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Functions that protect *Escherichia coli* from DNA-protein crosslinks

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Abstract

Pathways for tolerating and repairing DNA-protein crosslinks (DPCs) are poorly defined. We used transposon mutagenesis and candidate gene approaches to identify DPC-hypersensitive *Escherichia coli* mutants. DPCs were induced by azacytidine (aza-C) treatment in cells overexpressing cytosine methyltransferase; hypersensitivity was verified to depend on methyltransferase expression. We isolated hypersensitive mutants that were uncovered in previous studies (*recA*, *recBC*, *recG*, and *uvrD*), hypersensitive mutants that apparently activate phage Mu Gam expression, and novel hypersensitive mutants in genes involved in DNA metabolism, cell division, and tRNA modification (*dinG*, *ftsK*, *xerD*, *dnaJ*, *hflC*, *miaA*, *mnmE*, *mnmG*, and *ssrA*). Inactivation of SbcCD, which can cleave DNA at protein-DNA complexes, did not cause hypersensitivity. We previously showed that tmRNA pathway defects cause aza-C hypersensitivity, implying that DPCs block coupled transcription/translation complexes. Here, we show that mutants in tRNA modification functions *miaA*, *mnmE* and *mnmG* cause defects in aza-C-induced tmRNA tagging, explaining their hypersensitivity. In order for tmRNA to access a stalled ribosome, the mRNA must be cleaved or released from RNA polymerase. Mutational inactivation of functions involved in mRNA processing and RNA polymerase elongation/release (RNase II, RNaseD, RNase PH, RNase LS, Rep, HepA, GreA, GreB) did not cause aza-C hypersensitivity; the mechanism of tmRNA access remains unclear.

Keywords

azacytidine; cytosine methyltransferase; tmRNA; DNA helicases; DNA repair

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1. Introduction

DNA-protein crosslinks (DPCs) are induced by radiation and by many chemicals, including formaldehyde, multiple carcinogens, chemotherapeutic agents and metals (1,2). Most of these agents induce crosslinks between a large repertoire of DNA-binding proteins and DNA, and usually in a manner that is not site-specific with respect to DNA location. These aspects of DPC formation have made their study quite difficult, and our understanding of cellular responses to DPCs is correspondingly rudimentary.

A subclass of DPCs involves enzymes that become covalently attached to DNA as part of their reaction mechanism, and this subclass includes enzymes with DNA sequence specificity (1,2). Under normal conditions, the covalent protein-DNA intermediate is transitory, but specific inhibitors and/or DNA base modifications can favor the covalent intermediate and, in some cases, make the complex essentially irreversible. Thus, various DNA topoisomerases are stabilized in their covalent complexes with DNA by chemotherapeutic agents, certain DNA repair enzymes including glycosylases can become trapped at abasic sites or other aberrant bases, and DNA cytosine methyltransferases can be trapped when their target is an appropriately modified base such as 5-azacytidine (aza-C). Particularly in the case of restriction system methyltransferases, the enzyme can exert a high level of DNA sequence specificity even though the modified base is randomly inserted in place of normal cytidine by the process of DNA replication, giving a protein-specific and DNA-site-specific DPC. For this and other reasons, we and others have adopted aza-C-induced methyltransferase-DNA complexes as a useful model system to study cellular responses to DPC formation.

Given the great variety and efficiency of DNA repair mechanisms, one would expect repair pathway(s) that efficiently remove DPC lesions, presumably in all branches of life. One such pathway operates on small proteins that are crosslinked to DNA. The excision repair system has been shown *in vitro* to excise an oligonucleotide containing covalently linked proteins that are about 10–15 kDa or smaller (3–7). Furthermore, *uvrABC* mutants are hypersensitive to formaldehyde, as are mutants that lack the alternative excision nuclease *Cho* (7,8). What about DPCs involving larger proteins? Treatment of *E. coli* with aza-C leads to DPCs involving the endogenous 53-kDa Dcm methyltransferase (or other cytosine methyltransferases expressed in the cell). Strikingly, *uvrABC* mutants lacking excision repair show no hypersensitivity to aza-C, arguing against an involvement of excision repair for this DPC with a large protein (7–10). In contrast, *recA* and *recBC* mutants with defects in recombinational repair are quite hypersensitive to aza-C (7–10). This result has been interpreted to mean that recombination can repair the relevant DPC (7,8). However, there is no direct evidence for such a repair pathway, and instead the function of the recombination machinery could be to repair downstream DNA damage caused by DPCs, such as broken replication forks (see (11)). Defining the precise molecular pathway whereby DPC toxicity is mitigated by recombinational repair is an important goal.

Not surprisingly, unrepaired DPCs have been found to inhibit the processes of DNA replication and transcription. *In vivo*, aza-C-induced DPCs formed with methyltransferase EcoRII (M.EcoRII) were shown to block plasmid replication in a site-specific manner (11).

Correspondingly, DPCs have been shown to block both polymerases and replicative DNA helicases *in vitro* (12–15). Both *E. coli* and phage T7 RNA polymerases have been shown to stall at DPC sites, although in the latter case a very inefficient and mutagenic read through was also documented (12,15). Indirect evidence for inhibition of transcription *in vivo* comes from the finding that aza-C-induced DPCs trigger tagging by the tmRNA system, which releases and thereby recycles ribosomes that are stalled or have reached a premature RNA end (16) (also see below). tmRNA functions by binding to the empty A-site of a stalled ribosome and inducing the ribosome to translate the mRNA coding sequence of tmRNA. This segment of tmRNA encodes a degradation tag that is recognized by several different protease systems, resulting in the degradation of the abnormal truncated polypeptide (17).

Bacterial cells have multiple pathways to resolve replication/transcription complexes stalled at protein roadblocks or other blocking lesions. The DinG, UvrD, and Rep helicases have been implicated in preventing or mitigating the damage from collisions between the replication machinery and bound proteins (such as RNA polymerase), and at least Rep and UvrD have protein removal activity *in vitro* (13,18–20). Specific to blocked transcription complexes, Mfd, the transcription-coupled repair factor in bacteria, recognizes RNAP stalled at DNA damage such as a pyrimidine dimer, removes RNAP from the DNA, and recruits excision repair machinery (21,22). Mfd can also remove RNAP stalled by nucleotide starvation *in vitro* (23). Transcription terminator Rho has been shown to prevent double stranded DNA breaks, presumably by removing RNAP ahead of the replisome and preventing damaging collisions (24). Another transcription factor, HepA, has been shown to activate transcription by recycling RNAP, and potentially plays a role during DNA damage (25,26). GreA and GreB are elongation factors that travel with the transcription complex, and have been shown to induce cleavage of the 3' proximal dinucleotide from the nascent RNA by RNAP, allowing for restart of transcription at the new 3' end (27,28). GreA and GreB have also been shown to stimulate activation of backtracked elongation complexes (29). DksA, along with ppGpp, has numerous effects on elongation complexes and has also been shown to prevent replication/transcription collisions (19,30). Trailing RNA polymerases have also been shown to help push stalled elongation complexes past roadblocks (31).

