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Specific Activation of Open Complex Formation at an *Escherichia coli* Promoter by Oligo(*N*-methylpyrrolecarboxamide)s: Effects of Peptide Length and Identification of DNA Target Sites†

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Received November 21, 1988; Revised Manuscript Received February 1, 1989

ABSTRACT: It has previously been shown that open complex formation at a promoter containing a block substitution of nonalternating A-T sequences in the spacer DNA separating the contacted −10 and −35 regions could be accelerated by distamycin. No stimulation was observed at a promoter with a substitution of alternating A-T base pairs in the same region or at the promoter with wild-type spacer. Here we compare the effect of distamycin [tris(*N*-methylpyrrolecarboxamide), formally a P3] with that of its extended homologues P4, P5, and P6. It is found that the stimulatory potential of these synthetic oligopeptides which bind in the minor groove of DNA ranks in the order P4 > (distamycin, P5) > P6. The interaction of these peptides with the three promoters was studied by monitoring the positions of the promoter DNA protected from MPE–Fe(II) cleavage in the presence of different concentrations of ligand. The results suggest that a higher affinity of oligopeptide for the spacer DNA than for the −10 and/or −35 region is a necessary, but not sufficient condition for stimulation. Different patterns of protected DNA regions are seen with each of the three promoters; with distamycin, P4, and P5, a unique arrangement of protected regions is observed for the variant containing nonalternating A-T base pairs in its spacer DNA. These data support the hypothesis that differences in the ways the minor-groove binders interact with each of the promoter variants account for the observed differential stimulation. We further postulate that it is a ligand-induced structural change in the nonalternating A-T DNA which is responsible for the activation of open complex formation at the promoter containing this substitution.

In the process of transcription initiation at a bacterial promoter, RNA polymerase interacts specifically with two highly conserved hexamers located at positions −10 and −35 relative to the start site of transcription (von Hippel et al., 1984; McClure, 1985). These sequences are separated by a spacer DNA (consensus length 17 base pairs), which is devoid of contacts with polymerase (Siebenlist et al., 1980; Auble et al., 1986) over a stretch of about 10 base pairs. We have constructed a collection of promoter variants bearing, in this region of the spacer, substitutions with nine base pairs of various sequences that have the ability to adopt conformations other than typical B-DNA. Using these variants, we have been able to demonstrate that the intrinsic structure of the spacer region can affect the interaction between RNA polymerase and promoter DNA (Auble et al., 1986). In subsequent studies, promoter variants S(TT), S(AT), and S(wt) (Figure 1) were exposed to sequence-specific DNA-binding ligands, in the hope of targeting them to the spacer regions and thus extrinsically altering the structure of these promoters. The addition of the tripeptide distamycin to S(TT) resulted in a specific increase in functional complex formation at this promoter (Bruzik et al., 1987).

Distamycin, like the related antitumor antibiotic netropsin, binds along the minor groove of DNA in A,T-rich regions of DNA through hydrogen bonds and hydrophobic interactions (Zimmer & Wahnert, 1986; Kopka et al., 1985; Coll et al., 1987). The structures of distamycin and the synthetic homologues (P4–P6) studied in this paper are shown in Figure 2. The synthetic peptides differ from each other in the number of *N*-methylpyrrolecarboxamide residues. Like distamycin, P4, P5, and P6 bind preferentially to A,T-rich regions of DNA (Schultz & Dervan, 1983; Youngquist & Dervan, 1985). The binding site size on the DNA increases with increasing number

† P.B.D. and P.d.H. are grateful to the National Institutes of Health for research support (GM-27681 and GM-31808, respectively).
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0006-2960/89/0428-4455$01.50/0

FIGURE 1: Relevant features of promoter variants used in this study. Only the sequence of the nontranscribed strand is given. For reference, the various bases were assigned numbers on the basis of their position in the promoter. The number assigned to each position is given below the base which occurs at that position. The sequences of the block substitutions which were made at positions −18 to −26 in the spacer regions of S(TT) and S(AT) are shown below the sequence of the wild-type construct S(wt).

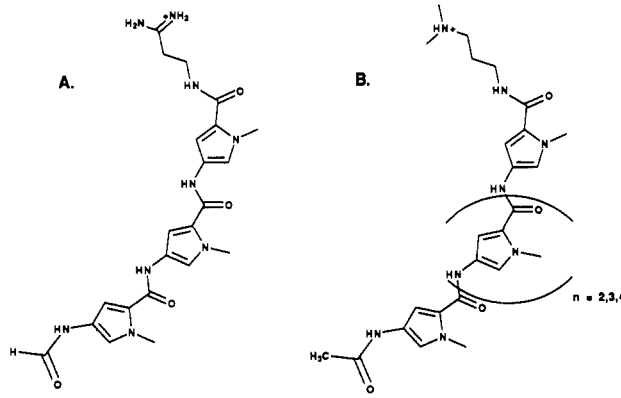

FIGURE 2: (A) Structure of distamycin (formally a P3) and (B) the distamycin analogues P4, P5, and P6, which differ in the number of *N*-methylpyrrolecarboxamide rings. n = 2, 3, and 4 for P4, P5, and P6, respectively.

of *N*-methylpyrrolecarboxamide residues, such that analogues with n residues bind to $n + 1$ successive base pairs (Youngquist & Dervan, 1985).

