the plasmids were confirmed by restriction mapping (Sambrook et al., 1989) and by phenotypic analysis, in vivo (Ikeda et al., 1992). Electrophoretic analysis of the plasmids showed that the DNA was at least 95% supercoiled DNA (data not shown). Little or no linear and/or open circular forms could be detected. The plasmids were used in their natural state at natural superhelical densities.

Methods

Screening for T7 Promoter Activity in Vivo. The plasmid pKGP1-1 is a derivative of pACYC177 (Chang & Cohen, 1978) that carries T7 *gene 1* ligated to the *tac* promoter (deBoer et al., 1982; 1983). The plasmids pCAT10-1 and pCM-X# are derivatives of pKK232-8 (Brosius & Lupski, 1987; Brosius & Holy, 1984) that carry the gene encoding CAT ligated to the T7 φ10 promoter or a mutant T7 φ10 promoter, respectively. In the presence of T7 RNA polymerase, pCAT10-1 and the pCM-X# plasmids that contain sequences recognizable as T7 promoters will express *CAT*. Consequently, *E. coli* harboring pKGP1-1 and pCAT10-1 or pCM-X# can be screened for cam resistance or cam sensitivity.

Competent *E. coli* JM101, 200 μL, is cotransformed with pKGP1-1 (40 ng) and the promoter assay plasmid (1 μL of a solution of DNA from a miniprep), either pCAT10-1 or a pCM-X# plasmid. SOC, 0.9 mL, is added to the cells, and the culture is grown at 37 °C for 1 h. Equal volumes of the transformation, 200 μL, are spread on three different types of LB–agar plates containing either (1) 50 μg/mL kan and 50 μg/mL amp, (2) 50 μg/mL kan, 50 μg/mL amp, and 1.0 mM IPTG, or (3) 50 μg/mL kan, 50 μg/mL amp, and 30 μg/mL cam. The plates are incubated at 37 °C for 16–20 h, and the number of colonies on each plate is counted.

Note: The *E. coli* JM101 must be positively *lac*IQ. The absence of a good *lac*IQ phenotype produces misleading results.

Preparation of Cell Extracts. Competent *E. coli* JM101, 200 μL, was transformed with 40 ng of pKGP1-1 (Ikeda et al., 1992) and 100 ng of a T7 promoter assay plasmid (pCM-X#) (Ikeda et al., 1992). SOC, 0.9 mL, was added to the cells, and the culture was grown at 37 °C for 1 h. A fifth of the transformation was then spread on LB–agar plates. If the promoter assay plasmid carried a strong or intermediate T7 promoter, the transformation was spread on plates containing 50 μg/mL kan, 50 μg/mL amp, and 30 μg/mL cam. If the promoter assay plasmid carried an inactive promoter or no T7 promoter, the transformation was spread on plates containing 50 μg/mL kan and 50 μg/mL amp. If pKGP1-1 was the only plasmid included in the transformation, the transformation was spread on plates containing 50 μg/mL kan. The plates were then incubated for 16 h at 37 °C.

Two colonies were picked from each transformation plate, and each colony was placed in 10.0 mL of LB that contained the same antibiotics as were present on the transformation plate. The cultures were grown at 37 °C to an A_{600} of 0.3–0.4, and the cells were collected by centrifugation. The cells were resuspended in 0.6 mL of 50 mM Tris-HCl, pH 7.8, and 0.3 mM DTT; the suspensions were separated into six samples of 100 μL and were stored at −70 °C.

To prepare the cell extracts, the 100-μL cell suspensions were thawed and sonicated at 4 °C. The sonicated mixtures were then centrifuged at 16000*g* for 15 min, and the supernatants were collected. The protein concentrations of these extracts were then determined according to the Coomassie blue assay of Bradford (1976) using bovine serum albumin as the standard.

Extracts were always prepared immediately prior to use.

Measurement of Chloramphenicol Acetyltransferase Activity. CAT activity can be measured spectrophotometrically as described by Brosius and Lupski (1987) and Shaw (1975) by monitoring the increase in A_{412} caused by the accumulation of 5-thio-2-nitrobenzoic acid produced from the reaction of CoA with DTNB. Briefly, 0.600 mL of a freshly prepared solution of 100 mM Tris-HCl, pH 7.8, 0.1 mM acetyl-CoA, and 0.4 mg/mL DTNB was warmed to 37 °C in a water-jacketed cuvette. Cell extract (5 μL) was added to the reaction mixture, and the absorbance at 412 nm was adjusted to zero. The A_{412} was then recorded at 10-s intervals for 30 s to determine background "activity". Cam (12 μL of a 5 mM solution in 70% ethanol) was added to the cuvette, and the A_{412} was recorded at 10-s intervals for 5 min.

