Altering the interaction between σ^{70} and RNA polymerase generates complexes with distinct transcription-elongation properties

Yvonne Berghöfer-Hochheimer*, Chi Zen Lu*, and Carol A. Gross*†‡

Departments of *Microbiology and Immunology and †Stomatology, Genentech Hall, 600 16th Street, University of California, San Francisco, CA 94143

Contributed by Carol A. Gross, December 9, 2004

We compare the elongation behavior of native *Escherichia coli* RNA polymerase holoenzyme assembled *in vivo*, holoenzyme reconstituted from σ^{70} and RNA polymerase *in vitro*, and holoenzyme with a specific alteration in the interface between σ^{70} and RNA polymerase. Elongating RNA polymerase from each holoenzyme has distinguishable properties, some of which cannot be explained by differential retention or rebinding of σ^{70} during elongation, or by differential presence of elongation factors. We suggest that interactions between RNA polymerase and σ^{70} may influence the ensemble of conformational states adopted by RNA polymerase during initiation. These states, in turn, may affect the conformational states adopted by the elongating enzyme, thereby physically and functionally imprinting RNA polymerase.

All multisubunit RNA polymerases use initiation factors to recognize their promoters, a strategy that allows tight base-specific binding during the initiation phase of transcription and nonspecific binding during elongation, after release of the initiation factor. This function is performed by σ in eubacterial cells (1). Almost all bacteria contain multiple σ factors, one directing transcription to housekeeping genes and the remainder directing transcription to genes encoding specialized functions (1).

Genetic, biochemical, and structural characterization of the interaction between σ and RNA polymerase (E) (2–4) reveals that the interface between the two proteins is both extensive, having at least four regions of interaction (5–9), and dynamic, with some interactions depending on the formation of the preceding ones (5). Conformational changes in both partners result from this interaction. The changes in σ unmask and reposition its DNA-binding domains to allow promoter recognition (6, 7, 10–14). Conformational changes in RNA polymerase may reposition portions of RNA polymerase in close contact with the nucleic acids, but the functional consequences of such changes are unknown.

Usually, σ factors dissociate from elongating RNA polymerase shortly after RNA polymerase leaves the promoter (15–18) but remain associated with RNA polymerase longer than normal at a special class of promoters (19–21). The predominant eubacterial promoter has two conserved recognition sequences, centered at −10 and −35 bp upstream of the starting point of transcription (+1). Promoters with a reiterated −10 motif in the initially transcribed region exhibit prolonged σ association. This motif was discovered first in promoters directing lambdoid phage late transcription. The σ recognition of the reiterated −10 region induces a transcription pause (19–23) that is required to load the λQ elongation factor that antiterminates transcription (24). Reiterated −10 regions were identified recently (21–23) in a subset of bacterial promoters, including *lac*UV5. It is thought that σ dissociates shortly after passing the reiterated −10 region (23). Eσ^{70} from stationary cells may be refractory to dissociation as a significant fraction (≈30%) of RNA polymerase purified from such cells retained σ during elongation (25).

In *Escherichia coli*, the average rate of elongation is 50 nt/s (ref. 26 and references therein); however, this speed is not constant. First, the template encodes two known types of pauses. Class I pauses (like *his*) are mediated by a stem–loop structure that interacts with RNA polymerase (27, 28). Class II pauses (like *ops*) involve backtracking due to a weak DNA/RNA hybrid (29, 30). Single-molecule studies reveal additional diversity during elongation. First, RNA polymerase often hesitates for a few seconds, which is a behavior that has been attributed to the transient assumption of a RNA polymerase conformation refractory to elongation (31, 32). Second, RNA polymerase can pause for a longer time and then backtrack (33), which is a behavior that may be part of the proofreading mechanism of the enzyme (34). Last, the diversity in transcription rates (≥5-fold) of single molecules far exceeds that expected for a single transcribing species (35). Because each molecule maintains its rate for the duration of the measurements (≈5 min), this variation is not due to rapidly interconverting conformers. It is unclear whether these are relatively stable conformational states or a consequence of posttranslational modification (35).

Given the extensive and dynamic interface between RNA polymerase and σ, we wondered whether subtle alterations in these interactions could influence the range of conformational states adopted by RNA polymerase and, thereby, alter its pausing and elongation behavior. We examined this issue with RNA polymerase holoenzyme containing σ^{70}, the *E. coli* housekeeping σ. We reasoned that holoenzyme reconstituted *in vitro* from RNA polymerase and σ^{70} (r-Eσ^{70}) might have slightly different contacts than that of native RNA polymerase holoenzyme (n-Eσ^{70}), assembled *in vivo*. Therefore, we compared the pausing and elongation properties of these two holoenzymes. Also, we compared the pausing and elongation properties of WT holoenyzme with those of holoenzyme with a σ^{70} mutant (E407K) that partially disrupts the strongest interaction between RNA polymerase and σ^{70} (5, 6, 8). We find reproducible differences in the elongation behavior of these populations. We suggest that altering the contacts between RNA polymerase and σ^{70} can alter the elongation properties of polymerase.

Materials and Methods

Proteins and DNA. Native Eσ^{70} or E$\sigma^{70(E407K)}$ were purified from *E. coli* strain BL21(DE3) that was transformed with pET15rpoD or pET15rpoD-E407K, encoding N-terminal HIS-tagged σ^{70}, and induced with 1 mM isopropyl β-D-thiogalactoside for 4 h. Native holoenzyme was purified as described (36, 37) with the following modifications. After polyethyleneimine precipitation and Ni^{2+}-nitrilotriacetic acid (NTA) chromatography, the eluate was precipitated with 40% $(NH_4)_2(SO4)$, and the pellet was resuspended in 0.5 ml of TGED (10 mM Tris, pH 7.9/5% glycerol/0.1 mM EDTA/1 mM DTT) and fractionated first on a Superdex 200 10/30 column (Pharmacia) and then on a MonoQ 5/5 column. For the experiments shown in Fig. 1, RNA

Abbreviations: NTA, nitrilotriacetic acid; TEC, transcription-elongation complex.

