Site-directed Mutagenesis of the −10 Region of the lacUV5 Promoter

INTRODUCTION OF dA₄·dT₄ TRACT SUPPRESSES OPEN COMPLEX FORMATION*

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Peter T. Chan‡ and Jacob Lebowitz§

From the Department of Microbiology, University of Alabama, Birmingham, Alabama 35294

Homopolymeric dA₄·dT₄ sequences, where n = 4 or greater, have special properties leading to increased duplex stability and DNA bending. The lacUV5 promoter was used to examine the functional consequences of changing the −10 TATAAT consensus sequence to the sequence TAATAT. The transversion mutation at the underlined site was accomplished with site-directed mutagenesis using translation termination as the selection procedure. For free DNA, structural differences at the 5′ and 3′ junction regions of the dA₄·dT₄ tract can be readily detected by DNase I digestion. However, site binding by Escherichia coli RNA polymerase appeared unaltered by the TAATAT sequence since identical DNase I footprints were obtained for the lacUV5 and mutant promoters. Binding competition studies under different ionic strengths revealed a significant reduction in mutant promoter open complex formation relative to the lacUV5 promoter. Mutant promoter open complexes also dissociated faster and to a greater extent than the corresponding lacUV5 promoter open complexes when challenged with heparin or a combination of heparin and increased KCl concentrations. Consequently, mutant promoter open complexes appear less stable than lacUV5 promoter open complexes.

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‡ Supported in part by Training Grant CA09467.
§ To whom all correspondence should be addressed: Dept. of Microbiology, UAB Station, 520 CHSB, University of Alabama, Birmingham, AL 35294.

The abbreviations used are: bp, base pair(s); MCS, multiple cloning sites.

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nating A-T hexamer sequences (8, 9). Furthermore, the unwound region of such an open complex may be less stable and would enhance dissociation, causing an increase in the backward rate constant, \( k_{-b} \). To examine the effects of introducing a dA₄,dT₄ sequence at the -10 region of a promoter it would be best to generate a mutation that would result in the conversion of a typical -10 promoter sequence to a homopolymeric dA₄,dT₄ sequence without any other changes in the promoter sequence. To accomplish this objective we turned to the well characterized lacUV5 promoter of E. coli (12, 13). The -10 sequence of the lacUV5 promoter is the canonical sequence “TATAAT.” A transversion of the middle T-A base pair would result in the sequence “TAAAT,” putting a dA₄,dT₄ sequence into the -10 region of the lacUV5 promoter. Therefore, the effects of this special structure on open complex formation can be examined in the lacUV5 promoter system with only a single base pair difference.

MATERIALS AND METHODS²

RESULTS

Site-directed Mutagenesis: Selection by Translation Termination—The M13 amber phage system was chosen to carry out the site-directed mutagenesis experiment. However, the only amber phage vector available has mp8 multiple cloning sites (MCS) (M13mp8am16 (14)). This vector contains a wild type lac promoter and translation start signals 5’ to the MCS. Because of the orientation of the MCS in M13mp8am16, cloning of the EcoRI-PstI fragment (115 bases on the top strand and 107 bases on the bottom strand, Fig. 1A, Miniprint) carrying the lacUV5 promoter into this vector would result in two opposing lac promoters. The formation of a 100-base pair hairpin structure would be obstructive to the site-directed mutagenesis process. Consequently, a new M13 amber phage vector with the restriction site linker from the even number M13 phage vector was constructed in order to reverse the orientation of the MCS region for insertion of a lacUV5 promoter fragment (see Miniprint for details).

It has been shown that the reversion of a stop codon or the restoration of the correct reading frame to allow the synthesis of the α peptide of β-galactosidase can serve to efficiently select for base pair substitution mutants for DNA cloned into M13 derivative vectors (15, 16). For the above studies the simple X-Gal color plaque assay (conversion of white to blue plaques) for the expression of the lacUV5 gene provided the basis for mutant selection. Conversely, if a mutation generates a stop codon which eliminates lacZ expression, then the conversion of blue to white plaques will be the basis for mutant selection. The target of the site-directed mutagenesis was the -10 T in the lacUV5 promoter (TATAAT) to generate a dA₄,dT₄ sequence within the -10 sequence. From the nucleotide sequence (Fig. 1A, Miniprint), we noticed that one of the three reading frames in the lacUV5 promoter fragment is open. The T to A transversion at -10 would introduce a translation termination codon (TAAAT to TAAAAAT) into this open reading frame. The lacUV5 promoter in an EcoRI to PstI fragment from pHC6241ac was used to produce an open reading frame fragment that would fit into the lacZ coding frame of the M13 vector. The lacUV5 insert increased the size of the lacZ fragment by 37 amino acid residues. As stated above, a T to A transversion at position -10 of the lacZ fragment reading frame at that position.