The specific activity of the CAT in the extract was determined by dividing the initial net rate of change of the A_{412} (the initial rate of change A_{412} corrected for background) by both the extinction coefficient of 5-thio-2-nitrobenzoic acid (ϵ = 13 690 cm^{-1} M^{-1}) and the amount of protein added to the reaction and multiplying by the volume of the reaction. One unit of CAT activity is defined as the amount of enzyme necessary to acetylate 1 nmol of cam/min.

Measurement of β-Lactamase Activity. Bla activity can be measured spectrophotometrically as described by Jones et al. (1982) by monitoring the increase in A_{415} caused by the accumulation of CENTA hydrolysis products. Reaction buffer (600 μL) containing 50 μg/mL CENTA and 0.1 M sodium phosphate, pH 7.0, was warmed to 37 °C in a water-jacketed cuvette. The A_{415} of the spectrophotometer was adjusted to zero, and 5 μL of cell extract was added to the cuvette. The A_{415} was recorded at 10-s intervals for 5 min.

The specific activity of the bla in the extract was determined by dividing the initial net rate of change of the A_{415} by both the extinction coefficient of the hydrolyzed CENTA (ϵ = 9900 cm^{-1} M^{-} as reported by Calbiochem) and the amount of protein added to the reaction and multiplying by the volume of the reaction. One unit of bla activity is defined as the amount of enzyme necessary to hydrolyze 1 nmol of CENTA/min.

Determination of Relative Promoter Strength in Vivo. To determine relative promoter strength in vivo, the specific CAT activity of a sample was first divided by its specific bla activity to give a relative abundance ratio. The relative abundance ratio was then normalized by division by the relative abundance ratio measured for extracts made from *E. coli* containing a selection plasmid carrying a wild-type T7 promoter (pCAT10-1). This defines the relative, in vivo, strength of a wild-type T7 promoter as 1.0; therefore, promoters that direct the synthesis of reduced quantities of CAT will have relative strengths less than 1.0.

Measurement of Promoter Strength in Vitro. As previously described by Burgess and co-workers (Chapman et al., 1988; Chapman & Burgess, 1987), promoter strength in vitro can be determined by measuring the production of RNA from a supercoiled plasmid containing a mutant T7 promoter (pCM-X#) in comparison to the production of RNA from a supercoiled plasmid containing a consensus T7 promoter (pCAT10-1). See Results for the discussion of the assay conditions.

Transcription assays were carried out in 60-μL reactions containing 50 mM Tris-HCl, pH 7.8, 60 mM NaCl, 2.5 mM spermidine, 10 mM $MgCl_2$, 1 mM DTT, 400 μM of each of the four rNTPs, approximately 0.64 μCi of [2,8-^{3}H]ATP, 30 μg/mL bovine serum albumin, 8 nM promoter, and 80 nM T7 RNA polymerase. The reactions were equilibrated at 37 °C, and RNA synthesis was initiated by addition of T7 RNA polymerase. At 6 and 12 min, 25-μL samples were removed from the reactions and were spotted on DE81 filters. The filters were washed three times with 0.3 M ammonium formate, pH 8.0, and once with ethanol (Brutlag & Kornberg, 1972). The RNA retained on the filters was then quantitated by liquid scintillation. The specific activity of the [2,8-^{3}H]ATP was 41.6 cpm/pmol.

The relative strength of a promoter on a pCM-X# clone was determined by dividing the amount of RNA synthesized in a reaction containing the pCM-X# clone by the amount of RNA synthesized in a reaction containing the pCAT10-1 consensus clone. This procedure defines the activity of pCAT10-1 to be 1.00.

