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Suppression of a *dnaKJ* Deletion by Multicopy *dksA* Results from Non-Feedback-Regulated Transcripts That Originate Upstream of the Major *dksA* Promoter

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DksA is an RNA polymerase (RNAP) binding transcription factor that controls expression of a large number of genes in concert with the small-molecule "alarmone" ppGpp. DksA also aids in the resolution of conflicts between RNAP and DNA polymerase (DNAP) during genome replication. DksA was originally identified as a multicopy suppressor of the temperature sensitivity caused by deletion of the genes coding for the DnaKJ chaperone system. Here, we address a longstanding question regarding the role of DksA in Δ*dnaKJ* suppression. We demonstrate that DksA expression from a multicopy plasmid is necessary and sufficient for suppression, that overexpression occurs despite the fact that the major *dksA* promoter is feedback regulated in wild-type cells, and that weak, non-feedback-regulated transcription originating upstream of the major promoter for the *dksA* gene accounts for overexpression. We tentatively rule out three potential explanations for suppression related to known functions of DnaKJ. Because a determinant in DksA needed for the regulation of transcription initiation, but not for resolution of RNAP-DNAP conflicts, is needed to bypass the need for DnaKJ, we suggest that suppression results from an unidentified product whose promoter is directly or indirectly regulated by DksA.

In conjunction with efforts to identify a function(s) of DnaKJ, the *dksA* (DnaKJ suppressor A) gene was originally identified as a multicopy suppressor of the temperature-sensitive growth and filamentation phenotypes of *dnaKJ* deletion mutants (14). Following this initial observation, studies on DksA focused primarily on its role as an RNA polymerase (RNAP) binding transcription factor that is necessary for the proper regulation of many genes, including those involved in translation, motility, pathogenesis, amino acid biosynthesis, and defense against antioxidant stress (13, 17–19, 21, 25–27). DksA exerts its effects by binding directly to the secondary channel of RNAP and allosterically modifying the kinetic properties of the enzyme, thereby sensitizing RNAP to changes in the concentrations of guanosine tetraphosphate (ppGpp) and the initiating nucleoside triphosphate (iNTP) (26, 29, 34, 35). D74, an aspartate residue at the tip of the coiled-coil domain, is necessary for DksA to function in transcription initiation (16). In addition, DksA aids in resolving conflicts between the active DNA replication machinery and stalled or elongating RNA polymerases, and it helps to ensure the proper resolution of double-strand breaks caused by these collisions (22, 42, 45). It appears that D74 is not required for these latter functions, suggesting a mechanistically distinct role of DksA in regulating transcription initiation and elongation (42).

DksA protein levels remain the same during different phases of growth (26, 34). Constant levels of DksA are maintained by a negative feedback loop in which expression from the *dksA* gene is regulated at the transcription initiation step by its own gene product, in conjunction with ppGpp (6). DksA levels do not increase even when transcription from the *dksA* gene originates from its native promoter on a multicopy plasmid. The RNA binding protein CsrA can weakly regulate DksA levels, but its effects are masked by the DksA autoregulatory loop (8).

DnaKJ is one of a number of heat shock proteins (HSPs) that are induced during the heat shock response (HSR), one of the most evolutionarily conserved regulatory responses in biology. As a response to elevated temperatures, a cohort of chaperones, cochaperones, and proteases are produced in response to an increase in the amount of unfolded or misfolded proteins in the cytoplasm (12). Expression of the genes encoding cytoplasmic HSPs is mediated primarily by the sigma factor σ^{32}, whereas expression of the genes encoding stress-responsive periplasmic or extracellular functions is mediated primarily by the extracytoplasmic sigma factor σ^{E} (1, 10, 33). The synthesis, level, and activity of σ^{32} are highly regulated at the levels of translation and protein stability (39). Upon induction of the HSR, σ^{32} levels increase as translation of the *rpoH* mRNA is derepressed and unfolded proteins titrate away chaperones and proteases (including those responsible for destabilizing σ^{32}), leading to an increase in σ^{32} holoenzyme levels and increased transcription from heat shock promoters (40). As cells adapt to elevated temperatures, unfolded proteins become less abundant, they no longer sequester the heat shock proteins, σ^{32} levels decline, and the activities of heat shock promoters decrease to a new steady state, leading to a decline in HSP synthesis (41).

In addition to its synthesis, the activity of σ^{32} is also regulated. Regulation of σ^{32} activity is carried out in large part by the chaperone DnaK and its cochaperone DnaJ (44), one of the major cytoplasmic chaperone systems responsible for promoting proper

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protein folding under normal and stress conditions (5). DnaKJ plays a role in the inactivation and degradation of σ^{32} through direct interactions with free σ^{32} (9, 11, 20). Strains without functional DnaKJ display increased HSP synthesis at 30°C and fail to turn off the HSR at 42°C (39, 43). These mutants also display temperature and cold-sensitive growth phenotypes, they are filamentous, and they are defective for λ phage growth (14).

We recently showed that a feedback system keeps DksA concentration constant even when the *dksA* gene is expressed from its native promoter on a multicopy plasmid (6). However, *dksA* was originally identified as a multicopy suppressor of the filamentation and growth defects of a Δ*dnaKJ* mutant at 42°C (14), suggesting that suppression might result from overproduction of DksA. We demonstrate here that overproduction of DksA does in fact occur in this context and that it is necessary and sufficient for suppression. We show that DksA overproduction results from transcripts originating upstream of the major *dksA* promoter in the original multicopy suppressor plasmid that are not feedback regulated, thereby bypassing control by the autoregulation system. Although we have not identified the specific function of DnaKJ, whose absence results in temperature-sensitive lethality and is suppressed by overproduction of DksA, our experiments suggest that DksA's role in regulation of transcription initiation is responsible for multicopy suppression.

MATERIALS AND METHODS

Strains, plasmids, and proteins. The strains and plasmids used in this work are provided in Table 1. Promoter fragments were inserted into plasmids and recombined into λRS415, and single-copy chromosomal promoter-*lacZ* fusions were created in VH1000 as λ lysogens (38) (referred to previously as system II) (30). Promoter endpoints for *lacZ* fusions are indicated in the figure legends and in Table 1.

Construction of the Δ*dksA* strains was performed by transduction of a *dksA*::*tet* insertion-deletion (28) with P1*vir* (24). The Δ*dnaKJ* strain, pK101, and the Δ*dnaKJ*-suppressing plasmid, pJK537, were gifts from Elizabeth Craig (University of Wisconsin—Madison [UW-Madison]). The construction of these plasmids and strains was described previously (14).

