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Oligonucleotide mutagenesis of the *lacPUV5* promoter

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ABSTRACT

Synthetic oligonucleotides were used to introduce mutations into the lacPUV5 promoter. Four mutations were obtained at positions -13, -14, and -15, with respect to the transcriptional start site. The effects of these mutations were measured in vivo and the results are discussed with respect to the consensus sequence and other promoter mutations located in this region.

INTRODUCTION

The efficiency at which RNA polymerase recognizes and interacts with a promoter can play a major role in determining the level at which a gene is expressed. In addition to transcriptional activators and repressors, the sequence of the promoter is a crucial element in modulating this process. Comparative analysis of promoter sequences and isolation of promoter mutations has indicated two regions which are extremely important for RNA polymerase interactions, centered around -35 and -10 with respect to the transcriptional start site (for review, see Hawley & McClure, 1983).

We have chosen to concentrate on a region that remains ambiguous in terms of its specificity of interaction with RNA polymerase. This region is located upstream relative to the -10 conserved sequence (the Pribnow box) and has been strongly implicated by chemical probe studies to be in close contact with RNA polymerase (reviewed by Rosenberg & Court, 1979; Siebenlist et al., 1980). In particular, when G residues in this region are methylated in the major groove or phosphates on the backbone are ethylated, RNA polymerase binds at a reduced efficiency, and prebound RNA polymerase protects (or enhances) these same sites from chemical modification. There have been a few mutations isolated in this region, but at a much lower frequency than expected if important contacts are being made here during transcription initiation. Comparison of promoter sequences has also shown that weak homologies exist at only a few of these sites (see Hawley and McClure, 1983, for a summary of these studies). In this paper we describe the incorporation of mutations in

the lacPUV5 promoter with synthetic oligonucleotides and their effect on lacZ expression in vivo.

MATERIALS AND METHODS

Reagents and Bacterial Strains

Large Fragment DNA Polymerase I was obtained from Bethesda Research Laboratories and T4 DNA ligase was a gift of R. Simoni. Universal Primer for DNA sequence analysis was obtained from New England Biolabs.

JM101 (F'traD36 lacI^{q}Zm15 proAB/Δ(lac-proAB) supE thi) was used as a host for the M13 vector (mRZ361). RZ201 (F$^-$ ara Δ(lac-pro) thi rpsL) was the host for the lambda prophage.

Mutagenesis

To introduce mutations in the region from -13 to -16, "mixed" oligonucleotides were used as primers for DNA synthesis in vitro. Four oligonucleotides were synthesized which are complementary to the anti-sense strand of the lacPUV5 promoter and span bases -7 to -21. Each oligonucleotide contained three non-complementary bases at one position (-13, -14, -15 or -16) and could theoretically be used to introduce three mutations at each of these sites. The oligonucleotides were synthesized on silica gel using appropriately protected deoxynucleoside 3'-phosphoramidites. The general synthesis and isolation procedures have been described previously (Caruthers, 1982; deHaseth et al., 1983) and the oligonucleotides were then purified by polyacrylamide gel electrophoresis under denaturing conditions (20% acrylamide, 7 M urea).

Each oligonucleotide was used as a primer for in vitro DNA synthesis as described by Zoller & Smith (1982), with the following modifications. mRZ361 was used as the template DNA (a M13mp8 derivative containing the lacPUV5 promoter; Munson et al., 1984). Additional DNA polymerase and ligase were added after an overnight incubation at 15°C, and the incubation continued for 4 hrs. Before loading the reaction mixture on the alkaline sucrose gradient, the sample was phenol and ether extracted. Phage which had incorporated the mutation were detected by the dot blot hybridization procedure (Zoller & Smith, 1982). DNA sequence analysis was done by the dideoxy chain termination method (Sanger et al., 1977).

