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Spacer mutations in the *lac* p^s promoter

(transcription/RNA polymerase/recombinant promoter)

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ABSTRACT **Mutations have been constructed that delete either one or two base pairs near position −19 in the *lac* p^s promoter. Deletion of either of two adjacent base pairs increases the rate of open complex formation by nearly an order of magnitude. Two promoters that have different single-base deletions are indistinguishable by either their rates of open complex formation or stability of the open complexes once formed. However, simultaneous deletion of both base pairs produces a promoter that forms complexes at a rate similar to that of the unmodified DNA sequence. The maximal rate of open complex formation is achieved at a spacer length of 17 base pairs, the most frequently occurring spacer length among promoters. These results suggest that the spacing between the two strongly conserved regions of sequence homology is an important determinant of the rate of open complex formation. A model is suggested that proposes that three important promoter elements, the −10 region, the −35 region, and the spacer region, act simultaneously to facilitate open complex formation by RNA polymerase.**

The promoter is a DNA sequence that directs RNA polymerase to bind and initiate transcription specificially. Comparison of prokaryotic promoter sequences has shown two regions of sequence homology located ≈10 and 35 base pairs prior to the start point of transcription (1, 2). The precise roles of each of these regions in promoter function are unknown. The importance of these homologies is supported by the concentration of promoter mutations in these sequences. In general, sequence changes that decrease homology to the consensus tend to be down promoter mutations.

The region between the −10 and −35 sequences has not been shown to be of importance. These sequences are poorly conserved and few mutations exist in this region. Nevertheless, the number of intervening nucleotides is highly conserved (1). This suggests that proper spacing between the −10 and −35 sequences may be functionally important and prompts us to identify this as a third region of the promoter, the "spacer" region.

To test the importance of this conserved spacer length, we have constructed mutations that change the spacer length in the *lac* p^s promoter. This promoter has a spacer region one nucleotide longer than the consensus length. Deletion *in vitro* yields promoter DNA that is of consensus length and shorter. The properties of these strains lend support to the hypothesis that the spacer region is a third important element in promoter function.

MATERIALS AND METHODS

RNA polymerase holoenzyme was prepared according to Gonzales *et al.* (3). Nuclease S1 was purchased from Miles and purified on sulfopropyl-Sephadex by a modification of Vogt (4). Bovine serum albumin (fraction V; Pentex) was purified by passage over a heparin agarose column. Other materials were as described (5).

For construction of deletion mutants (see Fig. 1), *Eco*RI restriction fragments containing the p^s promoter (5.5 μg; ref. 6) were ligated overnight at 15°C. The resultant polymer was cleaved with *Hpa* II. Half of the *Hpa*-ended product was treated with DNA polymerase I and dCTP to extend the 3′ termini one nucleotide. These partially filled products were precipitated and pooled with the unfilled fragments (final vol, 30 μl) in 50 mM NaOAc, pH 4.3/150 mM NaCl/5 mM $ZnSO_4$ containing S1 nuclease at 50 Vogt units/ml. After 1 hr at 25°C, the products were extracted with phenol and precipitated. Fragments of 120 and 80 pairs were removed by electrophoresis on 8% polyacrylamide gels. The larger fragments were extracted from the gels, pooled, and religated. The resultant polymer was recut with *Eco*RI, and the fragments were cloned into pBR322 as described (5).

To screen for properly constructed inserts, small-scale plasmid preparations (7) were tested for cleavage with *Eco*RI but resistance to *Hpa* II. Candidates were cut with *Hha* I, and the promoter-containing subfragments were subjected to partial guanosine cleavage (8) to reveal spacer mutants, later confirmed by sequence analysis. Other recombinant promoters were constructed as described (5). An L157 plasmid and an L241 *lac*-transducing phage were provided by W. S. Reznikoff.

Binding-rate assay mixtures, described previously (5), were modified to contain 3.25% glycerol, 1 μM [α-^{32}P]CTP at 80 Ci/mmol (1 Ci = 3.7×10^{10} becquerels), purified bovine serum albumin at 100 μg/ml, and 2–4 nM DNA fragment. Saturation values determined by titration with enzyme were combined with initial velocities to give semilogarithmic plots from which rate constants were calculated. Small reductions in saturation values were observed at high enzyme concentration. Apparent pseudo-first-order behavior was always observed. Concentrations are expressed in terms of active polymerase, determined by titration of the UV5 promoter (5).

The stability of the RNA polymerase–DNA complexes was measured by transferring aliquots (10 μl) from reaction mixtures containing preformed complexes that had been challenged with heparin at 100 μg/ml to 10-μl aliquots of buffer containing NTPs at twice the final stated concentration. These were incubated for 20 min at 37°C and the amount of RNA was determined (5).