To account for the increase in functional complex formation at S(TT) in the presence of distamycin, we hypothesized that the structural alteration introduced in the spacer region of the promoter due to the binding of the tripeptide brought the −10 and −35 regions into a conformation more compatible with polymerase binding. In order to test this hypothesis and to better elucidate the mechanism by which distamycin facilitates transcription from S(TT), we have used MPE footprinting and abortive initiation assays to study the binding and transcriptional activities of the distamycin analogues P4, P5, and P6 on promoter variants S(wt), S(TT), and S(AT).

In this paper we demonstrate that activation of open complex formation by distamycin analogues is a function of the number of *N*-methylpyrrolecarboxamide residues, with P4 having the greatest effect. The MPE–Fe(II) footprinting data lend support to a model where activation is a result of oligopeptide binding to the spacer DNA. One possible interpretation is that activation by bound oligopeptide is a result of realigning the −10 and −35 sequences to put them in an orientation optimal for polymerase binding.

Materials and Methods

Chemicals and Enzymes. UTP and UpA were from Sigma. [α^{32}P]UTP was purchased from New England Nuclear. Restriction enzymes and DNA polymerase I, large fragment, were purchased from New England Biolabs and Boehringer-Mannheim. All other chemicals were of reagent grade.

Escherichia coli RNA polymerase was purified by the method of Burgess and Jendrisak (1975). Our polymerase preparation was demonstrated to be 40% active in T7 transcription experiments (Chamberlin et al., 1979). The concentration of polymerase reported in the titration experiments has been corrected for the fraction of active enzyme.

DNA Binding Ligands. Distamycin A was purchased from Sigma Chemical Co. Distamycin analogues P4, P5, and P6 were synthesized and characterized as described (Youngquist & Dervan, 1985; Youngquist, 1988). For oligopeptide titration assays, all ligands were dissolved in 10 mM Tris-HCl, pH 7.5, and 10 mM NaCl to 10^{-2} M. For footprinting studies all peptides were dissolved in water to 10^{-3} M. Concentrations were checked spectrophotometrically (a_{308} for P4 = 40000 M^{-1} cm^{-1}, a_{312} for P5 = 46000 M^{-1} cm^{-1}, and a_{314} for P6 = 53000 M^{-1} cm^{-1}). Peptides were diluted to the appropriate concentration and were aliquoted prior to freezing at −20 °C. Unused portions of thawed aliquots were discarded.

DNA. The λ P_{RM} promoter variants used in these experiments were constructed as described (Auble et al., 1986). Plasmid DNA bearing the promoter of interest was isolated by the alkaline lysis method (Maniatis et al., 1982). Titration experiments were conducted on plasmid which had been linearized at a unique *Pst*I site. Footprinting experiments were done on 3′ end-labeled *Xmn*I–*Nar*I promoter-containing fragments isolated from the same plasmids. The *Nar*I site was used to generate the 3′ end of the fragment rather than *Sal*I [as used in Auble et al. (1986)] since the closer proximity of this site to the promoter region results in better resolution on footprinting gels. Fragments were prepared as described (Auble & deHaseth, 1988) with the exception that, following electroelution and isobutyl alcohol extraction, fragments were run over DE-52 columns for further purification instead of being phenol extracted. This was done to eliminate the possibility of phenol contamination which might interfere with the MPE cleavage reaction.

Abortive Initiation Assay. Formation of competent RNA polymerase–promoter complexes was monitored by their ability to catalyze the repeated synthesis of the aborted RNA product UpApU from UpA and UTP (McClure, 1980; Bruzik et al., 1987). With this assay, two different types of experiments were carried out as detailed in Bruzik et al. (1987): Titrations with different amounts of oligopeptide were done to rapidly evaluate the effect of peptide length on open complex formation. RNA polymerase and promoter DNA (to a final concentration of 12 and 1 nM, respectively) were preincubated in transcription buffer (Bruzik et al., 1987) in the presence of peptide for an amount of time that, in the absence of peptide, resulted in the formation of an open complex at about half the promoters (5 min). This was followed by the addition of UpA and UTP (1 mM and 20 μM), and the amount of UpApU synthesized in 10 min was determined in order to evaluate the actual extent of open complex formation. Alternatively, by monitoring the accumulation of UpApU versus time of incubation, the lag time τ (or its reciprocal, the observed rate constant k_{obs}) of open complex formation could be determined (Bruzik et al., 1987).

