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Segment-specific mutagenesis: Extensive mutagenesis of a *lac* promoter/operator element

(site-directed mutagenesis/sodium bisulfite/single-stranded phage vector/*lac*UV5 promoter)

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ABSTRACT **A method for highly efficient segment-specific mutagenesis is described. The method uses as target for sodium bisulfite mutagenesis the DNA single strands of a DNA restriction fragment that had been separated by cloning into base-complementary regions of a pair of phage fd vectors. After repair synthesis *in vitro*, the mutagenized DNA fragment is recovered by cloning into a nonmutated plasmid vector and analyzed for sequence and by functional tests. By using this method, the nucleotide sequence of a 109-base pair restriction fragment containing the *lac* promoter/operator from *Escherichia coli* was extensively modified. More than 90% of the 235 isolates obtained showed a change in phenotype; all of 22 analyzed for their nucleotide sequence were found to carry multiple C → T point mutations in up to 60% of the possible target positions. Nevertheless, few isolates showed major changes in promoter activity relative to the nonmutated promoter element, which indicates a high degree of flexibility in the promoter sequence.**

Molecular cloning methods have been used to develop a type of genetic analysis in which a cloned genetic element is first altered at predetermined sites and then assayed for changes in phenotype in the appropriate biological environment. This "reversed genetics" (1) allows construction and study of silent mutations that are often essential to correlate structure and function of a genetic element. In this approach, deletions or insertions can be introduced rather easily. However, methods to generate point mutations at predetermined sites in a DNA molecule by base-specific chemical treatment (2–5) or by incorporation of nucleotide analogs (1) give rise to only a few mutants among a large background of nonmutants. Because this yield is too low to permit saturation of a genetic element with point mutations, we developed an improved method for sodium bisulfite mutagenesis *in vitro*. This reagent has been used before for site-specific mutagenesis of sequences next to a unique restriction site in which a DNA sequence could be rendered single stranded to become a selective target for the mutagen (2). We have overcome these limitations by using a single-stranded phage vector system to clone either strand of the desired target DNA separately. Mutagenesis of the single-stranded recombinant DNA enabled us to introduce any number of C → T base changes into one strand of a defined restriction fragment. The present study exemplifies the scope of the method by an extensive variation of the nucleotide sequence of the *lac* promoter/operator, a regulatory signal that controls the expression of the *lac* operon in *Escherichia coli*.

MATERIALS AND METHODS

E. coli C600 r^-m^+ and KB35 and phages fd109 and fd109-2 were obtained from R. Herrmann (6). *E. coli* C600 *gal*K^- (*gal* $E^+T^+K^-$) and pKO1 were obtained from K. McKenney (7). Construction of plasmids and recombinant phages, including transformation and cultivation of bacteria, and isolation of recombinant DNA was done essentially as described by Herrmann *et al.* (6). DNA sequence analysis was carried out according to Maxam and Gilbert (8) using dimethyl sulfate (G), formic acid (A+G), and hydrazine (C+T, C) as base-specific reagents.

Mutagenesis of Single-Stranded DNA. Single-stranded DNA (50 μg, 18 pmol) from phage fd109-11 or fd109-21 was dissolved in 68 μl of TE buffer (10 mM Tris·HCl, pH 8/0.1 mM EDTA), and 320 μl of 4 M sodium bisulfite reaction mixture (156 mg of $NaHSO_3$, 64 mg of Na_2SO_3 in 0.43 ml of H_2O) and 12 μl of 50 mM hydroquinone were added. After 4, 8, and 21 hr at 37°C in the dark, 100-μl samples were taken and chromatographed on Sephadex G-100 columns (2 ml) previously equilibrated with 10 mM KPO_4, pH 6, to remove bisulfite. The void volume containing the DNA was collected and adjusted to 0.2 M Tris·HCl, pH 9.2/50 mM NaCl/2 mM EDTA. After 8–16 hr more at 37°C, the samples were desalted by filtration through Sephadex G-100 and lyophilized. The rate of conversion of cytosine to uracil was determined with pEx110 DNA radioactively labeled in dCMP by nick-translation (9). This DNA was heat denatured and mutagen treated as described above. Samples were digested with DNase I and snake venom phosphodiesterase to 5′-NMPs (10). After separation of the products by paper electrophoresis at pH 3.5 (11), the radioactivity in the dCMP and dUMP spots was quantitated in a scintillation counter.

