Role of Region C in Regulation of the Heat Shock Gene-Specific Sigma Factor of Escherichia coli, σ^{32}

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Expression of heat shock genes is controlled in Escherichia coli by the antagonistic action of the σ^{32} subunit of RNA polymerase and the DnaK chaperone system, which inactivates σ^{32} by stress-dependent association and mediates σ^{32} degradation by the FtsH protease. A stretch of 23 residues (R122 to Q144) conserved among σ^{32} homologs, termed region C, was proposed to play a role in σ^{32} degradation, and peptide analysis identified two potential DnaK binding sites central and peripheral to region C. Region C is thus a prime candidate for mediating stress control of σ^{32}, a hypothesis that we tested in the present study. A peptide comprising the central DnaK binding site was an excellent substrate for FtsH, while a peptide comprising the peripheral DnaK binding site was a poor substrate. Replacement of a single hydrophobic residue in each DnaK binding site by negatively charged residues (I123D and F137E) strongly decreased the binding of the peptides to DnaK and the degradation by FtsH. However, introduction of these and additional region C alterations into the σ^{32} protein did not affect σ^{32} degradation in vivo and in vitro or DnaK binding in vitro. These findings do not support a role for region C in σ^{32} control by DnaK and FtsH. Instead, the σ^{32} mutants had reduced affinities for RNA polymerase and decreased transcriptional activities in vitro and in vivo. Furthermore, cysteines inserted into region C allowed cysteine-specific cross-linking of σ^{32} to RNA polymerase. Region C thus confers on σ^{32} a competitive advantage over other σ factors to bind RNA polymerase and thereby contributes to the rapidity of the heat shock response.

The major heat shock proteins (HSPs) of Escherichia coli are molecular chaperones and proteases that constitute a cytosolic system for folding, repair and degradation of proteins (5, 6, 11). Their synthesis is induced as part of the cellular heat shock response after exposure to a large variety of stress conditions which appear to have in common the ability to cause protein misfolding (4, 7, 10, 16, 40). When induced by upshift of the cells to a nonlethal temperature (e.g., 42°C), the heat shock response is transient and consists of a rapid induction phase followed by a shutoff phase starting approximately 5 to 10 min. after upshift.

Expression of HSPs is positively controlled at the transcriptional level by the heat shock promoter-specific σ^{32} subunit of RNA polymerase, encoded by rpoH (4, 11, 42). Stress-dependent changes in heat shock gene expression are mediated by the antagonistic action of σ^{32} and negative modulators which act upon σ^{32} (34–36). These modulators are the DnaK chaperone and its DnaJ and GrpE cochaperones, which inactivate σ^{32} by direct association and mediate its degradation by proteases (8, 9, 20, 21, 34, 35, 38). Degradation of σ^{32} is mediated mainly by FtsH (HHIB), an ATP-dependent metalloprotease associated with the inner membrane (14, 37, 39, 40). FtsH degrades free σ^{32} but not RNA polymerase-bound σ^{32}, indicating that protease and RNA polymerase compete for binding to σ^{32} (40). The role of the chaperones in σ^{32} degradation is poorly understood. Inactivation of σ^{32} occurs by association of DnaK and DnaJ with the free form of σ^{32}, thereby preventing its binding to RNA polymerase (8, 9, 20, 22). There is increasing evidence that the sequestration of the DnaK chaperone system through binding to misfolded proteins is a direct determinant of the induction of the heat shock response (4, 7, 37, 40). Conversely, the shutoff of the heat shock response is assumed to result from HSP-mediated repair and degradation of misfolded proteins, which frees the DnaK chaperone system to inactivate σ^{32} and to promote its degradation. Furthermore, a competition may exist in vivo between σ^{32} and other sigma factors including σ^{70} for association with RNA polymerase. This competition is subject to stress-dependent changes and, consequently, leads to alterations in transcriptional activity of σ^{32} (2).

