A lac promoter with a changed distance between -10 and -35 regions

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ABSTRACT

    A lac promoter mutant was constructed by filling in the protruding
ends of the HpaII site located within the lac promoter. The mutation,
named M42, is a two base pair insertion that changes the distance between
the -10 and -35 regions from 18 to 20 residues. The activity of the
mutant promoter measured in vivo is 15% of the wild-type promoter. The
M42 promoter is sensitive to the catabolite repression in the manner
similar to that of the wild type. Sequences of several deletions within
the lac promoter are also given.

INTRODUCTION

    Most of the sequenced promoter mutations in bacteria map upstream from

the mRNA initiation site, in the -10 and -35 regions. These two regions are

presumed to form contacts with RNA polymerase that lead to the formation of

the closed and open complexes. Relatively little attention has been paid to

the significance, structure and function of DNA between the -10 and -35

regions. Two questions can be raised: is the sequence of that spacer DNA

important for promoter functions and, in particular, how does the distance

between these regions affect promoter activity. The comparison of over 50

promoter sequences indicates a variation of the distance by up to 3 base

pairs, the consensus interval being 17 base pairs (1, 2). The length of the

spacer DNA is defined here as the number of residues between the sequences

whose consensus approximations are TTGACA (-35 region) and TATAAT (-10

region).

    In this paper we present the data on the construction of a two base

pair insertion mutation at the HpaII site of the lactose promoter of

Escherichia coli. The properties of the mutant promoter are also discussed.

    An analogous study in which base pairs have been deleted from this

interval in the lacP^S promoter is presented in (3).

MATERIALS AND METHODS

Strains and plasmids

For transformations and growth of the plasmids, strain CSH26 (ara, Δ (lac pro), thi) was used.

For cloning of promoter fragments, pRZ5015 plasmid was used. The plasmid, whose construction and properties will be described in detail in a separate communication (4), is a pBR322 derivative in which the SalI-PvuII fragment carrying the tet gene was substituted by the W205 trp-lac fusion (5). The trp-lac junction point lays within the lac operator (5), hence the W205 fusion does not have the lac promoter. The EcoRI-SmaI-BamHI linker (6) substitutes in pRZ5015 for the original pBR322 EcoRI-BamHI fragment.

The trp-lac fusion carries the intact β-galactosidase gene. The β-galactosidase levels thus can indicate the activity of the promoter fragment cloned into the EcoRI, SmaI, BamHI or even SalI or HindIII sites.

To assay the activity of promoters the pRZ5605 plasmid was also used. This plasmid was constructed by deleting approximately 2 kb DNA fragment from pRZ5015, thus shortening the distance between the BamHI site and the beginning of the lacZ gene to about 200 bp. For unknown reasons, the latter plasmid gives three-fold higher expression of β-galactosidase from the Hae203 lac promoter than pRZ5015, while both plasmids have the same β-galactosidase background level in absence of a promoter within the EcoRI-BamHI region. The structure of the two plasmids is presented in Fig. 1.

The source of the Hae203 lac promoter fragment with EcoRI and BamHI ends was pRZ5012. pRZ5012 is the derivative of pRZ5015 which contains the Hae203 lacP$^+$ fragment between the EcoRI and BamHI sites (4). The EcoRI and BamHI ends of this fragment were generated by the procedure previously described (7, 8).

Media, reagents and enzymes

The media used for growing cells are the same as those described in (9).

Unlabeled deoxynucleoside and dideoxynucleoside triphosphates were obtained from PL Biochemicals, Inc. α-^{32}P dATP was purchased from Amersham.

T4 ligase and restriction enzymes were purchased from Bethesda Research Labs, Inc. E. coli DNA polymerase I (large fragment) was obtained from Boeringer-Manheim. M. luteus polymerase was purchased from PL Biochemicals, Inc.

The β-galactosidase assay technique and the definition of units of activity were described by Miller (10).
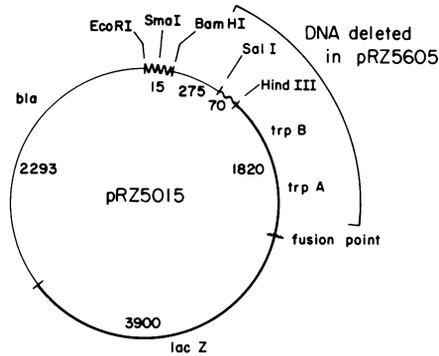
Fig. 1. The map of the pRZ5015 plasmid. The sites that can be used for cloning of the promoter fragments are shown. The numbers give the approximate sizes of the fragments.

