Selection and Characterization of a Mutant T7 RNA Polymerase That Recognizes an Expanded Range of T7 Promoter-like Sequences†

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ABSTRACT: The compatible plasmids pKGP1-1 and pCM-X# will confer chloramphenicol resistance to *Escherichia coli* harboring the two plasmids if the T7 RNA polymerase produced from pKGP1-1 can recognize the T7 promoter carried on pCM-X# and transcribe the CAT gene that is cloned behind the promoter [Ikeda et al. (1992) *Biochemistry 31*, 9073–9080]. When *E. coli* harbor pKGP1-1 and a pCM-X# plasmid that carries a point mutation in the T7 promoter that destroys promoter activity (an inactive pCM-X#), the T7 RNA polymerase will not utilize the T7 promoter point mutant, will not produce CAT, and will not induce chloramphenicol resistance. The selection of mutants of T7 RNA polymerase that exhibit altered promoter recognition was pursued by randomly mutagenizing pKGP1-1 with aqueous hydroxylamine, cotransforming *E. coli* with the mutagenized pKGP1-1 and a mixture of seven different inactive pCM-X# plasmids, and isolating and characterizing the RNA polymerase that was present in those colonies that exhibited chloramphenicol resistance. It was established that *E. coli* harboring the mutant plasmid pKGP-HA1mut4 and an inactive pCM-X# are chloramphenicol-resistant and that the mutation responsible for the expression of CAT from the inactive pCM-X# plasmid is a G to A transition at nucleotide 664 of T7 gene 1 that converts glutamic acid (222) to lysine. Apparently this mutation expands the range of T7 promoter sequences that can be utilized by the enzyme. The mutant T7 RNA polymerase, GP1(Lys222), utilizes all seven inactive T7 promoter point mutants more efficiently than wild-type T7 RNA polymerase both *in vivo* and *in vitro*. Furthermore, the correlation of *in vivo* and *in vitro* promoter utilization suggests that the restoration of chloramphenicol resistance in the cotransformed *E. coli* results from the ability of GP1(Lys222) to initiate transcription from T7 promoter point mutants that are normally inactive.

Gene 1 of bacteriophage T7 encodes a highly specific DNA-dependent T7 RNA polymerase that is responsible for the transcription of 80% of the T7 genome. The RNA polymerase is a simple single-subunit enzyme of 883 amino acids (98.6 kDa) that requires no auxiliary factors for accurate transcription *in vitro*. T7 RNA polymerase alone is able to recognize its promoters, initiate transcription, elongate the RNA transcript, and terminate transcription (Chamberlin & Ryan, 1982; Bautz, 1976; Chamberlin & Ring, 1973a,b). Comparison of the 17 natural T7 RNA polymerase promoters yields a 23-base-pair consensus sequence (Figure 1) that includes the site of the initiation of transcription (+1) and extends from −17 to +6 (Moffatt et al., 1984; Studier & Dunn, 1982; Oakley et al., 1975). *In vitro* studies of promoter-dependent T7 RNA polymerase activity have defined the kinetics of transcription (Ikeda et al., 1992c; Martin & Coleman, 1987), the stability of the promoter–polymerase complex (Muller et al., 1989; Shi et al., 1988; Gunderson et al., 1987; Basu & Maitra, 1986; Ikeda & Richardson, 1986; Smeekens & Romano, 1986), the contribution of abortive initiation to promoter efficiency (Ikeda, 1992), and the DNA contacts essential for promoter activity (Ikeda et al., 1992a,b). The data suggest that the T7 promoter is organized into two

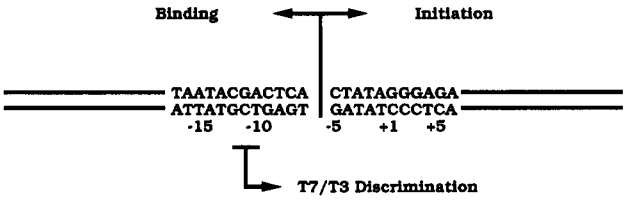

FIGURE 1: Consensus T7 promoter. The base pairs at −9, −10, and −11 have been implicated in the ability of T7 RNA polymerase to distinguish T7 and T3 promoters.

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 in the binding domain of the T7 promoter reduce or eliminate promoter binding but have little effect on the initiation of transcription. In contrast, single base changes in the initiation domain of the promoter have little effect on promoter binding but reduce the rate of initiation.

We recently 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., 1992a,b). 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#,[1] is a pKK232-8 (Brosius & Lupski, 1987; Brosius & Holy, 1984) derivative that carries the gene encoding CAT[2] ligated to potential T7 promoters. *E. coli* harboring these two plasmids are cam-

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[1] 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#.

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resistant if the pCM-X# plasmid carries an active T7 promoter and are cam-sensitive if the pCM-X# plasmid carries an inactive T7 promoter.[3] 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., 1992a,b).

Although much is known about the activity of T7 RNA polymerase and the structure of the T7 promoter, little is known about the structure–function relationships of T7 RNA polymerase itself. Several researchers have noted that limited proteolysis of T7 RNA polymerase yields a 20-kDa amino-terminal fragment and an 80-kDa carboxyl-terminal fragment (Ikeda & Richardson, 1987; Davanloo et al., 1984). The carboxyl-terminal fragment can initiate RNA synthesis but cannot extend the transcript (Muller et al., 1988). It has been suggested that the amino-terminal domain of T7 RNA polymerase contains a nonspecific RNA binding site that stabilizes the T7 transcription complex and allows for processive RNA synthesis. Other structural studies have shown that DNA binding and polymerase activity are separable functions in T7 RNA polymerase. Amino acid insertions into the reading frame of T7 RNA polymerase at residues 640, 648, or 881 inactivate polymerase activity but do not disrupt promoter binding, while insertions at residues 159, 222, 240, or 242 disrupt DNA binding but do not inactivate polymerase activity (Gross et al., 1993; Patra et al., 1992). Finally, replacement of Asn748 of T7 RNA polymerase by the corresponding residue found in T3 RNA polymerase (Asp) alters promoter recognition by the enzyme. The Asp748 T7 RNA polymerase prefers a promoter with C at positions −11 and −10—the bases normally found in the T3 promoter (Raskin et al., 1992; Joho et al., 1990; Klement et al., 1990).

Further characterization of promoter recognition and utilization by T7 RNA polymerase would be greatly aided by the identification and characterization of mutant T7 RNA polymerases with altered promoter recognition. We report here the use of the compatible pKGP1-1 and pCM-X# to select a mutant T7 RNA polymerase with an expanded range of promoter recognition and the characterization of the specificity of the mutant enzyme.

EXPERIMENTAL PROCEDURES

Materials

Cell Strains. *E. coli* DH5 and DH5αF′ were obtained from Gibco/BRL, *E. coli* JM101 was obtained from Stratagene, and *E. coli* BL21 was obtained from Novagen.

Chemicals. Acrylamide, agarose, ammonium persulfate, buffers, dithiothreitol, *N,N,N′,N′*-tetramethylethylenediamine, *N,N′*-methylenebis(acrylamide) and urea were electrophoresis grade. IPTG was molecular biology grade. Media were from Difco. Antibiotics and hydroxylamine were from Sigma. DTNB and CENTA β-lactamase substrate were from Calbiochem. All other chemicals were reagent grade.

Enzymes. Restriction endonucleases and Klenow fragment of *E. coli* DNA polymerase I were from New England Biolabs. Calf intestine alkaline phosphatase, Sequenase version 2.0, T7 polynucleotide kinase, and T4 ligase were purchased from U.S. Biochemical Corp.

T7 RNA polymerase was purified by standard methods (Ikeda et al., 1992c), and the mutant T7 RNA polymerase was purified by standard methods (Tabor & Richardson, 1985; Ikeda & Richardson, 1987) from *E. coli* BL21 harboring plasmids pMutA/B and pAGR-3R. The T7 RNA polymerase was greater than 98% pure and was estimated to have a specific activity of 43 700 units/mg, while the mutant T7 RNA polymerase was greater than 95% pure and was estimated to have a specific activity of 14 800 units/mg (Chamberlin et al., 1970).

