Tethering σ^{70} to RNA polymerase reveals high in vivo activity of σ factors and σ^{70}-dependent pausing at promoter-distal locations

Rachel Anne Mooney and Robert Landick[1]

Department of Bacteriology, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA

Bacterial σ factors compete for binding to RNA polymerase (RNAP) to control promoter selection, and in some cases interact with RNAP to regulate at least the early stages of transcript elongation. However, the effective concentration of σs in vivo, and the extent to which σ can regulate transcript elongation generally, are unknown. We report that tethering σ^{70} to all RNAP molecules via genetic fusion of *rpoD* to *rpoC* (encoding σ^{70} and RNAP's β′ subunit, respectively) yields viable *Escherichia coli* strains in which alternative σ-factor function is not impaired. $\beta'::\sigma^{70}$ RNAP transcribed DNA normally in vitro, but allowed σ^{70}-dependent pausing at extended –10-like sequences anywhere in a transcriptional unit. Based on measurement of the effective concentration of tethered σ^{70}, we conclude that the effective concentration of σ^{70} in *E. coli* (i.e., its thermodynamic activity) is close to its bulk concentration. At this level, σ^{70} would be a bona fide elongation factor able to direct transcriptional pausing even after its release from RNAP during promoter escape.

[*Keywords*: RNA polymerase; σ factor; transcriptional regulation; *E. coli*; pausing]

Supplemental material is available at http://www.genesdev.org.

Received August 8, 2003; revised version accepted October 1, 2003.

A fundamental paradigm in transcriptional regulation is the cyclical association of RNA polymerase (RNAP) with dissociable initiation, elongation, and termination factors (Burgess et al. 1969; Chamberlin 1976). In bacteria, σ factors bind tightly to free RNAP to program promoter recognition and initiation (Gill et al. 1991; Maeda et al. 2000), but are thought to release stochastically from elongation complexes (ECs) as, or shortly after, RNAP escapes promoters (Shimamoto et al. 1986). The avidity of σ-factor binding decreases dramatically in part because RNA chains >8 nt compete with σ for sites on RNAP (Krummel and Chamberlin 1989; Daube and von Hippel 1999; Murakami et al. 2002). The elongation factor NusA also may assist σ release by competitive binding to the EC (Gill et al. 1991). A similar cycle of initiation factor binding and release operates for eukaryotic RNAPs, although the details are less well understood (Kimura et al. 2002; Pokholok et al. 2002).

During development, differentiation, and changes in environment, binding, and release of initiation factors allow cells to alter patterns of gene expression by reprogramming RNAP. In bacteria, reprogramming is accomplished by switching among σ factors associated with RNAP (e.g., seven σs in *Escherichia coli*, 18 in *Bacillus subtilis*, and at least 65 in *Streptomyces coelicolor*; Ishihama 2000; Kunst et al. 1997; Bentley et al. 2002). Among these, one σ is the major or housekeeping σ and is typically present at the highest level (σ^{70} in *E. coli*).

Selection among σs for RNAP binding is thought to be mediated by concentration-dependent competition among available σ molecules for available RNAP molecules (Zhou and Gross 1992; Hicks and Grossman 1996; Farewell et al. 1998; Gross et al. 1998; Kolesky et al. 1999; Ishihama 2000; Maeda et al. 2000). The concentrations of available σs are in turn highly regulated by their expression levels, rates of degradation, and sequestration via binding to inhibitory proteins called anti-σs (Ishihama 2000). Although RNAP outnumbers total σ in *E. coli* (~1.7:1; Materials and Methods), σs must compete for the smaller pool of available RNAP (~0.6 per σ), which includes newly synthesized RNAP and RNAP not engaged in transcription or sequestered nonspecifically (Ishihama 2000; see Discussion).

The idea that σs are released from RNAP during transcript elongation has been challenged recently (Bar-Nahum and Nudler 2001; Mukhopadhyay et al. 2001). In one view, permanent association of σ^{70} with some

[1]**Corresponding author.**
E-MAIL landick@bact.wisc.edu; FAX (608) 262-9865.
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1142203.

RNAP is proposed to accelerate recycling of this RNAP for new rounds of transcription by circumventing the need to rebind σ^{70}, which could be rate-limiting in the transcription cycle (Bar-Nahum and Nudler 2001).

σ^{70} plays at least one regulatory role during transcript elongation. σ^{70} can remain bound to RNAP long enough to stimulate pausing at promoter-proximal sites that resemble –10 promoter elements (Ring et al. 1996). In this case, σ^{70}-dependent pausing was lost when the pause site was moved 20 bp downstream and tested in vitro (Ring et al. 1996). This could be explained by stochastic σ^{70} release after promoter escape (Shimamoto et al. 1986) or by resistance of mature ECs to σ^{70}-stimulated pausing.

To assess the effective concentration of σ^{70} in vivo and to gain insight into σ^{70}'s effect on the EC, we created an *rpoC*::*rpoD* gene fusion that tethered σ^{70} to all RNAP in cells via a covalent polypeptide linkage. Tethering proteins by genetic fusion fixes the local concentration of interacting proteins (Raag and Whitlow 1995; Timpe and Peller 1995; Robinson and Sauer 1998). Depending on the length of the tether and the location of binding sites relative to the tether-attachment points, tethering can generate local protein concentrations of 10^{-5} to 10 M (Robinson and Sauer 1998). The bulk concentration of σ^{70} in vivo is ~15 μM (Materials and Methods). However, a variety of factors, including macromolecular crowding, can dramatically alter the effective concentration of σ^{70} in cells (i.e., its thermodynamic activity). Tethering σ^{70} to RNAP makes it possible to examine the effects in vivo of known local concentrations of tethered σ^{70}, and thus gain insight into the effective concentration of σ^{70} in cells.

Results

E. coli *is viable with* σ^{70} *fused to RNAP at the* β′ *C terminus*

We initially tested the in vivo function of σ^{70} tethered to the C terminus of β′ or β when corresponding gene fusions were conditionally expressed from plasmids (Table 1). (RNAP tolerates alterations at the ends of β′ or β; σ^{70} tolerates alterations at its N terminus; Severinov et al. 1997; Sharp et al. 1999.) β::σ^{70} was unable to complement for loss of σ^{70} or β function. However, β′::σ^{70} complemented for loss of σ^{70} and, to a limited extent, β′. We considered this result promising and concentrated further study on β′::σ^{70}. As an initial control that β′::σ^{70} was incorporated into RNAP, we replaced region 4 of the tethered σ^{70} with the corresponding region of σ^{32} (Kumar et al. 1995). This eliminated the ability of β′ in the fusion to provide even weak β′ function (β′::$\sigma^{70/32}$; Table 1), suggesting that the weak complementation of *rpoC*^{TS} by β′::σ^{70} reflected incorporation into a functional RNAP. Based on these results, we next asked if the *rpoC*::*rpoD* fusion could replace *rpoC* in the chromosome (Materials and Methods).

The resulting strain, in which σ^{70} was tethered to all RNAPs, proved viable. We transduced the *rpoC*::*rpoD* allele into a strain in which *rpoD* expression can be shut off (Lonetto et al. 1998). This strain exhibited no defect in either growth or recovery from stationary phase when forced to rely on β′::σ^{70} for both β′ and σ^{70} function (Fig. 1A,B). The simplest interpretation of these results is that β′::σ^{70} RNAP is a fully functional enzyme and that the weak complementation of *rpoC*^{TS} by the plasmid-encoded β′::σ^{70} reflected partial overexpression toxicity (Table 1).

The structure of bacterial RNAP holoenzyme (Vassylyev et al. 2002) is consistent with this interpretation (Fig. 1C). Although σ region 1.1 is not resolved in the structure, if spherical, it would occupy a hydrated volume ~30 Å in diameter on the downstream face of RNAP. At least 27 amino acids may flexibly connect region 1.1 to β′: three amino acids from the gene fusion, plus 19 C-terminal amino acids of *E. coli* β′ that correspond to the disordered C-terminal segment in the RNAP structure, plus the last five visible amino acids of β′, which form a random coil. These 27 amino acids (~100 Å fully extended) are sufficient to span the 80 Å

Table 1. *Plating efficiency of tethered* σ^{70} *expressed from plasmids in wild-type and mutant strains*

		rpoD$^-$ strain[a]		*rpoB*$^-$ strain[b]	*rpoC*$^-$ strain[b]
Plasmid	Subunit	–IPTG	+IPTG	+IPTG	+IPTG
pTrc99c	None	$<10^{-6}$	$<10^{-6}$	$<10^{-6}$ [d]	$<10^{-6}$ [d]
pRL663	β′	—	—	—	1
pRL706	β	—	—	1	—
pCL391	σ^{70}	1	1	—	—
pRM314	β′::σ^{70}	$<10^{-6}$	0.5–1	—	Weak, $<10^{-6}$ sc[c]
pRM328	β::σ^{70}	$<10^{-6}$	$<10^{-6}$	$<10^{-6}$ [d]	—
pRM317	β′::$\sigma^{70/32}$	$<10^{-6}$	$<10^{-6}$	—	$<10^{-6}$ [d]

[a]Strain CAG20153 (Supplementary Table 1) in which *rpoD* transcription is conditionally repressed by TrpR. These strains were tested on plates lacking IAA to prevent *rpoD* transcription.
[b]*rpoC*$^-$ strain is RL602 (Supplementary Table 1; functionally *rpoC*^{TS}). *rpoB* strain is RL585 (Supplementary Table 1, functionally *rpoB*^{TS}). These strains were plated at 42°C, a nonpermissive temperature for *rpoC* or *rpoB* expression.
[c]Weak growth at 10^6 cells/mL with ITPG, but not without ITPG; no single colonies (sc) at $<10^6$ cells/mL. When struck on IPTG plates, this strain gave growth at 42°C in the heavy part of the streak, but not single colonies. No equivalent growth was observed in plates lacking IPTG.
[d]No growth at any cell density with or without IPTG.

