

Effects of Amino Acid Substitutions at Conserved and Acidic Residues within Region 1.1 of *Escherichia coli* σ^{70}

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Amino acid substitutions in *Escherichia coli* σ^{70} were generated and characterized in an analysis of the role of region 1.1 in transcription initiation. Several acidic and conserved residues are tolerant of substitution. However, replacement of aspartic acid 61 with alanine results in inactivity caused by structural and functional thermolability.

Core RNA polymerase ($\alpha_2\beta\beta'$) requires the variable specificity subunit, sigma (σ), to direct promoter-dependent transcription (1, 3, 4, 12, 18, 22, 23, 26). Following promoter binding, holoenzyme ($\alpha_2\beta\beta'\sigma$) progresses through several intermediate complexes, en route to a stable initiated open complex (2, 14). σ factor has been implicated in stages of initiation beyond promoter recognition (8, 9, 13, 15, 17). Recently, we showed that the conserved amino terminal domain (region 1) of *Escherichia coli* σ^{70} is important for the process of strand melting and initiated complex formation at the λp_R promoter (24).

Region 1 is unique to the primary σ factors, yet little is known of its function. Deletion of region 1.1 (amino acids 1 to 100) from σ^{70} has two major consequences for holoenzyme. The first is inefficient progression from the closed to the strand-separated open complex. This can be overcome by increasing the time allowed for formation of holoenzyme-promoter complexes and is lessened by addition of region 1.1 in trans. The second and more deleterious effect is impaired transition from the strand-separated open complex to a stable initiated complex (RP_{init}). According to this analysis, amino acids between positions 50 and 75 of σ^{70} are critical for proper initiation in vitro (24).

A comparison of region 1.1 among several primary σ factors revealed conserved residues at positions 52 (glycine [G]), 53 (isoleucine [I]), 55 (valine [V]), and 61 (aspartic acid [D]), as well as a high degree of acidity (40%) within the segment from amino acids 50 to 75 (24). Here, we test whether alterations at these conserved positions or in the overall acidity of the region influence initiation by holoenzyme ($E\sigma$).

Site-directed mutagenesis (10) and the Expand high fidelity PCR system (Boehringer Mannheim) were used to create substitutions at positions 52, 53, 55, 61, 57, 58, 63, 64, and 69 (Table 1). *rpoD* was mutagenized in M13 phage (10) and amplified with oligonucleotides that incorporated restriction sites at the 5' and 3' ends of the fragment. The restricted fragments were ligated into pQE30-T (24). PCR mutagenesis was used to

amplify a fragment corresponding to the 3' end of the *rpoD* gene with a 5' mutagenic oligonucleotide and a 3' oligonucleotide that incorporated a restriction site. A concurrent round of amplification included a 5' oligonucleotide complementary to the 5' end of *rpoD* and a 3' oligonucleotide with complementarity to an internal segment of *rpoD*, downstream from the genetic alteration(s). The 5' and 3' PCR fragments were mixed, and the full-length mutagenized *rpoD* gene was amplified, gel isolated, digested, ligated into pQE30-T, transformed into *E. coli* XL1 Blue (Stratagene), and sequenced to confirm the changes. The plasmids were transformed into *E. coli* 19284 (*rpoD800*, W3110 *srl::Tn10 recA lacI^q*) to test for function in vivo (24). Transformation mixtures were split and spread onto Luria-Bertani plates containing ampicillin (100 mg/ml), kanamycin (30 mg/ml), and 2% glucose and then incubated at 32 and 44°C, to evaluate complementation of the *rpoD800* temperature-sensitive growth defect at 44°C. Plasmids were likewise transformed into strain CAG20176 to test growth in the absence of chromosomal *rpoD* expression at 32°C (11, 24).