Fig. 2. The use of single dideoxy sequencing reaction to screen for site-directed mutants. The dideoxy A sequencing reaction was used to screen the 15 white plaques (lanes 1-15) and the two blue plaques, lanes marked B. The C lane is M13mp18lac1 used as control. Location of the -10 sequence and the A to T transversion is indicated on the right side of the photograph. Boxed numbers indicated the correct mutation had occurred.

The oligonucleotide (19-mer) 5’-CCACACATTTTACGAGCCG-3’ was used in the mutagenesis experiment. Fifteen white plaques, two blue plaques, and a control (M13mp18lac1) were screened for the desired mutation using a single dideoxy sequencing reaction (ddA). In this case, loss of a sequence band at the -10 position as a result of the T to A transversion (A to T in this case for the lower strand of the promoter DNA read by the sequencing reaction) would indicate a successful site-directed mutagenesis. The screening data are shown in Fig. 2. Out of the 15 white plaques screened, two appeared to be deletions (lanes 8 and 9), and two have an additional A band missing (lanes 10 and 11). Eleven out of the 15 white plaques screened carried the correct mutation.

One of the mutants (lane 13) was selected for further use and is identified as M13mp18lac2. This recombinant phage was further purified by two more passages, selected for single plaque, and checked by a single dideoxy sequencing reaction (ddA) before being used for preparation of double-stranded replicative form DNA.

Reconstruction of the lacUV5 and the Mutant lac Promoters—The lacUV5 promoter in pHC6244lac1 contains only 60 bp upstream of the transcription initiation site (Fig. 1A, ² Portions of this paper (including “Materials and Methods” and Figs. 1, 3, and 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

³ P. T. Chan, unpublished results.
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Miniprint) which may not comprise the complete upstream sequence necessary for optimal promoter function. Therefore, we decided to reconstitute both the lacUV5 and the mutant promoter using the upstream sequence from the wild type lac promoter. The experimental details of the promoter reconstructions are presented in the Miniprint (Fig. 3).

**DNase I Digestion and RNA Polymerase Footprinting**—To probe for differences between the lacUV5 and the mutant promoters, DNase I digestion patterns and RNA polymerase footprinting for both the top and bottom strands of the promoter DNAs were compared. The results are shown in Fig. 4A. On the top strand, the DNase I-sensitive phosphodiester bond in the lacUV5 promoter located between positions −11 and −10 became resistant to cleavage in the mutant promoter. In addition there appears to be significant suppression of cleavage of the phosphodiester bond between positions −5 and −6. For the bottom strand, cleavage of the phosphodiester bonds between the −11 and −10 bases was reduced and cleavage between the −13 and −12 bases was completely suppressed. These results are summarized in Fig. 4B. From the footprinting data shown in Fig. 4A, it is obvious that RNA polymerase binds to both promoters with identical footprints when excess enzyme is present. Therefore, RNA polymerase binding is conserved in the mutant.

**Open Complex Formation: Competition between lacUV5 and Mutant Promoters**—Binding competition provides an assay of the differences in open complex formation between the mutant and the wild type promoters. Increases in ionic strength will decrease the value of \(K_c\) (17). By performing the competition assay versus increasing KCl concentration, we can examine open complex formation over a range of binding constants, providing further discrimination between the mutant and wild type promoters. Nitrocellulose filter binding assay (18) was used to compare the efficiency of open complex formation between the lacUV5 and the mutant promoters; two sets of restriction fragments containing these two promoters were used in a competition assay. Both promoters were obtained in two different DNA fragment sizes: 190 bp (Fig. 1B, Miniprint) and 313 bp (Fig. 1C, Miniprint) as indicated in Fig. 5A. The 190-bp fragment was generated by EcoRI and HindIII digestion, and the 313-bp fragment was the result of HaeIII digestion. The smaller fragment containing one promoter was combined with the larger fragment containing the other promoter and vice versa. Formation of open complexes within each set of fragments was compared. The binding analysis of each promoter in two different fragment sizes allowed us to detect any effects of DNA fragment size or flanking sequences on the stability of the open complexes formed in the competition assay.