Plasmids used in the Δ*dnaKJ* suppression assay were constructed by amplification of the region of interest from pJK537 by PCR and insertion of the fragment of interest into the EcoRI and HindIII sites of pRLG770 (32). Site-directed mutagenesis of the *dksA* gene was carried out using the QuikChange Lightning mutagenesis kit (Stratagene). All constructs were verified by DNA sequencing.

Δ*dnaKJ* suppression assay. MG1655 or MG1655Δ*dnaKJ Escherichia coli* transformed with the plasmids listed in the legend to Fig. 1 or in Table 1 was grown on LB plates overnight at 30°C in the presence or absence of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Colonies were then scraped from these plates, diluted to an initial optical density at 600 nm (OD_{600}) of 0.02, and grown in the presence or absence of 0.2 mM IPTG with shaking at 30°C to an OD_{600} of ~0.3. Appropriate dilutions were plated on LB agar in the presence or absence of 0.2 mM IPTG and incubated at 30°C or 42°C overnight. Colonies were counted, and plating efficiency was calculated as the fraction of colonies formed at 42°C/30°C.

***In vivo* promoter activity assay.** Cells containing promoter-*lacZ* fusions were scraped from LB plates grown overnight at 30°C, suspended in LB to an initial OD_{600} of 0.02, and grown with shaking at 30°C to an OD_{600} of ~0.3. One-milliliter samples were removed to tubes on ice containing 4 ml of Z buffer (24), incubated on ice for 30 min, and lysed by sonication, and β-galactosidase activity was measured as described previously (3). Background activity was less than 1 Miller unit under these conditions (30).

RNA extraction and primer extension. RNA was extracted using a hot-phenol method from wild-type and Δ*dksA* cells containing pBR322 or pJK537, and transcription *in vivo* was analyzed by primer extension as described previously (31). Cells were grown in morpholinepropanesulfonic acid (MOPS) minimal medium supplemented with 0.4% glucose, 0.4% Casamino Acids, 40 μg/ml tryptophan, and 10 μg/ml thiamine at 30°C to an OD_{600} of ~0.3. Aliquots of cells were added to hot phenol and SDS at appropriate time intervals, and the aqueous phase was then precipitated with ethanol and suspended in 10 mM Tris-Cl, pH 8.0. For primer extension, 19 μl of RNA (from ~7 ml of the original culture), 5 μl of 5× FS buffer (Invitrogen), and 1 μl (~3 ng) of one of the ^{32}P-labeled primers (see Fig. 3) were incubated together at 80°C for 10 min and placed on ice for 1 min, and the primer extension reaction was initiated by incubation of 2.5 μl of 5 mM deoxynucleoside triphosphates (dNTPs), 1 μl of 0.1 mM dithiothreitol (DTT), 1 μl of 5× FS buffer, and 0.5 μl of SuperScript III reverse transcriptase (Invitrogen) with the RNA-primer mix at 48°C for 30 min. Reactions were stopped by addition of an equal volume of formamide stop solution (95% formamide, 20 mM EDTA, pH 8.0, 0.05% xylene cyanol, 0.05% bromophenol blue). To map the *dksA* transcription start site, the product of the reverse transcription reaction was examined on an 11% polyacrylamide-urea gel next to a sequencing ladder generated from pJK537 using the ThermoSequenase cycle sequencing kit (USB).

***In vitro* luciferase refolding assay.** Luciferase (13 mg/ml in 1 M glycylglycine, pH 7.4) was diluted 40-fold in denaturation buffer (25 mM HEPES, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, and 5 mM DTT). For experiments in which other components of the reaction were present prior to thermal denaturation of luciferase, wild-type DksA, D74N DksA, Ssa1 and Sis1, or bovine serum albumin (BSA) was incubated at 30°C for 10 min at the concentrations given in Fig. 4. Ssa1 and Sis1 were generous gifts from Elizabeth Craig (UW-Madison). Thermal denaturation was achieved by incubation at 42°C for 15 min. An 0.5-μl aliquot of the denatured luciferase was removed and added to 62 μl of refolding buffer (25 mM HEPES, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 2 mM ATP). When added after luciferase denaturation, wild-type DksA, D74N DksA, Ssa1 and Sis1, or BSA was incubated with the enzyme at 30°C, and then a 1.5-μl aliquot of each sample was removed at appropriate time intervals and added to 24 μl of dilution buffer (25 mM HEPES, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT). Samples were then mixed with 50 μl of luciferase assay reagent (Promega) at room temperature for 10 min, and luciferase activity was measured using a TD 20/20 luminometer (Turner).

Western blots. For experiments measuring DksA protein levels, cells were grown with shaking at 30°C in LB to an OD_{600} of ~0.3 in the presence or absence of 0.5 mM IPTG. Samples (1 ml) were added to cold 100% trichloroacetic acid (TCA) (7% final concentration), vortexed briefly, incubated on ice for at least 15 min, and then centrifuged at 20,000 × *g* for 15 min. After removal of the supernatant, the pellet was washed with 100% cold acetone, the pellet was suspended in 50 to 100 μl of SDS resuspension buffer (2% SDS, 20 mM sodium phosphate, pH 7.5, 10 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride [PMSF]), and protein concentration was determined by the Bradford assay (Bio-Rad). Five micrograms of total protein was loaded on a 10% Bis-Tris gel (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare) using a semidry transfer apparatus (Hoefer), and blots were probed with an anti-DksA polyclonal antibody (a gift from Diana Downs, UW-Madison) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnologies) and imaged using ECL+ (Amersham) and a Typhoon (GE Healthcare) scanner.

