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DksA involvement in transcription fidelity buffers stochastic epigenetic change

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ABSTRACT

DksA is an auxiliary transcription factor that interacts with RNA polymerase and influences gene expression. Depending on the promoter, DksA can be a positive or negative regulator of transcription initiation. Moreover, DksA has a substantial effect on transcription elongation where it prevents the collision of transcription and replication machineries, plays a key role in maintaining transcription elongation when translation and transcription are uncoupled and has been shown to be involved in transcription fidelity. Here, we assessed the role of DksA in transcription fidelity by monitoring stochastic epigenetic switching in the *lac* operon (with and without an error-prone transcription slippage sequence), partial phenotypic suppression of a *lacZ* nonsense allele, as well as monitoring the number of *lacI* mRNA transcripts produced in the presence and absence of DksA *via* an operon fusion and single molecule fluorescent *in situ* hybridization studies. We present data showing that DksA acts to maintain transcription fidelity *in vivo* and the role of DksA seems to be distinct from that of the GreA and GreB transcription fidelity factors.

INTRODUCTION

All organisms maintain their phenotypic identity by accurate replication of the information encoded in their genes but also by a high fidelity of the processes that generate the products expressed from those genes. Every level of information transfer from DNA to RNA to protein is prone to errors, but not all of these errors are considered to have significant and long-lasting consequences for the organism. Unlike the more permanent effects of genetic mutation and potentially detrimental consequences of errors in protein folding known to cause prion formation, the magnitude and consequences of errors in transcription are just beginning to be explored (1–7). One mRNA error can compromise the cell with the production of many faulty proteins; translation errors however, lead to only one compromised protein among many wild-type proteins produced from the same transcript and are therefore potentially of less consequence, even if translation may be more error-prone than transcription. Indeed, errors in RNA can impart a long-lasting and heritable impact on cellular phenotype when arising in a transcript of a transcription factor involved in a differentiation switch. Specifically, we have shown that alteration of transcription fidelity *in vivo* promotes heritable phenotypic change in the bistable *lac* operon system (Figure 1) (1). We showed that error-prone RNA polymerase (RNAP) mutants, as well as loss of the RNAP fidelity factors GreA and GreB, reduce transcriptional fidelity. We also showed that an error-prone message in the transcriptional regulator controlling the differentiation switch promotes transcriptional infidelity (2). Moreover, we showed that both the *cis* and *trans* components of the system act synergistically in the control of transcriptional fidelity (2). Thus, the bistability of the *lac* operon can be used to capture and monitor the consequences of transient transcription errors in living *Escherichia coli* cells, providing a sensitive tool to study proteins involved in the fidelity of RNA transcription.

Auxiliary transcription factors bind RNAP and can influence gene expression processes without directly interacting with DNA. This family of proteins includes GreA and GreB, Gfh1, Rnk, DksA, DksA2 and TraR (8–14). Each auxiliary factor has been shown, or predicted, to dock to the surface of RNAP and protrude their coiled-coil tip into the secondary channel of the RNAP complex. Among these auxiliary transcription factors, only GreA and GreB, the bacterial homologs of eukaryotic TFIIS transcription fi-

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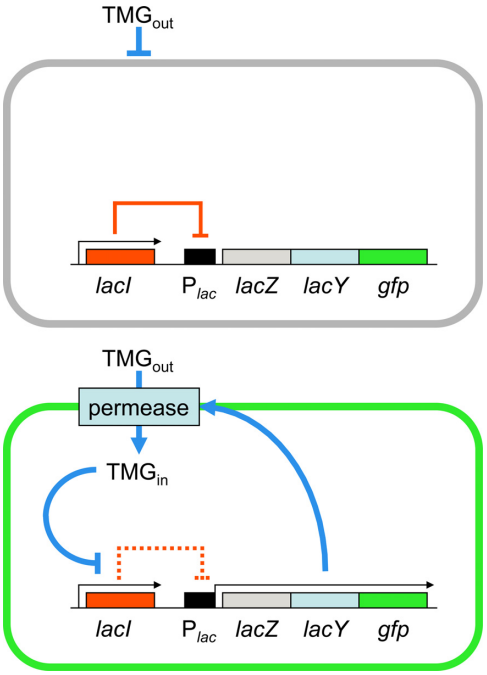


Figure 1. Stochastic switching in the *lac* bistable gene network. Under maintenance conditions [that concentration of inducer which does not activate transcription of the operon but allows an already induced cell to remain induced (44)], the *lac* operon is OFF when the *lac* repressor is bound to the *lac* operator (indicated by the solid red line) and the inducer TMG remains extracellular; stochastic events that lead to a transient derepression of the *lac* operon will result in a burst of *lac* operon functions and the appearance of permease will initiate an autocatalytic positive-feedback response (indicated by solid blue lines), which will heritably maintain the ON state (TMG induces an allosteric transition in *lac* repressor, indicated by the dashed red line, so that it no longer binds to the *lac* operator), and the cell will exhibit green fluorescence (1,2,4).

delity factor (15–17), are known to be required for high fidelity RNA synthesis *in vivo* and *in vitro* (1,2,5,18–22). The coiled-coil structures enable these factors to reach close to the active center of the RNAP complex where they promote endonucleolytic cleavage of the nascent transcript. Short pauses, associated with a backtracked RNAP of less than 5 bp and a pause of less than 20 s, are resolved by GreA, which stimulates the cleavage and allows RNAP to proceed with elongation. Longer pauses, which involve the extrusion of the 3′ end of the nascent RNA into the secondary channel, require GreB and its action is necessary to restart RNAP (19). Therefore, GreA and GreB are required to revive backtracked RNAP under different circumstances.

E. coli DksA is a structural homolog of the Gre factors. Although DksA is not related to the Gre proteins on a sequence similarity basis, it interacts with the same area on the surface of RNAP (23–26). DksA was first implicated in transcription initiation, and recently this protein was also unambiguously shown to have a substantial effect on transcription elongation as well where it prevents the collision of transcription and replication machineries (27–29). In addition, DksA has recently been shown to play a key role in maintaining transcription elongation when translation and transcription are uncoupled (30). The structural similarity between the fidelity factors GreA, GreB and DksA, in addition to recent discoveries regarding the role of DksA in transcription elongation *in vivo* (28) and transcription fidelity (31), encouraged us to investigate the role of DksA in maintaining high RNA quality and epigenetic change in living cells.