MPE Footprinting. Footprinting reactions were conducted essentially as described (Van Dyke & Dervan, 1983). Methidiumpropyl-EDTA (Hertzberg & Dervan, 1982, 1984) was dissolved in water at a concentration of 1 mg/mL and divided into aliquots that were stored frozen at −20 °C. For each 10-μL cleavage reaction 20000 cpm (Cerenkov) of end-labeled fragment (approximately 10 fmol) and calf DNA (final concentration 100 μM base pairs) were preincubated with peptide for 20 min at 20 °C in buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl) (in order to obtain footprints of P6 it was necessary to do the preincubation at 60 °C). Following

the preincubation period a freshly prepared solution of 100 μM MPE–Fe(II) was added to each reaction to a final concentration of 10 μM. The cleavage reaction was initiated by the addition of 1 μL of 1 mM DTT and was allowed to continue for 10 min at 37 °C. To stop cleavage, reactions were frozen on dry ice. The samples were then evaporated to dryness, resuspended in formamide loading solution, and heated for 2 min at 100 °C prior to electrophoresis on a 6% denaturing polyacrylamide gel. Autoradiography was usually performed on dried gels. In retrospect, the footprinting protocol employed may have been unfortunate, given the strong differential effect of temperature on the binding of distamycin to poly(dAdT)·poly(dAdT) and poly(dA)·poly(dT) sequences (Breslauer et al., 1987). We believe that the footprints obtained are characteristic of the situation at 37 °C (the temperature where the activity measurements were also performed), as the on- and off-rates for DNA binding are expected to be high. In addition, the spacers of S(AT) and S(TT) are found to have similar affinities for distamycin (see Figure 7), a situation representative of the relative binding constants at 37 °C, but not at the lower temperature, where S(AT) would be expected to have a 10-fold higher affinity for the peptide than S(TT).

Densitometry. Densitometry of autoradiographs from the footprinting experiments was conducted on an LKB Ultroscan XL scanning laser densitometer. The extent of cleavage at representative positions in the −10, spacer, and −35 regions was evaluated from the densitometer scans. Because peaks were uniform in width, the height of each peak in the scan is a true measure of the extent of cleavage at a particular position. Reduction in cleavage at positions −9 and −12 in the −10 region, positions −20 and −26 in the spacer region, and positions −32 and −34 in the −35 region were followed as a function of oligopeptide concentration (positions indicated in Figure 1 by asterisks). Interpolation was used to determine the concentration of oligopeptide which resulted in 50% protection of a given region of the promoter. These values were normalized to the concentration of oligopeptide which yields 50% protection of the −35 region.

Densitometry was also used to define the location and size of oligopeptide binding sites (protected regions) on each of the promoters. The densitometric scan generated in the presence of oligopeptide was subtracted from the scan generated in the absence of oligopeptide to produce a difference scan. Protected bases were identified from this difference scan as those positions at which the difference scan was positive.

Results

The ability of the distamycin analogues P4, P5, and P6 to stimulate transcription from promoter variants S(TT), S(AT), and S(wt) (Figure 1) was tested in titration experiments conducted as described under Materials and Methods. Previously we had reported that open complex formation at variant S(TT) is stimulated 2.5-fold by the addition of 0.05–0.10 μM distamycin as measured by this assay (Bruzik et al., 1987). As shown in Figure 3A, UpApU synthesis from variant S(TT), carried out by promoter-bound RNA polymerase, is stimulated almost 3-fold in the presence of 0.75 μM P4. No such stimulatory effect of P4 was observed in similar titration experiments with the other two promoters. At higher concentrations of P4, a decrease in UpApU synthesis was observed at all three promoters. The addition of P5 (optimal concentration 1 μM—data not shown) stimulated open complex formation at promoter S(TT) approximately 2-fold, but not at promoters S(wt) and S(AT). Unlike distamycin, P4, or P5, P6 did not enhance transcription from any of the promoter variants. As