RESULTS

Phenotype Classification of Mutant T7 Promoters. The family of promoter selection plasmids, pCM-X#, carries a promoterless *CAT* gene ligated to a potential T7 promoter, while the compatible plasmid *gene 1* expression plasmid, pKGP1-1, carries the gene encoding T7 RNA polymerase ligated to a *tac* promoter. When *E. coli* JM101 that contains pKGP1-1 and a promoter selection plasmid is spread on LB–agar plates containing (1) 50 μg/mL kan and 50 μg/mL amp, (2) 50 μg/mL kan, 50 μg/mL amp, and 1.0 mM IPTG, and (3) 50 μg/mL kan, 50 μg/mL amp, and 30 μg/mL cam, three types of results are observed. If the promoter selection plasmid carries a strong T7 promoter (e.g., pCAT10-1), there are approximately 200 colonies on the kan/amp and kan/amp/cam plates and 0 colonies on the kan/amp/IPTG plate [a strong T7 promoter in the presence of large amounts of T7 RNA polymerase is lethal in *E. coli* (Schneider & Stormo, 1989)]; these clones are designated "strong" clones. If the promoter selection plasmid carries a sequence that cannot be recognized or utilized by T7 RNA polymerase, there are approximately 200 colonies on the kan/amp and kan/amp/IPTG plates and 0 colonies on the kan/amp/cam plate; these clones are designated "inactive" clones. Finally, if the promoter selection plasmid carries a sequence that can be weakly recognized or utilized by T7 RNA polymerase, there are approximately 200 colonies on the kan/amp plates, 50–200 colonies on the kan/amp/IPTG plates, and 50–200 colonies on the kan/amp/cam plate. These clones are designated "intermediate" clones; however, the intermediate clones can be further subdivided. Some intermediate clones exhibit characteristics resembling strong clones. These strong intermediate clones produce less than 100 colonies on kan/amp/IPTG plates and 200 colonies on both kan/amp and kan/amp/cam plates. Conversely, some intermediate clones exhibit characteristics resembling inactive clones. These weak intermediate clones produce either very few or very small colonies on kan/amp/cam plates and 200 colonies on both kan/amp and kan/amp/IPTG plates.

The growth characteristics and phenotypic classification of *E. coli* harboring 23 different promoter selection clones that carry 22 different T7 promoter point mutants and a wild-type T7 promoter are listed in Table I. The data clearly show the three classifications discussed in the preceding paragraph.

Relative Promoter Strength in Vivo. The designation of the phenotypic classes defined in the previous section as strong, intermediate, and inactive implies that the growth characteristics of the *E. coli* harboring the plasmids pKGP1-1 and pCM-X# are dependent upon the activity of the promoter carried by the pCM-X# plasmid. This may or may not be true. Chloramphenicol resistance is dependent upon the

Table I: Phenotypic Classification of Mutant T7 Promoters

position	mutation	clone[a]	colonies[b] kan/amp/IPTG	colonies[b] kan/amp/cam	class[c]
	no promoter	pKK232-8	200	0	inact
	no mutation	pCAT10-1	0	200	strong
−19	G to A	pCM-B57	0	200	strong
−11	G to A	pCM-B282	40	200	inter[d]
	G to C	pCM-B121	200	150	inter
	G to T	pCM-T297	200	0	inact
−10	A to G	pCM-G3005	200	200	inter
	A to C	pCM-C2002	200	200	inter
	A to T	pCM-B233	200	200	inter
−9	C to G	pCM-P1160	200	0	inact
	C to A	pCM-T270	200	0	inact
	C to T	pCM-P1087	200	0	inact
−8	T to G	pCM-P1198	200	200	inter
	T to A	pCM-T286	200	0	inact
	T to C	pCM-B64	200	40	inter[e]
−7	C to G	pCM-P1208	200	0	inact
	C to A	pCM-P1031	200	0	inact
	C to T	pCM-T263	200	150 (s)	inter[e]
−6	A to G	pCM-T221	80	200	inter[d]
	A to C	pCM-B139	200	200	inter
	A to T	pCM-T231	200	200	inter
−5	C to A	pCM-B93	200	100 (s)	inter[e]
	C to T	pCM-T220	200	200	inter
+3	G to A	pCM-B14	0	200	strong
consensus T7 promoter			$^{-17}$TAATACGACTCACTATAGGGAGA^{+6}		

[a] Promoter selection plasmid cotransformed with pKGP1-1. [b] Numbers of colonies on plates containing the indicated antibiotics and/or IPTG per 200 colonies on an identical plate containing only kan and amp. "s" designates extremely small colonies. [c] Inter, intermediate; inact, inactive. [d] Classified as intermediate but exhibits characteristics of a strong clone. [e] Classified as intermediate but exhibits characteristics of an inactive clone.

synthesis of CAT, and the synthesis of CAT is dependent on both transcription and translation. Consequently, CAT abundance can be affected by the genotype of the host, the growth conditions of the culture, the state of the cell cycle of the host, the rate of protein turnover, the lifetime of the mRNA, and the copy number of the plasmid carrying the gene encoding CAT. Fortunately, it has been previously shown that the normalization of CAT synthesis to bla synthesis (an internal control) from the ampicillin resistance gene on the promoter selection vector yields a relative measurement of CAT abundance that minimizes these non-promoter-dependent contributions to the measurement of CAT synthesis (Brosius & Lupski, 1987; Klotsky & Schwartz, 1987; Li et al., 1984; Lupski et al., 1984). This allows relative CAT abundance to be interpreted as a measure of promoter strength (Brosius & Lupski, 1987).