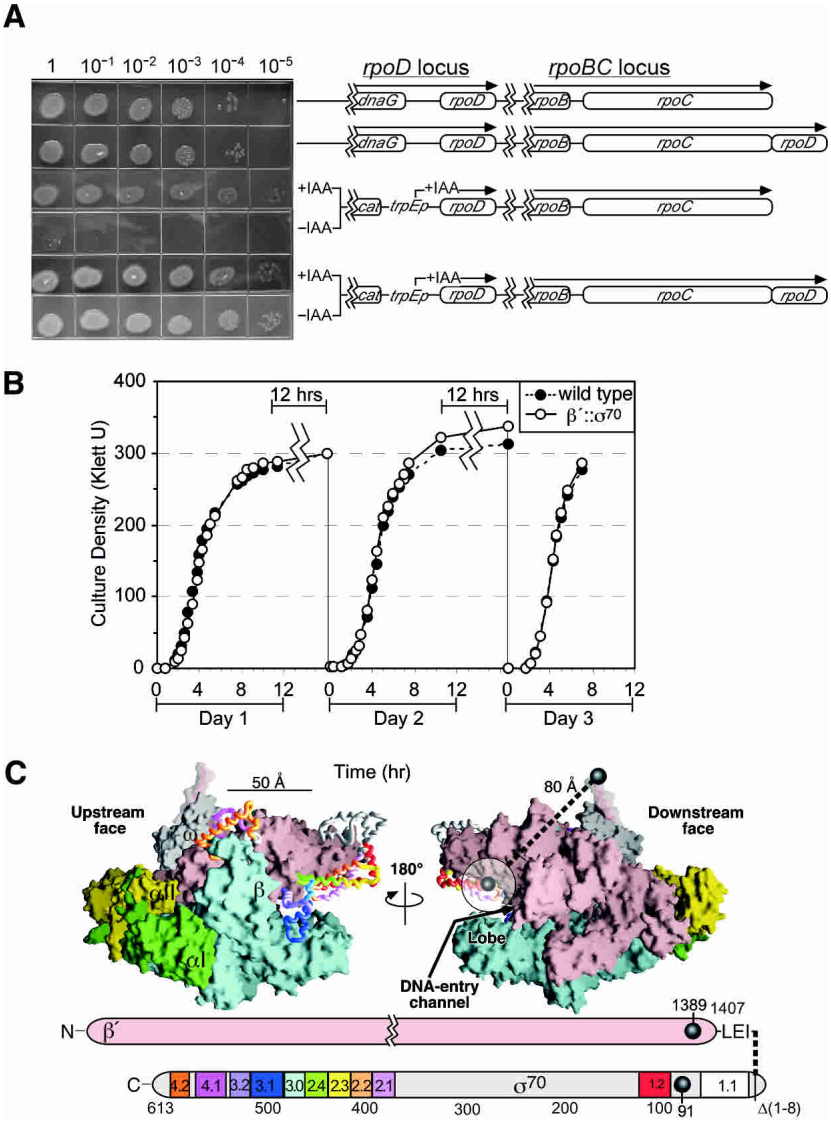

Figure 1. Strains and growth phenotypes. (*A*) Early log-phase cultures of strains with the *rpoD* and *rpoC* loci illustrated were serially diluted and plated onto LB plates or LB plates containing indole acrylic acid (IAA). Strains (*top* to *bottom*) are RL301, RL1374, CAG20153, and RL1094 (Supplementary Table 1). The efficiencies of plating minus IAA versus plus IAA were ~10^{-5} for the wild-type strain (CAG20153) and ~1 for the β′::σ^{70} strain (RL1094). (*B*) Wild-type or β′::σ^{70} with TrpR-repressed *rpoD* strains (C600K$^-$ and RL1094) were monitored during growth in LB at 37°C using a Klett colorimeter (data are averages for two independent cultures). After residing in stationary phase for ~12 h, the strains were diluted back to Klett <5. (*C*) Model of σ^{70} tethering to β′ based on the *Thermus thermophilis* holoenzyme structure (Vassylyev et al. 2002; β′ NCD removed). The upstream and downstream faces of RNAP are shown above schematics of the *E. coli* β′ and σ^{70} subunits. RNAP subunits are shown in spacefill; σ, is shown as a C_α-trace colored by regions shown in the schematic. The N-terminal and C-terminal residues of σ and β′ resolved in the structure are depicted as black spheres (corresponding to β′1389 and σ^{70}91 in *E. coli*). σ region 1.1 (semitransparent white circle) is positioned based on the hydrated volume of an 82-amino acid globular protein attached to σ^{70}91.

between the last resolved amino acids in β′ and the likely positions of region 1.1, proposed to be within the DNA-entry channel in the holoenzyme and at the outside edge of RNAP's lobe domain in initiation complexes (Mekler et al. 2002).

To verify that σ^{70} remained tethered to RNAP in the viable β′::σ^{70} strain, we examined the subunits present in cell extracts by immunoblotting with anti-β′ and anti-σ^{70} antibodies (see Materials and Methods). Only β′::σ^{70}, not β′ or σ^{70}, was present in a whole-cell extract from the β′::σ^{70} strain when *rpoD* expression was shut off (Fig. 2A, cf. lanes 1 and 2). Therefore, β′::σ^{70} was not cleaved to separate β′ and σ^{70} subunits in vivo, and this intact β′::σ^{70} polypeptide could serve as the sole source of both β′ and σ^{70} in viable *E. coli* cells.

A possible explanation for the viability of the β′::σ^{70} strain would be that one β′::σ^{70} polypeptide provides β′ and a second polypeptide provides σ^{70} in a single RNAP holoenzyme. If this were true, then purified, active β′::σ^{70} RNAP should contain two β′::σ^{70} polypeptides. However, highly purified, fully active β′::σ^{70} RNAP gave a stoichiometry of one β′::σ^{70} to one β and contained no β′ or σ^{70} (Fig. 2A, lanes 3,5; densitometry not shown). Another possibility would be that σ^{70} from one β′::σ^{70} RNAP provided σ^{70} function to a second β′::σ^{70} RNAP. If this were true, then open complexes (OCs) formed by β′::σ^{70} RNAP would be of significantly greater mass (920 kD vs. 460 kD, not including DNA), and would exhibit significantly slower electrophoretic mobility. To test this, we formed OCs with wild-type and β′::σ^{70} RNAPs on a λP_R-containing DNA fragment and compared them by native gel electrophoresis (Fig. 2B). The β′::σ^{70} OCs exhibited similar mobility to wild type (see legend to Fig. 4C, below). We conclude that β′::σ^{70} RNAP and its OCs contain a single β′::σ^{70} polypeptide.

The results described to this point confirm that RNAP tethered to σ^{70} supports cell growth and functions with the σ^{70} to which it is tethered. This suggests that β′::σ^{70} RNAP can use at least σ^{32} and σ^{E}, which are required for

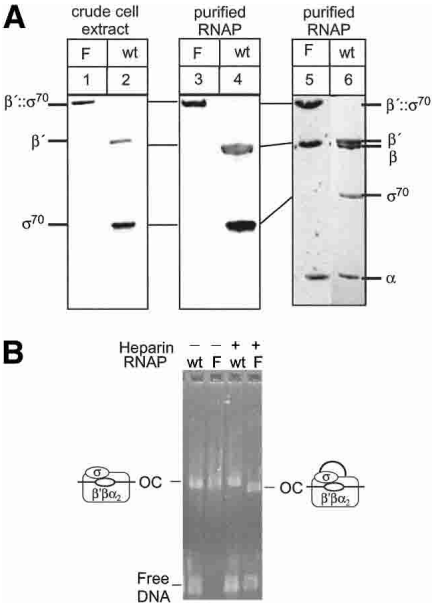

Figure 2. Purified RNAP and immunoblot analysis. *(A) (Left)* Cellular extracts of wild-type (wt) or β′::σ^{70} (F) strains (RL301 and RL1094) were separated by 3%–8% Tris-Acetate (Novex), transferred to nitrocellulose, and probed with a mixture of β′ and σ^{70} antibodies (Materials and Methods). Equal amounts of cellular protein were present in each lane. (*Center*) Western blot of wild-type (wt) and β′::σ^{70} (F) RNAPs. Samples were separated by 3%–8% Tris Acetate (Novex) and probed as in the *left* panel. (*Right*) β′::σ^{70} RNAP (F) purified from the strain containing the *rpoC*::*rpoD* fusion and *Trp*-repressed *rpoD* (RL1094) separated by 4%–15% PAGE gel (Pharmacia) shown next to wild-type (wt) RNAP for size comparison. (*B*) β′::σ^{70} (F) or wild-type (wt) RNAPs were mixed with λP_R template, allowing the formation of open complexes at 37°C for 20 min (heparin was present where indicated for the last 10 min), separated by native gel electrophoresis (4% NuSieve agarose, 0.5× TBE), and stained with EtBr. The absence of heparin masked the slightly faster mobility of β′::σ^{70} OCs (see legend to Fig. 4C) because β′::σ^{70} OCs appeared to aggregate more than wild-type OCs.

viability of *E. coli* at 37°C (Zhou et al. 1988; De Las Penas et al. 1997), and σ^S, which is necessary for wild-type recovery from the stationary phase (Ishihama 2000). We next examined use of alternative σs more carefully and tested for function of NusA, which also competes with σ^{70} for interaction with RNAP (Gill et al. 1991). In the presence or absence of chromosomally encoded, untethered σ^{70}, β′::σ^{70} strains plated a λ phage that requires function of NusA (and other Nus proteins; Friedman et al. 1976) equivalently to wild type (Table 2). The same was true for survival at 45°C (an even more stringent requirement for σ^{32} and σ^E), for growth on medium requiring σ^N function, for σ^F-dependent swarming motility, and for σ^S-dependent formation of peroxidase in the stationary phase (Table 2). Thus, alternative σs and NusA appear to exhibit high in vivo activity, sufficient to allow normal function in β′::σ^{70} strains despite the presence of σ^{70} tethered to all RNAP molecules. This remains true even in β′::σ^{70} strains also containing chromosomally encoded, untethered σ^{70}.