‡To whom correspondence should be addressed. E-mail: cgross@cgl.ucsf.edu.

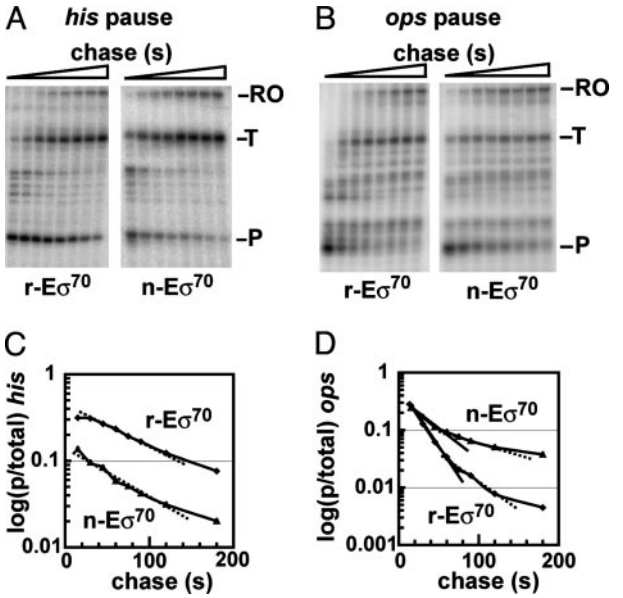

Fig. 1. Pausing of σ^{70}-dependent RNA polymerase holoenzyme complexes at the *his* and the *ops* pause sites. (*A* and *B*) Preformed [α-^{32}P]CMP-labeled TECs were chased with 10 μM GTP and 150 μM each of ATP, CTP, and UTP. Samples were removed at the following time points: 15, 30, 45, 60, 75, 90, 120, and 180 s, and they were then run on a 10% denaturing polyacrylamide gel. The autoradiogram shows the time-dependent escape of TECs at *his* (*A*) and at *ops* (*B*). The pause site (P), terminated transcripts (T) and runoff transcription (RO) are indicated. (*C* and *D*) Reaction profiles, quantitated by plotting the fraction of paused RNA vs. time. The calculated pause half-life at *his* (*C*) was 73 s for *r*-Eσ^{70} (◆) and 41 s for *n*-Eσ^{70} (▲). Pause efficiencies, determined by backextrapolation of the exponentials to time point 0, were 40% for *r*-Eσ^{70} and 18% for *n*-Eσ^{70}. At *ops* (*D*) solid and dashed straight lines indicate the two exponential phases for each holoenzyme complex. Half-lives for *r*-Eσ^{70} (◆) were 17 and 51 s, and for *n*-Eσ^{70} (▲), they were 27 and 106 s. Pause efficiencies, determined from the fast phase, were 40% for *r*-Eσ^{70} and 32% for *n*-Eσ^{70}.

polymerase core was obtained from BL21(DE3) culture, transformed with pGEMABC (S. Darst, The Rockefeller University, New York), purified as described above, except gel filtration was performed on Sephacryl 300 and Ni^{2+}–NTA chromatography was omitted. For all other experiments using reconstituted enzyme, native Eσ^{70} was depleted of σ^{70} on a BioRex70 column, 100–200 mesh (Bio-Rad); eluted core subunits were concentrated on MonoQ (38). We purified σ^{70} and $\sigma^{70\text{-E407K}}$ on Ni^{2+}–NTA resin. Templates for *in vitro* transcription were generated by PCR from pIA171 (*his*), pIA273 (*ops*) (29), or pIA146 (for elongation-rate determination; ref. 39) and gel-purified. For immobilized transcription, templates were 5′-labeled with Biotin-16-dUTP by Klenow fill-in and bound to Dynabeads (Dynal, Oslo) before transcription. Stalled A29-TECs were washed three or four times with TGED plus 300 mM KCl/0.1% Sarkosyl/0.1 mg/ml heparin/0.05 mg/ml BSA and two times with 1× transcription buffer (see below) before transcription was resumed.

***In Vitro* Transcription.** Core RNAP and σ^{70} were reconstituted on ice for 10 min; and reconstituted or native holoenzyme was added to their respective templates and shifted to 30°C for 15 min to form A29-halted complexes. The final transcription conditions were as follows: 20–50 nM template, 20–50 nM RNAP, 1× transcription buffer (20 mM Tris·HCl, pH 8.0/20 mM NaCl/10 mM $MgCl_2$/5% glycerol/0.1 mM DTT/0.1 mM Na_2·EDTA/0.05 mg/ml BSA), 150 μM ApU dinucleotide 2.5 μM each ATP and GTP, 1 μM CTP, and 1 μCi of [α-^{32}P]-CTP (1 Ci = 37 GBq; 3,000 Ci/mmol, 10 mCi/ml). Elongation was resumed by adding 150 μM each ATP, CTP, and GTP each; 5–10 μM GTP; and 0.1 mg/ml heparin (final concentrations). Elongation rate was measured by using 80 μM NTPs. Elongation rate under limiting NTP concentrations was measured similarly, except that UTP alone was added to halted A29-TECs to resume elongation. Samples (10 μl) were removed at defined time intervals and added to 5 μl of formamide-loading dye. All samples were heated for 3 min at 90°C and run on 6–10% sequencing gels. Transcripts were quantitated by using a PhosphorImager scan and IMAGEQUANT software (Molecular Dynamics). Retention of $\sigma^{70}{}_{His}$ in stalled A29-TECs was determined by an indirect method (25).

Table 1. Pause half-lives and pause efficiencies of two different native and reconstituted RNAP holoenzymes at the *his* and *ops* pause sites

	his		*ops*	
Enzyme	$T_{1/2}$, s	p.e., %	$T_{1/2}$, s	p.e., %
Native	40 ± 3	26 ± 6	27 ± 0.4	31 ± 2
Reconstituted*	70 ± 4	39 ± 2	17 ± 1	38 ± 3
Native†	38 ± 6	23 ± 5	33 ± 2	28 ± 3
Reconstituted†	64 ± 4	40 ± 2	18‡	26‡

SD was obtained from at least three independent experiments. p.e., Pause efficiency.
*Preparation of reconstituted enzyme from overexpressed RNAP core subunits.
†Preparation of native enzyme used for σ^{70} depletion and a new reconstituted enzyme.
‡Single measurement.