For experiments measuring σ^{32} or GroEL levels, cells were grown with shaking at 30°C in LB to an OD_{600} of ~0.3 and shifted to 42°C; aliquots were removed at appropriate time intervals; and gel electrophoresis, transfer, and blotting were performed as described above except using monoclonal anti-σ^{32} (Neoclone, Madison, WI) or polyclonal GroEL (from Elizabeth Craig, UW-Madison) antibodies and HRP-conjugated

TABLE 1 Strains, plasmids, and primers used in this study[a]

Strain, plasmid, or primer	Genotype, description, and/or sequence (5′–3′)	Plasmid	P*dksA-lacZ* endpoints	Reference
Strains				
RLG3499	VH1000 = MG1655 *pyrE*$^+$ *lacZ lacI*			30
RLG8169	MG1655			14
RLG8170 (pJK101)	MG1655 *dnaK14 dnaJ14*			14
RLG8173	MG1655 *dnaK14 dnaJ14*	pJK537		14
RLG8174	MG1655 *dnaK14 dnaJ14*	pRLG6333		This work
RLG10101	VH1000		−100 to +37	6
RLG10103	VH1000 *dksA*::*tet*		−100 to +37	6
RLG11308	MG1655 *dnaK14 dnaJ14*	pRLG11308		This work
RLG11310	MG1655 *dnaK14 dnaJ14*	pRLG11310		This work
RLG11314	MG1655 *dnaK14 dnaJ14*	pRLG11314		This work
RLG11316	MG1655 *dnaK14 dnaJ14*	pRLG11316		This work
RLG11317	VH1000		−1332 to +37	This work
RLG11318	VH1000		−630 to +37	This work
RLG11319	VH1000		−1332 to −100	This work
RLG11320	VH1000		−630 to −100	This work
RLG11321	VH1000 *dksA*::*tet*		−1332 to +37	This work
RLG11322	VH1000 *dksA*::*tet*		−630 to +37	This work
RLG11323	VH1000 *dksA*::*tet*		−1332 to −100	This work
RLG11324	VH1000 *dksA*::*tet*		−630 to −100	This work
RLG11325	MG1655 *dnaK14 dnaJ14*	pRLG8873		This work
RLG11326	MG1655 *dnaK14 dnaJ14*	pRLG11326		This work
RLG11327	MG1655	pBR322		This work
RLG11328	MG1655 *dnaK14 dnaJ14*	pBR322		This work
Plasmids				
pBR322	Cloning vector			
pJK537	1.7-kb fragment of originally isolated *dnaKJ* suppressor clone inserted into pBR322			14
pRLG770	pBR322-based *in vitro* transcription vector			32
pRLG6333	pINIIIA-based complementation vector with DksA expressed from an IPTG-inducible *lpp-lac* promoter			26
pRLG8873	pRLG6333 with DksA D74N			16
pRLG11308	pRLG770 with the full 1.7-kb insert from pJK537			This work
pRLG11310	pRLG770 with a 1.3-kb fragment of insert from pJK537			This work
pRLG11314	pRLG770 with 1.2-kb insert from pJK537 without *dksA* and *yadB* ORFs			This work
pRLG11316	pRLG770 with 0.7-kb fragment of insert from pJK537			This work
pRLG11326	pRLG11308 with DksA D74N			This work
Primers				
RLG4041	TTCTCCTTAACACGCACTATCGATCCCCATG, primer 1 (Fig. 3)			6
RLG4033	TGCGCACGCGACCAGAATGT, primer 2 (Fig. 3)			This work
RLG4043	AGAGGGGGAGAAAATTCCAT, primer 3 (Fig. 3)			This work
RLG5865	GTCAATACGGCTGCGTTCTGCGC, primer 4 (Fig. 3)			This work
RLG5866	CAACCGCTGCGGGCAGCCTGT, primer 5 (Fig. 3)			This work

[a] Promoter endpoints for P*dksA-lacZ* fusions are numbered relative to the transcription start site. All P*dksA-lacZ* fusions are on phage lambda prophage integrated in single copy on the bacterial chromosome. Plasmids were used for Δ*dnaKJ* complementation assays. Primers were used in transcription start site mapping experiments. ORF, open reading frame.

goat anti-mouse (σ^{32}) or anti-rabbit (GroEL) IgG secondary antibodies (Santa Cruz Biotechnologies).

RNAP-promoter complex decay assay. Promoter complex half-lives were determined by an *in vitro* transcription-based assay (3). Briefly, supercoiled plasmids (1 nM) containing the *rrnB* P1 or *dnaKJ* P1 promoter were preincubated with 10 nM Eσ^{70} or Eσ^{32} RNAP (σ^{32} was a gift from Richard Burgess, UW-Madison) in the presence or absence of DksA (0.2 μM) in transcription buffer containing 50 mM NaCl, 40 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mg/ml BSA at 30°C for 10 min. Double-stranded σ^{70} full consensus promoter DNA (700 nM) was added to the reaction mixture as a competitor for free RNAP (26), aliquots were removed at the indicated time intervals, and transcription was initiated by addition of NTPs (final concentrations, 500 μM ATP, 200 μM CTP, 200 μM GTP, 10 μM UTP, and 1.0 μCi [α-^{32}P]UTP). After 10 min at 30°C, the reactions were stopped by addition of an equal volume of formamide stop solution, and transcripts were separated on a 6% polyacrylamide gel containing 7 M urea, scanned on a Typhoon (GE Healthcare) scanner, and quantified using ImageQuant and Sigmaplot software.

RESULTS

Overproduction of DksA is necessary and sufficient to suppress the temperature-sensitive phenotype of a Δ*dnaKJ* mutant. The original multicopy suppressor pJK537 (see introduction) derived from a library constructed from a partial digest of genomic DNA inserted into pBR322 (14). pJK537 contains an ~1.7-kb insert