A central open question is the identity of the binding sites within σ^{32} for DnaK, DnaJ, FtsH, and the core of RNA polymerase and the functional interplay between these sites. Previous work showed that the in vivo half-life of fusions between N-terminal fragments of σ^{32} and β-galactosidase increased when a stretch of 23 residues (R122 to Q144), located between conserved regions 2 and 3 of σ^{32} and termed region C (Fig. 1), is deleted or replaced by other residues (27). Within region C, a segment of 9 amino acids between residues 132 and 140 of σ^{32} (QRKLFFNLR) is almost entirely conserved within σ^{32} homologs but not other sigma factors; it was therefore termed the RpoH box (28). This specific conservation strongly suggests a regulatory role for the RpoH box. Consistent with this assumption were the results of a study in which a σ^{32}-derived peptide library was screened for DnaK binding sites. A high-affinity DnaK binding site exists within the RpoH box in the center of region C, and a second binding site was found close to the RpoH box at the periphery of region C (between residues L118 and K125) (25). Based on this peptide analysis, the RpoH box, and possibly the peripheral DnaK binding motif, is a prime candidate for a regulatory site within the σ^{32} protein which allows binding of DnaK and possibly also degradation by FtsH (25).
The aim of the present study is to experimentally investigate the regulatory role of region C, in particular of the two DnaK binding motifs. In contrast to our expectations, we did not find evidence for a role of region C in chaperone binding and degradation by FtsH. Instead, we found that region C was involved in high-affinity binding of σ32 to RNA polymerase, thereby providing to σ32 a competitive advantage over other sigma factors in association with RNA polymerase.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** Cells of strains BB2019 [GW1000 recA441 ualA1 Δ(aroF-leu)U169 supE(Ts) pTiH165 (Am) pDM1(1)] (8) and BB7809 (C500 thr-1 leuB6 thi-1 lacY supE44 rfbD1 flaA2 lacF1 PpBAD-mdha) (40) were grown aerobically at 30 or 42°C in Luria broth or M9 minimal medium supplemented with 0.2% glucose (M-Glu) or 0.2% maltose (M9-Mal) as the carbon source, thiamine (20 μg/ml), and appropriate amino acids (50 μg/ml). The growth media were also supplemented with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) for BB7809 and kanamycin (40 μg/ml) and ampicillin (100 μg/ml) when required. The wild-type rpoH gene cloned into plasmid pHEU21-2ΔΔ12 (9) was used as template for mutagenesis by the method of Kunkel et al. (17). pBAD30 (rpoH) (wild type or mutant) was obtained by cloning the EcoRI-HindIII fragment from pHEU21-2ΔΔ12 into pBAD30 (12). For production of hexahistidine-tagged σ32 proteins, wild-type and mutant alleles of rpoH were subcloned into the EcoRI fragment of pHEU211-1 (amino-terminal histidine fusion, used for σ32) or the PstI fragment of pHEU212ΔΔ12 (rhoH) as the template. The P1 sequence comprises internal coding sequences of recA441 sulA11 (GW1000) (20) and recA441 sulA11 (argF-lac)U169 supC (Ts) pTiH165 (Am) pDM1(1) (8) into pBAD30 (12).

**Analysis of protein interactions.** Association of σ32 with DnaK core was determined by gel filtration on a Superose 12 column (Pharmacia) with a mobile buffer containing 40 mM HEPES-KOH (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 10% glycerol, and 1 mM β-mercaptoethanol. A final purification step was performed by using a MonoQ column (HR55; Pharmacia) and elution of the bound protein by a linear 100 to 1,000 mM KCl gradient. Untagged σ32 and σ32-1F137E were purified as fusion proteins with self-cleavable intein-chitin binding domains on affinity chromatography columns as specified by the supplier (New England Biolabs), except that a different running buffer was used (20 mM Hepes-KOH, 500 mM NaCl, 0.5% Triton X-100) and an additional washing step of the cell extract-loaded chitin column with 20 mM HEPES-KOH–500 mM NaCl–5 mM MgCl2–5 mM ATP was performed to elute DnaK bound to σ32. The chitin bound intein-σ32 was eluted from the intein moiety with running buffer containing 50 mM dihexylthiotrithiol (DTT), which induces self-slicing, and further purified using a MonoQ column. RNA polymerase (holoenzyme and core), σ32, DnaK, DnaJ, and FtsH were purified (purity of approximately 70% for σ32 and >90% for the other proteins) as described previously (3, 23, 32, 39). Protein concentrations were routinely determined by Bradford assay (26) and by bovine serum albumin (BSA) as the standard, and for σ32, calibrated by the bichonicinic acid protein assay (Pierce). The peptides used were synthesized by R. Franck (ZMBH, University of Heidelberg, Heidelberg Germany) and (for σ32-E115A) by Jerini Bio Tools (Berlin, Germany). Concentrations were determined by measurement of the absorption at 280 nm.

**β2 labeling of proteins and in vitro degradation assays.** To assay the degradation of σ32, the proteins were labeled with N-succinimidyl-[2,3-3H]propionate (Amersham) as described previously (9), except that free N-succinimidyl-[2,3-3H]propionate was removed by dialysis against transcription buffer (20 mM Tris-HCl [pH 8.0], 200 mM KCl, 5 mM MgCl2, 1 mM DTT, 5% glycerol). Degradation of labeled σ32 by FtsH was assayed in a purified system adapted from Pfeifer et al. (52). First, BSA (1 mg/ml) was preincubated at 42°C for 30 min with 50 μM ZnCl2–25 mM Tris-acetate (pH 8.0)–2.5 mM magnesium acetate (final concentration) was mixed with 1 μM final concentration in a final volume of 20 μl of reaction buffer (50 mM Tris-acetate [pH 8.0], 5 mM magnesium acetate, 2 mM β-mercaptoethanol, 50 mM KCl, 5 mM ATP) and incubated at 42°C. Aliquots of 2 μl were withdrawn at the indicated times, mixed with BSA (0.5 mg/ml) and EDTA (20 mM), and precipitated with TCA (10%, vol/vol). Radioactivity in the supernatant was determined in a scintillation cocktail (Aquasol 2, New England Nuclear). To assay the degradation of σ32, the final volume of the reaction mixture was 60 μl and the concentration of peptide was 60 μl at the indicated times. Aliquots of 18 μl were mixed with 92 μl of 6.5% trichloroacetic acid to stop the reaction. Products were analyzed by reverse-phase chromatography with a 5 to 80% acetonitrile gradient in 0.1% trifluoroacetic acid.

