Bacterial promoter architecture: subsite structure of UP elements and interactions with the carboxy-terminal domain of the RNA polymerase α subunit

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We demonstrate here that the previously described bacterial promoter upstream element (UP element) consists of two distinct subsites, each of which, by itself, can bind the RNA polymerase holoenzyme α subunit carboxy-terminal domain (RNAP αCTD) and stimulate transcription. Using binding-site-selection experiments, we identify the consensus sequence for each subsite. The selected proximal subsites (positions −46 to −38; consensus 5′-AAAAAARNR-3′) stimulate transcription up to 170-fold, and the selected distal subsites (positions −57 to −47; consensus 5′-AWWWWWTTTTT-3′) stimulate transcription up to 16-fold.

RNAP has subunit composition α2ββ′σ and thus contains two copies of αCTD. Experiments with RNAP derivatives containing only one copy of αCTD indicate, in contrast to a previous report, that the two αCTDs function interchangeably with respect to UP element recognition. Furthermore, function of the consensus proximal subsite requires only one copy of αCTD, whereas function of the consensus distal subsite requires both copies of αCTD. We propose that each subsite constitutes a binding site for a copy of αCTD, and that binding of an αCTD to the proximal subsite region (through specific interactions with a consensus proximal subsite or through nonspecific interactions with a nonconsensus proximal subsite) is a prerequisite for binding of the other αCTD to the distal subsite.

[Key Words: Promoter; RNA polymerase; α subunit; UP element; transcription initiation]

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Bacterial promoters consist of at least three RNA polymerase (RNAP) recognition sequences: The −10 element, the −35 element, and the UP element (Hawley and McClure 1983; Ross et al. 1993). The −10 and −35 elements are recognized by the RNAP σ subunit [Dombroski et al. 1992], and the UP element, located upstream of the −35 element, is recognized by the RNAP α subunit [Ross et al. 1993; Blatter et al. 1994]. The best-characterized UP element is in the rnb P1 promoter, in which the sequence determinants are located between positions −40 and −60 with respect to the transcription start site [Rao et al. 1994], and UP element-α interactions facilitate initial binding of RNAP and subsequent step[s] in transcription initiation [Rao et al. 1994; Strainic et al. 1998]. A consensus UP element sequence [referred to here as the consensus full UP element], derived from binding-site-selection experiments, consists almost exclusively of A and T residues and increases promoter activity >300-fold [Estrem et al. 1998]. UP elements have been identified upstream of many bacterial and phage promoters and can function with RNAPs containing different σ factors [e.g., Newlands et al. 1993; Ross et al. 1993, 1998; Fredrick et al. 1995].

Each RNAP α subunit consists of two domains connected by a long unstructured and/or flexible linker [Blatter et al. 1994; Jeon et al. 1997]. The 28-kD amino-terminal domain αNTD is responsible for dimerization of α and for interaction with the remainder of RNAP [Igarashi and Ishihama 1991; Busby and Ebright 1994]. The 8-kD carboxy-terminal domain αCTD is responsible for interaction with the UP element [Blatter et al. 1994] and with a number of transcriptional activators [Igarashi and Ishihama 1991; Busby and Ebright 1994; Savery et al. 1998]. The αCTD residues most crucial for DNA interaction are nearly invariant in bacteria [Gaal et al. 1996; Murakami et al. 1996], and therefore the DNA sequences recognized by α are also likely to be highly conserved. The interdomain linker presumably accounts for the ability of αCTD to interact with DNA and/or activator molecules at different locations upstream of the −35 element [Newlands et al. 1992; Blatter et al. 1994].

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promoters were cloned as phage λ-borne lacZ fusions, and transcription activities were assessed by plating on MacConkey-lactose indicator agar. On the basis of plaque color, at least 90% of the selected promoters were more active than the control promoter lacking an UP element, and remarkably, ~30% were even more active than the wild-type rrnB P1 promoter.

Nineteen clones with the darkest red plaque color were analyzed by DNA sequencing, and eight different proximal subsite sequences were identified (Figs. 1A–C). Six of the eight sequences contained a perfect A tract from −46 to −41, and the remaining two contained near-perfect A tracts (interrupted only by a T at position −42 or by a C at −46). There also was a bias for purines at positions −38 and −40. We quantified promoter activities by measuring β-galactosidase activities of strains monolysogenic for phages containing the promoter–lacZ fusions. The proximal subsites stimulated transcription 82- to 170-fold (Fig. 1B), which is more than the stimulation observed with the full UP element from rrnB P1 (69-fold), but less than the stimulation observed with the consensus full UP element (330-fold, Estrem et al. 1998).

To provide information about the relative importance of individual positions for function, we introduced single transversions into a representative selected proximal subsite [promoter 4547, 130-fold stimulation, Fig. 2]. Each substitution decreased transcription: substitutions at −41, −42, or −43 decreased proximal subsite function strongly [to 6- to 10-fold stimulation; 5%–8% of the effect of the parent proximal subsite]; substitutions at −44 and −45 decreased transcription moderately [to 34- to 37-fold stimulation; 26%–28% of the parent]; and substitutions at positions −38, −39, −40, and −46 decreased transcription modestly [to 68- to 96-fold stimulation; 52%–74% of the parent]. Taken together, the nucleotide frequencies from the binding-site-selection experiment [Fig. 1C] and the mutational analysis of a consensus proximal subsite [Fig. 2] suggest that positions −41 to −43 are most crucial for proximal subsite function.

