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Protein−Nucleic Acid Interactions during Open Complex Formation Investigated by Systematic Alteration of the Protein and DNA Binding Partners†

John D. Helmann*,‡ and Pieter L. deHaseth*,§

Section of Microbiology, Cornell University, Ithaca, New York 14853-8101, and Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106-4935

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BACKGROUND

In bacteria, transcription is catalyzed by a large multisubunit RNA polymerase (RNAP)[1] containing two dissociable components: the core enzyme (E), which catalyzes phosphodiester bond formation, and one of a family of sigma (σ) subunits, transcription factors which determine promoter specificity (*1*−*4*). In logarithmically growing *Escherichia coli* the prevalent form of the holoenzyme contains σ^{70} (Eσ^{70}). Major advances have been made in our understanding of the structure of RNAP over the past 4 years (*5*−*13*). Low-resolution structures, interpreted on the basis of the recently elucidated structures of DNA polymerases and T7 RNAP, indicate the presence of a DNA binding channel on the enzyme (*14*). Due to its very large size, however, there is still no high-resolution structure of the entire enzyme available, although structures have been determined for the two domains of α and for a large fragment of σ^{70} (*5*, *7*, *8*, *10*).

In this review, we focus on the molecular contacts that develop during the transition from the initial binary RNAP−promoter complex to the mature, strand-separated open complex. We will emphasize those studies reported since this topic was previously reviewed (*15*). In some studies, subunits of RNAP have been altered by site-directed mutagenesis, amino acid analogue incorporation, or chemical modification. In other studies, it is the nucleic acid component of the complex that has been systematically altered. For example, analyses of RNAP interactions with single-stranded, double-stranded, gapped, forked, heteroduplex, and depurinated DNA templates have provided a tremendous wealth of information. Taken together, these studies lend support to a bind−nucleate−melt model for open complex formation and point, in particular, to a crucial role in the early stages of this process for the adenine base at −11 on the nontemplate strand.

PATHWAY OF OPEN COMPLEX FORMATION

The process of open complex formation involves the sequential interaction of Eσ^{70} RNAP with several distinct regions of the promoter (Figure 1). The UP element region, located between −40 and −65, is contacted by the C-terminal domain of the α subunit (*16*). The strongest UP elements have a recognizable pattern of alternating A-rich and T-rich sequences (*17*, *18*), and DNA sequence studies suggest that many promoters retain sequences in this region that favor α subunit binding (*19*). However, strong UP elements are found in a relatively small number of promoters, including those for stable RNA and those in bacteriophage. At some promoters, contacts with the UP element may serve a role

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* To whom correspondence should be addressed. J.D.H.: telephone, 607-255-6570; fax, 607-255-3904; e-mail, jdh9@cornell.edu. P.L.d.H.: telephone, 216-368-3684; fax, 216-368-4544; e-mail, pld2@po.cwru.edu.

‡Cornell University.

§ Case Western Reserve University.

[1] Abbreviations: ss, single stranded; ds, double stranded; RNAP, RNA polymerase; E, core enzyme; PNA, peptide nucleic acid.

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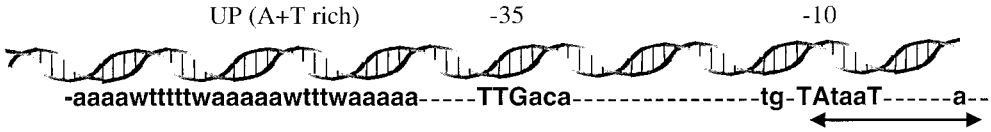

FIGURE 1: Elements of a generic σ^{70} promoter (*35*). The five elements of a consensus promoter are shown as the sequences of the nontemplate strand: the conserved hexamer sequences at −10 and −35 (*102, 103*), a spacer DNA separating them, a conserved TG dinucleotide just upstream of the −10 region (*23*), and an A+T-rich "UP" element between −40 and −65 (*16, 17, 19, 22, 104*). The most highly conserved bases are in upper case, the more weakly conserved positions in lower case. The start site of transcription, often an "A", is indicated, as well as the region of strand separation in an open complex (double-headed arrow), which extends 12−14 base pairs from positions −11 or −10 to +2 or +3. The UP element sequence is based on the observed base preferences in a compilation of *B. subtilis* σ^A promoters (*19*) [the UP consensus derived from SELEX experiments is very similar (*18*)].

