Insights into transcriptional regulation and σ competition from an equilibrium model of RNA polymerase binding to DNA

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To explore scenarios that permit transcription regulation by activator recruitment of RNA polymerase and σ competition *in vivo*, we used an equilibrium model of RNA polymerase binding to DNA constrained by the values of total RNA polymerase (E) and σ^{70} per cell measured in this work. Our numbers of E and σ^{70} per cell, which are consistent with most of the primary data in the literature, suggest that *in vivo* (i) only a minor fraction of RNA polymerase (<20%) is involved in elongation and (ii) σ^{70} is in excess of total E. Modeling the partitioning of RNA polymerase between promoters, nonspecific DNA binding sites, and the cytoplasm suggested that even weak promoters will be saturated with $E\sigma^{70}$ *in vivo* unless nonspecific DNA binding by $E\sigma^{70}$ is rather significant. In addition, the model predicted that σ s compete for binding to E only when their total number exceeds the total amount of RNA polymerase (excluding that involved in elongation) and that weak promoters will be preferentially subjected to σ competition.

nonspecific DNA binding | gene regulation promoter | systems biology | *Escherichia coli*

In bacteria transcription is initiated by RNA polymerase (RNAP) holoenzyme ($E\sigma$), which is formed when core RNAP (E) binds the transcription initiation factor σ (1). $E\sigma$ initially binds to promoter sites in a closed complex, which then transits to an open complex, competent for transcription. The number of intermediates between the closed and open complex is variable and promoter-dependent; each step may be subject to regulation *in vivo* (2, 3). At least for some promoters, $E\sigma$ binding to promoters is thought to be reversible on the time scale of transcription initiation *in vivo* (3); reversibility has also been demonstrated *in vitro* for several promoters (3–6). Even binding to the strong *lac* UV5 promoter is reversible *in vitro* when tested under conditions that approximate the *in vivo* situation (6).

Recruitment of $E\sigma$ to promoters *in vivo* is thought to depend on the intrinsic binding affinity of the promoter and is modulated by repressors that prevent and activators that stabilize interactions between $E\sigma$ and the promoter (3). Based on *in vitro* studies of the mechanism of activator function, it is believed that promoters that bind $E\sigma$ weakly require activators to recruit $E\sigma$. In addition, cells contain multiple σ s, which direct E to various sets of promoters specific to the σ factors (1). These σ s are believed to compete with each other for binding to E (7–10). By changing the relative levels of the σ s, *Escherichia coli* is thought to coordinate its transcriptional program with growth conditions (11–13). This view is based on observations indicating that (i) overexpressing one σ decreases expression of genes controlled by another σ (7), (ii) mutationally altering binding constants of one σ for E alters expression by another σ (14), and (iii) physiological effectors such as ppGpp may act by altering relative binding of σ s to E (8–10). In the present work, we use an equilibrium model of RNAP binding to DNA to explore *in vivo* scenarios that permit transcription regulation by activator recruitment of RNAP and σ competition.

Nonspecific binding of E and $E\sigma$ to DNA should be an important component of a model that describes RNAP binding to promoters. Nonspecific binding of both species has been demonstrated *in vitro* (15); however, the magnitude and extent of nonspecific binding *in vivo* are hard to evaluate experimentally. von Hippel *et al.* (16) were the first to develop a model that examined the role of nonspecific binding in regulating transcription. Using a simple equilibrium approach, they examined partitioning of the Lac repressor between specific and nonspecific sites in *E. coli*. Their results showed that the kinetics of induction of Lac repressor could be understood only when nonspecific DNA binding was considered, suggesting that nonspecific DNA binding may play an important role in the thermodynamics and kinetics of interaction of transcription factors (as well as RNAP) with their specific sites (16, 17).

Estimation of RNAP partitioning between promoters, nonspecific binding sites on DNA, and the cytoplasm requires knowledge of the levels of E, σ^{70} (the housekeeping σ factor), and at least some alternative σ s. However, published values have been measured by different techniques, in different strains, and under different physiological conditions (both during exponential phase and during entry into stationary phase) (18–26). In fact, discrepancies in these numbers have led to the common perceptions that (i) as most RNAP is elongating, only a minor fraction could be bound to DNA nonspecifically (27, 28), and (ii) cellular levels of σ^{70} are much less than that of RNAP (29); thus, σ s may compete because of their excess over free RNAP. We have remeasured the levels of E, σ^{70} , σ^{32} , and σ^E in *E. coli* K12 MG1655. Analysis of our data, which are consistent with most of the primary data in the literature, suggests that *in vivo* (i) only a minor fraction of RNAP (<20%) is involved in elongation and (ii) σ^{70} is in excess of total E.