**Analysis of protein interactions.** Association of σ32 with DnaK, DnaJ, and RNA polymerase core enzyme (RNAP core) was determined by gel filtration with a Superose 12 column equilibrated with 20 mM Tris-HCl–5 mM MgCl2–5 mM KCl (pH 8.0) and 300 μM ATP, 50 μM DnaK was incubated for 2 h at 30°C in transcription buffer (180 mM NaCl, 10 mM Tris-HCl–5 mM MgCl2–5 mM KCl (pH 8.0), the association of σ32 with RNAP core, 0.5 μM (in competition experiments) or 1 μM σ32 was incubated with 1.5 μM RNAP core for 10 min at 30°C in transcription buffer (20 μl, final volume). To determine the association of σ32 with DnaK, 5 μM DnaK was incubated for 2 h at 30°C in transcription buffer (to discourage oligomerization), mixed with 1 μM σ32 in a final volume of 20 μl and further incubated for 30 min at 30°C. These mixtures were shifted to ice, adjusted to 100 μl by addition of transcription buffer which for competition experiments contained a 10- or 30-fold excess of unlabeled σ32 or σ32, respectively, and loaded on a Superdex 200 column at 4°C. Labeled σ32 was detected in the elution fractions by liquid scintillation counting.

**Cross-linking experiments.** To couple the cysteine-specific cross-linker N-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidylpropionamide (TFPAM-3) to cysteine-containing σ32 mutant proteins, 50 μl of a 20 μM protein solution was dialyzed against buffer A (20 mM Hepes-KOH [pH 8.0], 200 mM KCl, 5 mM EDTA), incubated for 1 h at 30°C in the dark with a 10-fold molar excess of TFPAM-3 and then dialyzed against buffer B (20 mM Hepes-KOH [pH 8.0], 200 mM KCl, 5 mM MgCl2) to remove free TFPAM-3. All other proteins were dialyzed against buffer B before use. For cross-linking, the proteins were mixed as indicated (150 pmol each) in 20 μl of buffer B and incubated at 30°C for 2 h in the dark. After addition of 50 μl of buffer C (5 mM EDTA) were added and the mixtures were incubated for a further 10 min on ice. Cross-linking was induced on ice by illumination under UV light (360 nm) for 5 min. After addition of sample buffer (16) and boiling for 5 min, the samples were subjected to electrophoresis on SDS–10% polyacrylamide gels. The gels wereelectrophoresed onto polyvinylidene difluoride membranes (Amersham) for immunodetection. Immunoblots were developed with a Vistra EC Fluorescence
RESULTS

In vitro analysis of region C-derived peptides. To investigate whether region C comprises binding sites for DnaK and FtsH that are responsible for the control of σ^32 activity and stability, we designed amino acid alterations predicted to perturb these sites (Fig. 1). For DnaK, this approach is straightforward since two potential binding sites central and peripheral to region C had been identified (25). Furthermore, the consensus sequence motif recognized by DnaK and the sequence features which prevent DnaK binding have been elucidated. The binding motif consists of a hydrophobic core of up to five consecutive hydrophobic residues flanked by segments enriched in basic residues (30), features which are compatible with the architecture of the substrate binding cavity of DnaK (29, 44). Introduction of negatively charged residues into the hydrophobic core prevents DnaK binding (30). These findings provide a rational basis for introducing alterations into the two potential DnaK binding sites central and peripheral to region C. Recent results concerning the substrate specificity of FtsH (40a) led us to speculate that this protease also recognizes hydrophobic stretches within protein sequences. We therefore changed hydrophobic residues within the hydrophobic stretches located in region C to negatively charged residues in order to perturb DnaK binding and degradation by FtsH.

As an experimental starting point, we used peptides comprising either the RpoH box (including the central DnaK binding site) or the peripheral DnaK binding site to test the effects of sequence alterations in vitro. This approach since peptides can bind DnaK with high affinity in an ATP-dependent fashion (25, 31) and can be degraded efficiently by FtsH in the presence of ATP (40a). A 21-mer peptide comprising the wild-type sequence of the RpoH box (σ^32-Q132-Q151-C) has very high affinity for DnaK (K_d = 40 nM) and is rapidly degraded by FtsH in the presence of ATP (t_1/2 = 7 min) (Fig. 2). To perturb the single DnaK binding motif within the RpoH box, we replaced F137, located in the center of the hydrophobic core, by E (σ^32-Q132-Q151-C/F137E). This replacement increased the K_d of the σ^32-DnaK complex by 50-fold (K_d = 2 μM) and strongly reduced the efficiency of degradation by FtsH (t_1/2 = 28 min) (Fig. 2). A 18-mer peptide comprising the DnaK binding site located peripheral to region C (σ^32-E115-A131-C) has high affinity for DnaK (K_d = 0.2 μM) (Fig. 2) but is only slowly degraded by FtsH in the presence of ATP (t_1/2 = 60 min) (data not shown). A replacement I123 by D, predicted to prevent DnaK binding to its binding site within this peptide (σ^32-E115-A131-C/I123D), caused a strong decrease in affinity for DnaK (K_d = 3.4 μM) (Fig. 2) and no observable degradation by FtsH (data not shown).

