Transcription Initiation at the Flagellin Promoter by RNA Polymerase Carrying $\sigma^{28}$ from Salmonella typhimurium*

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The $\sigma$ subunit of RNA polymerase is a critical factor in positive control of transcription initiation. Primary $\sigma$ factors are essential proteins required for vegetative growth, whereas alternative $\sigma$ factors mediate transcription in response to various stimuli. Late gene expression during flagellum biosynthesis in Salmonella typhimurium is dependent upon an alternative $\sigma$ factor, $\sigma^{28}$, the product of the fliA gene. We have characterized the intermediate complexes formed by $\sigma^{28}$ holoenzyme on the pathway to open complex formation. Interactions with the promoter for the flagellin gene fliC were analyzed using DNase I and KMnO$_4$ footprinting over a range of temperatures. We propose a model in which closed complexes are established in the upstream region of the promoter, including the −35 element, but with little significant contact in the −10 element or downstream regions of the promoter. An isomerization event extends the DNA contacts into the −10 element and the start site, with loss of the most distal upstream contacts accompanied by DNA melting to form open complexes. Melting occurs efficiently even at 16 °C. Once open complexes have formed, they are unstable to heparin challenge even in the presence of nucleoside triphosphates, which have been observed to stabilize open complexes at rRNA promoters.

Additional DNA contacts are made at some promoters by the C-terminal domain of the $\alpha$ subunit of RNA polymerase at an AT-rich region between −38 and −59 called the UP element (9).

Comparison of the amino acid sequences of members of the $\sigma^{70}$ family reveals four highly conserved regions with subdomains that have been implicated in specific functions (Fig. 1). Region 1.1 inhibits $\sigma^{70}$ from binding to the DNA in the absence of the core subunits (10) and is also required for efficient progression from the earliest RNAP-DNA complex to a transcriptionally active complex during initiation (11). Deletion of regions 1.1 and 1.2 of $\sigma^{70}$ results in transcriptional arrest after initial binding of RNAP to the promoter (11). Regions 2.1, 2.2, and 3.2 are important for core binding (12–14), and region 2.3 has been implicated in promoter melting (15–18). Both holoenzyme and the $\sigma$ factor interact with non-template bases in the −10 element to stabilize the open complex (19–21). Regions 2.4 and 4.2 are responsible for contacting the −10 and −35 promoter recognition elements, respectively, and for positioning holoenzyme for initiation (10, 22–26). Regions 1.2, 2, and 4 are found in almost all $\sigma$ factors, but region 1.1 is found only in the primary $\sigma$ factors (4). Interestingly, both regions 1.1 and 1.2 are absent in the Salmonella typhimurium alternative $\sigma$ factor required for flagellum biosynthesis, $\sigma^{28}$, hinting at potential variation in the structure of Eo$\sigma^{28}$DNA complexes or in the mechanism of transcription initiation.

Initiation can be described as a series of sequential steps, which have been well characterized for both Eo$\sigma^{70}$ and Eo$\sigma^{28}$ from E. coli (27). By varying the conditions, several intermediates can be visualized using footprinting methods. RNAP (R) binds to the promoter (P) to form an initial closed complex, RP$_{C1}$, which protects the DNA from approximately −60 to −5 relative to the start point of transcription. RP$_{C1}$ isomerizes to a second closed complex (RP$_{C2}$) that maintains the upstream contacts and extends further downstream of the transcription start site to +20. RP$_{C2}$ then undergoes strand opening to form an open complex, RP$_{O}$, whereas the length of the footprint is unchanged. There is evidence for more than one open complex, which is dependent on the presence or absence of Mg$^{2+}$ (28, 29). RP$_{O}$ enters the initiation stage, or RP$_{init}$, in the presence of initiating nucleotides. Short transcripts of 2–12 nucleotides in length (abortive products) are synthesized while RNAP remains at the promoter. RNAP then enters into the elongation phase of transcription upon promoter clearance and $\sigma$ factor release. Several of these intermediate complexes have been visualized by performing DNase I and KMnO$_4$ footprinting experiments over a range of temperatures, with the rationale that temperature-dependent complexes represent time-dependent events (30–32).