β′::σ^{70} *RNAP displays wild-type enzymatic properties and activities*

We next sought to determine if β′::σ^{70} RNAP also behaved normally in vitro. For this purpose, we tested transcription by wild-type and β′::σ^{70} RNAPs of a linear DNA template encoding the well-characterized *his* pause site downstream of the T7 phage A1 promoter (Chan and Landick 1989). Upon initiation under conditions that allowed RNAP to transcribe only to A29, no difference was apparent between wild-type and β′::σ^{70} RNAPs in the rate of abortive initiation, the rate of productive initiation, or the ratio of abortive to productive products (as reflected by the AUC abortive RNA and A29 productive RNA; Fig. 3A). When transcription past A29 was allowed after 2 min, both RNAPs escaped the A29 position efficiently and exhibited indistinguishable kinetics of pausing and subsequent transcription to the end

Table 2. *Properties of wild-type and β′::σ^{70} fusion strains in various conditions*

Phenotype	Required factor	Wild-type σ^{70} level[a]	β′::σ^{70}, no add. σ^{70} [a]	β′::σ^{70}, plus σ^{70} [a]
λ growth[b]	NusA, B, E, G	1	0.8 ± 0.2	1.0 ± 0.4
Growth at 45°C[c]	σ^{32}, σ^E	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
Growth on Arg/Gln[d]	σ^N	1.2 ± 0.3	1.3 ± 0.1	1.0 ± 0.4
Motility[e]	σ^F	1	1.0 ± 0.1	1.0 ± 0.1
Catalase production[f]	σ^S	+	+	+

[a]The strains for the wild-type σ^{70} level were RL301 or C600 K$^-$. The β′::σ^{70}, no additional σ^{70} strain was RL1094 (chromosomally encoded σ^{70} not expressed). The β′::σ^{70}, plus σ^{70} strains were RL1374 or RL1390 (both contain additional, chromosomally encoded σ^{70}). All measurements are the averages of at least three determinations. The methods for determining phenotypes and plating efficiencies are in the Supplemental Material.
[b]The number of plaques formed by λcIc17 (requires *E. coli nus* functions; Friedman et al. 1976) on β′::σ^{70} strains divided by the number of plaques formed on a wild-type strain.
[c]EOP on rich medium of β′::σ^{70} strains at 45°C divided by EOP at 37°C.
[d]EOP on minimal medium containing Arg (requires σ^N-directed transcription of *glnA*) divided by EOP on Gln-containing medium (allows σ^N-independent growth).
[e]The swarm diameter of β′::σ^{70} strains on motility agar plates divided by the swarm diameter of a wild-type strain. Flagella synthesis requires σ^F function.
[f]Observation of O_2 evolution upon addition of 3% H_2O_2 to stationary-phase cultures; requires σ^S-dependent expression of catalase from *kat*.

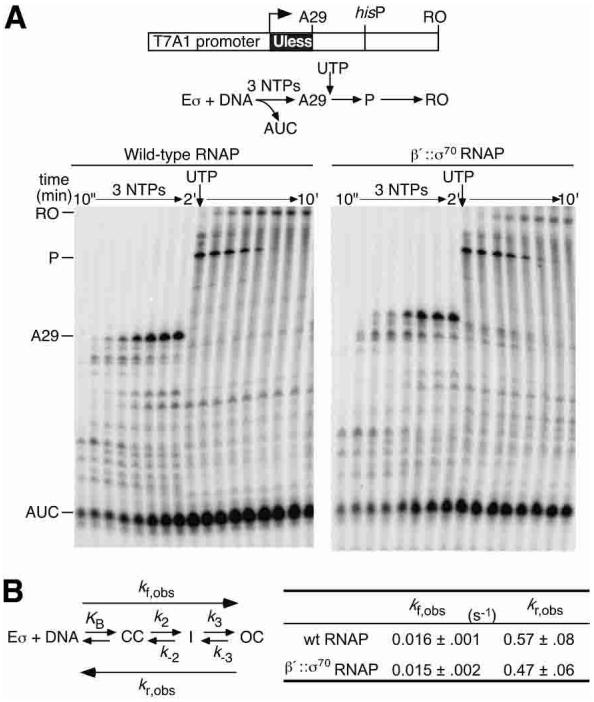

Figure 3. (*A*) In vitro transcription assay. $\beta'::\sigma^{70}$ or wild-type RNAPs (40 nM) were allowed to initiate transcription on a linear DNA template (25 nM) with ApU, ATP, [^{32}P]CTP, and GTP. Samples were mixed with 2× STOP buffer at 10, 20, 30, 45, 60, 75, 90, and 120 sec. UTP was then added to allow transcription past A29, and additional samples were taken 10, 20, 30, 45, 60, 120, 240, and 480 sec later (final sample 10 min after reaction started). The samples were separated by 15% denaturing PAGE. (AUC) Trinucleotide abortive product; (A29) A29 EC; (P) *his* pause RNA; (RO) run-off RNA. (*B*) Kinetics of promoter association, following the mechanism defined by Saecker et al. (2002). $k_{f,obs}$ and $k_{r,obs}$ were obtained for $\beta'::\sigma^{70}$ and wild-type RNAPs on the template shown in panel *A* (≥3 independent experiments; Materials and Methods).

of the template. We also tested the ability of NusA protein to increase the duration of pausing at the *his* pause site and the efficiency of termination by $\beta'::\sigma^{70}$ RNAP at several intrinsic terminators; no significant differences from wild-type RNAP were observed (data not shown).

To test whether σ^{70} tethering affects steps on the pathway of OC formation, we measured the overall rates of OC formation at the T7 A1 promoter ($k_{f,obs}$) and of preformed OC dissociation ($k_{r,obs}$; Fig. 3B; Materials and Methods). Both rates are composites of multiple steps (McClure 1980; Saecker et al. 2002) and would reveal any effects of the tether. Neither $k_{f,obs}$ nor $k_{r,obs}$ differed significantly between wild-type and $\beta'::\sigma^{70}$ RNAPs (Fig. 3B), consistent with wild-type function of the tethered σ^{70}.

Tethering σ^{70} to RNAP increases σ^{70} local concentration to ~55 μM

The lack of effect of tethering σ^{70} to RNAP caused us to consider whether tethering actually increased the local concentration of σ^{70} around RNAP. Such increased local concentrations have been measured for other cases of protein tethering (Timpe and Peller 1995; Robinson and Sauer 1998), and are well-grounded in polymer-chain theory (Flory 1969), but must be determined for each case.

To measure the local concentration of tethered σ^{70}, we performed competition binding assays using a ^{32}P-labeled derivative of σ^{70} (Materials and Methods). We compared the ability of [^{32}P]σ^{70} to displace untethered σ^{70} from wild-type RNAP and to displace tethered σ^{70} from $\beta'::\sigma^{70}$ RNAP using conditions in which σ^{70} binding to RNAP equilibrates (Sharp et al. 1999). To detect [^{32}P]σ^{70} bound to RNAP, we separated the reactions by nondenaturing PAGE and visualized RNAP and σ^{70} by protein staining and [^{32}P]σ^{70} using a PhosphorImager (Fig. 4A). [^{32}P]σ^{70} displaced untethered σ^{70} from the wild-type holoenzyme as predicted for equivalent K_ds of the prebound σ^{70} and [^{32}P]σ^{70}. However, only modest [^{32}P]σ^{70} binding to $\beta'::\sigma^{70}$ RNAP could be detected even at high concentrations of [^{32}P]σ^{70} (Fig. 4A,B). Assuming that all ^{32}P at or above the position of $\beta'::\sigma^{70}$ RNAP in the gels arose from [^{32}P]σ^{70} binding to $\beta'::\sigma^{70}$ RNAP (because binding of a second σ^{70} would slow migration), we calculated a local concentration of tethered σ^{70} of ~55 μM (Fig. 4B; Materials and Methods). This is >50-fold higher than the concentration of RNAP in the assay (1 μM), but significantly less than measured for optimal cases of tethering (Robinson and Sauer 1998). The extensive topography of σ^{70}–RNAP contacts relative to the tether attachment points on σ^{70} and RNAP, interference of the tether with some contacts, or the tether length may limit tether enhancement of local concentration.

This experiment unambiguously demonstrated weaker binding of free σ^{70} to RNAP in the presence of tethered σ^{70}. The local concentration estimate for tethered σ^{70} of 55 μM should be a lower limit because it included ^{32}P that was nonspecifically retarded. However, our estimate conceivably could be inflated if the complex of [^{32}P]σ^{70} and $\beta'::\sigma^{70}$ RNAP dissociated during electrophoresis. This seemed unlikely because release of [^{32}P]σ^{70} during electrophoresis would form a smear between the positions of holoenzyme and free σ^{70}. No such smear was evident; rather, the amount of [^{32}P]σ^{70} visible at the position of free σ^{70} was the same in the $\beta'::\sigma^{70}$ lanes and the σ^{70} alone lanes, but was reduced in the wild-type RNAP lanes by the amount bound to RNAP (Fig. 4A, cf. the PhosphorImager densities at the bottom of the 1 μM and 2.5 μM lanes).

To confirm that tethered σ^{70} produced a local σ^{70} concentration of ~55 μM, we sought to detect the binding of untethered σ^{70} to $\beta'::\sigma^{70}$ RNAP in a manner that would prevent its release during electrophoresis. We reasoned that if the untethered σ^{70} were engaged in OC formation by $\beta'::\sigma^{70}$ RNAP, two σ^{70}s would be bound to RNAP because untethered σ^{70} would be trapped in the network of RNAP–σ^{70}–DNA interactions in the OC (Murakami et al. 2002), and tethered σ^{70} would remain covalently attached to RNAP. To favor trapping untethered σ^{70} bound to $\beta'::\sigma^{70}$ RNAP, we used a promoter on which