These results show that at the peptide level, the RpoH box contains overlapping or identical recognition sites for DnaK and FtsH which are efficiently perturbed by the F137E exchange and that the N-terminal end of region C contains a binding site for DnaK which is perturbed by the I123D exchange.

In vivo activity of σ^32 mutant proteins with altered region C. The above results formed the basis for a rational design of mutational alterations of region C within the σ^32 protein (Fig. 1). rpoH was mutated to introduce the F137E (rpoH-F137E) or I123D mutation (rpoH-I123D), or the rpoH-WRI121,122,123ART mutation. This mutation generates a mutant protein with increased similarity to σ^32. The mutant rpoH genes were cloned into plasmid pUHE21-2fdΔ12 (9) such that their expression is controlled by the IPTG-regulatable P_airlac/ promoter. When produced to the levels used in the experiments described below, the three mutant σ^32 proteins were recovered in the soluble fractions of cells growing at 30 and 42°C (data not shown). The mutational alterations therefore did not cause structural changes in σ^32 leading to aggregation, allowing further analysis of the in vivo activities of the mutant proteins.

We first tested the ability of plasmids containing the rpoH mutant alleles to complement the temperature-sensitive growth of rpoH165(Am) mutant cells (BB2019) in liquid culture and on agar plates. The rpoH-WRI121,122,123ART and rpoH-I123D alleles allowed IPTG-dependent complementation of growth at 42°C, but the colonies were smaller than those formed by cells expressing wild-type rpoH. The rpoH-F137E mutant allele allowed only partial complementation of growth at 42°C, leading to formation of a reduced number of slow-growing colonies (data not shown).

We then determined in pulse experiments the ability of the plasmid-borne mutant alleles to restore the heat shock response in rpoH165(Am) cells after a shift from 30 to 42°C. The induction of expression of the rpoH-WRI121,122,123ART (data not shown) and rpoH-I123D (Fig. 3) alleles by IPTG allowed the induction of expression of heat shock genes at 30 and 42°C. However, the amplitude of the response was two- and fourfold

![FIG. 2. DnaK binding and FtsH-mediated degradation of peptides derived from region C. (A) Amino acid sequences of the peptides used. Mutated residues are boxed. (B) Dissociation constants (K_d) of the peptide-DnaK complexes. The K_d values were determined by peptide titration with fluorescently labeled peptide σ^32-Q132-Q151-C IAANS as competitor as described previously (25). (C) In vitro degradation of peptides by FtsH. Degradation is shown as a percentage of the amount of peptide remaining.](http://jb.asm.org/)
lower for cells expressing *rhoH-WRI121,122,123ART* and *rhoH-I123D*, respectively, than for cells expressing wild-type *rhoH*. Furthermore, the induction of the heat shock response was delayed by 5 to 10 min in cells expressing *rhoH-I123D*. The induction of expression of the *rhoH-F137E* allele allowed an increase in heat shock gene expression only after the temperature upshift to 42°C, and the induction of the heat shock response was delayed (10 min) compared to the response in cells expressing wild-type *rhoH*. However, in cells producing either one of the mutant proteins, a shutoff phase of the heat shock response was observed, suggesting that the DnaK-mediated inactivation of σ^32^ is operative in vivo. These results correlate well with the growth complementation profile of the mutant allele. Together, these data indicate that the mutational alterations I123D and WRI121,122,123ART of σ^32^ cause only partial regulatory defects of σ^32^ whereas the F137E mutation causes stronger regulatory defects in vivo.

**Proteolysis of σ^32^ mutant proteins in vivo.** We investigated the effects of the mutational alterations in region C on σ^32^ stability by performing pulse-chase experiments followed by immunoprecipitation of σ^32^.

The above experiments were complicated by our finding that the in vivo activity of σ^32^-F137E was low at 30°C (Fig. 3). Consequently, in the *rhoH165*(Am) mutant cells producing σ^32^-F137E, the synthesis of HSPs was reduced compared to that in cells producing wild-type σ^32^.

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**Proteolysis of σ^32^ mutant proteins in vitro.** To further substantiate these in vivo results, we investigated the half-life of each mutated σ^32^ protein in vitro by using purified histidine-tagged σ^32^ and FtsH. The three σ^32^ mutant proteins had wild-type-like elution profiles during gel filtration and ion-exchange chromatography. Furthermore, they were indistinguishable from the wild type with respect to the proteolysis pattern obtained by partial proteinase K and trypsin digestion (data not shown). We thus have no indication for changes in their overall tertiary structures.