***In Vitro* Synthesis of the Complementary Strand.** Mutagen-treated phage DNA (either fd109-11 or fd109-21 DNA) (2 pmol) was mixed with 0.5 pmol of a heat-denatured mixture of primer fragments (prepared by cleavage of pBR322 with *Alu* I and *Eco*RI) and incubated in 20 μl of DS buffer (40 mM Tris·HCl, pH 7.2/10 mM $MgCl_2$/0.2 mm dithioerythritol/100 mM KCl) for 10 min at 90°C. After cooling to 37°C (within 2 hr), [α-^{32}P]dNTPs (40 mCi of ^{32}P/mmol; 1 Ci = 3.7×10^{10} becquerels) were added in DS buffer to final concentrations of 0.2 mM each and a final volume of 30 μl. Six units of DNA polymerase I was added and the sample was incubated for 25 min at 20°C and 10 min at 37°C. The reaction products were isolated by ethanol precipitation and digested with restriction endonucleases *Eco*RI and *Hin*dIII, and the resulting DNA fragments were separated on a 6% polyacrylamide gel. The mutagenized double-stranded *lac*UV5 promoter DNA element was visualized by autoradiography and isolated as described (8).

Recloning of the Mutagenized Promoter DNA Element. Samples of mutagenized DNA fragment were ligated with excess (0.01 pmol) *Eco*RI/*Hin*dIII pBR322 DNA fragment. To eliminate self-ligation of the vector, the latter had been de-

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Abbreviation: bp, base pair(s).

phosphorylated by treatment with calf intestine alkaline phosphatase (Boehringer Mannheim) and purified twice by gel electrophoresis (4% polyacrylamide). Competent *E. coli* C600 were transformed with the ligation mixtures and transformants resistant to ampicillin were isolated. These were tested for growth in the presence of various concentrations of tetracycline.

Galactose Kinase Assay. *E. coli* C600 *gal*K$^-$ (*gal* E$^+$T$^+$K$^-$) was transformed with pKO110 and its various mutant derivatives. Clones harboring the plasmids were tested for galactose kinase activity in toluenized cells according to McKenney (7). Glucose was used as carbon source instead of fructose as the only modification of this protocol. About 700 units of galactokinase were obtained with pKO110, which is ≈20-fold that obtained with a promoterless pKO derivative.

GENERAL STRATEGY

The principle of our method is to selectively treat the isolated single strands of a cloned DNA segment with a highly efficient mutagen and then reclone the mutated DNA element into an unmutated vector molecule that allows assay for structural and functional changes. As outlined in Fig. 1, the experimental procedure has four steps.

Step I. The DNA fragment of interest is transferred to a single-stranded phage vector such as fd109 (6). Cloning in either orientation is desirable because it provides both complementary single strands.

Step II. Base changes are introduced into the single-stranded DNA of the recombinant phage by chemical mutagenesis. Treatment with sodium bisulfite was chosen because it can convert essentially all cytosine residues in a DNA molecule to uracil residues without doing significant damage to other bases (12–14) or the sugar phosphate backbone (see below).

Step III. A stable DNA copy is established from the altered DNA sequence of the fragment of interest by fragment-primed repair synthesis *in vitro*. This step creates U·A base pairs, which is essential to stabilize the newly formed nucleotide sequence against the highly efficient uracil excision–repair system of *E. coli* (15).

Step IV. The mutagenized DNA double strand is cleaved from the mutated vector and recloned in an unmutated recipient vector. Preferably, this vector allows assay of functional changes in the recloned DNA element. In addition, nucleotide sequences are determined from the mutated recloned DNA inserts in individual isolates. The usefulness of this method is exemplified by a 109-base pair (bp) DNA fragment (termed

