Modulation of DNA repair by mutations flanking the DNA channel through RNA polymerase

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The RuvABC and RecBCD proteins promote rescue of stalled or broken DNA replication forks in *Escherichia coli*. Strains lacking these proteins cope poorly with DNA damage and have problems with chromosome segregation and cell division. We show how these difficulties are overcome to varying degrees by a sub-class of RNA polymerase mutations selected for their stringent phenotype. Thirty-five mutations were sequenced. All but one change single amino acids in RpoB or RpoC that lie on or near the path taken by DNA through the enzyme, indicating they may affect the stability of transcription complexes. Four mutant enzymes are shown to form unstable open complexes at the λ cro promoter. At least one may also release stalled complexes or limit their formation, as it reduces the need for reactivation of transcription by GreA or GreB, and for transcription-coupled DNA repair of UV damage by Mfd. The results shed light on the interplay between DNA replication and transcription and suggest ways in which conflicts between these two vital cellular processes are avoided or resolved.

Keywords: *Escherichia coli* (p)ppGpp/recombination/replication/RNAP

Introduction

The rescue of broken or damaged replication forks is crucial for genome duplication, chromosome segregation and cell division. It relies on close interplay between DNA replication, recombination and repair, as highlighted by the phenotype of *Escherichia coli* mutants lacking PriA protein, a factor enabling replication fork assembly at sites removed from normal origins of replication. These mutants are defective in recombination, fail to resume replication following exposure to DNA damaging agents and have very low viability, even during normal growth (Kogoma, 1997; Sandler and Mariani, 2000).

The idea that recombination could repair a damaged replication fork is not new (Skalka, 1974), but only recently has the extent to which it underpins chromosome duplication been fully appreciated (Cox, 2001). It had also been long proposed (Hanawalt, 1966), but only recently proven (Kuzminov, 2001), that a fork collapses when it runs into a single-strand nick in the template DNA, though such events are probably rare during normal growth of a wild-type strain because DNA ligase is so very active.

However, recent studies indicate that replication forks arrested by lesions in the template strands or by DNA-bound protein complexes form Holliday junctions, and may be broken *via* the action of the RuvABC junction resolvase (Seigneur *et al.*, 1998; Flores *et al.*, 2001). But these studies also indicate that RecBCD nuclease may limit fork breakage by digesting the duplex DNA end extruded from the junction and displacing RuvAB(C). This would restore a fork structure and allow PriA to promote replication restart. When a fork is broken, the recombinase activities of RecBCD and RecA may catalyse recombination between the broken arm and the intact sister molecule, forming a D loop that can be targeted by PriA and converted to a replication fork. RuvABC-mediated resolution of the Holliday junction formed by this recombination restores a fully-functional fork structure. Thus, RuvABC resolvase may function both to break and restore replication fork structures, which may explain the sensitivity of *ruv* mutants to DNA damage.

McGlynn and Lloyd (2000) discovered that certain mutations (*rpo**) in RNA polymerase (RNAP) alleviate the UV-sensitive phenotype of *ruv* strains. These were found among stringent RNAP mutations that compensate for the lack of (p)ppGpp, the signalling molecules of the stringent response (Cashel *et al.*, 1996). Stringent mutations occur exclusively in *rpoB*, *rpoC* and rarely *rpoD* (Cashel *et al.*, 1996). The levels of (p)ppGpp alter gene expression, adapting it to conditions of stationary phase or starvation (Cashel *et al.*, 1996; Hengge-Aronis, 1996). Stringent mutations mimic this effect. However, altered gene regulation is unlikely to account for the increased UV resistance of *rpo** *ruv* strains. If it were, any stringent mutation would be expected to promote survival, which is not the case (McGlynn and Lloyd, 2000). Those that do (*rpo**) must therefore have some additional feature.

Both (p)ppGpp and stringent mutations destabilize RNAP complexes at all promoters tested (Barker *et al.*, 2001a,b). The *rpo** mutations may specifically reduce the incidence of stalled elongation complexes trapped by lesions in the template strand. In this paper we describe how they substitute amino acid residues in RpoB or RpoC that are located on or very close to the path of DNA through the enzyme. They form a major cluster within the RNAP structure, with many surface exposed on the DNA channel. Their properties support the idea that RNAP molecules stalled at lesions in the DNA are major obstacles to replication fork progression, especially in UV-irradiated cells. We discuss how destabilization of such complexes by the Mfd, GreA and GreB proteins, and by (p)ppGpp, may determine how often replication forks meet problems, what happens when they do and consequently what has to be done to resume replication.

