Conserved Region 3 of Escherichia coli σ70 Is Implicated in the Process of Abortive Transcription*

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Multiple-round in vitro transcription assays were performed using purified Escherichia coli RNA polymerase reconstituted with either wild-type or mutant σ70 proteins. These mutants, σ70(P504L) and σ70(SS06F), bear single amino acid changes in conserved protein region 3. Behavior of the mutant enzymes on three test templates, bearing either the T7 A1, T5 N25, or T5 N25antiDSR, promoter, were characterized. Transcription of all three promoter templates produced a pattern of specific abortive RNA species, which was qualitatively different for the mutants compared to the wild-type σ70 enzyme. Short abortive RNAs were produced at similar levels for mutant and wild-type enzymes. The production of longer abortive species was either reduced or increased by the mutant enzymes in a systematic manner that appears promoter-specific, and could be RNA length- or promoter distance-dependent. The process of abortive RNA transcription is thought to be tightly associated with that of promoter clearance. However, promoter clearance from these templates appears only slightly affected by the mutant enzymes. These mutants implicated region 3 of σ70 in the process of abortive transcription and suggest that the sequence of enzymatic events leading to the production of abortive or full-length RNA may be separable.

The initiation of transcription from a promoter by prokaryotic RNA polymerase has been characterized mainly at the level of promoter binding and open complex formation (1), each appears to be a multistep process (see Ref. 2 and references therein). Much less is known about subsequent initiation steps, termed the process of promoter clearance (1, 3). Promoter clearance like other steps in initiation involves the formation of a multistate series of RNA polymerase-DNA complexes generally termed initial transcribing complexes (ITC) (4). In the ITC, RNA synthesis begins, but the polymerase forms only short RNAs (generally 2–9 nucleotides) whose syntheses are aborted. These reiteratively synthesized abortive RNAs are released from the ITC without disengagement of RNA polymerase from the promoter (5–9). The production of abortive RNAs ceases upon transition of the ITC into a stable ternary elongation complex, which appears to occur concomitantly with the release of σ70 and promoter clearance (4, 10, 11).

The probability of an RNA polymerase molecule forming an abortive transcript while traversing the initiating template appears to have a great dependence on the specific initially transcribed sequence. This was demonstrated by substitution of the sequences from +3 to +20 of the T5 phage promoter N25, producing the semi-synthetic promoter T5 N25antiDSR (14). Both promoters, N25 and N25antiDSR, bind polymerase and form open complexes with equal efficiency; however, the N25antiDSR promoter is drastically reduced in activity (14). Recently, a detailed investigation of abortive transcription properties of three promoters, T7 A1, T5 N25, and T5 N25antiDSR, was completed. It was found that the reduction in promoter activity of N25antiDSR, resulting from alterations in the initial transcribed sequence, was attributed to a limitation at promoter clearance.

In achieving promoter clearance, it is unclear whether the release of σ70 is a passive consequence or an active prerequisite to a successful transition from the ITC to the elongation complex. Regardless of the nature of σ70 participation, there remains a fundamental necessity of RNA polymerase to relinquish high affinity promoter binding to establish a stable elongating complex (1). Consistent with this is the fact that promoter recognition occurs by direct binding of σ70 domains of RNA polymerase holoenzyme to DNA (12, 13) and is, therefore, concluded by the release of σ70 from the ITC. Progress toward understanding this transition has been slow due to the extreme instability of ITCs, which has hampered conventional biochemical analysis. Thus, analysis of mutant RNA polymerases altered in their abortive transcription properties might offer new insight into this poorly understood process.

We have recently reported the isolation of two Escherichia coli σ70 mutants bearing changes in conserved region 3 (15). This study revealed an apparent change in interaction of mutant σ70 proteins with core polymerase in vivo. In addition, a cross-linkable ATP analog bound at the initial nucleotide binding site of RNA polymerase in an ITC produces a strong cross-link of the initiating nucleotide to region 3 of σ70, placing it close to the catalytic center of the initiating polymerase (16). It is plausible, therefore, that mutants of σ70 bearing alterations in region 3 might be affected in transcription initiation and early RNA synthesis.

In this study we investigated whether mutational changes in region 3 of σ70 altered patterns of RNA synthesis in vitro. For this purpose we also utilized the three extensively characterized promoters T7 A1, T5 N25, and T5 N25antiDSR. By comparing wild-type and mutant reconstituted RNA polymerases, we find that mutant enzymes produced a strikingly different pattern of abortive RNAs from all three promoter templates.

