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Sigma and RNA Polymerase: An On-Again, Off-Again Relationship?

Review

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In bacteria, a fundamental level of gene regulation occurs by competitive association of promoter-specificity factors called σs with RNA polymerase (RNAP). This σ cycle paradigm underpins much of our understanding of all transcriptional regulation. Here, we review recent challenges to the σ cycle paradigm in the context of its essential features and of the structural basis of σ interactions with RNAP and elongation complexes. Although σs can play dual roles as both initiation and elongation regulators, we suggest that the key postulate of the σ cycle, that σs compete for binding to RNAP after each round of RNA synthesis, remains the central mechanism for programming transcription initiation in bacteria.

The σ Cycle

Despite its size and complexity, the ~400 kDa catalytic core of the bacterial RNAP (subunit composition $\alpha_2\beta\beta'\omega$) is incapable of promoter-specific initiation. Dissociable σ factors, which bind core RNAP to form holoenzyme, direct key aspects of the initiation process, including recognition of promoter DNA and melting of the DNA to expose the transcription start site. This process was originally described as a "σ cycle" (Figure 1), in which σ associates with RNAP to orchestrate initiation and then dissociates after the transition to a stable elongation complex (EC) is complete (Travers and Burgess, 1969; Chamberlin, 1976). Once RNAP finishes transcription and releases DNA and RNA, it is free to be bound anew by σ and begin another cycle of transcription. The key feature of the σ cycle is the ability of RNAP to be reprogrammed rapidly by different σs in each new round of transcription.

Thus, the σ cycle allows cells to adjust transcription patterns rapidly to optimize cellular metabolism in response to changing external conditions and cellular signals and to orchestrate developmental programs by using different σs with different promoter specificities to regulate discrete sets of genes. One σ is typically present at the highest level and is responsible for the cell's general housekeeping genes (σ^{70} in *E. coli*). Other σs direct specific transcriptional responses, such as a heat-shock σ that directs synthesis of gene products that stabilize or refold proteins (σ^{32} in *E. coli*), an alternative σ that directs synthesis of gene products for assimilation of nitrogen compounds (σ^{54} in *E. coli*), or a stationary phase σ that directs synthesis of gene products required during stationary phase and some stresses (σ^{S} in *E. coli*). The number of alternative σs varies from zero in some obligate endosymbionts to at least 65 (e.g., *Streptomyces coelicolor*; Bentley et al., 2002). In *Bacillus subtilis*, sequential cascades of σs drive gene expression in the tightly regulated developmental program of sporulation (Losick and Pero, 1981; Stragier and Losick, 1990; Kroos and Yu, 2000). Eukaryotic cells use a similar system of cyclical association of regulators with RNAP to regulate transcriptional initiation, although the number of regulators is much larger and the details are less well understood (Cramer, 2004; Sims et al., 2004; Svejstrup, 2004).

The central role of available σ pools in transcriptional regulation is reflected in the multiple mechanisms cells use to modulate σ availability. The relative amounts of different σs are determined by rates of synthesis and degradation, by posttranslational modifications that interconvert inactive and active forms of σs, and most ubiquitously, by the extent to which a given σ is sequestered from the active pool by binding to a cognate anti-σ (Hughes and Mathee, 1998; Ishihama, 2000; Kroos and Yu, 2000). Anti-σs have been identified for most known σs, and both the contacts between anti-σs and σs and the mechanisms that govern their interactions are surprisingly diverse (Campbell et al., 2002a, 2003; Sorenson et al., 2004; Campbell and Darst, 2005). Because these mechanisms allow cells to modulate the composition of σ pools easily, the σ cycle allows transcription to respond to changing conditions efficiently.

The σ cycle paradigm is supported by a significant body of biochemical evidence. Shortly after the identification of σs, Travers and Burgess (1969) found that one σ could program initiation by five or more core RNAP molecules during in vitro transcription of phage DNA, which could only be explained by recycling of σ. The most complete study of σ affinity for initiating versus elongating forms of RNAP was performed by Gill et al. (1991), who observed a drop in σ^{70} affinity between core RNAP and ECs (from K_d ~10^{-10} to ~10^{-6} M) by using the elongation regulator NusA in a competition binding assay. NusA and σ^{70} compete for binding to RNAP, although both NusA and σ^{70} may be bound to ECs in special situations (Yarnell and Roberts, 1992). Because NusA exhibits a reasonably constant affinity (K_d of ~3×10^{-8}) for both core RNAP and ECs, Gill et al. reasoned that NusA must displace weakly bound σ during elongation, whereas σ^{70} would displace NusA during initiation (Figure 1). Thus, 50-fold excess σ^{70} could not inhibit NusA function during elongation, and 300-fold excess NusA could not inhibit σ^{70} function during initiation. The lower σ^{70} affinity in ECs has been attributed to the presence of the RNA transcript (Krakow and von der Helm, 1971; Daube and von Hippel, 1999), a view that is supported by observation of structural clash between parts of σ in RNAP-σ crystal structures and the presumed path of the nascent RNA transcript (Mekler et al., 2002; Murakami et al., 2002a; Murakami and Darst, 2003; see below).

New results from several groups have challenged the traditional view of the σ cycle, suggesting instead that σ

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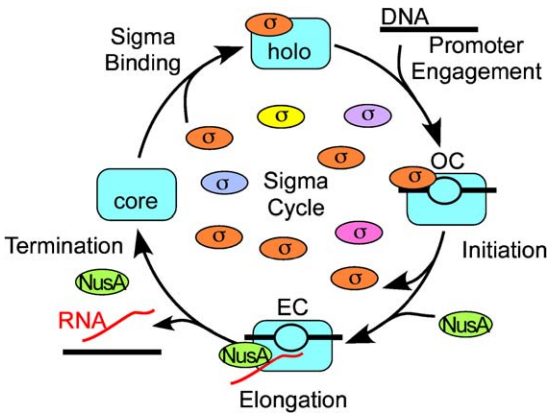

Figure 1. The σ Cycle

A pool of σ factors compete for binding to core RNAP to form a holoenzyme, which binds promoter DNA to form an open complex (OC). σ release from the elongating form of the enzyme (the EC) explains how σ can be reused to direct transcription initiation by multiple molecules of core RNAP. Competitive binding to the EC by NusA may help displace σ from the EC (after Travers and Burgess [1969], Chamberlin [1976], and Gill et al. [1991]).

may influence steps during elongation, either through transient association with ECs (Ring et al., 1996; Ko et al., 1998; Marr et al., 2001; Mooney and Landick, 2003; Brodolin et al., 2004; Nickels et al., 2004; Kapanidis et al., 2005), through persistent association with ECs (Bar-Nahum and Nudler, 2001; Mukhopadhyay et al., 2001), or potentially through conformational imprinting of the EC that persists after σ release (Berghofer-Hochheimer et al., 2005). Such effects would allow σ to help regulate steps in the transcription cycle other than initiation. Some of these recent results appear to contradict the σ cycle paradigm by implying that a tight RNAP-σ complex can persist through multiple rounds of transcription.