Like the single transversion mutants, the selected sequence 4542 also contains a single base pair change from the sequence in proximal subsite 4547 [Fig. 1]. In this case, however, the subsite has a T at position −42, yet exhibited full function in stimulating transcription. Furthermore, the rrnB P1 proximal subsite contains T at each of the three critical positions −41, −42, and −43, yet still stimulated transcription moderately (20-fold, Fig. 1B). We conclude that UP elements with T substitutions at these positions retain substantial function (see Discussion).

**Identification of optimal distal subsite sequences**

To determine whether the distal UP element subsite can function without a proximal subsite and to define the optimal sequence for the distal subsite, we performed binding-site-selection experiments analogous to those used to define the consensus full UP element [Estrem et al. 1998]. We constructed a library of DNA fragments containing the rrnB P1 core promoter, randomized DNA sequences in the proximal subsite region [−46 to −38], and a sequence shown previously to lack UP element function in the distal subsite region [Fig. 1A]. [Position −37 was not randomized, because it was shown previously that cytosine is critical at this position in rrnB P1 [Josaitis et al. 1990].]

We incubated RNAP with the DNA fragment library for a time limiting for RNAP-promoter complex formation, blocked further RNAP–promoter complex formation by addition of heparin, isolated RNAP–promoter complexes by nondenaturing PAGE, and amplified promoter DNA from RNAP–promoter complexes by PCR. After 13 cycles of selection and amplification by increasingly stringent conditions [see Materials and Methods], several lines of evidence suggest that the consensus full and rrnB P1 UP elements each contains two parts, that is, a proximal subsite, centered at about position −42, and a distal subsite, centered at about position −52 (Ross et al. 1993; Estrem et al. 1998). First, the rrnB P1 proximal subsite, in the absence of the rrnB P1 distal subsite, is protected by RNAP in hydroxyl radical DNA footprinting experiments and exhibits partial ability to stimulate transcription [Leirmo and Gourse 1991; Newlands et al. 1991; Rao et al. 1994]. Second, the proximal subsite of the rrnB P1 UP element, by itself, is able to cooperate with CAP [galactose activator protein] in CAP-dependent transcription [Czarniecki et al. 1997; Noel and Reznikoff, 1998; Law et al. 1999]. Third, the proximal and distal subsites of the rrnB P1 UP element can be separated by insertion of 11 bp without loss of protection of either subsite by RNAP and without loss of the ability to stimulate transcription [Newlands et al. 1992]. Fourth, in DNA affinity-cleaving experiments with an RNAP derivative containing Fe-EDTA incorporated into αCTD, two sets of cleavages are observed in the rrnB P1 UP element—one in the proximal subsite and one in the distal subsite [Murakami et al. 1997a].

Here we define consensus sequences for individual UP element subsites and determine the number of copies of αCTD required to interact with and respond to full UP elements and individual UP element subsites. The results have important implications for UP element structure/function and for promoter architecture.

**Results**

**Identification of optimal proximal subsite sequences**

To confirm that the proximal UP element subsite can function without a distal subsite and to define the optimal sequence for the proximal subsite, we performed binding-site-selection experiments analogous to those used to define the consensus full UP element [Estrem et al. 1998]. We constructed a library of DNA fragments containing the rrnB P1 core promoter, randomized DNA sequences in the proximal subsite region [−46 to −38], and a sequence shown previously to lack UP element function in the distal subsite region [Fig. 1A]. [Position −37 was not randomized, because it was shown previously that cytosine is critical at this position in rrnB P1 [Josaitis et al. 1990].]

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then performed binding-site-selection experiments and in vivo assays analogous to those used for the proximal subsite selection described above. On the basis of plaque color, ~50% of the resulting selected promoters exhibited activities greater than that of the control promoter lacking an UP element, but none of these promoters were as active as \textit{rrnB} P1. From 21 clones producing the darkest red plaques, 19 different distal subsite sequences were identified (Fig. 3B,C). The sequences had a high frequency of A residues at −57, A or T from −56 to −53, and T from −52 to −47 (Fig. 3C), and stimulated transcription 4- to 16-fold (Fig. 3B). This level of transcription stimulation is less than that observed with the consensus full UP element, the consensus proximal subsite, or even the \textit{rrnB} P1 proximal subsite.

The \textit{rrnB} P1 distal subsite closely matches the bind-
ing-site-selected distal subsites. We had previously con-structed overlapping triple substitutions in the \textit{rrnB} distal subsite and measured their effects as promoter–lacZ fusions to obtain information about individual residues important for function (Estrem 1998). All triple substitutions in the distal subsite decreased transcription at least threefold, and the substitution centered at position –52 decreased transcription the most (approximately six-fold).

Relationship between the consensus full UP element and the consensus subsite sequences

The distributions of nucleotides at each position in the selected proximal and distal subsite sequences are pictured in diagram form in Figure 4A and compared with the distribution obtained in the previously described full UP element selection (Estrem et al. 1998). Fig. 4B presents the derived consensus sequences.

The consensus proximal subsite sequence is related to the corresponding proximal region in the consensus full UP element, but differs in substantive ways. The consensus proximal subsite includes the three specified positions from the corresponding segment of the consensus full UP element, –41, –42, and –43, but it also contains five additional specified positions, with strong preference for A at –44, –45, and –46 and for purine at –38 and –40 (Fig. 4B).