CAT activity in a cell extract can be measured spectrophotometrically by monitoring the increase in A_{412} caused by the accumulation of 5-thio-2-nitrobenzoic acid produced from the reaction of CoA with DTNB (Brosius & Lupski, 1987; Shaw, 1975), while bla activity in the same extract can be measured spectrophotometrically, by monitoring the increase in A_{415} caused by the accumulation of CENTA hydrolysis products (Jones et al., 1982). The absolute CAT and bla activities present in extracts prepared from *E. coli* harboring plasmids pKGP1-1 and a promoter selection vector are listed in Table II. As expected, the absolute activities of both CAT and bla vary widely. CAT activities range from 21.6 ± 2.4 to 4089 ± 571 units/mg and, bla activities range from 15.9 ± 5.0 to 588 ± 20 units/mg; however, most of the intermediate clones produce more CAT than the inactive clones.

Division of the CAT activities by their respective bla activities produces a relative CAT abundance ratio that is normalized for such factors as the copy number of the promoter selection vectors, the state of the cell cycle, and the growth conditions of the culture. The relative abundance ratios clearly delineate the relative production of CAT that is required for cam resistance. While the wild-type T7 promoter and the T7 promoter point mutants that are phenotypically classified as strong give relative CAT abundance ratios of 23.9 ± 5.6 to 43.1 ± 5.9, the T7 promoter point mutants that are classified as inactive give relative CAT abundance ratios lower than 0.24 ± 0.07. Furthermore, the T7 promoter point mutants that are phenotypically classified as intermediate again fall between the strong and inactive clones. The intermediate mutants produce relative abundance ratios of 0.86 ± 0.24 to 20.8 ± 0.8.

Although there is a clear distinction in the relative abundance of CAT in *E. coli* harboring plasmid pKGP1-1 and either a strong, intermediate, or inactive promoter selection vector, and there is a precedent for interpreting relative CAT abundance as a measure of promoter strength (Brosius & Lupski, 1987; Klotsky & Schwartz, 1987; Li et al., 1984; Lupski et al., 1984), the interpretation of relative CAT abundance as a measure of promoter strength assumes that T7 RNA polymerase is present in relatively equivalent quantities in all of the cell strains. Equivalent quantities of T7 RNA polymerase might have been obtained with a strain of *E. coli* that contained a single integrated copy of the gene for T7 RNA polymerase [e.g., BL21(DE3 Studier & Moffatt, 1986)], but plans to utilize the same selection system to isolate mutants of T7 RNA polymerase dictated that the gene

Table II: Chloramphenicol Acetyltransferase and β-Lactamase Activity in Vivo[a]

promoter mutation	CAT act. ± σ (units/mg)[b]	bla act. ± σ (units/mg)[b]	ratio of CAT to bla act. ± σ
no promoter	11.7 ± 6.6	337 ± 94	0.03 ± 0.01
no mutation	781 ± 79	33.9 ± 6.7	23.9 ± 5.6
−19 G to A	1046 ± 84	24.4 ± 1.6	43.1 ± 5.9
−11 G to A	1470 ± 54	70.9 ± 4.4	20.8 ± 0.80
−11G to C	712 ± 47	415 ± 76	1.76 ± 0.34
−11 G to T	21.6 ± 2.4	160 ± 20	0.14 ± 0.01
−10 A to G	476 ± 97	189 ± 23	2.52 ± 0.36
−10 A to C	972 ± 219	193 ± 47	5.06 ± 0.48
−10 A to T	3076 ± 40	574 ± 59	5.41 ± 0.59
−9 C to G	14.0 ± 2.0	136 ± 42	0.11 ± 0.02
−9 C to A	40.8 ± 2.5	186 ± 37	0.22 ± 0.03
−9 C to T	12.7 ± 3.9	112 ± 10	0.11 ± 0.03
−8 T to G	1427 ± 36	337 ± 76	4.43 ± 1.11
−8 T to A	122 ● 47	588 ± 20	0.21 ± 0.09
−8 T to C	122 ± 39	149 ± 62	0.86 ± 0.24
−7 C to G	139 ± 122	505 ± 371	0.24 ● 0.07
−7 C to A	26.8 ± 5.0	184 ± 71	0.16 ± 0.06
−7 C to T	78 ± 47	85 ± 25	0.89 ± 0.32
−6 A to G	2581 ± 276	277 ± 34	9.49 ± 2.15
−6 A to C	942 ± 42	383 ± 43	2.48 ● 0.29
−6 A to T	4089 ± 571	241 ± 32	17.2 ± 3.4
−5 C to A	201 ± 108	208 ± 51	0.92 ± 0.33
−5 C to T	402 ± 97	239 ± 9	1.68 ± 0.19
+3 G to A	622 ± 75	15.9 ± 5.0	41.5 ± 10.6