Media. LB medium consists of 10.0 g/L of tryptone, 5.0 g/L yeast extract, and 10.0 g of NaCl/L of medium; the pH of the medium was adjusted to 7.5 with NaOH. SOC medium 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 medium was adjusted to 7.5 with NaOH.

Nucleoside 5′-Triphosphates. [2,8-^{3}H]ATP (25–40 Ci/mmol), [α-^{35}S]dATP (1000–1500 Ci/mmol), [α-^{32}P]UTP (800 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Dupont/New England Nuclear Research Products. Ribonucleoside triphosphates were obtained from Pharmacia/LKB. dNTPs and ddNTPs were from U.S. Biochemical Corp.

Oligodeoxyribonucleotides. Complementary 24-base-pair oligonucleotides containing the T7 promoter (5′dATTAATACGACTCACTATAGGACT3′ and 3′TAATTATGCTGAGTGATATCCTGA5′) were purchased from Genosys Biotechnologies, Inc. M13 sequencing primer (−40) was purchased from New England Biolabs. Sequencing primers TEMP −34 (5′dATA GGT ACG ATT TAC3′), 171 (5′dTCA ACT TAA AGC TGG3′), 298 (5′dCCG ACA GCC TTC CAG TTC CT3′), 365 (5′dCTC TGG CTT GCC TAA3′), and 563 (5′dCTG ACA TGC TCT CTA3′) were purchased from Genosys Biotechnologies, Inc. The names of the primers indicate the location with respect to T7 gene 1 (in base pairs) that the primers will anneal. Since ligation of the pKGP-HA1mut4 *Eco*RI/*Pst*I fragment into the *Eco*RI and *Pst*I sites of M13mp19 inserts the mutant T7 gene 1 into M13mp19 in counterclockwise direction, the noncoding (with respect to translation) strand of T7 gene 1 is the template for sequencing.

Plasmids. Plasmid pKK232-8 was obtained from Pharmacia. The plasmid pKGP1-1 (an expression clone of T7 gene 1) (Figure 2), the plasmid pCAT10-1 (a selection plasmid carrying the CAT gene cloned under the control of a wild-type T7 promoter), the plasmids pCM-T270, pCM-T286, pCM-T297, pCM-P1031, pCM-P1087, pCM-P1160, and pCM-P1208 (the plasmids carrying CAT genes cloned under the control of T7 promoter point mutants that are normally inactive in the presence of T7 RNA polymerase), and the plasmids pCM-P1198, pCM-B64, and pCM-T221 (the plasmids carrying CAT genes under the control of T7 promoter point mutants with moderate activity) have been previously described by Ikeda et al. (1992a,b) (Table I). Plasmid pSP64, a plasmid carrying an SP6 promoter, was obtained from Promega; plasmid pLM10, a pBR derivative carrying a T3 promoter, was a gift from Dr. William McAllister, State University of New York, Brooklyn; and plasmid pAGR3R,

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

[3] The pCM-X# plasmids that carry T7 promoter point mutants that destroy promoter activity are designated inactive pCM-X# plasmids, while pCM-X# plasmids that carry T7 promoter point mutants with moderate or wild-type activity are designated intermediate and strong pCM-X# plasmids, respectively.

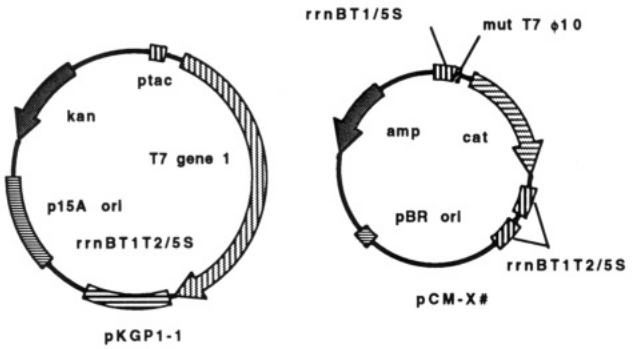

FIGURE 2: Plasmids pKGP1-1 and pCM-X#: a schematic representation of the two-plasmid selection system. Abbreviations: kan, kanamycin resistance gene; amp, ampicillin resistance gene; cat, chloramphenicol resistance gene; p15A ori, p15A origin of replication; pBR ori, Col E1 origin of replication; rrnBT1T2/5S, transcriptional terminators from the *E. coli* 5S rRNA gene; ptac, *tac* promoter; mut T7 *ϕ10*, wild-type or mutant T7 *ϕ10* promoter; T7 gene 1, gene encoding T7 RNA polymerase.

Table I: Selection Plasmids

promoter selection plasmid	type of promoter[a]	mutation carried by the promoter	phenotypic promoter strength[b]
pKK232-8	none		inactive
pCAT10-1	wild-type T7	none	strong
pCM-T297	point mutant	−11G to T	inactive
pCM-P1160	point mutant	−9C to G	inactive
pCM-T270	point mutant	−9C to A	inactive
pCM-P1087	point mutant	−9C to T	inactive
pCM-P1198	point mutant	−8T to G	intermediate
pCM-T286	point mutant	−8T to A	inactive
pCM-B64	point mutant	−8T to C	intermediate[c]
pCM-P1208	point mutant	−7C to G	inactive
pCM-P1031	point mutant	−7C to A	inactive
pCM-T221	point mutant	−6A to G	intermediate[d]

[a] Point mutant indicates that a T7 promoter containing a single point mutation is carried on the selection plasmid. [b] Phenotypic promoter strength is an estimate of *in vivo* promoter activity as assayed in plating experiments with wild-type T7 RNA polymerase (Ikeda et al., 1992a,b). [c] Classified as intermediate but borders on inactive. [d] Classified as intermediate but borders on strong.

a plasmid carrying the *lacI* gene, was a gift from Dr. William Jack, New England Biolabs. All plasmids were prepared by 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., 1992a,b). Electrophoretic analysis of the purified plasmids showed that the DNA was at least 95% supercoiled DNA (data not shown). Little or no linear or open circular forms could be detected. For the hydroxylamine mutations and the *in vitro* RNA polymerase assays (see below), the plasmids were used in their natural state at natural superhelical densities. For examination of run-off transcripts, the plasmids of pCAT10-1, pCM-P1198, pCM-T270, and pCM-P1208 were cleaved with *Nde*I, while the plasmid pLM10 was cleaved with *Pvu*II. Complete cleavage of these plasmids was confirmed by analytical electrophoresis, and the cleaved DNA was purified by phenol extraction and ethanol precipitation. The precipitated DNAs were then redissolved in buffer containing 10 mM Tris-HCl, pH 7.8, and 1 mM EDTA.

Methods

Mutagenesis of pKGP1-1 with Hydroxylamine. Mutagenesis of pKGP1-1 was performed in a 500-μL reaction containing 17–25 μg of pKGP1-1, 0.8 M hydroxylamine, and 0.1 M potassium phosphate buffer, pH 6.0. The reaction was incubated at 70 °C, and at 15, 30, 45, 60, and 75 min 100 μL samples were removed from the reaction and place on ice. The samples were then dialyzed extensively against 75 mM $CaCl_2$ at 4 °C and stored at −20 °C.

Screening for Mutant T7 RNA Polymerases with Altered Promoter Specificity. Competent *E. coli* JM101, 200 μL, were cotransformed with 4 μL of the hydroxylamine-treated pKGP1-1 (approximately 40 ng) and either 4 μL of a mixture of all of the inactive pCM-X# plasmids, where the concentration of each individual inactive pCM-X# is 4 ng/4 μL, or 40 ng of pCAT10-1. SOC, 0.9 mL, was added to the cells, and the culture was grown at 37 °C for 1 h. LB, 1.1 mL, containing 100 μg/mL amp, 100 μg/mL kan, and 60 μg/mL cam was then added to the SOC culture, and the cells were grown at 37 °C for an additional 4 h. Subsequently, equal volumes of the transformation, 200 μL, were 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 30 μg/mL cam, or (3) 50 μg/mL kan, 50 μg/mL amp, and 1.0 mM IPTG. The plates were incubated at 37° for 16 h, and the number of colonies on each plate was counted if any were present.