In contrast, the consensus distal subsite sequence is almost identical to that of the corresponding sequence

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Distal subsites. Sequences and relative activities of 19 promoters selected for binding of RNAP in vitro and screened for high transcription in vivo. Details are described in Fig. 1, except that the promoters contained different distal regions (filled rectangle; –59 to –46) and the SUB sequence in the proximal region (–45 to –38 CTAGGAAT). The randomized residues are indicated as N and displayed in context (non-template strand) below the schematic. The –35 hexamer is in boldface type. Distal region sequences are shown for the 19 promoters, for an \textit{rrnB} construct containing only the distal region of the \textit{rrnB} UP element (\textit{rrnB} distal; RLG3099), and for a construct lacking an UP element (No UP; RLG3097). Distal subsite 4513 is described in the text.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Consensus sequences. (A) Frequency diagrams of residues in the binding-selected full UP elements (from Estrem et al. 1998) and in the binding-selected proximal and distal subsites (from Figs. 1C and 3C). Each nucleotide is represented as a letter proportional in size to its frequency at that position in the selected population. The non-template strand positions protected by RNAP in hydroxyl radical footprints (Estrem et al. 1998; Fig. 6) are indicated by lines. (B) Consensus subsite sequences based on the nucleotide frequencies. One nucleotide is indicated when it is present in >70% of the population, or two when together they represent 95% or more of the population. W = A or T; R = A or G; N = no single base pair present in 70% of the population and no 2 bp make up 95% of the population.}
\end{figure}
from the *rrnB* P1 and consensus full UP elements [Estrem et al. 1998]. We constructed promoters containing the distal subsite from *rrnB* P1 or full UP element 4192 [Estrem et al. 1998] and containing a nonfunctional proximal region. The resulting UP elements stimulated transcription in vivo 9- and 16-fold, respectively, consistent with their sequence similarity to the binding-selected distal subsites (*rrnB* P1 Distal and 4513; Fig. 3B). The most active proximal and distal subsite sequences (4549 and 4513; Figs. 1 and 3), were combined to create a composite UP element [4541; −59 5′-GGAAAATTTTTT-TTAAAAAAGA-3′](Estrem et al. 1998). We constructed promoters containing a nonfunctional proximal region. The resulting UP elements stimulated transcription in vivo 9- and 16-fold, respectively (Fig. 5). (Under the same conditions, a consensus full UP element was 340-fold [data not shown], which is very similar to the effect of the consensus full UP element [330-fold; Estrem et al. 1998]. Nevertheless, the stimulatory effect is far below that expected for the product of the effects of the two individual subsites (16-fold × 170-fold = 2720-fold), suggesting that the observed 330- to 340-fold increase represents the limit for activation of the *rrnB* P1 core promoter in vivo and/or that in consensus full UP elements the two subsites do not function independently (see Discussion).

The consensus proximal and distal subsites stimulate transcription through interactions with αCTD

In vitro transcription experiments were performed to establish that individual consensus proximal and distal subsites, by themselves, stimulate transcription through interactions with αCTD. The consensus proximal and distal subsites increased transcription 10- and 9-fold, respectively (Fig. 5). Under the same conditions, a consensus full UP element and the *rrnB* P1 UP element stimulated transcription by 47- and 21-fold, respectively. The single base pair substitutions in the proximal subsite that decreased transcription in vivo (Fig. 2) also decreased transcription in vitro (data not shown). We conclude that the individual consensus subsites stimulate transcription and that this stimulation requires no components other than promoter DNA and RNAP.

We note that the consensus proximal subsite stimulated transcription less well in vitro than in vivo [10-fold vs. 130-fold, Figs. 5 and 1], whereas the consensus distal subsite stimulated transcription similarly in vitro and in vivo [9-fold vs. 16-fold, respectively, Figs. 5 and 3]. The quantitative difference in vitro versus in vivo for the effect of the proximal subsite may reflect differences in limiting steps to which the assays are sensitive, differences in solution conditions, differences in supercoiling, or the absence/presence of potential accessory factors.

To assess the dependence of transcription stimulation on αCTD–DNA interaction, we performed parallel in vitro transcription experiments with two mutant RNAP derivatives: αΔ235 RNAP, which completely lacks the αCTD, and αR265A RNAP, which has a single amino acid substitution that disrupts αCTD–DNA interaction [Gaal et al. 1996; Murakami et al. 1996]. The individual consensus proximal and distal subsites, like the *rrnB* P1 and consensus full UP element, failed to stimulate transcription with αΔ235 RNAP and αR265A RNAP (Fig. 5).

We conclude that transcription stimulation by individual consensus subsites absolutely requires αCTD–DNA interaction.

The consensus proximal and distal subsites are binding sites for αCTD

We performed hydroxyl radical DNA footprinting experiments using RNAP and promoters containing only a consensus proximal subsite or only a consensus distal subsite [Fig. 6A–D]. In each case, strong protection (i.e., protection comparable to that in the −35 element region) was observed in the consensus subsite, and only weak protection was observed in the nonconsensus subsite. We also performed hydroxyl radical DNA footprinting experiments using purified α and promoters containing only a consensus proximal subsite or only a consensus distal subsite [Fig. 6A, B, E, F]. In each case, preferential protection was observed in the consensus subsite. (Weaker protection was observed also in the nonconsensus subsite and −10 bp downstream from the consensus proximal subsite. α is a dimer, therefore, the weak protection may be attributable to nonspecific interactions with the second αCTD.) We conclude that a single consensus subsite is sufficient for binding αCTD, both with RNAP and with purified α.