[a] Activities are given as the average of at least four separate measurements of CAT and bla activity. The ratio of CAT to bla activity is the average of the ratios of the individual measurements. σ designates the standard deviation from the average. [b] Units of enzyme activity per milligram of extract protein.

encoding T7 RNA polymerase be provided on an extra chromosomal plasmid. Since the gene encoding T7 RNA polymerase is provided on a pACYC-based plasmid (pKGP1-1), the assumption that T7 RNA polymerase is present in relatively equivalent quantities in all of the cell strains may not be valid. Variations in the copy number of pKGP1-1 could change the gene dosage and could result in the production of variable quantities of T7 RNA polymerase. It might be argued that the quality of the CAT abundance data suggests that the amount of T7 RNA polymerase produced is relatively stable and equivalent, but a more convincing proof that the CAT abundance data reflect promoter strength and not variations in the levels of T7 and RNA polymerase would be the correspondence of the CAT abundance data to the relative in vitro strengths of the T7 promoter point mutants.

Relative Promoter Strength in Vitro. The relative in vitro strength of T7 promoters and T7 promoter point mutants is dependent upon the physical state of the DNA template (linear or supercoiled) and the composition of the transcription buffer. Burgess and co-workers have previously reported that the in vivo activities of T7 promoters that are carried on plasmids most closely correlated with their in vitro activities when the promoters were assayed, in vitro, on naturally supercoiled templates in the presence of NaCl and spermidine (Chapman et al., 1988; Chapman & Burgess, 1987). To demonstrate that the relative CAT abundance data reflect promoter strength and not variations in the levels of T7 RNA polymerase present in the cells, the T7 promoter point mutants were assayed in vitro for T7 promoter activity using the methods described by Burgess and co-workers (Chapman et al., 1988; Chapman & Burgess, 1987).

Figure 1A shows the in vitro promoter strengths of the 22 T7 promoter point mutants relative to the wild-type φ10 promoter of plasmid pCAT10-1. The relative in vitro strengths of the 2 strong point mutants are 0.72 ± 0.030 and 0.74 ± 0.084, while the relative strengths of the 13 intermediate point mutants range from 0.0048 ± 0.005 to 0.46 ± 0.020. Furthermore, all seven of the inactive point mutants show very little promoter activity. The relative in vitro strengths of the inactive point mutants range from 0.0049 ± 0.005 to 0.018 ± 0.005. Although it appears that the relative in vitro strengths of a few of the intermediate mutants are more similar to the strengths of the inactive mutants and that the relative strength of one of the inactive mutants appears to be more similar to the strengths of the intermediate mutants, this overlap in classes is not significant. The relative in vitro strength of the inactive mutants and the weak intermediate mutants border on the lower limits of the transcription assay, and the errors associated with these relative strengths are large in comparison to the magnitudes of the measurements. Consequently, the overlap in the relative in vitro strengths of the weak intermediate mutants and the inactive mutants is within the error limits of the in vitro assay.