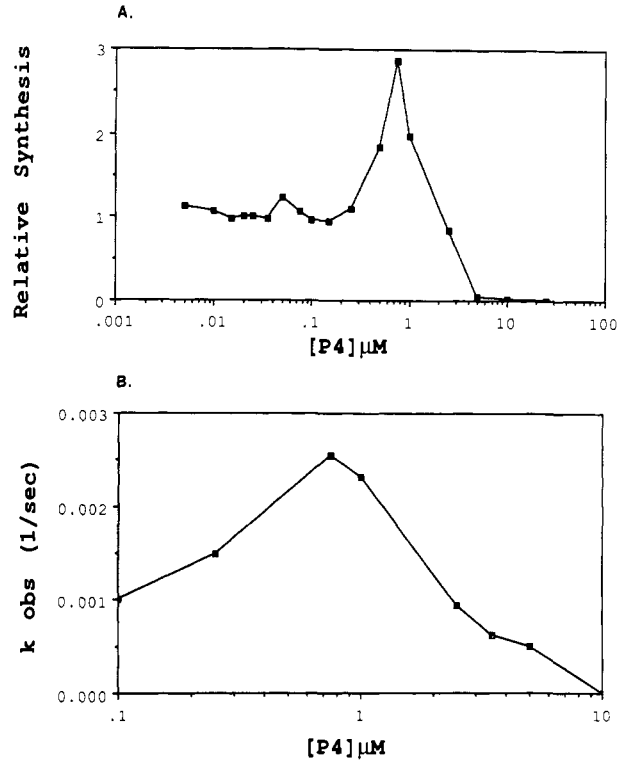

FIGURE 3: Concentration dependence of the effect of P4 on open complex formation at promoter variant S(TT)—see Figure 1. (A) Effect of P4 on the activity of the promoter. Various concentrations of oligopeptide (0–10 μM) were preincubated with promoter DNA prior to the addition of RNA polymerase. After allowing time for open complex formation in the presence of polymerase, nucleotides were added, and the amount of UpApU synthesized in 10 min at 37 °C from P_{RM} variant S(TT) was quantitated. The data are expressed relative to the amount of UpApU synthesized in the absence of added peptide. (B) P4 concentration dependence of the rate of open complex formation. RNA polymerase (22 nM) was added to a reaction mixture containing promoter DNA and the nucleotides [^{32}P]UTP and UpA. At various times, an aliquot was removed, and the amount of UpApU synthesis which occurred in that time was determined by ascending chromatography. From these data a value for k_{obs} was obtained (McClure, 1980; Bruzik et al., 1987).

was the case with distamycin and P4, P5 and P6 also had inhibitory effects at high concentrations (≥5 μM).

We previously demonstrated that the stimulatory effect of distamycin on S(TT) was primarily due to its effect on k_{obs}, the reciprocal of the lag time for functional complex formation (Bruzik et al., 1987), rather than on the steady-state rate of synthesis of UpApU by RNA polymerase bound at this promoter. In the presence of maximally stimulating concentrations of distamycin, the value for k_{obs} was shown to increase 2–3-fold. The data in Figure 3B show that, as with distamycin, the value of k_{obs} for open complex formation at S(TT) in the presence of P4 at maximally stimulating concentrations is almost 3-fold higher than that in the absence of added oligopeptide. A summary of the effect of the distamycin analogues on UpApU synthesis from each of the promoter variants is given in Figure 4, where the greatest relative level of stimulation resulting from the addition of each oligopeptide is recorded. For variant S(TT) the optimal number of *N*-methylpyrrolecarboxamide rings for stimulation of open complex formation is four.

In view of their similar behavior in these assays, it is likely that distamycin and P4 are affecting the same steps during the process of open complex formation. A simplified scheme for open complex formation (McClure, 1980) distinguishes a binding step to form the inactive closed complex, followed by an isomerization to yield the initiation-competent open

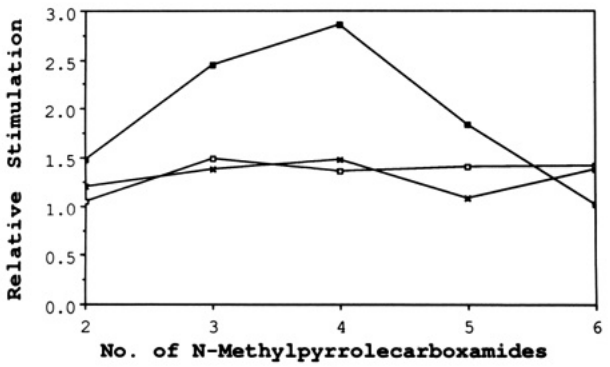

FIGURE 4: Optimal stimulation of promoter-dependent UpApU synthesis as a function of the structure of the distamycin analogues. The *x* axis indicates the number of *N*-methylpyrrolecarboxamide residues of each ligand. The relative level of stimulation resulting from the addition of each oligopeptide to transcription reactions was evaluated in titration experiments as described under Materials and Methods. The values for ligands with two and three *N*-methylpyrrolecarboxamide residues (netropsin and distamycin, respectively) are from Bruzik et al. (1987). S(TT) (■); S(AT) (×); S(wt) (□).

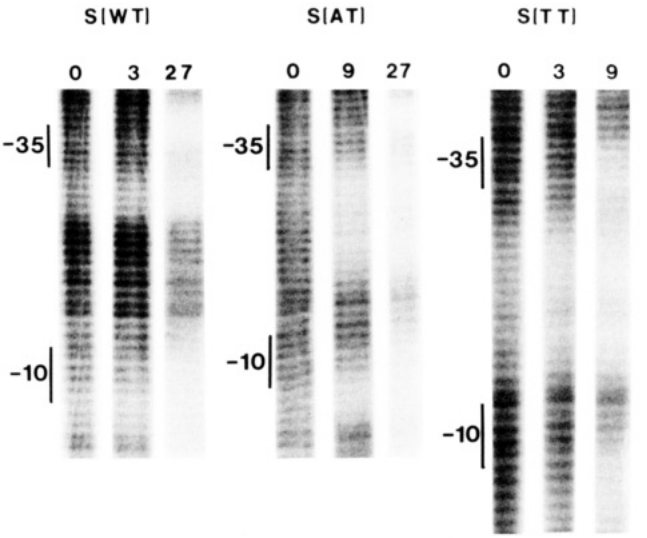

FIGURE 5: MPE–Fe(II) footprints of distamycin bound to the promoter variants S(wt), S(TT), and S(AT). *Xmn*I–*Nar*I promoter fragments bearing the promoter of interest were preincubated for 20 min at 20 °C with varying concentrations of distamycin. Cleavage (10 min at 37 °C) was initiated by the addition of a freshly prepared solution of MPE–Fe(II) and DTT. Cleavage products were resolved by electrophoresis using 6% urea gels. (Lanes 1–3) 0, 3, and 27 μM distamycin on S(wt); (lanes 4–6) 0, 9, and 27 μM distamycin on S(AT); (lanes 7–9) 0, 3, and 9 μM distamycin on S(TT). Relevant regions of the promoters are indicated.