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

Isolation of pKGP1-1 Plasmids Carrying Potential Mutants of T7 RNA Polymerase. Colonies that grew on the kan/amp/cam plates were transferred to 4.0 mL of LB containing 50 μg/mL kan, 50 μg/mL amp, and 30 μg/mL cam and were grown overnight at 37 °C. The plasmid DNA was isolated from the overnight cultures and the presence of pKGP1-1 and a pCM-X# plasmid was confirmed by restriction analysis (*Eco*RI plus *Pst*I, data not shown).

To separate the pKGP1-1 plasmid from the pCM-X# plasmid, either the DNA isolated from a cam-resistant colony was run on a low-melting agarose gel and the band corresponding to pKGP1-1 was isolated from the gel and used to transform *E. coli* JM101, or the DNA from the resistant colony was digested with *Pvu*II for 5 h at 37 °C (pKGP1-1 contains no *Pvu*II sites, and the pCM-X# plasmids contains 3 *Pvu*II sites; complete digestion of the pCM-X# plasmid was confirmed by gel analysis) and 3 μL of the digest was used to transform *E coli* JM101. In either case the transformed cells were then plated on LB-agar plates containing (1) 50 μg/mL kan, (2) 50 μg/mL kan and 50 μg/mL amp, and (3) 50 μg/mL amp. The absence of colonies on the kan/amp and amp plates was used to confirm the removal of the pCM-X# plasmids. Colonies from the kan plates were then grown overnight in 4.0 mL of LB containing 50 μg/mL kan, and the plasmid DNA was isolated; the identity and purity of the isolated pKGP1-1 plasmid was confirmed by restriction analysis (*Eco*RI plus *Pst*I).

To determine the promoter specificity of the T7 RNA polymerase mutants, *E. coli* JM101 was cotransformed with 2 μL of the isolated pKGP1-1 DNA and each of the following plasmids: 20–50 ng of each of the inactive pCM-X# plasmids separately, 40 ng of pCAT10-1, and 40 ng of pKK232-8. SOC, 0.9 mL, was added to the cells, and the culture was grown at 37 °C for 1 h. Equal volumes of the transformations, 200 μL, were 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 30 μg/mL cam, or (3) 50 μg/mL kan, 50 μg/mL amp, and 1.0 mM IPTG. The plates were incubated at 37 °C for 16 h, and the number of colonies on each plate was counted.

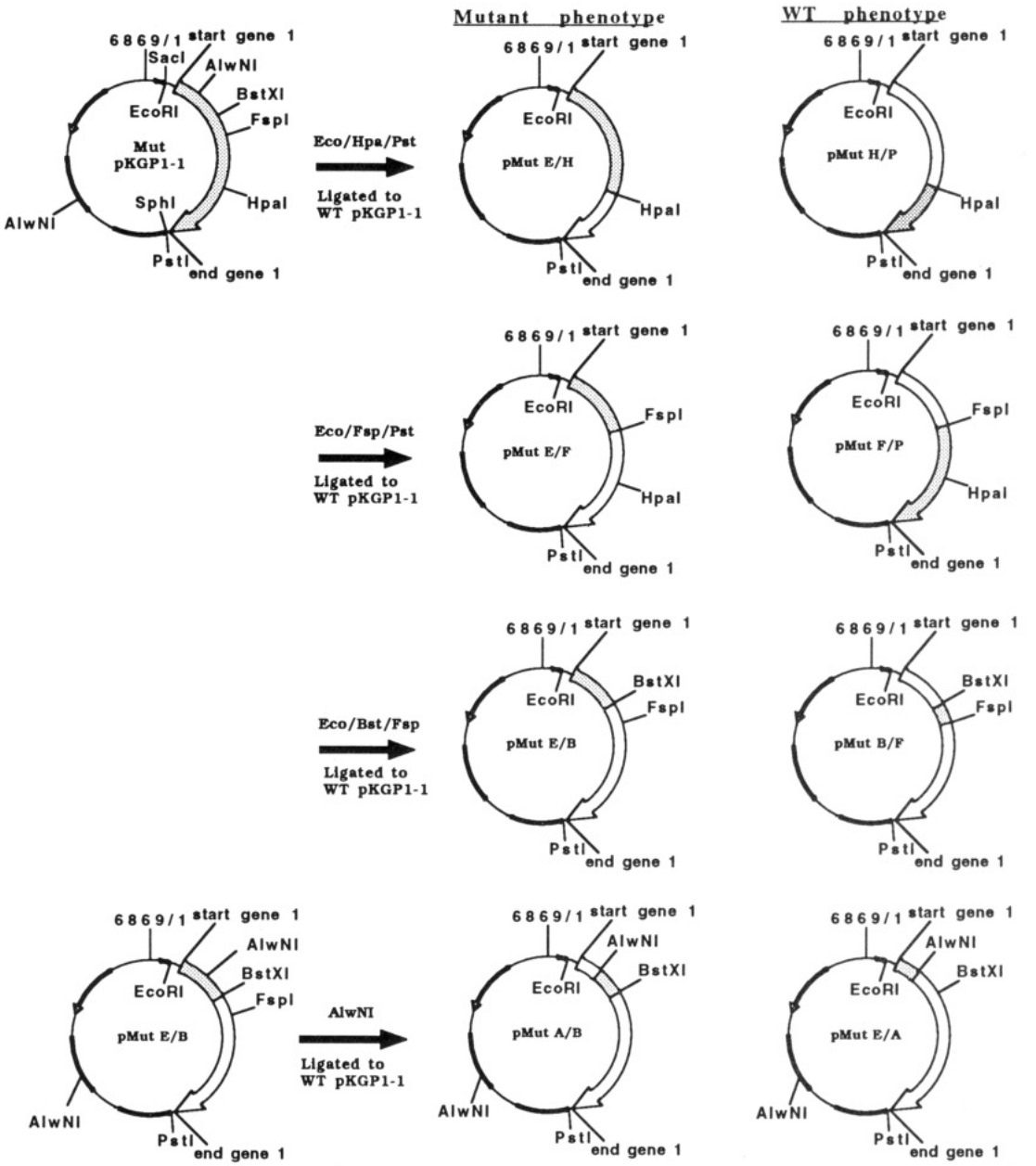

FIGURE 3: Localization of the mutation in pKGP-HA1mut4. As described under Experimental Procedures, T7 gene 1 restriction fragments from pKGP-HA1mut4 were cloned into the corresponding sites of wild-type pKGP1-1. The restriction enzymes used to generate each set of clones are listed above each arrow. The newly constructed recombinant plasmids are shown on the left side of the arrow, and the T7 gene 1 restriction fragments ligated into wild-type pKGP1-1 are shown as shaded segments on the maps. The clones listed under mutant phenotype produced cam-resistant *E. coli* in the cotransformation experiments. The clones listed under WT phenotype produced cam-sensitive *E. coli* in the cotransformation experiments.

Identification of the T7 RNA Polymerase Mutation Responsible for Altered Promoter Specificity. Approximately 100 μg of the mutant pKGP1-1 were isolated for cloning. The mutant pKGP1-1 was cut with either (1) *Eco*RI and *Hpa*I, *Hpa*I and *Pst*I; (2) *Eco*RI and *Fsp*I, *Hpa*I and *Fsp*I; or (3) *Eco*RI and *Bst*XI, *Bst*XI and *Fsp*I, and the restriction fragments containing the pieces of the potentially mutant T7 gene 1 were isolated on a low-melting agarose gel. These "mutant" fragments were then cloned into the corresponding sites of a wild-type pKGP1-1 plasmid (Figure 3). This generated the clones (1) pMutE/H and H/P, (2) pMutE/F and F/P, and (3) pMutE/B and B/F. *E. coli* JM101 was then cotransformed with each clone and either a mixture of all seven inactive pCM-X# plasmids or pCM-P1031. The cells were then 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 30 μg/mL cam, or (3) 50 μg/mL kan, 50 μg/mL amp, and 1.0 mM IPTG. The plates were incubated at 37 °C for 16 h, and the number of colonies on each plate was counted to determine which restriction fragment confers the altered promoter specificity.