RESULTS

Spacer Mutations. The construction of spacer mutations in the *lac* p^s promoter relied on deletion of bases within a *Hpa* II cleavage site between the −10 and −35 regions. The procedure was initiated by protecting the *Eco*RI termini of the parent promoter fragment by ligation at high concentration to form a polymer (Fig. 1). This polymer was then cleaved with *Hpa* II to produce a collection of fragments with *Hpa* II termini. Cleavage

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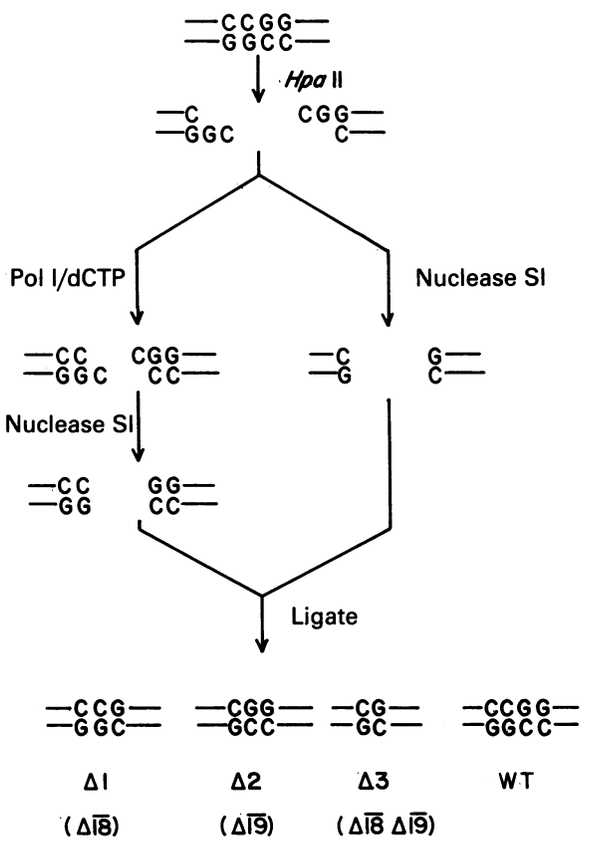

FIG. 1. Scheme for construction of deletion mutants. Numbers in parentheses refer to sequence positions, with respect to the major transcription start point, that were deleted. The parent strain was *lac* p^s. WT, wild type.

leaves the central two bases in the C-C-G-G recognition sequence in unpaired 5′ extensions. Half of the recessed termini were extended one nucleotide by using DNA polymerase I and dCTP. The remaining single-base extensions were removed by nuclease S1 treatment. The other half of the sample was rendered blunt ended with nuclease S1 without DNA polymerase I treatment. The entire mixture of blunt-ended molecules was again polymerized and then digested with *Eco*RI, leading to a collection of recombined promoters that included single- and double-base-pair deletions.

When these molecules were cloned into pBR322, screening showed that 9 of 12 clones contained appropriately sized inserts that failed to recut with *Hpa* II, indicating damage to the C-C-G-G recognition sequence. These were screened further by partial sequence analysis. Certain clones were chosen for complete sequence analysis, which revealed the mutants shown in Fig. 1. Strains Δ1 and Δ2 are single-base-pair deletions of adjacent base pairs, shortening the promoter to consensus length. Strain Δ3 deletes both of these base pairs, further shortening the spacer.

All promoters were found to be functional as tested by runoff transcription of isolated fragments. Each promoter led to the appearance of the *lac*-specific RNA doublet, as shown previously for p^s and other *lac* mutant promoters (5, 9). Recently, it has been shown that this doublet actually represents transcripts that are microheterogeneous at the 5′ terminus but arise from a common open promoter complex (A. Carpousis, personal communication).

Several tests showed that these deletions do not directly eliminate contact points for RNA polymerase. First, the stability of open complexes was measured for the Δ1, Δ2, and Δ3 promoters. In this experiment, open complexes were challenged with heparin, which inactivates free RNA polymerase, and the time-dependent decrease in capacity to synthesize runoff *lac* RNA was followed. Fig. 2 shows that deletion of either of the two central base pairs of the *Hpa* II site leads to open complexes of decreased stability. Note that the open complexes formed at Δ1 and Δ2 are identical by this criterion. This observation suggests that the change in open complex stability is not caused by deletion of specific contacts but rather by the change in spacer length; Δ1 and Δ2 have identical spacer lengths but have lost different base pairs (Fig. 1).