All three mutant proteins (σ^32^-F137E, σ^32^-I123D, and σ^32^-WRI121,122,123ART) were degraded by FtsH in presence of ATP, with similar kinetics to those of wild-type σ^32^ (Fig. 5 and data not shown). The histidine tags fused to the σ^32^ proteins were not responsible for the degradation, since the authentic σ^32^-F137E mutant protein, purified after cleavage from an intein-chitin fusion, was degraded with the same efficiency as the tagged derivative (data not shown).

Thus, consistent with the in vivo data, the alterations introduced within region C did not affect the efficiency of σ^32^ degradation by FtsH in vitro.

DnaK and DnaJ binding to σ^32^ mutant proteins in vitro. Several in vitro approaches were used to test whether DnaK and DnaJ binding to σ^32^ is impaired by the mutational alterations in region C. In one approach, we used gel filtration to detect complexes between σ^32^ and the chaperones. 3H-labeled wild-type and mutant σ^32^ proteins, all histidine tag fusions, were incubated with DnaK and subjected to gel filtration to
separate DnaK-$\sigma^{32}$ complexes from free $\sigma^{32}$ (Fig. 6). Under the conditions used, approximately 75% of wild-type $^{3}$H-labeled $\sigma^{32}$ was recovered in complex with DnaK (eluting in fractions 12 to 17). The $^{3}$H-labeled $\sigma^{32}$ mutant proteins ($\sigma^{32}$-F137E, $\sigma^{32}$-I123D, $\sigma^{32}$-WR121,122,123ART) showed similar efficiencies of complex formation, even under chase conditions in which the complexes were separated after the addition of a 30-fold molar excess of unlabeled wild-type $\sigma^{32}$. Thus, the mutations introduced into region C of $\sigma^{32}$ had no defect in binding of DnaK. For the $\sigma^{32}$-F137E mutant protein and wild-type $\sigma^{32}$, we verified that these results are also valid for authentic proteins lacking histidine tags (data not shown). Furthermore, no defect in chaperone binding was observed when the $\sigma^{32}$ mutant proteins were incubated with DnaK together with DnaJ in the presence of ATP (data not shown).

In a second approach, we tested the interaction of DnaK with $\sigma^{32}$ proteins by a functional assay which relies on the ability of substrates to stimulate the ATPase activity of DnaK (25, 31). In single-turnover ATPase assays in the presence of DnaJ, wild-type $\sigma^{32}$ and the $\sigma^{32}$-F137E mutant protein stimulated ATP hydrolysis to similar extents (approximately 10-fold) (data not shown). The $\sigma^{32}$-I123D mutant protein stimulated ATP hydrolysis by DnaK efficiently, although to a slightly lower level compared to the two other proteins. We do not consider this difference to be significant with respect to the DnaK-$\sigma^{32}$ interaction.

In a third approach, we used plasmon surface resonance spectroscopy to analyze the ability of the $\sigma^{32}$ mutant proteins to interact with DnaJ. This method was used previously to detect the interaction of DnaJ with wild-type $\sigma^{32}$ (9). We did not observe any difference between the wild type and the three $\sigma^{32}$ mutant proteins in affinity for DnaJ (data not shown). Taken together, these data indicate that none of the mutational alterations within region C affects the affinity of DnaK and DnaJ for $\sigma^{32}$.

**RNAP binding of $\sigma^{32}$ mutant proteins in vitro.** Our findings that the mutational alterations introduced into region C of $\sigma^{32}$ failed to show defects in the interaction with FtsH and DnaK-DnaJ led us to search for other roles for this region. Since several $\sigma^{32}$ mutant proteins analyzed in this study had defects in activity, we investigated whether the mutated segments of region C are involved in the interaction of $\sigma^{32}$ with the RNAP core enzyme. We determined the efficiency of association of $^{3}$H-labeled $\sigma^{32}$ with RNAP by using gel filtration. We focused on the $\sigma^{32}$-F137E and $\sigma^{32}$-I123D mutant proteins, since they showed functional defects in vivo. The relative amounts of the $\sigma^{32}$-core complexes were evident immediately after addition of the competitor, and 5 min after addition almost no