MATERIALS AND METHODS

Overexpression System—The σ70 overproduction plasmid pLHN12 (a kind gift of Lam Nguyen and Richard Burgess) is derived from the

1. The abbreviations used are: ITC, initial transcribing complex; PCR, polymerase chain reaction; bp, base pair(s); mAb, monoclonal antibody.
2. L. M. Hsu and M. J. Chamberlin, manuscript in preparation.
BL2(DE3)pLysS/pET system (Ref. 17; Novagen Inc.); it contains the rpoD structural gene cloned into the pET11T vector (18) under control of the T7 promoter. Mutated rpoD genes were amplified from chromosomal DNA by the polymerase chain reaction (PCR) as described previously (15). A naturally-occurring 281-base-pair (bp) BamHI-XhoI fragment spanning the region 3 coding sequence was excised from the PCR products and subcloned into the unique BamHI-XhoI sites of pHI12. The presence of mutant alleles and the absence of PCR-generated mutations were confirmed by DNA sequencing.

**Protein Preparations and Holoenzyme Reconstitution**—The α70 proteins were purified using a modification of the method of Gribkov and Burgess (19). Core RNA polymerase free of α70 was purchased from Epicentre Technologies (Madison, WI). Immunoprecipitations of RNA polymerase holoenzyme were carried out using the IgGα70 anti-β monoclonal antibody (mAb) NT63, which reacts with both core and holoenzyme RNA polymerase (20). A fixed amount of purified α70, 100 ng, was mixed in transcription buffer with 0, 50, 100, 200, 300, 400, or 500 ng of core RNA polymerase in a total volume of 15 μl for 15 min at room temperature. To each, 5 μl of mAb NT63 ascites fluid was added and immunoprecipitations were performed as described (21) using formalin-fixed protein A-positive Staphylococcus aureus cells and NET buffer (21). Samples were electrophoresed through a 7% SDS-polyacrylamide gel. The α70 protein was detected and quantitated by Western analysis as described previously (15), using α70 mAb 2F8 and 3D3 (22).

**Preparation of DNA Templates**—PCR templates, approximately 200 bp long, were made by PCR amplification of the plasmids pAR1707 (T7 A1 promoter), pDS3/PN25 (T5 N25 promoter), and pDS3/PN25<sup>s</sup> <i>antD</i> <i>D</i><sup>RS</sup> (N25anti promoter) using oligonucleotide primers as described by Hsu and Chamberlin. <sup>2</sup>Templates produced run-off transcripts of 50 nucleotides for T7 A1 and T5 N25 and 65 nucleotides for N25anti.

**Transcription Reactions**—Reactions were 20 μl, containing 20 ng template DNA and 100 ng RNA polymerase in 50 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 10 μM 2-mercaptoethanol, 10 μg/ml acetylated bovine serum albumin. Optimal KCl concentration <sup>2</sup> used was 190 mM at 20°C, and precipitates were collected by centrifugation and resuspended in 20 μl of formamide loading buffer (66% formamide, 100 mM Na<sub>2</sub>EDTA, 0.05% bromphenol blue, 1× Tris borate-EDTA buffer). Samples were analyzed on a standard 7 μm urea, 20% polyacrylamide gel in 1× Tris borate-EDTA (TBE) buffer. For electrophoresis 0.5× TBE buffer was placed in the top reservoir and 1.2× TBE in the bottom reservoir to stack low molecular weight RNAs. Bromphenol blue tracking dye was run 3/4 the length of the gel. Radioactivity was quantified using a Molecular Dynamics PhosphorImager and ImageQuant™ version 3.3 software.