A final pair of clones was generated by cleaving wild-type pKGP1-1 and the pMutE/B clone with *Alw*NI and ligating a wild-type restriction fragment to a pMutE/B restriction fragment. The resulting pMutE/A and A/B clones were then used to transform *E. coli* JM101, and the transformed cells were screened as outlined in the preceding paragraph.

To determine the identity of the mutation responsible for the altered promoter specificity of the mutant T7 RNA polymerase, the sequence of the region conferring the mutant phenotype was determined. To facilitate sequencing, the mutant pKGP1-1 plasmid was cut with *Eco*RI and *Pst*I, and

the restriction fragment containing T7 gene 1 was ligated into the *Eco*RI and *Pst*I sites of M13mp19. The clone was confirmed by restriction mapping (data not shown). The mutant region was then sequenced via the protocols recommended by U.S. Biochemical Corp. using Sequenase version 2.0 and the sequencing primers TEMP −34, 171, 298, 365, and 565 and the New England Biolabs M13 sequencing primer (−40).

Preparation of Cell Extracts. Extracts of *E. coli* JM101 harboring either pKGP1-1 and pCM-X# of mutant pKGP1-1 and pCM-X# were prepared as described by Ikeda et al. (1992a). 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 Ikeda et al. (1992a) 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. 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 Ikeda et al. (1992a) by monitoring the increase in A_{415} caused by the accumulation of CENTA hydrolysis products. 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 (Ikeda et al., 1992a).

Measurement of the Specific Activities of the T7 RNA Polymerases. The specific activities of GP1(Lys222) and T7 RNA polymerase were determined by measuring the amount of [2,8-^{3}H]AMP that is incorporated into DE81-retainable RNA during a 10-min incubation at 37 °C in 50-μL transcription reactions containing 50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 1.5 μg of T7 DNA, 400 μM each of the four rNTPs, [2,8-^{3}H]ATP (66.0 cpm/pmol), 1 mM DTT, 30 μg/mL BSA, and 0.150 or 0.030 μg of RNA polymerase (Ikeda & Richardson, 1987). One unit of activity is equal to the incorporation of 1 nmol of ATP into RNA in 1 h (Chamberlin et al., 1970).

In addition, the specific activities of GP1(Lys222) and T7 RNA polymerase were also determined under other nonstandard conditions to provide a direct comparison of the two polymerases activities in different assays. As noted in the text, different DNAs were used (1.5 μg of pCAT10-1 or 1.5 μg of pCAT10-1/*Nde*I) and different buffer conditions were used (50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 60 mM NaCl, and 2.5 mM spermidine).

Measurement of Promoter Strength in Vitro. As previously described by Burgess and co-workers (Chapman et al., 1988; Chapman & Burgess, 1987) and Ikeda et al. (1992a), 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).

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 a pCAT10-1 consensus clone. This procedure defines the activity of pCAT10-1 to be 1.00 (Ikeda et al., 1992a).

Confirmation of the Site of Initiation of Transcription. To confirm that GP1(Lys222) specifically initiates transcription at the T7 promoter carried on the various pCM-X# plasmids, run-off transcripts were examined. Run-off transcripts were produced 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 dithiothreitol, 125 μM UTP, 400 μM each of the other three rNTPs, 15 μCi of [α-^{32}P]UTP, 30 μg/mL bovine serum albumin, 8 nM plasmid (either pCAT10-1/*Nde*I, pCM-P1198/*Nde*I, pCM-T270/*Nde*I, pCM-P1208/*Nde*I, or pLM10/*Pvu*II), and 80 nM T7 RNA polymerase or 40 nM GP1(Lys222). The reactions were equilibrated at 37 °C, and RNA synthesis was initiated by addition of T7 RNA polymerase. At 60 min the reactions were stopped by the addition of an equal volume of loading buffer (90% formamide, 10 mM Tris-HCl, pH 7.8, 0.1% xylene cyanol, and 0.1% bromophenol blue). The samples were heated to 90 °C for 2 min, cooled on ice, and loaded on a 5% acrylamide (30:1 acrylamide to bisacrylamide)/50% urea denaturing gel. The samples were then electrophoresed for 18 h at 250 V. After electrophoresis, the gel was fixed by soaking in an aqueous solution containing 10% methanol and 10% acetic acid. The gel was then dried and visualized by autoradiography.

Estimation of Promoter Binding. The ability of T7 RNA polymerase and GP1(Lys222) to bind a T7 promoter was determined by the gel-retardation method of Muller et al. (1988). The promoter-containing oligonucleotide was identical to the one used by Muller et al., but the 25-μL binding reactions contained 10 mM potassium phosphate, pH 7.8, 1 mM EDTA, 20 mM NaCl, 10% glycerol, 5 μM promoter (approximately 3.9×10^7 cpm) or 2 μM promoter (1.57×10^7 cpm), T7 RNA polymerase or GP1(Lys222), and in some cases a nonspecific competitor DNA (3 μg of λ DNA). After a 10-min incubation at 25 °C, the samples were loaded onto a preelectrophoresed 5% acrylamide gel (30:1 acrylamide to bisacrylamide) and electrophoresed for 50 min at 12 W in 45 mM Tris–borate and 1 mM EDTA. The gel was then fixed by soaking in an aqueous solution of 10% methanol and 10% acetic acid, dried, and visualized by autoradiography. The promoter/polymerase complexes were then cut out of the gel, and the amount of promoter contained in the complexes was measured by liquid scintillation.

RESULTS

Mutagenesis of pKGP1-1 and Selection of Possible Mutants of T7 Gene 1. To improve the possibility that a T7 gene 1 mutation would be responsible for the expression of the CAT gene cloned behind the inactive T7 promoter point mutant, pKGP1-1 was exposed to aqueous hydroxylamine at 70 °C for 15, 30, 45, 60, and 75 min. The treated plasmid samples were then dialyzed to remove the hydroxylamine, and *E. coli* JM101 was transformed with the treated pKGP1-1 and either a mixture of the seven inactive pCM-X# plasmids or pCAT10-1 (Table I and Figure 2). After the transformed cells were allowed to recover in liquid culture in the absence of antibiotics, amp, kan, and cam were added to the liquid

Table II: Phenotypic Characterization of pKGP-HA1mut4

promoter selection plasmid[a]	mutation in the T7 promoter	T7 RNA polymerase plasmid	no. of colonies[b] kan/amp	kan/amp/cam	kan/amp/IPTG
pCAT10-1	none	pKGP1-1	200	200	0
mix of all 7 inactive	all 7 inactive	pKGP1-1	200	0	200
pKK232-8	no promoter	pKGP1-1	200	0	200
pCAT10-1	none	pKGP-HA1mut4	200	200	0
pKK232-8	no promoter	pKGP-HA1mut4	200	0	0
mix of all 7 inactive	all 7 inactive	pKGP-HA1mut4	200	200	0
pCM-T270	−9C to A	pKGP-HA1mut4	200	200	0
pCM-T286	−8T to A	pKGP-HA1mut4	200	200	0
pCM-T297	−11G to T	pKGP-HA1mut4	200	200	0
pCM-P1031	−7C to A	pKGP-HA1mut4	200	200	0
pCM-P1087	−9C to T	pKGP-HA1mut4	200	200	0
pCM-P1160	−9C to G	pKGP-HA1mut4	200	200	0
pCM-P1208	−7C to G	pKGP-HA1mut4	200	200	0

[a] Promoter selection plasmid cotransformed with pKGP1-1. [b] Number of colonies found on the indicated plates.

medium and the entire culture was incubated for 4 h at 37 °C. This step amplifies the abundance of those mutants that are cam-resistant and simplifies the isolation of mutants that might occur very infrequently. The transformed cells were then plated on LB-agar containing either (1) 50 μg/mL kan and 50 μg/mL amp (kan/amp) or (2) 50 μg/mL kan, 50 μg/mL amp, and 30 μg/mL cam (kan/amp/cam). It was observed that the pKGP1-1 sample that had been incubated with hydroxylamine for 60 min yielded approximately 200 colonies on the kan/amp and kan/amp/cam plates when cotransformed with pCAT10-1 and 100 colonies on the kan/amp plate and 26 colonies on the kan/amp/cam plate when cotransformed with the mixture of the seven different pCM-X# plasmids that carry the seven inactive T7 promoter point mutants. This suggested that the CAT gene on at least one of the pCM-X# plasmids was expressed in the presence of the hydroxylamine-treated pKGP1-1. (Note: Due to the early imposition of cam selection, the 26 "mutant" colonies do not reflect the frequency of mutation!)