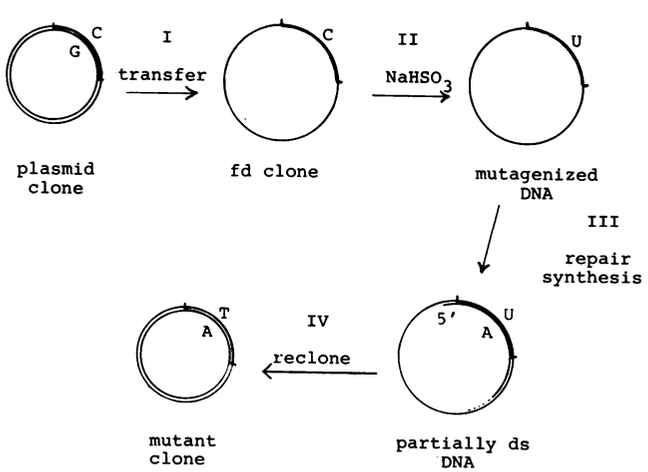

FIG. 1. Segment-specific mutagenesis procedure. ds, Double stranded.

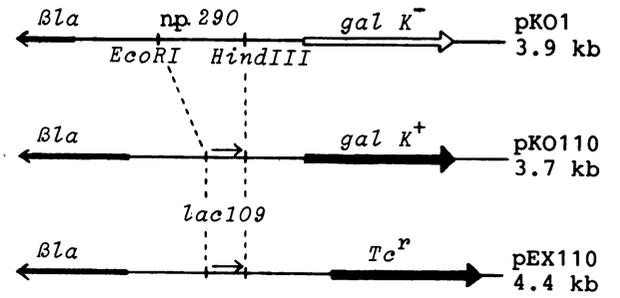

FIG. 2. Structure of pEx110 and the pKO plasmids. All plasmids are derivatives of pBR322 (18). pEx110 lacks the 31 bp between positions 1 and 31. In the pKO plasmids, the *tet* region (positions 1–2065) has been replaced by the promoterless *E. coli* galactokinase gene. Both systems allow directed cloning of *Eco*RI/*Hin*dIII restriction fragments next to an indicator gene. n.p. 290 is a promoterless DNA fragment (7). kb, kilobases.

*lac*109 below) containing the *lac* promoter/operator element from the CAP-independent *lac*UV5 mutation of *E. coli* (16, 17).

RESULTS AND DISCUSSION

Structure and Properties of the *lac*109 Fragment and Plasmid pEx110. The plasmid we started from is pEx110, a derivative of pBR322 in which the 31 bp between the *Eco*RI and *Hin*dIII sites have been replaced by the *lac*109 fragment (ref. 18; Fig. 2). This element consists of the *Alu* 95 fragment from the *lac*UV5 control region (19) flanked by linker sequences. As a consequence, pEx110 has the following properties relevant for this study. (*i*) The promoter element can be isolated by cleavage with *Eco*RI/*Hin*dIII and recloned in a defined orientation. (*ii*) Expression of tetracycline resistance is under control of the cloned *lac*109 element. Mutant plasmids that have an altered DNA sequence in the promoter element mediate levels of tetracycline resistance different from that of pEx110 (8 μg/ml) (20–22). (*iii*) *E. coli* cells containing the plasmid grow on X-gal indicator plates (23) as blue colonies, because the *lac* operator in the promoter element titrates the *lac* repressor, leading to constitutive synthesis of enzyme β-galactosidase (24). This provides an additional marker for successful mutagenesis of the target.

Recloning of *lac*109 in Single-Stranded Phage Vectors (Step I). To prepare its single strands for mutagenesis, the 109-bp promoter element was cloned in the single-stranded phage vectors fd109 and fd109-2. As shown in Fig. 3, insertion of *lac*109

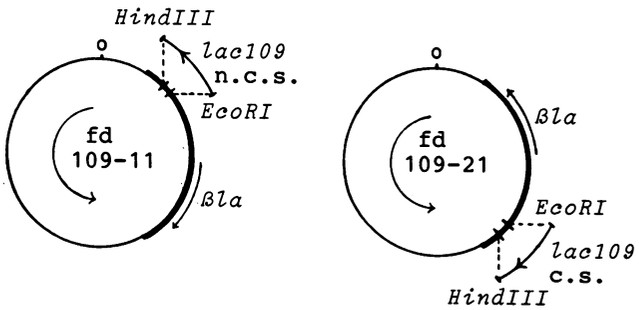

FIG. 3. Structure of the recombinant DNA from phages fd109-11 and fd109-21. The two vector phages (fd109 and fd109-2) carry an 1876-bp fragment from pBR322 in opposite orientations (▬). This fragment provides a selectable marker (the β-lactamase gene, *βla*; indicated by arrows outside the circles) and the two restriction sites used for cloning (*Eco*RI and *Hin*dIII). The cloned *lac*109 DNA segment is illustrated by the enlarged insert, in which c.s. and n.c.s. indicate the coding and noncoding strands, respectively, of the *lac*UV5 promoter on *lac*109. Inner arrows indicate direction of transcription of the *fd* genes.