As implied above, analyses of mutants that are hypersensitive to DPC-forming agents can be extremely useful in defining the intracellular consequences and responses to interruptions in processes such as replication and transcription. Several previous reports have characterized *E. coli* aza-C hypersensitive mutants, leading to the conclusions above about excision and recombinational repair (7–10,16,32). Additional mutants that have been shown to be hypersensitive include *lexA*, *recG*, *ruvABC*, *priA*, *priB*, *polA*, *uvrD*, *ssrA*, *smpB*, *hflC* and *dnaJ* (7–9,16,32). A limitation of some of these past studies of aza-C-hypersensitive mutants is that they relied on the endogenous Dcm protein for DPC formation, leaving some uncertainty about whether hypersensitivity was indeed due to DPC formation or some other effect of aza-C on cell metabolism. Indeed, while *recA* knockout mutants were among the first shown to be aza-C hypersensitive, a double *recA dcm* knockout is still hypersensitive, for reasons that have never been explained (9). This result raises the possibility that some aza-C-hypersensitive mutants are affected not because of DPC formation, but from some

entirely different toxic effect of aza-C. It should be noted that while the *recA dcm* mutant is still hypersensitive to aza-C, overexpression of a cytosine methyltransferase greatly increases the sensitivity of a *recA* knockout, clearly showing an involvement of RecA in some process related to DPC formation (9). In addition, a subset of the above mutants (*recB*, *recG*, *ssrA*, *smpB*, *hflC* and *dnaJ*) were shown to have DPC-dependent sensitivity to aza-C (8,9,16), and our approach in the current work explicitly demands DPC-dependent sensitivity.

In this study, we use both transposon mutagenesis and candidate gene approaches to further define *E. coli* functions that do or do not protect against the toxic effects of DPC formation, using aza-C treatment with overexpression of the M.EcoRII methyltransferase. We confirm the importance of *recA*, *recBC*, *recG*, and *uvrD* in survival after DPC formation, find that inactivation of a number of other functions in DNA metabolism, cell division and tRNA modification cause hypersensitivity to aza-C-induced DPCs, and rule out the involvement of several proteins that might be expected to play a role in survival after DPC formation. We also trace the defect in *miaA*, *mmnG* and *mmnE* mutants to a defect in the tmRNA ribosome recycling pathway and identify three hypersensitive transposon insertion mutants that apparently activate expression of the prophage Mu Gam protein, which binds to DNA ends and thereby blocks access to RecBCD enzyme.

2. Materials and Methods

2.1 Materials

Aza-C was obtained from Sigma-Aldrich, nitrocellulose membranes (Protran® BA 85) from Whatman, and polyclonal M.EcoRII antibody from Proteintech Group, Inc (custom generated). LB broth contained Bacto tryptone (10 g/L), yeast extract (5 g/L) and sodium chloride (10 g/L), supplemented with the appropriate antibiotics. Antibody to the SsrA tag was kindly provided by Tania Baker (MIT).

2.2 *E. coli* strains

The transposon mutagenesis screen utilized strain HK21 carrying plasmid pR215 (16). HK21 is a derivative of strain ER1793 (obtained from New England Biolabs) and has the following genotype: F⁻ *fhuA2* (*lacZ*)*r1* *glnV44* *e14*⁻ (*McrA*⁻) *trp-31* *his-1* *rpsL104* *xyl-7* *mtl-2* *metB1* (*mcrC-mrr*)*114::IS10* *sula* (*Keio deletion*) *dinD::lacZ*. Mutants that passed the sensitivity screening in the original background were further analyzed by transferring the transposon insertion mutation, via P1 transduction, into strain HK22 (same as HK21 except lacking the *dinD::lacZ* fusion). Note that insertions in the *dinD::lacZ* segment can be transduced along with the entire segment, because *dinD::lacZ* is less than half the size of DNA transduced by phage P1 (see Supplemental Table 2). All Western blot experiments were done using the transduced HK22 strains with the indicated plasmid. *E. coli* strains for the candidate gene approaches were constructed by P1 transduction of deletions from the Keio collection (33) into strain HK22, selecting for the kanamycin resistance marker inserted at the Keio deletion site. Plasmid pBAD-MEcoRII was then transformed into each HK22 strain following confirmation of the gene knockout.

2.3 Plasmids

Plasmids pR215 and pBAD-MEcoRII were used to express M.EcoRII (16). The pR215 plasmid is a pACYC184-derived plasmid containing the tetracycline resistance marker and the M.EcoRII gene under control of its own promoter (34). The pBAD-MEcoRII plasmid is a pBAD33 derivative (35) which carries chloramphenicol resistance and the M.EcoRII coding sequence under control of an arabinose-inducible promoter. Expression of M.EcoRII from this plasmid is repressed with glucose (0.2%) and activated with arabinose (0.05%).

2.4 Isolation of transposon mutants

The transposon mutagenesis screen was performed using the EZ-Tn5™ <KAN-2>Tnp Transposome™ Kit from Epicentre. The transposase-transposon complex was electroporated into HK21 cells carrying plasmid pR215, selecting for the kanamycin resistance gene on the transposon. The transposon mutants were spotted onto LB agar plates with and without 5 µg/mL aza-C for the primary screen. Mutants with an apparent growth defect in the presence of aza-C were subjected to a secondary screen by streaking onto aza-C-containing LB plates. Mutants that passed these initial screens were then subjected to a liquid end-point growth assay and spot assays of serially diluted cell suspensions on solid media with and without aza-C. In the liquid growth assay, overnight cultures were diluted to an OD₅₆₀ of 0.5, diluted 1:2000 in LB plus tetracycline (to maintain selection for the pR215 plasmid), and then mixed with an equal volume (75 µL each) of LB containing serial dilutions of aza-C (starting at 40 µg/ml) in 96-well plates. Total cell growth was measured after 18 h at 37°C. For spot assays on LB agar plates, overnight cultures were diluted to an OD₅₆₀ of 0.5, and 10-fold serial dilutions were spotted (5 µl each) onto LB agar plates containing aza-C (5 µg/ml) and incubated overnight at 37°C. The location of the transposon insertions within the *E. coli* chromosome was determined by genomic DNA sequencing using transposon-specific primers: KAN-2 FP-1 Forward Primer (5'-ACCTACAACAAAGCTCTCATCAACC-3') and KAN-2 RP-1 Reverse Primer (5'-GCAATGTAAACATCAGAGATTTTGAG-3').

2.5 Growth kinetics for aza-C sensitivity

Aza-C sensitivity was measured with continuous growth curves in a temperature-controlled ELx808™ Absorbance Microplate Reader. Overnight cultures in LB media at 37°C were diluted to an OD₆₃₀ of 0.5, diluted 1:2000 in LB with tetracycline if cells contained pR215 or chloramphenicol and 0.05% arabinose if cells contained pBAD-MEcoRII, and then mixed with an equal volume (75 µl each) of LB containing serial dilutions of aza-C (15 µg/ml) in 96-well plates. The cells were grown in the plate reader at 37°C with constant shaking, and OD₆₃₀ was measured every 15 minutes for 18 h.

2.6 Western blots for M.EcoRII and SsrA tagging

M.EcoRII and SsrA tagging levels were analyzed in mutant derivatives of the HK22 pBAD-MEcoRII strain background. Cells were pregrown overnight in LB media at 37°C in the presence of 0.2% glucose, and then diluted to an OD₅₆₀ of 0.1. The cells were then grown to an OD₅₆₀ of 0.5 in the same media, with or without aza-C (50 µg/ml). Cells were then harvested by centrifugation, washed once with LB, and finally resuspended in LB containing arabinose (0.05%) to induce M.EcoRII expression. After one hour at 37°C with shaking, cell

samples equivalent to 2 ml of $OD_{560} = 0.5$ were harvested, incubated in an ethanol/dry ice bath for at least 15 minutes, and then stored at -20°C .