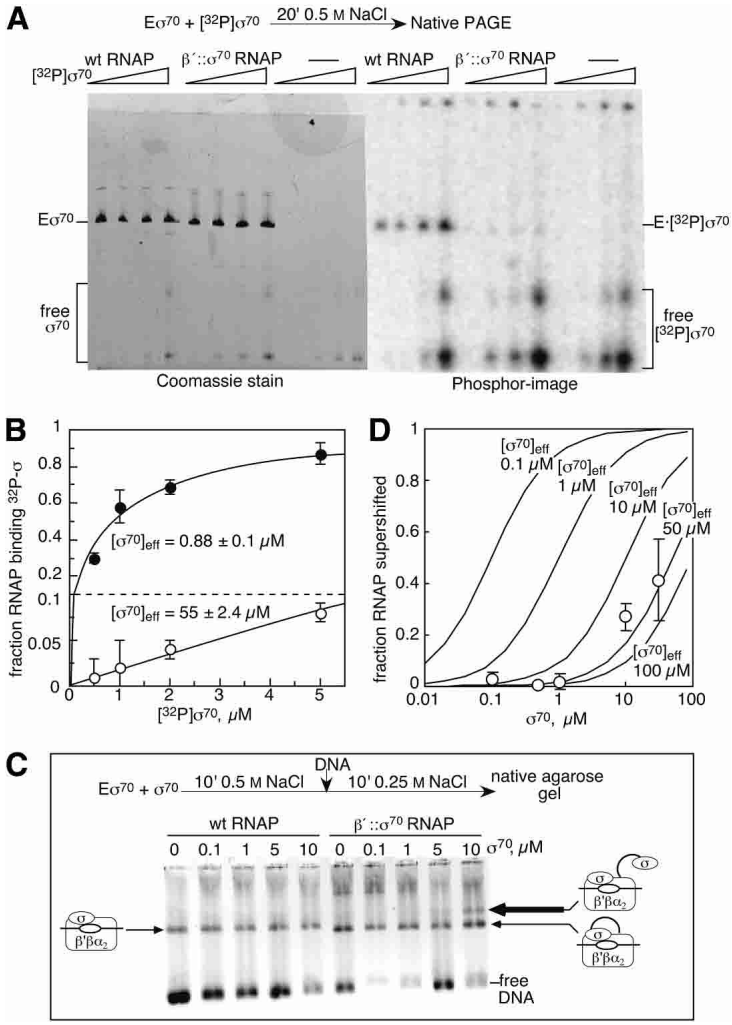

Figure 4. Effective local concentration of tethered σ^{70} measured by competitive binding. (*A*) Equilibrium σ^{70}-binding assay (see Materials and Methods). Samples are (*left* to *right*) 0.5, 1, 2, and 5 μM [^{32}P]σ^{70} with 1 μM wild-type RNAP, $\beta'::\sigma^{70}$ RNAP, or no RNAP. The positions of holoenzyme (E σ^{70}) and free σ^{70} are indicated; slower σ^{70} bands are σ^{70} dimers. (*B*) Quantitation of *A*. The fraction of RNAP binding ^{32}P-labeled σ^{70} is plotted against the amount of ^{32}P-labeled σ^{70} present in the reactions. (●) Wild-type RNAP; (○) $\beta'::\sigma^{70}$ RNAP. Effective concentrations of σ^{70} were estimated by nonlinear regression (Materials and Methods) from the averages of three experiments. (*C*) OC mobility assay. $\beta'::\sigma^{70}$ or wild-type RNAPs were incubated with the indicated amounts of σ^{70} and then with added promoter DNA (Materials and Methods). OCs and DNA were separated on a native agarose gel and stained with EtBr. The positions of the free DNA, the wild-type OC, the $\beta'::\sigma^{70}$ OC, and the supershifted $\beta'::\sigma^{70}$ OC formed with a second, untethered σ^{70} are indicated. Heparin was omitted in this experiment to minimize smearing of the supershifted OC band, although this caused variable nonspecific binding of free DNA (evident as aggregates near the top of the gel). Addition of heparin gave constant amounts of free DNA in gels and approximately the same amounts of supershifted OC, allowing use of up to 30 μM σ^{70} (Fig. 4D). The slightly increased mobility of $\beta'::\sigma^{70}$ OCs (see also Fig. 2B) and of $\beta'::\sigma^{70}$ RNAP (panel *A*) may reflect a slight structural change caused by tethering that is under study. ($\beta'::\sigma^{70}$ RNAP does, however, contain ω.) (*D*) Quantitation of OC supershifting as a function of σ^{70} concentration. The fraction of RNAP binding a second σ^{70} (the supershifted species in *C*) is plotted against the concentration of added, untethered σ^{70} (0.1, 0.5, 1, 10, and 30 μM; average of three experiments that included heparin). Predicted binding curves (assuming all σ^{70} species to be equivalent for binding to RNAP) for different effective local concentrations of the tethered σ^{70} are shown for comparison (Materials and Methods).

OCs were stable for many hours ($P_{UPFullcon}$; Materials and Methods). We equilibrated RNAPs with increasing concentrations of free σ^{70}, incubated them with $P_{UPFullcon}$ DNA for 10 min, and then separated the reactions on a nondenaturing agarose gel. After visualizing the locations of DNA by ethidium staining, OCs were readily apparent as retarded bands for both wild-type and $\beta'::\sigma^{70}$ RNAPs (Fig. 4C). A second, more slowly migrating species appeared when 5–10 μM σ^{70} was added to $\beta'::\sigma^{70}$ RNAP (Fig. 4C, thick arrow), but was not present with wild-type RNAP. Nonspecific binding and smearing prevented us from using concentrations of σ^{70} above 30 μM. Because the supershifted species was also not observed with DNA alone or DNA plus 5–10 μM σ^{70} (data not shown), we attribute it to $\beta'::\sigma^{70}$ RNAP complexed with a second, untethered σ^{70} that is engaged in promoter contacts. To estimate the local concentration of the tethered σ^{70}, we plotted the amounts of the supershifted species versus concentrations of added σ^{70} and compared the data with predicted curves (Materials and Methods; Fig. 4D). They matched reasonably to the prediction for ~50 μM local concentration of tethered σ^{70} and were more than the prediction for 100 μM. Thus, both assays give an estimate of tethered σ^{70} local concentration consistent with ~55 μM.

Tethering σ^{70} to RNAP modestly increases σ^{70} local concentration in vivo

To compare the local concentration of tethered σ^{70} with the effective concentration of wild-type σ^{70} in vivo, we tested the ability of chromosomally encoded $\beta'::\sigma^{70}$ or increased amounts of plasmid-encoded, untethered σ^{70} to protect wild-type cells (also containing chromosomally encoded σ^{70}) against the toxic effects of a plasmid-encoded mutant σ^{70} (RC584). σ^{70}RC584 binds RNAP and interferes with promoter recognition (Siegele et al. 1989). The presence of tethered σ^{70} significantly increased resistance to σ^{70}RC584 (Fig. 5, cf. closed and open circles; wild-type σ^{70} is not toxic when expressed at comparable levels, dashed line). Expression of untethered σ^{70} from a low-copy-number, compatible plasmid to about fivefold above the normal level conferred a

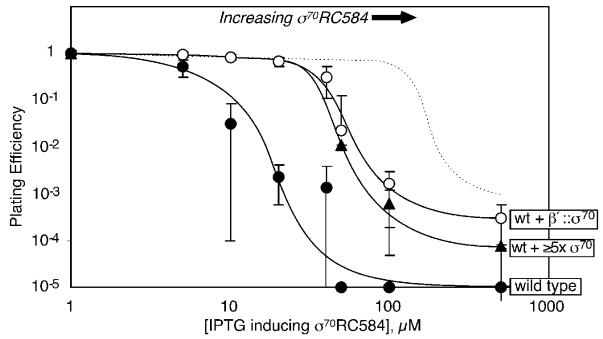

Figure 5. $\beta'::\sigma^{70}$ inhibits toxicity caused by σ^{70}RC584. RL113 (wild type, ●), RL113 + pBADσ^{70} (wt + ≥5× σ^{70}; ▲), or RL1366 (wt + $\beta'::\sigma^{70}$; ○) carrying pRM389 (σ^{70}RC584 expressed under LacI control) were plated with or without IPTG as described in Materials and Methods (both strains were deleted for *lacY*). Data are mean values of three to six independent experiments. The dotted line shows the approximate effect of wild-type σ^{70} expressed from pCL391 for comparison.

level of σ^{70}RC584 resistance similar to that conferred by tethered σ^{70} (Fig. 5, triangles).

These results confirm that $\beta'::\sigma^{70}$ RNAP generates a local σ^{70} concentration higher than that of untethered σ^{70} in wild-type cells. A straightforward interpretation would place the effective concentration of wild-type σ^{70} at ~11 μM because approximately fivefold overexpression gave σ^{70}RC584 resistance similar to ~55 μM local concentration of $\beta'::\sigma^{70}$. However, overexpressed wild-type and RC584 σ^{70}s could affect each other's levels in ways not measurable in plating experiments. Furthermore, we did not test whether a lower level of σ^{70} overexpression also could protect against RC584 σ^{70} as $\beta'::\sigma^{70}$. Thus, we conclude wild-type cells contain σ^{70} at effective concentrations ≥11 μM and significantly less than 55 μM.

Tethering σ^{70} to RNAP only modestly affects heat shock and σ^{32} function

We next wanted to examine the effect of tethered σ^{70} on function of the alternate σ factor σ^{32}, which is required for the well-defined heat-shock response in *E. coli* (Straus et al. 1987). When σ^{70} is overexpressed, σ^{32} function appears to be compromised by competition for binding to core RNAP (Zhou et al. 1992). However, σ^{32} is up-regulated in response, resulting in little if any inhibition of synthesis of heat-shock proteins or delay in the heat-shock response. We first confirmed that $\beta'::\sigma^{70}$ RNAP could use σ^{32} in vitro (transcription of *groE* by $\beta'::\sigma^{70}$ RNAP depended on added σ^{32}; data not shown). We next tested whether $\beta'::\sigma^{70}$ RNAP would affect expression of heat-shock proteins and σ^{32} levels similarly to overexpression of σ^{70}.

The heat-shock response in $\beta'::\sigma^{70}$ cells was slightly delayed relative to wild-type cells (Fig. 6A). The GroE synthesis rate was slower in $\beta'::\sigma^{70}$ cells 1 or 2 min after shifting cells to 42°C, but reached the same level as wild-type by 11 min post-heat shock (Fig. 6A, plot). The characteristic suppression of σ^{70}-dependent transcription was similar in $\beta'::\sigma^{70}$ and wild-type cells (Fig. 6A, cf. * bands).

To ask if heat shock was near normal because σ^{32} levels were up-regulated in the $\beta'::\sigma^{70}$ strain, we measured σ^{32} levels by immunoblotting. The σ^{32} level in $\beta'::\sigma^{70}$ cells was ~80% of that in wild-type cells, a statistically insignificant difference (Fig. 6B; Materials and Methods). The slight delay in GroE expression in heat-shocked $\beta'::\sigma^{70}$ cells is consistent with a local concentration of tethered σ^{70} higher than the wild-type σ^{70} effective concentration, but the nonelevated σ^{32} levels in $\beta'::\sigma^{70}$ cells and the inhibition of σ^{70}-directed transcription upon heat shock even when σ^{70} was tethered to RNAP were surprising (see Discussion).