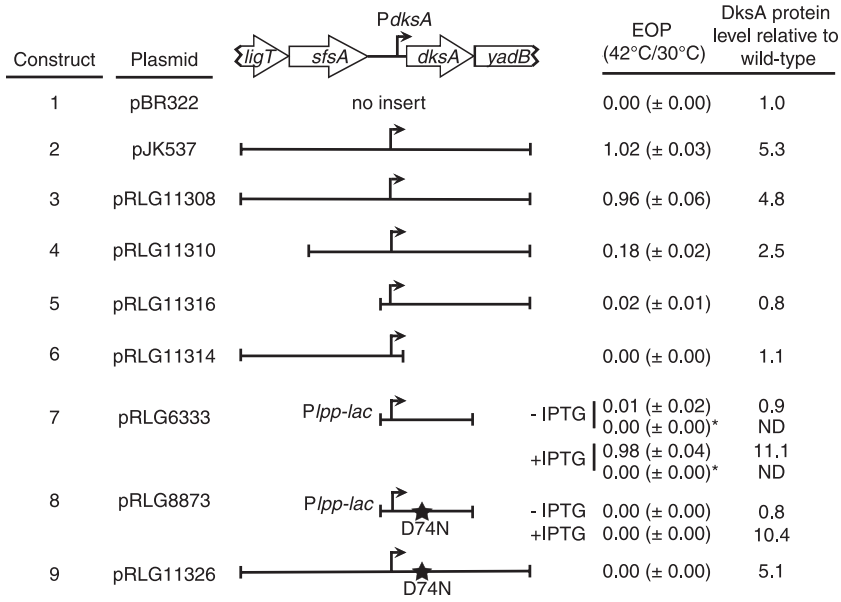

FIG 1 Overproduction of wild-type DksA is required to suppress the temperature-sensitive phenotype of a Δ*dnaKJ* mutant. Efficiencies of plating (EOP; mean and standard deviation) of Δ*dnaKJ* strains transformed with plasmids containing inserts with various upstream endpoints relative to the *dksA* gene at 42°C versus 30°C after growth in LB or in LB plus IPTG (lines 7 and 8). Values marked with an asterisk were grown in LB without IPTG before plating. ND, not determined. DksA protein level as measured by Western blotting relative to wild-type levels (averages of three independent experiments). The endpoints of the DNA fragments carried by the plasmid vector with respect to the primary *dksA* transcription start site (+1) are as follows: line 1, pBR322 control, no insert; line 2, −1332 to +769; line 3, −1332 to +769; line 4, −630 to +769; line 5, −100 to +769; line 6, −1332 to +37; lines 7 and 8, not applicable; line 9, −1332 to +769. The primary transcription start site for the *dksA* gene is 54 bp upstream of the translation start site (6).

that includes the last 486 nucleotides of *ligT*, the entire *sfsA* and *dksA* coding regions, and the first 224 nucleotides of *yadB* (Fig. 1). Deletion mapping of the plasmid led to the conclusion that the *dksA* gene was required for suppression, and it was proposed that overproduction of DksA might be responsible (14).

However, we showed recently that *dksA* expression is feedback regulated in its native context and that DksA is not overexpressed even when cells contain a multicopy plasmid with the *dksA* gene expressed from its native promoter (6). Therefore, we decided to investigate whether the *dksA* gene itself is sufficient for rescue of the *dnaKJ* mutant, whether DksA is really overproduced from pJK537, and, if so, what accounts for this potential overproduction.

As demonstrated in the original study (14), pJK537 suppressed the temperature sensitivity of a Δ*dnaKJ* mutant at 42°C (Fig. 1, line 2, efficiency of plating [EOP], ~1.02). To address the possibility that a mutation in the *dksA* promoter or coding region was responsible for DksA overproduction from pJK537, we resequenced the entire 1.7-kb insert. No mutations were found compared to the genomic sequence reported in EcoCyc (15; P. Chandrangsu and R. L. Gourse, data not shown). To rule out the possibility that suppression was somehow related to the identity of the plasmid backbone, we amplified the insert from the suppressor plasmid and cloned it into another pBR322-derived plasmid, pRLG770 (32), to form pRLG11308. This construct, which contains genomic DNA sequences 1,332 bp upstream to 769 bp downstream from the primary *dksA* transcription start site, retained the ability to suppress the Δ*dnaKJ* phenotype (EOP, ~0.96) (Fig. 1, line 3).

However, a construct with genomic sequences extending only 630 bp upstream from the *dksA* transcription start site suppressed the Δ*dnaKJ* phenotype only partially (EOP, ~0.18) (Fig. 1, line 4), and a construct that contained only 100 bp upstream from the transcription start site but included the primary *dksA* promoter, P*dksA*, was unable to suppress the Δ*dnaKJ* phenotype (EOP, ~0.02) (Fig. 1, line 5). These data indicated that suppression is dependent on DNA sequences upstream from the primary *dksA* promoter. A plasmid containing the upstream sequences alone without the *dksA* gene (−1332 to +37) was unable to suppress (Fig. 1, line 6), suggesting that effects of the upstream sequences on *dksA* expression were necessary for suppression.

We next addressed whether overproduction of DksA was sufficient for suppression by using a plasmid with an IPTG-inducible *lpp-lac* promoter fused to the *dksA* gene, pRLG6333 (24). A *dnaKJ* deletion strain carrying pRLG6333 was grown in LB in the presence or absence of 1 mM IPTG to an OD_{600} of ~0.3 and plated with or without 0.2 mM IPTG at 42°C. The EOP of cells grown and plated without IPTG was ~0.01, whereas the EOP of cells grown and plated with IPTG was ~0.98 (Fig. 1, line 7). When cells were grown without IPTG but plated on IPTG (so that DksA was overproduced only after plating), there was no suppression (values marked with an asterisk; Fig. 1, line 7), indicating that high levels of DksA expression are needed prior to heat shock for relief from temperature sensitivity.

Western blotting assays were used to address whether the amount of DksA produced in the Δ*dnaKJ* mutant at 42°C from the various constructs is correlated with the level of suppression. The plasmids that fully suppressed the Δ*dnaKJ* mutation, namely, the original suppressor, pJK537; the plasmid containing the same DNA fragment in a different plasmid backbone, pRLG11308; and the plasmid containing the IPTG-inducible promoter, pRLG6333, all greatly overproduced DksA (5.3-fold, 4.8-fold, and 11.1-fold,

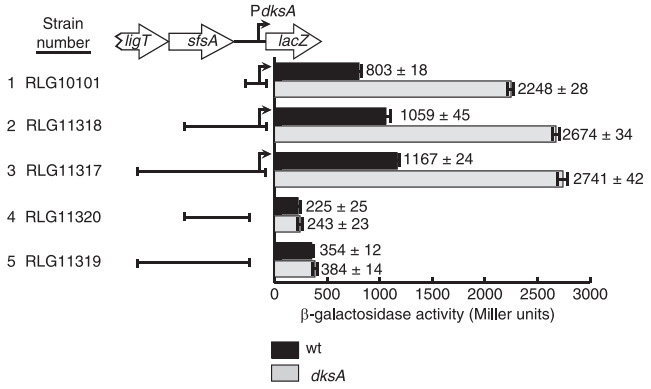

FIG 2 Overproduction of DksA from pJK537 is the result of transcription arising from DNA upstream of P*dksA*. β-Galactosidase activities were determined in wild-type (wt) and Δ*dksA* strains carrying P*dksA-lacZ* fusions with various upstream endpoints (see Materials and Methods). Values given are the average and standard deviation calculated from three independent experiments. The endpoints of the DNA fragments used for construction of the promoter-*lacZ* fusions are as follows: line 1, −100 to +37; line 2, −630 to +37; line 3, −1332 to +37; line 4, −630 to −100; line 5, −1332 to −100.

respectively) (Fig. 1, lines 2, 3, and 7, respectively). The plasmid with genomic sequences extending only 630 bp upstream (pRLG11310), which only partially suppressed the Δ*dnaKJ* mutant, overproduced DksA only 2.5-fold (Fig. 1, line 4), and the construct with only the primary *dksA* promoter (pRLG11316), which resulted in an EOP of only ~0.02, did not overexpress DksA (it produced only ~80% as much DksA as did the wild-type strain) (Fig. 1, line 5). Taken together, the data indicated that overproduction of DksA, resulting from the *dksA* gene in multicopy as well as the presence of sequences upstream of the major DksA promoter, is necessary and sufficient to suppress the temperature-sensitive phenotype of the Δ*dnaKJ* mutant.