Results

Reconstituted and Native RNA Polymerase Holoenzyme Complexes Have Distinct Pausing Properties. We tested whether *r*-Eσ^{70} and *n*-Eσ^{70} holoenzymes were functionally equivalent in their behavior at a Class I (*his*) and Class II (*ops*) pause. Strikingly, their behavior was distinct. Quantification of a representative pulse–chase experiment at *his* (Fig. 1*A*) revealed a pause half-life of 73 s for *r*-Eσ^{70} and 41 s for *n*-Eσ^{70} (Fig. 1*C*). The two preparations also differed in pause efficiency (40% for *r*-Eσ^{70} and 18% for *n*-Eσ^{70}). Quantification of a representative pulse–chase experiment at *ops* (Fig. 1*B*) revealed that the two enzyme preparations had similar pausing efficiencies (≈40%) but different kinetics (Fig. 1*D*). Escape from the *ops* pause is biphasic. *n*-Eσ^{70} showed a longer-pause half-life than *r*-Eσ^{70} in both phases, exactly opposite of their behavior at *his*. In the fast phase, *n*-Eσ^{70} showed a $T_{1/2}$ = 27 s and *r*-Eσ^{70} showed a $T_{1/2}$ = 17 s. In the slow phase, *n*-Eσ^{70} showed a $T_{1/2}$ of 106 s and *r*-Eσ^{70} showed a $T_{1/2}$ of 51 s. Results of three or more independent experiments at these two pause sites are given in summary in Table 1 (top two rows). We conclude that *r*-Eσ^{70} and *n*-Eσ^{70} possess distinct pausing behaviors at Class I and Class II pause sites.

The *n*-Eσ^{70} and *r*-Eσ^{70} preparations used in the above experiments differed in several respects. They were purified at different times with slightly different procedures; *n*-Eσ^{70} was purified from RNA polymerase specified by its chromosomal genes, whereas *r*-Eσ^{70} was made from overexpressed RNA polymerase (see core RNA polymerase preparation 1 in *Materials and Methods*); and *r*-Eσ^{70} was reconstituted from components that had been stored separately in high glycerol. Therefore, we repurified *n*-Eσ^{70} to apparent homogeneity by using multiple columns, starting with extracts made from exponentially growing cells; separated a portion of the preparation into E and σ^{70}; and immediately reconstituted it with purified σ^{70} (Fig. 2*A*). Purified *n*-Eσ^{70} and the core enzyme derived from it are shown in Fig. 2*B*. An overloaded 15% gel demonstrates that both preparations

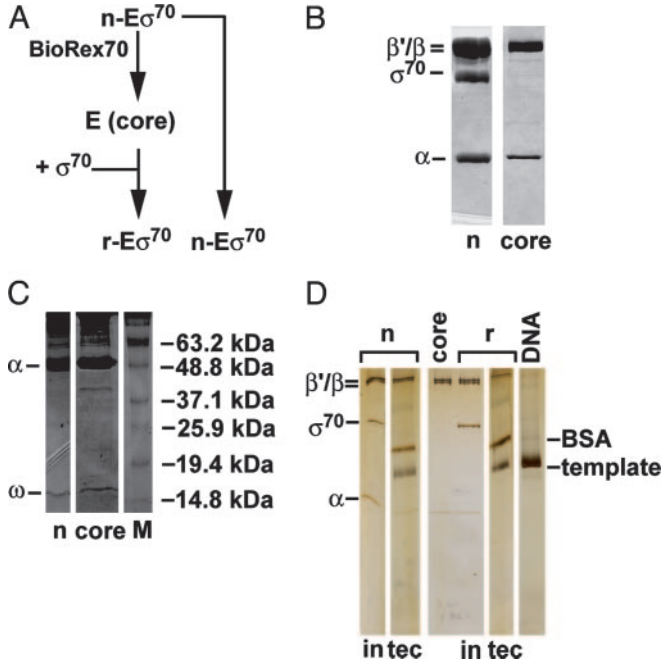

Fig. 2. SDS/PAGE of purified native holoenzyme Eσ^{70}, and RNA polymerase core. (*A*) Diagram of purification of core enzyme (E) and reconstituted enzyme (r) from native enzyme (n). (*B*) Coomassie blue staining of 1 μg of *n*-enzyme and of 1 μg of core RNA polymerase separated on a 10% SDS/PAGE gel shows the purity of holoenzyme preparation and efficient removal of σ^{70} (core). (*C*) Coomassie blue staining of 5 μg of native (n) or core separated on a 15% SDS/PAGE gel shows that both preparations have the ω subunit of RNA polymerase. The band above 37 kDa in the core preparation must be a degradation product of one of the core subunits. (*D*) The silver-stained 7–15% gradient SDS/PAGE gel shows input (in) of *n*-enzyme or *r*-enzyme and the washed TECs (tec), used for immobilized transcription.

retain the ω-subunit and that *n*-Eσ^{70} does not have visible contamination with other low-molecular-weight proteins (Fig. 2*C*). We compared the pausing properties of *r*-Eσ^{70} and *n*-Eσ^{70}. Newly purified *r*-Eσ^{70} and *n*-Eσ^{70} holoenzymes (Table 1, bottom two rows) showed properties identical to the original enzymes (Table 1, top two rows). Thus, transient separation of σ^{70} and core are sufficient to alter pausing behavior.

r-Eσ^{70} and *n*-Eσ^{70} Differ in Their Elongation Rates. We examined elongation rates by using a template in which the T7A1 promoter drives transcription of *rpoB*, which has no known terminators or regulatory pauses (32, 39). When stalled radiolabeled complexes of 29 nt in length (A29) were chased with 80 μM NTPs, *r*-Eσ^{70} and *n*-Eσ^{70} showed distinct elongation rates (Fig. 3*A*), Of the *r*-Eσ^{70} transcription-elongation complexes (TECs), 50% produced full-length transcripts after 50 s, whereas it took 100 s for 50% of the *n*-Eσ^{70} TECs to complete the runoff transcript (Fig. 3*B*), indicating that the two complexes differ significantly in their elongation rates.

