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Conserved Region 2.1 of *Escherichia coli* Heat Shock Transcription Factor σ^{32} Is Required for Modulating both Metabolic Stability and Transcriptional Activity

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***Escherichia coli* heat shock transcription factor σ^{32} is rapidly degraded in vivo, with a half-life of about 1 min. A set of proteins that includes the DnaK chaperone team (DnaK, DnaJ, GrpE) and ATP-dependent proteases (FtsH, HslUV, etc.) are involved in degradation of σ^{32}. To gain further insight into the regulation of σ^{32} stability, we isolated σ^{32} mutants that were markedly stabilized. Many of the mutants had amino acid substitutions in the N-terminal half (residues 47 to 55) of region 2.1, a region highly conserved among bacterial σ factors. The half-lives ranged from about 2-fold to more than 10-fold longer than that of the wild-type protein. Besides greater stability, the levels of heat shock proteins, such as DnaK and GroEL, increased in cells producing stable σ^{32}. Detailed analysis showed that some stable σ^{32} mutants have higher transcriptional activity than the wild type. These results indicate that the N-terminal half of region 2.1 is required for modulating both metabolic stability and the activity of σ^{32}. The evidence suggests that σ^{32} stabilization does not result from an elevated affinity for core RNA polymerase. Region 2.1 may, therefore, be involved in interactions with the proteolytic machinery, including molecular chaperones.**

When cells or organisms are exposed to high temperature, synthesis of a set of heat shock proteins (HSPs) is rapidly induced. This induction generally occurs at the transcriptional level and is mediated by specific transcription factors. In *Escherichia coli*, the level of heat shock transcription factor σ^{32} (encoded by the *rpoH* gene) increases rapidly and transiently upon a temperature upshift, directing RNA polymerase to transcribe heat shock genes that encode HSPs, such as molecular chaperones and ATP-dependent proteases (9, 10, 56). The increase in the σ^{32} level depends on both increased synthesis and stabilization of the normally unstable σ^{32} (half-life, ~1 min). The stabilization and enhanced synthesis of σ^{32} observed upon temperature upshift represent two distinct events that presumably involve different signaling pathways (15, 52).

Upon a temperature upshift from 30 to 42°C, the rate of σ^{32} synthesis increases 10-fold within 3 to 4 min at the translational level (41), as mediated by the secondary structure of the 5′ portion of *rpoH* mRNA (26, 31, 57). A high temperature directly disrupts the mRNA secondary structure, perhaps without involvement of other cellular factors, leading to enhanced ribosome entry and initiation of translation (27). Thus, the rate of synthesis of σ^{32} is primarily determined by the ambient temperature. Besides translational induction, marked stabilization of σ^{32} (eightfold stabilization) occurs for the first 4 to 5 min upon a heat shock, and this is followed by rapid destabilization (41). Transient stabilization of σ^{32} has been thought to occur by titration of DnaK and/or DnaJ chaperones away from σ^{32} by unfolded proteins accumulating at a high temperature, since these chaperones are involved in dealing with both σ^{32} and unfolded proteins (5, 7).

The DnaK chaperone team (DnaK, DnaJ, and GrpE) is required for rapid σ^{32} degradation because σ^{32} is markedly stabilized in *dnaK*, *dnaJ*, or *grpE* mutants (40, 47). The DnaK chaperone team is also involved in degradation of short-lived proteins, such as SulA and RcsA, and abnormal proteins, including puromycyl fragments, canavanine-containing proteins, and a nonsecreted form of alkaline phosphatase (12, 14, 18, 42). Two possible roles of the DnaK chaperone team in proteolysis have been proposed; one of these roles assumes that chaperones act in protein turnover only as accessory factors that help maintain substrates in a soluble form (14), and the other assumes that chaperones promote formation of protease-substrate complexes (12). Since σ^{32} stabilized in chaperone-deficient cells remains soluble and exhibits high transcriptional activity, the DnaK chaperone team may well play an active role, although the exact roles of chaperones in σ^{32} turnover, including their binding sites, remain unknown.

FtsH (HflB), a member of the AAA family of proteins (23), is the first protease that has been shown to degrade σ^{32} (11, 49). It is a membrane-bound ATP-dependent metalloprotease with an active site facing the cytoplasm, and it forms a ring structure consisting of six protomers (34). It degrades membrane proteins that are not assembled into functional complexes (1, 19) and cytoplasmic proteins (33, 38). Cytoplasmic ATP-dependent proteases, including HslUV (ClpYQ), also participate in σ^{32} degradation appreciably, although the relative contributions of FtsH and other proteases remain unknown (16, 17, 55).