DNA Preparation and sequencing

Procedures for ligation of DNA, transformation and plasmid preparation have been previously described (9). DNA fragments were isolated by elution from polyacrylamide gels (11). Protruding ends of DNA fragments were filled in with M. luteus DNA polymerase (8).

Sequencing was done by the dideoxy method (12). The templates were single strands of the Hae203 lac promoter fragment. To isolate them, pRZ5012 was cut with EcoRI and BamHI, the mixture was denatured by heating at 90°C for 2 min. and run on a 5% polyacrylamide gel. The single strands of the promoter fragment separated readily and were subsequently eluted from the gel (11). The sense (or transcribed) strand of the Hae203 fragment runs slower on the gel than the other strand. The primers were the Hha59 (-140 to -82) and Alu27 (+37 to +63) subfragments of Hae203. The two primers were used for sequencing in different directions.

RESULTS

To test how changes of the distance between the -10 and -35 regions influence the lac promoter function we constructed a two base pair insertion at the HpaII site of the lac promoter by filling in the protruding HpaII

ends. To overcome the difficulty of the presence of over 30 HpaII sites on
the pRZ5012 plasmid we designed a procedure outlined in Fig. 2.

First, to protect the EcoRI and BamHI protruding ends of the fragment,
the Hae203 promoter fragments were ligated to form multimers. Then, the
multimers were cut with HpaII, giving two kinds of fragments: short, 162 bp
pieces containing the BamHI site in the middle of the fragment, and the
longer 244 bp fragments with the EcoRI site. Subsequently, the HpaII pro-
truding ends were filled in and the fragments were ligated to form multimers
again. At this point the mixture was treated with HpaII restriction enzyme
to decrease the probability of cloning of the intact, wild-type promoter
fragment at a later step. The following EcoRI and BamHI digestion should
have led to the formation of three classes of fragments as shown in Fig. 2.
The fragments of only one class (containing fragments with desired mutation)
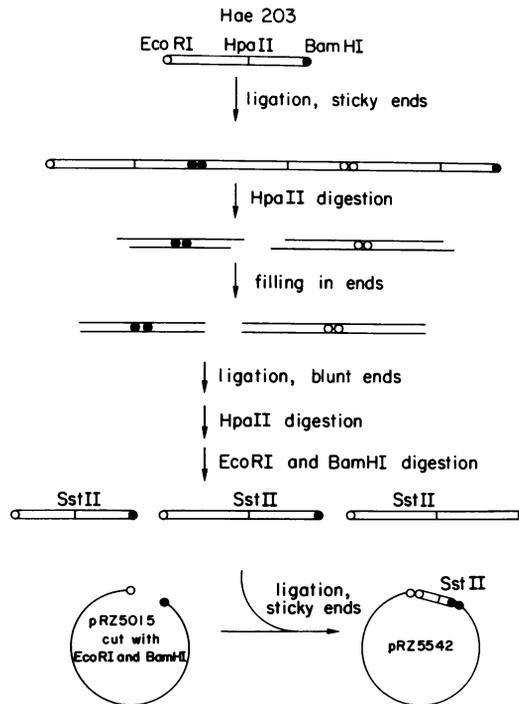would be expected to be cloned into pRZ5015 by sticky end ligation.

Fig. 2. A schematic presentation of the procedure used to generate the
M42 mutation. Open and filled-in circles indicate the EcoRI or BamHI ends
of the fragment, respectively.

The screening procedure was designed to pick up not only the constructed mutant but also deletions within the lac promoter fragment. Deletions could be formed due to traces of nuclease in the commercial enzyme preparations used. The procedure included testing on the indicator plates for the ability of the cells to synthesize β-galactosidase, estimation of the plasmid size on agarose gels and analysis of the HpaII digestion pattern of the plasmids. The replacement of the 581 bp HpaII band by the 674 bp band indicated the presence of the desired mutation (Fig. 3), displacement of the 581 bp band upwards (to less than 674 bp band position) or downwards indicated a deletion. Sample results of the screening are presented in Fig. 4. Among 80 clones screened, 24 had changes within the promoter fragment and 1 clone carried the desired mutation.

It was possible to test for the presence of the two base pair insertion by restriction digest since the filling in of the HpaII site ($\binom{\text{C CGG}}{\text{GGC C}}$) produces an SstII site ($\binom{\text{CCGC GG}}{\text{GG CGCC}}$). The site would be unique on the plasmid. The plasmid with these properties is pRZ5542. The mutation was named M42.

The M42 mutation and four deletion mutations were sequenced. The sequence of the M42 promoter was confirmed (Fig. 5). All deletions were found to originate from the HpaII site of the lac promoter. The deleted regions are -140 to -20 in M10, -46 to -20 in M41, -18 to +41 in M44 and -107 to -20 in M78 (Fig. 6).