Very Low Amounts of σ^{70} Remain in the TECs. The simplest explanation for these differences is that *r*-Eσ^{70} and *n*-Eσ^{70} differentially retain σ^{70} in the TEC and that the presence of σ^{70} alters the pausing and elongation properties of the enzyme. Therefore, we determined the amounts of σ^{70} present in each TEC halted upstream of the pause site by using a procedure described by Bar-Nahum and Nudler (25). Paused ternary complexes with short radiolabeled transcripts were immobilized on Ni–NTA resin by virtue of their σ^{70}_{His} (25), and the fraction of the radiolabeled transcript retained on Ni–NTA was determined.

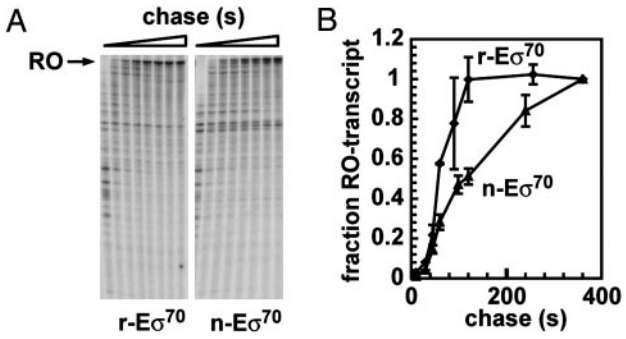

Fig. 3. Elongation rates of *r*-Eσ^{70} and *n*-Eσ^{70}. (*A*) Preformed [^{32}P]-CMP-labeled TECs were elongated on a template without strong pause sites by using 80 μM NTPs. Samples were taken at 10, 30, 45, 60, 90, 120, 240, and 360 s and analyzed on a 6% denaturing PAGE. (*B*) Reaction profiles of runoff transcription. The ratio of the runoff (RO) transcript to total transcripts was plotted against time. The calculated elongation rates were 50 s for *r*-Eσ^{70} and 100 s for *n*-Eσ^{70}.

This fraction is a measure of the fraction of ternary complexes that retain σ^{70}_{His}. This experiment indicated that $\leq$5% of the TECs retain σ^{70}_{His} (Fig. 4), which is similar to the previous determination for RNA polymerase purified from exponentially growing cells (25). Thus, the amount of σ^{70} in the TECs was too low to account for the differences in the pausing and elongation properties of *r*-Eσ^{70} and *n*-Eσ^{70}. Also, these reactions contain sufficient heparin to disrupt the σ^{70} function during elongation (21, 39, 40).

The Difference in *r*-Eσ^{70} and *n*-Eσ^{70} Behavior at the *his* Pause Is Maintained After a Stringent Wash Procedure. In a second protocol, we stringently washed the TECs to remove free σ^{70} and potential nonpolymerase contaminants from elongating complexes and examined transcription at *his* and during elongation. Stalled TECs of *n*-Eσ^{70} and *r*-Eσ^{70} were formed and templates then immobilized by means of a terminal biotinylated nucleotide to a streptavidin matrix. A fraction of the stalled, immobilized TECs was then washed three or four times with a high-salt buffer containing 0.1% sarkosyl to remove free components, and subsequently reequilibrated to normal salt conditions before resumption of elongation (see *Materials and Methods*). A silver-stained gradient gel of stalled *n*-Eσ^{70} and *r*-Eσ^{70} A29-TECs demonstrated that σ^{70} was removed by this procedure and that no other contaminating bands were present in *n*-Eσ^{70} (Fig. 2*D*). Behavior at *his* was determined for both washed and unwashed TECs. Importantly, *r*-Eσ^{70} still paused $\approx$50% longer than *n*-Eσ^{70}

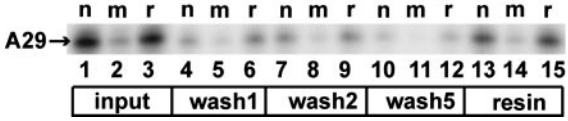

Fig. 4. Indirect quantitation of σ^{70}_{His} retention in TECs. WT native Eσ^{70} (n, lanes 1, 4, 7, 10, and 13), mutant *n*-Eσ^{70} (m; lanes 2, 5, 8, 11, and 14), and WT reconstituted Eσ^{70} (r; lanes 3, 6, 9, 12, and 15) were used to generate ^{32}P-labeled TECs at transcript position +29 (A29). The TECs were incubated with Ni–NTA resin, washed with transcription buffer, and eluted from the resin by using formamide loading dye. We ran 5% of the original input reactions, 2.5% of supernatants of wash steps, and 4% of bound TECs (resin) on a 6% denaturing gel. The final calculated amounts of A29-TECs bound to the resin were 2.6% (lane 13), 0.4% (lane 14), and 3.5% (lane 15). The following transcripts were detected in the supernatants: after the first washing step, 8% (lane 4), 10% (lane 5), and 11% (lane 6); after the second washing step, 4% (lane 7), 4% (lane 8), and 5% (lane 9); and after the fifth step, 0.5% (lane 10), 0.2% (lane11), and 0.7% (lane 12).

Table 2. Pause half-lives and pause efficiencies of native and reconstituted RNAP holoenzymes at the immobilized *his* pause template

RNAP holoenzymes	$T_{1/2}$, s (before/after wash)	p.e., % (before/after wash)
Native*	25/22	37/74
Reconstituted*	33/36	48/70
Native†	20/23	34/42
Reconstituted†	30/31	34/49

$T_{1/2}$ and pause efficiency (p.e.) were determined before and after washing A29-stalled TECs (see *Materials and Methods*).

*TECs washed three times with high-salt buffer and one time with transcription buffer.