For the M.EcoRII Western blots, frozen cell pellets were thawed at room temperature and resuspended in 25 μl of water and 25 μl of sample buffer (20% glycerol, 100 mM Tris pH 6.8, 2% SDS, 2% β -mercaptoethanol, and bromophenol blue), and boiled for 5 min in a water bath. An aliquot (15 μl) of each sample was loaded onto a 7.5% polyacrylamide (Tris-HCl) gel and run for approximately 2 h in 25 mM Tris-Glycine buffer containing SDS (0.1%). The portion of the gel containing proteins larger than about 75 kDa was cut off and stained with Coomassie blue dye to serve as a loading control. The remaining portion of the gel was transferred to a nitrocellulose membrane for 60 min at 12 V using a Genie Blotter transfer device (Idea Scientific Co.). The blot was blocked for 1 h in 20% non-fat milk powder solution (Biorad) in Tris-buffered saline (TBS). The membrane was incubated overnight at 4°C with polyclonal M.EcoRII primary antibody and Tween (0.1%), and then washed three times with TBS buffer at room temperature (10 min each). The membrane was incubated with secondary antibody (IRDye 800CW-conjugated goat anti-rabbit IgG (LI-COR®)) for 30 min, and the washes were repeated. After air-drying, the membrane was scanned on an Odyssey Imaging System (LI-COR Biosciences), and quantified using the Odyssey software.

The SsrA tagging Western blots were performed in the same manner as above, except that the primary antibody was against the SsrA tag. For the follow-up experiment examining SsrA tagging in the *recA* mutant, overnights of wild-type and *recA* mutant cells were diluted to $OD_{560} = 0.1$ in LB with chloramphenicol and grown for one and a half generations at 37°C , at which point arabinose (0.05%) was added to induce M.EcoRII expression. The cell cultures were split in half at about $OD_{560} = 0.5$, with one sample receiving aza-C (50 $\mu\text{g}/\text{mL}$), and both were incubated for 1 h at 37°C before harvesting.

3. Results

3.1 Transposon insertion mutants hypersensitive to aza-C

To identify genes involved in mitigating the damage caused by the M.EcoRII model DPC's, we began with a transposon insertion mutant screen. We mutagenized *E. coli* strain HK21 containing the pR215 plasmid, which overexpresses M.EcoRII, and screened for mutants that are hypersensitive to aza-C. HK21 contains a *sulA* deletion to prevent cell filamentation/growth inhibition due to SOS induction after DPC formation, and a *dinD::lacZ* fusion to monitor SOS induction (16). This strain is also deficient for the McrA and McrBC restriction systems, to prevent inadvertent DNA damage at non-cognate sites upon methyltransferase overexpression (36). In this genetic background, the expression of M.EcoRII from the pR215 plasmid causes a substantial increase in aza-C sensitivity, implying that growth inhibition is due to DPC formation (16).

We screened a collection of 6,888 colonies after electroporating the EZ-Tn5™ <KAN-2> transposon™ (Epicentre) into this strain and selecting for insertion mutants based on kanamycin resistance. Each insertion mutant was first tested for sensitivity to aza-C in a primary screen by spotting resuspended colonies onto LB plates containing aza-C (5 $\mu\text{g}/\text{ml}$).

Mutants that appeared aza-C hypersensitive in both this initial screen and a secondary screen on aza-C-containing plates were then subjected to a tertiary screen. This final screen consisted of a liquid (microtiter plate) end-point growth assay to compare inhibitory concentrations to that of wild-type cells and/or spot assays of serially diluted cell suspensions on solid media with and without aza-C. Following this series of screens, a total of 24 mutants qualified as aza-C hypersensitive.

The locations of the transposon insertions were identified using genomic sequencing with a primer complementary to transposon sequence. The 24 insertion mutants localized to 16 different genes, which included genes involved in recombination, cell division, translation, tRNA modification and chaperone pathways (Table 1; also see Supplemental Table 1 for context of the surrounding genes for each insertion). In a previous contribution, we analyzed the mutants with insertions in *ssrA*, *hflC* and *dnaJ*, providing evidence for the involvement of the tmRNA system in response to DPC formation ((16); also see below). To avoid redundancy with the previous publication, only limited data on these three mutants will be shown here.

A more robust comparison of aza-C sensitivity levels was conducted by measuring growth curves in the presence of varying concentrations of aza-C in 96-well plates using a Biotek ELx808 Absorbance Microplate Reader (which allows incubation at 37°C and agitation for aeration). Examples of growth curves of the wild-type and two hypersensitive mutants (Mu gene 9 and *dinG*) are shown in Figures 1A–C. Sensitivity levels were compared by processing the growth curves in a manner that corrects for differences in growth rates. For each mutant and the wild type, we first determined the growth rate of the drug-free culture during its exponential phase (OD₅₆₀ values from 0.01 to 0.1; in every case, these data points matched a simple exponential curve with R² values of greater than 0.99). We next determined the first time point at which the growth rate had fallen to half that value (or less), and plotted the accumulated cell density (as a fraction of the drug-free value) at that time point for the various drug concentrations (Figure 1D – 1F). Based on these growth curves, insertion mutants in genes *recA*, *recC*, *recG*, *ftsK*, *trmH*, *uvrD*, and three different bacteriophage Mu genes (see below) were the most hypersensitive, whereas mutants in *dinG*, *miaA*, *mnmE*, and *mnmG* showed more modest levels of sensitivity (for sensitivity data on *ssrA*, *dnaJ* and *hflC* mutants, see (16)).

It should be noted that while complete knockouts of *ftsK* are inviable, mutations that create FtsK truncations that retain the N-terminal 210 amino acids are viable, yet deficient for XerCD-mediated recombination (37). The insertion mutants we isolated would be expected to fall under this category given the location of the insertions (see Table 1). Therefore, we propose that the hypersensitivity of the *ftsK* mutants is due to a lack of XerCD recombination. To test this proposal, we made a *xerD* knockout strain by moving the deletion from the Keio collection via P1-mediated transduction and transforming the strain with the pBAD-MEcoRII plasmid. As predicted, the *xerD* knockout strain proved to be Aza-C hypersensitive, supporting the model that XerCD recombination is important for cell survival during DPC formation (Figure 2A).

One *trmH* knockout mutant was identified in the aza-C sensitivity screen. TrmH is a tRNA methyltransferase, and one could speculate that TrmH is thereby important in the activity of tmRNA. Alternatively, insertion mutants in *trmH* have been shown to have polar effects on *recG* (38). Because we identified *recG* knockout mutants in the screen, the aza-C sensitivity of the *trmH* insertion could be due to a similar polar effect (see Supplemental Table 1 for location of *recG* immediately downstream of *trmH*). To distinguish between these models, we transformed the *trmH::kan* cells with a plasmid from the Aska collection overexpressing either TrmH or RecG (pCA24N-*trmH* and pCA2N-*recG*, respectively; (39)). The *trmH::kan* cells with pCA24N-*recG* were no longer hypersensitive to aza-C, even in the absence of IPTG to induce high-level expression of RecG (data not shown). However, the *trmH::kan* cells with pCA24N-*trmH* were just as sensitive as the parental *trmH::kan* cells (data not shown). We conclude that the *trmH* insertion is hypersensitive due to a polar effect on RecG expression.