In considering implications of these results for the σ cycle paradigm, it is imperative to recognize the distinction between possible effects of σ on transcript elongation versus retention of σ through multiple rounds of transcription. σ effects on elongation are fully compatible with the σ cycle paradigm and require only that σ possess both initiation-programming and elongation-regulating activities (which could be related to each other or wholly distinct). In contrast, σ retention and obligate reuse through multiple rounds of transcription would violate the σ cycle paradigm and would require revision of the textbook view that RNAP initiation specificity is reset in each round of transcription by rebinding of a new σ.

Two Versions of the σ Cycle

As originally conceived, the σ cycle did not specify a precise point of σ release from the EC. Subsequently, two more detailed versions of the σ cycle were postulated. In the obligate-release model (Figure 2A), RNAP cannot form a stable EC until σ is released from the complex. (This transition is thought to occur when the RNA transcript reaches 8–9 nt in length; Korzheva et al., 1998, 2000; Sidorenkov et al., 1998). In an alternative view, called the stochastic release model (Figure 2B), the affinity of σ for RNAP decreases in the EC but release occurs stochastically after RNAP has initiated transcription.

Evidence for an obligate-release σ cycle in bacteria came from comparison of promoter bound (open) RNAP complexes and ECs that were subjected to chromatography or electrophoresis to distinguish tightly bound from easily dissociable σ (Hansen and McClure, 1980; Straney and Crothers, 1987; Krummel and Chamberlin, 1989; Metzger et al., 1993). Apparent release of σ from ECs led to an RNA length-dependent model in which the rate of σ release is stringently correlated with the length of the RNA. The consensus conclusion was that σ^{70} is released when the RNA transcript grows from 8 to 9 nt (Hansen and McClure, 1980; Metzger et al., 1993), although some promoter-dependent variability in the point of release was suggested (Krummel and Chamberlin, 1989). Straney and Crothers (1985, 1987) suggested that σ^{70} release and promoter escape were mechanistically coupled via a "stressed intermediate" that must either release σ^{70} or abort initiation by releasing the RNA transcript. A mechanistic requirement for σ release is the essence of the obligate release model.

The alternative stochastic release model (Figure 2B) was proposed by Shimamoto et al. (1986) based on kinetic measurements of the time required for σ release. They observed that changing the rate of RNA synthesis by altering NTP concentrations had no effect on the kinetics of σ release, which occurred in vitro at 0.2–0.4 s^{-1} (37°C) regardless of elongation rate once transcription began (equivalent to an average release position of ~60 nt for an elongation rate of 25 nt/s). These results suggest that σ is released stochastically, that release is independent of the rate of elongation once RNAP has initiated RNA synthesis, and in particular, that the length of the nascent RNA does not dictate the point of σ release.

Importantly, in both the obligate and stochastic release models, σ release occurs during each transcription cycle and, consequently, σ competition for rebinding RNAP is allowed, as specified by the σ cycle paradigm.

σ Nonrelease or Partial Release

In 2001, reports from the Nudler and Ebright groups raised questions about the generality of the σ cycle and the extent of σ release. Bar-Nahum and Nudler (2001) propose that, in vivo, a subpopulation of ECs retains σ through multiple rounds of transcription. These workers assayed σ release by using holoenzymes purified from exponential and stationary phase cells to form stalled ECs containing a 32 nt RNA. They found that ~7% of purified, 32 mer-containing ECs formed from exponential-phase RNAP and ~33% formed from stationary-phase RNAP still contained σ. Both the retention of σ by these ECs and the different behaviors of exponential-phase and stationary-phase RNAPs were unexpected, leading Bar-Nahum and Nudler to suggest that increased σ retention by stationary phase RNAP could allow continued transcription of some σ^{70}-dependent genes during stationary phase when σ^{S}-directed transcription predominates. Bar-Nahum and Nudler then used holoenzyme purified from cells after T7 RNAP-driven overexpression of

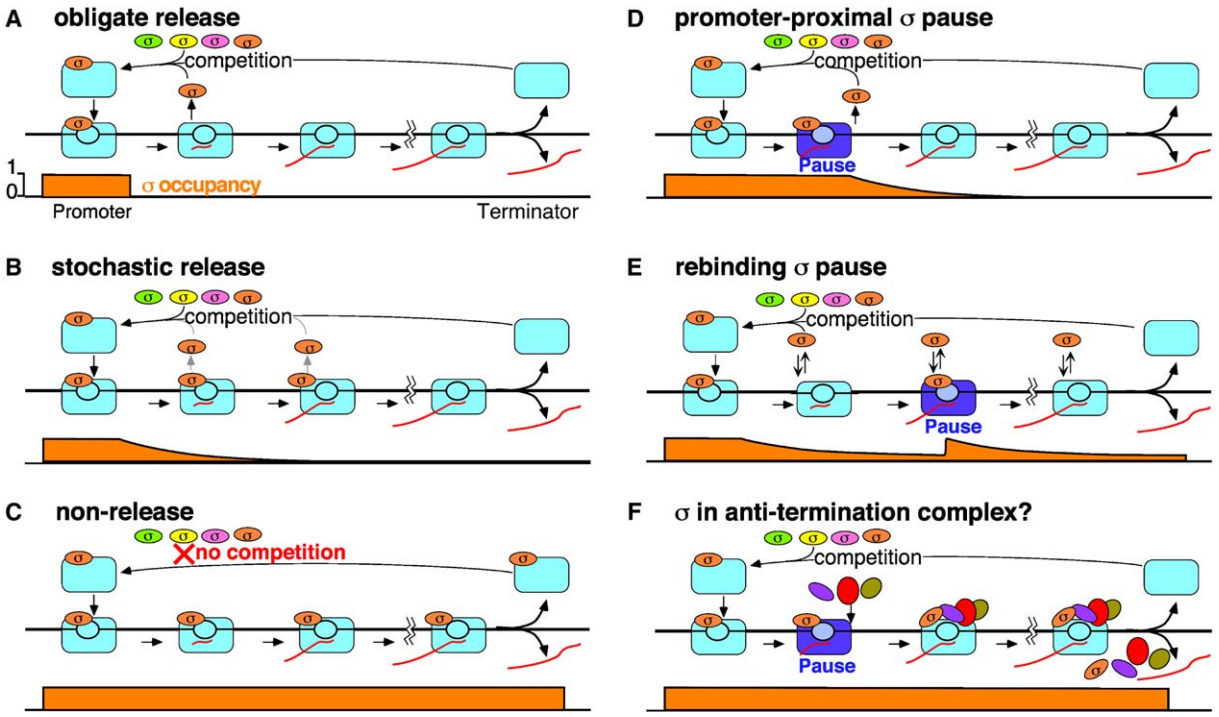

Figure 2. Models of σ Action

The orange plots below the models depict the fraction of RNAP bound by σ at a given point in the transcriptional units.