The S. typhimurium flagellar operon can be divided into three classes of genes (I–III) based on their transcriptional hierarchy in flagellar assembly (33). $\sigma^{28}$, encoded by the fliA gene, is expressed late in flagellum biosynthesis and is re-

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The abbreviations used are: RNAP, RNA polymerase; Eo$\sigma^{28}$, $\sigma^{28}$ holoenzyme.
required for transcription of all class III genes including *fliC*, encoding flagellin, the primary component of the flagellar filament (34), and *fliM*, encoding the σ^28^ anti-σ factor (35, 36). In this report, we have characterized transcription initiation by DNA polymerase carrying the *S. typhimurium* α factor, σ^28^, on variants of the flagellin (*fliC*) promoter. We detected intermediate complexes formed by Eσ^28^ during initiation that are distinct from those characterized for other holoenzymes. Initial binding to the promoter does not require the −10 element, but further extension of the RNA polymerase-DNA contacts and isomerization to the open complex demand the presence of the −10 sequence. We propose an alternative mode of promoter recognition and binding as compared with other well characterized holoenzymes.

**EXPERIMENTAL PROCEDURES**

**Overproduction and Purification of σ^28^ and Reconstitution of Holoenzyme**—The fltA gene, encoding σ^28^, was inserted into plasmid pET15b (Novagen, Inc.) to generate pKH439 (a gift from K. Hughes), which resulted in the addition of six histidines at the amino terminus. Hexahistidine-tagged σ^28^ was overproduced and purified using the method described by Wilson and Dombroski (11). Holoenzyme was reconstituted by adding 1.0 pmol of *E. coli* core RNA polymerase (E) (Epicentre Technologies Corp.) to 5.0 pmol of σ^28^ in protein dilution buffer (10 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.4 mg/ml bovine serum albumin, and 0.1% Triton X-100) and incubating on ice for 15 min. Because of the amino acid sequence conservation among the core subunits, heterologous holoenzymes have been used to assess the behavior of alternative σ factors (37–40). Additionally, we compared the amino acid sequence of the α and β subunits of *E. coli* and *S. typhimurium* RNA polymerases (11) (data not shown). There is 100% identity between the α subunits and 98% identity between the β subunits.

**Constructions and Generation of Promoter Fragments**—pflfIC35 was constructed by annealing two oligonucleotides (Integrated DNA Technologies) of 122 bases in length (41). The DNA was designed to incorporate HindIII and BamHI restriction sites near the 5′- and 3′-ends, respectively. The double-stranded DNA was digested with HindIII and BamHI, ligated into the same sites in pBluescript KS⁺ (Stratagene), and transformed into *E. coli* strain DH5α (Life Technologies, Inc.). The clones were sequenced to confirm the deletion using the fmol™ DNA sequencing system (Promega).

pflfICΔ10 was constructed using an oligonucleotide with a deletion of the −10 element as a polymerase chain reaction primer with plasmid pMC72, containing the wild-type fltC promoter, as the template. The resulting DNA fragment was ligated into pCR2.1 and transformed into Invitro™ One Shot competent cells from the original TA cloning kit (Invitrogen). The clones were sequenced to confirm the deletion using the fmol™ DNA sequencing system.