A residue essential for transcription regulation by DksA is critical for suppression of a Δ*dnaKJ* mutant. An aspartic acid residue near the tip of the coiled-coil domain (D74) is essential for DksA function as a regulator of transcription initiation (16) but not for DksA function in DNA replication (42). We introduced a D74N variant under the control of the IPTG-inducible *lpp-lac* promoter (pRLG8873; Fig. 1, line 8) or in the context of pJK537 (pRLG11326; Fig. 1, line 9) into the *dnaKJ* deletion strain. When D74N DksA was overexpressed more than 5-fold (pRLG11326) or more than 10-fold (pRLG8873), the Δ*dnaKJ* deletion strain was still unable to grow at 42°C (Fig. 1, lines 8 and 9). These results imply that suppression of the temperature sensitivity of a Δ*dnaKJ* strain requires DksA's ability to regulate transcription initiation but not to resolve RNAP-DNA polymerase (DNAP) collisions.

Transcription from DNA upstream from P*dksA* is not subject to feedback regulation by DksA. One possible explanation for the correlation between DksA levels and the amount of DNA upstream of the *dksA* gene in the multicopy plasmid constructs is that there is transcription that originates upstream from the major *dksA* promoter that leads to overexpression in the multicopy vector because it is not regulated by ppGpp/DksA. To test this hypothesis, we measured transcriptional activity from a series of *dksA* promoter-*lacZ* fusions containing the same extents of upstream DNA as shown in Fig. 1 (Fig. 2, black bars). At 30°C in LB, transcription from the promoter-*lacZ* fusion with only the primary *dksA* promoter (fragment endpoints, −100 to +37) was ~803 Miller units (line 1). As increasing extents of upstream DNA were included (to positions −630 and −1332 relative to the primary start site +1; lines 2 and 3), transcription activity increased to ~1,059 and ~1,167 Miller units, respectively. Fusions constructed with fragments containing only upstream DNA, without the primary *dksA* promoter (−630 to −100 and −1332 to −100; lines 4 and 5), had activities of ~225 and ~354 units, respectively. The *lacZ* fusions that did not include the primary *dksA* promoter were active enough to account for the differences between fusions 1 to 3, since background activity in this promoter-*lacZ* fusion system is very low (only ~1 Miller unit; Materials and Methods). Therefore, it is unlikely that the upstream DNA sites function by stimulating the primary *dksA* promoter. Rather, the data suggest that the additional transcription activity derives from transcripts that originate within the upstream DNA.

To address whether the weak, putative upstream transcription initiation events were subject to feedback regulation by ppGpp/DksA, we measured the activities of the promoter-*lacZ* fusions in a strain lacking the *dksA* gene. The β-galactosidase activities from constructs containing the primary DksA promoter increased ~3-fold in the Δ*dksA* strain (Fig. 2, gray bars, lines 1, 2, and 3), consistent with the results that we reported previously (6). In contrast, the Δ*dksA* mutation did not increase transcription activity from fusions containing only the upstream DNA sequences (Fig. 2, gray bars, lines 4 and 5), suggesting that those transcription events are not feedback regulated by DksA. These results suggest that transcription events originating from the upstream sequences, although somewhat infrequent, are sufficient to account for the overexpression of DksA, because they are not feedback regulated and they are present on a multicopy plasmid.

As a further test of the hypothesis that there are transcripts that originate upstream of P*dksA* in the multicopy suppressor plasmid, we performed primer extension using RNA isolated from a Δ*dnaKJ* strain containing either pJK537 or pBR322. Oligonucleotides that were complementary to different regions upstream of the *dksA* gene were used (positions where primers hybridized are pictured in Fig. 3A; the primer numbers correspond to the lane numbers at the bottom of Fig. 3B).

One moderately strong product resulted from extension of primer 1, specific to the strain containing pJK537 (Fig. 3B, asterisk, lane 7). Alignment with DNA sequence markers generated using the same primer (lanes 1 to 4) allowed identification of putative Eσ^{70} recognition hexamers corresponding to this extension product (DNA sequence of this region is at the left of the gel image). This potential promoter was also identified previously in two genome-wide transcription start site surveys (7, 23). We suggest that unregulated transcription activity that resulted from the −630 to −100 fragment (Fig. 2, line 4), as well as the corresponding partial suppression of the Δ*dnaKJ* mutation that resulted from this region in the complementation experiment (Fig. 1, line 4), might derive in part from this promoter.

Weaker extension products were also observed in the lanes containing pJK537 (Fig. 3B, arrows in lanes generated by primers 1, 2, 3, and 5). These products are consistent with the small incremental amount of transcription activity that resulted from inclusion of DNA upstream of −630 (Fig. 2, line 5). Alignment of these extension products with DNA sequence markers made from primers 1, 2, 3, and 5 identified the positions of putative transcription start sites (Chandrangsu and Gourse, data not shown). However, no obvious recognition elements for binding Eσ^{70} or alter-