To compare the in vitro data to the in vivo data, values designated as relative in vivo promoter strength were calculated from the relative CAT abundance ratios by normalization of the CAT abundance ratios to the CAT abundance measured for the promoter selection vector carrying the T7 φ10 promoter (pCAT10-1). The relative in vivo promoter strengths are shown below the relative in vitro promoter strengths in Figure 1. Generally, the trends noted for in vitro promoter strengths are duplicated by the relative in vivo data, but there are differences. The relative in vivo promoter strengths of the inactive promoter mutants (0.0046 ± 0.0023 to 0.010 ± 0.005) are nearly identical to their relative in vitro strengths (0.0049 ± 0.005 to 0.018 ± 0.007), while the relative in vivo strengths of the intermediate promoter mutants (0.036 ± 0.018 to 0.87 ± 0.24) are greater than their in vitro strengths (0.0048 ± 0.005 to 0.46 ± 0.02). Although the absolute magnitudes of the in vivo and in vitro strengths of the intermediate mutants differ, the in vivo and in vitro strengths show the same relative trends. Finally, the relative in vivo strengths of the strong promoters differ from their relative in vitro strengths. The in vivo strengths of the strong mutants (average 1.76 ± 0.76) are either greater than or comparable to that of the wild-type T7 φ10 promoter (1.0), yet the in vitro strengths of the strong mutants (approximately 0.73) are less than the in vitro strength of the φ10 promoter (1.0). This difference in the in vivo and in vitro data is probably due to the difficulties and variability encountered in measuring the very low levels of bla produced in competition with the high levels of CAT. Small errors in the measurement of bla abundance result in large differences in the abundance ratio. The extremely low levels of bla produced in the presence of strong T7 promoters further suggest that potential T7 promoters with relative in vitro strengths greater than approximately 0.50 are utilized so well in vivo that transcription overwhelms the host. This can make it difficult to differentiate strong T7 promoters in vivo, but in general, the in vivo and in vitro strengths of the strong T7 promoters are qualitatively similar.

In spite of the problems encountered in measuring the in vivo strengths of the strong T7 promoters, the correspondence of the relative in vivo and in vitro promoter strength measurements for the inactive and intermediate T7 promoter mutants suggests that the relative in vivo data reflects promoter