Using an equilibrium model based on our new values and relevant data from the literature, we explored the possible partitioning of E and $E\sigma$ between promoters, nonspecific DNA sites, and the cytoplasm. Our results suggest that even weak promoters will be saturated with $E\sigma^{70}$ *in vivo* unless nonspecific DNA binding by $E\sigma^{70}$ is rather significant. Thus, strong nonspecific binding is required for activators to work by recruiting $E\sigma$ and for the binding affinity of the promoter to contribute to regulation. In addition, our model predicts that σ s compete for binding to E only when their total number exceeds the total amount of RNAP (excluding those molecules involved in elongation), rather than the amount of free RNAP, and that weak promoters will be preferentially subjected to σ competition.

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Abbreviation: RNAP, RNA polymerase.

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Fig. 3. Decrease in $E\sigma^{70}$ promoter occupancy induced by σ competition. Calculated according to the equilibrium model. See *Materials and Methods* and *Supporting Text* for a complete discussion of the binding constants and approximations used in the model. E_T , the number of RNAP not included in elongation = 12,000 molecules per cell; σ^{70} promoters = 10,500; DNA binding sites = 11×10^6 (see Table 4, column 3). The concentration of free RNAP = free E + free $E\sigma^{70}$ is set at 1%. (A) Decrease in $E\sigma^{70}$ promoter occupancy calculated for various numbers of σ^{70} molecules per cell. $E\sigma^{70}$ promoter occupancy at $\sigma^A = 0$ is 48%. Dashed line: $\sigma^{70} = 8,000$ molecules per cell ($K_S = 1.19 \times 10^{-7}$ M, $K_{NS} = 4.01 \times 10^{-4}$ M). Competition starts when $\sigma_T = \sigma^{70} + \sigma^A > E_T = 12,000$ molecules. Solid line: $\sigma^{70} = 17,000$ molecules per cell ($K_S = 2.76 \times 10^{-7}$ M, $K_{NS} = 4.01 \times 10^{-4}$ M). An arbitrary line of perfect competition is calculated for $\sigma^{70} = 17,000$ molecules per cell for a "symmetrical" case when $E\sigma^A$ has the same number of specific binding sites and the same K_S as $E\sigma^{70}$. (B) Decrease in $E\sigma^{70}$ promoter occupancy calculated for promoters with various binding affinities to $E\sigma^{70}$. σ^{70} is set at 17,000 molecules per cell. The occupancy of σ^{70} promoters with $E\sigma^{70}$ (at $\sigma^A = 0$) is set at 99% for "strong" promoters ($K_S = 2.07 \times 10^{-9}$ M, $K_{NS} = 1.88 \times 10^{-3}$ M), 48% for "intermediate" promoters ($K_S = 2.76 \times 10^{-7}$ M, $K_{NS} = 4.01 \times 10^{-4}$ M), and 5% for "weak" promoters ($K_S = 5.01 \times 10^{-6}$ M, $K_{NS} = 2.42 \times 10^{-4}$ M).

titrates nonspecifically bound E to form holoenzyme ($E\sigma^A$) and only then competes with σ^{70} for E. Additional simulations of the model indicate that total σ must be in excess over E regardless of variations in the number of free RNAP or the number of promoters saturated with $E\sigma^{70}$ at $\sigma^A = 0$ (data not shown). It is noteworthy that σ^A competes more weakly than an equivalent amount of σ^{70} (Fig. 3A, "perfect competition" line). This asymmetry is due to our assumption that $E\sigma^{70}$ binds a large number of promoters, whereas $E\sigma^A$ has few promoter sites. We therefore ignored specific binding of $E\sigma^A$. As a result, equilibrium is slightly shifted so that E preferentially binds σ^{70} over σ^A (but see Figs. 4 and 5, which are published as supporting information on the PNAS web site).

That the total number of σ s must be higher than the number

of nonelongating E for σ competition *in vivo* rationalizes the higher number of σ^{70} per cell estimated in this work (Table 1). In this regard, it is interesting that, at low growth rates, the ratio of σ^{70}/E is greater than at high growth rates (Tables 1 and 4). Thus, the competition among σ s may be more prevalent at low than high growth rates.