Mutants were generated that replaced either acidic, conserved, or, at position 61, both conserved and acidic amino acids with alanine (A). Replacement of glutamic acid (E) at position 69 (E69) with A had no effect on σ^{70} function in vivo (Table 1). Double mutations replacing E at positions 57 and 58

TABLE 1. Region 1.1 amino acid substitution mutants^a

| Mutation | Complementation |
|--------------------------|-----------------|
| Conserved | |
| G52A..... | + |
| V55A..... | + |
| V55I..... | + |
| Acidic | |
| D61A ^b | - |
| D61E..... | + |
| D61S..... | + |
| E57,58A..... | + |
| D63,64A..... | + |
| E57,58A, D61,63-64A..... | - |

^a Results of in vivo analysis indicate complementation (+) and lack of complementation (-). The same results were obtained for two different strain backgrounds, 19284 and CAG20176.

^b Position 61 is both highly conserved and acidic.

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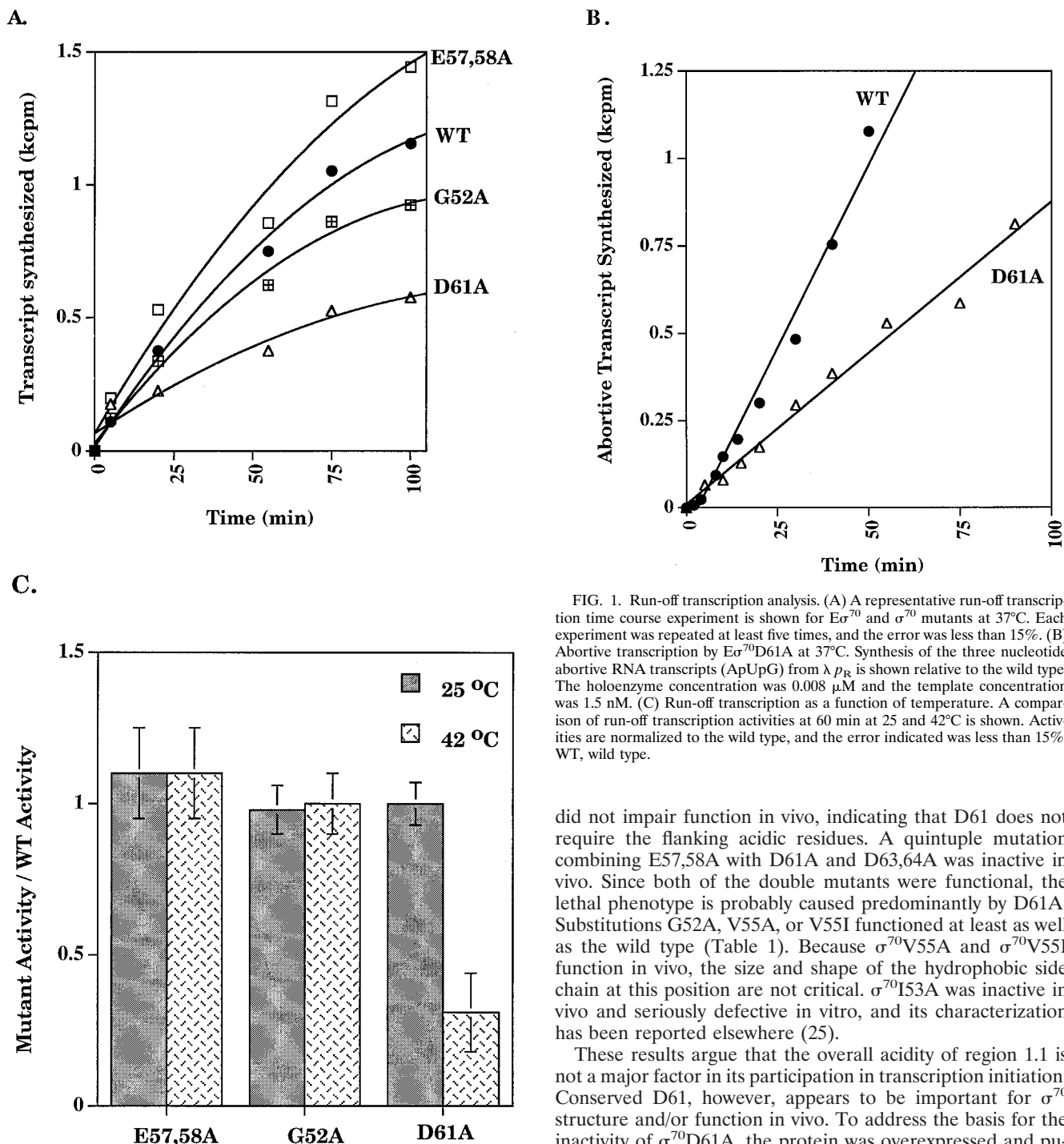