Here, we employed a variety of *in vivo* genetic assays to assess the role of DksA in transcription fidelity including stochastic epigenetic switching in the *lac* operon (with and without an error-prone transcription slippage sequence), partial phenotypic suppression of a *lacZ* nonsense allele as well as monitoring the number of *lacI* mRNA transcripts produced in the presence and absence of DksA *via* an operon fusion and single molecule fluorescent *in situ* hybridization (smFISH) studies. Our results show that DksA acts to maintain transcription fidelity and that the *in vivo* role of DksA seems to be distinct from that of the GreA and GreB fidelity factors. Our results corroborate a recent *in vitro* study indicating that DksA increases the fidelity of RNA synthesis by slowing down misincorporation events by RNAP (31) and our overexpression studies complement the *in vivo* partial phenotypic nonsense suppression that has been observed in Δ*dksA* cells (31).

MATERIALS AND METHODS

Bacterial strains

Strains are listed in Table 1 and plasmids are listed in Table 2. Unless otherwise stated, all strains used are derivatives of MG1655. Transduction with P1*vir* and other standard genetic methods were performed as described by Miller (32). To monitor the proportion of cells that are ON or OFF for *lac* operon expression, we have replaced the *lacA* gene in the wild-type *E. coli* MG1655 chromosome with a *gfp* cassette, so that when the *lacZYA*::*gfp* transcript is expressed, β-galactosidase, galactoside permease and green fluorescent protein are produced from the *lacZ*, *lacY* and *gfp* genes, respectively (Figure 1) (1,2).

Genetic manipulations

Genes to be overexpressed were cloned into pBA169 or pBR322 vectors by standard cloning and transformation techniques (33–35). Gene replacements were made as reported by Datsenko and Wanner (36,37). Specific mutations were transferred between different genetic backgrounds using P1 phage transduction (32,38).

Growth conditions and media

Bacteria were grown in LB broth and on LB and M9 glucose (0.2%) agar plates. Media were supplemented with the following antibiotics: ampicillin (Amp; 50 μg/ml) or carbenicillin (CB; 50 μg/ml), rifampicin (Rif; 50 μg/ml), tetracycline (Tet; 12.5 μg/ml), chloramphenicol (CM; 12.5 μg/ml) and kanamycin (Kan; 30 μg/ml). Thiamine (10 μg/ml) was added when necessary. Isopropyl-β-D-thiogalactoside (IPTG) was used as an inducer for overexpression of the respective constructs (Table 2). Methyl-β-D-thiogalactoside (TMG) was used in the epigenetic bistable switch assay. 5-Bromo-4-chloro-3-indolyl-β-D-thiogalactoside (Xgal) was

Table 1. Bacterial strains

Strain	Genotype	Reference
CH30	MG1655	laboratory stock
CH458	*lacZYA*::*gfp-cmR*	(1)
JW0141	Δ*dksA*::*kan*	(38)
CH645	*lacZYA*::*gfp-cmR* Δ*dksA*::*kan*	CH458 x P1 JW0141
CH568	*lacZYA*::*gfp-cmR* $\Delta greA_{FRT}$ $\Delta greB_{FRT}$	(1)
CH2193	*lacI*-A_9*lacZYA*::gfp_{FRT}	(2)
CH5106	*lacI*-A_9*lacZYA*::gfp_{FRT} *rpoC*-F1325L *thi*3178/Tn10*kan*	CH2193 x P1 CH2300
CH5110	*lacI*-A_9*lacZYA*::gfp_{FRT} *rpoC*-F1325L *thi*3178/Tn10*kan* Δ*dksA*::*tet*	CH5106 x P1 CH2300
CH543	*lacZ*-U118	(45)
CH580	*lacZ*-U118 Δ*dksA*::*kan*	(45)
CH2570	*lacZ*-U118 Δ*yjaZ*::*kan* Δ*btuB*3191::*Tn*10 *rpoC*-E677G	(45)
CH5035	*lacZ*-U118 *dksA*-NN Δ*yadD*::*kan*	CH543 x P1 CH4247
CH2417	*lacZ*-U118 Δ*dksA*::*kan* pBA169	CH580 + pBA169
CH2455	*lacZ*-U118 Δ*dksA*::*kan* pGreB	CH580 + pGreB
CH2418	*lacZ*-U118 Δ*dksA*::*kan* pDksA	CH580 + pDksA
CH2419	*lacZ*-U118 Δ*dksA*::*kan* pDksA-NN	CH580 + pDksA-NN
CH2454	*lacZ*-U118 Δ*dksA*::*kan* pGreA	CH580 + pGreA
CH2455	*lacZ*-U118 Δ*dksA*::*kan* pGreB	CH580 + pGreB
RG8527	Δ*dksA*::*tet* *rpoC*-F1325L *thi*3178/Tn10*kan*	RL Gourse
CH2263	*lacZYA*::*gfp-cmR* *rpoC*-F1325L *thi*3178/Tn10*kan*	CH458 x P1 RG8527
CH2300	Δ*dksA*::*tet* *lacZYA*::*gfp-cmR* *rpoC*-F1325L *thi*3178/Tn10*kan*	RG8527 x P1 CH2263
JD1337	*dksA*-NN	JD Wang
CH4207	*dksA*-NN pKD46	JD1337 + pKD46
JW5010	Δ*yadD*::*kan*	(38)
CH4247	*dksA*-NN Δ*yadD*::*kan*	CH4207 x P1 JW5010
CH2714	$lacI_{FRT}$*lacZYA*	(2)
CH5041	$lacI_{FRT}$*lacZYA* Δ*dksA*::*tet*	CH2714 x P1 CH2300
CH5045	$lacI_{FRT}$*lacZYA* Δ*yjaZ*::*kan* Δ*btuB*3191::*Tn*10 *rpoC*-E677G	CH2714 x P1 CH2570
CH5047	$lacI_{FRT}$*lacZYA* *rpoC*-F1325L *thi*3178/Tn10*kan*	CH2714 x P1 CH2300
CH5087	$lacI_{FRT}$*lacZYA* *rpoC*-F1325L *thi*3178/Tn10*kan* Δ*dksA*::*tet*	CH5047 x P1 CH2300