†TECs washed four times with high-salt buffer and one time with transcription buffer.

even after extensive washing (Table 2, column 1), as was found for reactions carried out in solution (Table 1, column 1), although pause and efficiency were affected by this protocol (compare Tables 1 and 2). In contrast, washing eliminated the difference between the elongation rate of *r*-Eσ^{70} and *n*-Eσ^{70}. Untreated *n*-Eσ^{70} elongated more slowly than *r*-Eσ^{70} on the immobilized template, whereas extensively washed *n*-Eσ^{70} elongated as rapidly as *r*-Eσ^{70} (Table 3). We consider the implications of these results in *Discussion*.

A Single Mutation in σ^{70} (E407K) Alters Elongation Properties of Native Eσ^{70}. We used the $\sigma^{70\ (E407K)}$ mutant to test whether altering a contact between σ^{70} and core RNA polymerase altered elongation properties. Mutant *n*-Eσ^{70} showed subtle, but reproducible, defects in elongation behavior. First, the pausing efficiency (fast phase) of mutant *n*-Eσ^{70} at the *ops* pause was significantly (≈2-fold) reduced compared with WT *n*-Eσ^{70} (Fig. 5). Second, although elongation rates of the two enzymes were the same at 80 μM NTPs (data not shown), they showed clear differences when assayed at very low NTPs (Fig. 6), a condition known to promote pausing (40, 41). At every examined time point, mutant *n*-Eσ^{70} had a significantly higher proportion of full-length transcripts than WT *n*-Eσ^{70} (shown as transcript ratio a/b; Fig. 6 *A* and *C*), and the same was true when comparing the early time points for mutant *r*-E$\sigma^{70}$and WT *r*-Eσ^{70} (Fig. 6 *B* and *D*). As expected from the elongation phenotypes presented in Fig. 3, WT *r*-Eσ^{70} also exhibited a higher proportion of full-length transcripts than WT *n*-Eσ^{70} (Fig. 6 *A* and *B*, compare light bars in Fig. 6*D* to gray bars in Fig. 6*C*). The most striking result was achieved with mutant *r*-Eσ^{70}, which had 40% long transcripts after a 30-s incubation, a fraction not achieved by any other enzyme until 2 min (Fig. 6*D*, black bars).

Discussion

The principal contribution of this article is the demonstration that altering the interactions between RNA polymerase and σ^{70} alters behavior of RNA polymerase during the elongation phase. We demonstrate this alteration in two ways, (*i*) by comparing the elongation properties of *n*-Eσ^{70} assembled *in vivo* with *r*-Eσ^{70} assembled *in vitro*, and (*ii*) by comparing elongation properties of transcription initiated by WT σ^{70} with that initiated by a mutant σ^{70} defective in a major contact with RNA polymerase. We argue below that these effects are not due to either differential retention or reassociation of σ^{70} with elongating RNA polymerase, and they are unlikely to result from chemical heterogeneity of the enzyme. Some differences cannot be due to the differential presence of elongation factors. We suggest that the interactions between σ^{70} and RNA polymerase influence the ensemble of conformational states adopted by RNA polymerase during initiation. These states, in turn, affect the conformational states adopted by the elongating enzyme, thereby physically and functionally imprinting RNA polymerase.

Table 3. Elongation rates of native and reconstituted RNAP holoenzymes at the immobilized *rpo*B template

RNAP holoenzyme	Without wash	With wash
Native	90 ± 14	53 ± 1.4
Reconstituted	40 ± 0	40 ± 0

Stalled TECs were washed four times with high-salt buffer and two times with transcription buffer (see *Materials and Methods*). Data are the mean of two experiments.

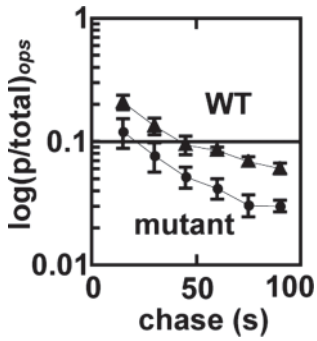

Fig. 5. Mutant *n*-Eσ^{70} has pausing properties distinct from WT *n*-Eσ^{70} at the *ops* pause site. Reaction profiles of WT and mutant *n*-Eσ (fast phase) are shown. Fractions of paused RNA generated by mutant *n*-Eσ and WT *n*-Eσ^{70} holoenzymes at *ops* were plotted against time. Back-extrapolation of the reactions to time 0 yielded pause efficiencies of 26% for WT *n*-Eσ^{70} and 16% for mutant *n*-Eσ^{70}. Data are the average of four independent experiments.

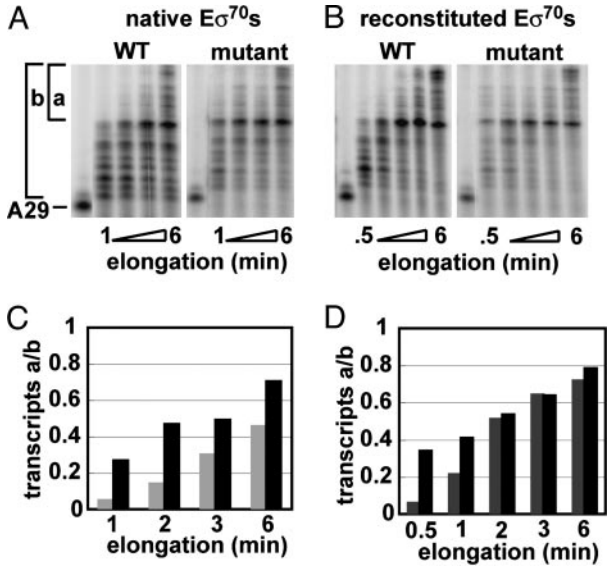

Fig. 6. Transcription elongation by WT and mutant Eσ^{70} under NTP limitation. (*A* and *B*) Autoradiograms of continuously labeled transcripts. A29-[^{32}P]-CMP-labeled TECs were generated by using a subset of 2.5 μM NTPs. Elongation was resumed by addition of 2.5 μM UTP and continued for the indicated times. (*C* and *D*) Distribution of transcripts. As a relative measurement of the synthesis rate, the ratio of transcripts longer than a prominent transcript (a) to the total number of transcripts (b) is plotted vs. time. (*C*) Transcript ratio a/b of WT *n*-Eσ^{70} (gray bars) and mutant *n*-Eσ^{70} (black bars). (*D*) Transcript ratio a/b of WT *r*-Eσ^{70} (gray bars) and mutant *r*-Eσ^{70} (black bars).