Equal mass of the lacUV5 and mutant promoter fragments (1 \(\mu\)g each) was used in order to produce equal staining intensity. \(^{32}\)P-end-labeled DNA was used in a control experiment for the recovery of DNA fragment intensity on the nitrocellulose filter. The average percentage of DNA not recovered from the filter was 3.03\%, with a standard deviation of 0.36\%, for 12 samples.

The binding competition was first performed under RNA polymerase limiting conditions: 1 \(\mu\)g of RNA polymerase per 1 \(\mu\)g of each of the two DNA fragments or a molar ratio of RNA polymerase to promoter of approximately 1:6. Formation of open complexes was examined for four different ionic strengths ranging from 0.05 to 0.2 M KCl. It is evident from Fig. 5B, section C, lanes 1-4, that the binding of RNA polymerase to lacUV5 promoter fragments far exceeds the binding to mutant promoter fragments. Open complexes were also formed at saturated binding conditions (10 \(\mu\)g of RNA polymerase per 1 \(\mu\)g each of the two DNA fragments). This represents a molar ratio of RNA polymerase to promoter of approximately 1:6. Fig. 5B, section D, lanes 1 and 2 (0.05 and 0.1 M KCl) shows that binding was essentially complete for both lacUV5 and the mutant promoters when one visually compares the amounts bound relative to the input DNA of lane A. As expected, differential binding between lacUV5 and the mutant promoter can be observed when the ionic strength is raised due to a sufficient decrease in the respective \(K_c\) values to generate differences in the dissociation of open complexes.

It should be noted that there appears to be a significant enhancement of binding affinity for the mutant promoter in
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Fig. 5. A, restriction map around the lacUV5 and mutant promoter in pHCG624lac2 and pHCG624lac3 showing how the two different sizes of the DNA fragments carrying the promoters were obtained and their respective sizes. B, nitrocellulose filter binding assay of competitive open complex formation between the lacUV5 and mutant promoters. The DNA fragment contains the promoter as indicated. Lane A contains 1 μg of each DNA fragment used in each binding competition assay, 48 and 79 nM of the 313- and 190-base pair fragments, respectively. Lane B is control assay without RNA polymerase added. Lanes under C were competitive binding assays with 1 μg of RNA polymerase (21.2 nM) per assay. Lanes under D were competitive assays with 10 μg of RNA polymerase (212 nM) per assay. The four ionic strengths used were: 1, 0.05 M; 2, 0.1 M; 3, 0.15 M; and 4, 0.2 M KCl.

the 313-bp fragment versus the 190-bp fragment although the molarity of the former is lower than the latter by a factor of 1.65. This is particularly evident in comparing band intensities for the top and bottom panels of section C, Fig. 5B. Although it appears that the location of the mutant promoter or the variation in length of the flanking sequences on the DNA fragment can affect the binding affinity, the evidence is still equivocal that the −10 transversion mutation reduced the capacity of the mutant promoter to form open complexes.

Dissociation of RNA Polymerase-Promoter Open Complex—The relative stability of open complexes can be examined by comparing the open complex dissociation rate. It has been demonstrated that the addition of heparin or other template competitive reagents promotes the dissociation of open complexes with dissociation following irreversible first order kinetics (19, 20). Fig. 7 shows that a heparin concentration of 0.5 mg/ml did not dissociate lacUV5 promoter open complexes over a 15-min time range, whereas approximately 40% of the mutant promoter open complexes were dissociated.

The combined action of both heparin and the increased salt concentration should substantially enhance the rate of dissociation. The results are shown in Fig. 8A. (Absorbance unit × mm²) values of the DNA bands in Fig. 8A were determined, and these values were used to calculate the fraction of open complexes remaining at each time point (Fig. 8B).

It is evident from the results of Figs. 7 and 8 that mutant promoter open complexes are less stable than lacUV5 promoter open complexes. We have also examined the relative stability of open complexes for the 190-bp promoter fragments and found that the mutant promoter open complexes are less stable than lacUV5 promoter open complexes.

DISCUSSION

We believe that the special features found for homopolymeric dA₄·dT₄ sequences can account for both the structural and functional effects observed due to a −10 transversion of the lacUV5 promoter, which replaced the consensus TATAAT sequence with the sequence TAAAT. The narrow minor groove geometry of dA₄·dT₄, sequences could change the structural accessibility of phosphodiester bonds or deoxyribose residues toward endonucleases or chemical reagents leading to changes in the cleavage patterns produced by those agents. In addition a narrow minor groove geometry should generate structural discontinuities at the 5′ and 3′ junctions between the homopolymeric structure and normal B DNA structure of the promoter. It has been proposed that phased junction effects are responsible for DNA bending (1, 2). The structural consequences of narrow minor groove geometry on nuclease and chemical attack of dA₄·dT₄ sequences could change the structural accessibility of phosphodiester bonds or deoxyribose residues toward endonucleases or chemical reagents leading to changes in the cleavage patterns produced by those agents.