(A) Obligate σ release. σ is completely released from RNAP before the enzyme can pass a critical stage in transcript elongation, for instance prior to formation of an EC containing an 8–9 bp RNA-DNA hybrid (Hansen and McClure, 1980; Straney and Crothers, 1987; Krummel and Chamberlin, 1989; Metzger et al., 1993).

(B) Stochastic σ release model. The affinity of σ for RNAP decreases significantly after promoter escape, making σ release a function of σ's off rate and the effective concentrations of free σ and of any competitors (Shimamoto et al., 1986; Gill et al., 1991).

(C) σ nonrelease. σ is incorporated as an integral subunit of the EC. Because σ remains bound when RNAP releases at a terminator, other σs are excluded and reinitiation is proposed to occur more rapidly (Bar-Nahum and Nudler, 2001).

(D) Promoter-proximal, σ-dependent pause. For at least some promoters (e.g., λ $P_{R'}$ and *lacUV5*), a promoter-like sequence just downstream of the transcriptional start site can reengage σ's DNA contacts and cause transcriptional pausing before σ is released from RNAP (dark blue, EC; Ring et al., 1996; Nickels et al., 2004).

(E) σ rebinding. Even after σ is released from ECs, σ may rebind the EC provided σ is present at high enough effective concentration. The viability of cells containing σ^{70} fused to all its RNAP suggests this is the case at least for *E. coli* σ^{70} (Mooney and Landick, 2003). Transient σ interaction with the EC allows formation of a paused EC (dark blue) when a σ binding DNA sequence is encountered.

(F) Hypothetical role of σ as antitermination factor. Continuous retention of σ might be explained by its incorporation into ECs as an antitermination factor, similarly to the proposed behavior of the antiterminator RfaH, which also loads onto ECs by recognition of a nontemplate-strand consensus sequence that becomes exposed at pause sites (Artsimovitch and Landick, 2002). Such a role of σ in antitermination has not been demonstrated.

His_6-tagged-σ to test whether the σ-retaining subpopulation had an advantage in multiround transcription. After forming ECs, they purified the subpopulation still containing the His_6-tagged-σ (using Ni^{2+}-NTA agarose) and observed that, on a T7A1-promoter template, but not on an *rrnB* P1-promoter template, the purified, σ^{70}-containing ECs were capable of more rounds of transcription than the unpurified, mixed EC population. Based on these results, Bar-Nahum and Nudler conclude that σ-retaining RNAP could account for "much of transcription in the exponential phase" and "most of σ^{70}-directed transcription in the stationary phase."

These intriguing results raise some important questions. First, what is the physical basis for persistent association of some of σ^{70} with core RNAP? Does it represent covalent attachment of σ^{70} to core RNAP (which would explain Bar-Nahum's and Nudler's observations)? If so, which σ^{70} and RNAP moieties form the covalent linkage and how are they altered in stationary phase? Second, and most importantly, does σ^{70}-retaining RNAP exist in vivo or is it formed during the process of cell lysis and RNAP purification? Third, would the purified, σ^{70}-retaining ECs still exhibit enhanced multiround synthesis if a competing σ factor were present (e.g., σ^{S}) or if they were added back to the original reaction mix (i.e., might increased activity reflect removal of an inhibitory impurity)? And fourth, why did the purified, σ^{70}-retaining ECs exhibit an improved capacity for multiround transcription on a T7 A1-promoter template, but not on an *rrnB*P1 promoter template? Bar-Nahum and Nudler suggest that the rate of σ rebinding to RNAP may affect transcription for T7 A1, but not for *rrnB* P1 because at *rrnB* P1, "open complex formation is likely to be rate-limiting." However, in similar conditions, the *rrnB* P1 promoter actually binds RNAP with higher initial affinity than T7 A1 ($K_B \approx 10^{10}$ M^{-1} versus 10^8 M^{-1}), isomerizes to an initiation-competent state about two times faster than T7 A1 ($k_i \approx 0.04 \pm 0.01$

s^{-1} versus 0.014 ± 0.01 s^{-1}), and avoids abortive initiation (Johnson et al., 1991; Rao et al., 1994; Johnson and Chester, 1998; Barker et al., 2001). Despite the short lifetime of the *rrnB* P1 open complex, these characteristics make it doubtful that the overall initiation rate at *rrnB* P1 is significantly slower than at T7 A1 and thus doubtful that σ retention should affect T7 A1, but not *rrnB* P1. Although the hypothesis that a subpopulation of ECs retain σ raises some interesting possibilities, more evidence will be needed before we conclude the σ cycle paradigm is contradicted.

Ebright and coworkers challenged the obligate release model and questioned whether the σ cycle was obligatory for all RNAPs by using fluorescence resonance energy transfer (FRET) between a fluorescent group in σ^{70} and one incorporated either into the upstream or downstream DNA or into RNAP to detect σ translocation during the early stages of transcription (Mukhopadhyay et al., 2001). These workers observed persistence of the FRET signal in ECs that had extended the nascent RNA to at least 50 nt, arguing that there is no mechanistic requirement to release σ^{70} from mature ECs. However, the FRET signal between σ and RNAP decreased with increasing transcript length, consistent with predictions of a stochastic release model.

In this issue, Kapanidis et al. (2005) extend these experiments by using single-molecule FRET and conclude that σ behavior is generally consistent with the stochastic release model. They confirm that σ^{70} need not be released when ECs form (e.g., an EC with an 11 nt transcript) but observe release of σ^{70} from most halted ECs with longer transcripts. Although σ^{70} release is incomplete and its rate is slow relative to that reported by Shimamoto et al. (1986), this may reflect the conditions used, especially the absence of excess core RNAP. Excess core RNAP was used in previous key σ cycle experiments (Travers and Burgess, 1969; Shimamoto et al., 1986), is present in vivo, and may accelerate σ release.

In summary, the experiments of Bar-Nahum and Nudler (2001) and Mukhopadhyay et al. (2001) provide strong arguments against the obligate release model. Determining the extent to which they may also be at odds with the stochastic release model of Shimamoto et al. (1986) will require additional work. It will be particularly important to test factors likely to influence σ release in vivo, for instance addition of other macromolecules (e.g., NusA, core RNAP, and other σs), addition of small molecules (e.g., ppGpp; Jishage et al., 2002), and solution conditions (e.g., macromolecular crowding).