Table 2. Plasmids

Plasmid	Construct	Reference
pBA169	pTrc99A Δ*ncoI* Ap^R	(34)
pCP20	FLP^+ λ $cI857^+$ λ p_R Rep^{ts} Ap^R Cm^R	(37)
pDksA	pBA169-*dksA*	(35)
pDksA-NN	pBA169-*dksA*-NN	(28)
pGreA	pBA169-*greA*	(28)
pGreB	pBA169-*greB*	(28)

used to monitor β-galactosidase levels in colonies on M9 glucose agar plates supplemented with casamino acids.

To demonstrate hysteresis and bistability in *lac* operon expression in single cells, a bacterial culture grown in minimal A salts (32) plus $MgSO_4$ (1 mM) with succinate (0.2%) and thiamine was diluted 1:5 in fresh medium with (ON culture) or without 1 mM TMG (OFF culture) and shaken at 37°C for 7 h. After this induction period, the two cultures were individually diluted and ∼200 cells were seeded to new tubes containing fresh medium that contained varying amounts of TMG and shaken at 37°C for 42 h. Flow cytometry was used to determine the percentage of cells that were induced for *lac* operon expression (ON cells).

To determine epigenetic switch frequencies, a bacterial culture grown in minimal succinate media, was diluted and ∼200 cells were seeded to new tubes containing fresh medium, with a maintenance level of TMG, and shaken at 37°C for 42 h, as previously described (1,2,39), and subjected to flow cytometry.

Flow cytometry

The analysis of phenotypic switching was performed as described previously (2), using BD FACSCanto™ II Flow Cytometer (Becton, Dickinson and Company, USA) with Diva™ acquisition software (Becton Dickinson), FloJo™ analysis software (Tree Star, Inc. USA) and Prism™ (GraphPad). All cultures were subjected to arbitrary gating, which encompassed approximately 70% of the most typical events (Supplementary Figure S1).

β-galactosidase activity assay

Cultures were grown overnight at 37°C in LB in the presence of IPTG (0.1 mM) to express the *lacZ*-U118 allele. Aliquots (0.5 ml) were assayed as described earlier (32), except that samples were centrifuged to pellet cell debris before determination of OD_{420}.

Single-molecule mRNA fluorescent *in situ* hybridization (smFISH)

The smFISH protocol has been described in detail (40,41). Fluorescently labeled oligonucleotide probe sequences designed against the *lacI* transcript (purchased from Biosearch Technologies, USA) are described in (39). Bacterial strains for smFISH analysis were grown in minimal A salts with succinate (0.2%) and thiamine at 37°C. The estimation of mRNA number in the cell relies on quantifying localized fluorescence and is not achieved by counting discrete spots. The number of bound probes is measured on the basis of the total fluorescence intensity (photon flux) of the spots, without requiring that individual mRNAs appear as separate spots. By performing a calibration step, the total intensity of spots in the cell can then be converted to the number of target mRNAs (41).

RESULTS

Effect of DksA on epigenetic switching in the *lac* bistable system

To analyze the impact of DksA on transcription fidelity, we employed a bistable *lac* system that captures transient errors in mRNA synthesis in the form of a stable and heritable phenotype (1,2). Briefly, the *lac* operon comprises a bistable switch controlled by the LacI repressor under a specific, maintenance concentration of inducer (TMG). The *lacI* mRNA is a rare transcript, made approximately once per cell cycle, and transcription errors in *lacI* can lead to a transient depletion of LacI repressor function that triggers an autocatalytic positive-feedback response that flips the epigenetic switch of the bistable network to the ON state (Figure 1). Previously, we showed that a 5-fold decrease in transcription fidelity due to an RNAP mutation (measured both *in vitro* and *in vivo*) (42) leads to a 4- to 6-fold increase in switching frequency in our bistable *lac* assay (1). Thus, the *lac* bistable system is sensitive to RNA mistakes. If DksA acts to ensure proper transcription, the absence of DksA should increase the phenotypic switching frequency in our *lac* bistable assay. In order to circumvent the inability of the Δ*dksA* strain to grow in the minimal medium required for the phenotypic switching assay, we used a known RNAP suppressor of Δ*dksA* auxotrophy, β' F1325L (43). To eliminate the possibility of the suppressor itself affecting our observations on DksA, we assessed whether the *rpoC* F1325L mutation alone had an effect in the bistable switch assay. The β' F1325L allele strain shows maintenance of the bistable switch (1,44) at a concentration of 8 μM TMG (Figure 2A and B). Thus, the system remains bistable in the presence of the β' F1325L allele. Cells with β' F1325L switch their phenotype from OFF to ON at a frequency of 2% (Figure 2C and D) and when the β' F1325L allele is combined with deletion of *dksA*, the phenotypic switch frequency is elevated approximately 7-fold ($P = 0.001$; Figure 2C and D). Moreover, this frequency fluctuates between the cultures indicating that stochastic events trigger the switching process, similar to that observed for mutation (Figure 2C) (1,2).