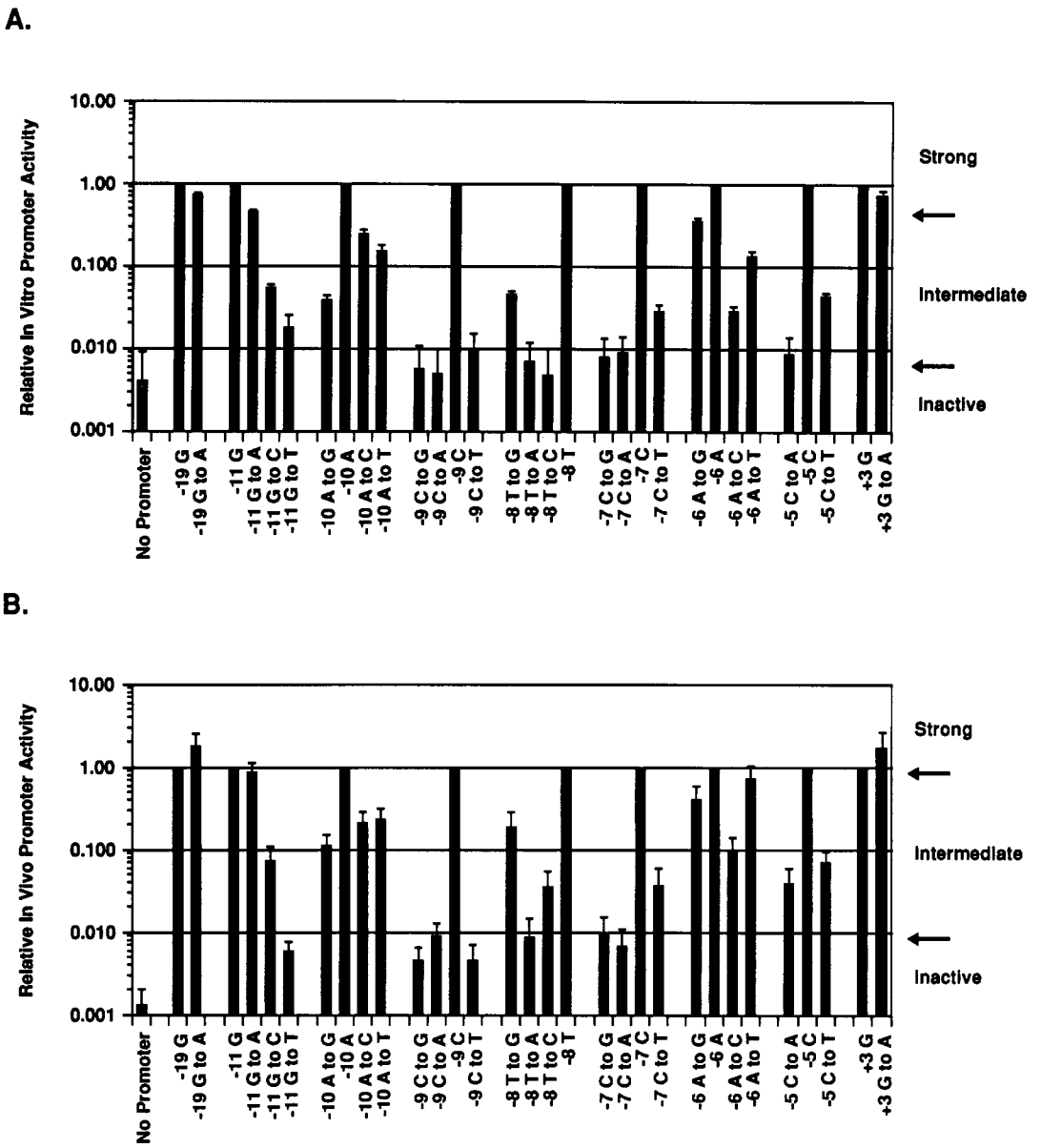

FIGURE 1: Relative in vitro and in vivo strength of mutant T7 promoters. The mutations are listed on the horizontal axis, and relative activity is represented along the vertical axis. The arrows on the right side of the graphs indicate the approximate activities that differentiate inactive, intermediate, and strong promoters. As previously noted, the –11 G to A, the –6 A to G, the –8 T to C, the –7 C to T, and the –5 C to A mutants are all classified as intermediate promoters, but the –11 G to A and the –6 A to G mutants show some characteristics of a strong promoter while the –8 T to C, the –7 C to T, and the –5 C to A mutants show some characteristics of an inactive promoter. For clarity, only the top halves of the error bars are shown; however, the error bars should extend below the tops of the data columns for a distance equivalent to the value that the error bars extend above the data columns on the logarithmic scale. No error bars are shown for the wild-type bases since the consensus T7 promoter has been defined to have a relative activity of 1.00. (A) Relative in vitro promoter strength, as defined under Experimental Procedures. The results are the average of the two time points of at least three different samples, and the error associated with the measurements is the greater of the standard deviation observed or the limit of accuracy of the assay (0.005). (B) Relative in vivo promoter strength, as defined under Experimental Procedures. The errors associated with these results were calculated by standard methods for the propagation of errors.

strength and not variations in the levels of T7 RNA polymerase.

DISCUSSION

The differences in growth characteristics and cam resistance exhibited by cultures of *E. coli* that harbor different pCM-X# promoter selection plasmids and identical pKGP1-1 plasmids suggest that different T7 promoter point mutations can affect promoter activity differently; however, no hierarchy of base preference based on these observations has been proposed previously because of the lack of a conclusive connection between the degree of apparent CAT expression and absolute T7 promoter activity.

In vivo, the synthesis of CAT in the presence of plasmids pKGP1-1 and pCM-X# is dependent upon the growth conditions of the culture, the state of the cell cycle of the host, the rate of protein turnover, the lifetime of the mRNA, the copy number of the pCM-X# plasmid carrying the *CAT* gene, the strength of the potential T7 promoter responsible for transcription of the *CAT* gene, and the abundance of T7 RNA polymerase. Brosius and Lupski (1987) previously showed that normalization of CAT synthesis from pKK232-8 derivatives to bla synthesis from the *bla* gene on the same plasmid minimizes the effects of the first five variables described above and gives a relative measure of the strength of the promoter transcribing the *CAT* gene if RNA poly-

Table III: Base Preferences for T7 Promoter Usage at Positions −11 to −5

consensus T7 promoter position	$^{-17}$TAATACGACTCACTATAGGGAGA^{+6}	
	consensus base	base preference[a]
−11	G	G > A > C > t
−10	A	A > C,T > G
−9	C	C ≫ g,a,t
−8	T	T > G > C > a
−7	C	C > T > g,a
−6	A	A > G,T > C
−5	C	C > T > A

[a] The hierarchy of base preference was determined from the relative in vivo and in vitro strengths and the phenotypic classifications. Lower case letters denote the substitutions that inactivate T7 promoter activity.