Tethered σ^{70} causes σ^{70}-dependent pausing independent of distance from the promoter

An additional consequence of tethering σ^{70} is that its high local concentration will be maintained during tran-

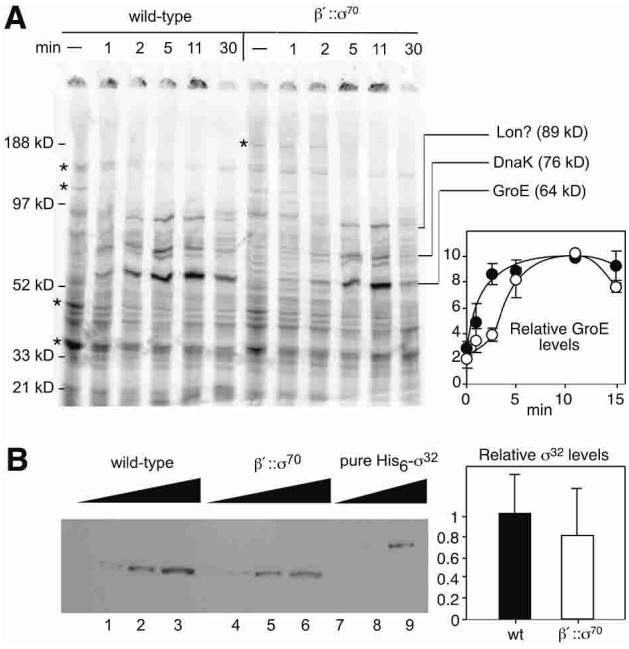

Figure 6. Heat-shock response. (*A*) Cells were grown in minimal media at 30°C and then shifted to 42°C. At the times indicated, samples were incubated with [^{35}S]methionine for 2 min. Samples were separated by 4%–12% PAGE. The positions of DnaK, GroE, and an 89-kD protein likely to be Lon are indicated. (*) Major proteins whose expression decreases on heat shock; the *top* band is $\beta'::\sigma^{70}$, and the *next-to-top* band is β, β′. Relative GroE levels are averages of four experiments. (●) Wild-type (RL301); (○) $\beta'::\sigma^{70}$ strain with chromosomally encoded σ^{70} (RL1374). (*B*) Immunoblot analysis using antibodies against σ^{32} (Materials and Methods). (Lanes *1–3*) Wild-type whole-cell lysate (MC1060). (Lanes *4–6*) $\beta'::\sigma^{70}$ whole-cell lysate (RL1454). (Lanes *7–9*) His_6-tagged σ^{32}. Equal amounts of total cellular protein were electrophoresed.

script elongation. Because untethered σ^{70} is able to stimulate pausing at –10-like sequences near a promoter (Ring et al. 1996), we wondered if tethering would allow σ^{70} to stimulate pausing at –10-like sequences generally. To test this possibility, we examined σ^{70}-dependent pausing on templates originally studied by Roberts and colleagues (+16/17 and +37 pauses), and on an additional template encoding a consensus extended –10 sequence near +450 of an artificial transcription unit (+462 pause). $\beta'::\sigma^{70}$ RNAP gave slightly increased pausing at the +16/17 site (ctcaAcgAT, –10-like σ^{70}-binding sequence; Fig. 7A). This strong pausing also occurred at the +37 site (TGcTATAAT, consensus extended –10-like σ^{70}-binding sequence; Fig. 7B), where wild-type RNAP pauses weakly if at all. This result confirms that wild-type RNAP fails to pause at +37 because its σ^{70} is released, rather than because the structure of the EC past +25 precludes pausing (Ring et al. 1996). Tethered σ^{70}, in contrast, stimulates pausing because it remains attached to RNAP in a functional state after completion of the transition to an EC.

To ask if the tethered σ^{70} could act at even greater distances from a promoter, we tested the +462 pause template (TGcTATAAT σ^{70}-binding sequence; Fig. 7C). As expected, wild-type RNAP was unable to recognize the +462 pause; however, $\beta'::\sigma^{70}$ RNAP exhibited strong pausing. If pausing at +462 occurred simply because the tethered σ^{70} was present at high local concentration, then elevated concentrations of untethered σ^{70} should cause wild-type RNAP to pause. Consistent with this interpretation, wild-type RNAP recognized the +462 pause when additional σ^{70} (1 μM) was added to ECs (Fig. 7C). We concluded that this pause depended on the consensus extended –10-like sequence because a mutant TGcTgTAAg site nearly eliminated pausing (data not shown). This effect of nonconsensus substitutions may explain why Marr et al. (2001) found that additional σ^{70} (0.8 μM) did not direct pausing at the nonconsensus +16/17 pause. Pause escape by $\beta'::\sigma^{70}$ RNAP was barely detectable at consensus extended –10-like sequences (e.g., plot in Fig. 7B). However, $\beta'::\sigma^{70}$ RNAP was paused, not terminated, because addition of GreA or GreB protein reduced pausing dramatically (data not shown), as shown for the +16/17 pause by Marr and Roberts (2000).

Because NusA is thought to displace σ^{70} from the EC (Gill et al. 1991), we next asked how NusA affected rec-

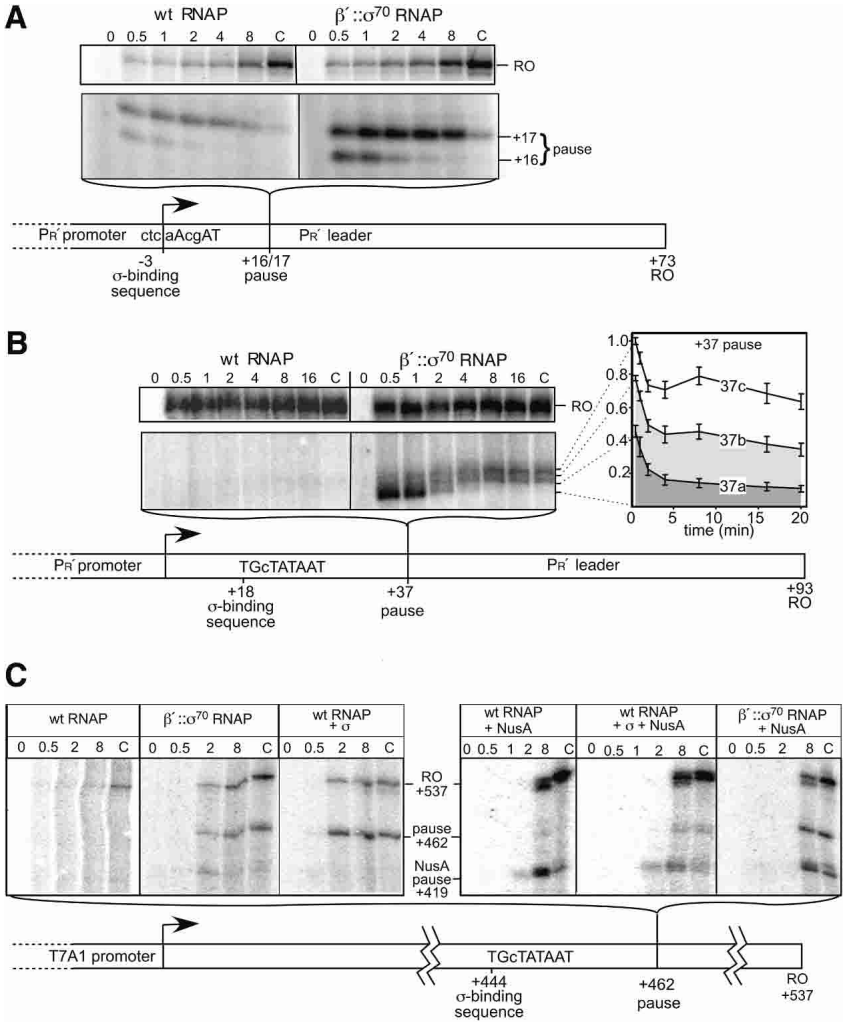

Figure 7. The effective concentration of tethered σ^{70} is sufficient to cause promoter-distal, σ^{70}-dependent pausing. The effective concentration of tethered σ^{70} is sufficient to cause promoter-distal, σ^{70}-dependent pausing. Synchronous in vitro transcription with $\beta'::\sigma^{70}$ or wild-type RNAPs (Materials and Methods; time of transcript elongation as indicated in min; C, incubation with additional 200 μM NTP for 8 min after the last indicated time point). (*A*) +16/17 pause template (Ring et al. 1996). The *top* panel shows the run-off (RO) product; the *lower* panel shows the pause. The schematic of the template indicates the σ^{70}-binding sequence and the position of the pause. (*B*) +37 pause template (+20 in Ring et al. 1996). Gel panels and schematic are as in *A*. The three pause positions on this template have not been mapped precisely and therefore are designated 37a, 37b, and 37c; the third pause band (relative to the +16/17 site) is probably caused by the stronger consensus σ^{70}-binding sequence. The time dependence of pause RNA levels (plot) is the average of three independent experiments. (*C*) +462 pause template. Gel panels and schematic are as in *A*. σ (1 μM) or NusA (10 μM) were added as indicated.

ognition of the +462 pause. Addition of NusA to 10 μM slowed elongation overall, generated a new pause at 419 nt, and reduced, but did not eliminate, σ^{70}-dependent pausing caused by untethered σ^{70} (Fig. 7C, wt RNAP + σ + NusA). However, NusA had little effect on +462 pausing by $\beta'::\sigma^{70}$ RNAP (Fig. 7C) or by wild-type RNAP when untethered σ^{70} was added to 10 μM (1:1 stoichiometry with NusA; data not shown). These results establish that tethered σ^{70}, and even untethered σ^{70} if added at higher concentration (but still well below that predicted to occur in vivo), can direct σ^{70}-dependent pausing irrespective of pause site location in a transcriptional unit. The findings are consistent with the paradigm that NusA competes with σ^{70} for interaction with an EC (Gill et al. 1991), but suggest that NusA may not eliminate σ^{70}-dependent pausing in vivo (see Discussion).

Discussion

Our study of tethering σ^{70} to RNAP leads to four main conclusions. First, a local σ^{70} concentration of ~55 μM (the measured value for $\beta'::\sigma^{70}$ RNAP) does not significantly perturb the physiology of *E. coli*, consistent with the effective concentration of untethered σ^{70} in wild-type cells being only slightly lower. Second, tethered σ^{70} at this modestly increased local concentration only slightly delays heat-shock gene expression and is still inactivated upon heat shock (like untethered σ^{70}), even though σ^{32} levels are not elevated. Third, tethered σ^{70} or even 1 μM untethered σ^{70} can stimulate pausing in vitro anywhere in a transcriptional unit. Fourth, the effective concentration of free σ^{70} present in cells appears sufficient to allow it to interact transiently with an EC even after σ^{70} release, and to stimulate pausing at extended –10-like sequences in vivo.

Effective concentration of σ^{70} in vivo

An accurate understanding of σ-factor binding by RNAP requires knowing the effective concentrations of σs and RNAP in vivo. However, many factors can cause the effective concentrations of molecules in cells to differ from their bulk concentrations. First, the cytoplasm of *E. coli* is a highly concentrated mixture of macromolecules, small molecules, and water more akin to a gel than to a dilute solution. Macromolecular crowding will increase the effective concentrations of σ^{70} and RNAP significantly, quite likely by a factor of 10 or more (Record et al. 1998; Ellis 2001). The magnitude of crowding effects may depend on growth conditions, as the water content of cells changes significantly in response to osmolytes in the growth medium (Cayley et al. 1991).