The oligonucleotide containing the non-complementary bases at -13 yielded one mutation in the 497 phage that were screened. At position -14, the same base change was obtained four times out of 511 phage. Two different mutations were introduced at -15 after screening 155 phage and at -16 a mutation was

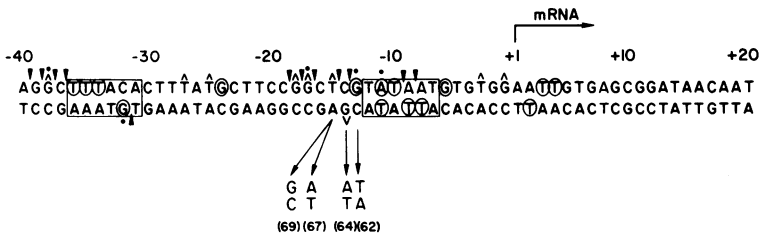

FIGURE 1. The sequence of the lacPUV5 promoter with the base pair changes of mutations 62, 64, 67 and 69 indicated. The conserved regions around -35 and -10 are boxed, and the start site of transcription is indicated. The results of the chemical probe studies are taken from Siebenlist et al. (1980). (▼) designate ethylated phosphates which interfere with binding of the polymerase; (o, ^) indicate purines that are protected from or enhanced by dimethyl sulfate attack, respectively; (o, ^) bromouracil substituted thymines that the polymerase protects from or opens to photochemical cleavage, respectively; (•) purines which when methylated interfere with binding of the polymerase.

incorporated once (out of 638 phage) but it also contained a deletion starting at -19. All but one of the mutations involved the hybridization of an oligonucleotide that contained a pymidine mismatched to a pyrimidine in the template strand.

β-Galactosidase Assays

The mutations obtained were recombined onto a λplac5 derivative (see Munson et al., 1984) which was phenotypically Lac$^-$ due to a promoter mutation at position -11 (T743; Reznikoff et al., 1983). Recombination between the λplac5 derivative and the M13 vector (mRZ361) changed the phenotype and introduced a Hind III site at +77. The frequency of recombination was consistent with the recombination endpoint being upstream of the promoter mutation being introduced and not in the 1-3 bp separating the two mutations. Single lysogens of the recombinant phage were obtained in RZ201 and β-galactosidase activity was measured as described by Miller (1972).

RESULTS AND DISCUSSION

Oligonucleotide mutagenesis was used to investigate the weakly conserved region located upstream relative to the Pribnow box in the lacPUV5 promoter. The four mutations obtained are shown in Figure 1 along with the results of several chemical probe studies. These mutations were recombined onto λplac5 and their effect on lacZ expression was measured in vivo.

Position -15

Comparison of 112 promoter sequences has shown that there is a pref-

TABLE I
β-Galactosidase Activity

Mutation	% UV5[a]	Relative Error
UV5	100	-
UV5-62	105	6.7
UV5-64	107	5.9
UV5-67	114	4.5
UV5-69	122	7.9

[a]The values shown are the average values of six independent sets of assays. Each assay was done in duplicate and normalized with respect to lacPUV5. The average value for lacPUV5 was 533 units.

erence of TA base pairs at position -15 (Hawley & McClure, 1983; summarized in Table II). There are two promoter mutations at this site (in gal and tyrT) which change TA base pairs to CG base pairs, resulting in 10-30% decrease in promoter activity (Table II). In the lacPUV5 promoter we have obtained two mutations at -15, a TA to AT transversion (mutation 67) which increased β-galactosidase activity 15% and a TA to GC transversion (mutation 69) which increased β-galactosidase activity 20% (Table I). We can tentatively rank the four possible base pairs at this position in terms of optimal promoter activity: GC > AT > TA > CG*. The discrepancy between the effects of these mutations on promoter activity and the frequency of the base pairs occurring at position -15 may be due to the fact that the effects are not large (i.e., the selective pressure is minimal).