pflfICΔlabeled primers were generated for use in synthesizing labeled fltC promoter DNA (10). Oligonucleotide primers were from BioServe Biotechnologies, Genosys Biotechnologies, Inc., or Integrated DNA Technologies. Plasmid pMC72 (a gift from K. Hughes) and the two plasmids containing pflfICΔ10 and pflfICΔ35, as described above, were used as the template DNA to generate the fltC promoter DNA and derivatives. Both radiolabeled and unlabeled fltC promoters were synthesized using the polymerase chain reaction to generate a 250-base pair fragment. Each polymerase chain reaction contained 10× Taq Buffer A (Fisher), 50 pmol of each primer, 40 ng of template DNA, and 2.5 units of Taq polymerase (Fisher) in a final volume of 100 μl. A Perkin-Elmer Thermocycler was set for 35 cycles with 95 °C for denaturation, 50 °C for annealing, and 72 °C for extension. The products were purified using the Qiaquick polymerase chain reaction DNA purification kit (QIAGEN Inc.).

**DNase I Footprinting**—P- and end-labeled DNA promoter fragment and DNase I buffer (20 mM sodium Hepes (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 200 μg/ml bovine serum albumin) were combined in a volume of 50 μl. Holoenzyme was added in 10-fold excess over the DNA unless noted otherwise. Additional processing of these samples has previously been described (40). Several variations of the DNase I footprinting are described below.

**Heparin Competition**—The Eσ^28^-promoter complexes were allowed to form at 37 °C for 15 min. In some cases, nucleoside triphosphates (NTPs) were added to a final concentration of 0.2 mM for 1 min, and then heparin was added (25 μg/ml final concentration), followed by incubation for another minute. The complexes were treated with 0.5 units of DNase I (Promega) for 30 s and processed as already described.

**Temperature Variation**—The Eσ^28^-promoter complexes were allowed to form at 0, 4, 16, 25, and 37 °C for 15 min. The complexes formed at 0 and 4 °C were subjected to DNase I digestion (2 units of DNase I) for 35 and 30 min, respectively. Complexes formed at 16 °C were digested for 4 min (1.5 units of DNase I), and the 25 and 37 °C samples were digested for 1 min (1.0 unit of DNase I).

**KmO₄ Footprinting—Eσ^28^ and 0.1 pmol of end-labeled DNA promoter fragment were combined in KmO₄ buffer (20 mM sodium Hepes (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, 0.1 mM EDTA, 0.2 mM dithiothreitol, and 200 μg/ml bovine serum albumin) to a final volume of 50 μl and then treated with 2.5 μl of 50 mM KmO₄ (Sigma) for 2 min at the temperatures indicated. Additional processing has been described (42).

**Nucleotide Stabilization Assay in Vitro**—The nucleotide stabilization assay was performed as outlined by Wilson and Dombroski (11) with the following modifications. The Eσ^28^-promoter complexes were allowed to form for 15 min, and then 0.1 mM ATP, CTP, and GTP were added to the preformed complexes for 30 s, prior to filtration and washing with 0.8 M NaCl.

**RESULTS**

**Promoter Binding and Transcription in Vitro**—RNAP was formed by mixing hexahistidine-tagged σ^28^ with purified *E. coli* core RNAP. Because of the high degree of similarity between the core subunits of *E. coli* and *S. typhimurium* RNAPs, a heterologous system was used as reported by others to assess the behavior of alternative σ factors (37–40). Runoff transcription assays were performed to analyze the overall efficacy of transcription initiation by Eσ^28^ (42). The promoter chosen for this analysis, fltC, drives the expression of flagellin, one of the late flagellar gene products. A transcript of the expected size was observed. No difference in behavior was noted in the presence or absence of the hexahistidine tag (data not shown). Deletion of the AT-rich sequence just upstream of the −35 element had a slight effect on transcription (2-fold reduction), but was not attributable to the presence of an UP element since holoenzyme containing a truncation of the carboxyl-terminal domain of the α subunit resulted in the same transcriptional behavior as the wild-type enzyme, independent of the upstream DNA sequence (data not shown).