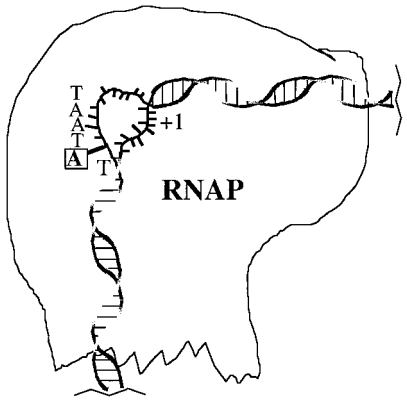

FIGURE 2: Proposed path of a portion of the promoter DNA (the interactions with the −35 region and UP element are not included) on RNAP in the open complex. The magnitude and location of the DNA bends thought to accompany open complex formation are unknown and are shown in a plausible orientation (modeled after data from ref *105*). The −11 A residue is boxed to emphasize its importance in establishing the open complex. To initiate transcription, RNAP must establish a catalytic center containing two template bases, two rNTPs, and a magnesium ion.

in the earliest stages of the binding reaction (*20*), perhaps even preceding recognition of the −35 element (*21*), while at others the UP element may affect a much later step (*22*). The most critical determinants of promoter function are the two highly conserved hexamers centered near −10 and −35 that are contacted by σ. In addition, both the sequence and the length of the spacer region are important for promoter function (*15*). Finally, many promoters have a TG dinucleotide upstream of the −10 element (together referred to as an "extended −10 region"), and in this case, the −35 region seems to be less critical for formation of a functional RNAP−promoter complex (*23*).

In the mature, initiation-competent open complex (Figure 2), the conformation of the DNA is altered in two important ways: the strands are separated over 12−14 base pairs (*15, 24−28*), from the upstream half of the −10 region to about position +2, and the DNA is bent, roughly centered at the −10 region (*29, 30*). Given the relative dimensions of RNAP (100 × 100 × 160 Å) and the length of promoter DNA protected by RNAP from DNase I cutting (at least 70 base pairs), it is likely that promoter-bound DNA is wrapped around RNAP (*31, 32*). The open complex is very stable at most promoters but exists only transiently at others. However, it is thought that in all cases it is responsible for the initiation of RNA synthesis (*33, 34*). That open complexes can be stable is remarkable, as the complementary strands of DNA must be kept separate under conditions that greatly favor base pair formation. To accomplish this, RNAP binds tightly to at least one of the two strands.

Open complex formation is a complicated process involving several intermediates, and both macromolecular components undergo conformational changes. The reaction shown in Scheme 1 reflects our current understanding of the process, as it occurs at the very well studied λ P_R promoter (*31, 35, 36*).

Scheme 1

$$R + P \overset{1}{\rightleftarrows} RP_{c1} \overset{2}{\rightleftarrows} I_1 \overset{3}{\rightleftarrows} I_2 \overset{4}{\rightleftarrows} RP_0$$

It is likely that open complex formation at many bacterial promoters proceeds via either this or a related mechanism (see refs *15* and *35* for reviews). Step 1 is the binding of RNAP (R) to promoter DNA (P), to form the closed complex, RP_{c1}, with a typical DNase I footprint extending from −55 to about −5. By analogy with the equivalent complex formed at *lacUV5* (*37*), RP_{c1} likely involves sequence-specific contacts at the −35 region only. Step 2 is a rearrangement, which is manifested in the downstream extension of the DNA footprint, to about +15/+20. At λ P_R, step 3 is the rate-limiting step [in kinetic terms, the transition state with the highest activation free energy (*38*) lies between I_1 and I_2]. It is believed to constitute a major conformational change in RNAP, involving the burial of nonpolar groups. The nature of the conformational change has been the subject of some conjecture. It may be the closure of a sliding clamp around the DNA (*12*) or a rearrangement of the two DNA binding domains of σ (*37*) during the realignment of the −35 and −10 regions (*15, 36*). No changes are observed in the size of the DNA footprint at this step, but the strand separation process is believed to be nucleated concomitantly with or immediately following the conformational change. In some representations, I_1 and I_2 are together written as RP_{c2}; then the RP_{c1} to RP_{c2} conversion includes the rate-limiting process. During step 4 the strand separation process is completed by a downstream expansion of the nucleated transcription bubble as discussed below.

The process of DNA melting has been studied by monitoring the susceptibility of thymine bases to oxidation by potassium permanganate. While strand separation is a cooperative process at several promoters, in other cases discrete intermediates are observed. These can be visualized by controlling reaction temperature or by omission of magnesium ion (*27, 35, 39−41*). In several cases, the process of DNA strand separation appears to involve an initial distortion or localized melting event at or near −11 (*28, 39, 40, 42, 43*). A similar low-temperature intermediate in the melting of the flagellin promoter by the *Bacillus subtilis* $E\sigma^D$ holoenzyme was visualized and designated RP_N (*39*). At slightly higher temperatures, the transcription bubble extends

