Articles

Promoter Recognition by *Escherichia coli* RNA Polymerase. Effects of Single Base Pair Deletions and Insertions in the Spacer DNA Separating the −10 and −35 Regions Are Dependent on Spacer DNA Sequence†

Sarah E. Warne and Pieter L. deHaseth*

Department of Biochemistry, School of Medicine, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106-4935

Received February 11, 1993; Revised Manuscript Received March 30, 1993

ABSTRACT: *Escherichia coli* RNA polymerase contacts promoter DNA at two upstream regions separated by a spacer DNA. We had previously studied the effects of substitutions of simple DNA sequences in a stretch of the spacer DNA devoid of any known specific contacts with RNA polymerase. It was found that substitution of nine consecutive nonalternating dG–dC base pairs, but not nine alternating dG–dC base pairs, impaired promoter function. We proposed that this effect was due to the fact that the oligo(dG)–oligo(dC) sequence adopted a conformation (possibly A-helical) resulting in a reduction in its length and twist as compared with the B-form DNA of the alternating sequence. Here we test this hypothesis by combining the substitutions with single base pair insertions and deletions in the spacer DNA, which affect the length and the twist in known ways. Deletions and substitutions equally affect the activities of promoters with the presumed B-DNA substitutions. However, for promoters bearing the oligo(dG)–oligo(dC) substitution, a deletion in the spacer DNA impairs promoter activity to a much greater extent than the insertion of a base pair. This asymmetry is consistent with our hypothesis that the deleterious effects of the substitution are due to its having the reduced twist and/or length characteristic of A-DNA. Additionally, we present data that concern the sequence requirements for adoption of this structure that leads to reduced promoter function.

Promoter recognition by *Escherichia coli* RNA polymerase is thought to involve two hexameric regions upstream of the start site of transcription (designated the −10 and −35 regions to indicate their approximate positions), separated by a spacer DNA (von Hippel et al., 1984; McClure, 1985). No evidence has been found for specific contacts between the spacer DNA and RNA polymerase beyond a stretch immediately upstream of the −10 region. The main role of the spacer is thought to be maintaining the −10 and −35 regions in the proper orientation for initial binding of RNA polymerase and subsequent formation of a complex that is competent to initiate RNA synthesis. This has been explicitly formulated in the "untwist and melt" model (Stefano & Gralla, 1982; Auble & deHaseth, 1988; Ayers et al., 1989) for formation of a functional RNA polymerase–promoter open complex where strand separation has taken place in the region around the start site of transcription (Siebenlist et al., 1980). It is envisaged that, in order to make initial contacts with both the −10 and −35 regions, RNA polymerase has to rotate them with respect to each other, putting torque on the spacer DNA. The free energy thus stored in the DNA would drive the nucleation of the strand separation process. The relative position of the −10 and −35 regions in the initial complex with RNA polymerase is determined by the binding sites for these regions on the enzyme and is therefore independent of their positions on the free promoter. The more the relative position of the two regions on a particular promoter differs from the optimal one, the more the promoter function will be impaired.

Experimental support for the above model has been derived from the observation that changes in the length of the spacer DNA as well as the introduction of DNA sequences with potentially altered structures affect promoter function. The dependence of promoter activity on the length of the spacer DNA has been well-documented (Mulligan et al. (1985) and references therein; Stefano & Gralla, 1980, 1982; Ayers & deHaseth, 1989). The pattern that has emerged is that a 17 base pair spacer is optimal and that promoters with even one base pair deletion or insertion display considerably less activity in vitro as well as in vivo. By targeting a region of the spacer DNA that appears to be devoid of RNA polymerase contacts, we have shown that substitutions with poly(dG)–poly(dC) in either one of the two possible orientations reduced promoter activity 2–3-fold both in vivo and in vitro (Auble et al., 1986). Other simple homopolymer sequences had essentially no effect. These results would be consistent with the model presented above, if the nonalternating oligo(dG)–oligo(dC) sequences adopted a structure with a reduced twist and/or rise as compared to B-helical DNA (Auble & deHaseth, 1988). We refer to this structure as A-helical, as the A-helix has these properties and has been observed for some dG–dC-rich sequences in the crystal (Dickerson, 1992) and for poly(dG)–poly(dC) in solution (Sarma et al., 1986). Due to the presence of the substituted region, the spacer would then not only be shorter but also have a reduced twist compared to a spacer that adopted the B-helical structure over its entire length.

† This work was supported by National Institutes of Health Grant GM31808.

* Address correspondence to this author. Telephone: (216) 368-3684. FAX: (216) 368-4544.