DNase I footprinting was used to characterize the interaction of Eσ^28^ with pflfIC (Fig. 2). A DNA fragment of 230 base pairs in
length containing the fliC promoter was 5’-end-labeled on the template strand with \( ^{32}P \). Increasing amounts of reconstituted E. coli were added, followed by DNase I digestion (Fig. 3). Continuous protection was observed from -24 to +17, with partial protection from -46 to -24 relative to the transcription start site. Additional weak interactions between +17 and +20 could be discerned. Overall, this footprint is shorter in the upstream region than those typical for E. coli or E. coli under similar conditions.

Intermediate Complexes Formed during Transcription Initiation—We characterized the intermediate E. coli-DNA complexes during the process of transcription initiation that can be visualized by manipulating the temperature of incubation. E. coli was incubated with 5’-end-labeled fliC (template strand) for 15 min, at 0, 4, 16, 25, and 37 °C. Protection was observed from -65 to -54 and from -48 to -19 at 0 and 4 °C (Fig. 4A). At 16 °C, a hypersensitive band appeared at -46, and partial protection began to extend downstream toward the -10 region and the start site (-46 to +17), with the reappearance of bands at -33 and -24. By 25 °C, E. coli fully occupied the region from -46 to +17. At 37 °C, the upstream contacts from -65 to -54 disappeared, but strong protection from -46 to +17 remained. Thus, E. coli appeared to initially bind primarily to the upstream region of the promoter since -10 protection was absent at lower temperatures and then shifted downstream to contact the -10 element up to the +17 region as the temperature was increased. We cannot rule out the possibility that some minor contacts in the upstream region are maintained. However, it is clear that a major rearrangement takes place as a function of temperature. The same pattern of contacts was observed with footprinting performed using DNA labeled on the non-template strand (data not shown).

Open Complex Formation—Extended DNase I footprints at 37 °C are usually indicative of open complexes for E. coli (27, 43–46). We used KMnO\(_4\) sensitivity to determine which E. coli promoter complexes in the DNase I temperature series were open complexes. KMnO\(_4\) chemically modifies thymine residues in single-stranded DNA, which renders modified positions sensitive to cleavage upon piperidine treatment (47). E. coli promote complexes were formed for 15 min at 0, 4, 16, 25, or 37 °C and subjected to KMnO\(_4\) treatment and piperidine cleavage (Fig. 4B).

From 0 to 4 °C, the DNA remained base-paired as demonstrated by lack of reactivity to KMnO\(_4\). Open complexes were fully established at 16, 25, and 37 °C with reactivity to KMnO\(_4\) at +1, -6, -7, and -9. Thus, similar to RP\(_D\) for E. coli and E. coli, the E. coli DNase I footprints that extend into the -10 region and start site represent open complexes. Surprisingly, however, E. coli formed open complexes at temperatures as low as 16 °C, whereas E. coli and E. coli form primarily extended closed complexes (RP\(_{C2}\)) under these conditions (27, 30–32, 46, 48). We did not observe an RP\(_{C2}\)-type complex for E. coli under any conditions.

Promoter Variants Lacking the -10 or -35 Element—If E. coli initially binds to the -35 region, releases upstream interactions, and shifts its contacts to include the -10 and downstream regions, then removal of the -35 element should preclude any binding by E. coli. Likewise, removal of the -10 consensus sequence should permit binding of only E. coli in the -35 region. We constructed deletions of the TAA sequence at -35 (pflIC10) and the GCCGATA sequence at -10 (pflIC35) (Fig. 2) and analyzed the DNase I footprints over a range of temperatures. Deletion of the -10 element still allowed normal binding of E. coli at 0 and 4 °C, as expected based on the presence of the predicted initial binding site at -35 (Fig. 5A). However, upon raising the temperature, binding to pflIC10 was gradually abolished, consistent with the idea that polymerase shifts its contacts downstream during open complex formation to interact with the -10 element and downstream sequences. In the case of pflIC10, these sequences have been removed, and E. coli is unable to remain stably bound to the upstream sequences and results in dissociation.