As indicated above, three insertions that led to strong aza-C hypersensitivity were localized within bacteriophage Mu genes (*kil*, 6 and 9). The HK21 parental strain is not a traditional Mu lysogen, but it does have a *dinD::lacZ* fusion construct that is known to contain Mu prophage DNA (40,41). For other reasons, we had subjected strain JH39, the *E. coli* strain from which the *dinD::lacZ* fusion was moved, to next-generation sequencing analysis. This confirmed the presence of phage Mu DNA, and allowed us to assemble the complete sequence of the *dinD::lacZ* fusion construct (Supplemental Table 2). The three transposon insertion mutants were in Mu genes *kil*, 6 and 9, with the transposon always in the same orientation, one that aligns the kanamycin-resistance gene with the Mu early open reading frames in this region (see Supplemental Table 1). Each of the three insertion mutants could therefore presumably activate expression of Mu gene *gam*, which is only 42 bp downstream of the most distal transposon (the one in gene 9), by read-through transcription. Gam is normally transcribed as part of an early operon during Mu infection, starting at the P_e promoter (42,43), but would not normally be expressed in lysogens (or presumably from the *dinD::lacZ* construct) (44). The Mu Gam protein inhibits nucleases, including RecBC, by binding to DNA ends (45). We therefore infer that these three insertions activate Gam expression, essentially creating a phenocopy of a RecBC knockout mutant. Shee *et al* (46) recently showed that Gam expression by other means induces *recBC* knockout phenotypes. Consistent with this model, the HK22 Mu 9::*kan* cells are hypersensitive to both nalidixic acid and ciprofloxacin, well-documented phenotypes of RecBC knockout mutants (Supplementary Figure 1). Expression of the toxic product of the Mu *kil* gene should not occur in any of the three mutants because the insertions are within or downstream of *kil*.

3.2 Expression of M.EcoRII is needed for maximal sensitivity of insertion mutants

The pR215 plasmid used in the initial screen contains the M.EcoRII coding sequence under control of its native promoter, precluding a simple test of whether aza-C sensitivity requires M.EcoRII expression. We therefore transferred each of the transposon insertion mutants by P1 transduction into a similar *E. coli* strain (HK22) that contains the M.EcoRII coding sequence in a pBAD33 vector, which is arabinose inducible (35). While HK22 does not originally contain the *dinD::lacZ* fusion, the P1 transduction of the insertion mutations in

Mu DNA results in transfer of the entire fusion, which presumably integrates at the native location in *dinD* via homologous recombination with the DNA flanking the fusion.

All of the insertion mutations caused hypersensitivity in this new background, and in general the levels of sensitivity in the HK22 background were similar with this pBAD expression system as with the pR215 strains. Most of the mutants were sensitive to aza-C when grown in the presence of arabinose but not in the presence of glucose (which represses expression from pBAD plasmids), confirming the need for M.EcoRII protein in aza-C sensitivity (Supplemental Figure 2). The remaining mutants are very sensitive mutants such as *recA* and *recC*; these were somewhat sensitive to aza-C even in the presence of glucose, but much more sensitive in the presence of arabinose (Supplemental Figure 2). Therefore, M.EcoRII (*i.e.*, DPC formation) is required for the major mode of sensitivity of all of the insertion mutants. These results rule out the possibility that aza-C incorporation into RNA is involved in the major mode of sensitivity for any of the mutants (also see (16)). However, in the very sensitive mutants like *recA*, some residual sensitivity is apparently generated either from expression of the endogenous Dcm methyltransferase, from leaky expression of M.EcoRII (in spite of glucose addition), or from some other pathway (e.g. effects of aza-C without DPC formation).

This and other experiments also confirm that the transposon insertions are causative for the aza-C sensitivity phenotypes, because sensitivity was transferred with the kanamycin resistance gene in the transposon during the P1 transduction event.

3.3 Transposon insertion mutations do not alter levels of M.EcoRII

One possible explanation for the aza-C hypersensitivity of a transposon insertion mutant is that the gene knockout increases M.EcoRII levels, for example by increasing expression or stability of the protein. To approach this possibility, we compared the levels of M.EcoRII between wild type and the various mutants by Western blotting. Cells harboring the pBAD-MEcoRII plasmid were grown in the presence of arabinose (0.05%; in the absence of aza-C) for 1 hour at 37°C in rich media (LB) prior to sample preparation. M.EcoRII levels in each of the mutants were found to be similar to that in the wild-type strain, eliminating this possible explanation for hypersensitivity (Figure 3).

3.4 Candidate approach: Functions potentially involved in DPC repair

The sensitivity screen above uncovered some interesting mutants, but it was cumbersome and we did not screen enough mutants to approach saturation of the genome. Therefore, we also conducted a candidate-based screen to directly address the involvement of proteins that might be expected to impact survival after DPC formation (as with the *xerD* strain above). Deletion mutations in a variety of genes were moved, usually from the Keio knockout collection (33), into the HK22 background by phage P1-mediated transduction. Transductants were verified by PCR and the pBAD-MEcoRII plasmid was transformed into the strain in order to test aza-C sensitivity.

One set of genes tested were those whose products might be expected to be directly involved in the repair or tolerance of DPCs or downstream DNA damage. The SbcCD complex has

been shown to remove proteins bound to DNA *in vitro* by introducing double strand breaks (47), and one could imagine a repair pathway similar to that used to trigger meiotic recombination near SPO11 complexes (48). UmuCD (also known as pol V) is a translesion polymerase that is expressed during the late phases of the SOS response (49), and perhaps base insertion opposite the DPC could be involved in some tolerance pathway. RecF is a part of the RecFOR recombination pathway, which is alternative to the RecBC pathway for RecA-mediated recombination (50). RecFOR is important in stalled fork processing after UV damage (51,52), and aza-C-induced DPCs cause fork stalling (11). Knockout mutants for each of these functions expressing M.EcoRII displayed essentially wild-type sensitivity to aza-C in cells in the microtiter plate assay (Figure 2B–2D). We conclude that none of these functions play important roles, or if they do, there are overlapping alternative functions that can take their place efficiently.

Both *dnaJ* and *hflC* knockouts are hypersensitive, raising the intriguing possibility that these proteins could be involved in proteolysis of the protein that is covalently linked to DNA (16). Since DnaJ and DnaK frequently act together (53,54), we also generated a *dnaK* knockout mutant and tested its sensitivity to aza-C. We were surprised to find that the *dnaK* mutant is somewhat resistant to aza-C treatment (Figure 2E; also see Supplemental Figures 3B, 4, and 5A, the latter of which shows that the level of resistance is higher when the extent of inhibition is measured later in the growth curve). This implies that the *dnaJ* mutant sensitivity is due to a DnaJ activity that is DnaK-independent, and perhaps DnaK normally sequesters DnaJ and reduces its ability to function in a DPC-relevant pathway (also see Discussion).

3.5 Transposon insertion mutants that compromise the tmRNA system

Knockout mutants of *ssrA*, which encodes tmRNA, and *smpB*, which encodes the protein partner of tmRNA, are hypersensitive to aza-C in cells expressing M.EcoRII (Table 1) (16). These and other results supported a model in which DPCs on the DNA template lead to blockage of coupled transcription/translation complexes, necessitating revival of stalled ribosomes by the tmRNA system (16). Because inactivation of the tmRNA system causes hypersensitivity to aza-C-induced DPC formation, some of the other transposon insertion mutants (besides *ssrA*) could be hypersensitive because they inactivate a gene necessary for proper functioning of the system. We therefore analyzed the levels of SsrA tagging in the transposon insertion mutants to query for functions involved in the tmRNA system.