**RESULTS**

Previous analysis of strains harboring the mutant α70 proteins, α70(P504L) and α70(S506F), indicated that compared to wild-type α70, a large fraction of the mutant α70 in the cell was in the free form, i.e. not in association with holoenzyme (15). This result was obtained by fractionating cell lysates on glyceral gradients and measuring the amount of α70 either sedimenting independently or with core RNA polymerase. A simple interpretation of this result is that the α70 mutant proteins have a lower affinity for core RNA polymerase or form an unstable holoenzyme. Given this uncertainty, we checked whether the purified mutant proteins could form stable holoenzyme. To test this, α70 was mixed with core RNA polymerase, and competence to form holoenzyme was assayed by coimmunoprecipitation of α70 with core-specific antibody. The ability of the purified wild-type and mutant α70 proteins to form holoenzyme was found to be essentially unaltered (Fig. 1). The mass of core enzyme is approximately 5 times greater than α70; thus, nearly all of the α70 present (100 ng) in the reconstitution reaction appears to be titrated by a molar equivalent of core RNA polymerase (500 ng) (Fig. 1B). Under subsequent in vitro transcription conditions, the α70 mutant and wild-type proteins are judged to be equally competent for stable holoenzyme formation. The exact cause of the previous in vivo observations (15), however, remains unknown. The immunoprecipitation assay used here was not sensitive enough to detect the extremely low levels of protein required to accurately determine the high affinity binding of α70 to core polymerase (data not shown).

![Fig. 1. Efficiency of holoenzyme reconstitution.](https://example.com/figure1.png)

**RNA Polymerase (ng of protein × 10<sup>-1</sup>)**

A constant amount of purified α70 (100 ng) was mixed with increasing amounts of purified core RNA polymerase, as indicated. The amount of α70 associated with holoenzyme was determined by coimmunoprecipitation with the anti-β subunit antibody, NT63 (see "Materials and Methods"). A Western analysis of α70 communoprecipitated with core RNA polymerase and detected by chemiluminescence. Remaining lanes contained an equal volume of buffer or a known amount of commercially obtained α70 saturated RNA polymerase, as internal controls. B), amounts of α70 communoprecipitated with different amounts of core RNA polymerase. Autoradiograms of gels from A were quantitated densitometrically and quantitated relative to the internal holoenzyme control to correct for recovery and variability in different assays.

**Possible effects of these mutants on the transcription cycle were investigated by multiple-round in vitro transcriptions.** In vitro transcription was carried out using PCR templates bearing either the T7 A1, T5 N25, or T5 N25<sup>s</sup> <i>antD</i> <i>D</i><sup>RS</sup> (N25anti) promoter as described by Hsu and Chamberlin. <sup>2</sup>These particular promoters were chosen since they represent promoters with variable abortive tendencies and we reasoned that changes in region 3 of α70 might affect the production of abortive RNAs (see Introduction). RNA polymerase holoenzyme was reconstituted (see "Materials and Methods"), and a 5-fold excess of holoenzyme to template was used in each assay. All four nucleotide substrates were present at concentrations of 20, 50, or 100 μM. In all reactions RNA was labeled with [γ<sup>32</sup>P]ATP, thus incorporating radioactivity only at the 5'-end of RNAs. This prevents obscuring of the pattern of abortive RNAs by the production of short RNAs resulting from Gre factor-mediated cleavage (24). In addition, incorporated radioactivity is directly proportional to the number of RNA molecules of each species regardless of size. RNA products were analyzed as described (see "Materials and Methods").

The pattern of abortive transcripts obtained for wild-type...
RNA polymerase on the three promoter templates is indistinguishable from that observed by Hsu and Chamberlin.

In Fig. 2, RNAs synthesized by transcription from the T7 A1 promoter are shown. It is immediately apparent that the production of abortive RNAs is lower for mutant s70 RNA polymerases than for the wild-type enzyme. Also lower is the amount of full-length RNA produced by the mutant RNA polymerases (Fig. 2, A and B). Fewer abortive RNAs may simply be produced because of lower enzyme activity. Closer inspection, however, reveals that the absolute amount of the early abortive tetrameric RNA (4-mer) produced is similar for all enzymes (Fig. 2B). Apparently, formation of the 4-mer RNA is not as affected as formation of longer abortive RNAs. This observation cannot be extended to dimer and trimer RNAs, since these were not sufficiently well resolved from unincorporated [γ-32P]ATP to allow reliable quantitation. In the case of the pentameric (5-mer) and heptameric (7-mer) length RNAs, which are prominent species for the wild-type enzyme, the mutants produce between 2- and 3-fold less (Fig. 2, A and B). Meanwhile, the full-length run-off transcript appears 30% less abundant for s70(P504L) and 65% less for s70(S506F) RNA polymerases. Thus, the reduction in abortive RNAs cannot be accounted for by a simple reduction in total enzyme activity in the case of s70 mutants compared to the wild type.