Several considerations suggest that neither differential σ^{70} retention nor rebinding explain the different elongation properties. First, an affinity-purification method showed that very little σ^{70} (<5%) was present in our TECs (Fig. 4). Bar-Nahum and Nudler (25) used both this method and an independent assay to show that very little σ^{70} remains with RNA polymerase purified from exponentially growing cells. Second, after stringent washing of TECs that eliminated σ^{70}, *n*-Eσ^{70} and *r*-Eσ^{70} still showed differential pausing at *his* (Fig. 2). Most importantly, even if σ^{70} were present, it is unlikely that it would act on our TECs. Our washing and transcription buffer contained heparin at a final concentration of 0.1 mg/ml. Brodolin (21), Ederth (39), and Neff (40) have shown that, at this concentration, heparin prevents σ^{70}-mediated pausing at the *lac*UV5 reiterated −10 region, dissociates σ^{70} from elongating complexes, and prevents σ^{70} rebinding. Although this same concentration of heparin does not inhibit the very strong λpR′ pause (42), it is likely to inhibit any weak interactions fortuitously found in our templates. Heparin dissociation of σ^{70} from elongating complexes is completely consistent with recent structural and biochemical studies positing that σ^{70} dissociates in several steps. Removal of σ^{70} from the RNA exit channel is thought to trigger release of σ^{70} from its interaction with the β flap, which in turn, results in destabilization of other σ^{70}–RNA polymerase contacts. Because heparin antagonizes the interaction between σ^{70} and the β flap domain (21, 39, 40), loss of this contact is probably sufficient to promote dissociation of weakly associated σ^{70} remaining in the elongation complex and to prevent its rebinding. Together, we propose that these considerations rule out differential σ^{70} action as a cause for the observed elongation differences.

Chemical damage to core RNA polymerase during handling could introduce heterogeneity that would result in elongation differences (35). Although it is difficult to conclusively disprove this possibility, we think it is unlikely to account for all differences observed because (*i*) to minimize oxidation damage, all steps were carried out in the presence of DTT or β-mercaptoethanol; and (*ii*) the same core RNA polymerase preparation reconstituted with WT or mutant σ^{70} showed elongation differences, indicating that any putative damage would have to occur after the reconstitution step.

Last, we consider whether these elongation phenotypes could result from differential presence of elongation factors. This explanation can be ruled out for the differences observed between WT and mutant σ^{70}. Mutant *n*-Eσ^{70} subjected to the same purification protocol as WT *n*-Eσ^{70} still showed decreased pausing at *ops* and increased elongation rate at a very low NTP concentration. Likewise, mutant *r*-Eσ^{70} subjected to the same purification protocol as WT *r*-Eσ^{70} elongated more rapidly at low NTPs. The situation is more complex when comparing WT *r*-Eσ^{70} and WT *n*-Eσ^{70}. Here, the BioRex chromatography step used to generate core RNA polymerase by removing σ^{70} might remove elongation proteins as well. Importantly, *n*-Eσ^{70}, the starting material for purification of core RNA polymerase, was itself very pure. Before BioRex chromatography, *n*-Eσ^{70} had been purified by using Ni–NTA affinity chromotography, high-resolution gel filtration, and anion-exchange chromatography. We also used high-salt treatment, which is known to remove identified elongation factors. The purity of the preparation is demonstrated by Coomassie blue-stained gels as well as by silver staining (Fig. 2 *B* and *D*). An overloaded 15% Coomassie blue-stained gel showed that both *r*-Eσ^{70} and *n*-Eσ^{70} have the small 9-kDa ω-subunit of RNA polymerase and failed to identify any proteins unique to *n*-Eσ^{70} (Fig. 2*C*). To deplete any potential contaminants further, we used immobilized templates, which enabled us to treat TECs with a stringent buffer containing high salt (300 mM KCl), detergent (0.1% Sarkosyl), and 0.1 mg/ml heparin. A silver-stained gel indicated that σ^{70} was removed by this procedure and that no proteins other than polymerase subunits were visible (Fig. 2*D*). Because silver staining may overrepresent proteins present in trace amounts, this result is our most critical test of the idea that *r*-Eσ^{70} and *n*-Eσ^{70} maintain their elongation differences in the absence of other proteins. Differential pause duration at *his* was maintained after washing (compare Tables 1, column 1, and 2), leading us to argue that this parameter truly depends on properties of the elongating polymerase. However, the elongation-rate differential disappeared (Table 3), which could mean that washing removed a protein contaminant from *n*-Eσ^{70} that was not present in *r*-Eσ^{70} because of the additional BioRex chromatography step. However, the fact that pause efficiencies of both *r*-Eσ^{70} and *n*-Eσ^{70} at *his* were altered by washing suggests that the washing procedure may cause conformational changes in elongating polymerase (Table 2, column 2). This could eliminate the elongation differences between *r*-Eσ^{70} and *n*-Eσ^{70}. In summary, we demonstrate that the elongation differences between mutant and WT Eσ^{70}, and the pause duration difference of WT *r*-Eσ^{70} and WT *n*-Eσ^{70} at *his* arise neither from contaminating elongation factors nor from differential σ^{70} binding or reassociation. These differences are likely to reflect different, metastable states in the enzyme that result from altered interactions during initiation.