Transcription stimulation by the consensus proximal subsite, but not by the consensus distal subsite, requires only one copy of αCTD

RNAP contains two α subunits: α[1] and α[II] (where α[1] is encoded RNA I transcript (Estrem et al. 1998)). We performed hydroxyl radical DNA footprinting experiments with two mutant RNAP derivatives: αD235 RNAP, which completely lacks the αCTD–DNA interaction, and αR265A RNAP, which has a single amino acid substitution that disrupts αCTD–DNA interaction [Gaal et al. 1996; Murakami et al. 1996]. The individual consensus proximal and distal subsites, like the *rrnB* P1 and consensus full UP element, failed to stimulate transcription with αΔ235 RNAP and αR265A RNAP (Fig. 5).

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defined as the subunit that interacts with the β subunit; see Heyduk et al. 1996). To determine whether transcription stimulation by UP element subsites requires a CTD of αI, a CTD of αII, or both CTDs, we prepared and analyzed two oriented-α RNAP derivatives: αI/αDII, in which only αI contains a CTD; and αDII/αII, in which only αII contains a CTD. To prepare oriented-α RNAP, we took advantage of the R45A substitution in α, which results in an α that is unable to interact with β, and thus is unable to serve as αI (Kimura and Ishihama 1995; Murakami et al. 1997a). We coexpressed genes encoding one α derivative with the R45A substitution and a hexahistidine affinity tag and a second α derivative without the R45A substitution and hexahistidine tag, lysed the cells, and isolated RNAP using metal-ion-affinity chromatography (see Materials and Methods; W. Niu and R.H. Ebright, in prep.).

We performed in vitro transcription experiments with the oriented-α RNAP derivatives and a promoter containing a consensus full UP element. Both αI/αΔII and αΔI/αII transcribed the promoter about one-third as well as wild-type RNAP (Fig. 7, left). The reduction in promoter activity in vitro on elimination of one αCTD was almost as much as the reduction in activity on elimination of one consensus subsite of the consensus full UP element (Fig. 5). We performed parallel experiments with the rrnB P1 UP element, which contains a moderately effective proximal subsite but a fully effective distal subsite (Figs. 1 and 3). The oriented-α RNAP derivatives transcribed the rrnB P1 promoter only about one-fourth as well as wild-type RNAP (data not shown). We conclude that both αCTDα and αCTDβ are required for maximal transcription of promoters containing two consensus or near-consensus UP element subsites.

Next, we performed in vitro transcription experiments with the oriented-α RNAP derivatives on promoters containing only a single consensus proximal subsite or a single consensus distal subsite. Both αI/αΔII and αΔI/αII...
transcribed the promoter with only a consensus proximal subsite nearly as well as wild-type RNAP (−80% as well as wild-type RNAP; Fig. 7, middle). In contrast, the oriented-α RNAP derivatives transcribed the promoter with only a distal subsite much less well than did wild-type RNAP (<25% as well as wild-type RNAP; Fig. 7, right). We conclude that only a single αCTD is required for efficient transcription stimulation by a consensus proximal subsite and that αCTDI and αCTDII can function interchangeably for this purpose. We also conclude that, in contrast, both αCTDI and αCTDII are required for efficient interaction of RNAP with the consensus distal subsite. These conclusions are consistent with the conclusions of the previous section that only a single αCTD is required for transcription stimulation by the consensus proximal subsite, but that both αCTDs are required for transcription stimulation by the consensus distal subsite.

Figure 7. In vitro transcription with oriented-α RNAP derivatives. Plasmids containing promoters with the indicated UP elements were transcribed with reconstituted wild-type RNAP (αI/αII) or with oriented-α RNAP derivatives (αI/αΔII and αΔI/αII). The transcription buffer was as described previously [Ross et al. 1993] except the reactions contained 160 mM NaCl instead of KCl. The templates were supercoiled plasmids containing the rrnB P1 core promoter with (left), consensus full UP element (4192; pRLG3278); (middle) consensus proximal subsite (4547; pRLG4213); or (right), consensus distal subsite (4513; pRLG4214). The rrnB P1 transcript and the vector-derived RNA I transcript are indicated. Transcriptional activities were quantified by PhosphorImager analysis and are expressed under each lane as a percentage (%) of transcription with the wild-type RNAP on the same template. (See Fig. 5 for relative activities of the three promoters with wild-type RNAP.)

We conclude that only a single αCTD is required for interaction with the proximal subsite, and that both αCTDI and αCTDII can function interchangeably for this purpose. We conclude that, in contrast, both αCTDI and αCTDII are required for efficient interaction of RNAP with the consensus distal subsite. These conclusions are consistent with the conclusions of the previous section that only a single αCTD is required for transcription stimulation by the consensus proximal subsite, but that both αCTDs are required for transcription stimulation by the consensus distal subsite.

Discussion

UP elements consist of subsites, each of which constitutes a binding site for αCTD

We demonstrate here that UP elements consist of proximal and distal subsites, and we define the consensus sequences for these subsites. The sequences of the consensus proximal and distal subsites are both A+T-rich but are significantly different (−46 5′-AAAAAARNR-3′−38 vs. −57 5′-AWWWWWTTTTT-3′−47). The relative tolerance for either A or T at some positions in both the proximal and distal subsites [see Results, Figs. 1 and 3] most likely reflects the binding of αCTD to DNA primarily in the minor groove [W. Ross and R.L. Gourse, unpubl.], where there is usually little discrimination between A and T residues [Seeman et al. 1976; see also Kielkopf et al. 1998]. Because each subsite binds an identical peptide (αCTD), the differences in the subsite consensus sequences must reflect the different locations of the two subsites within the RNAP–promoter complex, and thus the different potential molecular interactions for αCTD bound at the two locations. Factors that might differentially influence sequence preferences in the proximal subsite include requirements for possible interactions between αCTD and αNTD or between αCTD and σ region 4 bound at the −35 element [see below].