To gain further insight into the mechanisms of σ^{32} degradation, we isolated a number of σ^{32} mutants that were stabilized

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in vivo by screening for hyperactive molecules. Some of these stable mutants had amino acid changes in the N-terminal half of region 2.1, one of the regions highly conserved among bacterial σ factors (22).

MATERIALS AND METHODS

Bacteria, plasmids, and phage. *E. coli* K-12 strain MC4100 [F$^-$ *araD* Δ(*argF-lac*)*U169 rpsL relA flbB deoC ptsF rbsR*] and derivatives of this strain were used in most experiments. KY1612 [MC4100 Δ*rpoH30*::*kan zhf50*::Tn*10* (λpF13-*groEp-lacZ*)] lacks σ^{32} and cannot grow at temperatures above 20°C (58). Prophage λpF13-*groEp-lacZ* carried *lacZ* under control of the *groE* promoter (*groEp*) (53). All *rpoH* mutant alleles were cloned under the isopropyl-β-D-thiogalactopyranoside (IPTG)-dependent *trc* promoter (5′-TGTTGACAATTAATCATCCGGCTCGTATAATGTG-3′; −35 and −10 hexamer sequences are underlined) on derivatives of the multicopy pTrc99A vector (Amersham Biosciences). pTrc99A was cut with NcoI, blunt ended with ExoVII, and self-ligated to obtain pKV1142 lacking four nucleotides of the NcoI site. A derivative of pKV1142 containing a weaker *trc* promoter (5′-TGTTGACAATTAAATCCATCCGGCTCGTATAATGTG-3′) was obtained by inserting two nucleotides (indicated by boldface type) into the spacer between the −35 and −10 sequences by PCR (pKV1585). A promoterless intact *rpoH* gene was amplified by PCR by using pKV7 (29) as the template, an upstream primer (5′-CCGGAATTCCGATTGAGAGGATTTGAATGACTGACAAAATGC-3′) containing an EcoRI site (GAATTC; underlined) and the initiation codon (ATG; underlined), and a downstream primer (5′-CGCGGATCCACAATCTGCGTGGGGATTGGCGTTTTGCCGG-3′) containing a downstream region of *rpoH* and a BamHI site (underlined). Amplified DNA fragments were cut with EcoRI and BamHI and cloned into pKV1142 and pKV1585 cut with the same enzymes; the resulting plasmids carrying *rpoH* were designated pKV1425 and pKV1637, respectively. The mutation from GCG to GAG at the 76th codon of *rpoH* was introduced into pKV1425 and pKV1637 by site-directed mutagenesis, yielding pKV1693 and pKV1845, respectively. All *rpoH* mutant alleles were constructed with pKV1637 and pKV1845 by site-directed mutagenesis. The sequence of each *rpoH* allele was confirmed by DNA sequence analysis.

Media, chemicals, and reagents. L broth (48) was generally used for growing cells; ampicillin (50 μg/ml) was added when necessary. The synthetic medium used was medium E (48) supplemented with 0.5% glucose, 2 μg of thiamine per ml, and all of the amino acids (20 μg/ml each) except methionine and tryptophan; ampicillin was used at a concentration of 10 μg/ml. IPTG was added when necessary. L-[^{35}S]methionine (29.6 TBq/mmol) was obtained from American Radiolabeled Chemicals and Amersham Biosciences. Other chemicals were purchased from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemicals (Osaka, Japan).

Radioactive labeling of proteins. The assay for radioactive labeling of proteins was carried out essentially as described elsewhere (54). Cells were grown in synthetic medium at 30°C. Samples were taken and pulse-labeled with [^{35}S]methionine (100 μCi/ml) at the mid-log phase. To determine protein stability, pulse-labeled cells were incubated with excess unlabeled methionine (200 μg/ml), and samples taken at intervals were examined for radioactivities associated with σ^{32} by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Quantitation of σ^{32} bands was done with a BAS1800 BioImaging analyzer (Fuji Film, Tokyo, Japan).

Other methods. Nucleic acid manipulation (36), mutagenic PCR (6), SDS-PAGE (48), immunoblotting (16), and site-directed mutagenesis (37) were performed as described previously.