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an adenine tract (23). We suggest the possibility that different flanking sequences may change the structural features of the junction regions of a d\textsubscript{A\textsubscript{4}}-d\textsubscript{T\textsubscript{4}} tract, thereby affecting nuclease accessibility at these sites. The direction of bending at the junctions could be of considerable importance for RNA polymerase binding interactions and unwinding events.

In the context of the lacUV5 promoter sequence, we have provided qualitative evidence that both the rate of open complex formation and the stability of open complexes can be decreased by the replacement of a consensus TATAAT sequence with the more stable TAAAAT sequence. Given the enhanced stability of the d\textsubscript{A\textsubscript{4}}-d\textsubscript{T\textsubscript{4}} sequence, it would appear reasonable to postulate that the rate of open complex formation ($k_3$) is diminished. However, from the data it is not possible to separate the effect of the promoter mutation on $K_b$ and $k_2$. Furthermore, we have found that the location of the promoter and the length of the flanking sequences on the DNA fragment can affect both open complex formation and dissociation.$^3$ In order to develop a full understanding of the effects produced by the $-10$ d\textsubscript{A\textsubscript{4}}-d\textsubscript{T\textsubscript{4}} sequence and promoter location effects, a far more in-depth kinetic analysis is needed to obtain $K_b$, $K_a$, and $k_{2a}$ values under different ionic conditions for the mutant and the lacUV5 promoters for both the 190- and 313-base pair fragments.

We must be cautious about drawing a general conclusion regarding how a homopolymeric d\textsubscript{A\textsubscript{4}}-d\textsubscript{T\textsubscript{4}} sequence in the $-10$ region will affect promoter activity based on the following considerations. The p-Out promoter of the IS10 insertion sequence also contains a $-10$ hexamer TAAAAT sequence, yet this promoter is quite strong, with a $K_b$ value very close to that of lacUV5 and a $K_a$ value approximately 5-fold higher than the lacUV5 promoter (24). It would appear that different interactions of RNA polymerase with the p-Out promoter allow for opening of the more stable TAAAAT sequence at a rate close to that of the lacUV5 promoter. Consequently the effects of the special properties of a d\textsubscript{A\textsubscript{4}}-d\textsubscript{T\textsubscript{4}} sequence at the $-10$ site of a promoter must be considered in the context of variations in flanking sequences, i.e. junction and bending effects and also with respect to interactions with RNA polymerase that lead to promoter isomerization to the open state. The molecular events involved in this unwinding process and the interactions that stabilize the unwound region remain open questions.

In generating the lacUV5 transversion mutation, we added another translation termination strategy to screen for site-directed mutants. Although this method has its limitations, it is extremely efficient. Requirements for using this selection method include the following: 1) The sequence to be mutated must be located within an open reading frame which can be inserted into the reading frame of the lacZ fragment of the M13 vector at any restriction site or combination of restriction sites within the multiple cloning site. 2) The DNA fragment inserted into the M13 vector should not be too long as not to cause a complete loss of lacZ function due to fusion of extra amino acids to the $\beta$-galactosidase fragment (the upper limit of the DNA sequence length has not been determined). 3) The change in DNA sequence(s) must result in the generation of a translation termination codon in the open reading frame. This method should be extremely useful in generating mutations in regulatory sequences such as the promoter sequence reported here.

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REFERENCES

14. Biochemicals for Molecular Biology, pp. 33-38, Boehringer Mannheim Biochemicals, Indianapolis, IN
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As shown in Fig. 3, a gel shift was observed between the −5 and −4 regions of the lac promoters. The −10 portion of the promoter was observed in the form of a −30 base-pair fragment in the dA₅/T₅ sample in lane 13. The −30 and open complex was obtained from all these base pair (bp) fragments by the addition of 10 ng of the lacUV5 promoter to the reaction mixture. The DNA samples were added to each lane in the gel. The gel was run for 1 hour at 100 volts. The gel was stained with ethidium bromide and visualized under ultraviolet light. The bands were then visualized using Typhoon gel documentation system.

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