The sequence of the consensus proximal subsite differs not only from that of the consensus distal subsite, but also from the sequence of the corresponding segment of the consensus full UP element. The fact that the corresponding sequences within the consensus proximal subsite and the consensus full UP element differ indicates that binding of an αCTD at the proximal subsite is altered by binding of the other αCTD at the distal subsite [see also W. Ross and R.L. Gourse, unpubl.]. Factors that might differentially constrain the proximal subsite sequence in the context of a full UP element include sequence requirements for potential αCTD–αCTD interactions and/or for DNA bending in or adjacent to the proximal subsite [see below].

Both consensus subsites include A or T tracts that are likely to deviate somewhat in structure from standard B-form DNA [Koo et al. 1986; Young et al. 1995], and we suggest that some aspect of A-tract structure may contribute to α recognition. The stimulatory effect of A tracts on transcription when fused upstream of core promoters often has been attributed to effects of DNA struc-
We recently demonstrated that A-tract–αCTD interactions account for the observed stimulation (Aiyar et al. 1998). Our results establish that transcription stimulation by, and protection of, the consensus proximal subsite requires only a single αCTD (Figs. 7–9). We infer that the consensus proximal subsite constitutes a binding site for a single copy of αCTD. Our results further establish that two copies of αCTD are required for maximal transcription stimulation by, and protection of, a consensus full UP element (Figs. 7–9). We infer that the consensus distal subsite also constitutes a binding site for a single copy of αCTD. We note that the observation that function of a consensus proximal subsite requires only one copy of αCTD rules out the possibility that αCTD dimerization (Blatter et al. 1994; Jeon et al. 1997) is required for sequence-specific αCTD–DNA interaction.

The proximal subsite is preferentially occupied by αCTD

Several observations suggest that the proximal subsite region, by virtue of its location within the RNAP-promoter complex, is the preferred binding site for αCTD. First, the consensus proximal subsite is more effective than the consensus distal subsite in transcription stimulation in vivo. Second, the consensus proximal subsite, but not the consensus distal subsite, can stimulate transcription with RNAP derivatives containing only a single copy of αCTD. Third, αCTD preferentially occupies the proximal subsite region in RNAP–promoter complexes containing only one copy of αCTD, even in a promoter with a nonconsensus proximal subsite and a consensus distal subsite (Figs. 7–9).

We suggest four (not mutually exclusive) possible explanations for preferential occupancy of the proximal subsite region by αCTD. All four derive from the fact that the proximal subsite is located closer to the core promoter than the distal subsite (rather than from a difference in intrinsic affinity of the two subsite DNA sequences for αCTD). First, binding of αCTD to the proximal subsite may place less constraint on the linker connecting αCTD to the remainder of RNAP. Second, binding of αCTD to the proximal subsite may demand less DNA bending to bring the subsite close to the core promoter. Third, binding of αCTD to the proximal sub-

Figure 8. Hydroxyl radical footprints with oriented-α RNAP derivatives. DNA fragments containing the rrnB P1 core promoter and different UP elements were labeled in the template strand at position –66. (A) rrnB P1 UP element. (B) consensus full UP element (4192). [C] consensus distal subsite (4513). Vertical lines to the right of each panel indicate the positions of the core promoter and UP element subsites. [Lane 1] A-G sequence markers; [lane 2] no RNAP; [lane 3] wild-type RNAP [αI/αII,22 nM]; [lane 4] oriented-α RNAP [αI/αΔII, 8 nM]; [lane 5] oriented-α RNAP [αΔI/αII, 4 nM]. PhosphorImager scans of the footprints with the three RNAPs are superimposed in D [rrnB P1]; lanes from A, E (consensus full), lanes from B, and F (consensus distal); lanes from C. Each line is the ratio of radioactivity with RNAP/without RNAP. [Dashed line] Wild-type RNAP, [solid line] oriented-α RNAP [αI/αΔII]; [dotted line] oriented-α RNAP [αΔI/αII]. The scans of the footprints with the wild-type and two oriented-α RNAP derivatives are superimposed (normalized) in the core promoter region. The top and bottom of the gel are indicated in D–F.
aCTD at the proximal subsite assists binding of aCTD to the distal subsite

Our results establish that two copies of aCTD are required for function of a consensus distal subsite (Figs. 7 and 8). We propose that binding of a first copy of aCTD in the proximal subsite region cooperatively assists a second copy of aCTD in binding to a consensus distal subsite (Fig. 9A,C). This proposed cooperativity does not require a sequence-specific interaction of the first copy of aCTD with proximal subsite DNA; thus, the phenomenon is observed even with a promoter having a nonconsensus proximal subsite (Fig. 6). We suggest two (mutually exclusive) models to explain the proposed cooperativity. First, aCTD in the proximal subsite region may position aCTD to make favorable protein–protein interactions with σ specifically bound to DNA, that is, DNA binding of the aCTD–σ interaction could provide an explanation for the observation that UP elements can affect not only the initial binding of RNAP to promoter DNA to form the closed complex, but also the isomerization of the closed complex to the open complex (Rao et al. 1994; Strainic et al. 1998), because σ is involved in both of these processes (Hochschild and Dove 1998; Helmann and deHasedt 1999).