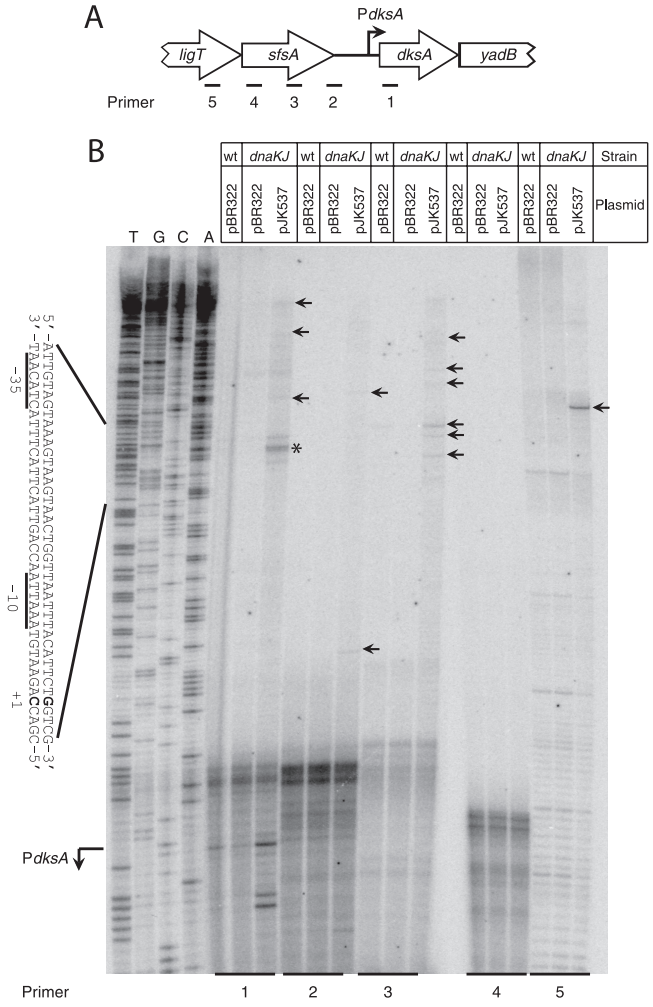

FIG 3 Primer extension analysis of transcripts initiating upstream of P*dksA*. RNA was isolated at an OD_{600} of ~0.3 from wild-type (wt) or Δ*dnaKJ* strains transformed with pBR322 or pJK537, and primer extension was performed using the five ^{32}P-labeled primers indicated below the map at the top of the figure. The numbers under the gel image refer to the primers used in the indicated lanes. Sequence markers for the reactions with primer 1 are in lanes 1 to 4. The band indicated with an asterisk in the primer 1 lane with pJK537 corresponds to a previously described start site described in the text. The sequence of the putative σ^{70}-dependent promoter upstream of this start site is at the left of the gel image, along with the possible corresponding RNAP recognition elements (underlined) and the transcription start site (bold). The position of the major promoter responsible for expression of the *dksA* gene (P*dksA*) is also indicated at the left of the gel image. Weak bands marked with arrows indicate that there may be additional weak transcription start sites upstream of P*dksA*. Alignment with sequence markers generated by primers 1 to 5 did not identify likely RNAP recognition elements correlating with these potential start sites (see Results). Dark bands near the bottom of the gel are incomplete extension products.

native holoenzymes were identifiable upstream of these potential start sites, perhaps because some represent very weak promoters with correspondingly poor consensus elements for binding RNAP and/or because the bands resulted not from transcription initiation events but rather from incomplete extension or RNA processing events.

Taken together, the Δ*dnaKJ* suppression data, the activities of the promoter-*lacZ* fusions, and the primer extension analysis are consistent with the model that multiple, weak, unregulated transcription events originating from the DNA upstream of the primary *dksA* promoter in the multicopy *dksA* plasmid account for DksA overproduction in the Δ*dnaKJ* strain.

DksA does not appear to exhibit chaperone function. Although non-feedback-regulated transcription from the multicopy plasmids described above accounts for DksA overproduction, it does not explain why high levels of DksA result in Δ*dnaKJ* suppression. Our ability to define the mechanism of suppression is limited by the fact that the basis for lethality of Δ*dnaKJ* mutant strains after exposure to heat shock is not known. Nevertheless, we generated three hypotheses for the mechanism of suppression by DksA and performed preliminary tests to address their validity.

First, DksA might suppress the Δ*dnaKJ* mutation by acting as a chaperone itself, compensating for the absence of DnaKJ chaperone function even though DksA and DnaKJ have no obvious structural similarity. We used a luciferase *in vitro* refolding assay to measure chaperone activity. Ssa1 and Sis1, the *Saccharomyces cerevisiae* homologs of DnaK and DnaJ, respectively, have been shown previously to exhibit chaperone activity comparable to that of DnaKJ/GrpE in this assay and served as a positive control (2, 36). After thermal denaturation of luciferase, incubation with Ssa1 and Sis1 resulted in restoration of ~60% of activity within 15 min, whereas wild-type DksA and D74N DksA exhibited no ability to restore luciferase activity, even after 60 min of incubation (Fig. 4A). The DnaKJ homologs also resulted in ~60% luciferase activity when they were preincubated with luciferase prior to thermal denaturation (Fig. 4B). In contrast, only ~30% of the luciferase activity was retained when DksA was preincubated with luciferase prior to denaturation, this activity did not increase with time, and it was the same with the wild-type and D74N DksA (Fig. 4B), a mutant protein that was incapable of suppression of the *dnaKJ* temperature-sensitive phenotype (Fig. 1). We therefore conclude that this modest, D74-DksA-independent effect of DksA on activity *in vitro*, observed only when DksA was preincubated with luciferase, is unrelated to the *in vivo* suppression. However, we recognize that we have not ruled out that DksA has a chaperone-like activity that functions on some other substrates.

GroEL protein levels do not change when DksA is overexpressed. It was reported previously that overproduction of a different chaperone, GroEL, can complement a Δ*dnaKJ* mutant (39, 46). Therefore, we tested whether DksA's ability to suppress a Δ*dnaKJ* mutant could be explained by an effect on GroEL levels.

As expected, GroEL levels measured in Western blot assays increased in wild-type cells exposed to heat shock (Fig. 5A and black bars in Fig. 5B). In the Δ*dnaKJ* strains without a heat shock, GroEL levels were elevated ~2-fold compared to the wild-type strain (Fig. 5A and light gray bars in Fig. 5B at 0 min), consistent with the role of DnaKJ in regulation of σ^{32}. However, overproduction of DksA from pJK537 did not significantly affect the level of GroEL (Fig. 5A and dark gray bars in Fig. 5B). Thus, an increase in *groEL* expression is not responsible for suppression of the Δ*dnaKJ* strain by multicopy *dksA*.