complex. Our previous experiments (Bruzik et al., 1987) indicated that the addition of distamycin facilitated the initial binding of RNA polymerase to the promoter without affecting the isomerization step. It appears reasonable that P4 and P5 operate in a fashion similar to distamycin to stimulate formation of an open complex.

The availability of a collection of similar peptides that differed in their ability to facilitate open complex formation, and of promoters that differed in their ability to be stimulated, offered the possibility of correlating ligand binding mode with stimulatory behavior. Therefore MPE–Fe(II) footprinting studies (Van Dyke & Dervan, 1983) were undertaken in order to probe the binding behavior of distamycin, P4, P5, and P6 on the promoter variants. Because single base pair resolution can be obtained, this is a useful method for determining the location and sizes of DNA binding sites of these peptides. DNA regions protected from MPE–Fe(II) cleavage by bound oligopeptide were defined in titration experiments on specifically end-labeled *Xmn*I–*Nar*I fragments bearing the promoter of interest. Representative footprints of distamycin bound to S(TT), S(AT), and S(wt) are shown in Figure 5. Several observations can readily be made from these data. In the absence of added distamycin, cleavage by MPE–Fe(II) is attenuated in the spacer region of S(TT), but not in that of S(AT). Such attenuation of cleavage by the agent Fe-EDTA in stretches of nonalternating AT base pairs has been reported (Burkhoff & Tullius, 1987) and is thought to reflect a narrowing of the minor groove (Nelson et al., 1987; Yoon et al., 1988). Other studies have demonstrated that intercalators, like methidium, interact differently with these two sequences (Wilson et al., 1985). It is likely that this differential reactivity with respect to cleavage by MPE–Fe(II) is indicative of a structural difference between the stretches of nonalternating and alternating AT base pairs in our promoter constructs. Inspection of the gel patterns shown in Figure 5 also shows that at low concentrations of distamycin the spacer regions of S(TT) and S(AT) are protected from cleavage but the bracketing −10 and −35 regions are not, indicating preferential binding of distamycin to the spacer DNA. At higher concentrations of oligopeptide, protection of the −10 and −35 regions of these promoters becomes visible. In contrast, no such preferential oligopeptide binding to the spacer DNA of S(wt) can be detected.

In order to quantitatively assess promoter-specific differences in cleavage protection patterns, densitometry was conducted on autoradiographs of gels from the footprinting experiments. From a comparison of the cleavage patterns in the presence and absence of added oligopeptide (see Materials and Methods), the regions of DNA protected by the oligopeptides could be assigned. Representative difference scans are shown in Figure 6. As is evident from this figure, the regions of spacer DNA protected by the oligopeptides are different for the three promoters under investigation. Two clearly defined regions in the spacer DNA of S(TT) are protected from MPE cleavage by distamycin (and also by P4 and P5—data not shown), each large enough to be a binding site [each molecule covers one more base pair than its number of *N*-methylpyrrolecarboxamide rings (Youngquist & Dervan, 1985)]. For each of the three oligopeptides these two regions are separated by a stretch of DNA, located between positions −20 and −25, that is not protected from cleavage; with P6 a contiguous region of protection is observed, large enough to accommodate several molecules (Figure 6 and data not shown). On S(AT), a contiguous region of protection is seen for distamycin (Figure 6), P4, P5, and P6 (data not shown), without being interspersed with a less protected stretch. Unlike the situation for either of these two promoter variants, only a region of the spacer DNA of S(wt) large enough to represent the binding site of one peptide molecule is protected: even at high concentrations, no protection is detected in the DNA between the −10 region and position −20.

To determine the relative affinities of the ligands for the −10 and −35 regions and for the spacer DNA, six positions were selected for study on the basis of the regions of protection found: two in the −10 region, two in the spacer region, and two in the −35 region. These positions are indicated by arrows in the densitometer tracings of Figure 6. The height of each peak analyzed in the scan served as a measure of the amount of protection by bound oligopeptide in a given region of the promoter. From the extents of protection at these positions at several peptide concentrations, the concentration at which the spacer, the −10 region, and the −35 region of each variant was half-maximally protected was determined by interpolation.