How might the distinct elongation behavior of mutant Eσ^{70} be generated? All alterations in elongation can be explained by a single functional change: a decreased propensity of mutant Eσ^{70} to backtrack. Thus, mutant *n*-Eσ^{70} has a decreased efficiency of pausing at *ops* (Fig. 2, Table 1) and at $\lambda P_{R'}$ (8), where backtracking is central to the pause (29, 43, 44), but not at *his* (data not shown), in which backtracking is not important for pause behavior. Likewise, mutant Eσ^{70} gives an increase in elongation at very low NTP concentrations, which are believed to induce RNA polymerase to adopt unactivated states that are prone to backtracking and arrest (45), but not at high NTP concentrations (data not shown), at which backtracking is minimized.

What structural alteration in RNA polymerase could explain the decreased propensity of mutant Eσ^{70} to backtrack? $\sigma^{70(E407K)}$ weakens the interaction between σ^{70} region 2.2 and a coiled-coil at the N terminus of the β′-subunit (5, 46, 47). This interaction is essential for σ^{70} recognition of the nontemplate strand of −10 region in both standard promoters and the reiterated −10 element that defines the $\lambda P_{R'}$ pause site (20, 48–53). However, the effect of this interaction on RNA polymerase has not been determined. The coiled-coil supports the rudder, which interacts with nascent RNA at the upstream edge of the DNA/RNA hybrid (54), an interaction believed to stabilize the elongation complex (55). The altered interaction between the coiled-coil and region 2.2 of $\sigma^{70(E407K)}$ could change either the initial positioning or conformation of the rudder. When set, this parameter might be maintained during elongation. Such a change could affect backtracking by elongating RNA polymerase, thereby resulting in the spectrum of functional changes observed when the mutant Eσ^{70} enzyme enters the elongation phase. $\sigma^{70(E407K)}$ and other similar σ^{70} mutants in region 2.2 confer a defect in Q-mediated antitermination. This defect has been attributed solely to poor recognition of the reiterated −10 region at the $\lambda P_{R'}$ pause site (48). However, pausing is not sufficient for Q function in (19, 56). Might the alteration in the rudder position postulated for mutant Eσ^{70} partially underlie this defect in Q mediated antitermination? This idea is consistent with the postulated role of Q-proteins to alter elongating RNA polymerase in the vicinity of the NTP-binding site so that it maintains its active conformation (43, 57, 58).

Regardless of the particular mechanism(s) involved, our data suggest that RNA polymerase has a memory of its extensive and dynamic interactions with its initiation factors and that altered interactions can result in altered elongation behavior. These

studies are consistent with recent single-molecule studies of diverse enzymes, which reveal memory landscapes consistent with more than one reaction path (59, 60). The idea that interactions during the initiation can be propagated during elongation suggests an interesting mechanism for altering elongation. In eubacteria, using different σ factors as well as other initiation proteins may alter the spectrum of states of elongating polymerase, thereby modulating its intrinsic behavior as well as its response to various elongation factors. In eukaryotes, in which the initiation of RNA polymerase PolII serves as a platform to coordinate all subsequent processing and transport of mRNA, the ability to influence the repertoire of RNA polymerase states may be crucial to promoter-specific directions to the elongating polymerase.

We thank K. Severinov (Rutgers, The State University of New Jersey, Piscataway) for pET15rpoD; R. Landick (University of Wisconsin, Madison) for pIA171, pIA273, and pIA146; all members of the C.A.G. laboratory for technical advice and helpful discussions; and A. Hochheimer, R. Landick, and J. Roberts for critical reading of the manuscript. This work was supported by National Institutes of Health Grant GM057755.