σ Can Regulate Elongation

Regardless of the details of stochastic σ release, groundbreaking discoveries by the Roberts group show that σ^{70} can function not only as an initiation factor but also as an elongation factor once RNAP relinquishes its contacts with the promoter (Ring et al., 1996; Ko et al., 1998; Marr et al., 2001). This finding reveals a second function of σ^{70} but does not contradict the essential features of the σ cycle paradigm. Roberts and coworkers found that σ^{70} can remain bound to RNAP long enough to stimulate pausing at promoter-proximal sites at which promoter-like −10 elements are present in the exposed nontemplate strand of ECs (Figure 2D). At the $P_{R'}$ promoter of bacteriophage λ, σ^{70} causes RNAP to pause at a −10-like sequence located just downstream of the transcription start site; these paused ECs contain a stably bound nascent RNA that is 16 or 17 nt in length. Consistent with a gradual release of σ^{70}, shifting the −10-like sequence further downstream resulted in considerably less pausing in vitro. However, a consensus −10 element (including an extended −10 sequence) allowed σ-dependent pausing even when placed ~10 bp further downstream such that the paused ECs contained a nascent RNA 27 or 28 nt in length. Thus, σ^{70} must persist as a functional part of the EC after release of promoter contacts or be able to rebind ECs (see below).

More recently, a similar promoter-proximal, σ-dependent pause was identified at the *lacUV5* promoter (Brodolin et al., 2004; Nickels et al., 2004; Kapanidis et al., 2005). This finding explains why so much σ retention was observed in the FRET experiments of Mukhopadhyay et al. (2001), who used the *lacUV5* promoter (Kapanidis et al., 2005). Further experiments demonstrated that when the −10-like element was altered away from consensus to reduce σ^{70} binding, the amount of σ^{70} detected by FRET in ECs decreased and the rate of σ^{70} release increased more than 4-fold (Nickels et al., 2004; Kapanidis et al., 2005). These results are consistent with a stochastic release model in which the rate of release can be altered by sequences in the early transcribed region.

Not only can σ^{70} reestablish DNA contacts when it is transiently retained in ECs, but it also can bind de novo to ECs and cause pausing when a suitable pause sequence is exposed in the transcription bubble (Mooney and Landick, 2003; Brodolin et al., 2004; Figure 2E); such pausing has been observed even when the pause signal is located at a great distance from the promoter (e.g., ~450 nt after the transcription start site; Mooney and Landick, 2003). The apparent inability of σ^{70} to cause pausing when σ-dependent pause sequences were displaced from promoters in earlier in vitro experiments (Ring et al., 1996) appears simply to reflect the relatively weak affinity of σ^{70} for ECs. At 0.5–1 μM, σ^{70} can bind to σ-free ECs artificially halted on promoter-proximal pause sequences (Brodolin et al., 2004). Even more remarkably, at 10 μM, σ^{70} can bind actively transcribing RNAP and cause pausing at a consensus −10-like sequence even in the presence of 10 μM NusA (Mooney and Landick, 2003). Thus, σ^{70} must be in rapid binding equilibrium with ECs such that even the transient presence of pause sequences during active elongation can tip the balance from NusA binding to σ^{70} binding.

Taken together, these results are consistent with stochastic σ release followed by an ability of σ to rebind RNAP and engage promoter-like elements that appear in the exposed nontemplate strand of ECs (Figure 2E). Interestingly, this same mode of binding was recently found for the elongation regulator RfaH, which recognizes an "*ops*" element (unrelated to promoter sequences) when the *ops* sequence is exposed in the nontemplate strand of ECs (Artsimovitch and Landick, 2002). Thus, once RNAP escapes a promoter, it is possible, although not yet demonstrated, that σ^{70} may become an elongation factor capable of joining specialized ECs like antitermination complexes (Weisberg and Gottesman, 1999; Figure 2F). The extent to which this happens,

the composition of such specialized ECs, and whether a σ present in such ECs is retained after transcription initiation or joins the EC subsequently all remain important questions for future investigations.

Structural Basis of σ Release and Rebinding

Recent structural studies are consistent with the idea that some, but not all, contacts between σ and RNAP can be retained or reestablished in ECs (Murakami et al., 2002a, 2002b; Vassylyev et al., 2002; Mekler et al., 2002). Housekeeping σs are divided into four conserved sequence regions (σR1–σR4; with subregions 1.1, 1.2, 2.1, 2.2, 2.3, 2.4, 3.0, 3.1, 3.2, 4.1, and 4.2) that fold into four independent structural domains ($\sigma_{1.1}$, σ_2, σ_3, and σ_4; Figures 3A and 3B). The domains are separated by flexible linkers, two of which can span significant distances (the $\sigma_{1.1-2}$ linker and the σ_{3-4} linker, which contains conserved sequence region 3.2). In holoenzyme, these domains and linkers contact an extensive surface of RNAP (~10^4 Å^2) in at least five discrete sets of contacts: (1) $\sigma_{1.1}$ in or near to the downstream DNA channel; (2) σ_2 to the β′ coiled coil; (3) σ_{2-3} linker and σ_3 to the β′ zipper, β protrusion, and β flap; (4) σ_{3-4} linker to the RNAP main channel, β saddle, and RNA exit channel; and (5) σ_4 to the β flap tip, β′ ZBD, and β′ dock (Figure 3C; Mekler et al., 2002; Murakami et al., 2002a; Vassylyev et al., 2002; Murakami and Darst, 2003). These contacts include both stabilizing and destabilizing interactions of more than 100 separate amino acid side chains (Borukhov and Nudler, 2003).

The simultaneous but independent binding of distinct structural elements of σ to different parts of the core RNAP results in the high-affinity σ-RNAP complex but allows for stepwise structural transitions that induce the dissociation of individual σ domains one by one (Figures 3C and 4A). So, for example, the $\sigma_{1.1}$/RNAP contacts appear to be lost on the transition from RNAP holoenzyme to the open promoter complex (Mekler et al., 2002). The σ_{3-4} linker and σ_4 contacts are sequentially lost as RNAP transitions to an EC, whereas retention of the σ_2, σ_{2-3} linker, and σ_3 contacts is sterically compatible with current models of EC structure and consistent with the observed ability of these domains to stimulate pausing after promoter escape by making contacts to a −10-like DNA element (Figure 4). As RNAP extends the nascent RNA chain, steric clash between the σ_{3-4} linker and the growing RNA chain is thought to contribute to abortive initiation and to cause displacement of the linker upon formation of a stable EC (Mekler et al., 2002; Murakami et al., 2002a; Murakami and Darst, 2003). As the RNA chain grows past 16–17 nt, it clashes with σ_4 and eventually causes displacement of σ_4 from the outlet of the RNA exit channel (Murakami and Darst, 2003; Nickels et al., 2005).