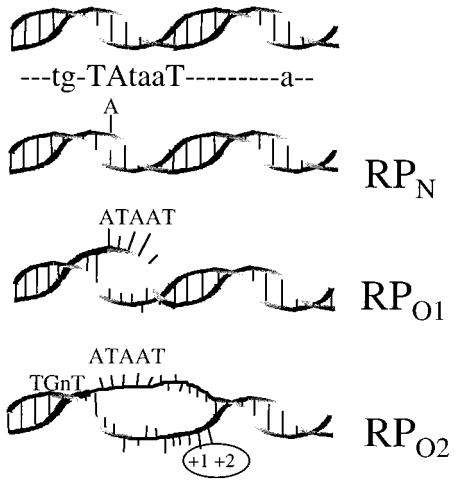

FIGURE 3: Bind−nucleate−melt model for open complex formation. The earliest event in the process of strand separation appears to correspond to a distortion or localized melting near −11. The DNA here is shown straight, although it is likely that nucleation is accompanied by, and may even depend on, bending of the DNA duplex (as in Figure 2). Subsequent downstream expansion of the transcription bubble can occur in either a stepwise or concerted fashion. In this example, two distinct open complexes are illustrated. Similar intermediates have been seen in several systems. Since no single nomenclature can be adapted to all promoters, and since bubble expansion generally proceeds in an upstream to downstream direction with different intermediate states, we have previously proposed that distinct open complexes (here arbitrarily designated RP_{O1} and RP_{O2}) be designated by the position of the most downstream melted base pair (e.g., RP_{-4}, RP_{-1}, RP_{+3}; see ref *39*).

to near, but does not extend past, the start site (*39*−*41*). Finally, at yet higher temperatures and in the presence of magnesium ion, the fully melted open complex forms (*27*, *39*). While the precise intermediates are specific to the promoter and holoenzyme combination, a general pattern is seen that includes a nucleated complex RP_N, an incompletely melted open complex RP_{O1}, and a fully mature, open complex RP_{O2} (Figure 3). The precise relationship between these intermediates and the kinetically defined intermediates of Scheme 1 has not been established.

The disposition of the strands in the mature open complex is not known, but it is thought that, in addition to the preferential interactions of RNAP with the nontemplate strand to stabilize the open complex and prevent reannealing of the two strands, there likely is a *physical* barrier to renaturation as well. While the template strand remains in the original channel, and near the catalytic site on RNAP, the nontemplate strand would be rearranged to follow a different path close to the surface of the RNAP (*44*); regions of RNAP would establish a wedge between these two strands (*14*). The relationship of this process and the kinetically detected conformational change in RNAP is unclear.

PROTEIN DETERMINANTS OF OPEN COMPLEX FORMATION

The σ subunit plays a determining role in the process of DNA strand separation. Early experiments demonstrated that σ^{70} and the nontemplate strand of promoter DNA can be efficiently cross-linked by UV light (*45*). Together with the known involvement of σ in determining −10 recognition, this provided evidence that σ was physically near the strand-separated DNA and suggested that σ might actually catalyze the strand separation event. Thus, σ should have a ssDNA binding surface capable of stabilizing the melted intermediate (*2*). However, unlike helicases, σ does not hydrolyze ATP nor does purified σ bind tightly to either dsDNA or ssDNA (*46*, *47*), properties expected of a DNA unwinding protein. Subsequent studies have led to a model in which ssDNA interacts with a surface of σ which becomes exposed only upon association with the core enzyme (*48*). Binding of ssDNA to the holoenzyme may also require contacts to core subunits. Indeed, a mutant holoenzyme with a deletion in β appears to be locked in an early intermediate stage of promoter melting (*49*). However, it is not clear if the deleted region of β contacts DNA or σ. Contacts between this general region of β and σ are consistent with recent mapping studies of the σ-core interface (*50*).

The current model postulates that σ factors, in solution, fail to bind to DNA because an autoinhibitory domain near the amino terminus (conserved region 1) interacts with either or both of the DNA binding domains (conserved regions 2 and 4). Upon binding to the core enzyme, the conformation of σ is altered and the DNA binding surfaces, including those important for stabilizing ssDNA, become exposed. This model emerged from the observation that removal of conserved region 1 from several σ factors increases their affinity for binding dsDNA (*46*, *51*). Indeed, a *B. subtilis* σ factor that lacks this conserved region binds promoter DNA selectively (*52*). Strong support for this inferred σ factor conformational change has been provided by recent biochemical experiments. Using site-directed mutagenesis, cysteine residues have been introduced into various regions of σ^{70} (*53*). When the rate constants for chemical modification at each cysteine were determined in both free σ and σ bound to the core, striking position-specific effects were observed, indicating regions with both increased and decreased exposure to solvent. Fluorescence measurements, using probes attached to each of the introduced Cys residues, also support a large conformational change (*53*). Using luminescence resonance energy transfer, it was possible to measure the approximate magnitude of the interdomain motion induced by the core: region 1 shifts by ∼20 Å and region 4 by ∼15 Å relative to region 2 (*54*). Genetic analysis has identified a point mutation in region 1 of σ^{70} that inhibits holoenzyme function although not, apparently, the binding of σ to the core (*55*). Interestingly, this defect is partially suppressed by a small deletion in the carboxy terminus of σ near region 4. An interaction between region 1 and regions 2 and/or 4 may also account for the aberrantly slow mobility of σ^{70} in SDS−PAGE since mutations in both region 2 and region 4 have been found to restore the expected mobility (*53*, *56*). Together, these data suggest that σ undergoes complex conformational changes upon interaction with the core enzyme.