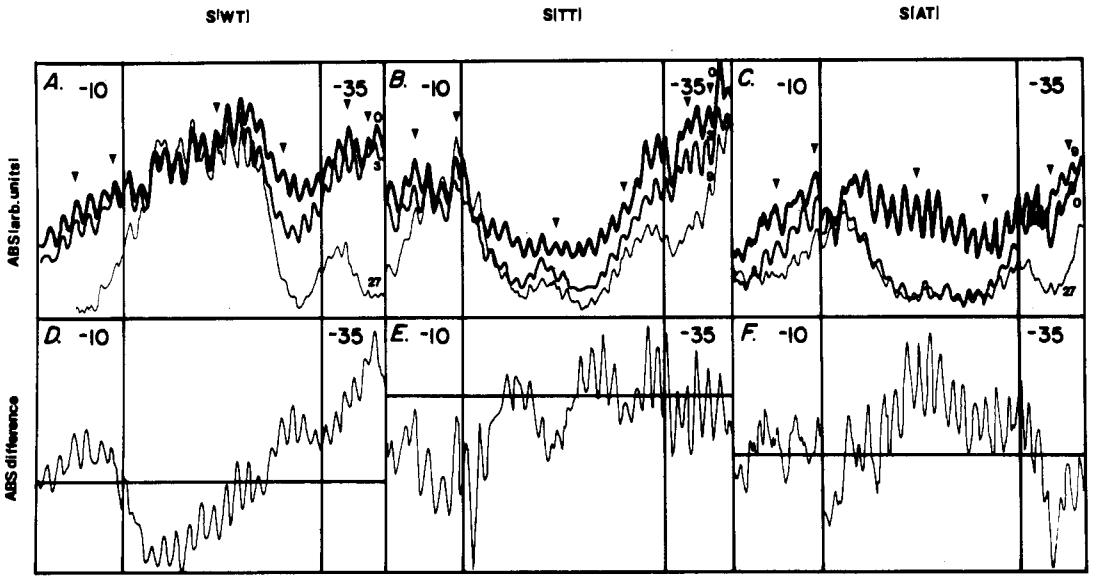

FIGURE 6: Densitometric analysis of the MPE–Fe(II) footprinting experiments. (A–C) Densitometric scans of autoradiographs of gels such as that shown in Figure 5 for distamycin binding to S(TT), S(wt), and S(AT). Scans were obtained on an LKB laser densitometer. The regions of each scan corresponding to the −10 region, the spacer, and the −35 region of the promoter variant are indicated. Those positions where cleavage was quantitated as a function of peptide concentration as described under Materials and Methods are marked with an arrow. The ordinate and the abscissa represent absorbance and gel position, respectively. To facilitate comparisons, the scans for each promoter were constrained to go through the same point at position −27 (arbitrarily chosen); consequently, the ordinate represents a different absorbance scale for each scan. (A) S(wt), 0, 3, and 27 μM distamycin; (B) S(TT), 0, 3, and 9 μM distamycin; (C) S(AT), 0, 9, and 27 μM distamycin. (D–F) Difference scans. The scans obtained in the absence of peptide were subtracted from those obtained at the indicated distamycin concentration for each of the promoters. The height of a peak in the resulting difference scan is a measure of the extent to which a particular base in the promoter was protected from cleavage. (D) S(wt), 0–27 μM distamycin; (E) S(TT), 0–3 μM distamycin; (F) S(AT), 0–27 μM distamycin.

Positions −32 and −34 in the −35 region were equally sensitive to the presence of peptide, but in almost every case (for each promoter and each oligopeptide tested) position −9 in the −10 region was protected at a lower concentration than was position −12. The data presented in Figure 7 are for the position where protection occurred more readily. The values for the concentration of peptide affording half-maximal protection were then normalized to that determined for the −35 region; this value was independent of the position and similar for all three promoters at 20 ± 10 μM. The data in Figure 7 represent the inverse of the values for each region; this parameter was chosen as it should be proportional to the relative affinities of each ligand for a particular site.

Again it is apparent that only one binding site in the spacer region of S(wt) is filled by any of the peptides prior to protection of the −10 or −35 region of this promoter; no stimulation of RNA polymerase binding to this promoter is observed. On the other hand, distamycin, P4, and P5, which can enhance the rate of open complex formation at S(TT), can bind to both sites in the spacer DNA of S(TT) without binding to the −10 or −35 region. P6, which does not stimulate transcription from this promoter, protects the −35 region of the promoter at a lower concentration of oligopeptide than required for protection of the spacer region. Analogously to S(TT), distamycin, P4, and P5 can bind in the spacer region of S(AT) independent of binding to either the −10 or −35 region. However, transcription from this promoter variant is not stimulated by distamycin or its homologues. Thus preferential occupancy of the spacer DNA by these ligands might be required but is certainly not sufficient for stimulation of open complex formation.