To confirm that the cam resistance observed with *E. coli* harboring inactive pCM-X# plasmids required the presence of a hydroxylamine-treated pKGP1-1 plasmid, "mutant" pKGP1-1 plasmid DNA was isolated from the 26 colonies that grew on the kan/amp/cam plate, and fresh *E. coli* JM101 was cotransformed with the mixture of the seven inactive pCM-X# plasmids and each of the different "mutant" pKGP1-1 plasmids. All of the isolated "mutant" pKGP1-1 plasmids allowed *E. coli* to grow on kan/amp/cam plates in the presence of the mixture of inactive pCM-X# plasmids. Since the 26 isolates behaved identically in this assay, one representative isolate (pKGP-HA1mut4) was chosen for further characterization.

E. coli JM101 was cotransformed with pKGP-HA1mut4 and pCAT10-1, pKK232-8, or each of the seven different inactive pCM-X# plasmids separately. As shown in Table II, all seven inactive pCM-X# plasmids, and pCAT10-1 allowed *E. coli* to grow on kan/amp/cam plates in the presence of pKGP-HA1mut4, while *E. coli* harboring pKK232-8 and pKGP-HA1mut4 were not resistant to chloramphenicol. This demonstrated that a T7 promoter-like sequence must be present on the CAT plasmid for expression of the CAT gene in the presence of pKGP-HA1mut4.

Identification of a Mutation That Alters the Specificity of T7 RNA Polymerase. To demonstrate that the mutation (or mutations) that alters the apparent promoter specificity of the T7 RNA polymerase encoded on pKGP-HA1mut4 is within T7 gene 1, restriction fragments from pKGP-HA1mut4 were ligated into a wild-type pKGP1-1 plasmid and the new plasmids were tested for their ability to confer chloramphenicol resistance to *E. coli* harboring the inactive promoter selection plasmid pCM-P1031. The first restriction fragments that were cleaved from pKGP-HA1mut4 and ligated into wild-type pKGP1-1 were the T7 gene 1 *Eco*RI/*Hpa*I and *Hpa*I/*Pst*I restriction fragments. On pKGP-HA1mut4 and on pKGP1-1, *Eco*RI cleaves between the *tac* promoter and the ATG of gene 1, *Hpa*I cleaves near the middle of gene 1, and *Pst*I cleaves just after the stop codon of gene 1. This allows the amino and carboxyl halves of gene 1 to be independently ligated into a wild-type pKGP1-1 and generates the new plasmids pMutE/H and pMutH/P that contain the amino and carboxyl halves of the gene 1 from pKGP-HA1mut4, respectively (Figure 3).

E. coli JM101 was cotransformed with pCM-P1031 and either pMutE/H or pMutH/P, and the transformations were spread on kan/amp, kan/amp/IPTG, and kan/amp/cam plates. Only cells containing pMutE/H and pCM-P1031 were able to grow in the presence of chloramphenicol. This confirmed that the mutation responsible for altered promoter specificity was within gene 1 and suggested that the mutation was located in the amino half of the gene.

The same strategy was used to construct and test the plasmids shown in Figure 3, and altered promoter specificity always segregated with the amino-terminal restriction fragment. This allowed us to localize the promoter specificity mutation to the 832-bp *Eco*RI/*Bst*XI restriction fragment cloned into pMutE/B; however, further localization of the promoter specificity mutation required manipulation of pMutE/B.

The restriction enzyme *Alw*NI cleaves pMutE/B and pKGP1-1 twice—between the *Eco*RI and *Bst*XI sites within gene 1 and near the origin of replication of the plasmids. By ligating the two *Alw*NI fragments of pMutE/B to the two reciprocal *Alw*NI fragments of pKGP1-1, the clones pMutE/A and pMutA/B are generated (Figure 3), and the *Eco*RI/*Bst*XI fragment of pMutE/B is further subdivided. When the pMutE/A and pMutA/B were tested for altered promoter specificity, only pMutA/B conferred chloramphenicol resistance to *E. coli* harboring pCM-P1031. In addition, *E. coli* JM101 cotransformed with pMutA/B and pCAT10-1, pKK232-8, or each of the seven different inactive of pCM-X# separately showed the same growth characteristics on kan/amp, kan/amp/cam, and kan/amp/IPTG plates as was observed with pKGP-HA1mut4 (data not shown). This suggested that the T7 gene 1 mutation responsible for altered promoter specificity was located on the 383-bp *Alw*NI/*Bst*XI restriction fragment.

Finally, sequencing of the 383-bp region using an M13mp19 clone of the pKGP-HA1mut4 mutant gene 1 revealed that the only difference between the wild-type T7 gene 1 and the mutant gene 1 was a G to A transition at position 664 of T7 gene 1 (Figure 4). Since the phenotypic assays done with the mutant gene 1 subclones showed that the mutation affecting the specificity of the mutant T7 RNA polymerase was located between positions 753 and 1143 of pKGP-HA1mut4, the mutation at 1105 of pKGP-HA1mut4 (position 664 in relation to T7 gene 1) that changes glutamic acid 222 to lysine is

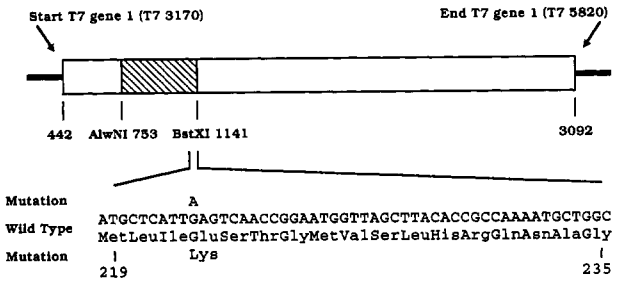

FIGURE 4: Sequencing of pMutA/B reveals a single mutation. The location and identity of the mutation in pMutA/B that is responsible for producing cam resistance in the cotransformation experiments is shown. The "mutant" restriction fragment ligated into the T7 gene 1 region of pMutA/B is shaded. The position of the start and end of T7 gene 1 is listed with respect to T7 (above the map) and with respect to pMutA/B (below the map). The amino acid sequence shown lists the amino acid positions within T7 gene 1.

probably responsible for the altered promoter specificity of the mutant RNA polymerase [GP1(Lys222)].

In Vivo and in Vitro Specificity of GP1(Lys222). In previous work with the compatible plasmids pKGP1-1 and pCM-X#, equivalent bacterial growth on kan/amp and kan/amp/cam plates accompanied by no growth on kan/amp/IPTG plates indicated that the pCM-X# plasmid carried a strong T7 promoter (Ikeda et al., 1992a,b). With pKGP-HA1mut4 and pMutA/B, the seven inactive pCM-X# plasmids and pCAT10-1 all showed equivalent bacterial growth on kan/amp and kan/amp/cam plates and no growth on kan/amp/IPTG plates (Table II), but control experiments also showed that *E. coli* JM101 harboring pMutA/B would not grow on amp/IPTG plates (data not shown). Apparently, the lack of growth on kan/amp/IPTG plates is due to the overproduction of the mutant T7 RNA polymerase and is not an indication of a strong promoter on the pCM-X# selection plasmid. The mutant RNA polymerase may be toxic, or the mutant RNA polymerase may utilize cryptic T7-like promoters within the cell that express proteins that kill the host *E. coli*.