The possibility that the two base pairs are fortuitously identical contact points for RNA polymerase is inconsistent with the properties of Δ3. If this possibility were true, then Δ3, which has lost both base pairs, should be further destabilized. Instead, Δ3 shows increased stability over that of Δ1 and Δ2. These data suggest that the differences between these promoters arise from their "spacing class" rather than the deletion of specific contacts for the enzyme.

The rates of polymerase binding by these promoters also support their identification as spacer length mutations. We report elsewhere (10) that Δ1 and Δ2 appear to form open complexes with RNA polymerase at identical rates. These rates are in fact increased over that of the parent; loss of a base pair has accelerated the reaction. This increase in rate disappears in Δ3 when both base pairs are deleted. These effects are explicable if it is the spacing, rather than specific contacts, that is altered by deletion. Those rates were measured at a single polymerase concentration. Since two, presumably independent, factors govern the rate of complex formation (5, 11), experiments carried out at a single concentration may, in principle, be misleading. We have extended this analysis to the complete accessible range of polymerase concentration. As shown below, Δ1 and Δ2, p^s parent, and Δ3 continue to behave as distinct spacer classes by this stringent test.

Rate of Open Complex Formation at Promoters that Have Spacer Mutations. We have combined the productive-transcription rate assay of Stefano and Gralla (5) with McClure's (11) τ method of analysis to yield a composite rate assay (unpub-

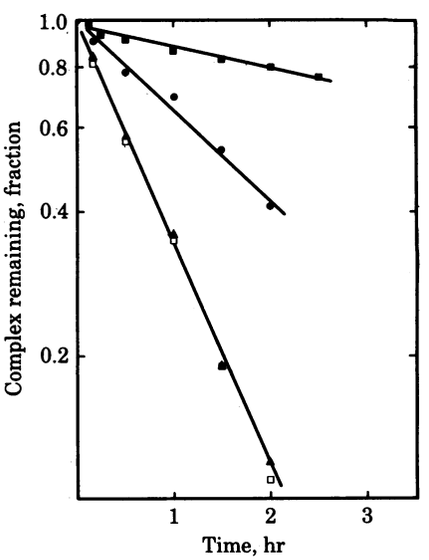

FIG. 2. Stability of open complexes formed at spacer mutant promoters. Open complexes were formed for 30 min and then challenged with heparin (100 μg/ml); aliquots were removed subsequently and assayed for ability of complexes to support transcription. ●, p^s; ▲, Δ1; □, Δ2; ■, Δ3.

lished). The principle of the method is as follows. The initial rate of production of heparin-resistant open promoter complexes is determined by a transcriptional assay as described (5), except that the reaction is always run with excess polymerase. Separately, the maximum amount of transcript that can be produced at polymerase excess is determined. A semilogarithmic plot of fractional activity against time then allows calculation of the reaction time (τ). This procedure is repeated at various concentrations of RNA polymerase and a plot of τ versus the reciprocal of the polymerase concentration is constructed. The resultant plot is formally analogous to a double-reciprocal plot of enzyme kinetics and the slope and intercept can yield rate constants for assumed substeps in the binding mechanism (11).

The application of this analysis to the promoters containing spacer mutations is shown in Fig. 3. Several qualitative points can be made without detailed interpretation of these curves. First, Δ1 and Δ2 are indistinguishable, consistent with their identical spacer lengths. A simple point-to-point comparison with the p^s parent shows that, at every concentration of RNA polymerase, the mutants are bound more rapidly (shorter reaction times) than the parent. A similar comparison shows that the binding rate of the Δ3 promoter is only very slightly different from that of the parent at every concentration. These data show that, over a range of conditions, the promoters that have the consensus spacer distance (Δ1 and Δ2) form open complexes much more rapidly than a promoter one base pair longer (p^s) or one base pair shorter (Δ3).

In principle, the data in Fig. 3 should allow separation of the mechanistic substeps that occur during open complex formation (11). However, we will show elsewhere that the behavior of several mutant *lac* promoters *in vitro* suggests that this method of analysis may not separate the roles of sequence when open complex formation proceeds through an unstable intermediate. Such is the case with these deletion mutants (Fig. 3) and several other mutant *lac* promoters (ref. 5; unpublished results). However, these data do demonstrate that the hierarchy of promoter strength *in vitro* is unaltered by changes in polymerase concentration.