Effect of DksA on the P_{lacI} promoter

To test whether the absence of DksA affects the *lacI* promoter, we employed a *lacI-ZYA* operon fusion (2). In this fusion, the P_{lac} promoter sequence between *lacI* and *lacZ* has been deleted in-frame so that *lac*Z is only expressed from the P_{lacI} promoter (Figure 3A). Interestingly, the absence of DksA, or the presence of an RNAP mutation that reduces binding of DksA to RNAP (β' E677G) (45), significantly affects transcription of this operon fusion. Cells lacking DksA exhibit a very low level of β-galactosidase, indicating a down regulation in P_{lacI} activity (Figure 3B). Importantly however, β' F1325L fully restores the level of β-galactosidase to the wild-type level in the absence of DksA, showing that β' F1325L is a valid suppressor of Δ*dksA* for transcription initiation (43). These data suggest that the increase in epigenetic switching observed in the β' F1325L Δ*dksA* strain is not due to a change in P_{lacI} promoter activity. This is important since a recent study showing a role for DksA in transcription fidelity used a plasmid-based *lacZ* fused to an early T7A1 promoter that was differentially expressed in the presence or absence of DksA in *E. coli*, and all *in vivo* results had to be normalized to take differential expression into account (31); no such accounting is needed here (see also Supplemental Figure S2).

Effect of DksA on *lacI* transcript number in single cells

To confirm that the increase in epigenetic switch frequency observed in the Δ*dksA* strain is not due to lower levels of *lacI* mRNA molecules, the number of *lacI* mRNA in β' F1325L cells lacking DksA compared to β' F1325L cells was measured at the single cell level using smFISH (39–41). smFISH was also performed on Δ*greA,B* cells for comparison.

Briefly, *lacI* mRNA was hybridized to a series of fluorescent probes and visualized by fluorescence microscopy (39–41). The total intensity of spots in the cell is then converted to the number of *lacI* transcripts in the cell (41). Using smFISH it has previously been shown that fully 86% of wild-type *lacI* cells do not exhibit any *lacI* mRNA, 11% of cells have one *lacI* mRNA and 3% have two or more *lacI* mRNA per cell at any given time (0.18 ± 0.01 transcripts per cell; 2081 cells monitored) (39). All strains analyzed here (Figure 4) exhibit similar *lacI* mRNA levels, and no strain exhibits lower *lacI* mRNA levels than the wild-type strain. Compared to the β' F1325L control, β' F1325L cells lacking DksA did not show any reduction in *lacI* mRNA levels: fully 81% of β' F1325L cells do not exhibit any *lacI* mRNA and 16% of cells have one *lacI* mRNA at any given time; similarly for β' F1325L Δ*dksA* cells, fully 78% of these cells do not exhibit any *lacI* mRNA and 18% of cells have one *lacI* mRNA at any given time (Figure 4). Therefore, the 7-fold increase in *lac* epigenetic switching observed in the β' F1325L Δ*dksA* strain, compared to the β' F1325L control, is not due to an overall decrease in *lacI* mRNA (Figure 4). Cells lacking GreA and GreB exhibited the same number of *lacI* mRNA per cell as the β' F1325L and β' F1325L Δ*dksA* cells: fully 76% of these cells do not exhibit any *lacI* mRNA and 19% of cells have one *lacI* mRNA at any given time. Therefore, the greater than 40-fold increase in epige-