Conserved region 2.3 of σ factor participates in forming the ssDNA binding site and includes four conserved aromatic amino acids (*15*, *57*, *58*). This region includes the sequence YKFSTYATWW, where the underlined amino acids represent respectively the σ^{70} aromatic residues Y425, Y430, W433, and W434 (the corresponding positions in the *B. subtilis* homologue, σ^A, are Y184, Y189, W192, and W193). Substitutions in these residues of both the *B. subtilis* and *E. coli* primary σ factors result in holoenzymes with defects in open complex formation (*57*; Panaghie and deHaseth,

unpublished results) and altered ssDNA binding properties (*47*). Specifically, mutant holoenzymes still recognize promoter DNA but are impaired in carrying out the subsequent strand separation process. This defect is apparent especially at low temperatures, where strand separation would be more difficult, and is suppressed by negative supercoiling of the template DNA (*40, 57, 59*).

UV-mediated cross-linking has demonstrated that ssDNA bound to holoenzyme is in physical contact with σ, whereas little cross-linking is detected to other subunits (*47, 60*). UV cross-linking is also detected to fragments of σ containing conserved region 2, suggesting a contact site in or near region 2 (*13*). This assignment is supported by protease mapping studies using radiolabeled *B. subtilis* σ^A and by the observation that the Y189A and W193A σ^A mutants display greatly reduced UV cross-linking to ssDNA (*47*). A direct, physical interaction between the conserved Trp residues in σ^{70} and ssDNA is revealed by elegant biophysical studies using σ factors carrying 5OH-Trp for Trp substitutions (*48*). Interaction with ssDNA specifically quenches the fluorescence of 5OH-Trp substituted for both W433 and W434, consistent with a direct role in binding ssDNA (*48*). These contacts may be near the site of nucleation of melting (near −11) since σ^A mutations in this region, most strikingly Y189A and W192A, lead to a large decrease in $KMnO_4$ reactivity at −11 on the P_{trnS} promoter (*40*).

Genetic data suggest that there is likely to be a collinear arrangement between the amino acid residues in σ important for DNA binding and the −10 recognition element (*61*). Inspection of the high-resolution structure of a fragment of σ^{70} containing DNA binding region 2 indicates that the aromatic residues important for strand separation project into solution from an extended α helix, helix 14 (*10*). Helix 14 also contains Q437, implicated in recognition of the conserved −12 T-A base pair (*62*). Although not present in the fragment used for crystallization, a nearby residue (E458) contacts the G in the TG dinucleotide of extended −10 elements (*63*). Thus, one can envision an arrangement in which the −10 region nontemplate DNA is aligned along this α-helical segment with the amino-terminal end of helix 14 nearest the transcription start site (*10*).

To test this model, DNA footprinting studies have been conducted using a chemical nuclease covalently attached to σ^{70} at several sites in and near region 2 (*64, 65*). These studies support a close juxtaposition between the σ^{70} DNA binding helix and the −10 promoter region, but the details of the binding interaction are difficult to discern and two quite distinct models have been proposed (*64, 65*). The relative orientation of the nontemplate DNA strand along helix 14 has also been studied using fluorescence energy transfer from protein-coupled donor molecules to oligonucleotide-coupled acceptors (*66*). These studies provide strong support for the collinear arrangement inferred from the genetic (*57, 61−63, 67*) and structural (*10*) studies.

NUCLEIC ACID DETERMINANTS OF OPEN COMPLEX FORMATION

The process of open complex formation involves manifold conformational changes in the DNA including bending, localized melting (or base flipping; *68*) of one or more bases to nucleate melting, and either a stepwise or concerted expansion of the melted region to establish the final open complex (Figure 3). These contortions must be driven by energetically favorable interactions with the protein partner and involve both nonspecific and sequence-selective components (*15*). Since RNAP interacts nonspecifically with polyanions, including heparin and ssDNA (*69*), it is likely that ion-pairing interactions play a significant role in this process. The overall process is also sequence selective, consistent with a role for oriented hydrogen bonds. To define the energetic basis of open complex formation, it will be necessary to identify the bonding partners in these interactions and their roles in stabilizing the melted DNA.

To identify the structural features recognized by the nucleic acid binding sites of RNAP, various substrates have been tested both for their ability to bind RNAP and to support the process of transcription initiation (Figure 4). The substrates discussed below are, to greater or lesser extent, all single stranded, have single-stranded regions, or facilitate strand separation. Some are recognized in sequence-specific fashion and others merely by virtue of their stretches of single-stranded DNA. Most can be considered as containing one or more of the following regions of promoter DNA: an upstream duplex, a nontemplate strand, a template strand, and a downstream duplex.

Tailed Templates. Among the earliest examples of modified nucleic acid structures that allow specific transcription initiation were 3′ tailed templates (Figure 4a). These are generated by addition of a single-stranded DNA extension, usually oligo(dC), to the 3′ end of the template strand. Initiation occurs at the ss/ds boundary of the template, not unlike the site of initiation of RNA synthesis at a promoter. These templates have been employed mostly to obtain elongation-like complexes with the eukaryotic transcription machinery for studies of elongation and termination (*70−74*). One problem especially prevalent with these templates, but to some extent with all mismatch complexes, is the nondisplacement of RNA from the dsDNA template (*70, 71, 74, 75*), presumably because there is no re-formation of the DNA duplex in the region of transcription initiation to drive the RNA displacement process. Interestingly, *E. coli* and wheat germ RNAP give mostly the displaced RNA product (*70*).