A conformation of the EC with displaced σ_{3-4} linker and σ_4 but bound σ_2 and σ_3 appears completely compatible with the paths of RNA and DNA in the EC (Figure 4B) and likely corresponds to σ bound ECs detected in vitro (Mukhopadhyay et al., 2001; Kapanidis et al., 2005). This σ bound EC may correspond to the state from which σ is released stochastically during transcript elongation and that is formed upon σ rebinding. This EC model in which σ maintains a subset of the holoenzyme contacts is consistent with the reduced affinity of σ^{70} for ECs (from ~10^{-10} to ~10^{-6} K_d; Gill et al., 1991) and also with the ability of NusA to displace σ. NusA appears to contact both the outlet of the RNA exit channel (displacing σ_4; Toulokhonov et al., 2001) and the β′ coiled coil (Traviglia et al., 1999; Borukhov et al., 2005), thus out competing key σ contacts once the σ_{3-4} linker is displaced. Additionally, anti-σs and core RNAP may be able to displace σ from ECs by initiating contacts with σ_4 and then "peeling" σ_2 and σ_3 off the EC by capturing new contacts as they become transiently exposed by thermal fluctuations (Chadsey et al., 1998; Sorenson et al., 2004).

This picture of σ/EC contacts is both consistent with and supported by available data on σ^{70}-dependent pausing. An intermediate EC state with a displaced σ_{3-4} linker but bound σ_4 (Figure 4C) appears to exist in the σ^{70}-dependent, promoter-proximal paused EC that forms downstream of the $\lambda P_{R'}$ promoter (Marr et al., 2001; Murakami and Darst, 2003; Nickels et al., 2005). In this paused EC, σ_2 makes sequence-specific contacts with a −10-like element (as described above), and σ_3 and σ_4 appear to be positioned where they could contact canonically spaced extended −10-like and −35-like elements, although sequence-specific interactions have not been demonstrated. Another intermediate EC state, which appears to form subsequently once λQ binds to the paused EC, contains both a displaced σ_{3-4} linker and a displaced σ_4 (Figure 4D; Marr et al., 2001; Murakami and Darst, 2003). At this point, the σ_2 pause contacts are retained and σ_4 appears to relocate to a −35-like sequence that is directly adjacent to the −10-like pause-inducing sequence, which interferes with pausing (Nickels et al., 2002).

Thus, available data suggest σ^{70} can interact with the EC through the normal σ_2 and σ_3 contacts seen in the RNAP holoenzyme. If DNA sequences with affinities for σ_2 and σ_3 are present, these domains may reestablish sequence-specific DNA contacts similar to those observed in promoter complexes. If a −35-like sequence is also present, σ_4 might also reestablish sequence-specific DNA contacts, although this has only been detected in the context of the promoter-proximal $\lambda P_{R'}$ pause (Nickels et al., 2002). In the σ-dependent paused conformation (Figure 4D), the EC may be unable to forward translocate because the DNA bubble is held open at the upstream edge, which would explain how σ^{70} binding can induce transcriptional pausing.

σ Is Released Stochastically In Vivo but May Rebind ECs

Most in vitro results paint a picture of stochastic σ release and rebinding that is consistent with the basic precepts of the σ cycle modified to include σ function as a pause-enhancing elongation regulator. The key questions now are whether, in vivo, σ is released stochastically and whether it is present at concentrations that would allow rebinding. Recent results suggest that the answer to both questions is yes.

The first of an impending wave of chromatin immunoprecipitation (ChIP) experiments to map RNAP and σ association with DNA gives results strikingly consistent with the stochastic release model (Wade and Struhl, 2004; Raffaelle et al., 2005). ChIP reports a snapshot of protein-DNA interactions in cells by quantitation of