Table 1. Properties of *E. coli* clones harboring mutagen-treated type pEx110 plasmids

Exp.	Mutagen-treated DNA*	Chemical treatment, hr	Clones, no.							
						Tetracycline (μg/ml) resistant				
			Total	op$^+$	op$^-$	5	5–10	10–20	20–40	40
1	fd109-11	4 and 8†	66	10	56	13	33	12	6	2
2	fd109-21	8	60	0	60		13	5	39	3
3	fd109-21	8	109	0	109		27	25	53	4

Samples of mutagen-treated *lac*109 DNA were ligated with 0.01 pmol of vector DNA and transfected into competent *E. coli* C600. No clones were obtained in a control experiment without *lac*109 fragment. *lac* operator function in the plasmids was tested on X-gal agar plates.

* fd109-11 and fd109-21 contain the noncoding and coding strands, respectively, of the *lac*109 promoter DNA fragment.

† A mixture of *lac*109 from two separate reactions was used.

results in the formation of fd109-11 and fd109-21, which carry the complementary strands of this DNA fragment.

Sodium Bisulfite Mutagenesis (Step II). The rate of C → U conversion under our experimental conditions was determined with ^{32}P-labeled plasmid DNA. After 4, 8, and 21 hr of treatment, conversion of cytosine to uracil was 30%, 50%, and 60%, respectively. In parallel, identical conditions were used to mutagenize the single-stranded circular DNA of the recombinant phages fd109-11 and fd109-21. As tested by agarose gel electrophoresis, no strand breaks occurred in these 8.4-kilobase molecules, even during 21 hr of mutagen treatment. This demonstrates that the majority of the cytosine residues can be deaminated even in a large single-stranded DNA molecule without significant damage to the backbone of the DNA chain.

Synthesis of the Complementary Strand and Isolation of the Mutagenized Promoter Element (Step III). Uracil-substituted DNA can serve as template for DNA polymerase I-catalyzed *in vitro* DNA synthesis (14). We carried out several *in vitro* DNA synthesis reactions using as template mutagen-treated phage DNA from fd109-11 or fd109-21 (Table 1). An *Eco*RI/*Alu* I digest of pBR322 DNA was included in the reaction mixture to provide the appropriate primer DNA, because this fragment mixture contains two DNA fragments that hybridize to the pBR322 sequences flanking the *lac*109 insert in the recombinant phages (18).

Recovery of the Mutagenized Double-Stranded Promoter Elements in a Plasmid Vector (Step IV). During synthesis, the DNA was labeled by incorporation of [α-^{32}P]dNTPs. This allowed identification and isolation of the mutagenized 109-bp *Eco*RI/*Hin*dIII fragment by cleaving the reaction products with these two restriction enzymes and separation from the partially double-stranded vector molecules on a polyacrylamide gel. The yields of mutagenized *lac*109 fragment decreased with increasing time of incubation of the DNA with the mutagen (22). This can be explained by C·G → U·A changes in the nucleotide sequence of the recognition sites for the enzymes used for fragmentation. Both sites contain a cytosine residue in either DNA strand (25). Their conversion to uracil in one DNA strand apparently suffices to cause resistance against cleavage by these enzymes.

For recloning, the mutated promoter elements were ligated between the *Eco*RI and the *Hin*dIII sites of plasmid pBR322. After transformation of *E. coli* C600, ampicillin-resistant clones were screened for changed tetracycline resistance and *lac* operator function. From three such experiments (Table 1), 235 clones were obtained. No ampicillin-resistant transformants could be isolated in a control in which the *lac*109 fragment was omitted from the ligation reaction, suggesting that all isolates contained inserts of the added mutated *lac*109 fragment.