Heteroduplex (Bubble) Templates. A variety of templates have been constructed bearing stretches of single-stranded DNA that would emulate the single DNA strands of an RNAP-generated open complex. This class of "bubble" template has found use in the characterization of prokaryotic as well as eukaryotic RNAP. By circumventing the need for strand separation, simplified systems can be employed, lacking components necessary for the strand opening step. For example, on bubble templates, site-specific initiation of transcription can be obtained with the core enzyme (*76*). We will here review the many different kinds of bubble templates that have been constructed, including bubbles introduced into promoters and in essentially random DNA sequence. In some experiments, RNA synthesis is primed from the 3′OH of a hybridized RNA oligonucleotide. Of the possible combinations, only primed synthesis from a bubble in a promoter region has not yet been described.

Mismatch bubbles in random sequence (nonpromoter) DNA, with disruptions of about 10 bp (Figure 4b), allow bidirectional initiation of RNA synthesis by both the core

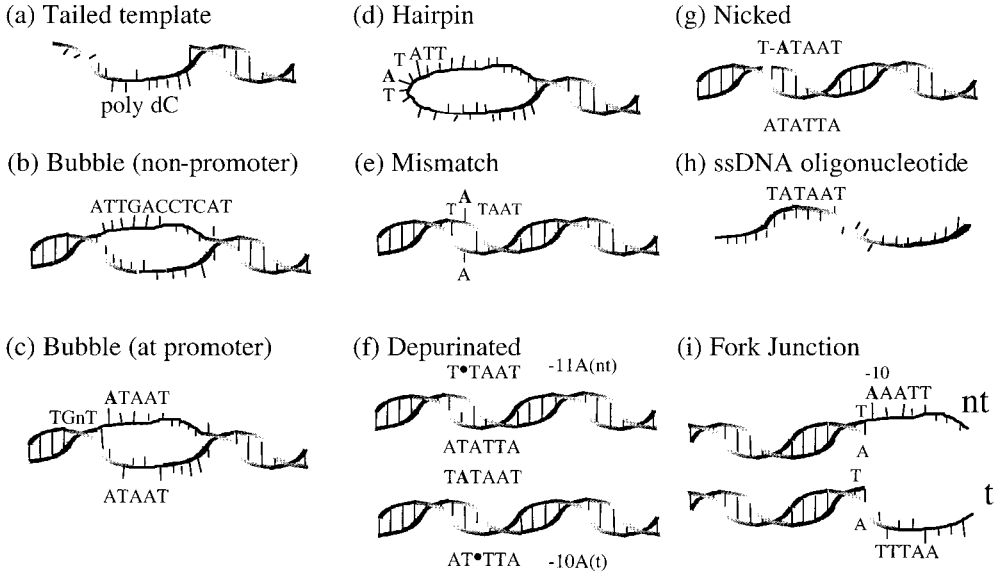

FIGURE 4: Various nucleic acid structures used for the investigation of transcription processes. The regions shown only include the extended −10 and downstream regions and do not explicitly include the −35 element. Constructs discussed in the text include (a) 3′-tailed templates, (b) bubble (heteroduplex) regions designed to lack promoter-like sequences, (c) bubble templates containing promoter recognition elements, (d) hairpin DNA, (e) single base-pair DNA mismatches, (f) depurinated DNA, illustrated here by depurination of −11A on the nontemplate (nt) strand or −10A on the template (t) strand, (g) nicked DNA, (h) nontemplate single-stranded oligonucleotides, and (i) fork junction templates containing either nontemplate (nt) or template (t) ssDNA extensions.

and holoenzyme. This indicates that the core recognizes some aspect of the bubble template, perhaps the ss/ds DNA junctions, and is consistent with the idea that a major function of the σ factor is to facilitate the *formation* of this structure on duplex, promoter DNA. Initiation of RNA synthesis takes place at both ss/ds DNA borders (*76*). The direction of RNA synthesis is likely determined by the polarity with which the RNAP happens to bind to the bubble template. In contrast to observations on tailed templates (and bubble templates with primer, below), RNA is efficiently displaced on these templates.