αCTD and αCTD′′ can function interchangeably

Our results with oriented-α RNAP derivatives indicate that aCTD^α and aCTD^α′′ are interchangeable for UP element subsite recognition. Furthermore, aCTD^β and aCTD^β′′ are also interchangeable for CAP-dependent
transcription of the lac promoter (W. Niu and R.H. Ebright, unpubl.). These results support and extend previous indications (Newlands et al. 1992; Zhou et al. 1994; Murakami et al. 1997b; Aiyar et al. 1998; Belyaeva et al. 1998) that there is a remarkable degree of flexibility in the positioning of αCTD\textsuperscript{I} and αCTD\textsuperscript{II} with respect to the rest of the RNAP–promoter complex, a phenomenon that likely results from the long unstructured linker between the two domains of α (Blatter et al. 1994; Jeon et al. 1997).

Our findings contradict the proposal of Murakami et al. (1997a) that there is a fixed relationship of αCTD\textsuperscript{I} and αCTD\textsuperscript{II} relative to the proximal and distal subsites. These investigators based their proposal on the results of DNA affinity cleaving experiments with an RNAP-derivative containing acetimido-benzyl-EDTA:Fe incorporated at residue 269 of αCTD\textsuperscript{II}. Because cysteine 269 is within the DNA-binding helix of αCTD (Gaal et al. 1996), and because even conservative amino acid substitutions [e.g., C269A, C269S] severely reduce αCTD-DNA binding and UP element-dependent transcription (Gaal et al. 1996; T. Gaal, H. Tang, R.H. Ebright, and R.L. Gourse, unpubl.), we suspect that incorporation of the DNA cleaving agent interferes with sequence-specific DNA interaction by αCTD\textsuperscript{II}. Therefore, we suggest that Murakami et al. (1997a) inadvertently created the functional equivalent of the oriented-α RNAP α/Δ\textsuperscript{I/II}, an RNAP derivative that (unlike wild-type RNAP) binds with the underivatized αCTD (αCTD\textsuperscript{II}) preferentially in the proximal region. These investigators did not report DNA experiments with an RNAP derivative having the cleaving agent incorporated in αCTD\textsuperscript{I}. We predict that such experiments would likewise indicate preferential binding of the underivatized αCTD, in this case αCTD\textsuperscript{I}, in the proximal subsite region.

**Implications for promoter architecture**

We have analyzed the *Escherichia coli* genome sequence to estimate the frequency of promoters that contain near-consensus subsites or full UP elements. For the purposes of this discussion, we define near consensus as 0–2 differences from consensus per subsite or 0–4 differences from consensus per full UP element. Table 1 presents the statistics for *E. coli* mRNA, tRNA, or rRNA promoters having near-consensus subsites or full UP elements. Table 2 provides the identities of these promoters.

Several conclusions can be drawn from this analysis. First, numerous *E. coli* promoters contain single near-consensus subsites. Second, promoters with a single near-consensus subsite are significantly more common than promoters with a near-consensus full UP element. Third, near-consensus proximal and distal subsites and full UP elements occur significantly more frequently in stable RNA (tRNA and tRNA) promoters.

It is important to emphasize that several UP element subsites with only a moderate match to consensus have been shown to stimulate transcription by an amount that correlates generally with similarity to consensus [e.g., see Fig. 1, *rrnB* P1 proximal; Ross et al. 1998].

| Subsite structure of UP elements | mRNA | tRNA | rRNA | Total |
|----------------------------------|------|------|------|-------|
| Consensus full\textsuperscript{b} | 10 (0.4%) | 3 (9.1%) | 3 (21%) | 16 (0.63%) |
| Proximal subsite\textsuperscript{c} | 76 (3.0%) | 8 (24%) | 5 (36%) | 89 (3.5%) |
| Distal subsite\textsuperscript{c} | 28 (1.1%) | 1 (3%) | 3 (21%) | 32 (1.3%) |

\textsuperscript{a}The numbers in parentheses refer to the numbers of promoters searched from the *E. coli* promoter database [provided by A. Huerta and J. Collado-Vides, Universidad Nacional Autónoma de México, Cuernavaca]. This database contains confirmed promoters and promoters predicted from sequence analysis [for sequences, see http://www.cifn.unam.mx/Computational_Biology/E.coli-predictions/].

\textsuperscript{b}Four or fewer mismatches to consensus.

\textsuperscript{c}Two or fewer mismatches to consensus.

Therefore, Tables 1 and 2 (which include only those promoters with near-consensus subsites) underestimate the number of promoters with sequences that are likely to function as UP elements.

The fact that each of the two copies of αCTD in RNAP can interact with an UP element subsite, together with the fact that the two copies of αCTD are flexibly tethered to the remainder of RNAP (Blatter et al. 1994; Jeon et al. 1997), allows for the evolution of additional, more complex classes of UP element-dependent promoters. Thus, promoters exist with functional subsites further upstream than the positions described here [Newlands et al. 1992; Aiyar et al. 1998], with multiple alternative functional distal subsites [Aiyar et al. 1998], or with UP element subsites and adjacent activator protein-binding sites that function cooperatively through αCTD-activator interactions [Murakami et al. 1997b; Belyaeva et al. 1998; Noel and Reznikoff 1998; Law et al. 1999]. The modular quality of promoter structure thus provides the potential for multiple input signals to be received by a single transcription initiation complex.