For the T5 N25 promoter, a different array of abortive transcripts is obtained with the wild-type enzyme and effects observed for the mutant polymerases are different from those seen on T7 A1 (Fig. 3A). Like T7 A1, production of short abortive RNAs (4- and 5-mers) are similar for the mutants, while longer products (6-8-mers) are reduced at least 2-3-fold (Fig. 3B). Unlike T7 A1, the mutant polymerases produce the late abortive 9-mer RNA to about 2-fold higher abundance. Also, the level of full-length run-off products (50-mers) is es-
properties of abortive RNAs. Both unprecedented length (10–15-mers), which exhibit all of the promoter is unusual in producing "oligomeric" transcripts of essentially unchanged for wild-type and mutant sequence. C run-off transcript is 65 nucleotide residues long. downstream sequences was transcribed as in Fig. 2. The full-length promoter.

(Fig. 3 N25 appears to be specific for sites of uridine incorporation N25 anti, which differs from the wild-type T5 N25 promoter by the presence of base substitutions from positions 1 to 20 (14). Here again, only minor effects are observed for short abortive products (4- and 5-mers), while longer products (6–9-mers) are drastically reduced (Fig. 4, A and B). The N25anti promoter is unusual in producing "oligomeric" transcripts of unprecedented length (10–15-mers), which exhibit all of the properties of abortive RNAs. Both α70 mutants produce more abortive ITC has been reported for wild-type RNA polymerase addition at an increased rate. An example of a stable non-abortive products, 5–8-mers for T7 A1, 6–8-mers for N25 promoter, and 6–9-mers for N25anti promoter, are drastically reduced. Furthermore, longer abortive products are more prevalent for N25 and N25anti promoters. Clearly the mutant enzymes respond to different properties associated with the production of these different length abortive RNAs. These different properties most likely result from distinct physical states characteristic of individual ITC, which may be either RNA length- or promoter distance-dependent. A reduction in abortive RNA production could in theory lead to an increased efficiency of promoter clearance. This, however, was not the case for these promoters since an increase in abundance of full-length RNA was not observed. Alternatively, these results could suggest that the mutant polymerases establish more stable ITCs at positions of midsize length RNAs, which do not readily abort, but which do not advance to the next nucleotide addition at an increased rate. An example of a stable non-abortive ITC has been reported for wild-type RNA polymerase at position +6 on the initial transcribed region of a T7 ES hybrid template (4).

The observed changes in production of abortive transcripts for all three promoters are complicated by apparent differences in specific enzymatic activity of the wild-type and mutant polymerases for production of full-length RNAs. It could be argued that the observed low abundance of abortive RNAs for mutant enzymes results from a simple reduction in overall enzymatic activity. In an attempt to address this issue, we estimated relative specific activity by summing the phosphorimaging counts for all of the [γ-32P]ATP-labeled RNA species produced by each polymerase for the three templates. This determination differs from the classic measurement of specific activity, since it quantitates the amount of RNA 5'-ends. Thus, it is a measure for the number of initiations attempted by each enzyme, i.e. formation of the first phosphodiester bond, during the course of the assay. In the classic specific activity determination short abortive RNAs are excluded from quantitation (25). Results in Table I indicated that the total number of RNA molecules produced was reduced for the α70(P504L) mutant enzyme only on T7 A1 but for the α70(S506F) on all templates. It must be noted that this evaluation is only an estimate of the

**DISCUSSION**

Cumulatively, the results presented here are consistent with a difference in the potential of forming abortive transcripts by RNA polymerases bearing the α70 mutants P504L and S506F. The behavior of these mutant enzymes also differs intrinsically from that of the wild-type in a manner that is independent of specific nucleotide affinities. Most interestingly, the abortive properties of these mutants appear to be influenced by the length of the nascent RNA. Short abortive products, 4-mer on T7 A1 and 4- and 5-mers on N25 and N25anti promoters, are produced with equal or slightly reduced abundance. Midsize abortive products, 5–8-mers for T7 A1, 6–8-mers for N25 promoter, and 6–9-mers for N25anti promoter, are drastically reduced. Furthermore, longer abortive products are more prevalent for N25 and N25anti promoters. Clearly the mutant enzymes respond to different properties associated with the production of these different length abortive RNAs. These different properties most likely result from distinct physical states characteristic of individual ITC, which may be either RNA length- or promoter distance-dependent. A reduction in abortive RNA production could in theory lead to an increased efficiency of promoter clearance. This, however, was not the case for these promoters since an increase in abundance of full-length RNA was not observed. Alternatively, these results could suggest that the mutant polymerases establish more stable ITCs at positions of midsize length RNAs, which do not readily abort, but which do not advance to the next nucleotide addition at an increased rate. An example of a stable non-abortive ITC has been reported for wild-type RNA polymerase at position +6 on the initial transcribed region of a T7 ES hybrid template (4).