FIG. 1. Run-off transcription analysis. (A) A representative run-off transcription time course experiment is shown for $E\sigma^{70}$ and σ^{70} mutants at 37°C. Each experiment was repeated at least five times, and the error was less than 15%. (B) Abortive transcription by $E\sigma^{70}D61A$ at 37°C. Synthesis of the three nucleotide abortive RNA transcripts (ApUpG) from λp_R is shown relative to the wild type. The holoenzyme concentration was 0.008 μM and the template concentration was 1.5 nM. (C) Run-off transcription as a function of temperature. A comparison of run-off transcription activities at 60 min at 25 and 42°C is shown. Activities are normalized to the wild type, and the error indicated was less than 15%. WT, wild type.

or D at positions 63 and 64 reduced the overall acidity of region 1.1, but neither affected σ^{70} function in vivo (Table 1). Replacement of conserved D61 with A, however, rendered σ^{70} nonfunctional.

Additional substitutions at position 61 addressed the contribution of the amino acid side chain. Both E and serine (S) could functionally substitute for D, indicating that polarity rather than side chain charge at this position is more important for function (Table 1). D61 is found in a cluster of acidic residues; however, simultaneous substitution of D63 and D64

did not impair function in vivo, indicating that D61 does not require the flanking acidic residues. A quintuple mutation combining E57,58A with D61A and D63,64A was inactive in vivo. Since both of the double mutants were functional, the lethal phenotype is probably caused predominantly by D61A. Substitutions G52A, V55A, or V55I functioned at least as well as the wild type (Table 1). Because $\sigma^{70}V55A$ and $\sigma^{70}V55I$ function in vivo, the size and shape of the hydrophobic side chain at this position are not critical. $\sigma^{70}I53A$ was inactive in vivo and seriously defective in vitro, and its characterization has been reported elsewhere (25).

These results argue that the overall acidity of region 1.1 is not a major factor in its participation in transcription initiation. Conserved D61, however, appears to be important for σ^{70} structure and/or function in vivo. To address the basis for the inactivity of $\sigma^{70}D61A$, the protein was overexpressed and purified (24) for characterization in vitro.

Run-off transcription analysis was performed to assess the overall effect of the D61A substitution on RNA synthesis (24). A time course at 37°C indicated that $E\sigma^{70}D61A$ was impaired in transcription, while $E\sigma^{70}G52A$ and $E\sigma^{70}E57,58A$ exhibited transcription rates similar to $E\sigma^{70}$ (Fig. 1A). $E\sigma^{70}D61A$ was also defective for abortive transcription (24) at 37°C (Fig. 1B). One explanation for the inactivity of $\sigma^{70}D61A$ is thermolability of the protein. Thus, we examined the effect of temperature on run-off transcription. At 25°C, $E\sigma^{70}D61A$ activity was indistinguishable from $E\sigma^{70}$. At higher temperatures, a transcriptional defect became apparent (Fig. 1C), with loss of activity as the