Most isolates displayed significant changes in phenotype relative to the parent plasmid pEx110 in the preliminary screening tests described above: More than 90% of the transformants (100% in experiments 2 and 3, see Table 1) gave rise to white colonies on X-gal plates, indicating that they carried a mutation in the *lac* operator region of *lac*109. In addition, the level of tetracycline resistance differed in the majority of clones from that of the parental plasmid pEx110 (Table 1). Both observations indicate a high level of mutagenesis.

Sequence Analysis of the Mutant Plasmids. From the 235 plasmids (see Table 1), 22 were chosen for DNA sequence analysis. Starting from the unique *Eco*RI site, the DNA sequence of the 109-bp insert was determined by the chemical degradation method (8). The sequences obtained are presented in Fig. 4. Eight are derived from mutagenesis of the noncoding strand of the promoter element (M3, M6–M8, M10, M11, M15, and M16; experiment 1, Table 1). As expected, they contain C → T transitions in this DNA strand. G → A transitions were detected in the remaining 14 mutants (M21–M38), which originated from experiments 2 and 3, in which the complementary strand had been mutagen treated. In summary, all 22 isolates analyzed show a high number of base changes corresponding to the level of C → U conversion observed after chemical treatment. Thus, 12 out of 23 potential target sites are affected on the average in the isolates from experiment 3 (M28–M38). In this experiment, the mutagen-treated strand was treated for 8 hr with sodium bisulfite, which corresponds to ≈50% C → U conversion. This indicates that the U·A pairs present in the double-stranded plasmid DNA (after step III) were quantitatively converted to T·A pairs *in vivo* after transformation into *E. coli*. This is several orders of magnitude greater than the yields of C → T changes obtained in experiments in which the complementary strand was not synthesized *in vitro* but rather provided by annealing of mutated *lac*109 single strands (22). These results emphasize the importance of step III in our protocol, which avoids the reversion of U·G to C·G pairs in the heteroduplex DNA by the uracil excision–repair system (15).

Sodium Bisulfite as a Probe for Secondary Structure. The mutagen does not act at random. As with other base-specific reagents (28) regions that have a potential for intramolecular base pairing are apparently protected from attack, whereas portions forming single-stranded loops are preferentially exposed. This is particularly evident from the distribution of base changes in the *lac* operator region in plasmids M21–M38, which were randomly selected for sequence analysis from a total of 169 coding-strand mutations. In a DNA single-strand, most of the *lac* operator sequence can base pair to form a stem loop structure (Fig. 4). Accordingly, the targets for the mutagen in the stem portion (positions −6, −4, −2, −1, and +5) are affected much more rarely (3 out of 70 possible events; Fig. 4) than in the average of the whole molecule in which one out of two possible sites has been mutated. In contrast, the probability for mutation