RESULTS

Initial screening of σ^{32} mutants with increased stability. The cellular level of σ^{32} is adjusted by regulation of its stability, as well as by regulation of its translational efficiency. To isolate σ^{32} mutants that specifically had increased stability, we used parental *rpoH* containing several undefined mutations that released the *rpoH* mRNA from translational inhibition. We expected that stabilization of σ^{32} would result in an increased level of protein, which in turn would increase transcription from heat shock promoters. The *rpoH* coding region amplified by PCR under error-prone conditions was ligated with a vector, placing *rpoH* under control of the IPTG-dependent *trc* promoter. The resulting plasmids were transformed into an *rpoH* deletion strain lacking σ^{32} (KY1612) and carrying λpF13-*groEp-lacZ* prophage to monitor σ^{32} activity, and cells were plated on L agar without IPTG and incubated at 30°C. Since KY1612 cells cannot grow at temperatures above 20°C (58), selection of transformants at 30°C guaranteed that potential σ^{32} mutants had transcriptional activity at least capable of supporting growth at 30°C, thus excluding mutants that had undergone drastic structural changes. β-Galactosidase activity expressed from the *groE* heat shock promoter served as an indicator of the transcriptional activity of σ^{32}. In the absence of IPTG, the rate of synthesis of σ^{32} expressed in cells harboring wild-type *rpoH* on plasmid (pKV1425) was about fourfold higher than the rate of synthesis in the wild-type strain carrying only the chromosomal *rpoH* gene (data not shown). Darker blue colonies on L agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were picked at 30°C, because transformants that produced stable σ^{32} mutants were expected to exhibit higher β-galactosidase activity.

Screening of over 20,000 transformants resulted in 55 initial candidates with apparently higher σ^{32} activity. When the σ^{32} level was examined by immunoblotting, it was significantly elevated in 27 of the transformants tested. Many of these transformants showed slower growth even in rich media, possibly due to increased σ^{32} levels. To minimize deleterious effects on cell growth, some *rpoH* mutants were recloned into a plasmid carrying a weaker *trc* promoter that contained two additional nucleotides between the −35 and −10 sequences (pKV1585). In spite of these efforts, however, growth of the Δ*rpoH* strains carrying mutant *rpoH* on the plasmid was slow in synthetic medium, which prevented pulse-labeling experiments.

The resulting plasmids were, therefore, transformed into a wild-type *rpoH*$^+$ strain (MC4100), and the stability of each mutant σ^{32} was examined by performing pulse-chase and immunoprecipitation experiments. Two *rpoH* mutant alleles encoding σ^{32} with particularly longer half-lives had several mutations that caused amino acid changes. Mutations responsible for σ^{32} stabilization were identified by swapping the restriction fragments between wild-type *rpoH* and each of the mutant alleles, and the mutation identified in this way was individually introduced into wild-type *rpoH* on plasmid pKV1637 by site-directed mutagenesis. One of the mutants containing two adjacent mutations that changed leucine residues to glutamine residues at positions 47 and 55 (designated L47Q-L55Q) produced σ^{32} with a half-life that was more than 10-fold longer than that of the wild type (Fig. 1). The half-life of σ^{32} containing the L47Q mutation alone and the half-life of σ^{32} containing the L55Q mutation alone were about three- and twofold longer than the half-life of the wild-type σ^{32}, respectively (Fig. 2). Another mutant had an A50S substitution (alanine replaced by serine) at position 50 and produced about fourfold more stable σ^{32} than the wild type produced (Fig. 1).

Further screening of stable σ^{32} mutants. Since the parental *rpoH* used in the experiments described above contained several uncharacterized mutations which caused amino acid changes that made it difficult to identify and characterize mutations responsible for σ^{32} stabilization, further screening was carried out with parental *rpoH* containing three well-defined mutations expected to disrupt the *rpoH* mRNA secondary