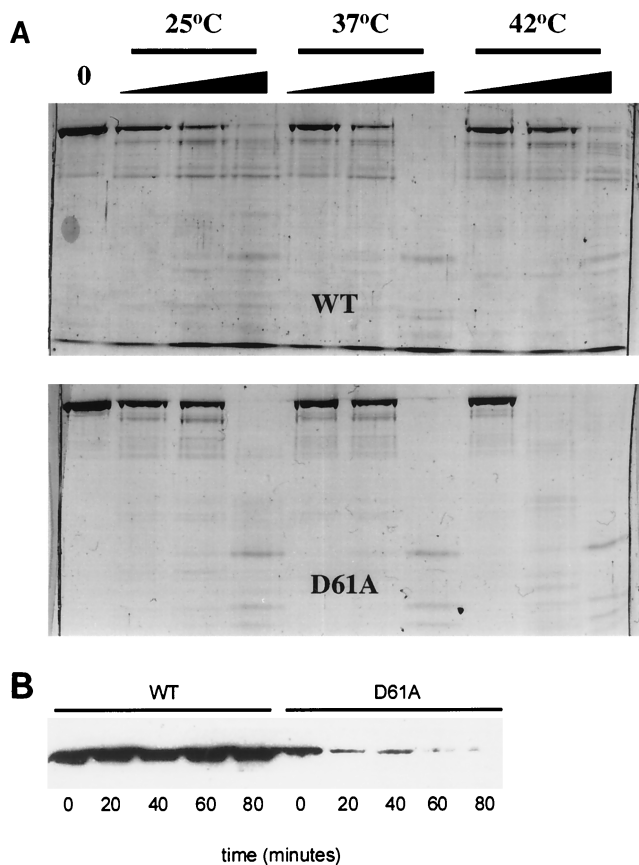


FIG. 2. Stability of σ^{70} D61A in vitro and in vivo. (A) Limited trypsinolysis. σ^{70} D61A (2 μ g) was subjected to trypsin digestion at three temperatures, as indicated, to assess possible structural defects. Wedges indicate increasing trypsin concentration (0.0125, 0.0625, and 0.025 μ g). Digestion of wild-type (WT) σ^{70} is shown for reference in the top panel. Fragments were resolved on a sodium dodecyl sulfate–8% polyacrylamide gel and visualized with Coomassie brilliant blue staining. (B) Immunoblot analysis of culture lysates of strain 19284 (wild-type [WT] σ^{70} and σ^{70} D61A). Exponentially growing cells at 37°C were upshifted to 44°C. Lysates were prepared from cells harvested at the indicated time points following temperature upshift, and proteins were resolved on a sodium dodecyl sulfate–8% polyacrylamide gel. Following Western transfer, histidine-tagged σ^{70} proteins were detected by using a six-His-tagged monoclonal antibody (Clontech).

temperature increased. The functional mutants $E\sigma^{70}$ G52A and $E\sigma^{70}$ E57,58A were not affected by increasing temperature, as compared to $E\sigma^{70}$.

Because σ^{70} D61A was functionally thermolabile in vitro, the possibility that it was structurally thermolabile was assessed by comparing susceptibility to trypsin digestion at 25, 37, and 42°C. Consistent with its transcriptional activity, σ^{70} D61A exhibited increased sensitivity to trypsin digestion at 42°C (Fig. 2A). The failure of σ^{70} D61A to complement *rpoD* mutant strains in vivo may therefore be caused by instability of the protein. To test this idea, we used immunoblotting to compare the levels of the wild type and σ^{70} D61A after a shift from 37 to 44°C (Fig. 2B). At the time of the upshift, there was significantly more wild-type σ^{70} present, and it remained stable for longer than 80 min (Fig. 2B). Conversely, σ^{70} D61A was much less stable, becoming nearly undetectable by 80 min. The D61A substitution appears to cause a structural disruption in σ^{70} that results in proteolytic instability both in vivo and in vitro and functional instability during transcription initiation. Other mutations in region 1.1 have also been reported to result in structural instability (5).

A more thorough evaluation of the initiation properties of the mutants was conducted to determine if a particular step in the process was affected by the substitutions. The first step in initiation is promoter recognition and binding by RNA polymerase. Nitrocellulose filter retention has been used to evaluate DNA binding at λp_R , and the complexes retained are open complexes (6, 7, 16, 19, 20). Holoenzyme (1 nM) was incubated with a 32 P-5'-end-labeled DNA fragment containing λp_R (0.1 nM). The binding of $E\sigma^{70}$ D61A to λp_R was indistinguishable from $E\sigma^{70}$ as well as the functional mutants $E\sigma^{70}$ G52A and $E\sigma^{70}$ E57,58A (data not shown).