Although the toxicity of the overproduced mutant T7 RNA polymerase makes it impossible to judge the efficiency of promoter usage in plating experiments, it does not seem to interfere with the selection of possible promoter recognition mutants of T7 RNA polymerase in the absence of IPTG. However, to estimate how efficiently GP1(Lys222) RNA polymerase utilizes point mutants of T7 promoters, it is necessary to directly measure promoter activity *in vivo* and *in vitro*.

We have previously shown that *in vivo* usage of the potential T7 promoters carried on the CM-X# plasmids can be estimated by measuring CAT activity relative to β-lactamase activity in extracts of *E. coli* harboring pKGP1-1 and a pCM-X# plasmid (Ikeda et al., 1992a,b). From these measurements, if *in vivo* usage of the wild-type T7 promoter (pCAT10-1) by wild-type T7 RNA polymerase (pKGP1-1) is defined as 1.0, then *in vivo* usage of the inactive T7 promoter point mutants by wild-type T7 RNA polymerase ranges from 0.005 ± 0.003 to 0.01 ± 0.005, and *in vivo* usage of the three intermediate-strength T7 promoter point mutants ranges from 0.04 ± 0.018 to 0.40 ± 0.18 (Figure 5, top). In contrast, while *in vivo* usage of the wild-type T7 promoter (pCAT10-1) by GP1(Lys222) is comparable to usage of the same promoter by wild-type T7 RNA polymerase, in vivo usage of the inactive T7 promoter point mutants by GP1(Lys222) ranges from 0.024 ± 0.019 to 0.22 ± 0.12, and *in vivo* usage of the three intermediate T7 promoter point mutants ranges from 0.22 ± 0.048 to 0.91 ± 0.39 (Figure 5, top). This showed that GP1-

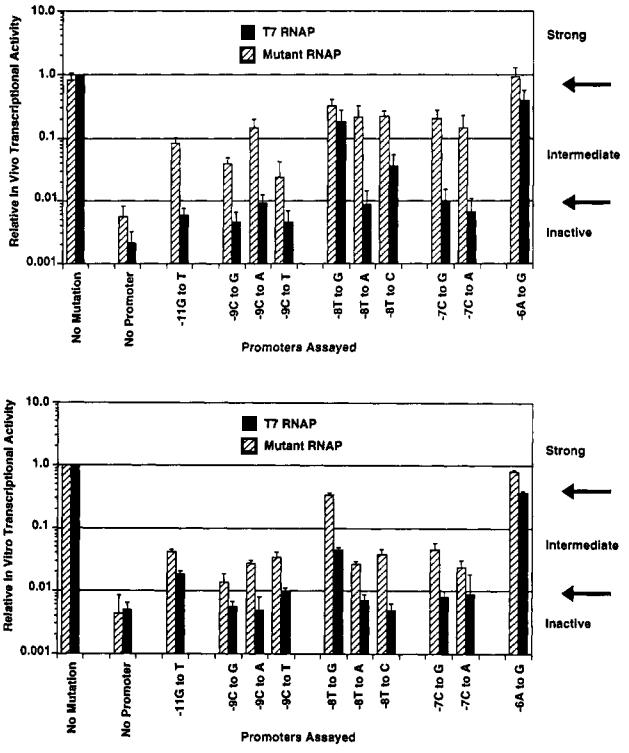

FIGURE 5: Relative *in vitro* and *in vivo* utilization of potential T7 promoters by GP1(Lys222) and wild-type T7 RNA polymerase. The promoters are listed on the horizontal axis, and relative activity is represented along the vertical axis. The arrows on the right side of the graphs indicate the approximate activities that differentiate the inactive, intermediate, and strong promoters. As previously noted, the –8 T to G mutant is classified as an intermediate promoter, while the –6 A to G mutant shows some characteristics of a strong promoter, and the –8 T to C mutant shows 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. (Top panel) Relative *in vivo* promoter utilization. The relative in vivo transcriptional activity (or promoter strength) is defined under Experimental Procedures. The errors associated with these results were calculated by standard methods for the propagation of errors. No error bar is shown for use of the wild-type promoter by wild-type T7 RNA polymerase since the consensus T7 promoter has been defined to have a relative *in vivo* activity of 1.00. (Bottom panel) Relative *in vitro* promoter utilization. The relative in vitro promoter strength (or promoter strength) is 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 deviations observed. No error bar is shown for use of the wild-type promoter by either wild-type T7 RNA polymerase or GP1(Lys222) since the consensus T7 promoter has been defined to have a relative *in vitro* activity of 1.00.

(Lys222) uses the inactive and intermediate T7 promoter point mutants 5–25 times and 2–6 times more efficiently than wild-type T7 RNA polymerase, respectively (Figure 5, top).

To confirm the *in vivo* measurements, promoter usage was also measured *in vitro*. Although T7 RNA polymerase and GP1(Lys222) exhibit different absolute activities in these *in vitro* reactions (Table III), relative comparisons are informative. If *in vitro* usage of the wild-type T7 promoter (pCAT10-1) by both T7 RNA polymerase and GP1(Lys222) is defined as 1.0, the *in vitro* usage of the inactive and intermediate T7 promoter point mutants by T7 RNA polymerase ranges from 0.005 ± 0.005 to 0.018 ± 0.005 and from 0.005 ± 0.005 to 0.36 ± 0.03 (Figure 5, bottom), respectively, while the *in vitro* usage of the inactive and intermediate T7 promoter point mutants by GP1(Lys222) ranges from 0.014 ± 0.005 to 0.05 ± 0.01 and from 0.04 ± 0.01 to 0.81 ± 0.03,

Table III: Assay Conditions Affect the Specific Activities of T7 RNA Polymerase and GP1(Lys222)

enzyme	DNA	specific activity (units/mg) buffer A[a]	buffer B[b]
GP1(Lys222)	T7	14800	26300
	pCAT10-1	2500	1900
	pCAT10-1/*Nde*I	2200	2600
T7 RNA polymerase	T7	43700	6600
	pCAT10-1	18900	5900
	pCAT10-1/*Nde*I	15100	1600

[a] Buffer A: 50 mM Tris-HCl, pH 8, and 10 mM $MgCl_2$. [b] Buffer B: 50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 60 mM NaCl, and 2.5 mM spermidine.

respectively. Although the absolute magnitudes of *in vivo* and *in vitro* promoter usage differ, the trends noted for *in vivo* promoter usage are almost duplicated in the *in vitro* measurements; however, some differences are noted. For example, while the *in vitro* data show that GP1(Lys222) uses the intermediate T7 promoter point mutants 2–8 times more efficiently than T7 RNA polymerase, the data also show that the mutant enzyme uses the inactive T7 promoter point mutants only 2–6 times more efficiently than the wild-type RNA polymerase. This difference in the *in vivo* and *in vitro* data is probably due to the difficulties encountered in measuring the low *in vitro* usage of the inactive T7 promoter point mutants by T7 RNA polymerase. Since background is difficult to subtract from these measurements, it is likely that *in vitro* usage of the inactive T7 promoter point mutants by T7 RNA polymerase is overestimated and that the relative increase in the efficiency of *in vitro* usage of these promoters by GP1(Lys222) is underestimated.