Also as expected, E. coli was unable to bind to pflIC35 even at 0 °C, presumably due to lack of the initial binding site in the -35 region and upstream portions of the promoter (Fig. 5B). KMnO\(_4\) footprinting showed no open complexes at either pflIC10 or pflIC35 at any temperature (data not shown). Taken together, these results support the idea that initial binding occurs from -65 to -19, followed by isomerization to relinquish major contacts between -65 and -54 and establishment of new contacts extending to +20 with concomitant DNA melting.

Stability of E. coli-Promoter Complexes—Previous studies have shown that E. coli typically forms a heparin-stable open complex. E. coli, also forms heparin-sensitive complexes in the presence or absence of NTPs (53).

RP\(_{init}\) complexes for E. coli can be distinguished from RP\(_D\) complexes by resistance to challenge with high salt in the presence of NTPs (11, 50, 54). We used an NTP stabilization assay to determine whether RP\(_{init}\) complexes formed by E. coli on pflIC were stable to salt challenge. E. coli-Ppromoter complexes were formed in the absence and presence of NTPs and were then filtered through nitrocellulose and subjected to a 0.8 M NaCl wash. In the presence of NTPs, 52% of the complexes were retained on the filter, as expected since RP\(_{init}\) is in equilibrium with RP\(_D\), whereas in the absence of NTPs, only 5% were retained. Therefore, E. coli RP\(_{init}\) complexes on pflIC, like E. coli-DNA complexes, are stable to 0.8 M NaCl, indicating an equivalent conformational change and stabilization upon NTP binding.
DISCUSSION

Transcription initiation has been characterized as a multi-step process in studies in which intermediates on the pathway to formation of an initiated complex have been detected by manipulating the temperature of incubation and varying the level of Mg$^{2+}$ for the RNAP-promoter interactions (30, 31, 43, 46, 50, 51). There is evidence for two different closed complexes (RPC1 and RPC2), which differ in the extent of their contacts with the promoter DNA. RPC1, which extends from approximately -60 to -5, is only observed at low temperatures (0 °C) for Es$^{70}$ (30) and Es$^{32}$ (31, 32). However, we have recently shown that derivatives of σ$^{70}$ with region 1.1 or both regions 1.1

FIG. 3. Binding of Es$^{28}$ to pftiC using DNase I footprinting. 5'-Radiolabeled pftiC (template strand) was incubated with increasing amounts of Es$^{28}$ for 15 min at 37 °C and subsequently treated with DNase I. All samples were analyzed by electrophoresis on a denaturing 8% polyacrylamide gel. The extent of each footprint is indicated on the side of each footprinting ladder. Lane 1, DNA alone; lane 2, 0.01 pmol of Es$^{28}$; lane 3, 0.02 pmol of Es$^{28}$; lane 4, 0.04 pmol of Es$^{28}$; lane 5, 0.08 pmol of Es$^{28}$; lane 6, 0.1 pmol of Es$^{28}$; lane 7, 0.2 pmol of Es$^{28}$.

FIG. 4. DNase I and KMnO$_4$ footprinting of Es$^{28}$ promoter complexes as a function of temperature. 5'-Radiolabeled pftiC (template strand) was incubated with Es$^{28}$ at the temperatures indicated below for 15 min, and the complexes were subsequently treated with DNase I or KMnO$_4$ and piperidine. DNA Alone shown here was obtained at 37 °C; however, the same pattern of digestion was obtained at 0 °C (data not shown). All samples were analyzed by electrophoresis on a denaturing 8% polyacrylamide gel. A, DNase I protection. Arrows on the side of each footprint indicate the extent of protection. First lane, DNA alone; second lane, 0 °C; third lane, 4 °C; fourth lane, 16 °C; fifth lane, 25 °C; sixth lane, 37 °C. B, KMnO$_4$ reactivity. Arrows indicate the reactive bases in the open complex. First lane, DNA alone; second lane, 0 °C; third lane, 4 °C; fourth lane, 16 °C; fifth lane, 25 °C; sixth lane, 37 °C.
and 1.2 removed slow the initiation process such that RP \text{C1} can be detected at 37 °C (11).