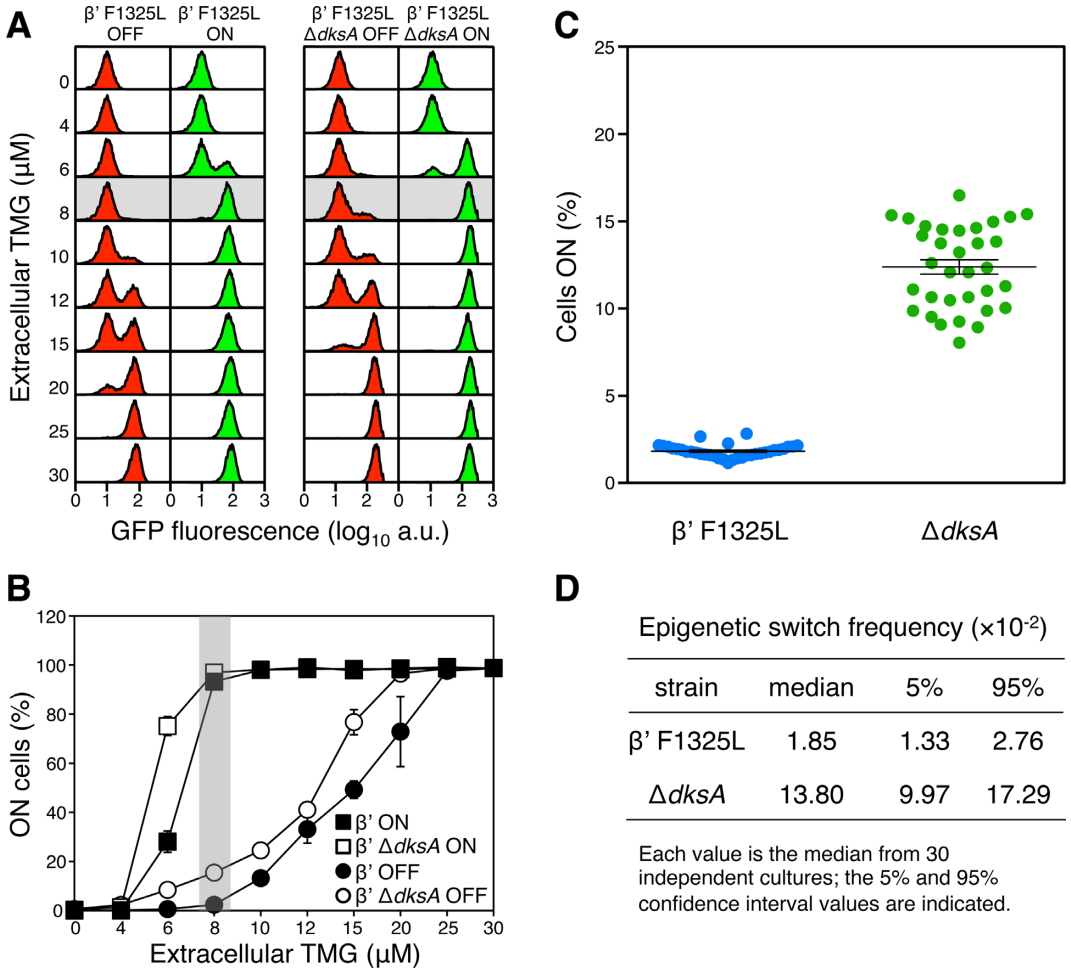


Figure 2. The absence of DksA increases stochastic epigenetic switch frequency in the *lac* bistable gene network. Loss of DksA confers auxotrophy, and our phenotypic switching assay requires cultivation in minimal media with succinate and thiamine. Therefore, we use a suppressor of the auxotrophy observed in Δ*dksA* cells, β' F1325L, as the appropriate control. (**A**) Representative flow cytometry GFP fluorescence histogram series of β' F1325L (CH2263) and β' F1325L Δ*dksA* (CH2300) cells that were originally ON (green histograms) or OFF (red histograms) were sub-cultured and grown in media containing various concentrations of TMG indicated on the vertical axis (10^4 cells interrogated). Below 6 μM TMG and above 20 μM TMG, the previous history of the cell (ON or OFF) does not affect the current state of the cell. However, between these TMG concentrations, the system exhibits hysteresis. The shaded area highlights the maintenance concentration of 8 μM TMG for these strains, the TMG concentration at which an ON cell remains ON and an OFF cell remains OFF but has a probability to stochastically switch ON (1, 2). (**B**) Cells that were originally ON or OFF were sub-cultured and grown in media containing various concentrations of TMG, as above. Filled symbols denote β' F1325L cells; open symbols denote β' F1325L Δ*dksA* cells. Each value is the average ± SD from four independent cultures. The shaded area highlights the maintenance concentration of 8 μM TMG for these strains. (**C**) OFF β' F1325L cells (blue dots), β' F1325L Δ*dksA* cells (green dots) were diluted and grown in media containing 8 μM TMG. After 42 h growth, flow cytometry was performed to determine the frequency of epigenetically ON cells in 30 independent cultures of each strain. Each dot represents the value for an independent culture. Means ± standard error of the mean are indicated. (**D**) The β' F1325L Δ*dksA* epigenetic-switch frequency is significantly increased over the β' F1325L value (Mann-Whitney Rank Sum Test, $P = 0.001$). All cultures analyzed carry the β' F1325L allele.

netic switching observed in Δ*greA,B* cells can also not be explained by a decrease in *lacI* mRNA number (1,2).

The two complementary methods to assess P_{lacI} activity in the absence of DksA (in the presence of the RNAP β' F1325L suppressor), the *lacI-ZYA* operon fusion and *lacI* mRNA smFISH, provide a consistent result: β' F1325L Δ*dksA* cells are not compromised for P_{lacI} activity nor *lacI* mRNA production compared to wild-type and β' F1325L cells.

Effect of DksA on mistranscription of a *lacZ* nonsense mutation

To further study transcription fidelity *in vivo*, we employed a previously described allele of the gene encoding β-galactosidase, *lacZ*-U118 (42,46–48), that encodes a nonsense UAA (ochre) codon at amino acid position 18 in β-galactosidase (Figure 5A) (47). This mutation stops translation early thereby unmasking transcription terminator sites in the untranslated mRNA and terminates transcription of the full operon. Partial phenotypic suppression ('leakiness') of *lacZ*-U118 depends principally on transcription errors that correct the ochre mutation at the mRNA level, allowing translation to proceed and yield a full-length transcript,

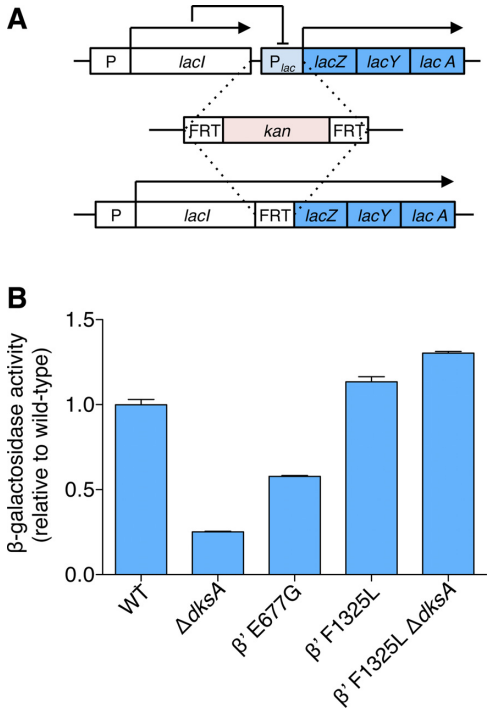


Figure 3. The RNA β' F1325L polymerase restores P_{lacI} activity to the wild-type level in Δ*dksA* cells. (**A**) In this *lacI-ZYA* operon fusion, the *lacI* gene encoding the *lac* repressor is fused with the *lac* operon genes and therefore the amount of functional *lacI* mRNA can be measured by assaying the β-galactosidase level in cultures grown in LB (2). (**B**) Loss of DksA causes a decrease in β-galactosidase level indicating that this transcription factor is required for the initiation of P_{lacI}. The effect of RNAP β' E677G falls between the wild-type and Δ*dksA* values indicating incomplete penetrance of the phenotype. Importantly, RNAP β' F1325L restores P_{lacI} activity to the wild-type level in Δ*dksA* cells. Average ± standard deviation values are indicated.