Second, the effective concentrations of σ^{70} and RNAP will be reduced by interactions that sequester them from participation in the competitive binding equilibria among σ factors and RNAP. ECs sequester about two-thirds of RNAP (Ishihama 2000). Additional RNAP binds nonspecifically to DNA and possibly to RNA; these contributions depend on the binding constants, target sizes, and amounts of DNA or RNA available for nonspecific interaction. Most σs in *E. coli* also specifically bind anti-σs (FlgM for σ^{F}, RseA for σ^{E}, DnaK for σ^{32}, and possibly Rsd for σ^{70}; Ishihama 2000 and references therein). 6S RNA specifically sequesters holoenzyme (Wassarman and Storz 2000). Some free σ or RNAP-bound σ could be sequestered nonspecifically. Promoter complexes also will sequester some σs, but it is difficult to estimate how many because some σ may release slowly after initiation (Shimamoto et al. 1986; Bar-Nahum and Nudler 2001; Mukhopadhyay et al. 2001) and because some OCs appear unable to initiate transcription (Susa et al. 2002). The extent to which these various interactions reduce σ^{70} and RNAP availability depends on their avidity, but together they likely reduce substantially the effective concentrations of σ^{70} and RNAP molecules.

Finally, the association of σs with RNAP is unlikely to be in equilibrium in vivo. RNAP constantly enters the pool available for σ binding both by release from DNA at terminators and by new synthesis. σs bind RNAP tightly, which means that the off-rates may be slower than the time it takes RNAP to bind and initiate at a promoter.

Given this complexity, the prospects for calculating the effective concentration of σ^{70} in vivo are poor. However, the effects of tethering σ^{70} to RNAP at known local concentration provides some insight. Both enhanced competition against σ^{70}RC584 (Fig. 5) and the slight delay in the heat-shock response (Fig. 6) of tethered σ^{70} suggest that the normal effective concentration of untethered, wild-type σ^{70} is modestly less than the local concentration of tethered σ^{70} (55 μM). Comparison to the effects of overproducing σ^{70} suggest it is ≥11 μM, close to σ^{70}'s bulk concentration (~15 μM). This is reminiscent of a similar conclusion reached for a cellular protein ordinarily present at very different bulk concentration, *lac* repressor (Law et al. 1993). For *lac* repressor, the balance of crowding and sequestration effects also yields an effective concentration near its bulk concentration (~1 nM in wild-type cells).

Competition of σ^{70} and σ^{32}

Despite the modest increase in the effective concentration of σ^{70} caused by tethering, σ^{32} levels are not elevated in the $\beta'::\sigma^{70}$ strain as expected for increased σ^{70} concentration (Zhou and Gross 1992), and the tethered σ^{70} is still inactivated upon heat shock. We cannot absolutely exclude the possibility that a small fraction of proteolytically cleaved $\beta'::\sigma^{70}$ RNAP allows the near normal σ^{32} function. However, we favor σ^{32} function with intact $\beta'::\sigma^{70}$ RNAP for three reasons. First, we did not detect cleavage of $\beta'::\sigma^{70}$ RNAP after heat shock (data not shown). Second, even if some cleavage occurred, σ^{32} must compete in these strains against additional, chromosomally encoded σ^{70} that also could bind any available core RNAP. Third, σ^{32} functions with uncleaved $\beta'::\sigma^{70}$ RNAP in vitro and this cannot be explained by proteolytic fragmentation of $\beta'::\sigma^{70}$. Rather,

we suggest the explanation for the lack of σ^{32} overexpression when σ^{70} is tethered to RNAP lies in the difference between increasing σ^{70} concentration locally versus globally. Overexpression of σ^{70} produces inclusion bodies and becomes toxic to *E. coli* at high levels of σ^{70} (data not shown; see Fig. 5). Aggregates of σ^{70} (the precursor to inclusion bodies) may be bound to the chaperone DnaK and thus could induce *E. coli*'s stress response and elevate the σ^{32} level by releasing σ^{32} from DnaK. Increasing σ^{70} concentration locally for RNAP, as occurs in the $\beta'::\sigma^{70}$ strain, would not provoke this stress response because it would not elevate the global σ^{70} level. In essence, competition of σ^{70} and σ^{32} may occur for both RNAP and DnaK upon global σ^{70} overexpression, but should be limited to RNAP for σ^{70} tethering.

The inactivation of tethered σ^{70} upon heat shock suggests something other than simple competition for σ^{32} shuts off σ^{70}-dependent gene expression. Indeed, even in wild-type cells, something in addition to an increase in σ^{32} level seems necessary to explain the decrease in σ^{70}-directed gene expression upon heat shock (Fig. 6A; Straus et al. 1987). Although σ^{32} levels are elevated fivefold 15 min after shift of *E. coli* to 42°C, σ^{70} levels themselves increase 2.5-fold (Taylor et al. 1984; Straus et al. 1987). This is equivalent to ~60 σ^{32} and ~900 σ^{70} per 1000 RNAP, suggesting that an unknown factor actively inhibits σ^{70} during heat shock. Whatever the mechanism of σ^{70} inhibition, it must also act on tethered σ^{70} and allow σ^{32} to dominate during heat shock despite its lower level. Such a requirement for additional factors has been a general conclusion in most studies of σ-factor switching (Fujita and Sadaie 1998; Kolesky et al. 1999; Lord et al. 1999; Maeda et al. 2000; Jishage et al. 2002; Rollenhagen et al. 2003). The fact that this mechanism operates on tethered σ^{70} rules out explanations involving physical segregation of σ^{70} away from sites of RNAP function because σ^{70} is fixed to RNAP by covalent linkage.

σ^{70} may function as an elongation factor in vivo

The textbook view of σ^{70} participation in the transcription cycle may need revision, although our results suggest that when or whether σ is released is not the relevant question. Even 1 µM σ^{70} can interact from solution with ECs in vitro and cause pausing anywhere in a transcriptional unit; this behavior is similar to that of σ^{70} permanently tethered to RNAP. Because σ^{70}'s effective concentration in cells is significantly higher than 1 µM, continuous transient interactions must occur with ECs regardless of when or whether it is released after promoter escape (Fig. 8).

Although NusA competes with σ^{70} for binding to the EC (Gill et al. 1991) and may temper the effects of σ^{70}-stimulated pausing, it is unlikely to eliminate this pausing in vivo. In vitro, a 10-fold excess of NusA reduced, but did not eliminate, σ^{70}-stimulated pausing (Fig. 7C). Although the in vivo concentration of NusA has not been reported, a 10-fold excess over σ^{70} would correspond to >110 µM or more than 2.6% of total cellular protein. Thus, it is unlikely there is enough NusA in cells to eliminate σ^{70}-dependent pausing at extended –10-like sequences.

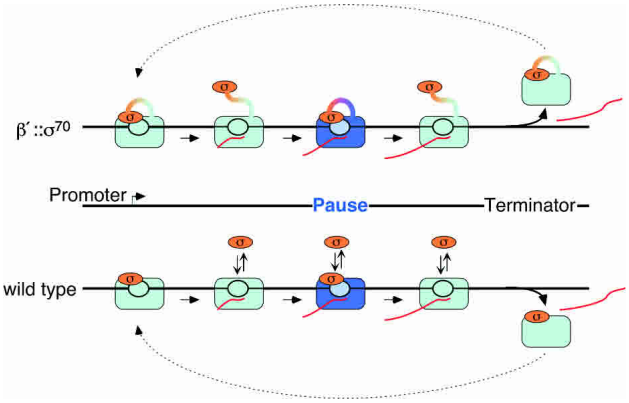

Figure 8. Model of σ^{70}–RNAP interactions during transcription in *E. coli*.

The view that emerges from this study is that the effective concentration of σ^{70} in vivo is sufficient to allow interaction with the EC and stimulation of pausing at extended –10-like sequences, making σ^{70} a bona fide elongation factor. Arndt and Chamberlin (1988) discovered that even 300 nM σ^{70} stimulates release of RNAP from terminators up to 20-fold during multiround transcription in vitro. They concluded that σ^{70} loads directly onto RNAP during the process of termination. Analysis of the time required for each round of RNA synthesis suggested that the rate-limiting step for recycling of RNAP for new rounds of transcription is an isomerization in this RNAP–σ^{70} complex (previously reported by Wu et al. 1976), rather than RNAP–σ^{70} reassociation. Given these observations and our results, we suggest that σ^{70} could be released from ECs upon promoter escape, but remain able to interact transiently with the EC during transcript elongation. If RNAP encounters an extended –10-like sequence (several of which are present within *E. coli*'s transcriptional units), transiently interacting σ^{70} could rebind to stimulate pausing. When RNAP reaches a terminator, σ^{70} could rebind tightly, making the overall behavior of wild-type σ^{70} similar to the behavior of σ^{70} tethered to RNAP without requiring continuous physical retention of σ^{70} by the EC.

Materials and methods

Strains, strain construction, plasmids, protein purification, plating efficiencies, and strain phenotypes are described in the Supplemental Material and Supplementary Table 1.

Templates for in vitro transcription and mobility shift assays

Templates were generated by PCR amplification (Supplementary Table 1) and purified by phenol extraction and spermine precipitation (Hoopes and McClure 1981). The template for mobility-shift assays (Fig. 4C) was isolated from a 1.5% GTG low-melting agarose gel, melted at 65°C in 5 vol of TE, extracted

twice with phenol, extracted with $CHCl_3$, and EtOH-precipitated.

Cell extracts

Cellular extracts were prepared from early exponential-phase cells by centrifugation for 2 min at 10,000*g*, suspension in SDS buffer (4% SDS, 10% glycerol, 62.5 mM Tris at pH 6.8, 5% β-mercaptoethanol), and incubation at 100°C for 10 min.

Immunoblot analysis

Immunoblotting and detection were performed using ECL Plus reagents (Pharmacia), following the manufacturer's instructions and using primary antibodies against β′ (NT73; 1:10,000), σ^{70} (2G10; 1:5000), and σ^{32} (3RH1; 1:3000), which was kindly provided by R. Burgess (University of Wisconsin, Madison, WI, USA), and secondary antibody (horseradish peroxidase-coupled mouse IgG) from Pharmacia. Proteolytic cleavage of $\beta'::\sigma^{70}$ to yield wild-type-length β′ or σ^{70} was undetectable in $\beta'::\sigma^{70}$ strains, with detection limits of 0.5 ng of β′ and 1.2 ng of σ^{70} per microgram of cell lysate. Levels of σ^{32} in wild-type and $\beta'::\sigma^{70}$ strains (Fig. 6B) were obtained from multiple independent cell lysates and blots (average ratio of σ^{32} in the $\beta'::\sigma^{70}$ strain relative to wild type of 0.8 ± 0.4). The estimated error in protein concentration loaded on gels (determined by Bradford assay) was 20%.