We have investigated the mechanism of transcription initiation by holoenzyme carrying \( \sigma^{28} \), the alternative \( \sigma \) factor required for flagellum biosynthesis in \( S. typhimurium \). Our analysis was conducted using the promoter for \( fliC \), encoding flagellin, a late gene in this pathway. The identity and stability of transcription complex intermediates that we identified during transcription initiation by \( \sigma^{28} \) are distinctive from those previously identified for \( \sigma^{70} \) and \( \sigma^{32} \).

Based on our results, we suggest the following pathway for the mechanism of \( \sigma^{28} \) transcription initiation (Fig. 7). \( \sigma^{28} \) forms a short complex with \( fliC \) at 0 °C that protects primarily the upstream region of the promoter (−65 to −19) from DNase I digestion. This suggests that the initial binding of \( \sigma^{28} \) is mediated mainly through interactions in the vicinity of the −35 consensus sequence. \( \sigma^{28} \) then isomerizes to make additional contacts that extend to +20. During this progression, some upstream contacts are lost as evidenced by the reappearance of bands from −65 to −54 on the DNase I footprints at 25 and 37 °C. Thus, \( \sigma^{28} \) appears to initially bind in the −35 region

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**Fig. 5.** DNase I footprinting of \( \sigma^{28} \) on \( fliC \) variants. 5′-Radiolabeled \( fliC\Delta 10 \) (template strand) or \( fliC\Delta 35 \) (non-template strand) was incubated with \( \sigma^{28} \) at the temperatures indicated below for 15 min and subjected to DNase I treatment. All samples were analyzed by electrophoresis on a denaturing 8% polyacrylamide gel. Arrows denote the locations of the protected regions. A, \( fliC\Delta 10 \). The position of the deletion of the −10 element is indicated by an asterisk. First lane, DNA alone; second lane, core RNAP; third lane, 0 °C; fourth lane, 4 °C; fifth lane, 16 °C; sixth lane, 25 °C; seventh lane, 37 °C. B, \( fliC\Delta 35 \). The position of the deletion of the −35 element is indicated by an asterisk. First lane, DNA alone; second lane, core RNAP; third lane, 0 °C; fourth lane, 4 °C; fifth lane, 16 °C; sixth lane, 25 °C; seventh lane, 37 °C.

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**Fig. 6.** Determination of \( \sigma^{28} \)-promoter complex stability. 5′-Radiolabeled \( fliC \) (template strand) was incubated with \( \sigma^{28} \) for 15 min at 37 °C, and the complexes were subsequently treated with DNase I. All samples were analyzed by electrophoresis on a denaturing 8% polyacrylamide gel. Arrows show the locations of the protected regions. First lane, DNA alone; second lane, \( \sigma^{28} \); third lane, \( \sigma^{28} + 25 \mu g/ml \) heparin (Hep); fourth lane, \( \sigma^{28} + 2 \mu M \) ATP, CTP, and GTP (ACG); fifth lane, \( \sigma^{28} + 2 \mu M \) ATP, CTP, and GTP + heparin.
and then shifts downstream to contact the −10 region, start site, and downstream sequences to +20 while relinquishing some of the original upstream contacts. This represents a notable difference in binding intermediates from those observed for Eσ70 or Eσ28.

The identity of RPc versus RPo complexes was assessed by KMnO4 sensitivity, which probes for DNA strand separation. Typically, significant open complex formation for Eσ70 or Eσ28 requires temperatures above 16 °C (27, 30, 31). One distinctive characteristic of Eσ28/fliC complexes is that they are optimally strand-separated between −9 and +1 even at 16 °C. Thus, the extended DNase I protection observed at 16 °C is representative of RPo, rather than an extended closed complex like RPc2.