FIG. 4. In vivo stability of the $\sigma^{32}$-F137E mutant protein at 30°C. Wild-type $\sigma^{32}$ and the $\sigma^{32}$-F137E mutant protein were produced from plasmids containing rpoH and tested for their stabilities in both BB2019 cells expressing the dnaK and dnaJ genes from authentic $\sigma^{32}$-dependent heat shock promoters (left panel) and BB7089 cells expressing the dnaK and dnaJ genes from the IPTG-regulated PA1/lacO-1 promoter. After pulse-labeling with $[^{35}$S]methionine and a chase step, aliquots were taken at the indicated time points followed by immunoprecipitation of $\sigma^{32}$ (top). The bottom panels show quantification of the precipitated proteins relative to time zero. Mean values of the results of at least two experiments are given.
holoenzymes containing the $\sigma^{32}$-F137E and $\sigma^{32}$-I123D mutant proteins were recovered (Fig. 7B). We therefore chose shorter chase times to determine the half-lives of the holoenzymes (Fig. 7C, left panel). The half-lives of the holoenzymes containing $^3$H-labeled $\sigma^{32}$-F137E (0.7 ± 0.07 min) and $^3$H-labeled $\sigma^{32}$-I123D (0.8 ± 0.2 min) were five- and fourfold reduced, respectively, compared to the half-life of the holoenzyme containing the wild-type $^3$H-labeled $\sigma^{32}$ (3.5 ± 0.2 min). For the $^3$H-labeled $\sigma^{32}$-F137E mutant protein, we performed additional experiments with a 10-fold excess of $\sigma^{30}$ as competitor and found a 6-fold decrease in the half-life (6.4 ± 0.6 min for the holoenzyme containing $^3$H-labeled $\sigma^{32}$ and 1 ± 0.1 min for the holoenzyme containing $^3$H-labeled $\sigma^{32}$-F137E) (Fig. 7C, right panel). These results show that the mutational alterations in region C decrease the affinity of $\sigma^{32}$ for RNAP by four- to sixfold.

**Transcriptional activity of $\sigma^{32}$ mutant proteins in vitro.** The reduced affinity of the $\sigma^{32}$ mutant proteins for RNAP may have consequences for their activity in the transcription of heat shock genes, in particular in a situation of competition with other sigma factors. This possibility was tested by runoff transcription assays with the $\sigma^{32}$-dependent P2 heat shock promoter of the dnaK dnaJ operon as a template. The reactions were performed in the presence or absence of $\sigma^{30}$ as competitor, with the $\sigma^{32}$-F137E and $\sigma^{32}$-I123D mutant proteins, since they had reduced activities in vivo and reduced ability to compete with $\sigma^{30}$ for RNAP binding in vitro. In the absence of competitor, both $\sigma^{32}$ mutant proteins had strongly reduced activities compared to wild-type $\sigma^{32}$ (Fig. 8). Moreover, the addition of equimolar concentrations of $\sigma^{30}$ was sufficient to strongly reduce the activities of both $\sigma^{32}$ mutant proteins, whereas the activity of wild-type $\sigma^{32}$ was affected only in the presence of a fivefold excess of $\sigma^{30}$ (Fig. 8). The $\sigma^{32}$-F137E and $\sigma^{32}$-I123D mutant proteins thus had reduced activities in heat shock gene transcription, which were further reduced in the presence of $\sigma^{30}$ as competitor.

**Cross-linking of region C of $\sigma^{32}$ to RNAP.** To obtain physical evidence for a role of region C in the association of $\sigma^{32}$ with RNAP, we determined by cysteine-specific cross-linking whether region C is surface exposed within $\sigma^{32}$ and in proximity to the RNAP in the holoenzyme. This approach was facilitated by the fact that $\sigma^{32}$ lacks cysteine, which allowed us to specifically engineer cysteines into region C. Plasmid-borne
**FIG. 7.** Binding of σ^{32} mutant proteins to RNAP. ³H-labeled σ^{32} proteins (wild type [WT], σ^{32}-F137E, and σ^{32}-I123D) (A) were incubated with RNAP, and a 30-fold excess of wild-type unlabeled σ^{32} (B and C, left) or a 10-fold excess of unlabeled σ^{70} (C, right) was added. At the indicated times, samples were subjected to gel filtration, and the amount of labeled protein was quantified and is expressed as the percentage of the total labeled protein (B) or as a relative amount of RNAP-bound σ^{32} recovered at time zero after the addition of competitor (C). (B) Open circles, 0-min chase with competitor; solid squares, 5-min chase; solid triangles, 60-min chase. (C) Open squares, σ^{32}-F137E; solid circles, wild-type σ^{32}; solid triangles, σ^{32}-I123D.

*rpoH* was mutagenized to encode a σ^{32} mutant protein (σ^{32}-TN128,138CC) which has two residues within region C replaced by cysteines (Fig. 1). The σ^{32}-TN128,138CC protein retained full activity in vivo, was a soluble monomer when purified as His tag fusion, and showed similar efficiencies in complex formation with DnaK and RNAP to those of wild-type σ^{32} (data not shown).

After coupling of the cysteine-specific, heterobifunctional cross-linker TFPAM-3 with purified σ^{32}-TN128,138CC, we tested whether DnaK, DnaJ, and RNAP can be specifically cross-linked. To control for nonspecific cross-linking of proteins to σ^{32}-TN128,138CC we tested several unrelated proteins (BSA, lysozyme, immunoglobulin G [data not shown]) and wild-type σ^{32} treated with TFPAM-3 (Fig. 9B). In the presence of a high concentration of DnaK, DnaJ, and DnaK plus DnaJ and ATP, no specific cross-linking product was
observed in silver-stained gels (Fig. 9A) or after immuno-
staining (data not shown). In contrast, three low-abundance
cross-linking products of more than 100 kDa (CL1, CL2,
and CL3) formed in the presence of RNAP. These products
were not generated when wild-type $\sigma^{32}$ lacking cysteines
was used (Fig. 9B). Immunostaining revealed that the cross-
linking products contain RNAP and $\sigma^{32}$-TN128,138CC (Fig.
9). The amount of the lower-molecular-weight band (CL3)
strongly decreased in the presence of an equimolar amount
of wild-type $\sigma^{32}$ as competitor and thus shows specificity
(Fig. 9A). The amounts of the other two cross-linking prod-
ucts (CL1 and CL2) showed only a slight but detectable
reduction upon addition of $\sigma^{32}$. These results are consistent
with a direct role of region C in the association of $\sigma^{32}$ with
RNAP but not with DnaK and DnaJ.