To test how the M42 mutation affects the promoter activity, the Hae205 M42 promoter fragment was cloned into pRZ5605, thus the lacZ gene on the plasmid was under control of the M42 promoter. The β-galactosidase levels
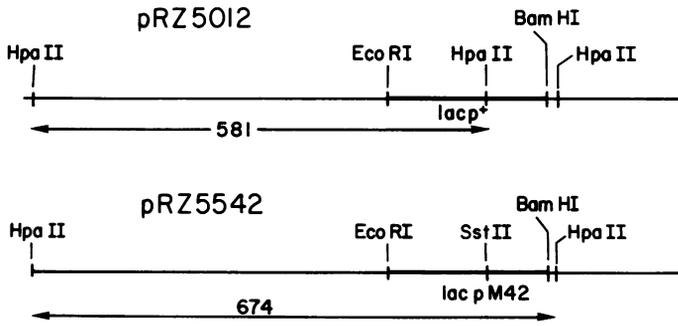
Fig. 3. Rationale for the screening prodedure. The closest HpaII sites on the both sides of the EcoRI-BamHI fragment are shown. The generation of the M42 mutation changes the length of the HpaII fragment from 581 bp to 674 bp.
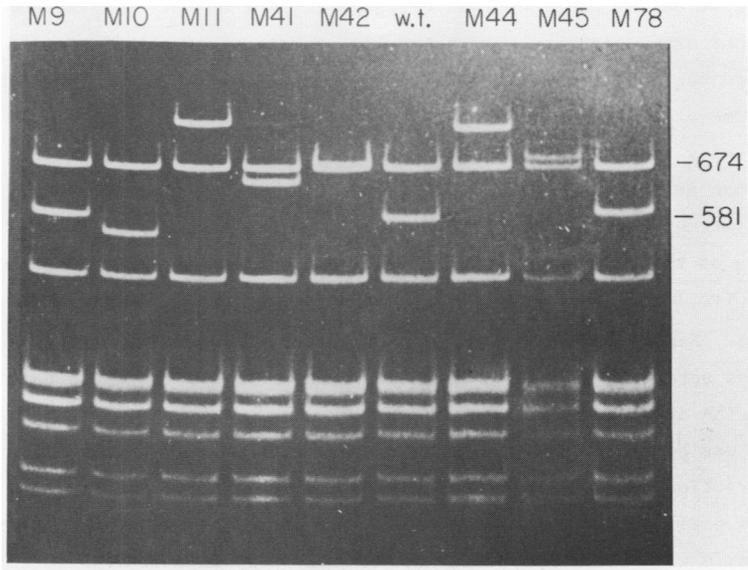
Fig. 4. Screening for the M42 mutation and deletions of the lac promoter
fragment. A polyacrylamide gel is shown with the HpaII digests of the
plasmid DNA from different clones obtained in the mutagenesis procedure
(see text and Fig. 2). The presence of the M42 mutation is indicated by
the disappearance of the 581 bp band and by the double band at position
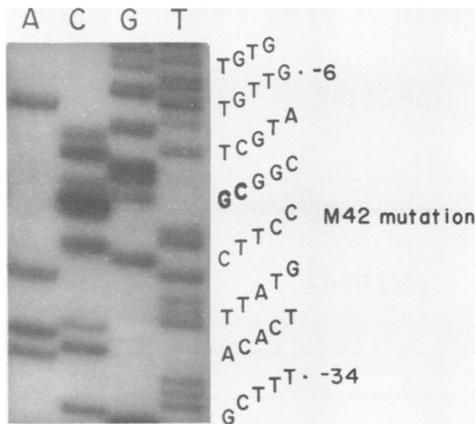corresponding to 674 bp DNA fragment. w.t. - digest of pRZ5012.

Fig. 5. Sequencing gel that demonstrates the generation of the M42 muta-
tion. The inserted at the HpaII site base pairs are indicated by bold
letters. The numbering of the residues is with respect to the mRNA start
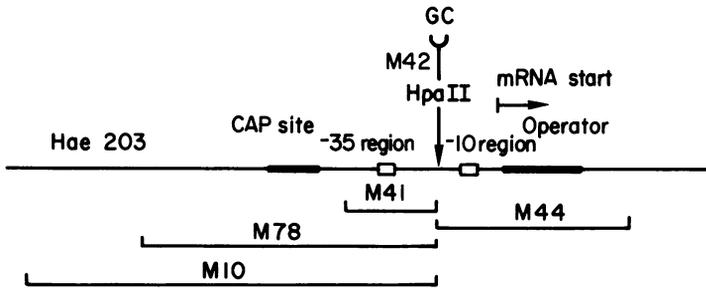site (+1).