HK22 cells with the pBAD-MEcoRII plasmid were grown for just over two generations at 37°C in glucose-containing LB with aza-C (50 µg/mL), and then expression of M.EcoRII was induced for one hour by washing the cells into LB media containing arabinose (0.05%). Although this strain background is proficient in downstream proteases that degrade SsrA-tagged proteins, significant levels of SsrA-tagged proteins accumulate upon DPC formation indicating saturation of the proteolysis systems (16). Tagged proteins were detected by Western blotting using an antibody to the SsrA tag sequence. As expected, the wild-type strain showed strong tagging while the *ssrA* insertion mutant showed only background levels that are due to cross-reactivity of the antibody (Figure 4A) (16). In addition, the insertion

mutants in *miaA*, *mmE* and *mmG* showed little or no SsrA tagging after DPC induction (Figure 4A) (see Discussion).

The products of *dnaJ* and *hflC* each have a potential connection to the tmRNA pathway, since they can modulate the activity of the downstream protease FtsH (HflB) (55–57). However, the ribosome clearing function, rather than induced proteolysis, is the critical function of the tmRNA system involved in aza-C sensitivity (16). Furthermore, double *dnaJ smpB* and *hflC smpB* mutants were more sensitive to aza-C than any of the single mutant parents (16). We also found that both the *dnaJ* and *hflC* insertion mutants showed robust aza-C-induced SsrA tagging (Figure 4A). These results all argue that inactivation of DnaJ or HflC causes hypersensitivity to DPC formation by mechanism(s) other than defects in the tmRNA system (see Discussion).

We were initially surprised to find that the *recA* transposon insertion mutant showed little or no SsrA tagging in the above protocol. That protocol involves growing cells in the presence of aza-C for two generations prior to induction of the M.EcoRII, and *recA* mutants are quite hypersensitive to aza-C even without overexpression of a methyltransferase (see above; also see (7,9,10)). Therefore, we considered the possibility that this extreme hypersensitivity caused such high levels of cell death and/or perturbation of metabolism that SsrA tagging could not take place for trivial reasons at such a late time. Indeed, the viable cell count of the *recA* mutant was reduced by about 100 fold or more during the pre-growth, consistent with this explanation, and in some repetitions, the *recA* culture did not even reach the target density ($OD_{560} = 0.5$). We therefore tested SsrA tagging in the *recA* mutant using a different protocol, one that queries tagging after a shorter aza-C exposure time. Actively growing cultures of wild-type and *recA* mutant cells were treated with arabinose (0.05%) at an OD_{560} of 0.3 to induce M.EcoRII expression. When the cultures reached OD_{560} 0.5 the cells were treated with (or without) aza-C (50 μ g/mL) for 1 hour. The *recA* mutants showed a substantial increase in SsrA tagging with aza-C treatment, although still not as strong an induction as with the wild-type control cells (Figure 4B). We conclude that SsrA tagging is triggered by aza-C-induced DPCs in *recA* mutant cells, but that the levels of tagging can be decreased by the extreme aza-C sensitivity of the mutant.

3.6 Candidate approach: Functions potentially involved in clearing DPC-stalled transcription/translation complexes

In the well-studied tmRNA pathway, the first step of ribosome release involves tmRNA binding to the empty A-site of the ribosome (58). For this to occur, the ribosome normally translates to the end of a truncated mRNA transcript (lacking a stop codon), or the transcript is cleaved to generate an empty A-site (59,60). In the case of DPCs and blocked transcription/translation complexes, how does tmRNA access the stalled ribosomes? One proposed model is that a ribonuclease cleaves the nascent transcript near the A-site of a stalled translation complex, generating a truncated transcript and a premature 3' mRNA end for any upstream ribosomes on the same transcript (61). Another model is that the blocked RNA polymerase, along with the nascent (truncated) transcript, is somehow released from the DNA template, and ribosomes then translate forward till they get stuck at the premature 3' mRNA end. We sought genetic data to distinguish between these two models.

If A-site cleavage is critical for tmRNA function at DPC-blocked transcription/translation complexes, we would expect to observe aza-C hypersensitivity in cells that are deficient in RNase II, which is implicated in A-site cleavage (see (62) and Discussion). We also tested mutants lacking other exonucleases with known roles in tRNA processing (RNase D, PH, and LS). However, knockout cells for each of these were no more sensitive to aza-C treatment than wild-type cells (Figure 2F and data not shown). These results argue that A-site cleavage, at least by the known pathway, is not necessary for tmRNA activity in this situation.

Turning to the model in which RNAP and nascent transcript are released from DNA after blockage by the DPC, two candidates arose in the transposon screen above, DinG and UvrD. These two helicases, along with Rep, were implicated as having overlapping roles to avoid transcription-replication conflicts, particularly when the collisions were from opposite directions (18), and could potentially be releasing DPC-stalled RNAP. If this release activity is responsible for tmRNA tagging, we would expect to see a decrease in tagging in these mutants. We found no such decrease in spite of the clear hypersensitivity of each mutant (Figure 1E, 1F and 4A).

The third helicase implicated in transcription-replication conflicts by Boubakri et al (18), Rep, was not identified in the transposon screen above, but could have been missed since the screen was not saturating. Rep is of particular interest because it was recently shown to play a major role in minimizing replication pausing at sites of bound protein, particularly transcription complexes, presumably by displacing the (non-covalently) bound protein (63). In the microtiter plate liquid growth assay, *rep* cells showed slight resistance to aza-C treatment (Figure 2G; also see Supplemental Figure 3D), however spot tests of *rep* cells on solid media showed no change in aza-C sensitivity compared to the wild type (Supplemental Figure 4). The slight resistance in the microtiter plate assay could be an indirect effect of the relatively poor growth of the *rep* cells. Overall, these experiments provided no support for the model that the activity of the accessory helicases is required for the tmRNA system, although it remains possible that the helicases are releasing DPC-stalled RNAP (also see Discussion).

A number of additional factors can release blocked elongation complexes under various circumstances, including Mfd, Rho, and HepA (for review, see (64)). Previously we showed that inactivation of Mfd or inhibition of Rho does not change sensitivity to aza-C-induced DPCs, arguing that they play no unique role in releasing RNAP stalled at a DPC (16). Here, we found that *hepA* cells were no more sensitive to aza-C than wild-type (Figure 2H; perhaps even slightly resistant). Furthermore, treating *hepA* with bicyclomycin did not lead to sensitivity, arguing against redundant roles of Rho with HepA (Supplemental Figure 6A).

Since no potential release factor emerged from these tests, we turned to factors known to stimulate elongation past blocking lesions, namely GreA, GreB, and DksA. Strains carrying *greA* or *greB* mutations showed no change in sensitivity to aza-C, implying that neither factor plays a unique role in relieving DPC-induced elongation blockage (Figure 2I and 2J). In addition, treating *greA* or *greB* cells with bicyclomycin (Rho inhibitor) also did not lead to aza-C hypersensitivity (Supplemental Figure 6B and 6C).