1. Gruber, T. M. & Gross, C. A. (2003) *Annu. Rev. Microbiol.* **57,** 441–466.
2. Borukhov, S. & Nudler, E. (2003) *Curr. Opin. Microbiol.* **6,** 93–100.
3. Darst, S. A. (2001) *Curr. Opin. Struct. Biol.* **11,** 155–162.
4. Gross, C. A., Chan, C., Dombroski, A., Gruber, T., Sharp, M., Tupy, J. & Young, B. (1998) *Cold Spring Harbor Symp. Quant. Biol.* **63,** 141–155.
5. Gruber, T. M., Markov, D., Sharp, M. M., Young, B. A., Lu, C. Z., Zhong, H. J., Artsimovitch, I., Geszvain, K. M., Arthur, T. M., Burgess, R. R., *et al*. (2001) *Mol. Cell* **8,** 21–31.
6. Murakami, K. S., Masuda, S. & Darst, S. A. (2002) *Science* **296,** 1280–1284.
7. Murakami, K. S., Masuda, S., Campbell, E. A., Muzzin, O. & Darst, S. A. (2002) *Science* **296,** 1285–1290.
8. Sharp, M. M., Chan, C. L., Lu, C. Z., Marr, M. T., Nechaev, S., Merritt, E. W., Severinov, K., Roberts, J. W. & Gross, C. A. (1999) *Genes Dev.* **13,** 3015–3026.
9. Vassylyev, D. G., Sekine, S., Laptenko, O., Lee, J., Vassylyeva, M. N., Borukhov, S. & Yokoyama, S. (2002) *Nature* **417,** 712–719.
10. Dombroski, A. J., Walter, W. A., Record, M. T., Jr., Siegele, D. A. & Gross, C. A. (1992) *Cell* **70,** 501–512.
11. Callaci, S., Heyduk, E. & Heyduk, T. (1999) *Mol. Cell* **3,** 229–238.
12. Campbell, E. A., Muzzin, O., Chlenov, M., Sun, J. L., Olson, C. A., Weinman, O., Trester-Zedlitz, M. L. & Darst, S. A. (2002) *Mol. Cell* **9,** 527–539.
13. Mekler, V., Kortkhonjia, E., Mukhopadhyay, J., Knight, J., Revyakin, A., Kapanidis, A. N., Niu, W., Ebright, Y. W., Levy, R. & Ebright, R. H. (2002) *Cell* **108,** 599–614.
14. Sorenson, M. K., Ray, S. S. & Darst, S. A. (2004) *Mol. Cell* **14,** 127–138.
15. Burgess, R. R. (1971) *Annu. Rev. Biochem.* **40,** 711–740.
16. Hansen, U. M. & McClure, W. R. (1980) *J. Biol. Chem.* **255,** 9564–9570.
17. Shimamoto, N., Kamigochi, T. & Utiyama, H. (1986) *J. Biol. Chem.* **261,** 11859–11865.
18. Stackhouse, T. M., Telesnitsky, A. P. & Meares, C. F. (1989) *Biochemistry* **28,** 7781–7788.
19. Ring, B. Z., Yarnell, W. S. & Roberts, J. W. (1996) *Cell* **86,** 485–493.
20. Marr, M. T. & Roberts, J. W. (1997) *Science* **276,** 1258–1260.
21. Brodolin, K., Zenkin, N., Mustaev, A., Mamaeva, D. & Heumann, H. (2004) *Nat. Struct. Mol Biol.*, **11,** 551–557.
22. Ring, B. Z. & Roberts, J. W. (1994) *Cell* **78,** 317–324.
23. Nickels, B. E., Mukhopadhyay, J., Garrity, S. J., Ebright, R. H. & Hochschild, A. (2004) *Nat. Struct. Mol Biol.*, **11,** 544–550.
24. Roberts, J. W., Yarnell, W., Bartlett, E., Guo, J., Marr, M., Ko, D. C., Sun, H. & Roberts, C. W. (1998) *Cold Spring Harbor Symp. Quant. Biol.* **63,** 319–325.
25. Bar-Nahum, G. & Nudler, E. (2001) *Cell* **106,** 443–451.
26. Uptain, S. M., Kane, C. M. & Chamberlin, M. J. (1997) *Annu. Rev. Biochem.* **66,** 117–172.
27. Landick, R., Wang, D. & Chan, C. L. (1996) *Methods Enzymol.* **274,** 334–353.
28. Toulokhonov, I., Artsimovitch, I. & Landick, R. (2001) *Science* **292,** 730–733.
29. Artsimovitch, I. & Landick, R. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 7090–7095.
30. Artsimovitch, I. & Landick, R. (2002) *Cell* **109,** 193–203.
31. Davenport, R. J., Wuite, G. J., Landick, R. & Bustamante, C. (2000) *Science* **287,** 2497–2500.
32. Neuman, K. C., Abbondanzieri, E. A., Landick, R., Gelles, J. & Block, S. M. (2003) *Cell* **115,** 437–47.
33. Shaevitz, J. W., Abbondanzieri, E. A., Landick, R. & Block, S. M. (2003) *Nature* **426,** 684–687.
34. Erie, D. A. (2002) *Biochim. Biophys. Acta* **1577,** 224–239.
35. Tolic-Norrelykke, S. F., Engh, A. M., Landick, R. & Gelles, J. (2004) *J. Biol. Chem.* **279,** 3292–3299.
36. Burgess, R. R. & Jendrisak, J. J. (1975) *Biochemistry* **14,** 4634–4638.
37. Nudler, E., Avetissova, E., Markovtsov, V. & Goldfarb, A. (1996) *Science* **273,** 211–217.
38. Lowe, P. A., Hager, D. A. & Burgess, R. R. (1979) *Biochemistry* **18,** 1344–1352.
39. Ederth, J., Artsimovitch, I., Isaksson, L. A. & Landick, R. (2002) *J. Biol. Chem.* **277,** 37456–37463.
40. Neff, N. F. & Chamberlin, M. J. (1980) *Biochemistry* **19,** 3005–3015.
41. Levin, J. R. & Chamberlin, M. J. (1987) *J. Mol. Biol.* **196,** 61–84.
42. Roberts, C. W. & Roberts, J. W. (1996) *Cell* **86,** 495–501.
43. Marr, M. T. & Roberts, J. W. (2000) *Mol. Cell* **6,** 1275–1285.
44. Marr, M. T., Datwyler, S. A., Meares, C. F. & Roberts, J. W. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 8972–8978.
45. Foster, J. E., Holmes, S. F. & Erie, D. A. (2001) *Cell* **106,** 243–252.
46. Arthur, T. M., Anthony, L. C. & Burgess, R. R. (2000) *J. Biol. Chem.* **275,** 23113–23119.
47. Arthur, T. M. & Burgess, R. R. (1998) *J. Biol. Chem.* **273,** 31381–31387.
48. Ko, D. C., Marr, M. T., Guo, J. & Roberts, J. W. (1998) *Genes Dev.* **12,** 3276–3285.
49. Young, B. A., Anthony, L. C., Gruber, T. M., Arthur, T. M., Heyduk, E., Lu, C. Z., Sharp, M. M., Heyduk, T., Burgess, R. R. & Gross, C. A. (2001) *Cell* **105,** 935–944.
50. Anthony, L. C., Dombkowski, A. A. & Burgess, R. R. (2002) *J. Bacteriol.* **184,** 2634–2641.
51. Kulbachinskiy, A., Mustaev, A., Goldfarb, A. & Nikiforov, V. (1999) *FEBS Lett.* **454,** 71–74.
52. Malhotra, A., Severinova, E. & Darst, S. A. (1996) *Cell* **87,** 127–136.
53. Severinova, E., Severinov, K., Fenyo, D., Marr, M., Brody, E. N., Roberts, J. W., Chait, B. T. & Darst, S. A. (1996) *J. Mol. Biol.* **263,** 637–647.
54. Zhang, G., Campbell, E. A., Minakhin, L., Richter, C., Severinov, K. & Darst, S. A. (1999) *Cell* **98,** 811–824.
55. Kuznedelov, K., Korzheva, N., Mustaev, A. & Severinov, K. (2002) *EMBO J.* **21,** 1369–1378.
56. Yarnell, W. S. & Roberts, J. W. (1992) *Cell* **69,** 1181–1189.
57. Yarnell, W. S. & Roberts, J. W. (1999) *Science* **284,** 611–615.
58. Santangelo, T. J., Mooney, R. A., Landick, R. & Roberts, J. W. (2003) *Genes Dev.* **17,** 1281–1292.
59. Edman, L. & Rigler, R. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 8266–8271.
60. Lerch, H. P., Mikhailov, A. S. & Hess, B. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 15410–15415.