We next explored whether extent of promoter occupancy affects competition between σ s. In these simulations, we (i) fixed $\sigma^{70}_t > E_t$, (ii) fixed the concentration of free RNAP to 1% (33), and (iii) varied K_S and K_{NS} to achieve initial $E\sigma^{70}$ promoter occupancies (at $\sigma^A = 0$) of 99%, 48%, or 5%. Calculation of σ^A competition for each initial condition indicates that an increase in σ^A always results in a decrease in $E\sigma^{70}$ promoter occupancy (Fig. 3B). However, the extent of competition depends inversely on the promoter saturation: promoters close to saturation are weakly competed, whereas promoters far from saturation show profound competition (Fig. 3B). These results can be understood by considering how the ability of σ^A to pull $E\sigma^{70}$ from its promoters is affected by the assumed K_S and K_{NS} of $E\sigma^{70}$. High promoter saturation by $E\sigma^{70}$ requires strong affinity for promoters ($K_S \ll K_{NS}$; see Fig. 2A and B). In this condition, σ^A primarily pulls $E\sigma^{70}$ from its nonspecific interactions with DNA, leading to a very small decrease in the occupancy of strong promoters. In contrast, low promoter saturation is achieved when K_S is weak ($K_S \approx K_{NS}$). Here σ^A competes with promoter-bound $E\sigma^{70}$ almost as readily as with nonspecifically bound $E\sigma^{70}$, accounting for the profound competition observed.

Further simulations indicated that when initial promoter occupancy ($\sigma^A = 0$) was fixed altering the free RNAP concentration (5%, 1%, or 0.1%) did not affect competition-induced changes in promoter occupancy (data not shown). Therefore, even if <1% of RNAP is free *in vivo*, we predict that the relationship between σ competition-induced changes in promoter saturation and promoter binding strengths (Fig. 3B) would not be altered. We therefore propose that only promoters far from saturation with $E\sigma^{70}$ will be efficiently regulated by σ competition *in vivo*. This prediction of the model is consistent with experimental data. V. Shingler (personal communication) showed that a weak promoter was more subject to σ competition than a strong promoter *in vivo*.

How would variation in the relative affinities of $E\sigma$ s for DNA and σ s for E affect predictions of the model? Our calculations suggest that when σ^A affinity for E is higher than that of σ^{70} , or when $E\sigma^A$ has tighter nonspecific DNA binding than $E\sigma^{70}$, equilibrium would shift toward binding to σ^A (Figs. 5 and 6, which is published as supporting information on the PNAS web site), thereby decreasing $E\sigma^{70}$ promoter occupancy more than indicated by the simulations in Fig. 3. However, strong nonspecific binding of $E\sigma^A$ to DNA is unfavorable for $E\sigma^A$ binding to its cognate promoters. In fact, because $E\sigma^A$ is present in much lower amounts than $E\sigma^{70}$, for significant binding of $E\sigma^A$ to its cognate promoters either $E\sigma^A$ nonspecific binding to DNA should be weaker than that of $E\sigma^{70}$ or promoter binding of $E\sigma^A$ should be much tighter than that of $E\sigma^{70}$ (Figs. 5 and 6).

Concluding Remarks. In this work, we remeasured the levels of E, σ^{70} , σ^E , and σ^{32} in *E. coli* and developed an equilibrium model that describes the partitioning of RNAP between promoters, nonspecific DNA binding sites, and the cytoplasm. By exploring how critical parameters of the model affect promoter saturation with $E\sigma^{70}$ and the competition between σ s, we have obtained important insights into each of these processes.

Our model suggests that nonspecific binding of holoenzyme to DNA plays a critical role in the extent of promoter saturation. It predicts that strong nonspecific DNA binding is required to keep promoters far from saturation. For their binding affinities to contribute to variation in promoter strength, and for activators to function by recruiting holoenzyme, weak promoters must be

relatively free of holoenzyme *in vivo*, which requires strong nonspecific DNA binding of RNAP. Likewise, nonspecific binding of *lac* repressor to DNA was predicted to control equilibrium binding at the operator (16). Thus, in both cases nonspecific DNA binding is predicted to be necessary to understand the observed *in vivo* regulation.

The large pool of E σ bound to DNA nonspecifically can effectively buffer weak promoters against changes in transcription by the strongest promoters in the cell. For example, if nonsaturating occupancy of promoters were achieved by having “just enough” E σ to bind to promoters, decreased expression of the strongest promoters (e.g., rRNA promoters) would lead to very large increases in expression of weaker promoters. However, because much of the released E σ will bind nonspecifically to DNA, the increase in transcription at weaker promoters is more modest, which may be necessary to allow appropriate regulation at such promoters.