Position -14

Comparison of promoter sequences has indicated that at position -14 there is a preference for GC base pairs, with the other three possibilities occurring at approximately equal frequencies (Table II). Four mutations have been isolated at this site in other promoters, one mutation increasing promoter activity (an AT to GC transition) and the other three mutations decreasing promoter activity (all three being GC to AT transitions). The E37 mutation in the λPrm promoter has been studied in vitro and was found to affect only the rate of isomerization and not the equilibrium binding constant (Shih & Gussin, 1983).

Table I shows that in the lacPUV5 promoter, a CG to AT transversion at -14 has no effect on transcription initiation (mutation 64). In this case

TABLE II

Sequence and Mutational Analysis of Positions -15, -14 and -13

Position	% bp occurrence[a] A T	G C	C G	T A	Mutation	Phenotype[b]	Promoter	Reference
-15	14	25	22	38	T → G / A → C	+	lac	This work
					T → A / A → T	+	lac	This work
					T → C / A → G	-	gal	Busby et al., 1982
					T → C / A → G	-	tyrT	Berman & Landy, 1979
-14	20	38	18	23	A → G / T → C	+	ant	Hawley & McClure, 1983
					C → A / G → T	0	lac	This work
					G → A / C → T	-	arg	Piette et al., 1982
					G → A / C → T	-	λPre	Rosenberg et al., 1978
					G → A / C → T	-	λPrm	Rosen et al., 1980
-13	17	31	24	28	G → A / C → T	+	trp	Miozzari & Yanofsky, 1978
					G → T / C → A	0	lac	This work

[a] Percentage of occurrence, based on 112 promoter sequences. Data taken from Hawley & McClure (1983).

[b] In vivo phenotype: +, increases promoter activity; 0, no change in activity; -, decreases promoter activity.

the mutation changes a less preferred base to another less preferred base. It is possible that both CG and AT base pairs at this site have an equally negative effect on transcription initiation and hence no change in lacZ expression is detected in vivo.

Position -13

At position -13 there is a random distribution of TA, GC and CG base pairs, with a slightly lower frequency of AT base pairs (Table II). We have made a GC to TA transversion (mutation 62) in the lacPUV5 promoter and have seen no effect on lacZ expression in vivo (Table I). There is, however, one previously isolated mutation that is located at this position. It is a GC to AT transition in the trp promoter of Shigella dysenteria, which resulted in a 10 fold increase in promoter activity in Escherichia coli (Miozzari & Yanofsky, 1978). Thus, the trp promoter mutation remains as the only evidence that the contact that RNA polymerase makes at this site is base specific.

CONCLUSIONS

The mutations described here, in conjunction with previous studies, suggest that at position -13 there may be a preference for AT base pairs (based on the trp promoter mutation), and that GC and TA base pairs are interchangeable in terms of their ability to interact with RNA polymerase. At position -14, a GC base pair appears to be optimal, with AT and CG base pairs having an equally negative effect. At position -15 we have been able to create a hierarchy of base pairs according to their interactions with RNA polymerase (GC > AT > TA > CG).

This analysis rests upon two assumptions. One assumption is that RNA polymerase contacts all promoters in an analogous manner. This has been shown to be true in a general way, but it is not clear whether the sequences around each position may influence the specificity of the contact at that site. The overall promoter arrangement (e.g., the distance between the -35 and -10 regions) may also influence the importance of a particular base at a certain site.

The second assumption is that a mutation which affects only one step in the transcription initiation process will alter the overall rate of initiation in vivo. In E. coli initiation of transcription is thought to involve at least two steps: an initial binding step, followed by an isomerization step in which the double helix is unwound (Chamberlin, 1976). A mutation which subtly alters a step following the rate limiting step would presumably not affect the overall rate of transcription initiation. The weak homology between promoters in this region and the low frequency at which mutations have been obtained at these sites, might be explained by contacts that are only used in a latter step of the initiation process.

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* For each base pair mentioned, the first base is that found on the non-coding strand of DNA.

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