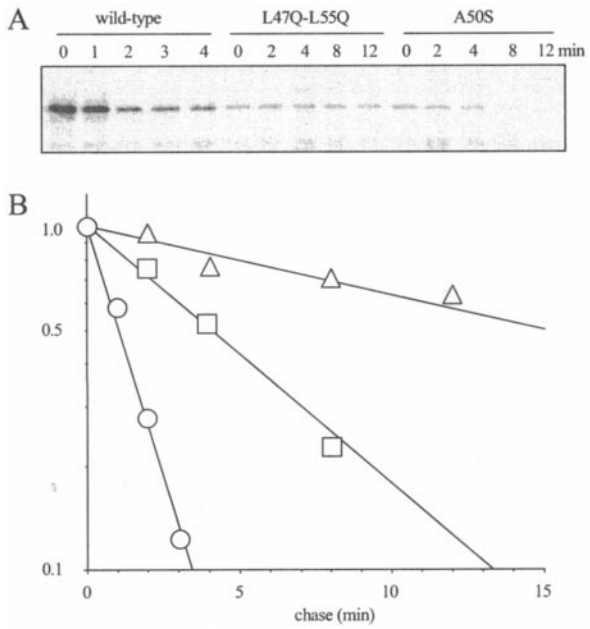

FIG. 1. Stability of σ^{32} in some mutants obtained by initial screening. MC4100 cells expressing wild-type σ^{32} from the *trc* promoter (pKV1425) or mutant σ^{32} from the weak *trc* promoter (pKV1637 derivatives) were grown in synthetic medium containing IPTG (100 μM for the wild type and 10 μM for the mutants) at 30°C to the mid-log phase and then were pulse-labeled with [^{35}S]methionine (100 μCi/ml) for 1 min and chased with excess unlabeled methionine (200 μg/ml). To exclude the possibility that the mutant σ^{32} was stabilized as the result of an increased level, wild-type σ^{32} (control) was expressed from the intact *trc* promoter with a higher inducer concentration. Samples were taken at intervals starting at 1 min (wild type) or 3 min (mutants) (defined as time zero), and radiolabeled σ^{32} was analyzed by immunoprecipitation, followed by SDS-PAGE (10% polyacrylamide gel). Under these conditions, small amounts of wild-type σ^{32} expressed from the chromosomal *rpoH* were not detectable due to both a low synthesis rate and rapid degradation. (A) Typical results of SDS-PAGE. (B) Plot of average values calculated from three experiments. Symbols: ○, wild-type σ^{32}; △, L47Q-L55Q; □, A50S.

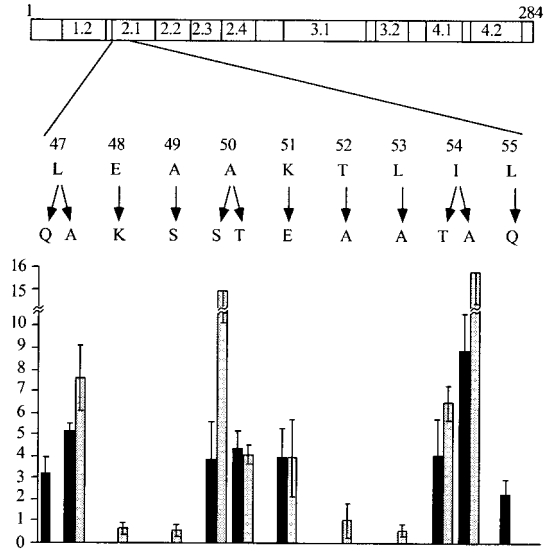

FIG. 2. Summary of stabilities (half-lives) of σ^{32} mutants localized in the N-terminal half of region 2.1. The stabilities of various σ^{32} mutants were determined by pulse-chase experiments essentially as described in the legend to Fig. 1. Synthetic medium with 500 μM IPTG (E48K-A76E, A49S-A76E, T52A-A76E, L53A-A76E) or without IPTG (the rest of the mutants) was used to grow cells. All σ^{32} mutants were expressed from pKV1637 or pKV1845 derivatives in *rpoH*$^+$ MC4100 cells. The averages of three experiments are shown, and the wild-type value was defined as 1. Solid bars, σ^{32} mutants without the A76E mutation; shaded bars, σ^{32} mutants with the A76E mutation. Conserved regions of σ^{32} and amino acid changes in the N-terminal half of region 2.1 are shown at the top.

structure appreciably. These mutations changed the G residues at positions 123 and 177 (A in the initiation codon was defined as position 1) to A and the T at position 180 to C; none of these changes altered the amino acids encoded. Indeed, the rate of synthesis of σ^{32} encoded by this *rpoH* allele was about 2.5-fold higher than rate of synthesis of the wild-type σ^{32} at 30°C, and the synthesis was induced only slightly (~twofold) upon a shift to 42°C (synthesis was induced about fivefold in the case of wild-type σ^{32}) (data not shown).

Error-prone random mutagenesis was carried out by using the parental *rpoH* gene described above as the template in PCR. To minimize deleterious effects of excessive σ^{32} on cell growth, PCR-amplified fragments were cloned under control of the weak *trc* promoter on pKV1585, and the resulting plasmids were transformed into Δ*rpoH* strain KY1612. Through analyses similar to the initial screening, several σ^{32} mutants that contained the A50T, K51E, or I54T mutation and exhibited three- to fivefold-higher stability than the wild-type σ^{32} were obtained (Fig. 2). Remarkably, these amino acid residues, as well as those found earlier, were all localized in the N-terminal half of region 2.1, a region highly conserved among bacterial σ factors, indicating that this region is directly or indirectly involved in modulating degradation of σ^{32}.