We also measured the effect of DksA on a σ^{32}-dependent promoter complex *in vitro* to address whether this class of promoters might be particularly susceptible to the effects of DksA. We found that the lifetime of a σ^{32}-dependent promoter complex (Eσ^{32}-*dnaKJ* P1) was actually affected much less by DksA than was that of a σ^{70}-dependent promoter complex under the same solution conditions (1.5-fold reduction in the half-life of the Eσ^{32}-

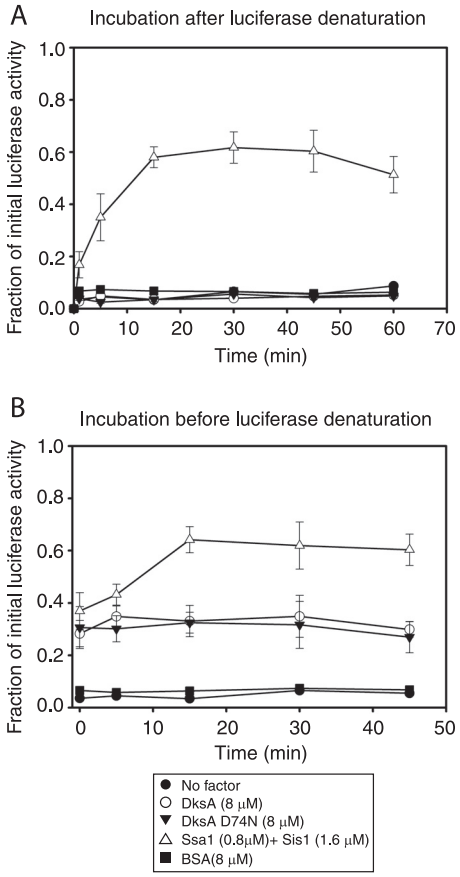

FIG 4 DksA does not complement the chaperone activity of DnaKJ. (A) A plot of an *in vitro* luciferase refolding assay where thermally denatured luciferase was allowed to refold at 30°C in the presence of wild-type or D74N DksA (8 μM), Ssa1 and Sis1 (0.8 and 1.6 μM, respectively), or BSA (8 μM). (B) A plot of an *in vitro* luciferase refolding assay where luciferase was preincubated with wild-type or D74N DksA (8 μM), Ssa1 and Sis1 (0.8 and 1.6 μM, respectively), or BSA (8 μM) prior to thermal denaturation and allowed to refold at 30°C. Values given represent the average and standard deviation from three independent experiments.

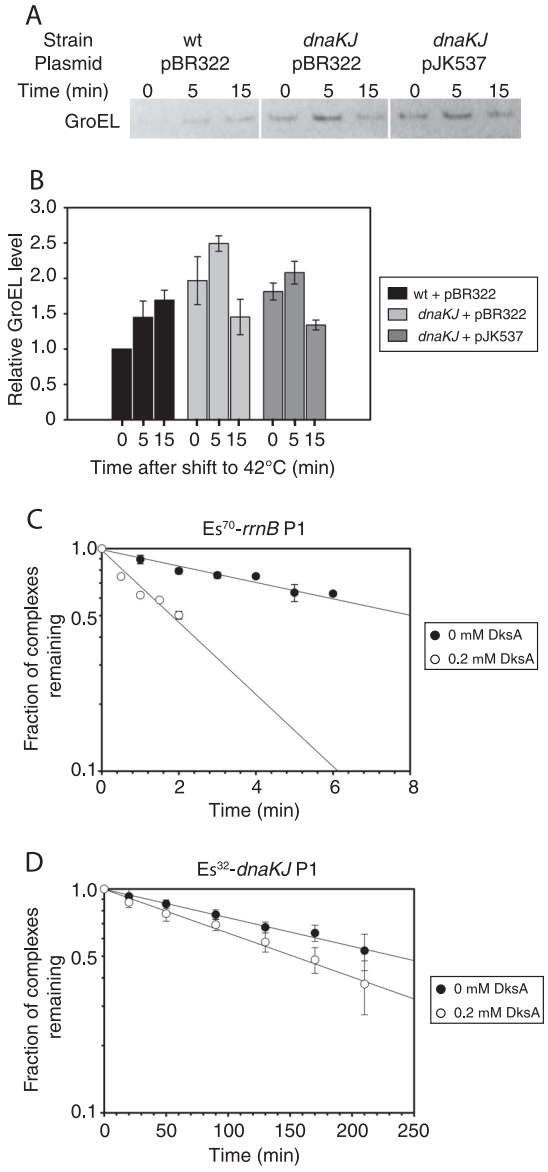

FIG 5 GroEL levels are not altered by overproduction of DksA. (A) Representative Western blots of GroEL from cell lysates of wild-type (wt) or Δ*dnaKJ* strains transformed with pBR322 or pJK537 grown at 30°C to an OD_{600} of ~0.3 and then shifted to 42°C. (B) Results (averages and standard deviations) from three independent experiments like those shown in panel A. Bars represent the amount of GroEL relative to that from the wild-type strain at time zero (i.e., 30°C). (C) Half-life of the complex formed by Eσ^{70} and *rrnB* P1 challenged with double-stranded consensus DNA competitor, with and without DksA. (D) Half-life of the complex formed by Eσ^{32} and *dnaKJ* P1 challenged with double-stranded DNA competitor, with and without DksA. Plots represent the averages and standard deviations of measurements from three independent experiments.

dependent *dnaK* promoter complex versus 4.4-fold reduction in the half-life of the Eσ^{70}-dependent *rrnB* P1 promoter complex [Fig. 5C and D]), even though DksA bound to Eσ^{32} and Eσ^{70} with similar affinities (L. Wang and R. L. Gourse, data not shown) when measured by the Fe^{2+}-mediated cleavage assay reported previously (19). These results suggest that Eσ^{32} is not particularly susceptible to the effects of DksA and are consistent with the conclusion that effects of DksA on heat shock promoter activity are not responsible for suppression of the Δ*dnaKJ* phenotype.

Overproduction of DksA does not restore proper regulation of σ^{32} levels to Δ*dnaKJ* mutants. As reported previously, the σ^{32} level increases transiently in cells exposed to elevated temperatures, reaches its peak ~5 min after temperature shift, and then gradually decreases to a new steady-state level that is characteristic of the elevated temperature (41). Several regulatory mechanisms control the amount and activity of σ^{32} (11, 12). Strains without DnaKJ fail to reduce σ^{32} levels following peak expression (10, 39). We reasoned that if a reduction of σ^{32} levels during the recovery phase of the heat shock response were the essential function supplied by DnaKJ, overproduction of DksA might suppress the Δ*dnaKJ* mutation by mimicking this function.