**Implications for transcription regulation**

Our results establish that consensus proximal subsites, consensus distal subsites, and full UP elements are differently affected by functional inactivation of one αCTD, with consensus proximal subsites showing almost no change in function, consensus distal subsites showing almost complete loss of function, and full UP elements showing partial loss of function [Fig. 7]. These differences in effects of functional inactivation of one αCTD potentially can be exploited for differential promoter regulation. For example, bacteriophage T4 Alt catalyzes ADP ribosylation of Arg-265 of one copy of αCTD in RNAP, a post-translational modification that functionally inactivates that copy of αCTD (K. Severinov, W. Ross, H. Tang, L. Snyder, A. Goldfarb, R.L. Gourse, and R.H. Ebright, unpubl.). We expect that Alt-mediated ADP-ribosylation would differentially affect promoters with UP elements containing consensus proximal and/or distal subsites. Furthermore, we speculate that there could be other post-translational modifi-
promoter fragments [0.5 µg; et al. 1991). In the first round of selection, radioactively labeled protein-binding sites on nucleic acids (Blackwell and Weintraub [49x253].

Table 2. Promoters in E. coli genome with near consensus UP elements

| mRNA | Full UP elements | Proximal subsites | Distal subsites |
|------|-----------------|-------------------|----------------|
| add as1A, cspB, cspE, envR, heml, hisL, ilvGMEDAp1, rpmFp1, 2118180 | cspAp1, cspB, dinG, cco, fadL, gcvcR, gidB, glnS, gut, hisL, hisS, hpaA, hupA, ilvGMEDAp2, lit, lpp, metG, polA, ppa, purH, recA, rob, srmB, sulA, syd, tdcR, thfD, tipA, tsp, tsr, ugppl, xylE, yadD, ybbB, ybeD, yehA, yifD, yifG, yigE, yigI, yhdW, yhiS, yihD, yidC, ylbA, yigP, yifD, yidD, yifT, yiJN, yohJ, yidL, 332725, 333657, 389475, 886646, 889312, 914128, 1168296, 1214698, 1341698, 1445540, 1627239, 1631644, 1732459, 1908123, 2183937, 2280877, 2783031, 2890237, 2903664, 2983617, 3107570, 3170227, 3203897, 3578769 | alpA, cipR, envR, hcdD, hisL, ilvGMEDAp1, narU, ndk, phnA, ppsA, recN, rpmFp1, tsr, ycgB, yhal, yhbY, yhiX, yifK, yifZ, 242496, 535810, 675934, 851820, 851282, 1215012, 1218824, 1906572, 2118180 | tRNA | argX, metT, valU | argX, asnU, aspV, glyW, metT, metZ, serT, serV | valU |
| rRNA | rmpAp1, rmpBp1, rmpCp1 | rmpAp2, rmpBp2, rmpCp2, rmpDp1, rmpGp1 | rmpAp1, rmpBp1, rmpCp1 |

For predicted promoters of unnamed genes, the numerical designations refer to the first position in the open reading frame. For actual promoter sequences, refer to http://www.cifi.unam.mx/Computational_Biology/E.coli-predictions/.

Materials and methods

Synthesis of promoter populations containing randomized proximal or distal upstream sequences

rrnB P1 promoter fragments used in the first round of in vitro selection were synthesized by annealing partially complementary top and bottom strand oligonucleotides and by use of T7 DNA polymerase as described (Estrem et al. 1998). Oligonucleotides were purchased from Integrated DNA Technologies (Corvalle, IA) or the University of Wisconsin Biotechnology Center, or were donated by NSC Technologies (Mt. Prospect, IL).

The top strand oligonucleotide contained random sequences in the upper 37 nucleotides, an EcoRI site, and the P1 sequence from −66 to −17 (see also Fig. 1). The bottom strand oligonucleotide contained random sequences in the lower 59 nucleotides, a HindIII site, and the P1 sequence from +50 to +17. Seventeen proximal and eight distal promoter fragments were sequenced without selection after cloning into the random regions were approximately equal.

UP element selection and screen

The selection was modeled after previous in vitro selections for protein-binding sites on nucleic acids [Blackwell and Weintraub 1990; Pollock and Treisman 1990; Tuerk and Gold 1990; Wright et al. 1991]. In the first round of selection, radioactively labeled promoter fragments [0.5 µg, −3 × 10^13 DNA molecules, that was in excess of the 5 × 10^6 (4^9) or 6.4 × 10^9 (4^14) molecules needed to ensure that all sequence combinations were represented in the proximal subsite or distal subsite selections, respectively] were incubated with RNAP for 4 min, and bound fragments were separated from unbound by gel electrophoresis as described previously (Estrem et al. 1998). For the second and subsequent rounds of selection, promoter fragments were amplified by PCR from gel-isolated RNAP-promoter complexes [Estrem et al. 1998]. The PCR primers contained all of the nonrandomized promoter positions to reduce the frequency of PCR-generated mutations in the core promoter region that might increase binding by RNAP [Estrem et al. 1998]. RNAP-binding reactions were carried out under progressively more stringent conditions [lower RNAP concentration and shorter reaction times]. The progress of the selection was monitored by sequencing representatives of the selected populations following eight (for the proximal) and six (for the distal) rounds of selection. A total of 13 cycles of RNAP binding, separation on gels, and PCR were carried out for each selection.

In vitro-selected promoters were fused to lacZ in phage λ and screened for high promoter activity on MacConkey lactose indicator plates [Estrem et al. 1998]. The promoter regions of the selected lacZ fusions were sequenced after PCR of DNA obtained directly from plaques. Three promoters from the proximal subsite selection and nine from the distal subsite selection were discarded, because they contained deletions or core promoter mutations. β-Galactosidase activities were determined from monolysogens of strain NK5031 that had grown exponentially at least three generations in Luria–Bertani medium [LB; Ross et al. 1998].