merase abundance is relatively constant (Brosius & Lupski, 1987). To show that relative CAT abundance in *E. coli* harboring pKGP1-1 and a pCM-X# is not a measure of the possible variations in the abundance of T7 RNA polymerase within the host, relative CAT abundance in the form of relative in vivo promoter strength was compared with relative in vitro promoter strength. As discussed under Results, relative in vivo and in vitro promoter strengths of the inactive and intermediate T7 promoter point mutants correlated very well, while the relative in vivo and in vitro strengths of the strong T7 promoters differed slightly because the strong promoters apparently overwhelmed the host cells. The correlation of the relative in vivo and in vitro measurements suggests that the growth characteristics of the *E. coli* harboring pKGP1-1 and a pCM-X# and the relative CAT levels produced by these cells are not predominantly determined by the amount of T7 RNA polymerase available within the cells but are determined by the strength of the potential T7 promoter responsible for transcription of the *CAT* gene on the promoter selection plasmid. This not only allows for a precise biochemical description of the three phenotypic classes of T7 promoter point mutants but also permits the determination of a hierarchy of sequence preference for the usage of the different T7 promoter point mutations in vivo.

Although the simpler in vitro assays provide an accurate measure of the in vivo activity of the T7 promoter mutants studied in this work, in vivo and in vitro promoter activities can be different for other T7 promoter mutations. Burgess and co-workers (Chapman & Burgess, 1987; Chapman et al., 1988) previously reported that some point mutations between −4 and +1 of the T7 promoter that do not affect in vitro activity decrease the in vivo activity of the promoter. Consequently, both in vitro and in vivo measurements of promoter strength are necessary to fully characterize mutant T7 promoters.

Since the single base substitutions that inactivate the T7 promoter are found at positions −11, −9, −8, and −7, this work focused on the effect of the different promoter point mutants between −11 and −5 on relative T7 promoter strength. Table III lists the hierarchy of base preference between positions −11 and −5 of the T7 promoter. Earlier work by Jorgensen et al. (1991) showed that contacts within the major groove of the T7 promoter between positions −12 and −5 are important for T7 RNA polymerase binding. Specifically, methylation of the G residues at positions −11, −9, and −7 interferes with binding of the promoter by T7 RNA polymerase, and methylation of the G residue at position −5 enhances binding by T7 RNA polymerase. While noting that the 5-methyl group of the T and the 7-methyl group of 7-methyl-G are sterically equivalent, the major groove functionality of the T7 promoter point mutants between −11 and −5 was analyzed for unique specificity sites (McClarin et al., 1986; Seeman et al., 1976).

Our previous analysis (Ikeda et al., 1992) of just the inactive T7 promoter point mutants suggested that close association with the N7 position of the G at −11, a hydrogen bond donor at the 4-amino group of the C at −9, a hydrogen bond acceptor at the 6-keto group at the G at −9, close association with the N7 position of the G at −9, close association with the N7 position of the A at −8, and a hydrogen bond acceptor at the N7 position of the G at −7 are important for T7 promoter activity. The base hierarchy further suggests that a hydrogen bond acceptor at the N7 position of the G at −11, either a hydrogen bond acceptor and a hydrogen bond donor at the 4-keto group of the T and the 6-amino group of the A at −9 or the 5-methyl group of the T at −9, and either hydrophobic interactions at the 5-hydrogen of the C at −7 or a hydrogen bond acceptor at the N7 of the G at −7 are necessary for efficient utilization of a potential T7 promoter.

The remaining three positions, −10, −6, and −5, all show a definite hierarchy of base preference, but only two of the three possible mutants at position −5 are shown. The data at position −5 can be completed by noting that Burgess and co-workers (Chapman & Burgess, 19087; Chapman et al., 1988) previously measured the in vitro and in vivo activities of a −5 C to G T7 promoter point mutant and reported that the relative in vitro (60 mM NaCl) and in vivo activities of this point mutant are 0.90 ± 0.10 and 0.66 ± 0.02, respectively; therefore, the hierarchy of base preference at position −5 is C,G > T > A. Although there is a definite hierarchy of base preference at positions −10, −6, and −5, T7 promoter activity does not correlate with the presence or absence of specific functional groups at the specificity sites in the major grooves of the wild-type and mutant promoters. As a consequence, it is difficult to suggest what base contacts at positions −10, −6, and −5 are important for T7 promoter activity.

In conclusion, correlation of the cam resistance of *E. coli* carrying pKGP1-1 and a promoter selection vector (pCM-X#) with the normalized levels of CAT expressed by the cells and the in vitro activities of the potential T7 promoters carried on the promoter selection vectors has allowed us to measure the in vivo and in vitro strengths of T7 promoter point mutants and suggest possible contacts necessary for efficient usage of the T7 promoter by T7 RNA polymerase.

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