Discussion

We have demonstrated that distamycin and the distamycin analogues P4 and P5 stimulate open complex formation at S(TT). As the S(TT) variant has a similar rate of open complex formation as its parent, S(wt) (Auble et al., 1986; Bruzik et al., 1987), the observed effect is a true stimulation rather than the correction of a defect introduced by the substitution of the A-T base pairs in the spacer region. The fact that there are differences in activation with different oligopeptides and that not all the oligopeptides serve to stimulate transcription from S(TT) suggests that we are not detecting a nonspecific effect of these oligopeptides on open complex formation. One model to account for the stimulation at variant S(TT) is that the peptide binds to the spacer region such as to introduce a conformational change in the promoter which results in a more favorable interaction with polymerase. This model, which emphasizes the role of DNA structure in mediating transcriptional activation (Bruzik et al., 1987), is supported by the results of ligand titration and footprinting experiments reported in this paper.

While distamycin, P4, and P5 can all stimulate open complex formation at promoter variant S(TT), maximal stimulation by P4 and by P5 is achieved at a 10-fold higher concentration than that by distamycin (0.75 μM vs 0.08 μM). Protection from MPE cleavage also requires a proportionally higher amount of P4 and P5 as compared to distamycin. The requirement for lower concentrations of distamycin in both footprinting and titration experiments suggests that the promoters have a higher affinity for distamycin than for P4 and P5. In contrast to these observations, methyl green displacement studies have demonstrated that the binding affinity of the distamycin analogues for DNA increases with increasing numbers of *N*-methylpyrrolecarboxamides (Zunino & DiMarco, 1972). One explanation for this difference in apparent binding affinity is that the relative binding affinities of P3–P6 could be sequence dependent.

We consistently observe that a 10-fold higher concentration of oligopeptide is required to see an effect in the binding assays (by footprinting) than in the functional assays (abortive synthesis). This is observed for all oligopeptides tested and for all promoters. The higher concentrations of ligand required in the footprinting experiments are most likely needed to ov-

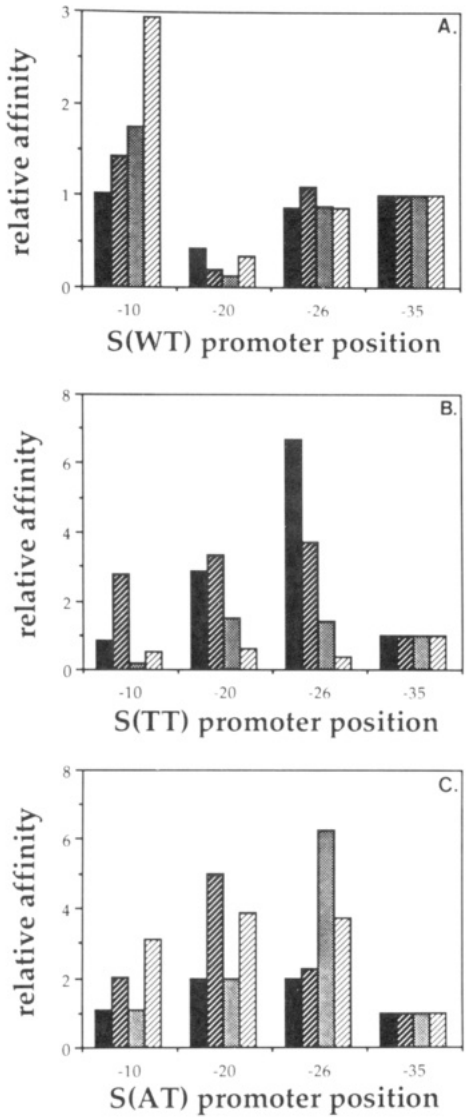

FIGURE 7: Relative affinities of the various ligands tested for promoter variants S(wt), S(TT), and S(AT). Densitometric scans, such as those shown in Figure 6, were used to evaluate cleavage at positions −9 and −12 in the −10 region, positions −20 and −26 in the spacer, and positions −32 and −34 in the −35 region as a function of peptide concentration. From these scans the concentrations of peptide which afforded half-maximal protection to each of the regions were determined as described under Materials and Methods. These concentrations were expressed relative to the value determined for the −35 region of the promoter to give a relative concentration for half-maximal protection. The parameter shown as the "affinity" on the ordinate is the reciprocal of this, e.g., the inverse relative concentration of peptide. This parameter should allow a comparison of the relative affinities of distamycin, P4, P5, and P6 for the various regions. Each group of four bars represents, from left to right, the values for distamycin, P4, P5, and P6, respectively.

ercome binding of the oligopeptides to the calf thymus DNA (concentration of base pairs 100 μM) included in the cleavage reactions although displacement of the ligand by the added MPE itself might also be a factor. The finding that distamycin not only binds DNA at a lower concentration than the other oligopeptides but also has a lower optimal concentration for stimulation of open complex formation supports the notion that the stimulatory behavior derives from DNA-bound ligand.