Bubbles have also been introduced in promoter DNA (e.g., Figure 4c) to mimic the RNAP-induced strand separation in open complexes (*41, 77*−*80*). Like the random sequence bubble templates, bidirectional initiation is possible from these templates, both with the core and with the holoenzyme (*77*). However, in many cases, transcription in one direction is preferred, even in the absence of σ (*80*). Factors that may account for this directionality include the availability of a pyrimidine appropriately positioned on the template strand (initiation preferentially begins with a purine) and UP-element-like sequences. However, a strong UP element upstream of the *B. subtilis* flagellin promoter was inefficient at orienting the core enzyme on the corresponding bubble templates (*80*). In the case of the holoenzyme, the presence of −10-like sequences within the ssDNA region, and −35-like sequences in the upstream region, may contribute to directional selectivity and start site localization (*80*; see also the section on filamentous phages, below). Indeed, σ^{70} alone, provided region 1 was removed, binds sequence-specifically to a bubble promoter (*79*). In addition to their use in investigating the roles of σ in determining the site of transcript initiation (*80*), bubble templates have been used to study the activation of transcription (*41, 80*) and to identify determinants of RNAP pausing (*78*). Heteroduplex promoters have also been used to address similar problems in eukaryotic systems (*81*−*83*).

An alternative method for the generation of the bubble template, and one that allows the use of completely self-complementary DNA strands, is strand invasion by PNA (peptide nucleic acid) (*84*). A short PNA strand, consisting of oligopyrimidine sequences, can hybridize to oligopurine stretches in dsDNA, leaving the pyrimidine-containing DNA unpaired and able to function as the template for transcription. It is speculated that such PNA displacement loops could function as novel regulators of gene expression, with potential pharmacological uses, by either inhibiting or activating transcription of specific target genes. Note that initiation of RNA synthesis is not primed: PNA does not possess a 3′-hydroxyl group.

Bubble templates, with an annealed RNA primer, have proven useful as models for transcription elongation complexes. By annealing an RNA primer in the bubble region so that the 3′OH of the primer is positioned upstream of the ss/ds boundary, an artificial elongation complex is obtained. Elongation of the annealed primer can take place with high efficiency (*85*−*87*) although the persistence of a hybrid between the nascent RNA and the template DNA has been observed. The RNA is thought to be displaced by RNAP normally but then would rehybridize when RNA strand invasion occurs due to base pairing between the primer sequence and the exposed template strand within the bubble. Indeed, addition of a large excess of an oligonucleotide "trap" complementary to the exposed template strand in the bubble region increases strand displacement (*87*). This is in contrast to nonprimed bubble templates, where transcription initiation takes place near the ss/ds boundary. On these templates the RNA is complementary to only a small stretch of the heteroduplex region, and strand invasion by the product RNA does not take place. Similar bubble templates, pioneered by Kashlev and co-workers (*88*), can be constructed from entirely complementary DNA strands. In this case, a short RNA oligonucleotide is first hybridized to the template strand, followed by the annealing of the complementary DNA

strand, apparently without displacement of the annealed oligonucleotide. RNA synthesis is primed from the hybridized RNA to generate model elongation complexes.

A variety of other nucleic acid structures have been tested for their ability to serve as templates for RNAP. Hairpin constructs function as templates for the initiation and elongation of RNA synthesis (Figure 4d). Here, the bubble is the single-stranded loop formed by an intact DNA strand, noncovalently closed on one side by a dsDNA stem (*76*). The template is the strand leaving the bubble in the 3′ to 5′ direction, a direction consistent with the utilization of a −10-like element upstream. However, this sequence is not essential for site selection since the core enzyme initiates from this same position (*76*). A relative of the hairpin is the dumbbell template, which consists of one DNA strand with the 3′OH end covalently linked to a short RNA sequence that hybridizes to a stretch of DNA near the 5′ end to prime RNA synthesis (*89*).

Promoter DNAs with Helix Defects. Disruptions in nucleic acid structure, such as mismatches, abasic sites, nicks, and gaps, can either stimulate or inhibit RNAP activity, depending on their location. In general, those changes that decrease helix stability within the region that is ultimately melted increase the rate of open complex formation and can stimulate either RNAP binding, transcription initiation, or both. As shown below, many subtleties of the RNAP−DNA interaction have been revealed from such analyses.

Recently published studies from Roberts' laboratory have shown that reduction of the stability of the −10 region by base mismatches accelerates open complex formation, likely by facilitating nucleation of DNA melting (*90*). While the interpretation of the effects of the mismatches themselves is complicated due to the sequence dependence of the interaction of RNAP with nucleotides on the nontemplate strand, rate enhancements of 5-fold or more were observed for mismatches at the −11 and −7 positions within the −10 element (e.g., an A-A mismatch at −11; Figure 4e). Interestingly, no such effects were observed for a mismatch at −12, the most upstream position of the −10 region, which may not become strand-separated in open complexes at most promoters (*90*). These experiments also served to demonstrate that the sequence identity of the nontemplate strand base was a major determinant of the rate of open complex formation.

Helix stability within the promoter region can also be reduced by removal of a base or introduction of a nick. For example, measurements of the rate of open complex formation revealed a large stimulation (greater than 15-fold in some cases) caused by depurination (Figure 4f) and nicking (Figure 4g) of promoter DNA in either strand between −12 and −4, therefore including the −10 recognition element (*91*). Significantly, the single exception was depurination of the nontemplate strand −11A. For example, depurination at −10A (on the template strand) stimulates the rate of open complex formation 8-fold, while depurination at −11A (on the nontemplate strand) actually inhibits open complex formation (Figure 4f). This strongly suggests that the −11 adenine base establishes favorable interactions with RNAP during the rate-limiting step of open complex formation (step 3 in Scheme 1; perhaps corresponding to formation of RP_N or RP_{O1} in Figure 3). Loss of this favorable protein−base interaction may impose a substantial energetic penalty that cannot be compensated even by the more favorable energetics of melting in a region lacking a base pair.