Site-directed promoter mutations

rrnB P1 promoters [−66 to +50] containing only the rrnB P1 proximal or distal subsite sequences [RLG3098 and RLG3099, respectively] or containing proximal subsite 4547 with single base pair substitutions were synthesized by PCR with mutagenic top strand oligonucleotides and bottom strand oligonucleotides complementary to the plasmid vector as described previously [Ross et al. 1998].

In vitro transcription

Promoter fragments were cloned into pRLG770 [Ross et al.
Supercoiled DNA concentrations were determined both spectrophotometrically and by quantitation of the vector encoded RNA I transcrips under conditions of RNAP excess [40 nM]. Transcription was carried out as described previously (Ross et al. 1993), except that reactions contained 0.6 nM DNA and different salt concentrations (see Figs. 5 and 7). Reconstituted RNAPs (Gaal et al. 1996; Tang et al. 1996) were used at concentrations that resulted in equivalent transcription from the lacUV5 promoter (2.7 nM for RNAP containing wild-type α, 9 nM for α265A, 17.4 nM α235), and oriented-α RNAP derivatives (see below) were used at concentrations that resulted in equivalent transcription from the rrrB P1 promoter lacking UP element sequences (2 nM [α3/α1], 3.5 nM [α1/α2D], 24 nM [α2D/ α1]). Gels were analyzed by PhosphorImager (Molecular Dynamics).

Hydroxyl radical footprinting

rrnB P1 promoter templates with different UP elements were generated by PCR from plasmids pRLG4213 (UP element 457), pRLG4214 (4513), pRLG3278 (4192), and pRLG4238 (−66 to +50 rrnB), and oriented-α RNAP derivatives (see below) were used at concentrations that resulted in equivalent footprinting from the rrrB P1 promoter lacking UP element sequences (2 nM [α3/α1], 3.5 nM [α1/α2D], 24 nM [α2D/ α1]). Gels were analyzed by PhosphorImager (Molecular Dynamics).

Construction of oriented-α RNA polymerases

Plasmids pREII-NHα, pREII-NHα[1–235], pREII-NHα45A, and pREII-NHα45A[1–235] encode amino-terminally hexahistidine-tagged α, α235, [Ala-45]α, and [Ala-45]α235, respectively, under control of the tandem lppP−lacPUV5 promoter, confer ampicillin resistance, and have pBR322-derived origins of replication [Niu et al. 1996; W. Niu, and R.H. Ebright, in prep.]. Plasmid pWN-Neo[1–235] encodes amino-terminally Flag [Kodak]-tagged α235 under the control of the tandem lppP− lacPUV5 promoter, confers kanamycin resistance, and has a pSC101-derived origin of replication (W. Niu and R.H. Ebright, in prep.).

Wild-type RNAPα3/α1 and oriented-heterodimeric RNAP α1/[Ala-45]α235 III RNA α3/α2D was prepared from strains XL1-Blue/pREII-NHα and XL1-Blue/pREII-NHα45A235, respectively, using Ni2+−NTA agarose chromatography and Mono-Q chromatography as described by Niu et al. (1996), followed by Anti-Flag M2 immunoadfinity chromatography as follows. Samples were dialyzed against 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 5% glycerol, adsorbed onto 1 ml of anti-Flag M2 affinity gel [Kodak]; washed with 5 x 12 ml of the same buffer, and eluted with 2 x 0.5 ml each of the same buffer containing 50 µg/ml, 75 µg/ml, 100 µg/ml, and 200 µg/ml Flag peptide [Kodak]. Peak fractions were pooled, dialyzed against 25 mM Tris-HCl [pH 7.9], 100 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiotreitol, and 50% glycerol and stored in aliquots at −20°C.

E. coli genome sequence analysis

Sequences of 253 confirmed mRNA promoters, 2248 predicted mRNA promoters, 33 tRNA promoters, and 14 rRNA promoters from E. coli were obtained from Arcelci Huerta and Julio Collado-Vides (http://www.cifn.unam.mx/Computation_al_Biology/Ecoli-predictions/). Each promoter sequence was 31 nucleotides in length, including the proposed −35 hexamer and 25 nucleotides upstream of the −35 hexamer. Sequences were searched in GCG version 9.0 using the command FINDPATTERNS with the parameters −DAT = AAAAAA-RNR/[N][7,7] −ONE−MIS = 2 for the proximal subsite, −DAT = AWWWWWTTTTT[N][16,16] −ONE−MIS = 2 for the distal subsite, and −DAT = AAAAAWTTTTTTNAAAA-NN[N][7,7] −ONE−MIS = 4 for the full UP element.

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Subsite structure of UP elements

Sequences of 253 confirmed mRNA promoters, 2248 predicted mRNA promoters, 33 tRNA promoters, and 14 rRNA promoters from E. coli were obtained from Arcelci Huerta and Julio Collado-Vides (http://www.cifn.unam.mx/Computation_al_Biology/Ecoli-predictions/). Each promoter sequence was 31 nucleotides in length, including the proposed −35 hexamer and 25 nucleotides upstream of the −35 hexamer. Sequences were searched in GCG version 9.0 using the command FINDPATTERNS with the parameters −DAT = AAAAAA-RNR/[N][7,7] −ONE−MIS = 2 for the proximal subsite, −DAT = AWWWWWTTTTT[N][16,16] −ONE−MIS = 2 for the distal subsite, and −DAT = AAAAAWTTTTTTNAAAA-NN[N][7,7] −ONE−MIS = 4 for the full UP element.

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