0006-2960/93/0432-6134$04.00/0

The correlation between DNA structure and DNA sequence is by no means clear-cut (Heinemann et al., 1992), and other interpretations of these substitution studies are possible. Instead of altering the twist and length of the spacer DNA, the substituted DNA sequence could have affected promoter function by altering the torsional or lateral flexibility of the spacer DNA or its curvature (Heumann et al., 1988; Lozinski et al., 1991). The effects of the substituted DNA would then have to be explained by a mechanism (e.g., affecting the introduction of polymerase-induced curvature) other than that outlined in the model. It was thus of interest to devise a method to determine whether the effect of the oligo(dG)–oligo(dC) substitution was indeed best explained by its adoption of a structure with A-helical characteristics. To accomplish this, we combined the substitution with the deletion or insertion of a base pair, producing promoter variants with 16 and 18 base pair spacers, respectively. These manipulations should decrease and increase, respectively, the length and twist of DNA, but not its flexibility or curvature. If the oligo(dG)–oligo(dC) substitution reduced the twist and/or length of the spacer, the combined effects of the substitution and the alteration of the spacer length would enforce each other for the deletion, but not the insertion, of a base pair. Thus the variant with the 16 base pair spacer would be a weaker promoter than the one with the 18 base pair spacer. Such an asymmetry is observed for promoters bearing the substitution with nonalternating dG–dC base pairs, but not for those with the alternating dG–dC sequence. This observation supports our original suggestion (Auble et al., 1986; Auble & deHaseth, 1988) that the former, but not the latter, sequence affects promoter function by adopting an A-helical structure. Finally, we also demonstrate that single T substitutions in a string of dC's (see Figure 1c) do not abolish the effect of the substitution on promoter function.

MATERIALS AND METHODS

(a) Chemicals and Enzymes. ATP, CTP, GTP, and UTP were purchased from Sigma. [α-^{32}P]UTP was from New England Nuclear. Restriction enzymes were purchased from New England Biolabs. *Taq* DNA polymerase was from Promega. All other chemicals were reagent grade. RNA polymerase was prepared as described (Burgess & Jendrisak, 1975; Gonzales et al., 1977). All concentrations given have been corrected for the fraction of active enzyme (40%).

(b) Promoter Variants Differing in the Lengths and Sequences of Their Spacers. A series of promoters with altered spacer sequences that had been constructed for earlier studies (Auble et al., 1986; Ayers et al., 1989) were the point of departure for the variants investigated here. These constructs consist of 84 base pair promoter fragments cloned into the *Eco*RI and *Bam*HI sites of a region of pRZ5202 (Munson, 1983) derived from pBR322. In order to change the spacer length of these constructs, we subcloned them as *Pst*I–*Sal*I fragments in the polylinker of phagemid pBluescriptII KS- (Stratagene). Mutagenesis was conducted essentially as described by Kunkel (1985), except that primer extension was performed at 25 °C rather than at 37 °C. This enabled the use of the same mutagenic oligonucleotide with a short region of complementarity (6 base pairs) on the 5'-side of the mismatch for converting the –35 region from TAGACA to TTGACA for all of our promoter variants, regardless of the sequence or length of the spacer DNA. Base changes were confirmed by dideoxy chain termination sequencing.

(c) DNA Fragments. For in vitro transcription assays, plasmids were digested with *Pst*I and *Sal*I to generate a 1.1-kb promoter-containing fragment, which was separated from the vector by agarose gel electrophoresis and purified by electrophoretic transfer onto DEAE–cellulose paper (Sambrook et al., 1989). A section of the fragment was amplified by the polymerase chain reaction (PCR). The reaction conditions were as follows: DNA, 50 ng; primers, 80 pmol; 1.5 mM $MgCl_2$; 250 μM dNTPs; 1× buffer (supplied by manufacturer); and 2.5 units of *Taq* DNA polymerase in a volume of 100 μL. One primer matched the sequence of pBR322 from positions 4334 to 4349, the other from 497 to 476 (both in the 5' to 3' direction); with these primers an amplified promoter fragment of 180 base pairs is generated.

PCR fragments used for runoff assays were gel-purified by DEAE–cellulose. The concentrations of purified fragments were determined by fluorimetry using Hoechst dye no. 33258 (Sigma). For start site analysis, the PCR-amplified DNA was digested with *Hae*II to yield a 153 base pair fragment terminating at position +66 with respect to the start site of the parental promoter.

(d) β-Galactosidase Assays. For determination of in vivo activities the promoter constructs were subcloned into the vector pRZ5202, which features a promoter-less β-galactosidase gene (Munson, 1983; Auble et al., 1986). β-Galactosidase assays (Miller, 1972) were carried out as previously described (Auble et al., 1986). A background value of β-galactosidase activity, measured on cells transformed with a promoter-less pRZ5202 plasmid, has been subtracted from all values. To facilitate the comparison of data gathered over a period of several weeks, the promoters S(CC) or FS(CC) (see Figure 1) were included in the assays, and for each assay their activities were used to normalize those of the other promoters.