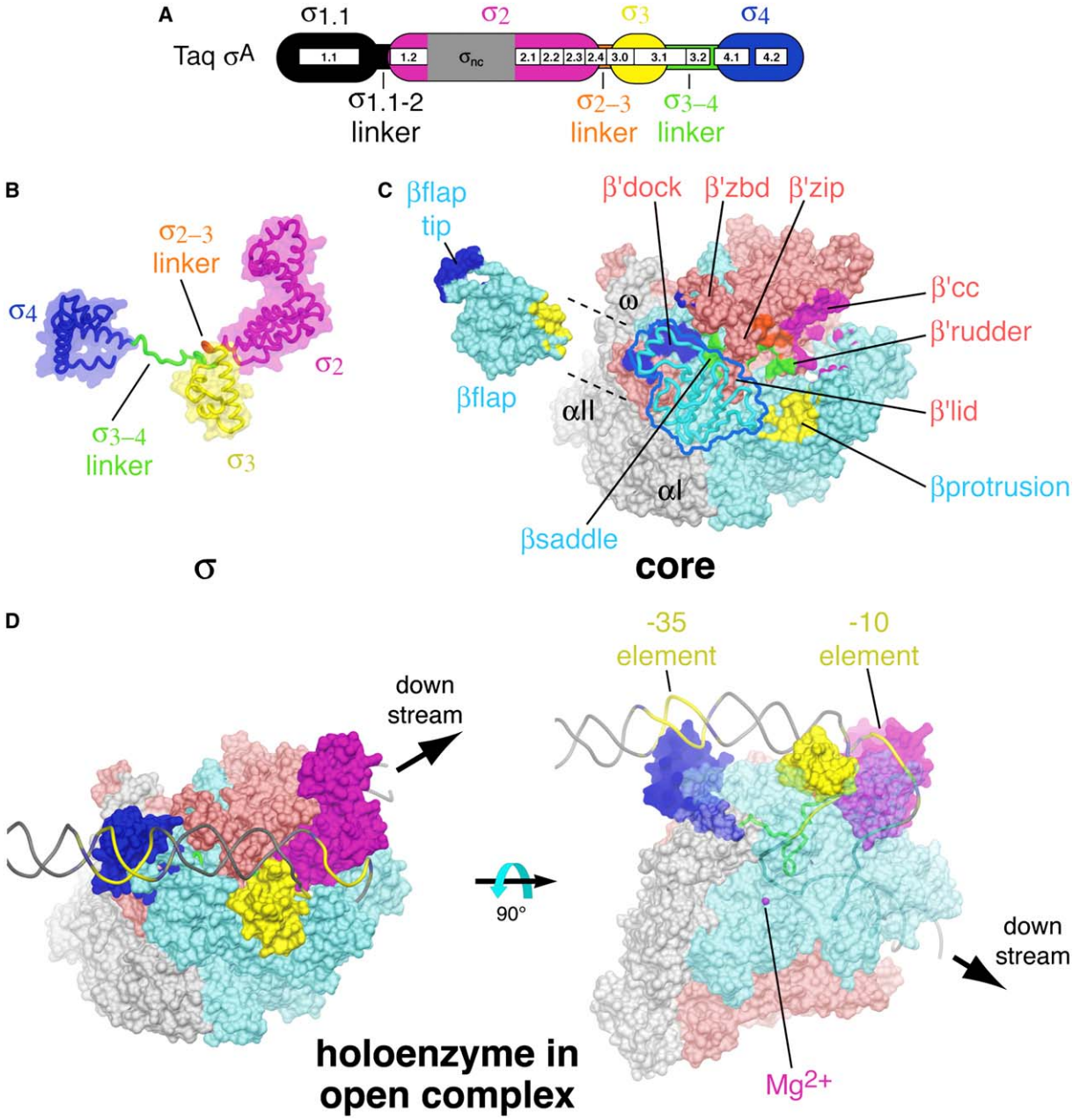

Figure 3. σ Contacts to RNAP and ECs

(A) σ regions. Conserved σ sequences (1.1–4.2) (Helmann and Chamberlin, 1988; Lonetto et al., 1992) are shown relative to structural domains of *T. aquaticus* σ^A ($\sigma_{1.1}$, black; σ_2, magenta; σ_3, yellow; and σ_4, blue) and linkers ($\sigma_{1.1–1.2}$ linker, black; $\sigma_{2–3}$ linker, orange; and $\sigma_{3–4}$ linker, green) between the domains (Malhotra et al., 1996; Campbell et al., 2002b; Murakami et al., 2002a; Vassylyev et al., 2002). $\sigma_{1.2}$ and $\sigma_{2.1}$ are separated by a nonconserved sequence (σ_{nc}).

(B) Structure of σ in holoenzyme. The arrangement of σ domains and linkers observed in the RNAP holoenzyme (Murakami et al., 2002b) is shown, colored as in (A). Transparent molecular surfaces of the structured domains σ_2, σ_3, and σ_4 are shown along with the backbone worm. $\sigma_{1.1}$ and the $\sigma_{1.1–2}$ linker, absent or disordered in all of the holoenzyme structures (Murakami et al., 2002a, 2002b; Vassylyev et al., 2002), are not shown.

(C) σ contacts on core RNAP. RNAP (Murakami et al., 2002b) is shown as a molecular surface, colored as follows: αI, αII, and ω, gray; β, cyan; and β′, pink. RNAP surfaces contacted by σ (<4.5 Å) are colored according to the σ structural element they contact (colored as in [A]). The β flap is shown as a backbone worm, with its surface displaced to reveal σ contacts behind it. Key structural features of RNAP involved in σ contacts are labeled by using nomenclature described previously by Kornberg, Cramer, and Darst (Cramer et al., 2001; Darst, 2001; Murakami and Darst, 2003; Cramer, 2004). Abbreviations: β′ zbd, β′ zinc binding domain; β′ zip, β′ zipper; and β′ cc, β′ coiled-coil. Again, $\sigma_{1.1}$ contacts are not shown, because $\sigma_{1.1}$ is not resolved in any crystal structures; biochemical data suggest $\sigma_{1.1}$ contacts RNAP downstream of the active site in holoenzyme and is displaced by downstream DNA upon open complex formation (Mekler et al., 2002; Murakami et al., 2002a; Vassylyev et al., 2002; Murakami and Darst, 2003).

(D) Holoenzyme contacts to DNA in the open complex. The view on the left corresponds to (B) and (C). The view on the right is rotated 90° toward the viewer to highlight the active site and main channel (seen through the transparent β subunit) and corresponds to the orientation of the diagrams in Figure 4.

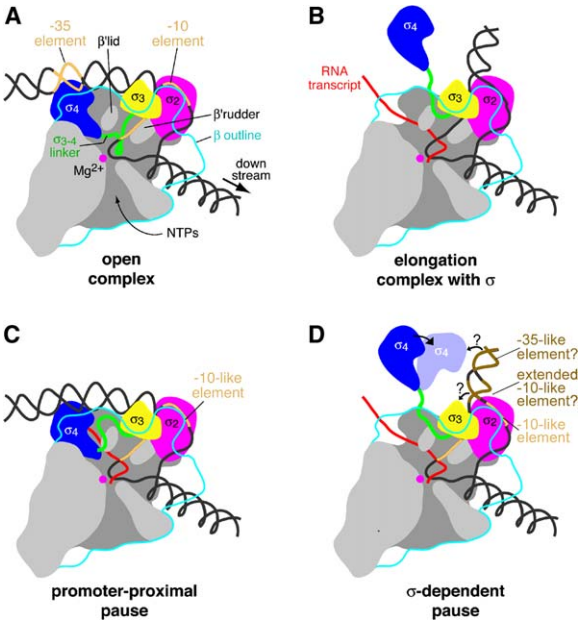

Figure 4. Models of σ Contacts in Various Transcription Complexes

Coloring and nomenclature are as in Figure 3.

(A) Open complex. See Murakami et al. [2002b] and Murakami and Darst [2003].

(B) σ bound EC model. σ is depicted with open-complex contacts sterically compatible with the EC structure (σ_2, σ_{2-3} linker, and σ_3) and contacts that are incompatible with the EC eliminated (σ_{3-4} linker and σ_4). This EC configuration likely exists when σ associates with non-paused ECs prior to σ release (Shimamoto et al., 1986; Mukhopadhyay et al., 2001; Kapanidis et al., 2005; Raffaelle et al., 2005) or upon σ rebinding (Mooney and Landick, 2003).

(C) Promoter-proximal σ-dependent paused EC model. In this state, the σ_{3-4} linker has been displaced from the main channel and RNA exit channel, but other σ contacts to RNAP remain. This EC configuration forms at the $\lambda P_{R'}$ promoter-proximal pause (Marr et al., 2001; Nickels et al., 2002) and also appears to form at a related promoter-proximal site in the *lac* operon (Nickels et al., 2004; Brodolin et al., 2004). Retention of σ_4 contacts to RNAP becomes disfavored once the RNA transcript grows past 16 nt (Murakami and Darst, 2003; Nickels et al., 2005), so this configuration may be restricted to promoter-proximal locations. Although σ_4 appears to be near DNA in the $\lambda P_{R'}$ paused EC, there is currently no evidence for sequence-specific interaction with a −35-like element (Marr et al., 2001); rather, σ_4 appears to relocate to a −35-like element 1 bp upstream from the −10-like element upon λQ binding (Nickels et al., 2002).