leading to the production of many active β-galactosidase molecules. The leakiness of this non-functional allele can be observed by assessing the level of β-galactosidase either by growing cells on media supplemented with the inducer IPTG and an excess of the lactose analogue Xgal, or more quantitatively by a β-galactosidase assay (32). Wild-type *E. coli* carrying the *lacZ*-U118 allele forms white colonies on Xgal plates, and strains with decreased transcription fidelity, such as *ack*-1 [*rpoB* P564L (1)] form colonies that are light blue (42). To test whether the absence of DksA has an effect on the leakiness of the nonsense codon in the *lacZ*-U118 allele, we streaked Δ*dksA* cells on IPTG Xgal M9 glucose casamino acids plates and observed that the absence of DksA clearly results in darker blue colonies as compared to wild-type (Figure 5B). In addition, we tested an RNAP mutant that has previously been shown to be insensitive to DksA, *rpoC* E677G (45), as well as a *dksA* allele that is defective in initiating transcription from DksA-dependent promoters *in vitro,* DksA-NN (28). We observed that, like the absence of DksA, the presence of the *rpoC* E677G allele also results in clearly increased β-galactosidase levels, attributed to increased leakiness of the *lacZ*-U118 allele. The presence of the DksA-NN allele also results in slightly bluer colonies compared to wild-type suggesting that DksA-NN acts during elongation since we have previously shown that DksA-NN has no effect on initiation of the P_{lac} promoter (28). The amount of functional β-galactosidase was then directly measured in the same strains. We have determined that the P_{lac} promoter is unaffected by the presence or absence of DksA which allows a straightforward comparison of β-galactosidase levels in different strains (see Supplemental Figure S2). This is important since a recent study showing a role for DksA in transcription fidelity used a plasmid-based *lacZ* fused to an early T7A1 promoter that was differentially expressed in the presence or absence of DksA in *E. coli*, and all *in vivo* results had to be normalized (by decreasing β-galactosidase levels observed by 2.3-fold) to take differential expression into account (31); no such accounting is needed here. The absence of either DksA, or the lack of sensitivity of RNAP to this transcription factor (*rpoC* E677G), resulted in a 2-fold increase in β-galactosidase levels (Figure 5C). Again, the presence of the initiation-deficient allele of *dksA*, DksA-NN, resulted in an intermediate activity of β-galactosidase, indicating only a slight but consistent decrease in transcription fidelity. This result of increased leakiness of a nonsense codon in the absence of DksA is similar to that found in a plasmid-based partial phenotypic suppression assay (31).

Moreover, we tested the suppression of the Δ*dksA*-related increase in *lacZ*-U118 leakiness by overexpressing auxiliary transcription factors that interact with the secondary channel of RNAP (Figure 5D). We used plasmids carrying DksA, DksA-NN, GreA and GreB (45). Overexpression of DksA has a dramatic effect on the leakiness of the *lacZ*-U118 allele in Δ*dksA* cells and nearly completely prevented (below wild-type levels) the production of functional β-galactosidase. Overexpression of the transcription initiation-deficient DksA-NN allele restored leakiness to the wild-type level, indicating that partial elongation activity is maintained for this protein. Overexpression of the transcription factor GreA, but not GreB, modestly decreased the effect on *lacZ*-U118 leakiness in Δ*dksA* cells (Figure 5D). These data suggest that the absence of DksA promotes base substitutions that increase the leakiness of the *lacZ*-U118 ochre mutation and overexpression of DksA reduces base substitution misincorporation. The weaker effect of GreA or GreB overexpression suggest that either the level of these factors is not enough to allow full rescue or that these factors act on a different step of transcription fidelity (see Discussion).

Effect of DksA on transcriptional slippage *in vivo*

We have previously shown that the combined absence of transcription fidelity factors GreA and GreB results in increased transcriptional slippage as measured by phenotypic switching in our *lac* bistable system in *E. coli* that was engineered to have a run of nine adenine residues immediately after the translation start codon (2). Here, we employed the same system to test whether DksA actively prevents RNAP slippage, similarly to GreA and GreB. Specifically, we used our slippage-prone allele of the *lac* repressor, *lacI*-A_9; this altered gene encodes a fully functional *lac* repressor, and the nine consecutive adenine residues are 'slippery' for RNAP (2,7,49,50) (Figure 6A). Both β' F1325L *lacI*-A_9 and β' F1325L Δ*dksA lacI*-A_9 strains exhibit bistability and simi-

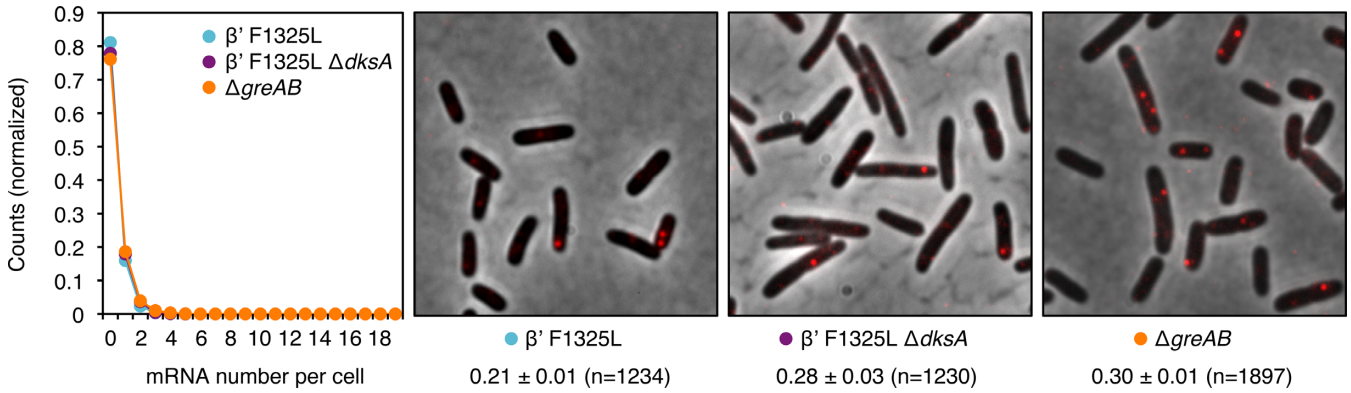


Figure 4. Absence of DksA does not affect the number of *lacI* transcripts in a cell. Single molecule fluorescence *in situ* hybridization (smFISH) analysis of *lacI* mRNA numbers in *E. coli* cells grown in minimal media with succinate and thiamine was performed (39–41). *lacI* mRNA was hybridized to a series of fluorescent probes that visualize the transcript. The strains analyzed were β' F1325L, β' F1325L Δ*dksA* and Δ*greAB*. The right panels represent microscopy images of the respective strains with *lacI* transcripts labeled using red fluorescent probes. The left panel shows the distribution of *lacI* transcripts per cell in the respective strains. Mean and standard error values for absolute *lacI* mRNA numbers per cell are shown for each strain; *n* is the number of cells analyzed. For comparison, wild-type *E. coli* cells exhibit an average of 0.18 *lacI* transcripts per cell, with fully 86% of cells not containing a *lacI* transcript at any given time (39).