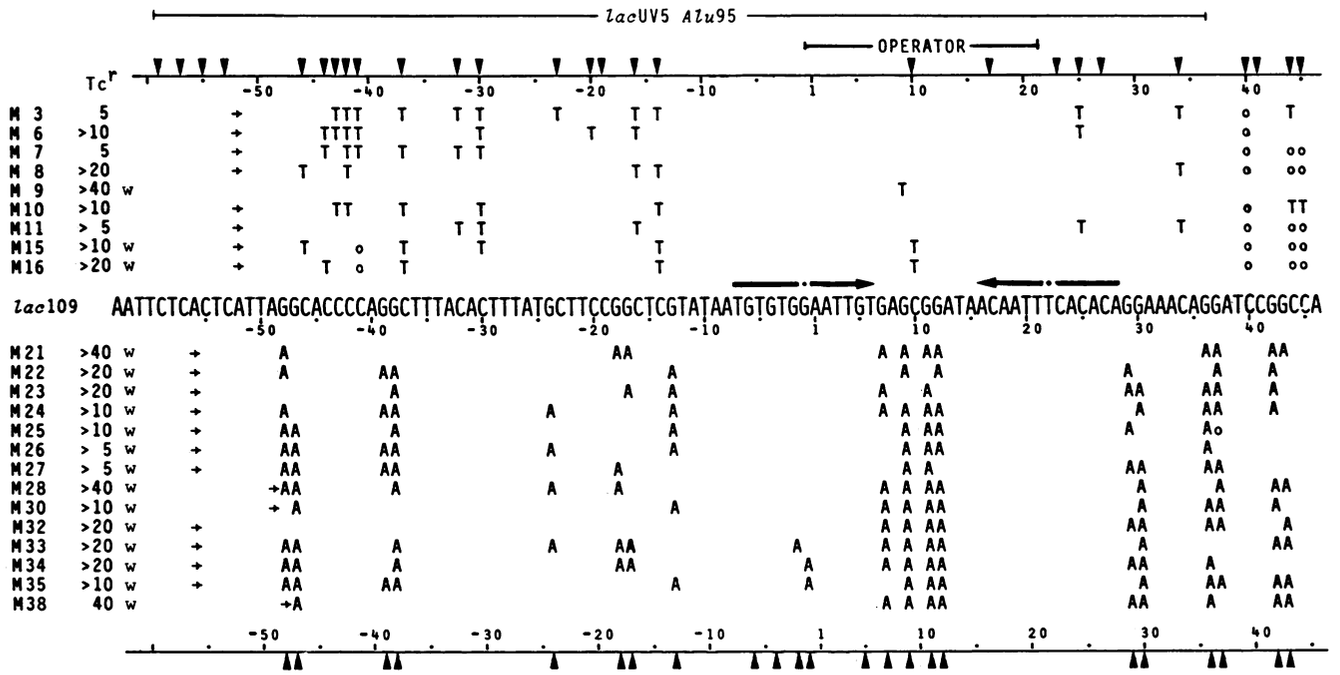

FIG. 4. Nucleotide sequence of *lac*109 and 23 mutant derivatives. The noncoding strand of *lac*109 is shown. The fragment starts at the left with an *Eco*RI cleavage site and ends with a *Hind*III cleavage site. Heavy arrows indicate the symmetry region containing the *lac* operator (26). Limits of the functional part of the operator and of the cloned *lac*UV5 DNA are delineated by bars. ▮, Positions of potential target sites for mutagenesis in the noncoding (upper) and coding (lower) strands; only bases changed are shown for the mutants. →, First nucleotide readable from the sequence analysis gels; o, ambiguous nucleotides. M9 results from random NH_2OH mutagenesis of pEx110. It carries the same G·C → T·A transversion as the *lac* operator mutant O^c5 (27). Variations of tet phenotype (Tc^r) and changes to op^- phenotype (w) are listed.

events in the loop portion (positions +7, +9, +11, and +12) is significantly enhanced (47 out of 56 potential events).

***lac* Operator Mutations.** Almost all of the clones isolated showed changes in *lac* operator phenotype, as displayed by the formation of white colonies on X-gal indicator plates (Table 1). As shown in Fig. 4, the nucleotide sequences of the 17 variants with this phenotype all have base changes in the functional part of the *lac* operator region (position +1 to position +21; refs. 24 and 26).

Promoter Function of the *lac*109 Mutants. The relative strengths of different promoters can be determined by using as a signal the expression of a neighboring promoterless indicator gene such as tetracycline resistance (20) or galactokinase. During cloning of the *lac*109 elements and the initial screening for successful mutagenesis, changes in the expression of the *tet* gene were used as a phenotypic marker (Fig. 4). However, these variations only in part reflect promoter properties of the *lac*UV5 variants as such (22, 29). Therefore, several of the *lac*109 elements were transferred into another promoter probe plasmid, pKO1 (to yield derivatives of pKO110; Fig. 2), which allows measurement of their transcriptional activity via expression of the *E. coli* galactokinase gene.