In the course of the initial screening, we found that the mobility of the σ^{32} mutant containing the A76E mutation on SDS-polyacrylamide gels was distinctly lower. To distinguish the mutant σ^{32} proteins from wild-type σ^{32} on the gel and to examine possible effects of mutant σ^{32} on the stability of wild-type σ^{32}, the A76E mutation was introduced into each of the σ^{32} mutants. The half-life of wild-type σ^{32} expressed from the chromosomal DNA was found to be 1 to 2 min in cells producing any of the σ^{32} mutants tested, indicating that production of σ^{32} mutants did not affect the stability of wild-type σ^{32}. Although the A76E mutation alone hardly affected the stability (Fig. 3), some stable σ^{32} mutants, such as the A50S mutant, were further stabilized by having both the original and A76E mutations simultaneously (Fig. 2 and 3). The reasons why only some of the specific mutants were further stabilized by the A76E mutation are unknown.

Directed mutagenesis in the N-terminal half of region 2.1. The results described above indicated that at least some amino acid residues in the N-terminal half of region 2.1 are critically involved in degradation of σ^{32}. To examine the specificity of the mutation in this region, additional σ^{32} mutants with mutations affecting adjacent amino acid residues (E48K, A49S, T52A, and L53A) were constructed, and their stabilities were examined by performing pulse-chase and immunoprecipitation experiments. However, none of these mutants appeared to produce σ^{32} that was significantly more stable than the wild-type σ^{32}, although precise half-lives could not be determined

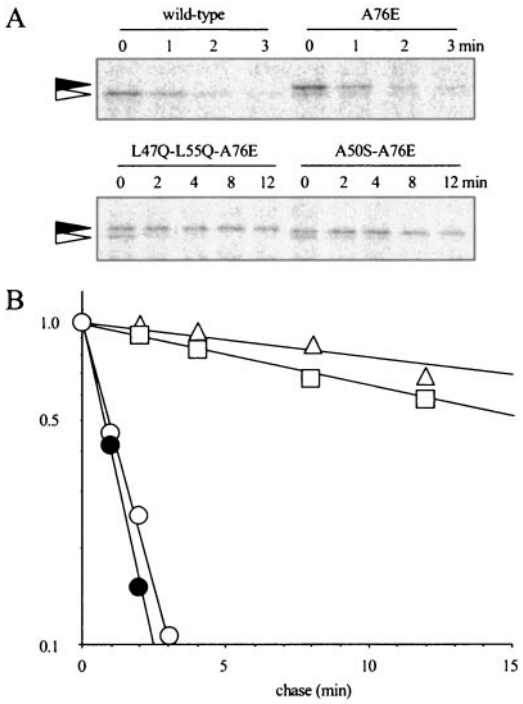

FIG. 3. Stability of σ^{32} containing the A76E mutation. The stabilities of wild-type σ^{32} and mutant σ^{32} proteins containing the A76E mutation were determined in wild-type (*rpoH*$^+$) MC4100 cells by pulse-chase experiments. Synthetic medium without IPTG was used to facilitate comparison of the stabilities of mutant σ^{32} protein expressed from *rpoH* on the plasmid and wild-type σ^{32} protein expressed from the chromosomal *rpoH* gene. (A) Results of a typical experiment. Since the rate of synthesis of mutant σ^{32} from plasmids was low in the absence of IPTG, wild-type σ^{32} expressed from the chromosomal *rpoH* gene could be detected simultaneously. The open arrowheads indicate the position of wild-type σ^{32} expressed both from the chromosomal *rpoH* gene and from pKV1425; the solid arrowheads indicate the position of mutant σ^{32} containing the A76E mutation expressed from plasmids (pKV1693 or pKV1845 derivatives). (B) Plot of average values calculated from three experiments. Symbols: ○, wild type; ●, A76E; △, L47Q-L55Q-A76E; □, A50S-A76E.