Under our standard sensitivity test conditions, the *dksA* knockout strain showed similar aza-C sensitivity as the wildtype (Figure 2K). However, when the effect of aza-C was measured later in growth (when the no-drug control reached 10% of its maximal growth rate), the *dksA* cells appeared modestly hypersensitive (Supplemental Figures 3F and 5B). One caveat is that the very poor growth of the *dksA* mutant might somehow skew the sensitivity results. Assuming that the modest hypersensitivity is meaningful, the simplest explanation is that, under those conditions, DksA releases RNAP and the transcript from DPC-stalled complexes. If so, inactivation of DksA should decrease or eliminate SsrA tagging. However, Western blots for SsrA tagging levels (in late-growth cells) revealed slightly elevated levels of tagging induced by aza-C in *dksA* cells relative to wild-type cells (Figure 4C). This result argues against a role of DksA in releasing RNA polymerase and the transcript. The slight increase in tagging is consistent with a speculative alternative model, in which DksA helps RNAP bypass the DPC lesion without removing it from the DNA, while some competing process releases the polymerase and transcript (see Discussion). *E. coli* RNAP can transcribe through reversibly bound proteins *in vitro* (31,65), and T7 RNAP can transcribe through a 44 kDa DPC *in vitro*, albeit with low frequency (15). While these results are consistent with a novel and interesting role for DksA in DPC biology, we were unable to identify any function required for tmRNA access after transcription/translation blockage by DPC (see Discussion).

4. Discussion

Through a combination of a transposon mutagenesis screen and a candidate gene approach, we have identified a number of *E. coli* mutants that are hypersensitive to DPC formation, and shown that certain other mutants are not hypersensitive in spite of a reasonable expectation that they might be. Aza-C hypersensitivity was shown to depend on expression of the M.EcoRII protein, and was not an artifact of reduced M.EcoRII expression levels. As described in the Introduction, several previous reports have characterized *E. coli* aza-C hypersensitive mutants (7–10), and our results generally confirm and extend these prior studies. We uncovered several mutants that had not been previously identified as aza-C hypersensitive, including *dinG*, *miaA*, *mnmE*, *mnmG*, *ftsK*, and *xerD* knockouts (as well as *hflC*, *dnaJ*, *smpB* and *ssrA*, analyzed by (16)). For some or all of these, the levels of DPC formed by endogenous Dcm may be insufficient to reveal an involvement of these functions in survival. Indeed, Salem et al (2009) were unable to detect Dcm-DNA crosslinks after aza-C treatment, presumably due to very low levels of endogenous Dcm (8). In a related vein, several of the past studies failed to explicitly test the involvement of DPC formation in aza-C hypersensitivity, whereas our experimental design explicitly requires the involvement of DPC formation by testing sensitivity upon M.EcoRII overexpression and verifying that overexpression increases sensitivity. Thus, even for mutants that had been shown previously to be aza-C hypersensitive, our results verify that hypersensitivity depends on DPC formation. Also, we were able to provide evidence against the involvement of multiple gene products whose known activities suggested that they might be involved in cell survival after DPC formation.

4.1 Role of recombination proteins in survival after DPC formation

Several of the genes identified from the transposon screen were previously implicated in genome stability, including the genes that encode recombination proteins RecA, RecC and RecG (Table 1). These proteins could play a direct role in resolving DPCs, or alternatively, they could be involved in repairing DNA ends that are secondary effects of DPCs (e.g. breaks generated at stalled replication forks; see (11,66,67)). We previously detected RecA-dependent Holliday junctions in plasmids with aza-C-induced DPCs, and argued that DPC-stalled replication forks were broken and then repaired in a RecA-dependent fashion, or alternatively, that these stalled forks underwent RecA-dependent fork regression (11). Either of these pathways could thereby reflect a role for recombination proteins in dealing with the downstream consequences of DPC formation, rather than a direct role in repairing DPCs. Even though aza-C-induced DPCs lead to fork stalling, we detected little or no effect on aza-C sensitivity from knocking out the RecF protein, which plays a key role in replication restart after UV (51,52).

On the other hand, recombination proteins could potentially play a more direct role in DPC repair. An attractive model is provided by the pathway of meiotic recombination, where Mre11/Rad50 complex cleaves DNA near an SPO11-DNA covalent complex to initiate a double-strand break repair event (48). This pathway was also implicated in repair of topoisomerase cleavage complexes (48). The homolog of the Mre11/Rad50 complex in *E. coli* is SbcCD, although the role of this protein is quite distinct from that of Mre11/Rad50 (68,69). Strikingly, SbcCD can cleave DNA *in vitro* near proteins that are very tightly bound to the end (47), and so we asked whether SbcCD might be involved in repair of DPCs. However, we found no change in aza-C sensitivity in a SbcC knockout mutant, strongly arguing against this possibility.

The functional homolog of Mre11/Rad50 in *E. coli* is the RecBCD enzyme, and we (and others) found that knockouts of this RecBCD are strongly hypersensitive to aza-C-induced DPCs. The involvement of the RecBCD enzyme clearly indicates the generation of double-strand breaks or ends after DPC formation, as does the identification of hypersensitive mutants that apparently express the Mu Gam protein (which inhibits RecBCD by binding to DNA ends). The role of RecBCD presumably involves the processing of double-strand ends for recombinational repair, and these ends could be generated as an indirect consequence of fork collision and/or as an essential step in a DPC repair pathway (see above).

The aza-C sensitivity of the FtsK partial-function mutant and the *xerD* knockout mutant implies that at least some of the protective recombinational activity that occurs after DPC formation is inter-chromosomal. Homologous recombination between chromosomes leads to chromosomal dimers that require XerCD-mediated site-specific recombination for proper resolution (70). This resolution occurs at the *dif* site in the terminus region and requires the N-terminus of FtsK for proper localization (71). Therefore, these data support the model that RecBCD and RecA catalyze homologous recombination between daughter chromosomes, which somehow protects the cell from DPCs or their downstream consequences.

Helicases play key roles in various aspects of DNA replication, recombination, and repair. We found that strains lacking helicases RecG, UvrD or DinG are hypersensitive to aza-C-

induced DPCs, while Rep knockout mutants are not. Our results thus far do not identify the molecular roles played by the three involved helicases in survival after DPC formation. However, several possibilities are worth pursuing. First, one or more of these helicases could be directly involved in a DPC repair pathway. Bacterial helicases including Rep and UvrD have been shown to remove non-covalently bound proteins from DNA (13,18,20), and a DNA-tracking protein like a helicase could perhaps serve both to efficiently recognize covalent adducts along the DNA length and facilitate their removal. Second, the RecG helicase could participate in the above-mentioned double-strand break repair reaction with RecA and RecBC by facilitating branch migration. Third, one or more of these helicases could play key roles in the behavior of the replication forks stalled at the aza-C-induced DPCs, perhaps modulating fork regression in some way and/or preventing aberrant re-initiation events (see (72)). Notably, Kumari et al. showed that covalently bound proteins can block UvrD translocation, and also that UvrD protein plays a key role in the replication of plasmids containing DPCs (13). Fourth, as will be discussed below, one or more of these helicases could be reducing sensitivity to aza-C-induced DPCs by disengaging RNA polymerase complexes stalled at DPCs.