Fig. 6. The position of the M42 mutation and the range of deletions on the lac promoter Hae203 fragment. The exact numbers of the residues deleted are given in the text.

in the strain harboring that plasmid were an indication of the promoter activity of the fragment. The results presented in Table 1 show that activity of the M42 promoter is roughly 15% of the wild-type. The M42 promoter responds similarly to the wild-type when the catabolite repression is relieved (medium supplemented with glycerol as compared to the medium with glucose). The expression is stimulated 3-4-fold. Similar stimulation was

Table 1

Comparison of activities of the M42 and wild-type lac promoters

Plasmid	Promoter	β-galactosidase activity, units [a]		Stimulation [b]
		Glucose	Glycerol	
pRZ5606 [c]	p^+	6650	18000	2.9
pRZ5607 [c]	M42	1600	4360	4.6
pRZ5605	no prom.	940	1280	1.4
CA8000 (HfrH str^s thi)		1350	2920	2.5

[a] Cells were grown on the M9 medium supplemented with casamino acids and the carbon source indicated. Multiple assays were performed. The probable error of the mean activities given in the Table is less than 5%.

[b] Stimulation is defined as the ratio of the net β-galactosidase activitiy (i.e. units measured - pRZ5605 units) on glycerol to that on glucose.

[c] pRZ5606 and pRZ5607 were constructed by cloning the Hae203 or Hae205 promoter fragments, respectively, into pRZ5605.

obtained when the catabolite repression was minimized by the addition of
cyclic AMP to the cells grown on the minimal medium supplemented with
glucose-6-phosphate (data not shown).

The promoter activity of different deletion mutants was tested in the
pRZ5012 cloning vehicle. β-galactosidase levels of the promoter deletions
were less than 10% of the levels measured in pRZ5012, i.e. the deletions did
not exhibit any detectable promoter activity.

DISCUSSION

It was shown that the M42 two base pair insertion in the spacer region
of the lac promoter strongly reduces the promoter activity. This demon-
strates that the length of the spacer is an important factor in the function-
ing of the promoter. There are several possible ways that this change in
the spacer region might have this effect:
(a) by altering the optimal spatial arrangements between the sequences
     required for formation of the closed and/or open complexes;
(b) by affecting the CAP action through a change of the distance between
     the CAP site and other promoter regions (such as -10 region);
(c) by changing the interaction of RNA polymerase with the spacer region
     itself.

Since in the M42 mutant the effect of the catabolite repression on the
β-galactosidase synthesis seems to be the same as in the wild-type (Table 1),
the action of the CAP protein does not seem to be affected by the two base
pair insertion. Alternative (b) can be therefore excluded.

One and two base pair deletions at the HpaII site of the lacP^S promoter
were recently constructed (3). It was shown that one base pair deletions
within the spacer region (deleted G at position -18 or C at position -19 of
the lac promoter) increase the rate of the open complex formation of the P^S
promoter in vitro, while if these two residues are both deleted the rate
decreases. Stefano and Gralla (3) concluded from these observations that it
was the length of the spacer DNA rather than the sequence around residue -19
that is of prime importance in determining lacP^S expression in these constructs.
Our data do not allow us to distinguish between hypotheses (a) and (c);
however, based upon the properties of the lacP^S constructs described above,
it is most plausible that the phenotype of the M42 is also due to the change
in the -10 to -35 spacing (hypothesis (a)).

Another example of a small deletion within the spacer region is a down
promoter mutation in the tyr tRNA promoter (Tp11-4) that reduces the spacer

length by one base pair (13).

The fact that the M41 deletion mutant, as well as M10 and M78, do not have any detectable promoter activity demonstrates that the -35 region is indeed necessary for the promoter activity (9). These deletions can be also useful for the transfer of the lac promoter mutations from the chromosome on to the plasmid by recombination in vivo. If the mutant promoter being transferred has some promoter activity, the transfer of the mutation can be screened for on lac indicator plates. The lack of promoter activity for mutation M44, on the other hand, confirms that the -10 region is necessary for promoter activity.

The construction of the M42 mutation substituted the HpaII site of the lac promoter by an SstII site which is unique on the pRZ5542 or pRZ5607 plasmids. This provides an opportunity for the site-specific mutagenesis of the lac promoter similar to that used by Shortle and Nathans (14). If the mutation constructed does not destroy the SstII site, it is possible to come back to the wild-type background by cutting out the protruding SstII ends and ligation of the linearized plasmid.

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