We tested this hypothesis by measuring σ^{32} levels in a wild-type strain, a Δ*dnaKJ* mutant containing a control plasmid, and a Δ*dnaKJ* mutant containing the DksA overproduction plasmid pJK537 (Fig. 6). Consistent with previous studies (41), σ^{32} increased upon shift from 30°C to 42°C in the wild-type strain and then returned to a new steady-state level (Fig. 6A and B). As expected from previous studies, σ^{32} was elevated in the absence of heat shock in the Δ*dnaKJ* mutant strain and remained elevated

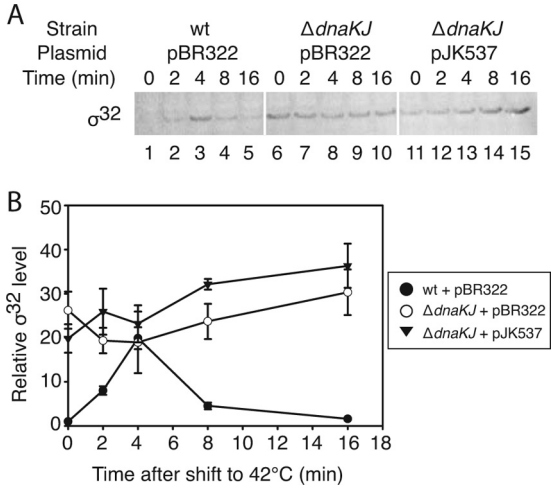

FIG 6 σ^{32} levels are not altered by overproduction of DksA. (A) Representative Western blots of σ^{32} from cell lysates of wild-type (wt) or Δ*dnaKJ* strains transformed with pBR322 or pJK537 grown at 30°C to an OD_{600} of ~0.3. (B) Plots of the amounts of σ^{32} relative to the wild-type strain grown at 30°C. Values given are the average and standard deviation calculated from three independent experiments.

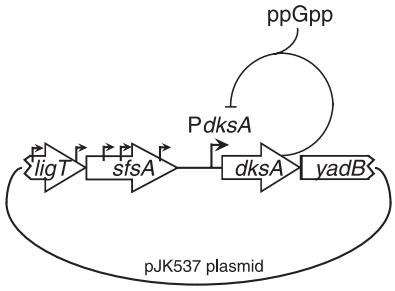

FIG 7 Weak, non-feedback-regulated transcription initiating upstream of the major *dksA* promoter accounts for DksA overexpression from a multicopy plasmid. For the original suppressor plasmid pJK537, transcription from the *dksA* promoter (P*dksA*, large arrow) is regulated by DksA and ppGpp, whereas transcription originating from within the *ligT* and *sfsA* open reading frames (small arrows) is not subject to regulation by DksA and ppGpp.

after a shift to 42°C (39) (Fig. 6A and B). pJK537 did not reduce steady-state σ^{32} levels before the temperature shift or prevent continued overproduction of σ^{32} at later times following the shift (Fig. 6A and B). These data suggest that DksA overproduction does not suppress the Δ*dnaKJ* mutation by regulating σ^{32} levels.

DISCUSSION

DksA expression is subject to negative feedback regulation, preventing overproduction of DksA from its native promoter on a multicopy plasmid (6). This feedback system maintains a constant level of DksA sufficient to allow RNAP to respond quickly to fluctuations in nutritional availability signaled by changes in the levels of ppGpp and NTPs and to ensure genome integrity during DNA replication (28, 42). We found that overproduction of DksA was needed for suppression of the Δ*dnaKJ* temperature-sensitive phenotype. Therefore, the primary goal of this study was to identify the basis for the subversion of the feedback control system that normally controls DksA expression, i.e., to account for the overproduction of DksA from plasmid pJK537, which was identified originally for its suppression of the Δ*dnaKJ* mutation.

We found that weak promoters located upstream of the major *dksA* promoter, apparently lacking the kinetic properties shared by promoters regulated by DksA and ppGpp, are responsible for the rescue of Δ*dnaKJ* mutants by pJK537 at high temperature (Fig. 7). These weak non-feedback-regulated promoters apparently provide enough transcription when present on a multicopy plasmid to overproduce DksA. In theory, these upstream promoters could provide a source of DksA expression in their natural chromosomal context (i.e., in the absence of a multicopy *dksA* plasmid) in response to some unidentified growth condition, but because we have no evidence for such a situation, the physiological relevance of responses to DksA overproduction is questionable.

Nevertheless, understanding the mechanism of suppression could help identify the activity responsible for the essentiality of *dnaKJ* or could uncover a previously unsuspected activity of DksA. We have tentatively ruled out potential roles for DksA as a cellular chaperone itself, as an inducer of σ^{32}-dependent gene expression, or as a regulator of σ^{32} levels. However, we acknowledge that these conclusions are based on negative results and therefore could be subject to revision. For example, we examined the effects of DksA *in vivo* on expression of only one of the many σ^{32}-dependent products, GroEL, albeit the one that was shown previously to be able to compensate for the loss of *dnaKJ*.

Previous work suggests that the magnitude of the heat shock response is dependent not only on the level of σ^{32} but also on the amount of active σ^{70}, implying a possible role for competition between these sigma factors in binding to core RNAP (4). The extent of this competition depends not only on the relative amounts of the two sigma factors and core RNAP but also on the relative affinities of the sigma factors for core RNAP and on the amounts of the chaperones, cochaperones, and proteases that affect the activities of the two sigma factors (41). Thus, overproduction of DksA could affect σ^{32}-dependent promoters directly, or it could affect the competition between σ^{70} and σ^{32} indirectly by affecting σ^{70}-dependent transcription. Consistent with a potential role for sigma factor competition in suppression, the gene encoding σ^{70}, *rpoD*, like that encoding DksA, was also isolated as a multicopy suppressor of some of the phenotypes of a Δ*dnaKJ* strain (37).

We suggest that the function of DksA in suppression likely is linked to its role in regulation of transcription initiation, based on the inability of the D74N DksA variant to restore growth to the Δ*dnaKJ* mutant at high temperature. Although we favor the model that the sensitivity of some unidentified promoters to DksA overproduction directly or indirectly is responsible for the Δ*dnaKJ* suppression, the precise mechanism remains unclear. Because ppGpp/DksA affects a large number of cellular promoters (see introduction), we do not mean to imply that the effect of overexpression of DksA on rRNA promoters is necessarily responsible for the Δ*dnaKJ* suppression. In fact, reduction of rRNA transcription by growth on minimal medium did not suppress the temperature sensitivity of the Δ*dnaKJ* mutation (Chandrangsu and Gourse, data not shown). Genome-wide approaches could be utilized to identify a promoter target(s) that responds to overexpressed DksA in a Δ*dnaKJ* mutant exposed to high temperature. However, we note that overproduction of DksA exacerbates the inhibition of transcription from rRNA promoters by ppGpp/DksA (34), and a reduction in rRNA transcription would release RNAP from

rRNA operons, making RNAP available for redistribution to other transcription units, including those transcribed by other holoenzymes. Thus, the mechanism of suppression could be quite indirect and complex, and simply identifying transcripts genome-wide whose amounts change when DksA is overexpressed in a Δ*dnaKJ* mutant exposed to high temperature would not distinguish the direct targets responsible for the suppression.

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