Nucleation of melting near −10 is consistent with the known properties of the consensus −10 sequence, TATAAT: TpA, which occurs twice in this sequence, has been found to be the most thermally labile (*92*) dinucleotide step in dsDNA. It has been shown that the −10 region is more thermally labile than other DNA sequences even in the absence of RNAP (*93*−*95*). An upstream to downstream direction of propagation of strand separation upon nucleation (*15*) is supported by several lines of evidence. These include the temperature dependence of permanganate sensitivity (*28*, *39*, *40*, *43*), dependence of the rate of RNAP-induced fluorescence enhancement on the position of 2-AP in the melted region (*22*), and identification of an RNAP mutant (*49*), which only melts base pairs proximal to the −10.

Single-Stranded DNA Oligonucleotides. RNAP can bind specifically and with high affinity (measured dissociation constants of near 4 nM) to oligonucleotides containing the nontemplate strand sequence of the −10 region (Figure 4h). The selectivity of the interaction is demonstrated by the fact that RNAP can bind to a nontemplate strand oligonucleotide even in the presence of a >100-fold molar excess of a nonspecific competing ssDNA or of the cognate dsDNA duplex (*47*, *60*). Direct comparison of the affinity of the RNAP holoenzyme for nontemplate and template oligonucleotides using 5-OH-Trp-substituted σ^{70} reveals an ∼200-fold selectivity for the nontemplate strand (*48*). As noted above, this interaction requires σ (*47*, *48*, *60*) and is affected by mutations in region 2 that affect recognition of −12 (*60*) and DNA melting (*47*, *48*). In contrast, free σ, even if containing the deletion in region 1 that allows DNA binding, interacts weakly, and without apparent sequence selectivity, with ssDNA (*47*, *48*). As discussed above, fluorescence energy transfer experiments demonstrate that the nontemplate strand ssDNA oligonucleotides bind preferentially in one orientation to RNAP (*66*), consistent with the models derived from genetic and structural data. Thus it is likely that the binding site that associates with nontemplate strand ssDNA oligonucleotides is the same site that binds this region in the open complex.

There is a vast literature documenting the sequence requirements in the −10 region for promoter function, but it is not yet known whether this sequence selectivity is due to interactions with dsDNA or ssDNA. To address this question, insight into the sequence selectivity of ssDNA binding to the holoenzyme is of great importance. In the first study of this type, Marr and Roberts (*60*) demonstrated that an oligonucleotide with a T → C change at −12 bound holoenzyme with an affinity 5-fold less than that of the consensus oligonucleotide. Moreover, this decreased affinity was partially compensated by a change in σ^{70} (Q437H) that is known to partially suppress T → C mutations in promoter DNA (*60*). A −12T → A change also affects ssDNA binding as observed in studies using oligonucleotides containing 2-aminopurine (2-AP) fluorescent base analogues, but only when the 2-AP substitutions were downstream of the −10 element: oligonucleotides containing 2-AP at positions −14 and −15 did not show such an effect (*96*). These results suggest that at least part of the sequence selectivity observed at the −12 position can be accounted for by interactions at the ssDNA binding site of RNAP.

In a systematic study of ssDNA binding selectivity using competition experiments with labeled consensus oligonucleotides and all 18 possible point mutations in the −10 consensus, it has been found that the three adenines in the −10 region are the most critical for binding of ssDNA to either *E. coli* σ^{70} or *B. subtilis* σ^{A} holoenzymes (Qiu and Helmann, unpublished data). For example, any of the three possible substitutions for −11A essentially eliminate binding and thereby support the notion that this base plays a critical role by virtue of its interaction in the ssDNA binding site. Although sequence-dependent effects are observed at all six −10 consensus positions, the critical sequence determinants for ssDNA binding (tAtAAt) differ from those for promoter activity (TAtaaT), with the single exception of −11A. Unexpectedly, oligonucleotides containing substitutions for either T−12 or T−7 still bound the holoenzyme, albeit with somewhat reduced affinity, as also observed in another study on ssDNA binding (*96*). Since both the −12 and −7 positions are highly conserved, and important for promoter strength, the relatively modest effects on interaction with the ssDNA binding site suggest that these positions may be recognized in dsDNA; note that at many promoters the −12 base pair is probably not melted. Conversely, substitution of the A's at −9 and −8 had drastic effects on ssDNA binding, despite the fact that these bases are, in general, less critical for promoter function. This raises the possibility that they may only be recognized after DNA melting, in which case changes would be unlikely to affect promoter strength unless DNA melting were the rate-limiting step.