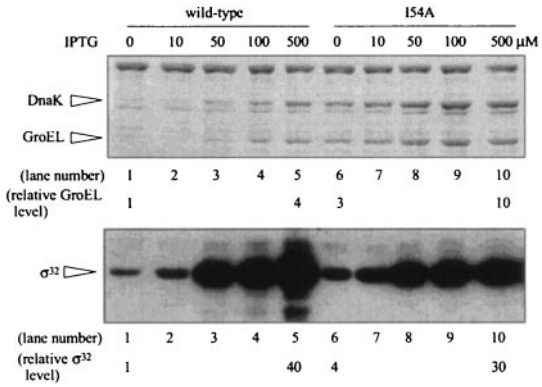

FIG. 4. Higher activity of stable mutant σ^{32}. MC4100 cells harboring plasmids were grown at 30°C in synthetic medium without IPTG (lanes 1 and 6) or with IPTG at a concentration of 10 μM (lanes 2 and 7), 50 μM (lanes 3 and 8), 100 μM (lanes 4 and 9), or 500 μM (lanes 5 and 10). Whole-cell proteins were analyzed by SDS-PAGE (10% polyacrylamide gel), followed by staining with Coomassie blue (upper panel) or immunoblotting (lower panel). The values shown below the lane numbers indicate the levels of GroEL (upper panel) or σ^{32} (lower panel) relative to the level in lane 1 and were determined by immunoblotting serial dilutions of samples on separate gels. Lanes 1 to 5, MC4100 expressing wild-type σ^{32} from the *trc* promoter (pKV1425); lanes 6 to 10, MC4100 expressing I54A from the weak *trc* promoter (pKV1637 derivative).

due to bands that overlapped wild-type σ^{32} bands resulting from the chromosomal *rpoH* gene on SDS-polyacrylamide gels. When these mutant σ^{32} proteins containing the A76E mutation were constructed and their half-lives were examined, they were all unstable, like the wild-type protein (Fig. 2). These results, combined with those presented above, indicate that only some specific amino acid residues in the N-terminal half of region 2.1 are required for rapid degradation of σ^{32}.

Since in the stable σ^{32} mutants obtained up to this point hydrophobic amino acids were replaced by polar amino acids (L47Q, A50S, A50T, and I54T) or a basic amino acid was replaced by an acidic amino acid (K51E), these changes might potentially alter the σ^{32} structure. We therefore constructed mutants with L47A and I54A mutations which still exhibited hydrophobicity at the corresponding amino acid residues and examined the protein stability. Surprisingly, both of these mutant proteins were found to be even more stable than the proteins isolated by screening for higher σ^{32} activity (Fig. 2), suggesting that the bulky hydrophobic amino acid side chains at positions 47 and 54 are important for the rapid turnover characteristic of σ^{32}.

Stable mutant σ^{32} proteins exhibited higher transcriptional activity. When HSP synthesis was examined with the mutants producing stable σ^{32}, the level of HSPs (DnaK and GroEL) was much greater in most mutants than in wild-type cells. To quantitatively assess σ^{32} activity, the intracellular level of σ^{32} was varied by varying the IPTG concentration (0 to 500 μM), and the levels of GroEL and σ^{32} in the mutant and wild type were compared by immunoblotting. As shown for a typical mutant in Fig. 4, the levels of DnaK and GroEL increased in parallel with an increased σ^{32} level for both wild-type and mutant σ^{32}, but the levels were much higher in the mutant (I54A) than in the wild-type at every IPTG concentration tested. In the absence of IPTG, the I54A level was fourfold higher than the wild-type σ^{32} level due to stabilization, which led to increased levels of the heat shock proteins. However, when the concentration of wild-type σ^{32} was increased to a similar level with 10 μM IPTG, the levels of the heat shock proteins hardly changed, indicating that the activity of wild-type σ^{32} was repressed by an unknown mechanism (see below) and that I54A was partially liberated from repression. Repression of σ^{32} activity clearly occurred even in the case of I54A, because while the I54A level with 500 μM IPTG was 7.5-fold higher than the level without IPTG, the levels of the heat shock proteins were only 3.3-fold higher with 500 μM IPTG. While the levels of heat shock proteins produced in cells expressing I54A with 10 μM IPTG were comparable to the levels produced in cells expressing wild-type σ^{32} with 500 μM IPTG, the levels of the I54A mutant were about 10-fold less than the level of the wild-type σ^{32}. Similar results were obtained for the L47Q-L55Q mutant (data not shown). These data strongly suggest that some amino acid residues (such as Ile^{54}) in the

N-terminal half of region 2.1 participate in regulation of both σ^{32} activity and stability. Under the conditions used here, no significant amount of wild-type σ^{32} expressed from the chromosomal DNA was detected.