This conclusion is strengthened by the footprinting results of the ligands on the various promoters. If DNA-bound ligand is responsible for the observed stimulation of open complex formation at S(TT), it would be expected that the ligands preferentially bound to the spacer DNA, as compared to the −10 and −35 regions. The reason for this expectation is as follows: the differences in S(TT), S(AT), and S(wt) reside here, and the −10 and −35 regions are sites of direct contact with RNA polymerase (Siebenlist et al., 1980), while the spacer DNA is not (Auble et al., 1986). Bound ligand at the −10 and −35 regions would interfere with polymerase binding. This is what is observed: distamycin, P4, and P5 cover the spacer DNA of S(TT) at lower concentrations than those required for binding to the −10 and −35 regions (see Figure 7). As the same relative (and absolute) affinity for the spacer DNA is also evident for the binding to S(AT), where no stimulation is observed, the interaction of the peptides with the spacer DNAs of the two promoters would have to be dissimilar.

Differences in the way the peptides interact with the different promoters become apparent after analysis of the distribution of protected regions in our promoter variants (see Results). With S(TT) discrete regions of protection are evident, while the binding at S(AT) seems to be delocalized. We speculate that the difference in binding at the two promoters might be due to the reported (Breslauer et al., 1987; C. Grygon, personal communication) differences in the interaction of distamycin with the two polymers poly(dAdT)·poly(dAdT) and poly(dT)·poly(dA), which are themselves structurally different (Peck & Wang, 1981; Rhodes & Klug, 1981; Nelson et al., 1987; Coll et al., 1987; Yoon et al., 1988). Possibly the two DNA sequences are also differentially sensitive to the influence of flanking DNA regions. The influence of flanking DNA on the binding of netropsin has previously been demonstrated (Schultz & Dervan, 1984). To what extent the observed differences in the binding regions of the oligopeptides on S(TT) and S(AT) are related to the differential activation of these two promoters is unclear.

While we postulate that the spacer-bound ligands exert their stimulatory effect by altering the relative position of the −10 and −35 regions so that they are now more favorably positioned to bind RNA polymerase, we do not know the nature of the oligopeptide-induced structural change in the DNA. Possibilities include bending the DNA (Kopka et al., 1985) and changing its pitch (Snounou & Malcolm, 1983). Elsewhere, we (Auble & deHaseth, 1988; Ayers et al., 1988) and others (Amouyal & Buc, 1987) argue that open complex formation involves the distortion of the spacer DNA early in the process, followed by the use of the stored up free energy to drive a later step. We have demonstrated that distamycin facilitates the initial binding step, but subsequent processes leading to open complex formation are not affected (Bruzik et al., 1987). Thus bound ligand would not affect the introduction and release of distortion free energy. We speculate that rather the spacer-bound peptide would subtly alter the way the −10 or −35 region is presented to RNA polymerase, enabling at these regions the formation of additional favorable contacts between the enzyme and the DNA.

The fact that an optimum is observed in the stimulation of UpApU synthesis can be explained if the extent of structural alteration introduced by the peptides is related to their size. Then P4 would induce the most favorable orientation of the −10 and −35 regions for RNA polymerase binding while P5 and P6 position the −10 and −35 regions beyond their most favorable orientation. To explain the differential activation of S(TT) as compared to that of S(AT), we envisage that, although the peptides have the capacity to bind both promoters, only the conformational change which would occur when they were bound to the spacer region of S(TT) would promote a favorable interaction with polymerase.

ACKNOWLEDGMENTS

We appreciate the careful review of the manuscript by the referees.

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Comparison of the Data, Analysis, and Results of X-ray Absorption Studies of Cytochrome *c* Oxidase

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Received February 4, 1988; Revised Manuscript Received November 14, 1988

ABSTRACT: Differences in the methods of analysis of X-ray absorption data used by Powers et al. [Powers, L., Blumberg, W. E., Chance, B., Barlow, C., Leigh, J., Jr., Smith, J., Yonetani, T., Vik, S., & Peisach, J. (1979) *Biochim. Biophys. Acta 547*, 520–538; Powers, L., Chance, B., Ching, Y., & Angiolillo, P. (1981) *Biophys. J. 34*, 465–498] and Scott et al. [Scott, R., Schwartz, J., & Cramer, S. (1986) *Biochemistry 25*, 5546–5555] are clarified. In addition, we compare the X-ray absorption data and results for resting cytochrome *c* oxidase reported by both groups using the same analysis method and conclude apart from any assumptions that the data are not identical.

In order to clarify the differences and disagreements between our results (Powers et al., 1979, 1981) and those of Scott et al. (1986) (SSC) apart from any assumptions in the analysis methods, this paper compares directly the data and results of X-ray absorption studies of resting cytochrome *c* oxidase from both groups. We point out that SSC have not represented correctly the use of the site modeling method (Powers et al., 1981). Taking into account the reduction in number of degrees of freedom in Fourier-filtered EXAFS data, application of fitting methods to the published data of both groups shows that the data are not identical. Comparison of the analysis methods of both in view of the assumptions inherent in each clarifies the differences in the proposed respective models.

Cytochrome *c* oxidase contains four metal redox sites, two copper and two iron. One iron and copper pair functions as an electron reservoir, Cu_a and Fe_a, while the other, Cu_{a_3} and Fe_{a_3}, comprise the active O_2 binding site. When the active site

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0006-2960/89/0428-4461$01.50/0