Thus, one major difference between Eσ28 intermediates and those found for Eσ70 or Eσ28 from E. coli is the absence of a detectable RPc2-like complex under the conditions that we used. Eσ28 appears to form a single closed complex characterized by a short DNase I footprint at 0–4 °C and then progresses to RPo. If an RPc2 complex forms, it may be too rapid or unstable to be detected with the methods employed in this study.

Because Eσ28 is functionally similar to the B. subtilis holoenzyme that is required for flagellin biosynthesis, Eσ70, we compared the transcription initiation complexes formed by these two enzymes at their respective flagellin promoters. The flagellar gene promoter (phag) from B. subtilis is very similar to pflIC, except it contains an UP element between −60 and −40 that provides additional contact sites for RNAP through the α subunit (57). Eσ28 forms complexes that are more similar to Eσ70 and Eσ28 because, at 4 °C, the DNase I footprint occupies from −73 to +1 (55). This protection then extends to +9 and +21 at 23 and 37 °C, respectively. Disappearance of the upstream contacts, which is observed for Eσ28, does not occur. Thus, DNA binding primarily in the −35 and upstream regions in the earliest detectable complex, with lack of contact in the −10 region, appears to be novel for Eσ28.

With respect to open complex formation, however, Eσ28 and Eσ70 share the ability to generate strand-separated promoter regions at low temperatures. At 16 °C, Eσ28 displays the same degree of open complex formation that is observed at 37 °C, but forms only closed complexes at 4 °C. Eσ70 begins to show DNA distortions in the −10 region at temperatures as low as 4 °C; however, the region of strand melting propagates unidirectionally as a function of temperature, forming three distinct open complex intermediates (55), whereas the reactivity of hases in the Eσ28 open complex remains the same from 16 to 37 °C, with no indication of directional movement.

We used challenge with the polyanionic competitor heparin as a tool to assess the relative stability of open (RPc) and initiated (RPin) complexes. Eσ28/fliC open complexes are unstable to even low levels of heparin in the presence or absence of initiating NTPs. In contrast, many Eσ70 open complexes are stable to heparin even in the absence of NTPs (11, 48–51). Eσ28 appears to form transcriptionally competent complexes that remain dissociable throughout the initiation process. We cannot rule out that Eσ28 may form heparin-resistant complexes on other σ28-dependent promoters or that heparin is actively destabilizing these complexes. However, the information from these studies is useful in comparing the relative stability of holoenzymes carrying different σ factors.

In the case of σ70, it has been shown that amino-terminal region 1.1 is involved in inhibition of DNA binding by σ (10, 56) and is required for efficient open complex formation by holoenzyme. Region 1.2, which is typically present in all primary and alternative σ factors, may also be involved in open complex formation for Eσ70 (11). σ28 is a very unusual member of the σ70 family of proteins since it lacks any homology to region 1.2, leading to some speculation regarding how this difference in structure may translate into a difference in function. The flagellar biosynthesis σ factor from B. subtilis, σD, retains homology to region 1.2. Eσ70 forms two closed complexes and isomerizes through several open complex intermediates (55). σ28, on the other hand, lacks both regions 1.1 and 1.2. Eσ28 forms an unusually short RPc1, and we did not observe an RPc2-like intermediate. Additionally, many Eσ70 open complexes are stable to heparin either in the absence or presence of NTPs, and open complexes do not form efficiently at low temperatures, whereas the opposite is true in both cases for Eσ28.

The composition of the amino terminus of the σ factor may affect the nature of the RNAP-DNA complexes that can be discerned during initiation. σ28 appears to facilitate transcription initiation at pflIC very efficiently by utilizing a mechanism with few intermediate complexes, but with reduced stability of the open complex. Whether this mechanism is a general phenomenon for all σ28-dependent promoters or whether it is characteristic for the flagellin gene promoter remains to be determined.

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