σ^{32} stabilization does not result from elevated affinity for core RNA polymerase. The increased levels of HSPs observed with the I54A and L47Q-L55Q mutants imply that the number of σ^{32} molecules associated with core RNA polymerase during steady-state growth increases in cells containing these stable mutant σ^{32} proteins. Stabilization of σ^{32} might therefore result from elevated affinity for core RNA polymerase, since region 2.1 has been thought to be one of the sites involved in binding to core RNA polymerase (21, 28, 39, 51) and σ^{32} bound to polymerase is hardly degraded by proteases in vitro (4, 17). To exclude this possibility, we constructed and examined the stability of stable σ^{32} mutants containing the Q80R mutation, which is known to reduce the affinity for core RNA polymerase (13) and not to affect the σ^{32} stability (44). When the Q80R mutation was introduced into two stable σ^{32} mutants, L47Q-L55Q and I54A, the resulting σ^{32} mutants (L47Q-L55Q-Q80R and I54A-Q80R) showed reduced DnaK and GroEL levels compared to wild-type σ^{32} (Fig. 5A and data not shown), unlike I54A (Fig. 4), presumably as a result of reduced affinity for core RNA polymerase. Nonetheless, both of these mutants remained stable and seemed to be more stable than the corresponding mutants without Q80R (Fig. 2, 3, and 5B), although the reason for further stabilization is unclear (see below). These results are consistent with the notion that these mutations in region 2.1 stabilize σ^{32} by reducing the affinity for some component(s) of the proteolytic machinery, leading to a higher σ^{32} level, which in turn promotes binding to RNA polymerase and increases HSP synthesis.

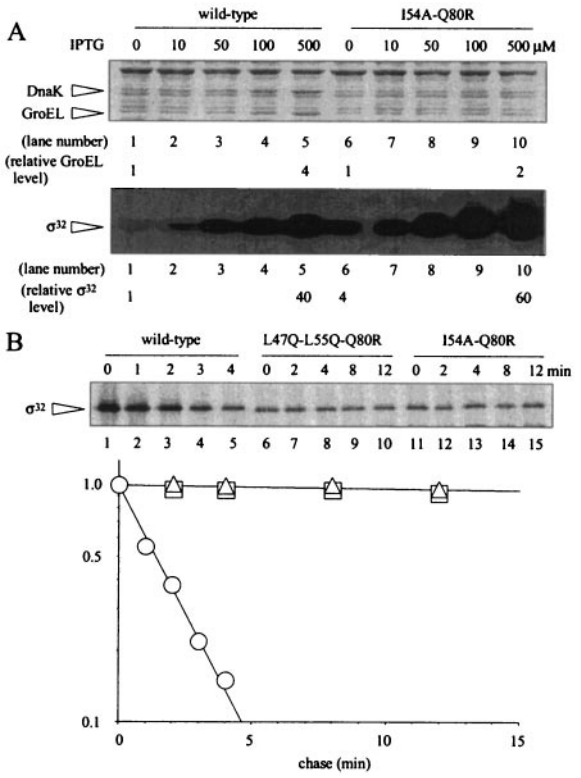

FIG. 5. Stabilization of σ^{32} mutants with reduced affinity for core RNA polymerase. (A) MC4100 cells harboring plasmids were grown at 30°C in synthetic medium without IPTG (lanes 1 and 6) or with IPTG at a concentration of 10 μM (lanes 2 and 7), 50 μM (lanes 3 and 8), 100 μM (lanes 4 and 9), or 500 μM (lanes 5 and 10). Analyses of whole-cell proteins (upper panel) and immunoblotting (lower panel) were performed as described in the legend to Fig. 4. Lanes 1 to 5, MC4100 expressing wild-type σ^{32} from the *trc* promoter (pKV1425); lanes 6 to 10, MC4100 expressing I54A-Q80R from the weak *trc* promoter (pKV1637 derivative). (B) Stability of L47Q-L55Q-Q80R and I54A-Q80R as determined by pulse-chase experiments essentially as described in the legend to Fig. 1, except that in the case of the wild type, the time-zero sample was taken 3 min after addition of unlabeled methionine, like mutant σ^{32}. Cells were grown in synthetic medium with IPTG at a concentration of 500 μM (wild type) or 10 μM (mutants). Results for a typical experiment are shown. (Upper panel) Lanes 1 to 5, wild type; lanes 6 to 10, L47Q-L55Q-Q80R; lanes 11 to 15, I54A-Q80R. (Lower panel) Symbols: ○, wild type; △, L47Q-L55Q-Q80R; □, I54A-Q80R.