(e) Runoff Transcription Assays. DNA fragments were diluted to a final concentration of 2 nM in transcription buffer [30 mM Tris-HCl (pH 8.2), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10 μM dithiothreitol, 45 μg bovine serum albumin/mL]. The DNA mix was prewarmed at 28 °C, and then RNA polymerase was added to a final concentration of 20 nM and incubation was continued for another 5 min to allow for the formation of open complexes. At that point, 5 μL of an NTP mix containing 1 mM GTP, 1 mM ATP, 1 mM CTP, 10 μM UTP, 15 μCi of [α-^{32}P]UTP, and 0.25 mg/mL heparin (to limit transcription to one round) was added. Transcription was allowed to proceed for 15 min at 28 °C. Reactions were terminated by the addition of one reaction volume of 2 mM UTP in 6 M urea; the mixtures were heated for 2 min at 90 °C prior to being loaded on to a denaturing 6% polyacrylamide gel. Assignment of the RNA bands on the gel was accomplished by comparison with a marker lane containing DNAs of known lengths. The relative amounts of radioactivity in each band (Figure 4) were determined by phosphorimaging of the gels (Molecular Dynamics) or by densitometry of films resulting from autoradiography (USB ScyScan 5000). The results of both treatments are presented as the percentage of radioactivity (or density) in the band of interest, as compared to the total amount of radioactivity (or density) which was detected in the particular run (comparison studies showed excellent correlation between the two methods).

RESULTS

We have previously demonstrated that promoter S(CC) was weaker than S(CT) (Figure 1c) and S(CG) (which has a CG substitution in the spacer at the same position as FS(CG); see Figure 1a) (Auble et al., 1986). Initial studies on the effects of changing the spacer DNA length of promoters bearing spacer substitutions indicated that some of the

	PROMOTER	-35	SPACER	-10	START-SITE
					+1 →
a)	FS(CC)16	**TTGACA** TT	**CCCCCCCCC**	GGCGA	**TAGATT**TAACGT**A**TGAGCA
	FS(CC)17	TTT	**CCCCCCCCC**		
	FS(CC)18	TTTT	**CCCCCCCCC**		
	FS(C_4TC_4)17	TTT	**CCCCTCCCC**		
	FS(CG)17	TTT	**CGCGCGCGC**		
	FS(CTC_7)17	TTT	**CTCCCCCCC**		
b)	Pbn 16	**TTGACA** TTTA	**CCCTGGG**	GGCGA	**TAGATT**TAACGT**A**TGAGCA
	Pbn 17	TTTAT	**CCCTGGG**		
	Pbn 18	TTTATT	**CCCTGGG**		
	Psm 16	TTTA	**TCCCGGG**		
	Psm 17	TTTAT	**TCCCGGG**		
	Psm 18	TTTATT	**TCCCGGG**		
c)	S(CT)	**TAGACA** TTT	**CTCTCTCTC**	GGCGA	**TAGATT**TAACGT**A**TGAGCA
	S(CC)		**CCCCCCCCC**		
	S(C_4TC_4)		**CCCCTCCCC**		
	S(C_3TC_5)		**CCCTCCCCC**		
	S(C_3TCTC_3)		**CCCTCTCCC**		
	S($C_3(TC)_3$)		**CCCTCTCTC**		
	S(TC_8)		**TCCCCCCCC**		
	S(C_8T)		**CCCCCCCCT**		

FIGURE 1: Promoter constructs used in this work. The –10 and –35 regions, the start site of transcription, and the region of the spacer DNA in which substitutions were made are indicated. The nomenclature used provides an indication of the sequences of the spacer DNAs, as well as their lengths. (a) Promoters with consensus –35 regions (differentiated from those with nonconsensus –35 regions (Figure 1c) by the prefix F) and spacers which had been manipulated at an upstream cluster of dA–dT base pairs to yield lengths of 16, 17, or 18 base pairs. (b) Promoters with consensus –35 regions, originally constructed for a different purpose (Ayers et al., 1989). The spacer length has been altered at positions immediately downstream from the cluster of T's mentioned above. (c) Promoters with 17 base pair spacers and a –35 region that differs from the consensus sequence at one position.