(D) Promoter-distal σ-dependent paused EC model. In this state, both the σ_{3-4} linker and σ_4 have been displaced from RNAP; sequence-specific contacts of σ_2 and possibly of σ_3 cause transcriptional pausing. This EC configuration appears to form at promoter-distal, σ^{70}-dependent pause sites (Mooney and Landick, 2003). Involvement of σ_3 contact to an extended −10-like element is untested but likely, based on the presence of an extended −10-like element at the +25 σ-dependent pause of phage 82 (Ring et al., 1996). Interaction of σ_4 with a nearby −35-like sequence also could affect promoter-distal pausing, but neither this interaction nor possible effects of its spacing have been tested. After λQ binding, interaction of σ_4 with a −35-like sequence 1 bp upstream from the −10-like sequence of the $\lambda P_{R'}$ paused EC destabilizes pausing, possibly due to clash between σ_2 and σ_4 (Nickels et al., 2002).

sheared DNA fragments recovered after immunoprecipitation of formaldehyde-crosslinked, nucleoprotein complexes. Wade and Struhl report that, during exponential phase growth, σ^{70} is associated with the promoter DNA, but not with the DNA in the downstream coding regions of *melAB*, *lacZYA*, and eight additional operons in *E. coli*. Although these data do not distinguish obligate versus stochastic release, a new report in this issue of *Molecular Cell* by Raffaelle et al. (2005) takes advantage of the high transcription level and faster elongation rate in the *E. coli* rRNA operons to measure gradual σ^{70} release unambiguously at an estimated rate of 0.1–0.2 s^{-1} (versus 0.2–0.4 s^{-1} estimated in vitro by Shimamoto et al. [1986]). Coupled with their observation of faster release of σ^S, which can program some rRNA operon transcription but binds RNAP more weakly, these findings clearly establish that a stochastic release mechanism operates in vivo in the rRNA operons (which are responsible for ~70% of all transcription in rapidly growing *E. coli*). Thus, in vivo, bulk in vitro, and single-molecule experiments all now show that most σ is released stochastically as RNAP moves through the early stages of transcription, suggesting that this is the principal mechanism by which the σ^{70} cycle operates (Shimamoto et al., 1986; Kapanidis et al., 2005; Raffaelle et al., 2005).

Although most of their results also show σ release from ECs consistent with stochastic release, Wade and Struhl (2004) detected persistent σ^{70} signal on the *melAB* operon (although not on the *lacZYA* operon) during stationary phase. This could indicate partial σ^{70} retention by a subset of ECs during stationary phase, along the lines proposed by Bar-Nahum and Nudler (2001). However, the stationary phase *melAB* ChIP signal clearly shows stochastic release of at least 80% of σ^{70}. Further, the residual σ^{70} signal within the *melAB* operon could easily reflect σ^{70} binding at cryptic promoters in *melAB*, at σ^{70}-dependent pause sites, or to RNAP bound nonspecifically in *melAB*, rather than σ nonrelease. σ^{70} may be more available for such interactions in stationary phase, because σ^{70} levels do not decrease in stationary phase even though σ^{70}-dependent transcription decreases. Indeed, ChIP detects RNAP within some operons even when transcription is blocked by rifampicin (Herring et al., 2005). Thus, additional work will be required to tease out whether a small subset of RNAPs retain σ, whether σ rebinding occurs at σ-dependent pause sites, and whether these events, if they occur, are physiologically important.

That the effective concentration of σ in vivo is sufficient to allow σ rebinding was recently made clear by the effects of tethering σ^{70} to RNAP in *E. coli* (Mooney and Landick, 2003). Remarkably, cells in which all RNAP molecules contain σ^{70} covalently fused to the C terminus of the β′ subunit not only remain viable but also exhibit only subtle effects even in growth conditions known to require the function of alternative σs. The likely explanation for this unexpected result is that the effective concentrations of σs in vivo are quite high, probably owing to the effects of molecular crowding in cells. The effective concentration of σ^{70} in vivo appears to be 10–15 μM, compared to the ~50 μM effective concentration of the tethered σ^{70} (Mooney and Landick, 2003). At such concentrations, σ^{70} binding to ECs is rapid, making the importance of whether or not σ is released from ECs doubtful. Thus, the behavior of the tethered-σ^{70} RNAP suggests that at the high effective concentration of σ^{70} in vivo, σ^{70} maintains sufficient interaction with ECs to respond whenever promoter-like

sequences become exposed in the nontemplate DNA strand (Mooney and Landick, 2003). In other words, in vivo, σ^{70} continues to interact transiently with ECs even after its initial release following promoter escape.

σ release and rebinding at such sites is consistent with the known properties of σ^{70}, of elongating RNAP, and of protein interactions in the *E. coli* cytoplasm. For σ at ~15 μM effective concentration (Mooney and Landick, 2003) to bind more than 50% of ECs during a ~20 ms EC-dwell time at a single template position (based on the average in vivo elongation rate of 50 nt/s; Vogel and Jensen, 1994), the effective on rate of σ must be >4 $\times$ 10^6 M^{-1} s^{-1}. Elowitz et al. (1999) measured the diffusion constant of an ~70 kDa protein in the *E. coli* cytoplasm at ~2.5 $\times$ 10^{-8} cm^2 s^{-1}. If the target size for σ-EC binding is similar to that of operator-site recognition on DNA, Debye-Smoluchowski theory predicts a σ on rate of ~2.5 $\times$ 10^7 M^{-1} s^{-1} (Richter and Eigen, 1974; Bruinsma, 2002). This would be fast enough to permit σ rebinding to ECs at specific template positions within operons in vivo.

To illustrate what this means for σ rebinding to an EC, consider a simple kinetic model in which σ transiently binds ECs at this rate, releases ten times more slowly than normally when it encounters a promoter-like sequence, and, at such a site, converts ECs to a paused state that elongates 100 times more slowly than average (Figure 5; i.e., σ off rate of 2.5 s^{-1} at the site versus 25 s^{-1} predicted from the σ^{70}-EC K_d of 10^{-6} M [Gill et al., 1991], and a pause dwell time of 2 s versus the 20 ms average dwell time). Even if the effective concentration of σ^{70} was as low as 3 μM, 50% of ECs would be trapped at the pause; at the predicted 15 μM concentration in vivo, more than 80% of ECs would be trapped (Figure 5; this percentage would be even higher if a more complex pause mechanism was used). Put simply, this simulation demonstrates that, in vivo, σ^{70} can act as an elongation factor to modulate transcription without requiring continuous physical retention of σ^{70} as part of the EC.