Utilization of Homologous Phage Promoters. The ability of GP1(Lys222) to utilize the seven different T7 promoter point mutants that are not utilized by wild-type T7 RNA polymerase showed that the mutant T7 RNA polymerase is less specific than the wild-type enzyme, but these measurements do not define a limit to the sequence variations accommodated by GP1(Lys222). To determine if GP1-(Lys222) still requires the context of a T7 promoter, transcription from plasmids containing no T7 promoter, an SP6 promoter, or a T3 promoter was measured *in vitro*. Figure 6 shows that little or no RNA is produced in the presence of templates carrying no promoter or an SP6 promoter and that a T3 promoter is poorly utilized by either wild-type T7 RNA polymerase (activity = 0.009 ± 0.003) or GP1(Lys222) (activity = 0.027 ± 0.008). However, GP1(Lys222) uses a T3 promoter more efficiently than wild-type T7 RNA polymerase, and the *in vitro* utilization of the T3 promoter by GP1(Lys222) is comparable to the *in vitro* utilization of a number of the inactive T7 promoter point mutants. Nevertheless, this result may not actually reflect specific initiation at the T3 promoter of pLM10 since few specific transcripts are seen with GP1(Lys222) in the run-off assays shown below. The *in vitro* activity of GP1(Lys222) on pLM10 may simply reflect nonspecific initiation.

Initiation of Transcription Occurs at the Potential T7 Promoters. To show that transcription initiates at the potential T7 promoters on the pCM-X# plasmids, run-off transcripts produced by T7 RNA polymerase and GP1(Lys222) were compared. A plasmid carrying a wild-type T7 promoter (pCAT10-1), a plasmid carrying an intermediate-strength T7 promoter point mutant (pCM-P1198), two plasmids carrying inactive T7 promoter point mutants (pCM-T270 and pCM-P1208), and a plasmid carrying a T3 promoter (pLM10)

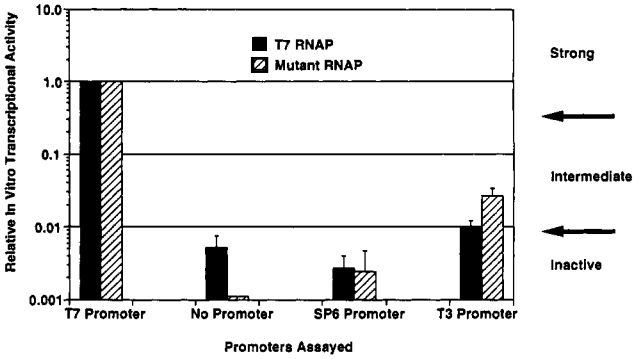

FIGURE 6: Relative *in vitro* utilization of bacteriophage SP6 and T3 promoters by GP1(Lys222) and wild-type T7 RNA polymerase. The promoters are listed on the horizontal axis, and relative activity is represented along the vertical axis. The relative in vitro promoter strength is defined under Experimental Procedures. The arrows on the right side of the graph indicate the approximate activities that differentiate the inactive, intermediate, and strong promoters. The results are the average of two time points of at least three different samples, and the error associated with the measurements is the greater of the standard deviations observed. 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 bar is shown for use of the wild-type promoter by either wild-type T7 RNA polymerase or GP1(Lys222) since the consensus T7 promoter has been defined to have a relative *in vitro* activity of 1.00. In these assays the concentration of T7 RNA polymerase was 80 nM while the concentration of GP1(Lys222) was 40 nM. All other conditions were as previously described (Ikeda et al., 1992a).

were linearized by cleaving the plasmids with either *Nde*I (the pCM-X# plasmids) or *Pvu*II (pLM10). Cleavage of the pCM-X# plasmids with *Nde*I places the potential T7 promoters 2905 nucleotides from the end of each template, while cleavage of pLM10 with *Pvu*II places the T3 promoter 2371 nucleotides from the end of its template. The linearized plasmids were then used in transcription reactions containing either 80 nM T7 RNA polymerase or 40 nM GP1(Lys222). Figure 7 shows that the lengths of the run-off transcripts produced by T7 RNA polymease are identical to the lengths of the run-off transcripts produced by GP1(Lys222). In addition, specific run-off transcripts are easily observed in reactions containing T7-like promoters, while specific run-off transcript are barely detectable in reactions containing a T3 promoter (pLM10/*Pvu*II). (Note: The run-off doublets seen in the pCM-X# lanes of Figure 7 are due to the presence of a sequence near the end of the pCM-X# templates that acts as a terminator of T7 transcription). This would seem to indicate that transcription by GP1(Lys222) is promoter-dependent and that the promoter must resemble a T7 promoter.

Promoter Binding by GP1(Lys222). A two amino acid insertion at position 222 of T7 RNA polymerase has been previously reported to disrupt promoter binding without affecting polymerase function (Gross et al., 1993); however, the opposite phenomenon, tighter promoter binding, would be a plausible mechanism that might explain the ability of GP1(Lys222) to recognize an expanded range of T7 promoter-like sequences. The positively charged lysine side chain could stabilize the binding of the RNA polymerase to the negatively charged DNA template and improve the ability of the mutant enzyme to recognize promoter-like sequences. To test whether the expanded specificity of GP1(Lys222) is due to stabilization of the promoter/enzyme complex, the binding of T7 RNA polymerase and GP1(Lys222) to an oligonucleotide containing a T7 promoter was measured by gel retardation. Figure 8 shows that GP1(Lys222) is still capable of forming promoter-

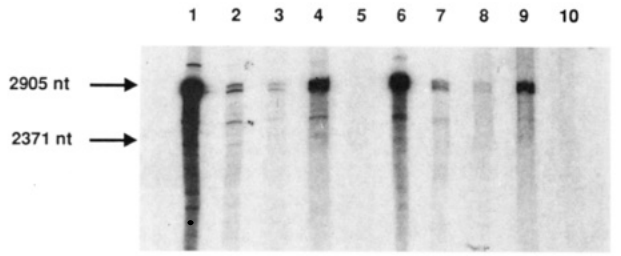

FIGURE 7: Comparison of the run-off transcripts synthesized by T7 RNA polymerase and GP1(Lys222). The autoradiograph of a denaturing 5% acrylamide gel shows that the run-off transcripts synthesized by T7 RNA polymerase and GP1(Lys222) are identical in length. Lanes 1–5 show the run-off transcripts produced by 40 nM GP1(Lys222) in the presence of pCAT10-1/*Nde*I (0.2 μL), pCM-P1198/*Nde*I (2.0 μL), pCM-T270/*Nde*I (10.0 μL), pCM-P1208/*Nde*I (10.0 μL), and pLM10/*Pvu*II (10.0 μL), respectively, while lanes 6–10 show the run-off transcripts produced by 80 nM T7 RNA polymerase in the presence of pCAT10-1/*Nde*I (0.2 μL), pCM-P1198/*Nde*I (2.0 μL), pCM-T270/*Nde*I (10.0 μL), pCM-P1208/*Nde*I (10.0 μL), and pLM10/*Pvu*II (10.0 μL), respectively. All other conditions are described under Methods. The volume in parentheses following each template is the volume of sample that was loaded onto the gel. Different volumes were loaded onto the gel to try to equalize the amount of run-off transcript in each lane. The transcripts synthesize in the presence of the pCM-X# templates are approximately 2900 nucleotides long, while the barely perceptible transcripts synthesized in the presence of the pLM10 templates are approximately 2370 nucleotides long. The run-off doublets seen in lanes 1–4 and 6–9 are produced by a partially effective transcriptional terminator near the ends of the pCM-X# templates.