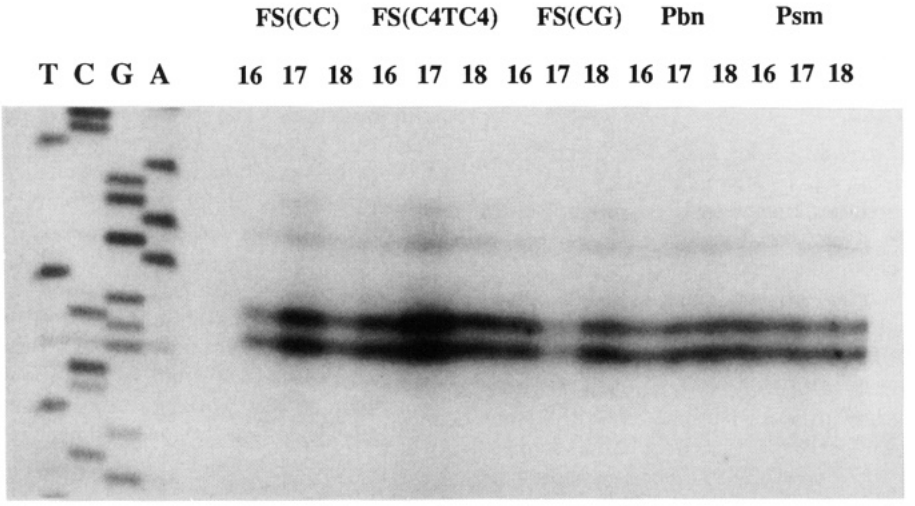

FIGURE 2: Spacer DNA sequence and length do not affect start site selection. Runoff transcription was carried out on *Hae*II-cut PCR-amplified fragments, the amounts of which were adjusted to yield approximately equally intense product RNA bands. The main (upper) bands represent RNA products 66 nucleotides in length. A set of sequencing lanes is shown to indicate the resolution of the gel.

resultant promoter variants were so weak that accurate determinations of their activities became difficult (data not shown). Therefore, we improved the overall strength of the promoter variants by engineering a base pair change in the –35 region to give a perfect match to the consensus sequence (von Hippel et al., 1984; McClure et al., 1985; Ayers et al., 1989) in this region. This generated the FS series of promoter variants shown in Figure 1a. In order to maintain constant boundaries between the substitutions and the immediately flanking DNA, the spacer lengths were altered by manipulating the number of nucleotides in an upstream cluster of T–A base pairs, as shown. We had not previously detected any effects of spacer DNA sequence or length on the start site selection of the promoter variants shown in Figure 1b (Ayers et al., 1989) or in Figure 1c (Auble et al., 1986); a similar observation was made by Lozinski et al. (1991). To determine whether this would also be the case with the variants shown in Figure 1a, we checked the sizes of the runoff transcripts initiated at these promoters on a high-resolution polyacrylamide gel. The results (Figure 2) clearly demonstrate that the lengths of the runoff products are not affected by spacer length or sequence. While in Figure 2 the transcripts show up as doublets, this is the case with the parental promoter (P_{RM} of phage λ) as well. We have not further studied this phenomenon.

To compare promoter strengths as a function of spacer sequence and length in vivo, the activities of the promoter-

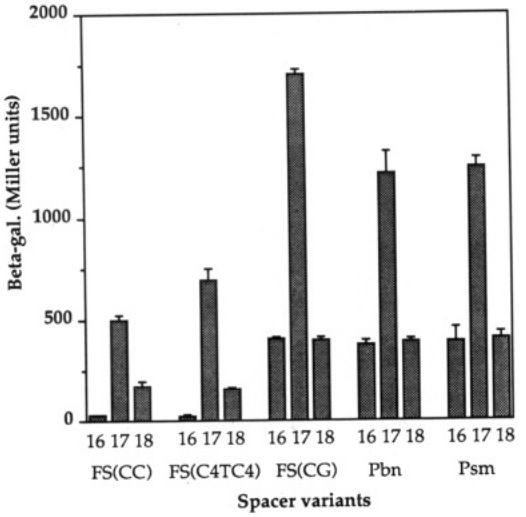

FIGURE 3: In vivo activities of promoter constructs. The values shown are averages of at least 24 determinations, with standard deviations. The names (including spacer DNA lengths) are indicated on the *x*-axis.

driven reporter gene, β-galactosidase, were determined in cells transformed with plasmid pRZ5202 (see Auble et al. (1986)) bearing the cloned promoter variants. The results of these experiments are shown in Figure 3. A comparison of the activities of promoters with spacer lengths of 17 base pairs shows that both FS(CC)17 and FS(C4TC4)17 (with consensus –35 regions) are less active than the other three promoters, including FS(CG). This effect is similar to that observed by Auble et al. (1986) (see also Figure 5) for promoters with one nonconsensus base pair in the –35 region (the –35 sequence shown in Figure 1c); thus, the deleterious effect of substitution of nine dG–dC base pairs is not dependent on the sequence of the –35 region. The fact that FS(CC)17 and FS(C4TC4) behave similarly is taken to indicate that the single dA–dT base pair interrupting the string of G–C base pairs does not substantially alter the structure of the substituted sequence (see also Figures 4 and 5). The spacers of Pbn17 and Psm17 are similar to those of promoters that have reduced activities (see Figure 5) and are also similar to sequences that adopt the A-helical structure (Dickerson, 1992). Therefore, the relatively high activities of Pbn17 and Psm17 (which had been constructed for another purpose (Ayers et al., 1989)) were surprising.