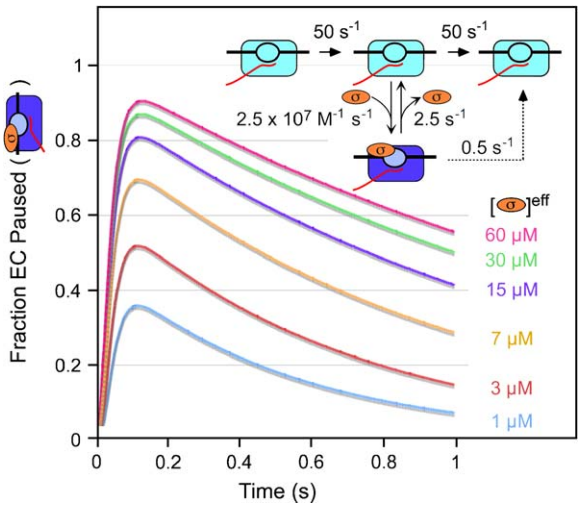

Figure 5. Kinetic Simulation of In Vivo Behavior of σ^{70} as an Elongation Regulator

σ is depicted as a single domain for simplicity (see Figures 1 and 3). In this kinetic model, ECs (10 nM) were allowed to elongate for two steps at 50 nt/s before encountering the pause site. The concentration of EC trapped in the σ bound, paused conformation (dark blue) as a function of time was calculated by numerical integration using the program Berkeley Madonna (v8.1β12; http://www.berkeleymadonna.com) with the rates and σ^{70} concentrations shown in the inset. The amount of paused EC is shown as a fraction of the total EC in the simulation.

Can σ Imprint RNAP?

A recent report from the Gross lab (Berghofer-Hochheimer et al., 2005) hints that the role of σ^{70} during elongation could be even more complicated. These workers detected differences in the pausing behavior of ECs generated from native holoenzyme (i.e., RNAP-σ^{70} holoenzyme purified directly from cells) versus ECs generated from purified core RNAP to which σ^{70} had been added back. Berghofer-Hochheimer et al. (2005) postulated that ECs retain a "memory" of the σ molecule that was bound during initiation and that this memory could alter the ability of the EC to recognize and respond to pause and termination signals. Although they were unable to eliminate all differences in pausing behavior, it is notable that the extent of these differences decreased with increasing purification of the ECs.

Long-lived conformational states of ECs have been considered previously (Goliger and Roberts, 1989; Telesnitsky and Chamberlin, 1989; de Mercoyrol et al., 1990; Davenport et al., 2000). However, strong evidence exists that the conformational state of ECs is reset with each round of nucleotide addition (Pasman and von Hippel, 2002; Greive and von Hippel, 2005). At least some of these suggested cases of long-lived conformational heterogeneity likely result from alternative RNA folding or from subtle chemical differences among RNAP molecules such as amino acid misincorporation or amino acid modification in vivo or in vitro (Toli-Nørrelykke et al., 2004). As noted by Berghofer-Hochheimer et al. (2005), such chemical differences, which could arise for instance during purification of RNAP without an associated σ, might explain the altered elongation properties of ECs. Distinguishing true conformational heterogeneity in RNAP from possible chemical heterogeneity and from conformational heterogeneity in nascent RNA is a significant experimental challenge. As in the case of σ nonrelease models, more definitive exclusion of alternative explanations will be needed before the idea of RNAP imprinting can be accepted.

Conclusion and Prospects

Recent studies of σ dynamics both in vitro and in vivo confirm that the vast majority of σ is stochastically released from ECs, allowing facile reprogramming of the transcriptional machinery and thus optimal regulatory flexibility, as originally envisioned in the σ cycle paradigm. Obligate σ release during promoter escape is not required for this purpose, perhaps explaining why obligate σ release has not evolved. Nevertheless, the overarching evolutionary lesson in bacterial gene regulation is that nature seizes every conceivable opportunity to couple gene expression with available information about a cell's internal and external environment. It would be surprising if special mechanisms that govern σ release, retention, or rebinding did not operate in certain cases to modulate gene expression. The search for

these specialized functions offers exciting avenues for future study.

Effects of σ on transcript elongation, like the now well-documented ability of σ^{70} to stimulate transcriptional pausing whether it is retained after initiation or rebound by ECs, offer a clear example of nature's regulatory efficiency and also raise important questions for future study. Nickels et al. (2004) note that 5.1% of *E. coli* promoters contain recognizable −10-like sequences at locations that could direct promoter-proximal, σ^{70}-dependent pausing. To what extent does σ^{70} direct pausing at these sites? Are there additional examples of such sites that escape recognition by sequence analysis? Do these sites modulate the rate of initiation by promoter occlusion? Do they allow recruitment of other transcriptional regulators before RNAP encounters critical checkpoints downstream? Given that σ^{70} can rebind ECs at any point in a transcriptional unit, do σs direct transcriptional pausing within transcription units in vivo? If so, what is the function of these pause events? What roles may −35-like and extended −10-like elements play at various σ-dependent pause sites?

The similarity between the way σ^{70} directs pausing and the action of the elongation regulator RfaH (Artsimovitch and Landick, 2002) raises a more general question of whether σs may sometimes become integral subunits of specialized ECs. Specialized ECs, which are usually referred to as antitermination complexes, are well characterized in phage λ transcription and have been unambiguously demonstrated to form in the *E. coli* ribosomal RNA operons (Torres et al. [2004] and references therein). However, the subunit compositions and the functions of non-λ specialized ECs are poorly understood at best. Even in λ, it is uncertain at what point σ^{70} is released from λQ-modified ECs in vivo (Ring et al., 1996). This and the extent to which σs may function as components of specialized cellular ECs deserve much greater attention. ChIP experiments (Wade and Struhl, 2004; Raffaelle et al., 2005), especially using microarrays to quantify signals (i.e., ChIP-chip experiments), as well as assays of in vivo protein-protein interaction networks such as recently reported by Butland et al. (2005) promise to yield insight into this fascinating subject.

We emphasize that function of σs as components of specialized ECs, if documented, would not, a priori, contradict the σ cycle paradigm. Even if σ were to stably associate with an EC, it clearly cannot retain all RNAP contacts required to function as an initiation factor (e.g., the σ_{3-4} linker and σ_4 contacts); in principle, σ could use entirely different contacts during elongation. Thus, free σs might still compete effectively against an EC bound σ for initiation-specific contacts or even outcompete it depending on the geometry of σ-EC contacts. Pioneering experiments by Arndt and Chamberlin (1988) suggest that free σ binds RNAP during the process of transcription termination and thereby accelerates termination; if σ loads onto RNAP this way in vivo, then it might displace an EC bound σ that used different contacts.

Finally, although the σ cycle paradigm appears secure as a fundamental mechanism by which transcription is programmed in bacteria, experiments to test whether, in special cases, σs may persist in functional association with RNAP through multiple rounds of transcription, as proposed by Bar-Nahum and Nudler (2001), are warranted. The possibility of such recycling transcriptional machines was recently suggested in yeast by the observation that the 5′ and 3′ ends of genes are sometimes bound in a single complex (O'Sullivan et al., 2004). Clearly, much additional data on the behavior of σs within the intracellular milieu of bacteria will be required to evaluate these ideas meaningfully.

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