Our model demonstrates that, for σ competition to occur, σ s must be in excess over all E not involved in elongation (rather than in excess of only free E). Previously, the number of σ^{70} (29) was thought to be lower than the number of nonelongating E (34), although there was a great deal of evidence for σ competition (7–10, 14). Our new estimates and careful consideration of the previously published data (18, 20, 23, 24, 29, 31, 35) indicate that σ^{70} and thus total σ s exceed nonelongating E, and our model rationalizes this finding: this condition is necessary for σ competition.

The model predicts that σ competition preferentially affects promoters that are far from saturation. This seems to be a sensible strategy. Promoters can be far from saturation because they have been designed to be intrinsically weak because only small amounts of their products are necessary for growth. Alternatively, under the particular conditions tested, the activator for that promoter may be nonfunctional. In either case, when remodeling transcription, it makes sense to shut off such promoters first. This has been experimentally validated in one case (V. Shingler, personal communication). The generality of this idea can now be subjected to experimental test.

Materials and Methods

Protein Purification and Quantification. His-tagged σ^{70} , σ^E , σ^{32} , and the periplasmic domain of RseA, as well as E and GST-tagged σ^E , were overproduced and purified as previously described (36–40). E was quantified by UV $A_{280\text{ nm}}$ absorbance [$E_{280\text{ nm}}^{\%} = 5.5$ (41)]. σ^{70} was quantified by Coomassie staining because degradation products were visible on the gel. Because the degradation products would be included in the UV $A_{280\text{ nm}}$ measurement, the amount of intact σ^{70} would be overestimated. Indeed, concentration of His-tagged σ^{70} based on the UV $A_{280\text{ nm}}$ measurement ($E_{280\text{ nm}}^{\%} = 8.07$ for His $_{6-}\sigma^{70}$) was 4.1 mg/ml, 1.5-fold higher than estimated by Coomassie. In the Coomassie staining protocol, purified proteins and known amounts of BSA were coelectrophoresed on the Tris-glycine SDS gels and then stained with Coomassie brilliant blue R-250 for 20 min and destained overnight. Protein staining was quantified by densitometric analysis using the AlphaImager 2200. A linear calibration curve, obtained from Coomassie staining of the BSA standards, was used to quantify the concentrations of the purified proteins. His $_{6-}\sigma^E$ and His $_{6-}\sigma^{32}$ were also quantified by Coomassie staining.

Strains and Media. The bacterial strains used in this work were *E. coli* CAG51114 (MG1655 $\phi\lambda$ [*rpoHP3-lacZ*] Δ *lacX74*), wild-type cells (wt); CAG19193 (ML 20035 *rpoH::kanR* λ P_{HS}-*lacZ*, kan^R), the $\Delta\sigma^{32}$ cells (42); CAG22216 [Δ *lacX74 galK galU* Δ (*araABC-leu*)7679 *araD139 hsdR rpsL mcrB rpoE*/ Ω λ P3-*lacZ*], the $\Delta\sigma^E$ cells; and RL1120 (W3110 *trpR tnaA2 rpoB5201*) (43). Cells were grown at 30°C in M9 glucose

medium, prepared as described (44) and supplemented with 0.2% glucose, 1 mM MgSO₄, and 2 μ g/ml thiamine (M9 minimal) or in M9 glucose plus amino acids (M9 complete), which contained the previous components and all amino acids (40 μ g/ml). Wild-type, RL1120, and CAG19193 cells were grown in both M9 minimal and M9 complete, and CAG22216 cells were grown in M9 complete only.

Preparation of Whole-Cell Extracts and Protein Level Measurements by Quantitative Western Blots. At OD₄₅₀ $\approx$ 0.16 (for M9 minimal) or OD₄₅₀ $\approx$ 0.275 (for M9 complete), 1 ml of culture was added to 111 μ l of ice-cold 50% trichloroacetic acid, incubated on ice for >15 min, centrifuged, resuspended, and boiled (5 min) in Laemmli sample buffer. These extracts were used for measuring the intracellular levels of σ^{70} , σ^E , σ^{32} , and E.