While the FS(CG), Pbn, and Psm variants have a symmetric distribution of activity with spacer length, this is clearly not the case for the FS(CC) and FS(C4TC4) promoters, for which the activity of the variant with the 16 base pair spacer is very low indeed, resulting in an asymmetric spacer length–activity profile. In both cases, the promoter with a spacer length of 18 bases is 3–4 times as active as that with the 16 base pair spacer. We have obtained a similar profile with the S(CC) promoter, where the activity of the promoter variant with the 16 base pair spacer was barely detectable (data not shown). A qualitatively similar result was obtained when the same promoters were tested for in vitro activity by a runoff assay. An autoradiogram of a runoff transcription gel is shown in Figure 4a; the quantitation of data from several such experiments is shown in Figure 4b,c. Again, symmetric profiles are observed with FS(CG), Pbn, and Psm, but asymmetric ones are seen with FS(CC) and FS(C4TC4) as a consequence of the very low amounts of RNA synthesis for the variants with 16 base pair spacers.

A discrepancy between the in vivo and in vitro results is apparent for the FS(C4TC4)17 and FS(C4TC4)18 promoters, whose activities are comparable to those of FS(CG)17 and FS(CG)18, respectively, in vitro but are significantly lower in vivo. In the in vitro experiments, the extent of open complex formation over a 5-min period determines the amount of runoff RNA that is produced. Saturation of promoters with RNA polymerase binding due to fast rates of complex formation, and a concomitant lack of discrimination among these promoters, can occur during pre-incubation with the RNA polymerase. This would explain the lack of difference for a spacer length of 17 base pairs but not for the promoters with 18 base pair spacers, where saturation with RNA polymerase would not be a factor. An obvious additional difference between the in vivo and in vitro experiments is that the promoters are contained on supercoiled DNA in the former and linear fragments in the latter. We have not determined whether the various promoter variants are differentially sensitive to DNA supercoiling.

To better define the DNA sequences that lead to reduced promoter activities when substituted in the spacer DNA, we employed the constructs shown in Figure 1c. Compared to those of Figure 1a,b, these promoters have an additional nonconsensus base pair in their –35 regions; in this respect they are identical to the constructs we have previously described (Auble et al., 1986). The results of in vivo measurements of their activities are shown in Figure 5a. In agreement with our previous studies (Auble et al., 1986) and with the experiments reported above, S(CC) is found to be less than half as active as S(CT). Substitutions of a single T at various positions within the stretch of dC's lead to slight increases in activity, independent of the exact positions of the substitutions. Surprisingly, the substitution of three T's does not further enhance promoter activity, even though the difference between S(C3(TC)3) and S(CT) is only one T (at position –25). That this T does not play a special role is demonstrated with the results shown in Figure 5b, which were obtained with promoters containing consensus –35 regions. In addition to some of the promoters shown in Figure 1a, the activity was measured for FS(CTC7)17, which has been derived from FS(CC) by a single dC to dT substitution at position –25. Both this substitution and the one in FS(C4TC4) have only small effects on promoter activity, comparable to the effects of other single T substitutions shown in Figure 5a. Therefore, we believe that a T substitution at this position is no different from those at other positions.

DISCUSSION

We have presented experiments designed to test our hypothesis that the substituted DNA in promoter variants S(CC), FS(CC), S(C4TC4), and FS(C4TC4) (see Figure 1) has an A-helical structure which is the cause of the reduced promoter activity associated with the substitutions. Upon combination of the substitution with a single base pair deletion or insertion, it is found that the promoter variants with 16 base pair spacers are much less active than those bearing 18 base pair spacers. Apparently, in the case of the deletion of a base pair, the effects of the substitution and the deletion reinforce each other, while this is not the case if the substitution is combined with the insertion of a base pair. We discuss below the implications of the asymmetric profile of promoter length versus promoter activity that we have observed and show that it is in qualitative agreement with the hypothesis that some substitutions adopt an A-helical DNA structure.