Addition of nucleoside triphosphates (NTPs) to open complexes allows progression to RP_{init} , which are stable to an 0.8 M NaCl wash (16, 21). The ability of NTPs to stabilize $E\sigma^{70}$ D61A- λp_R open complexes was assessed. $E\sigma^{70}$ Δ 100, previously shown to be defective in RP_{init} formation (24), was compared for reference. Under low-stringency wash conditions (0.1 M NaCl), at 2 and 30 min after adding holoenzyme to DNA, each $E\sigma^{70}$ derivative bound to λp_R as well as the wild type (Fig. 3). Under high-stringency wash conditions (0.8 M NaCl), the $E\sigma^{70}$ E57,58A complexes were retained as well as the wild type. Interestingly, the $E\sigma^{70}$ G52A complexes were less

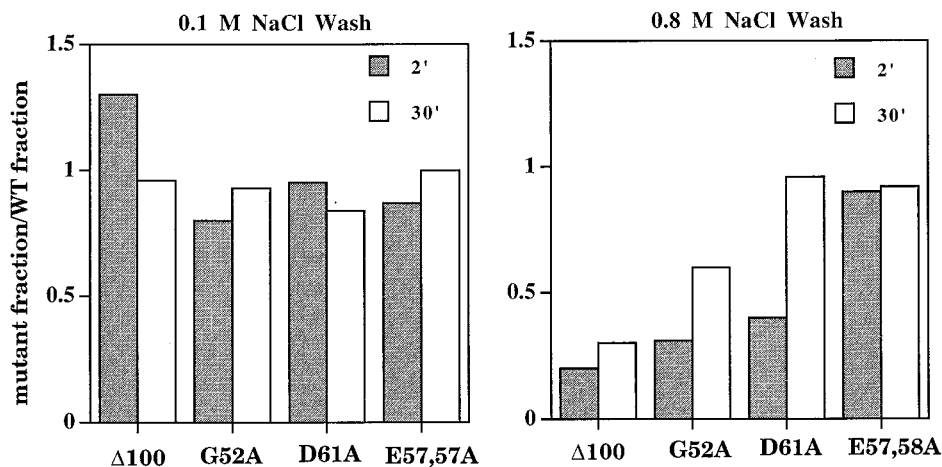


FIG. 3. Stability of initiated complexes. Nitrocellulose filter retention under low (0.1 M NaCl)- and high (0.8 M NaCl)-stringency wash conditions is shown. The fraction of mutant complexes retained is normalized to the fraction of wild-type (WT) complexes retained after allowing formation for 2 and 30 min following mixing of RNA polymerase with DNA. Amino acid substitutions are indicated.

stable than $E\sigma^{70}$, but this mutant was still able to complement in vivo. $E\sigma^{70}D61A-\lambda p_R$ complexes were unstable at 2 min, but by 30 min they were indistinguishable from $E\sigma^{70}$, indicating a slower rate of RP_{init} formation.

Impaired run-off transcription, combined with the slow rate of RP_{init} formation by $E\sigma^{70}D61A$, could be caused by difficulty in open complex formation. $KMnO_4$ footprinting analysis was performed to assess the ability of $E\sigma^{70}D61A$ to form open complexes (24). Strand melting for $E\sigma^{70}D61A$ occurred as efficiently as for $E\sigma^{70}$ in the absence and presence of NTPs, even at the times when RP_{init} formation was impaired (data not shown). Open complex formation by $E\sigma^{70}G52A$ was also examined, since the RP_{init} were slightly less stable than they were for $E\sigma^{70}$, but no differences relative to $E\sigma^{70}$ were detected.

In summary, the D61A mutation renders σ^{70} nonfunctional in vivo and functionally and structurally thermolabile in vitro, manifested in a slow rate of RP_{init} formation. Alanine substitution at acidic residues 57, 58, 63, 64, and 69 has no effect on σ^{70} function in vivo or in vitro. Therefore, acidity of region 1.1 is not a major contributing factor to the initiation properties of σ^{70} , but amino acids, including D61, are very important for structural stability.

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