The efficiency of protein staining with antibodies after transfer to poly(vinylidene difluoride) membrane differed for purified proteins alone and these same proteins mixed with whole-cell extract (Fig. 7, which is published as supporting information on the PNAS web site). To approximate conditions of the protein to be measured, we generated calibration curves by mixing known amounts of purified His $_{6-}\sigma^{70}$, His $_{6-}\sigma^E$, His $_{6-}\sigma^{32}$, or E, respectively, with cellular extracts from wild type, Δ *rpoE* (CAG22216), Δ *rpoH* (CAG19193), or RL1120 (whose β -subunit is modified by addition of protein A so that it runs at a higher molecular weight than authentic β). Samples with equal amounts of the total cellular protein but different amounts of the purified protein standards were loaded onto Tris-glycine SDS gels, and separated proteins were directly electroblotted onto poly(vinylidene difluoride) membranes. Blots were probed with dilutions of polyclonal antibodies specific to σ^{70} , σ^E , σ^{32} , or β and then incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-mouse antibodies. Blots were developed with the SuperSignal West Dura Extended Duration Substrate from Pierce. The signal was visualized by a charge-couple device, which eliminates nonlinearities due to properties of the film.

For σ^{70} , intracellular levels were determined directly from the calibration curve samples, which contained both His $_{6-}\sigma^{70}$ and σ^{70} (Fig. 8A, which is published as supporting information on the PNAS web site). For σ^E (Fig. 8B) and σ^{32} , intracellular levels were determined from extracts of wild-type cells run on the same gel as the calibration samples (which contained His $_{6-}\sigma^E$ or His $_{6-}\sigma^{32}$ mixed with extracts of strain lacking, respectively, σ^E and σ^{32}). For the β -subunit of E, intracellular levels were determined from extracts of wild-type cells on the same gel as the calibration samples [E mixed with extracts of RL1120 (Fig. 8C)].

The signals of known amounts of the purified proteins within the linear range were fitted to a straight line and used to measure intracellular concentrations using an appropriate dilution of the cell extract (for example, see Fig. 8). All quantification experiments were repeated at least three times.

Measurement of the Total Cellular Protein. At OD₄₅₀ of 0.16 (M9 glucose) and 0.275 (M9 complete), 0.9 ml of cell culture was added to 0.1 ml of ice-cold 50% trichloroacetic acid, incubated on ice for 15 min, centrifuged, resuspended in 0.1 ml of 5% SDS/0.1 M TrisBase by vortexing and boiling for 5 min, and used to measure total cellular protein. The calibration curve was constructed from trichloroacetic acid-precipitated 0.9-ml samples of varying concentrations of BSA in M9 glucose or M9 complete medium. A total of 0.1 ml of either the resuspended cellular protein samples or the BSA standards was mixed with 2 ml of BCA working reagent (from BCA protein kit) and incubated for 30 min at 37°C. The signal was measured at OD₅₉₀ versus water. The signals of the BSA standards were fitted by a straight line (in the linear range), which was used to calculate total cellular protein concentration. Experiments were repeated twice.

Measurement of the Number of Cells. A total of 0.1 ml of consecutive dilutions of cells in M9 minimal ($OD_{450} = 0.16$) or M9 complete ($OD_{450} = 0.275$) was plated on LB plates and incubated at 37°C, and cells were calculated from the numbers of colonies on the plates. Experiments were repeated three times.

Development of Equilibrium Model. A mathematical model was developed to study partitioning of RNAP between specific (promoters) and nonspecific sites on DNA and a pool of free RNAP in the cytoplasm. The model considers one major σ factor, labeled as σ^{70} , and one alternative σ factor, labeled as σ^A . It describes equilibrium binding between (i) free E and the σ s, (ii) E σ s and promoters, and (iii) E and E σ s binding to nonspecific DNA sites (Fig. 1). The following assumptions are used in the equilibrium model: (i) binding of E and E σ s to DNA is reversible; (ii) σ^{70} promoters can be occupied only by E σ^{70} (and σ^A promoters only by E σ^A); (iii) σ^{70} and σ^A have the same binding affinity for free E; (iv) all σ^{70} promoters have the same binding affinity for E σ^{70} (and all σ^A promoters have the same affinity for E σ^A); and (v) E, E σ^{70} , and E σ^A bind to nonspecific DNA sites with the same affinity. For simulation

of RNAP partitioning between various pools, we set $\sigma^A = 0$, because under “normal” growth conditions the alternative σ factors available for binding to E comprise a small fraction of total σ s. For simulation of σ competition, we set σ^A promoters = 0, because there are many more promoter sites for σ^{70} than for alternative σ s (45). Simulations varying specific and nonspecific binding of E σ^A are presented in *Supporting Text*. Parameters used in calculations are summarized in Table 4, column 3, and in *Supporting Text*. Details of model’s equations, parameter selection and calculation, and discussion of the assumptions are given in *Supporting Text*.

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