4.2 Heat shock proteins and sensitivity to DPCs

We isolated aza-C-hypersensitive mutants with transposon insertions in *hflC* and *dnaJ* from our screen, and analyzed these mutants in detail previously (16). The HflC gene product modulates the FtsH (HflB) protease (55), while DnaJ is a chaperone that can promote degradation of certain proteins by the FtsH protease (73). In our previous study, we showed that the hypersensitivities caused by the *hflC* and *dnaJ* mutations were independent of the tmRNA system, because these mutations further sensitized an *ssrA* knockout mutant (16). This result led to a speculation that HflC and DnaJ could possibly be involved in proteolysis of the covalently bound M.EcoRII as part of a DPC repair pathway. This idea is further supported by exciting new research from eukaryotic systems that shows proteolysis of the protein component of DPCs plays a key role in DPC repair (74,75).

DnaJ functions as a co-chaperone to the *E. coli* Hsp70 protein DnaK but also has DnaK-independent activities (53,54). To further explore the role of DnaJ in aza-C sensitivity, we also generated and tested a *dnaK* knockout mutant. The *dnaK* mutant turned out to be modestly resistant to aza-C treatment (Figure 2E and Supplemental Figure 5A). This indicates that the role of DnaJ in aza-C sensitivity is DnaK-independent, and perhaps also that DnaK can sequester DnaJ and reduce its ability to function in a DPC-relevant pathway. A different interpretation is that *dnaK* mutants overproduce heat shock proteins (56), and perhaps one or more heat shock proteins assist in survival after aza-C. In general, given the broad roles of HflC, DnaJ and DnaK in protein metabolism, it will be important to decipher whether aza-C sensitivity is modulated by direct or indirect effects.

4.3 Processing of stalled transcription/translation complexes and the tmRNA system

We have previously shown that inactivation of tmRNA or its protein cofactor SmpB leads to aza-C hypersensitivity, and that aza-C-induced DPCs trigger extensive SsrA tagging (16). This led us to propose a “chain-reaction” model in which RNA polymerase is blocked by the DPC, and then translating ribosomes on the attached nascent transcript are blocked by the

stalled polymerase and potentially backed up in an array of stalled ribosomes behind the RNA polymerase. The tmRNA system would then function to clear the stalled ribosomes, while the mechanism of clearance of the blocked RNA polymerase remains unknown. This pathway could potentially be coupled to a DPC repair pathway, but no evidence for such coupling is yet evident.

We tested the various aza-C hypersensitive mutants for possible defects in SsrA tagging to identify candidate activities in the tmRNA pathway and provide a molecular explanation for the sensitivity of those mutants. Indeed, the transposon insertion mutants in genes *miaA*, *mnmE*, and *mnmG* were found to be defective in SsrA tagging (Figure 4A). The products of these three genes are involved in tRNA modification, and mutants lacking any of these modifying enzymes show altered efficiencies of tRNA utilization and/or ribosomal frameshifting (76,77). Because tmRNA does not contain *miaA*- or *mnmE*-dependent modifications (78), the apparent defect in SsrA tagging after aza-C treatment most likely is due to an indirect role of these modifications in altering the mechanics of translation.

Regarding the chain reaction model for blockage of coupled transcription-translation complexes, it is not yet clear how tmRNA is able to access the empty A-site on the ribosome for the trans-translation process. It was first proposed that A-site cleavage was required for tmRNA tagging as it was believed that a ribosome stalled at the 3' end of a nonstop mRNA was the signal for tmRNA recruitment (79). Furthermore, *in vitro* assays showed that tmRNA tagging is most efficient when the 3' end of the transcript was within 6 nucleotides of the P-site codon, implying that A-site cleavage would facilitate tmRNA activity (61). However, it has since been shown that tmRNA activity occurs in the absence of A-site cleavage, showing the A-site cleavage is not a requirement for tmRNA tagging (79). We also found that knockouts of RNase II, which is required for A-site cleavage, are not deficient in tmRNA tagging, arguing against the A site cleavage model for tmRNA function after DPC formation.

The second model proposes that RNA polymerase and nascent transcript are released by some active process, to license tmRNA action on the released transcript. Presumably, release and reutilization of RNA polymerase would be important even if tmRNA gains access to the transcript through A site cleavage, so the fate of the RNA polymerase is relevant in any case. *In vitro* studies have shown that RNA polymerase is blocked by aza-C-induced DPCs (12), and also that RNA polymerase blocked by a tightly bound non-cleaving mutant EcoRI endonuclease is stable for up to 1 hour (80). These results imply that release of RNA polymerase requires some additional release factor(s), but its identity remains obscure. We previously found that sensitivity to aza-C-induced DPCs was unaffected by inactivation of transcription-coupled repair factor Mfd or by inhibition of transcription terminator Rho, arguing for a lack of involvement of these two factors (16).

In the current study, we found that mutational inactivation of helicase DinG or UvrD causes hypersensitivity to aza-C-induced DPCs, while inactivation of Rep does not (Figures 1, 2G, and Supplemental Figure 3D). While *dinG* and *uvrD* mutants are hypersensitive, the amount of DPC-induced SsrA tagging was not reduced in these mutants (Figure 4A). It remains

possible that DinG and/or UvrD releases stalled RNA polymerase at a DPC, but that this step is not needed for tmRNA action.

HepA (RapA), a member of the SWI/SNF superfamily of helicase-like proteins, is an RNA polymerase binding protein that allows recycling of the polymerase at poorly characterized post-transcription/post-termination complexes that form *in vitro* (25,81). Given its recycling function, HepA presented as a candidate for releasing RNA polymerase and the nascent transcript from the DPC-blocked complexes. However, a HepA knockout strain was found to be no more sensitive to DPC formation than wild type cells (Figure 2H), even with concomitant inhibition of Rho activity (Supplemental Figure 6A), arguing against an involvement of HepA.

E. coli RNAP can transcribe through reversibly bound proteins *in vitro* (31,65), and T7 RNAP can transcribe through a DPC *in vitro*, albeit with low frequency and in a mutagenic fashion (15). We were therefore interested in the possible role of factors that might promote polymerase read-through at blocking lesions. Transcription factors GreA, GreB and DksA interact with the secondary channel of RNA polymerase, and have been shown to decrease pausing and to stimulate elongation past a lesion or protein roadblock (29,31,82). We found that *greA* and *greB* mutants are no more sensitive to DPC formation than wild type cells, even when termination is decreased by decreasing Rho activity (Figures 2I–2J and Supplemental Figure 6).

The one transcription factor that we did find to be somewhat protective against aza-C-induced DPCs is DksA, but only when measured late in growth (Figure 2K and Supplemental Figure 5B). DksA modulates transcription elongation outside of its transcription initiation roles, although the specific mechanism is not well defined. Perhaps most relevant to the present study, DksA can prevent transcription-replication conflicts, perhaps by destabilizing paused transcriptional elongation complexes (19,30). This factor does not appear to be important in releasing nascent transcripts from the DPC-stalled complex for tmRNA action, because the knockout strain had increased rather than decreased SsrA tagging (Figure 4C). The increased tagging observed in the DksA knockout mutant suggests that DksA activity might instead be in competition with the factor(s) responsible for creating the substrates for the tmRNA system. One intriguing possibility is that DksA promotes transcription through DPCs, allowing bypass of the lesion when it is located on the template and/or the non-template strand.