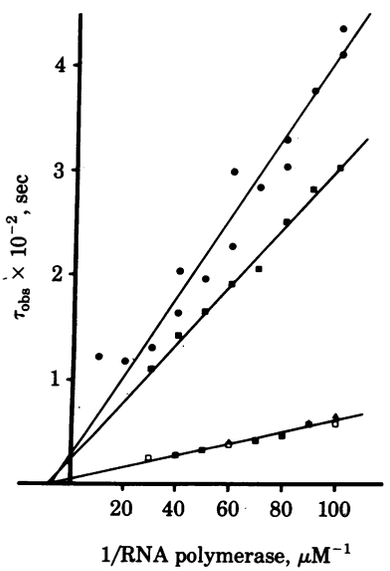

FIG. 3. Kinetic analysis of open complex formation. Each point represents a separate determination from the initial rates of heparin-resistant open complex formation as assayed by runoff transcription of the spacer mutant promoters. Symbols are as in Fig. 2. The apparent K_d for the A* intermediate (K_{A^*}) was $\approx 3 \times 10^{-7}$ M for all promoters. The overall K values for formation of the open complexes, derived from these and data in Fig. 2, were 2.1×10^9, 6.7×10^8, 6.7×10^9, and 1.2×10^{10} M^{-1} for p^s, Δ1, Δ2, and Δ3, respectively.

DISCUSSION

Our experiments have shown an important role *in vitro* for the length of a promoter element termed the spacer that separates the −10 and −35 sequences in the *lac* p^s promoter. Consensus sequences for the −10 (T-A-T-A-A-T) and −35 regions (T-T-G-A-C-A) and a consensus length of the spacer element have been derived by comparison of promoter sequences (1). The properties of the spacer mutations described above suggest that the maximal rate of open complex formation *in vitro* is attained when the spacer length matches this consensus of 17 base pairs, as in the Δ1 and Δ2 strains. Either lengthening (p^s) or shortening (Δ3) the spacer from the consensus length leads to nearly an order of magnitude decrease in rate. Although it is not known whether these results can be generalized, the properties of single-base-pair deletions in this region in tyrosine tRNA (12) and β-lactamase (13) promoters support this view.

It has been shown that mutations near the −10 and −35 regions of promoters lead to a decrease in the rate at which polymerase can form open complexes (5, 9, 14, 15). We will show elsewhere that, for several single-base changes in the *lac* promoter, sequence changes that substitute a less conserved element also lead to reductions in this rate. This apparent rule of decreasing rate with decreasing match to the consensus applies to sequences in the −10 and −35 regions and, as suggested by our data, the length of the spacer region between the two. It remains to be established whether this rule, derived from this limited data set, can be generalized to all promoters, although no exceptions are now known.

A Possible Model for Promoter Binding. How does deletion in the spacer region accelerate the rate of open complex formation? Each of these promoters forms initial closed or "A*" complexes that are unstable to dissociation (Fig 3; ref.5). This instability may be due to inability of the enzyme to make simultaneous contact with both the −10 and −35 regions of the promoter during the early steps that lead eventually to open complex formation. Thus the effect of changing the spacer length could be to facilitate such simultaneous contact with these separated regions.

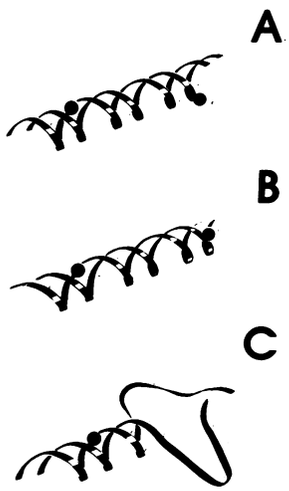

FIG. 4. Model for the state of DNA during promoter binding. (*A*) Either region of sequence homology (●, point of contact) can be bound transiently by RNA polymerase. (*B*) Fluctuations in the helical twist of the DNA bring contacts into proper alignment for simultaneous binding. (*C*) The resulting torsional stress is relieved by melting, leading to open complex formation (the downstream contact is not shown because the actual disposition of strands in space is not known).

This possibility is illustrated in Fig. 4. Initially, the enzyme fails to make full contact with both regions due to an unfavorable orientation of one region relative to the other in the initial complex. A more favorable orientation may be achieved by rotation of the contacts by untwisting the DNA. The probability of achieving this stressed state must depend on the length of the spacer which, through the DNA pitch, determines the relative orientation of the two contact regions. The consensus spacer length could simply be optimal for formation of this stressed state during the reaction. Open complex formation would result when the torsional stress is relieved by DNA melting. Thus, the separation of two homology regions in the promoter could be used by the enzyme to facilitate the melting process.

This model differs significantly from previous models for promoter binding. It does not require a stable closed complex. Nor does it involve an entry or recognition complex with any particular region of the promoter. Instead, the model allows similar roles for each of the promoter elements, except of course that it is the −10 region that is ultimately melted (1). One virtue of this hypothetical untwist-and-melt model is that it suggests a novel mechanism for transcriptional activation by either supercoiling or an activation protein such as catabolite activator protein. Either could act by inducing small changes in the helical periodicity of the DNA.

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