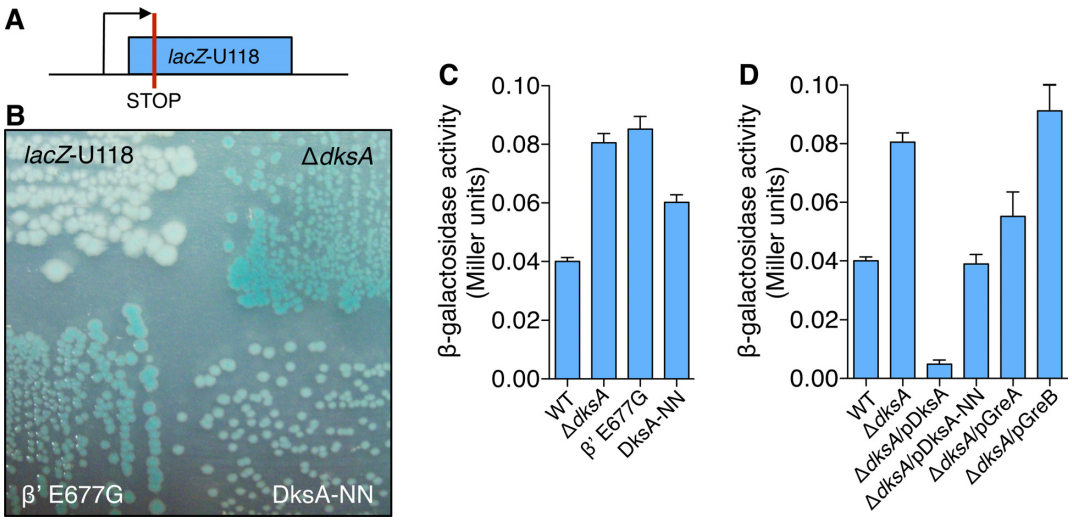
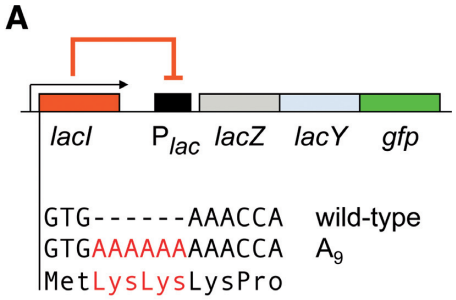
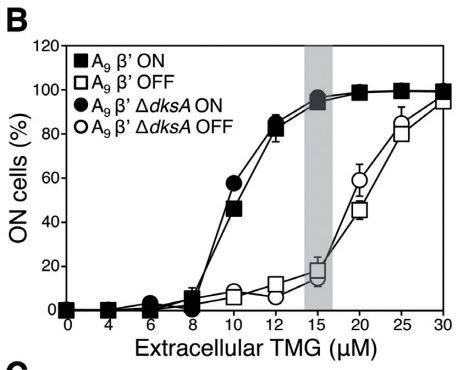


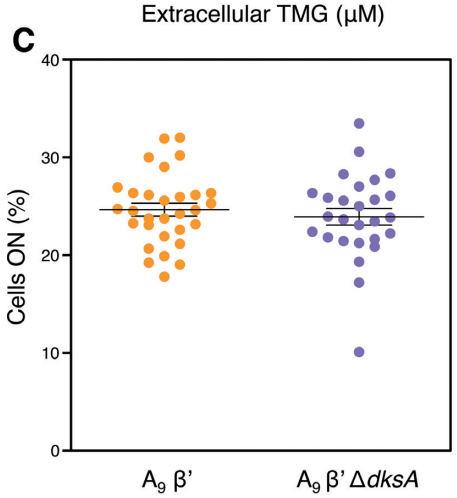
Figure 5. The effect of DksA on transcription fidelity in a partial phenotypic suppression system. (**A**) The *lacZ*-U118 polar nonsense allele encodes a truncated non-functional β-galactosidase enzyme. Due to the coupling of transcription and translation, transcription is terminated when the first ribosome cannot match the ochre codon in the mRNA. When RNAP makes an error and phenotypically suppresses the DNA-encoded nonsense mutation, the ribosome does not stall and transcription continues. The wild-type transcription error rate allows for synthesis of a very small amount of β-galactosidase. (**B**) A wild-type strain carrying the *lacZ*-U118 allele forms white colonies on M9 IPTG glucose casamino acids Xgal plates. The absence of DksA results in blue colonies, indicating a larger amount of β-galactosidase present (possibly due to an increased transcription error rate). Δ*dksA* cells can grow on these plates without a suppressor. The RNAP mutant β' E677G produces blue colonies as well, which agrees with the previously reported insensitivity to DksA (45). A transcription initiation-deficient allele of *dksA* (DksA-NN) does not mimic the phenotype of complete loss of DksA; therefore, the absence of the active tip could affect DksA-dependent transcription initiation but not necessarily elongation. (**C**) Quantification of the amount of functional β-galactosidase indicates that Δ*dksA* and β' E667G result in a 2-fold increase in *lacZ*-U118 leakiness, presumably from an elevated transcription error rate. The β-galactosidase activity observed for DksA-NN mutant is intermediate, which indicates partial elongation/fidelity activity of the mutated transcription factor. (**D**) Overexpression of DksA decreases *lacZ*-U118 leakiness below the wild-type level suggesting that DksA actively prevents transcription errors in this allele. Overexpression of DksA-NN has a weaker effect but it restores the error rate to the wild-type level. In addition we overexpressed two transcription fidelity factors GreA and GreB and observed only partial suppression by GreA suggesting these fidelity factors have only a mild effect on *lacZ*-U118 leakiness.

lar hysteresis patterns, and the maintenance concentration of 15 μM TMG is the same for both strains (Figure 6B).