The near-perfect symmetry obtained for the activity–spacer length relationship for S(CG) indicates that a spacer length

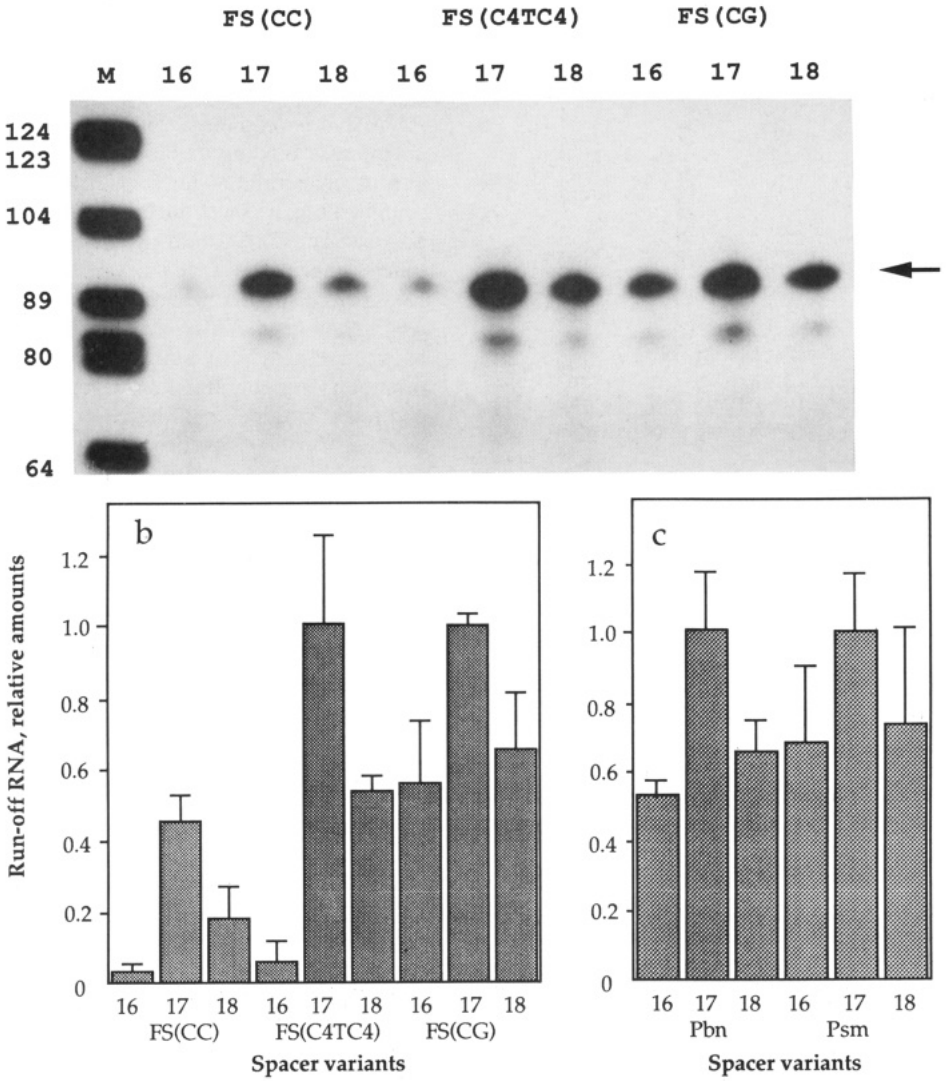

FIGURE 4: In vitro determination of promoter activities by runoff RNA synthesis. After a 5-min pre-incubation of promoter DNA and RNA polymerase, a single round of RNA synthesis was initiated by the addition of nucleoside triphosphates and heparin. The predicted size of the runoff RNA products is 93 nucleotides. The amounts of DNA added in each reaction are based on determinations of stock concentrations by a fluorescent dye–binding assay. We estimate that the DNA concentration in each reaction is 2.0 ± 0.2 nM. (a, top) Autoradiogram of a representative gel. The full-length runoff product used for the quantitations in b and c is indicated by the arrow; lane M is *Hae*III-cut pBR322 as size marker. The identities of the promoters as well as the lengths of their spacer DNAs are shown above the lanes. (b) and (c) Relative band intensities corresponding to the products made by the promoters indicated; individual gels contained either the promoters in b or those in c. To enable the comparison of data from different experiments, obtained by different methods of scanning the gels (see Materials and Methods), the data have been normalized to the intensity of the FS(C4TC4)17 band in b and to that of the Pbn17 band in c. The data presented are averages of three determinations.

of 17 base pairs having the structure of B-DNA apparently provides the optimal distance and relative rotation of the –10 and –35 regions. The results of published experiments on the effects of base pair deletions and insertions in the spacer DNAs of various promoters (as surveyed by Mulligan et al. (1985)) are difficult to interpret, as most of the observed effects are quite small. An exception is the study of Stefano and Gralla (1980, 1982) with the *lac* P^s promoter. It is found that both the deletion and the insertion of a base pair reduce promoter function in vivo to about 15% of that of a promoter with a 17 base pair spacer, bestowing upon this promoter a symmetric spacer length–activity profile.