In summary, our studies have not uncovered the pathway by which the tmRNA system gains access to ribosomes within DPC-stalled transcription/translation complexes. They have provided evidence against involvement of several RNases, Rep, Mfd, Rho, GreAB and HepA in any pathway that is protective for aza-C-induced DPCs. They have also identified DinG and UvrD as important factors for this protection, and these proteins might be involved in releasing stalled RNA polymerase (with some other mechanism of tmRNA access), or they might instead play roles in DPC repair and/or the response of replication fork encounters with DPCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

aza-C	azacytidine
DPC	DNA-protein crosslink
M.EcoRII	EcoRII methyltransferase
RNAP	RNA polymerase

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Highlights

Model DNA-protein crosslinks (DPCs) from EcoRII methyltransferase acting on azaC-DNA

Homologous recombination proteins RecA/RecBCD/RecG required for protection from DPCs

Chromosome dimer resolution by XerCD and FtsK important for protection from DPCs

Novel DPC hypersensitive mutants: *ftsK*, *xerD*, *dinG*, *dnaJ*, *hflC*, *miaA*, *mnmEG*, *ssrA*

Explores the processing of transcription/translation complexes stalled by DPCs

Figure 1. Aza-C hypersensitivity of mutants expressing M.EcoRII

HK22 (WT) or mutant derivatives (all containing the M.EcoRII-expressing plasmid pR215) were tested for aza-C sensitivity using a microtiter plate sensitivity assay. Overnight cultures were diluted to OD₅₆₀ = 0.5, then diluted 1:2000 in LB with tetracycline and mixed with an equal volume (75 μl each) of LB containing the indicated amounts of aza-C in 96-well plates. Cells were grown for 12 hours at 37°C and cell turbidity was measured every 15 minutes. Panels A through C are representative growth curves for the wild type and mutant cell lines respectively. For the comparative titration curves in panels D–F, a standardized point in the growth curve was first determined for each cell line, namely the time at which the growth rate of the no-drug culture dropped to 50% of the earlier maximum exponential rate. The OD₆₃₀ value at this time was then taken for each drug concentration and divided by the no-drug control to account for differences in growth rate. Sensitivity tests for all mutants were conducted multiple times, and the results in this figure are typical.

Figure 2. Aza-C hypersensitivity of potential repair protein and transcription-altering knockout strains

The indicated cells were grown in a similar manner as Figure 1, with the exception that the cells contained pBAD-MEcoRII and therefore the cells were grown in the presence of 0.05% arabinose and chloramphenicol. Drug titration curves were calculated as in Figure 1. For each experiment, wild-type (blue diamonds) and the indicated mutant (red squares) was grown in three independent cultures with the exception of the *rep* and *sbcC* mutants, where 6 independent cultures were grown and measured due to the poor growth and slight variability of these strains.

Figure 3. Expression of M.EcoRII in wild-type and mutant cells

Extracts from HK22 (WT) or the indicated HK22 derivatives, with M.EcoRII plasmid pBAD-MEcoRII, were analyzed by Western blotting with polyclonal antibodies to M.EcoRII. Cells were grown to an OD_{560} of 0.5, harvested by centrifugation, washed, and resuspended in LB containing arabinose (0.05%) to induce M.EcoRII. Cell samples were collected after incubating for one hour. The top portion of the gel was excised and stained with Coomassie blue as a total protein loading control. The Western antibody signals and total protein controls were quantitated using an Odyssey Imaging System. The calculated ratio of M.EcoRII to total protein is expressed as percentage of wild-type levels. Error bars were calculated by taking the standard deviation of three independent cultures. The experiment was conducted with only one insertion mutant even for those genes where we isolated two or three independent insertions; in each case, the mutant used was the one labeled (1) in Table 1.

Figure 4. SsrA tagging in wild-type and mutant cells

Cell lysate collection and Western blot analysis for HK22 and the indicated derivatives in panel A were conducted as in Figure 3 except that the primary antibody used was to the degradation tag of SsrA. In panel B, HK22 or HK22 *recA* cells, each containing pBAD-MEcoRII, were treated with arabinose (0.05%) at an OD₅₆₀ of 0.3 to induce methyltransferase expression, and then with (or without) aza-C (50 µg/mL) for 1 hour when the cells reached OD₅₆₀ 0.5. Cell lysis and Western blots were carried out as above. In both panels A and B, the data is from 3 independent cultures. In panel C Western blots for HK22 and HK22 *dksA* cells were conducted as in panel A with the exception that the data is from 5 independent cultures. For all three panels, the differences in SsrA tagging levels between plus and minus aza-C were calculated, and are shown relative to that of the wild-type. In panels B and C, the measured tagging levels were first adjusted for total protein loading (measured as in Figure 3). The experiment was conducted with only one insertion mutant even for those genes where we isolated two or three independent insertions; in each case, the mutant used was the one labeled (1) in Table 1.

Table 1

Summary of transposon-insertion mutants hypersensitive to DPC inducer aza-C

Gene	Gene coordinates	9-bp repeat ^a	Last nucleotide ^b	Transposon direction	Function
<i>dinG</i>	833070 → 835220	CATTATTGT	834439	→	DNA helicase
<i>dnaJ</i>	14168 → 15298	GCCATGAAA	14260	←	Chaperone
<i>ftsK (1)</i>	933224 → 937213	GCTTCCATC	937143	→	Cell division
" " (2)	" "	CTGGCAGCC	934308	←	
<i>hflC</i>	4403300 → 4404304	GTATTGAAG	4403912	→	Protease regulator
<i>miaA</i>	4399252 → 4400202	ACGCTCTTC	4388831	→	tRNA modification
<i>mmuE (1)</i>	3886828 → 3888192	GGGCTAAGT	3887200	→	tRNA modification
" " (2)	" "	GGCCGGGAA	3887126	←	
<i>mmuG (1)</i>	3925633 ← 3923744	CCCATGATG	3924896	→	tRNA modification
" " (2)	" "	AATCTGACC	3924521	→	
Mu 6	4542 → 4772	GGTAAAAGG	4732	→	Unknown
Mu <i>kil</i>	4315 → 4539	GTCTTAATG	4508	→	Host cell death
Mu 9	5482 → 5781	GCATTGTAT	5759	→	Unknown
<i>recA (1)</i>	2823769 ← 2822708	GGTGAGAAAG	2822884	→	Recombination
" " (2)	" "	GGCTCATCA	2823258	→	
<i>recC (1)</i>	2962428 ← 2959060	TCCTGGCAC	2959669	→	Recombination
" " (2)	" "	GCTTTGACC	2960353	→	
" " (3)	" "	GGCTATGGC	2961293	→	
<i>recG (1)</i>	3825210 → 3827291	GGCTTATGG	3825559	→	Recombination
" " (2)	" "	GTGTAGCTC	3826850	→	
<i>ssrA</i>	2755593 → 2755955	GCTTAGAGC	2755729	→	tmRNA
<i>trmH</i>	3824515 → 3825204	GCTGGGTAC	3824728	←	tRNA modification; polar effect on <i>recG</i>
<i>uvrD (1)</i>	3997983 → 4000145	GATCCACGC	3998683	→	DNA helicase
" " (2)	" "	CGTCTTACC	3998092	→	

^aThe 9-bp repeat was determined using a single primer that anneals within the transposon. Only one of the two junctions was sequenced, and we inferred the other junction because this transposon creates 9-bp repeats. The last nucleotide of the inferred repeat shows the last intact nucleotide of the gene, reading in the 5' to 3' direction with respect to the reference strand of the genome (regardless of the direction of the gene).

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^q Positions are given with reference to MG1655 sequence accession number U00096.3, with the exception of the Mu genes, which are given with reference to the sequence of bacteriophage Mu accession number AF083977.1.

, Direction of the transposon kanamycin-resistance gene with respect to the *E. coli* genome sequence.