Analysis of *lac* bistable switching at the maintenance concentration of TMG for the strains *lacI*-A_9 β' F1325L with or without DksA revealed no difference in epigenetic switch frequency, indicating that, unlike the GreA,B factors, slippage at the *lacI*-A_9 sequence is independent of DksA (Figure 6B and C). This lack of induction of RNA transcriptional frameshifting supports the idea that DksA specifically influences nucleotide misincorporation (31) as we observed with the leaky behavior of the *lacZ*-U118 allele in the absence of DksA, and the suppression of leakiness when DksA was overexpressed in Δ*dksA* cells (Figure 5D).







D

Epigenetic switch frequency (×10^{-2})

strain	median	5%	95%
A_9 β'	25.05	19.3	32.7
A_9 β' Δ*dksA*	24.30	16.3	31.8

Each value is the median from 30 independent cultures; the 5% and 95% confidence interval values are indicated.

Figure 6. DksA does not prevent transcriptional slippage. (**A**) The *lacI*-A_9 'slippery' sequence is prone to transcriptional slippage (2). (**B**) A hysteresis graph of strains carrying the β' F1325L mutation, which is a suppressor of Δ*dksA*-induced auxotrophy, and the modified *lacI*-A_9 allele, with or without DksA. The method used is that of Figure 2B. (**C**) The epigenetic switch frequency in presence of the *lacI*-A_9 sequence is identical with or without DksA. (**D**) The switch phenotype of *lacI*-A_9 is not significantly altered in the presence of DksA. All experiments were done using the *lacI*-A_9 β' F1325L background.

DISCUSSION

This work provides *in vivo* evidence that the auxiliary factor DksA is a *bona fide* transcription fidelity factor and supports the recent finding that DksA reduces misincorporation during transcription *in vitro* and in an *in vivo* plasmid T7A1-promoter partial phenotypic nonsense *lacZ* suppression system (31). Here, we employed a variety of *in vivo* assays to monitor transcription fidelity in living cells and observed a significant decrease in transcription fidelity when DksA was absent. First, we used the epigenetic *lac* switch system that is sensitive to transcription errors and found an increase in the frequency of epigenetic switching in the absence of DksA, indicating a decrease in transcription fidelity (Figures 2 and 5)(1,2). This increase in epigenetic switching is not due to a decrease in P_{lacI} activity (Figure 3) or the number of *lacI* transcripts in Δ*dksA* cells (Figure 4). Moreover, we show that the rate of misincorporation at the *lacZ*-U118 locus (that allows for synthesis of fully functional β-galactosidase) is increased in the absence of DksA as compared to the wild-type strain (Figure 5). This assay specifically detects base-pair misincorporation events by RNAP, which indicates that DksA diminishes nucleotide misincorporation into mRNA. Thus, our *in vivo* results are in agreement with the recent *in vitro* data showing that DksA/ppGpp decreases misincorporation by RNAP (31) and complement the *in vivo* partial phenotypic nonsense suppression that has been observed in Δ*dksA* cells (31).

Mechanistically, the GreA/B factors and DksA are different. Both factors have been shown to bind and compete for the secondary channel of RNAP, but unlike GreA/B, DksA is incapable of promoting endonucleolytic cleavage of the nascent RNA chain positioned in the secondary channel (29). Transcription fidelity factors GreA and GreB assist and stimulate RNAP to cleave nucleotides at the 3′ end of the growing RNA of backtracked RNAP in order to eliminate the mismatch error and restart transcription, and no such activity has been associated with DksA. Therefore, it is most likely that DksA acts at a different step than GreA/B during nucleotide incorporation. *In vitro,* DksA has been shown to have diverse effects on transcription elongation (29), including inhibition of transcript elongation. Thus, it is possible that DksA might affect fidelity by slowing the rate of transcription elongation, however there are no data supporting that a slowing down of transcription elongation rate affects transcription errors (1,51). *In vivo*, we show that overexpression of GreA or GreB cannot fully rescue the increased leakiness of the *lacZ*-U118 in Δ*dksA* cells, whereas overexpresssion of DksA reduces such *lacZ*-U118 leakiness below wild-type levels in Δ*dksA* cells. DksA has been shown to interact with the trigger loop of RNAP (26), a mobile domain involved in nucleotide selection during RNA synthesis and this suggests, as recently proposed (31), that DksA acts on the prevention of errors rather than error correction. Moreover, we show that unlike GreA/B, DksA does not play a role in preventing RNAP slippage at a track of nine adenines, again suggesting that DksA may only affect base substitution misincorporations.

The DksA factor has been shown to play a crucial role in the resolution of conflicts between replication and tran-

scription upon nutritional stress. Recent *in vitro* data suggest that DksA/ppGpp increases fidelity of transcription by slowing down misincorporation of nucleotides by RNAP (31). Misincorporation is known to cause RNAP backtracking, which may lead to formation of a RNAP roadblock on DNA. Thus, it was proposed that DksA may exert its anti-collision property by preventing formation of backtracked RNAP due to nucleotide misincorporation (31). This explanation may not be the cause of conflict for the following reasons: firstly, the effect of DksA on collision avoidance is ppGpp independent (28), while its role in fidelity *in vitro* is stimulated by ppGpp (31); secondly, we would like to note that a RNAP infidelity mutant, which decreases fidelity by 6-fold (42), has been shown to suppress the replication collision defect in the absence of DksA (28). Finally, the effect of DksA on resolution of replication/transcription conflicts during starvation is exacerbated by uncoupling transcription and translation (30), without invoking misincorporation of nucleotides by RNAP. The idea that transcription infidelity promotes replication conflict is interesting, but additional experiments will need to be performed in order to elucidate the exact role of DksA in resolving conflicts between replication and transcription, and how DksA affects transcription fidelity.

From our *in vivo* results, we find that DksA, along with a role in transcription initiation and elongation, also participates in maintaining transcription fidelity in *E. coli*. It is clear that maintaining transcription fidelity is important to ensure the proper propagation of the phenotypic state of the *lac* bistable switch and this is likely true for other gene networks. Transcription fidelity should therefore be considered a driving force of epigenetic change.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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