Promoter kinetics

To determine $k_{f,obs}$, 50 nM RNAP ($\beta'::\sigma^{70}$ or wild-type) was incubated with 25 nM T7 A1 template at 22°C in transcription buffer (Artsimovitch and Landick 2002). Samples were removed from 10 sec to 8 min, mixed with 0.1 vol of 2.5 mM ApU; 25 µM each ATP, [^{32}P]CTP, and GTP; and 1 mg/mL heparin; incubated at 37°C for 10 min; and then mixed with an equal volume of 2× STOP buffer (8 M urea, 98 mM Tris-borate at pH 8.3, 10 mM Na_2 EDTA, 0.2% bromophenol blue, 0.2% xylene cyanol). The RNA products were separated by 15% denaturing PAGE and quantified using a PhosphorImager. The results were fit to a single-exponential of formation by nonlinear regression using Kaleidagraph. At the concentrations of RNAP and DNA used, this assay would detect a decrease in the equilibrium binding of RNAP and DNA to form CC, but likely not an increase. To determine $k_{r,obs}$, OCs were first formed for 10 min as described for $k_{f,obs}$ determination. At time 0, heparin was added to 100 µg/mL, and sampled from 15 sec to 100 min. Samples were mixed with 0.1 vol of 2.5 mM ApU and 25 µM each ATP, [^{32}P]CTP, and GTP; incubated at 37°C for 10 min; and then mixed with an equal volume of 2× STOP buffer. The RNA products were quantified as described for $k_{f,obs}$ determination, and the results were fit to a single-exponential decay of OC by nonlinear regression using Kaleidagraph.

Equilibrium binding assay

HMKσ^{70} (250 pmole) was ^{32}P-labeled as described previously (Artsimovitch and Landick 2002). $\beta'::\sigma^{70}$ or wild-type RNAPs (1 µM) were incubated with [^{32}P]σ^{70} at 0.5, 1, 2.5, and 5 µM in equilibrating conditions using E buffer (0.5 M NaCl, 40 mM Tris-HCl at pH 8.0, 0.1 mM EDTA, 3 mM DTT, 0.2% Tween) plus 20% glycerol at 30°C for 20 min (Severinova et al. 1996; Sharp et al. 1999). Samples were then separated on a 4%–15% native Phast gel (Pharmacia) for 125 Vh at 15°C. Gels were Coomassie blue R-stained, analyzed using a PhosphorImager (Fig. 4A), and quantified (Fig. 4B). Effective σ^{70} concentration was obtained by nonlinear regression of fraction RNAP containing [^{32}P]σ^{70} versus concentration of [^{32}P] σ^{70} using the equation $y = x/(x + E)$, where x is the concentration of [^{32}P]σ^{70} and E is the effective concentration of tethered σ^{70}.

Open complex mobility assay

$\beta'::\sigma^{70}$ or wild-type RNAPs (100 nM) were mixed with increasing amounts of σ^{70} (amount added σ^{70} in micromolar indicated above gel panel) under equilibrium conditions (E buffer + 5% glycerol) at 37°C for 10 min. Linear DNA containing $P_{UPFullcon}$ (Supplementary Table 1) was added to 150 nM as the reaction was diluted to 0.25 M NaCl and incubated at 37°C for 10 min. OCs formed on this promoter are exceptionally stable ($t_{1/2}$ > 18 h; T. Gaal, pers. comm.). Reactions were electrophoresed on 4% NuSieve (FMC), 0.5× TBE, and stained with EtBr. Effective σ^{70} concentration (Fig. 4D) was modeled using the equation $y = x/(x + E)$, where x is the concentration of untethered σ^{70} and E is the effective concentration of tethered σ^{70} (set arbitrarily to 0.1, 1, 10, 50, and 100 µM).

Heat-shock response assay

Wild-type (RL301) or $\beta'::\sigma^{70}$ strain (RL1374) cells were grown in supplemented glucose medium at 30°C and labeled with [^{35}S]methionine (10 mCi/mL, Amersham; 0.2 mCi/mL final concentration) for 2 min at various times after shift to 42°C as described (Zhou et al. 1988). The cells were recovered by centrifugation, washed twice, suspended in SDS buffer, boiled for 10 min, and separated by 3%–8% Tris-Acetate (Novex) beside molecular weight markers. The gel was dried down and visualized using a PhosphorImager.

In vitro σ^{70}-dependent pause assay

Transcription on the +16/17 and +37 pause templates was performed as described (Ring et al. 1996). For the +462 pause template, A29 ECs were formed at 37°C by mixing 7.5 nM RNAP, 250 nM template, 150 µM ApU, and 10 µM ATP, GTP, and ^{32}P-CTP. The high excess of template was used to ensure only one RNAP binding per template. σ^{70} (1 µM), NusA (10 µM), or both were added to ECs as indicated. All 4 NTPs (150 µM) and 100 µg Rif/mL were then added to allow elongation to resume. Samples were removed at the times indicated, mixed with equal volume 2× STOP buffer, separated by denaturing PAGE (5%; 0.5× TBE), and analyzed using a PhosphorImager. The size of the σ^{70}-dependent pause band (462 nt, predicted) was measured by comparison to size markers generated by transcription of restriction endonuclease-treated +462 pause templates and found to be 465 ± 0.5 nt (data not shown).

In vivo concentrations

The in vivo concentrations of σ^{70} and RNAP are central to understanding the effects of tethering σ^{70}. Based on quantitative immunoblotting, Jishage and Ishihama (1995) report a constant in vivo concentration of σ^{70} of 50–80 fmole/µg cellular protein regardless of growth phase in rich medium at 37°C. At a growth rate of 40 min/generation in LB medium (Fig. 1), an *E. coli* cell contains ~7×10^{-13} mL of water (composing 70% of the cell mass) and ~2.3×10^{-13} g of protein (Cayley et al. 1991; Bremer and Dennis 1996). Assuming a partial specific volume for protein of 0.72 (Cantor and Schimmel 1980), this gives a total cell volume of ~1×10^{-12} mL. Based on these numbers, we calculate a bulk protein concentration of 230 mg/mL [$(2.3 \times 10^{-13}$ g of protein$)/(1 \times 10^{-12}$ mL)] and a bulk concentration of σ^{70} of 12–19

μM (12–19 nmole/mL). The molar ratios of total σs to σ^{70} and of RNAP to σ^{70} are reported to be 1.7 and 2.8 in the same conditions, with 65% of RNAP stably bound to DNA (e.g., as ECs; Ishihama 2000). Thus, the bulk concentration of free RNAP (available to bind σ) is also 12–19 μM and of total σs, 19–31 μM. Based on this, we derive the average bulk concentrations of free RNAP, σ^{70}, and total σs of ~15 μM, ~15 μM, and ~25 μM, respectively.

Acknowledgments

We thank R. Saecker and T. Record for suggesting the experiment shown in Figure 4C and for many helpful discussions; and C. Gross, J. Roberts, W. Ross, R. Gourse, I. Artsimovitch, and members of the Gourse and Landick labs for sharing ideas and encouragement during the course of this work. We also thank C. Vrentas for assisting with some experiments not shown; R. Gourse, R. Saecker, W. Ross, H. Murray, M. Palangat, and S. Kyzer for insightful comments on the manuscript; and T. Gaal, C. Chan, M. Sharp, D. Friedman, C. Gross, T. Arthur, R. Burgess, M. Marr, and J. Roberts for gifts of plasmids, strains, phage, and antibodies. This work was supported by NIH grants GM38660 (to R.L.) and, for support of R.A.M, T32 GM008349.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Arndt, K.M. and Chamberlin, M.J. 1988. Transcription termination in *Escherichia coli*. Measurement of the rate of enzyme release from Rho-independent terminators. *J. Mol. Biol.* **202:** 271–285.

Artsimovitch, I. and Landick, R. 2002. The transcriptional regulator RfaH stimulates RNA chain synthesis after recruitment to elongation complexes by the exposed nontemplate DNA strand. *Cell* **109:** 193–203.

Bar-Nahum, G. and Nudler, E. 2001. Isolation and characterization of σ^{70}-retaining transcription elongation complexes from *Escherichia coli*. *Cell* **106:** 443–451.

Bentley, S.D., Chater, K.F., Cerdeno-Tarraga, A.M., Challis, G.L., Thomson, N.R., James, K.D., Harris, D.E., Quail, M.A., Kieser, H., Harper, D., et al. 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417:** 141–147.

Bremer, H. and Dennis, P. 1996. Modulation of cell parameters by growth rate. In *Escherichia coli and Salmonella: Cellular and molecular biology*, 2nd ed. (eds. F. Neidhardt et al.), pp. 1553–1569. ASM press, Washington, DC.

Burgess, R.R., Travers, A.A., Dunn, J.J., and Bautz, E.K. 1969. Factor stimulating transcription by RNA polymerase. *Nature* **221:** 43–46.

Cantor, C.R. and Schimmel, P.R. 1980. *Biophysical chemistry.* Freeman, New York.

Cayley, S., Lewis, B.A., Guttman, H.J., and Record Jr., M.T. 1991. Characterization of the cytoplasm of *Escherichia coli* K-12 as a function of external osmolarity. Implications for protein–DNA interactions in vivo. *J. Mol. Biol.* **222:** 281–300.

Chamberlin, M. 1976. RNA polymerase—An overview. In *RNA polymerase* (eds. R. Losick and M. Chamberlin), pp. 17–68. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Chan, C. and Landick, R. 1989. The *Salmonella typhimurium his* operon leader region contains an RNA hairpin-dependent transcription pause site. *J. Biol. Chem.* **264:** 20796–20804.

Daube, S.S. and von Hippel, P.H. 1999. Interactions of *Escherichia coli* σ^{70} within the transcription elongation complex. *Proc. Natl. Acad. Sci.* **96:** 8390–8395.

De Las Penas, A., Connolly, L., and Gross, C.A. 1997. σE is an essential σ factor in *Escherichia coli*. *J. Bacteriol.* **179:** 6862–6864.

Ellis, R.J. 2001. Macromolecular crowding: Obvious but underappreciated. *Trends Biochem. Sci.* **26:** 597–604.

Farewell, A., Kvint, K., and Nystrom, T. 1998. Negative regulation by RpoS: A case of σ factor competition. *Mol. Microbiol.* **29:** 1039–1051.

Flory, P. 1969. *Statistical mechanics of chain molecules.* Interscience, London.

Friedman, D.I., Baumann, M., and Baron, L.S. 1976. Cooperative effects of bacterial mutations affecting λ N gene expression. I. Isolation and characterization of a nusB mutant. *Virology* **73:** 119–127.