Deletion of one base pair of B-DNA in the spacer reduces the relative rotation of the –10 and –35 regions by 34° and their distance by 3.4 Å, while insertion of a base pair leads to commensurate increases in these parameters. The magnitude of the expected effect of substituting a stretch of A-DNA for B-DNA in solution is less clear, as most of the relevant data on these structures are for crystals of deoxyoligonucleotides (summarized by Dickerson (1992)). In addition, there is a rather large variability in the values of the parameters obtained, both as functions of crystal structure and the nature of the oligomers, and between individual base steps within oligomers. However, all comparisons indicate that the values for the rise and twist are smaller for A-DNA than B-DNA. Substitution of A-DNA for B-DNA over a stretch of nine base pairs (or 8 base pair steps) would reduce the twist and rise of DNA by amounts comparable to the deletion of a base pair, on the basis of the average values compiled from X-ray diffraction experiments (Dickerson, 1992). Although the observed effect of deletion of a base pair observed here is smaller than that seen in other experiments (e.g., Auble et al. (1986)), the observed consequences of the two manipulations are similar (compare FS(CC)17 with FS(CG)16 in Figures 3 and 4a), commensurate with the above estimates. These results therefore support our contention that the stretch of nine dG–dC base pairs adopts the A-helical conformation in solution.

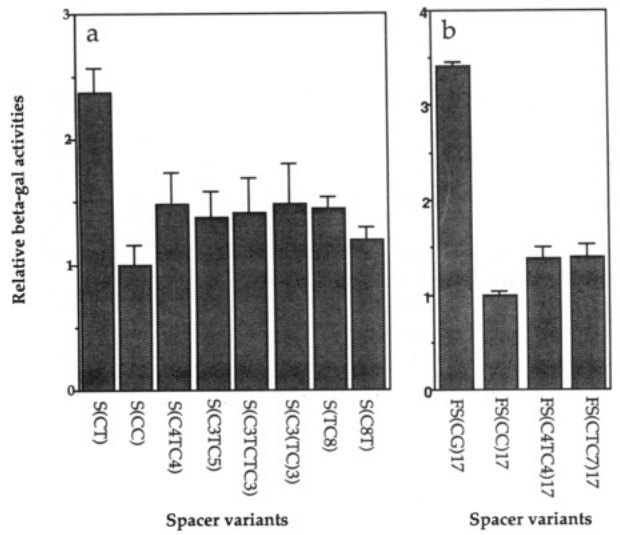

FIGURE 5: Effects of T substitutions in the stretch of nine dC residues on the in vivo activities of promoter variants. The data presented are averages of 24 or more determinations. (a) Promoter variants with –35 regions that differ in one position from the consensus sequence. (b) Promoter variants with consensus –35 regions. The data for FS(CG)17, FS(CC)17, and FS(C4TC4)17 are from the experiment shown in Figure 3 and are included to enable comparison with the FS(CTC7)17 data.

The results presented in Figures 3 and 4 show that the combination of the substitution of nine nonalternating dG–dC base pairs in the spacer DNA of a promoter with a one base pair deletion results in a greater reduction of promoter function than expected from the separate effects of either manipulation. This observation is consistent with both the A-helical structure of the substituted nonalternating dG–dC region and with the untwist and melt model described in the introduction. Focusing on rotational rather than length effects, we postulate that upon combination of the substitution with a deletion, the –10 and –35 regions become under-rotated with respect to their optimal position (17 base pair spacer, B-DNA) by an extent equal to the sum of the angles characteristic of each of the two manipulations. In the simplest possible case, the angle over which the spacer DNA is untwisted by RNA polymerase can then also be reduced by the same extent.

The energy required to put torque on the DNA is dependent on the square of the angle over which the DNA is twisted (e.g., Depew and Wang (1975)). Therefore, the loss of torque free energy generated by the RNA polymerase in a promoter for which the –10 and –35 regions are under-rotated by the sum of the angles is greater than the sum of the losses for the two cases where the substitution and deletion are present individually. With the assumption that the ability to nucleate the strand separation process inversely correlates with the loss of energy stored in the spacer DNA, this provides an explanation for the low activities of the promoters with both the oligo(dG)–oligo(dC) substitution and the one base pair deletion. A more quantitative treatment is not feasible, as not only are the magnitudes of the effects of the substitution on twist and length unknown but their relative importance for promoter function is also.