FIG. 9. Cysteine-specific cross-linking of $\sigma^{32}$-TN128,138CC with RNAP, RNAP, DnaK, wild-type $\sigma^{32}$, or $\sigma^{32}$-TN128,138CC proteins were mixed as indicated (+, 150 pmol; ++, 450 pmol). After exposure to UV light, the samples were separated by SDS-PAGE followed by silver staining or immunostaining with $\sigma^{32}$- or RNAP-specific antiserum. Cross-linking products (CL1, CL2, and CL3), DnaK, DnaJ, $\sigma^{32}$, and subunits of RNAP ($\alpha, \beta, \beta^\prime$) are indicated.

DISCUSSION

The aim of this study was to establish whether region C plays
a role in the regulation of $\sigma^{32}$. Such a role had been suggested by (i) the high conservation of region C, in particular of the
nonamer RpoH box, specifically among $\sigma^{32}$ homologs (28);
(ii) demonstration that the in vivo stability of protein fusions
between $\sigma^{32}$ segments and $\beta$-galactosidase strongly increases
when region C is deleted or replaced by other residues (27);
and (iii) identification at the peptide level of two high-affinity
binding sites for DnaK within the RpoH box and peripheral to
region C (25).

In view of the above evidence, it seemed plausible to postu-
late that region C provides recognition sites for DnaK and
FtsH which allow the regulation of $\sigma^{32}$ activity and stability. To
analyze this possibility, we mutagenically altered region C by

![Graphical representation of experimental data showing transcriptional activity and immunostaining results.]

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FIG. 8. In vitro transcriptional activity of $\sigma^{32}$ mutant proteins. Runoff tran-
scription assays were performed in transcription buffer (20 mM Tris-HCl [pH
8.0], 200 mM KCl, 5 mM MgCl$_2$, 1 mM DTT, 5% glycerol) as described previ-
ously (9), with a template consisting of a linear 360-bp DNA fragment (blunt
ends) containing the P2 promoter of dnaK. The transcription assay mixtures
contained 120 nM RNAP, $\sigma^{32}$, wild-type $\sigma^{32}$, $\sigma^{32}$-F137E, or $\sigma^{32}$-I123D as indi-
cated. +, fivefold molar excess (600 nM) of the corresponding protein over the
other proteins in the assay. Transcripts were analyzed by polyacrylamide-urea gel
electrophoresis (9) followed by autoradiography. The relative amounts of tran-
scripts were quantified and are expressed as a percentage of the transcript
obtained with wild-type $\sigma^{32}$ in the absence of $\sigma^{32}$ (defined as 100%).

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<table>
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rational design. Using peptides, McCarty et al. found that a 31-mer peptide comprising the wild-type sequence of region C is a high-affinity substrate for DnaK (K_d ≈ 80 nM) (25). This qualifies region C as recognition site for DnaK. We found that the 21-mer peptide comprising only the RpoH box is an excellent substrate for DnaK (K_d ≈ 40 nM), in accordance with our earlier findings (25), but also for FtsH (t_1/2, 7 min). Replacement of a single hydrophobic residue in this 21-mer peptide, positioned in the hydrophobic core segment of the DnaK binding motif, by a negatively charged residue (F137E) strongly decreased the affinity for DnaK (K_d ≈ 2 μM) and the efficiency of degradation by FtsH (t_1/2, 28 min). To our knowledge, this is the first evidence that a protease and a chaperone recognize the same sequence stretch within a substrate, possibly establishing a competitive relationship allowing kinetic partitioning of the substrate between the chaperone and the protease.