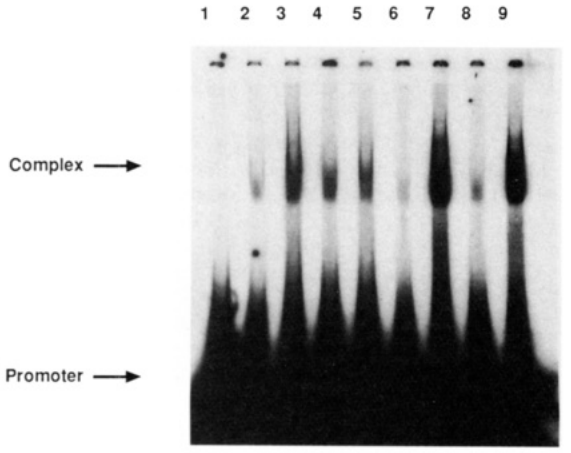

FIGURE 8: Binding of a T7 promoter by wild-type T7 RNA polymerase and GP1(Lys222). The autoradiograph of a 5% acrylamide gel shows the binding of wild-type T7 RNA polymerase (lanes 6–9) and GP1(Lys222) (lanes 2–5) to a ^{32}P-labeled oligonucleotide that carries a T7 promoter. Lane 1, no RNA polymerase, 5 μM promoter; lane 2, 26.5 nM GP1(Lys222), 5 μM promoter; lane 3, 132.7 nM GP1(Lys222), 5 μM promoter; lane 4, 26.5 nM GP1(Lys222), 5 μM promoter, 3 μg of λ DNA; lane 5, 132.7 nM GP1(Lys222), 5 μM promoter, 3 μg of λ DNA; lane 6, 12.9 nM T7 RNA polymerase, 5 μM promoter; lane 7, 64.7 nM T7 RNA polymerase, 5 μM promoter; lane 8, 12.9 nM T7 RNA polymerase, 5 μM promoter, 3 μg of λ DNA; lane 9, 64.7 nM T7 RNA polymerase, 5 μM promoter, 3 μg of λ DNA. Complex = position of the enzyme/promoter complexes. Promoter = position of the unbound promoter oligonucleotide.

specific complexes but that the affinity of the mutant enzyme for a T7 promoter is diminished. The data obtained from two different trials suggest that the promoter binding affinity of GP1(Lys222) ($1.4 \times 10^4 \pm 5 \times 10^3$ M^{-1}) is 20 times less than the promoter binding affinity of wild-type T7 RNA polymerase ($2.8 \times 10^5 \pm 2.2 \times 10^5$ M^{-1}). In addition, it is also observed that a GP1(Lys222)/T7 promoter complex is more easily disrupted by nonspecific competitor DNA (Figure 8, lanes 4 and 5) than a wild-type T7 RNA polymerase/T7 promoter complex (Figure 8, lanes 8 and 9). The susceptibility of GP1(Lys222)/promoter complexes to disruption by nonspecific DNA could be due either to the decreased stability of the specific enzyme/promoter complex or to an increased affinity for nonspecific DNA. Whatever the case, the Glu to Lys substitution in GP1(Lys222) produces an enzyme with reduced affinty for T7 promoters and an ability to utilize an expanded range of T7 promoter-like sequences.

DISCUSSION

The selection of promoter recognition mutants of T7 RNA polymerase was pursued by randomly mutagenizing pKGP1-1 with aqueous hydroxylamine, cotransforming *E. coli* with the mutagenized pKGP1-1 and a mixture of the seven different inactive pCM-X# plasmids, and isolating and characterizing the RNA polymerase that was present in those colonies that exhibited chloramphenicol resistance. It was established that *E. coli* harboring the mutant plasmid pKGP-HA1mut4 and an inactive pCM-X# are chloramphenicol-resistant and that the mutation responsible for the expression of CAT from the inactive pCM-X# plasmid is a G to A transition at nucleotide 664 of T7 gene 1 that converts glutamic acid (222) to lysine (Figures 3 and 4). To determine if the observed growth of the cotransformed *E. coli* in the presence of chloramphenicol reflects the ability of the mutant T7 RNA polymerase to utilize the T7 promoter point mutants found on the pCM-X# plasmids, *in vivo* and *in vitro* promoter utilization were measured and compared (Figure 5). *In vivo* promoter utilization was determined by measuring the relative abundance of CAT in extracts of *E. coli* that harbored pMutA/B (the variant of pKGP1-1 that carries a single G to A transition at nucleotide 664 of T7 gene 1) and a pCM-X# plasmid, while *in vitro* promoter utilization was determined by measuring RNA synthesis in the presence of purified RNA polymerase and purified template. Furthermore, the location of the initiation of transcription by GP1(Lys222) and T7 RNA polymerase was confirmed by comparing the lengths of the run-off transcripts synthesized by the two enzymes in the presence of linearized pCM-X# and pLM10 templates. Although the absolute magnitudes of *in vivo* and *in vitro* promoter utilization differ, the *in vivo* and *in vitro* data show the same relative trends. The mutant T7 RNA polymerase, GP1(Lys222), utilizes the seven inactive T7 promoter point mutants and three intermediate T7 promoter point mutants more efficiently than wild-type T7 RNA polymerase. The correlation of the *in vivo* and *in vitro* data and the observation that GP1(Lys222) and T7 RNA polymerase initiate transcription at the same location and synthesize run-off transcripts of identical lengths suggest that the restoration of chloramphenicol resistance in the cotransformed *E. coli* results from the ability of GP1(Lys222) to initiate transcription from T7 promoter point mutants that are normally inactive.

The observed changes in the promoter specificity of GP1(Lys222) and the location and identity of the mutation in GP1(Lys222) are notable. First, the Glu to Lys substitution at amino acid 222 of T7 RNA polymerase is located near the amino-terminal domain of the enzyme (amino acids 1–179) and alters promoter recognition by the mutant RNA polymerase. Similarly, it had been previously reported that a two amino acid insertion at position 222 disrupts DNA binding while preserving polymerase function (Gross et al., 1992). Apparently, regions near the amino-terminal domain of T7 RNA polymerase are involved in promoter binding. Second, the Glu to Lys substitution allows GP1(Lys222) to utilize all seven of the inactive T7 promoter point mutants. Since the

inactive T7 promoter point mutations occur at four different positions in the T7 promoter, it is unlikely that the single Glu to Lys substitution results in specific recognition of the seven inactive T7 promoter point mutants. In fact, the observation that GP1(Lys222) used intermediate T7 promoter point mutants more efficiently than wild-type T7 RNA polymerase suggests that the Glu to Lys substitution expands the specificity of the RNA polymerase. Conversely, the inability of GP1-(Lys222) to utilize an SP6 promoter (Figure 6), the inability of GP1(Lys222) to specifically initiate at a T3 promoter (Figure 7), and the ability of GP1(Lys222) to specifically utilize the T7 promoter point mutant indicates that promoter specificity is not eliminated and that at a minimum GP1-(Lys222) requires the context of a T7-like promoter.

Mechanistically, the Glu(222) to Lys substitution could reduce the specificity of GP1(Lys222) by at least two plausible mechanisms. First, the Lys substitution could cause a global structural change in the RNA polymerase that alters promoter binding, and second, the substitution of a positively charged amino acid side chain for a negatively charged amino acid side chain could stabilize the binding of the small negatively charged RNA initiation products within the transcription complex and increase the efficiency of utilization of a weak promoter. A third plausible mechanism seems, however, to be excluded by the observation that GP1(Lys222) binds a T7 promoter less tightly than the wild-type T7 RNA polymerase. This observation suggests that it is unlikely that the positively charged lysine side chain stabilizes the binding of the RNA polymerase to the negatively charged DNA template and improves the ability of the mutant enzyme to recognize promoter-like sequences. Ultimately, determination of the mechanism(s) responsible for the altered specificity of GP1-(Lys222) will require further characterization of the mutant enzyme.

In conclusion, mutant T7 RNA polymerases that exhibit altered promoter specificity can be isolated by screening for chloramphenicol resistance in *E. coli* harboring a plasmid that expresses T7 RNA polymerase (pKGP1-1) and a promoter selection vector that carries an inactive T7 promoter point mutant (an inactive pCM-X#). Furthermore, the mutation responsible for the altered promoter specificity of the mutant T7 RNA polymerase can be easily identified, and the effect of the mutation on promoter recognition can be measured. By isolating and characterizing mutations that alter promoter recognition by T7 RNA polymerase, it should be possible to identify the regions of the RNA polymerase that can contribute to promoter recognition.

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