Fujita, M. and Sadaie, Y. 1998. Promoter selectivity of the *Bacillus subtilis* RNA polymerase σA and σH holoenzymes. *J. Biochem. (Tokyo)* **124:** 89–97.

Gill, S.C., Weitzel, S.E., and von Hippel, P.H. 1991. *Escherichia coli* σ 70 and NusA proteins. I. Binding interactions with core RNA polymerase in solution and within the transcription complex. *J. Mol. Biol.* **220:** 307–324.

Gross, C.A., Chan, C., Dombroski, A., Gruber, T., Sharp, M., Tupy, J., and Young, B. 1998. The functional and regulatory roles of σ factors in transcription. *Cold Spring Harb. Symp. Quant. Biol.* **63:** 141–155.

Hicks, K.A. and Grossman, A.D. 1996. Altering the level and regulation of the major σ subunit of RNA polymerase affects gene expression and development in *Bacillus subtilis*. *Mol. Microbiol.* **20:** 201–212.

Hoopes, B.C. and McClure, W.R. 1981. Studies on the selectivity of DNA precipitation by spermine. *Nucleic Acids Res.* **9:** 5493–5504.

Ishihama, A. 2000. Functional modulation of *Escherichia coli* RNA polymerase. *Annu. Rev. Microbiol.* **54:** 499–518.

Jishage, M. and Ishihama, A. 1995. Regulation of RNA polymerase σ subunit synthesis in *Escherichia coli*: Intracellular levels of σ 70 and σ 38. *J. Bacteriol.* **177:** 6832–6835.

Jishage, M., Kvint, K., Shingler, V., and Nystrom, T. 2002. Regulation of σ factor competition by the alarmone ppGpp. *Genes & Dev.* **16:** 1260–1270.

Kimura, H., Sugaya, K., and Cook, P.R. 2002. The transcription cycle of RNA polymerase II in living cells. *J. Cell Biol.* **159:** 777–782.

Kolesky, S., Ouhammouch, M., Brody, E.N., and Geiduschek, E.P. 1999. σ competition: The contest between bacteriophage T4 middle and late transcription. *J. Mol. Biol.* **291:** 267–281.

Krummel, B. and Chamberlin, M.J. 1989. RNA chain initiation by *Escherichia coli* RNA polymerase. Structural transition of the enzyme in early ternary complexes. *Biochemistry* **28:** 7829–7842.

Kumar, A., Grimes, B., Logan, M., Wedgwood, S., Williamson, H., and Hayward, R.S. 1995. A hybrid σ subunit directs RNA polymerase to a hybrid promoter in *Escherichia coli*. *J. Mol. Biol.* **246:** 563–571.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessieres, P., Bolotin, A., Borchert, S., et al. 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* **390:** 249–256.

Law, S.M., Bellomy, G.R., Schlax, P.J., and Record Jr., M.T.

1993. In vivo thermodynamic analysis of repression with and without looping in lac constructs. Estimates of free and local lac repressor concentrations and of physical properties of a region of supercoiled plasmid DNA in vivo. *J. Mol. Biol.* **230:** 161–173.

Lonetto, M.A., Rhodius, V., Lamberg, K., Kiley, P., Busby, S., and Gross, C. 1998. Identification of a contact site for different transcription activators in region 4 of the *Escherichia coli* RNA polymerase σ70 subunit. *J. Mol. Biol.* **284:** 1353–1365.

Lord, M., Barilla, D., and Yudkin, M.D. 1999. Replacement of vegetative σA by sporulation-specific σF as a component of the RNA polymerase holoenzyme in sporulating *Bacillus subtilis*. *J. Bacteriol.* **181:** 2346–2350.

Maeda, H., Fujita, N., and Ishihama, A. 2000. Competition among seven *Escherichia coli* σ subunits: Relative binding affinities to the core RNA polymerase. *Nucleic Acids Res.* **28:** 3497–3503.

Marr, M.T. and Roberts, J.W. 2000. Function of transcription cleavage factors GreA and GreB at a regulatory pause site. *Mol. Cell* **6:** 1275–1285.

Marr, M.T., Datwyler, S.A., Meares, C.F., and Roberts, J.W. 2001. Restructuring of an RNA polymerase holoenzyme elongation complex by lambdoid phage Q proteins. *Proc. Natl. Acad. Sci.* **98:** 8972–8978.

McClure, W.R. 1980. Rate-limiting steps in RNA chain initiation. *Proc. Natl. Acad. Sci.* **77:** 5634–5638.

Mekler, V., Kortkhonjia, E., Mukhopadhyay, J., Knight, J., Revyakin, A., Kapanidis, A.N., Niu, W., Ebright, Y.W., Levy, R., and Ebright, R.H. 2002. Structural organization of bacterial RNA polymerase holoenzyme and the RNA polymerase–promoter open complex. *Cell* **108:** 599–614.

Mukhopadhyay, J., Kapanidis, A.N., Mekler, V., Kortkhonjia, E., Ebright, Y.W., and Ebright, R.H. 2001. Translocation of σ^{70} with RNA polymerase during transcription: Fluorescence resonance energy transfer assay for movement relative to DNA. *Cell* **106:** 453–463.

Murakami, K.S., Masuda, S., Campbell, E.A., Muzzin, O., and Darst, S. 2002. Structural basis of transcription initiation: An RNA polymerase holoenzyme/DNA complex. *Science* **296:** 1285–1290.

Pokholok, D.K., Hannett, N.M., and Young, R.A. 2002. Exchange of RNA polymerase II initiation and elongation factors during gene expression in vivo. *Mol. Cell* **9:** 799–809.

Raag, R. and Whitlow, M. 1995. Single-chain Fvs. *FASEB J.* **9:** 73–80.

Record Jr., M.T., Courtenay, E.S., Cayley, S., and Guttman, H.J. 1998. Biophysical compensation mechanisms buffering *E. coli* protein–nucleic acid interactions against changing environments. *Trends Biochem. Sci.* **23:** 190–194.

Ring, B., Yarnell, W., and Roberts, J. 1996. Function of *E. coli* RNA polymerase σ factor σ^{70} in promoter-proximal pausing. *Cell* **86:** 485–493.

Robinson, C.R. and Sauer, R.T. 1998. Optimizing the stability of single-chain proteins by linker length and composition mutagenesis. *Proc. Natl. Acad. Sci.* **95:** 5929–5934.

Rollenhagen, C., Antelmann, H., Kirstein, J., Delumeau, O., Hecker, M., and Yudkin, M.D. 2003. Binding of σ(A) and σ(B) to core RNA polymerase after environmental stress in *Bacillus subtilis*. *J. Bacteriol.* **185:** 35–40.

Saecker, R., Tsodikov, O., McQuade, K., Schlax Jr., P., Capp, M., and Record Jr., M. 2002. Kinetic studies and structural models of the association of *E. coli* σ^{70} RNA polymerase with the λP_R promoter: Large scale conformational changes in forming kinetically significant intermediates. *J. Mol. Biol.* **319:** 649–671.

Severinov, K., Mooney, R., Darst, S., and Landick, R. 1997. Tethering of the large subunits of *Escherichia coli* RNA polymerase. *J. Biol. Chem.* **272:** 24137–24140.

Severinova, E., Severinov, K., Fenyo, D., Marr, M., Brody, E.N., Roberts, J.W., Chait, B.T., and Darst, S.A. 1996. Domain organization of the *Escherichia coli* RNA polymerase σ 70 subunit. *J. Mol. Biol.* **263:** 637–647.

Sharp, M.M., Chan, C.L., Lu, C.Z., Marr, M.T., Nechaev, S., Merritt, E.W., Severinov, K., Roberts, J.W., and Gross, C.A. 1999. The interface of σ with core RNA polymerase is extensive, conserved, and functionally specialized. *Genes & Dev.* **13:** 3015–3026.

Shimamoto, N., Kamigochi, T., and Utiyama, H. 1986. Release of the σ subunit of *Escherichia coli* DNA-dependent RNA polymerase depends mainly on time elapsed after the start of initiation, not on length of product RNA. *J. Biol. Chem.* **261:** 11859–11865.

Siegele, D.A., Hu, J.C., Walter, W.A., and Gross, C.A. 1989. Altered promoter recognition by mutant forms of the σ 70 subunit of *Escherichia coli* RNA polymerase. *J. Mol. Biol.* **206:** 591–603.

Straus, D.B., Walter, W.A., and Gross, C.A. 1987. The heat shock response of *E. coli* is regulated by changes in the concentration of σ 32. *Nature* **329:** 348–351.

Susa, M., Sen, R., and Shimamoto, N. 2002. Generality of the branched pathway in transcription initiation by *Escherichia coli* RNA polymerase. *J. Biol. Chem.* **277:** 15407–15412.

Taylor, W.E., Straus, D.B., Grossman, A.D., Burton, Z.F., Gross, C.A., and Burgess, R.R. 1984. Transcription from a heat-inducible promoter causes heat shock regulation of the σ subunit of *E. coli* RNA polymerase. *Cell* **38:** 371–381.

Timpe, L.C. and Peller, L. 1995. A random flight chain model for the tether of the Shaker K^+ channel inactivation domain. *Biophys. J.* **69:** 2415–2418.

Vassylyev, D.G., Sekine, S., Laptenko, O., Lee, J., Vassylyeva, M.N., Borukhov, S., and Yokoyama, S. 2002. Crystal structure of a bacterial RNA polymerase holoenzyme at 2.6 Å resolution. *Nature* **417:** 712–719.

Wassarman, K.M. and Storz, G. 2000. 6S RNA regulates *E. coli* RNA polymerase activity. *Cell* **101:** 613–623.

Wu, F.Y., Yarbrough, L.R., and Wu, C.W. 1976. Conformational transition of *Escherichia coli* RNA polymerase induced by the interaction of σ subunit with core enzyme. *Biochemistry* **15:** 3254–3258.

Zhou, Y.N. and Gross, C.A. 1992. How a mutation in the gene encoding σ 70 suppresses the defective heat shock response caused by a mutation in the gene encoding σ 32. *J. Bacteriol.* **174:** 7128–7137.

Zhou, Y.N., Kusukawa, N., Erickson, J.W., Gross, C.A., and Yura, T. 1988. Isolation and characterization of *Escherichia coli* mutants that lack the heat shock sigma factor σ 32. *J. Bacteriol.* **170:** 3640–3649.

Zhou, Y.N., Walter, W.A., and Gross, C.A. 1992. A mutant σ 32 with a small deletion in conserved region 3 of σ has reduced affinity for core RNA polymerase. *J. Bacteriol.* **174:** 5005–5012.