An explanation of the observed effect of the insertion of a base pair in combination with the oligo(dG)–oligo(dC) substitution is more difficult. It had been expected that the A structure of the substituted DNA would at least partially compensate for the effect of insertion of a base pair. However, no such compensation is detected upon comparison of the ratios of the activities of promoters with 17 and 18 base pair spacers between promoters with various spacer substitutions (Figures 3 and 4b,c). It is possible that the compensation is too small to be detected given the experimental uncertainty in the data. Alternatively, the simple model employed above does not sufficiently account for some important features of the RNA polymerase–promoter interaction, possibly by not taking into account length effects.

Given the similarities of the spacer sequences of Pbn and Psm (Figure 1b) to that of FS(C4TC4) or to sequences that adopt the A-helix in the crystal (Dickerson, 1992), it is surprising that they show neither the reduced activity nor the asymmetry characteristic of promoters with the nonalternating dG–dC substitution. We have no explanation for this observation. In view of this result with the Psm and Pbn promoters, we tried to determine how critical the sequence requirements were for the reduction of promoter activity by a substitution in the spacer DNA. The results of the experiments on the T substitutions in the block of dG–dC base pairs show that such substitutions have a surprisingly small effect. In terms of our model that the substituted DNA adopts an A-helical structure, this is taken to indicate that the structure is but little disturbed by the presence of a limited number of T base pairs. This was also demonstrated by the observation that the FS(C4TC4) variants behaved very similarly to the FS(CC) ones with respect to the deletion or insertion of a base pair. We have not investigated whether transversions of the dG–dC base pairs in FS(CC) or S(CC) would have similarly small effects.

As pointed out before (Ayers et al., 1989), the untwist and melt model in its present form does not adequately deal with the way the deletion or insertion of a base pair in the spacer DNA affects the stability of the initial complex with RNA polymerase (Mulligan et al., 1985; Ayers et al., 1989) as inferred from kinetic studies. It is also possible that the interpretation of kinetic data in terms of these affinities is unwarranted in view of the complexity of the process of open complex formation (see also Roe et al. (1985)). In conclusion, it is not yet possible to quantitatively account for the effects of spacer DNA manipulations on promoter activity. However, the new results presented here, in conjunction with our previous work (Auble et al., 1986; Auble & deHaseth, 1988; Ayers et al., 1989), strongly suggest that an alteration in the rotational orientation of the –10 and –35 regions with respect to each other is an integral step in the pathway to formation of an open complex between RNA polymerase and a promoter.

REFERENCES

Auble, D. T., & deHaseth, P. L. (1988) *J. Mol. Biol. 202*, 471–482.

Auble, D. T., Allen, T. L., & deHaseth, P. L. (1986) *J. Biol. Chem. 261*, 11202–11206.

Ayers, D. G., Auble, D. T., & deHaseth, P. L. (1989) *J. Mol. Biol. 207*, 749–756.

Burgess, R. R., & Jendrisak, J. J. (1975) *Biochemistry 14*, 4634–4638.

Depew, R. E., & Wang, J. C. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4275–4279.

Dickerson, R. E. (1992) *Methods Enzymol. 211*, 67–111.

Gonzales, N., Wiggs, J., & Chamberlin, M. J. (1977) *Arch. Biochem. Biophys. 182*, 404–408.

Heinemann, U., Alings, C., & Bansal, M. (1992) *EMBO J. 11*, 1931–1939.

Heumann, H., Ricchetti, M., & Werel, W. (1988) *EMBO J. 7*, 4379–4381.

Lozinski, T., Adrych-Rozek, K., Markiewicz, W., & Wierchowski, K. Z. (1991) *Nucleic Acids Res. 19*, 2947–2953.

McClure, W. R. (1985) *Annu. Rev. Biochem. 54*, 171–204.

Miller, J. H. (1972) *Experiments in Molecular Genetics*, pp 352–355, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mulligan, M. E., Brosius, J., & McClure, W. R. (1985) *J. Biol. Chem. 260*, 3529–3538.

Munson, L. (1983) Ph.D. Thesis, University of Wisconsin, Madison, WI.

Roe, J.-H., Burgess, R. R., & Record, M. T., Jr. (1985) *J. Mol. Biol. 184*, 441–453.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sarma, M. H., Gupta, G., & Sarma, R. (1986) *Biochemistry 25*, 3659–3665.

Siebenlist, U., Simpson, R. B., & Gilbert, W. (1980) *Cell 20*, 269–281.

Stefano, J. E., & Gralla, J. D. (1980) *J. Biol. Chem. 257*, 13924–13929.

Stefano, J. E., & Gralla, J. D. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1069–1072.

von Hippel, P. H., Bear, D. G., Morgan, W. D., & McSwiggen, J. A. (1984) *Annu. Rev. Biochem. 53*, 389–446.