The identification of amino acid substitutions within region C peptides that affect the recognition of DnaK and FtsH provided a rational basis for specific mutagenesis of σ^32. It was surprising that σ^32 mutant proteins carrying the I123D and F137E substitutions, as well as a third mutant protein carrying the WR121,122,123ART substitution which renders the σ^32 sequence more similar to σ^31, showed no defects in affinity for DnaK or in degradation by FtsH. With respect to DnaK, wild-type-like interactions with the σ^32 mutant proteins were found in vitro by gel filtration, surface plasmon resonance spectroscopy, and a functional assay for substrate binding to DnaK. Furthermore, in cells producing σ^32-F137E, σ^32-I123D, or σ^32-WR1121,122,123ART, a DnaK-mediated shutdown of the heat shock response was still observed. However, in these cells the amplitude of the heat shock response was reduced, and in cells producing the σ^32-F137E and σ^32-I123D mutant proteins the induction kinetics were slower than in cells expressing wild-type σ^32. These differences are assumed to result from reduced affinities of the σ^32 mutant proteins for RNAP (see below). With respect to proteolytic susceptibility, the half-life of each σ^32 mutant protein was almost normal in vivo at 30°C, provided that the DnaK and DnaJ levels were adjusted, and in vitro in FtsH- and ATP-dependent degradation assays. It is important to note that the amino acid substitutions in the three σ^32 mutant proteins do not appear to cause overall structural changes, as evidenced by the unaltered partial proteolysis pattern and solubility of the proteins. It is therefore unlikely that the ability of DnaK and FtsH to recognize the σ^32 mutant proteins is caused by exposure of novel recognition sites induced by unfolding. Together, our data indicate that region C is not a regulatory site in σ^32 that is essential for binding of DnaK and degradation by FtsH. Recently, it was proposed that the C termini of protein substrates including λC1 and σ^32 are determinants for degradation by FtsH (1, 13), although recent experiments from our laboratory question the importance of the C terminus of σ^32 for degradation (unpublished results).

In retrospect, the identification of DnaK and FtsH recognition sites at the peptide level did not lead to elucidation of essential DnaK and FtsH recognition sites within the σ^32 protein. Several reasons may account for this failure. It is possible that region C plays a nonessential role in the DnaK- and FtsH-dependent regulation. Alternatively, and perhaps more probably, the segments within region C that act as recognition sites for DnaK and FtsH at the peptide level may not be accessible to interactions with these ligands in the context of the folded σ^32. For DnaK, this may be caused by helix formation by the respective segments in the folded state of σ^32, a conformation that is incompatible with the architecture of the substrate binding cavity (29, 44). It should be emphasized that the approach of using peptides as first indicators for potential chaperone and protease binding sites in protein substrates is not devalued by our findings. However, as expected for folded protein substrates, peptides do not provide information on the accessibility of such sites in the context of a folded protein. Validation of peptide data by transfer of corresponding mutations into the protein substrate is therefore an essential step of this approach. Here we investigated two of approximately seven major DnaK binding regions within the σ^32 sequence. Further experiments will be performed to investigate the regulatory potential of these regions.

Our experimental results provide evidence for a role of region C in the interaction of σ^32 with the core enzyme of RNAP. This is consistent with a similar conclusion obtained from an independent study of mutational alterations within the entire σ^32 protein (15). By glycerol gradient sedimentation analysis, Joo et al. showed that a σ^32 mutant protein altered in region C (F136L) has decreased affinity for RNAP and only partial transcriptional activity in vitro. However, this study did not provide quantitative data on the dissociation rate of the RNAP holoenzyme and did not investigate the in vivo activity of the σ^32 mutant protein and the interaction with FtsH and DnaK. Our present work thus considerably extends the findings of Joo et al. We quantified the RNAP binding defects of the σ^32-F137E or σ^32-I123D mutant proteins by measuring the half-lives of the RNAP holoenzymes in the presence of competitor. For both proteins, a sixfold-decreased half-life was obtained compared to that of the holoenzyme containing wild-type σ^32. These defects in core binding are sufficient to account for the reduced activities of the mutant proteins in vivo at 30 and 42°C and in runoff transcription in vitro at 30°C. However, the in vivo activity of the σ^32-F137E mutant protein is more dramatically affected than that of the σ^32-I123D mutant protein. The reason for this difference is unclear. We speculate that the σ^32-F137E mutant protein has additional defects in promoter recognition.

The ability of region C to increase the affinity of σ^32 for RNAP has physiological consequences. Region C may provide competitive advantages of both σ^32 over other sigma factors for RNAP binding and RNAP over DnaK, DnaJ, and FtsH for σ^32 binding. Region C thereby may increase the efficiency and speed by which the heat shock response is induced upon temperature upshift and the efficiency of heat shock gene transcription under steady-state conditions. In fact, only 10 to 30 molecules of σ^32 that exist in a cell growing at 30°C (35) are sufficient to produce HSPs that account for at least 5% of the total cytosolic protein (11). The observed delay in the induction of the heat shock response in cells producing the σ^32, F137E and σ^32-I123D mutant proteins is consistent with this proposed role of region C.

Our cross-linking experiments provide the first indication that region C is in close proximity to the RNAP. It is possible that region C provides directly the physical contacts which increase the affinity of σ^32 for RNAP core. However, since the sequence of region C is absent in other sigma factors not belonging to the σ^32 branch, it is clear that this region cannot be involved in essential contacts to RNAP but that it seems to be required to enhance affinity. Several regions within the polypeptide chains of σ^32 and σ^70, including the conserved regions 2.1., 2.2., 3, and 4, have been implicated in core interaction (15, 19, 24, 26, 33, 43). This multitude of potential interacting sites suggests a high structural complexity of the sigma factor-RNAP interaction which may be fully understood only upon elucidation of the atomic structures of the involved proteins.
ROLE OF REGION C IN REGULATION OF $\alpha^{32}$

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