The kinetics of the ssDNA binding reaction have been followed using oligonucleotides substituted with 2-AP. When the interaction of RNAP with such DNA is studied, the fluorescence of the 2-AP is seen to increase as a result of the binding, consistent with the unstacking of bases of the ssDNA upon binding to RNAP (*96*). The temperature dependence of the interaction (*96*) is similar to that for the formation of open complexes (*98*), suggesting both that RNAP has to undergo a similar conformational transition in order to interact with the two types of DNA substrates and that DNA melting itself is not the origin of the entire temperature dependence observed for RNAP−promoter interactions (*96*). Interestingly, the kinetics of the interaction of the RNAP holoenzyme with single-stranded oligonucleotides (*96*) are considerably slower than those observed with the fastest promoters (*22*), although ssDNA oligonucleotides are smaller (diffuse faster) and do not need to be melted. A major difference between an oligonucleotide and a promoter is the absence of a −35 region in the former. Indeed, effects of the −35 region on recognition of bubble promoters by fragments of σ^{70} have been observed (*79*), while Guo and Gralla (*97*) have reported that the presence of a double-stranded −35 region greatly stabilized the binding of partially single-stranded templates to RNAP.

Fork Junction Templates. Gralla and colleagues have recently pointed out that a major difference between the complexes of RNAP with ssDNA, and open complexes, is the resistance of the latter, but not the former, to heparin challenge (*97*). In a systematic dissection of the λ P_R' promoter, two important determinants of heparin resistance were found: the −35 region and a single-stranded "fork" containing the −10 region. While a ssDNA extension containing either the nontemplate or the template strand (Figure 4i) was bound tightly by holoenzyme, only the fragments with the nontemplate strand extension were highly resistant to heparin challenge. Remarkably, maximal resistance to heparin challenge was observed in constructs where −12T was in the duplex region, while −11A was single stranded. This is an important observation, as the location of the ds/ss border in these templates mimics that found for open complexes, and this again points to a critical role for the −11A position. Indeed, a single overhanging A residue allows high-affinity binding by the holoenzyme, and no other base could substitute for this A (in contrast, there was no base preference observed for the −7T position using longer fork junction templates). Addition of the next base, to generate an AA tail, decreases heparin resistance, while further extension of the ssDNA tail gradually restores the high-affinity interaction. This led to the proposal that the −10 position acts as a "gate" that limits the downstream extension of the transcription bubble. However, this result was obtained with constructs in which the −10 position is the nonconsensus A (Figure 4i); it would be interesting to test these ideas with a −10T-containing oligonucleotide.

The role of σ^{70} in interaction with the fork junction region has not been demonstrated directly but is supported by several observations. For example, transcription from bubble templates (which contain a fork junction) is also heparin resistant, but only if the σ factor is present (*76*). Although fork binding is difficult to demonstrate for σ^{70}, due to the poorly understood conformational changes required to unmask the DNA binding determinants, a tight interaction between σ^{54} and a fork junction DNA was observed (*97*).

Naturally Occurring Heteroduplex Promoter Elements: Replication Origins of Filamentous Phage. Upon infection of *E. coli* by a filamentous phage (e.g., M13, f1, or fd), the infecting DNA strand is converted to a DNA duplex, which is an important intermediate in the expression and replication of the phage genome. Second-strand DNA synthesis is primed by RNA transcribed from the infecting ssDNA by the host RNAP, Eσ^{70} (*99*). Examination of the secondary structure of the ssDNA in the transcription initiation region reveals a double-stranded −35-like element and a putative −10 region with multiple mismatches (*100*). It was shown that increasing the complementarity in the −10 region was deleterious to promoter function. Similarly, another ssDNA sequence capable of directing RNA synthesis has three mismatches in the putative −10 region and two in the −35 region in the predicted secondary structure (*101*). While in both cases transcription is markedly stimulated by the SSB protein, which is an integral player in initiation of replication in vivo, it is clear that the ability of RNAP to selectively recognize partially melted (mismatch) structures has been exploited in the evolution of promoters for the priming of DNA synthesis from ssDNA templates.

SUMMARY

Despite the absence of high-resolution structures for either bacterial RNA polymerase or an open complex, a fairly detailed picture has emerged of the intricate structural transitions that accompany DNA melting. Biochemical approaches, involving site-selective modifications of both the protein and DNA partners, indicate that strand separation nucleates at or near the second base pair (the A at −11) of

the −10 region. The evidence reviewed here establishes that this A plays a unique role in open complex formation that involves significant favorable interactions with RNAP and presumably with the σ subunit. Once nucleation occurs, strand separation proceeds, in either a concerted or stepwise manner, to one or two bases past the start site for RNA synthesis. The resulting strand-separated ("open") complex is stabilized by interactions with both the core enzyme and the σ factor. The σ subunit plays a key role in the process of initiation, including the sequence-specific recognition of both double-helical DNA (−35 region, TG sequence, and the first T-A bp of the −10) and single-stranded regions (the nontemplate strand of the −10 region). Interactions between specific aromatic amino acid side chains in σ region 2.3 and the nontemplate strand are implicated in both the nucleation and propagation of the transcription bubble.

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