DISCUSSION

The *E. coli* heat shock transcription factor, σ^{32}, is extremely unstable in vivo, with a half-life of about 1 min during steady-state growth. We isolated a number of σ^{32} mutants that are highly or moderately stabilized (two- to ninefold), and in the present study we scrutinized the effects of amino acid changes exclusively in the N-terminal half of conserved region 2.1. The amino acid residues that were specifically shown to be involved in modulating σ^{32} stability are Leu^{47}, Ala^{50}, Lys^{51}, Ile^{54}, and Leu^{55}. The results are consistent with previous data which indicated that an internal region (residues 36 to 122) of σ^{32} is critical for rapid degradation (3). Although some of the amino acid changes at these positions, such as L47Q and I54T, might alter the local protein structure with changing hydrophobicity of side chains, the σ^{32} mutant containing the L47A or I54A mutation, perhaps not leading to an appreciable structural alteration, was also found to be quite stable (Fig. 2). It appears likely that the side chains of Leu^{47} and Ile^{54} are particularly important for controlling the in vivo stability of σ^{32}. On the other hand, our data suggest that several adjacent residues (Glu^{48}, Ala^{49}, Thr^{52}, and Leu^{53}) are not critically involved in determining σ^{32} stability. Stable mutants similar to those reported here were isolated by Gross and colleagues (C. A. Gross, personal communication).

Although we focused on amino acid residues in the N-terminal half of region 2.1, we did obtain other stable σ^{32} mutants, each of which had several amino acid changes outside this region. These mutants are currently being analyzed to identify residues crucial for controlling stability. Previous studies revealed that region C of σ^{32} (amino acids 122 to 144) is involved in the control of σ^{32} stability (30). However, we did not obtain any mutants containing mutations in region C in the present screening analysis. The latter region overlaps one of the regions involved in the binding to DnaK (25) and to core RNA polymerase (2). The potential role of region C in σ^{32} stability remains unresolved. In any event, it appears likely that other regions of σ^{32} also participate in modulating σ^{32} stability. This is consistent with the fact that the stabilization of mutant σ^{32} observed in the present work was at most 15-fold (in the case of L47Q-L55Q) (Fig. 1), and the most stable mutants were less stable than most other proteins in *E. coli*. On the other

hand, we should not underestimate the role of region 2.1, particularly because some stable σ^{32} mutants with mutations in region 2.1, when combined with the Q80R mutation, became more stable than the corresponding original mutants. It is worth noting that while production of any of the stable mutant σ^{32} proteins resulted in higher levels of HSPs, production of stable mutant σ^{32} proteins also carrying the Q80R mutation did not result in higher levels of HSPs. The results imply that the increased synthesis of HSPs, including proteases, in these mutants (without Q80R) should in turn accelerate degradation of σ^{32} by changing the equilibrium of the association between stable mutant σ^{32} and the proteolytic machinery.

The finding that stable σ^{32} mutants containing the additional Q80R mutation that reduces the affinity for core RNA polymerase are more stable than the original mutants suggests that stabilization caused by mutations in region 2.1 results from reduced affinity of σ^{32} for components of the proteolytic machinery, although much work is required to determine the nature of the involvement of this region in modulating σ^{32} stability. In addition, this region might also be a contact site for factors regulating the transcriptional activity of σ^{32}, as suggested by the fact that cells expressing a mutant σ^{32} protein, such as I54A, exhibit higher levels of HSPs than cells expressing wild-type σ^{32} (Fig. 4). This is not unexpected, since the DnaK chaperone team has been thought to modulate the transcriptional activity as well as the stability of σ^{32} (8, 43, 45, 46, 50). Specifically, region 2.1 might be a recognition and/or contact site for the chaperones. This idea is supported by the fact that a 13-mer synthetic oligopeptide corresponding to amino acids 47 to 59 shows high affinity for DnaK (25). According to the results presented by Rüdiger et al. (35), DnaK has a tendency to associate with oligopeptides containing a hydrophobic core surrounded by positive charged amino acids. The N-terminal half of region 2.1 of σ^{32} contains Leu^{53}, Ile^{54}, Leu^{55}, and Lys^{51}, which seems to be an ideal sequence for DnaK binding (Fig. 2). It has been reported that substrate polypeptides accommodated by DnaK are in an extended conformation (20, 59). In line with the structure of σ^{70}-type σ factors, however, it is likely that region 2.1 of σ^{32} assumes an α-helix structure (24, 28, 32, 51). It is therefore tempting to speculate that the α-helix structure of region 2.1 undergoes alteration upon binding with DnaK, which in turn inhibits σ^{32} transcriptional activity and promotes σ^{32} degradation. In vitro experiments focusing on the structure of region 2.1 are necessary to explore this possibility further.

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