The results obtained in this system are presented in Fig. 5 together with the nucleotide sequences of nine multiple-point mutations and one single mutation, M9, that had shown a drastic change in *tet* phenotype (Fig. 4). In this collection, 24 out of

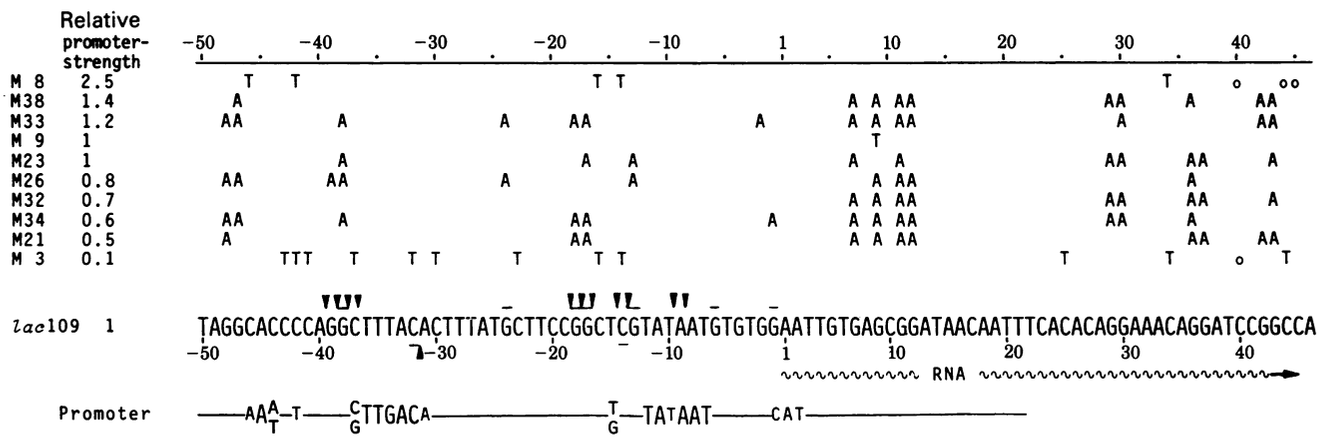

FIG. 5. Promoter mutations and promoter strength. Ten *lac*109 variants are ordered according to relative strength of the promoter signals as determined in the pKO1 system (Fig. 2 and *Materials and Methods*). The nucleotide sequence of the *lac*UV5 promoter is presented in full together with a generalized consensus promoter sequence (30). Contacts of the RNA polymerase to guanosines of the *lac*UV5 promoter sequence are indicated by overbars and underlines for the noncoding and coding strands, respectively; ▼, similar contacts to phosphate residues.

32 G·C pairs in the promoter region (position −50 to +20), which are potential targets for the mutagen used, are changed to A·T pairs. However, when the promoter activity of these variants was compared with *lac*UV5, a major change was observed in only one variant, M3, which is a mutation to 1/10 activity. This change in phenotype is most likely related to the C·G → T·A transition in position −32, which is part of the homology sequence around position −35 in most promoters (30, 31). A less prominent effect was observed with the up mutation M8, which shows two base changes in the −16 region, which has been identified as being in contact with the enzyme (30) but shows little sequence homology between promoters from various sources (31). Even more subtle, but still significant, changes in *gal*K expression were observed with other variants (Fig. 5), despite the fact that these carry a variable number of point mutations in their promoter sequences. No simple relationship could be established between nucleotide sequence and promoter activity among these mutants. These results show that there is a high degree of flexibility in the nucleotide sequence of a promoter element outside of two homology regions around positions −10 and −35. However, many base changes in the nonhomologous parts seem also to exert minor influences on promoter function.

Our results may also contribute to understanding the topography of the RNA polymerase/promoter complex, which has been investigated in detail with the *lac*UV5 promoter (for review, see ref. 30). Assuming that mutants that show similar promoter activity to *lac*UV5 (e.g., M23, M26, and M32) also show similar contact patterns, one can infer that these contacts do not necessarily involve a particular base at some of these positions. Thus, for instance, the guanine residues at positions −38 and −24 can be changed to adenine residues without affecting apparent promoter function. Similarly, the contacts to the phosphate residues around position −38 appear to be unaffected by base alterations in this part of the promoter.

Concluding Remarks. In principle, the method described can be used to change any fraction of G·C pairs into A·T pairs in an exactly specified target sequence of any size. The scope of the method may be improved: Self-complementary nucleotide sequences could be treated with mutagen in the presence of denaturing agents; certain segments in a target sequence could be protected from the action of the mutagen by added base-complementary nucleotide sequences; other base changes could be intoduced with other mutagens, provided that the mutagen-treated DNA single strand can serve as a template for repair DNA synthesis *in vitro*.

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