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**TABLE I**

| Type of α70 | Relative activity* |
|------------|-------------------|
| T7 A1      | 1.00              |
| T5 N25     | 1.00              |
| N25anti    | 1.00              |

*Transcription conditions were as described under "Materials and Methods" with 50 μM of each NTP. Total radioactive counts incorporated into RNA chains longer than 3 nucleotide residues were obtained by summing the individual counts determined by phosphorimaging of abortive and full-length RNAs.

The total phosphorimage counts for the wild-type reconstituted RNA polymerase were 6.80 × 10^7 for the T7 A1 template, 1.43 × 10^7 for the T5 N25, and 1.26 × 10^7 for N25anti.

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**Fig. 4. Production of abortive transcripts from the N25anti promoter.** A 225-bp PCR fragment bearing the N25anti promoter and downstream sequences was transcribed as in Fig. 2. The full-length run-off transcript is 65 nucleotide residues long. A and B, as in Fig. 3. C, as in Fig. 3 except representing the N25anti initial transcribed sequence.
total enzyme activity since it fails to include dimer and trimer RNAs, which could not be accurately quantitated. Given this caveat, however, differences in the activities of mutant polymerases appear insufficient to account for the much larger reduction in the level of midsize length abortive RNAs. It is dear that similar amounts of early abortive RNA species (e.g., 4- or 5-mers) are produced by all enzymes, which is consistent with the polymerases having approximately equal occupancy at these positions. Moreover, the observation that mutant enzymes produce an increased abundance of “long” abortive products (e.g., 9- and 10-mers) compared to wild-type enzyme is difficult to explain by simply assuming a lower overall RNA initiation rate.

It is also unlikely that the mutant RNA polymerases have acquired different affinities for specific nucleotide addition within the context of a specific initial transcribing sequence. Specific initial transcribed sequence positions displaying low affinity for nucleotide addition, high \(K_m\) values, have been determined for the three promoters, T7 A1, T5 N25, and N25anti.\(^2\) These sites do not correlate with sites of altered abortive behavior of the mutant enzymes. Thus, straightforward sequence-specific determinants that can account for the behavior of the \(\alpha^{70}\) mutants cannot be attributed simply to changes in specific \(K_m\) values.

In conclusion, it appears that mutational changes in region 3 of \(\alpha^{70}\) can alter the production of abortive transcripts and, thus, properties of the ITC of RNA polymerase (Figs. 2–4). To our knowledge, this is the first example of a mutational change in \(\alpha^{70}\) that affects abortive transcription and implicates region 3 in this process. This finding is consistent with the cross-linking studies of Severinov et al. (16), which physically places region 3 of \(\alpha^{70}\) near the active center of an initiating RNA polymerase. We find it intriguing that the only known homology, though quite divergent, between region 3 of \(\alpha^{70}\) and an eukaryotic basal transcription factor is found in the \(\beta\)-subunit of the general transcription factor TFII E (26). It has been proposed that TFII E recruits to the preinitiation complex, TFII H, which then mediates efficient promoter clearance of eukaryotic RNA polymerase II (27). It is not clear, however, that this is the sole function of TFII E and it is conjectured that TFII E may provide other essential functions during promoter clearance (27).

The process of abortive initiation has been thought to be tightly associated with the process of promoter clearance (1). Thus, the finding that abortive transcription can be significantly changed without influencing the production of full-length transcripts is perhaps surprising. For example, in the case of the N25 and N25anti promoters, the \(\alpha^{70}(P504L)\) mutant enzyme changes dramatically the pattern of abortive RNA products without affecting the abundance of full-length products (Figs. 3 and 4). These results could indicate that for the mutant enzymes the processes of abortive transcription and promoter clearance are separable, that is, not directly coupled nor sequential.

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