Results

Analysis of stringent RNAP mutations in a *recB* background

Stringent mutations modify RNAP such that genes are expressed as if (p)ppGpp were present. Although altered transcription is the defining property of stringent mutations, it does not seem sufficient to confer an *rpo** phenotype; not all stringent mutations promote survival of UV-irradiated *ruv* strains (McGlynn and Lloyd, 2000). The studies reported by McGlynn and Lloyd indicated that *rpo** mutations also promote survival of irradiated *recB* strains. However, the one mutation studied in detail (*rpoB**35) was found to have a much stronger effect on *ruv* than on *recB*. To investigate whether this is a feature of stringent phenotype in general, we studied the effect of (p)ppGpp and stringent mutations in a *recB* mutant background. First, we used a strain carrying null alleles of both *relA* and *spoT* to eliminate synthesis of (p)ppGpp (Cashel *et al.*, 1996). RelA protein is the main factor responsible for synthesis of (p)ppGpp during the stringent response, but a minor contribution is made by SpoT protein. We found the absence of (p)ppGpp in this *relA spoT* null background reduces the ability of *recB* cells to

survive irradiation with UV light. In contrast, the increased (p)ppGpp in a strain carrying *spoT1*, which eliminates the (p)ppGppase activity of SpoT, promotes survival (Figure 1A). However, the effects seen with *recB* are less dramatic than those seen previously with *ruv* (McGlynn and Lloyd, 2000).

To obtain stringent mutations, samples of a *relA spoT recB* construct were spread on minimal agar, on which only derivatives carrying stringent RNAP mutations can grow (Cashel *et al.*, 1996). Independent stringent clones were then screened for sensitivity to UV and those showing some increase in survival relative to the parent were kept for further analysis (Table I). Examples illustrating the range of effect observed are shown in Figure 1B. A number also proved resistant to rifampicin and were thus identified immediately as having a mutation in *rpoB* (Table I). Five of these *rpoB* alleles (*rpo**148, 551, 563, 571 and 1260) were transferred to a *relA⁺ spoT⁺* background and tested to see whether they suppress both *recB* and *ruv*. All five do (Figure 1C; data not shown). However, *rpo**148, 563 and 1260 clearly suppress *ruv* much better than *recB* (Figure 1C, RpoB proteins Q148P and T563P; data not shown). Thus, the *rpo**35 allele encoding RpoB H1244Q described previously by McGlynn and Lloyd (2000) is by no means an exception, although such strong suppressors are in the minority. Surprisingly, the *rpo**571 allele encoding RpoB L571Q proved a weaker suppressor of *recB* in this background than in the original isolate (compare Figure 1B with C). We cannot exclude the possibility that the original strain has a second mutation promoting survival.

Morphology, viability and growth of *rpo** cells

Cells of wild-type *E. coli* grown to mid-exponential phase are fairly uniform in size, with most having one or two compact nucleoids (Figure 2A). *rpo**148 cells resemble the wild type, but are generally shorter (Figure 2E). The same is true for cells carrying *rpo**571 or *rpo**551 from strains BT130 and BT132, respectively, or the prototypic *rpo**35 allele (data not shown). The smaller size of *rpo** cells is consistent with our finding that broth cultures grown to the same optical density as an *rpo*⁺ strain contain about twice as many cells (data not shown). Cells of *recB*, *ruv* and $\Delta relA \Delta spoT$ strains are more variable in morphology and have a reduced ability to form colonies, as described previously (Capaldo-Kimball and Barbour, 1971; Otsuji *et al.*, 1974; Xiao *et al.*, 1991). A significant fraction form long filaments, which in the case of *ruv* often show aberrant nucleoid segregation (Figure 2B; data not shown) (Ishioka *et al.*, 1998). Combining *recB* or *ruv* with $\Delta relA \Delta spoT$ leads to very extensive filamentation and the formation of anucleate cells or long filaments with unevenly distributed DNA (Figure 2C and D). Not surprisingly, <15% of the cells observed are able to form colonies. Introduction of *rpo**35, 148, 551 or 571 restores cell morphology to near wild type in all these strains (Figure 2F–H; data not shown). They also substantially improve viability. In the case of $\Delta relA \Delta spoT$ strains carrying *ruv* or *recB*, viable cells increase from <15% of the total population to >70% (data not shown). The associated improvement in cell morphology is quite dramatic (Figure 2G and H). Indeed, a substantial improvement in cell morphology and viability was found

Fig. 1. Effect of stringent factors on survival of UV-irradiated *recB* and *ruv* cells. (A) Effect of $\Delta relA \Delta spoT$ and *spoT1* on *recB*. (B) Effect of stringent *rpo* mutations in a *relA spoT recB* strain. (C) Comparisons of the effect of four different stringent mutations affecting RpoB (β subunit of RNAP) on *recB* and *ruv* strains. The strains used are identified by genotype and in each panel are (A) MG1655, N5034, N4278 and N4315; (B) MG1655, BT130, BT132, BT133, BT134 and N4315; (C) *recB*: BT235, BT181, BT175, BT236 and N4278; *ruv*: BT321, BT230, BT163, BT164 and N4583.

Table I. *Escherichia coli* strains and effect of *rpo* mutations on UV survival and Rif resistance

Strain	Relevant genotype ^a	Fraction surviving 45 J/m ² UV light ^b	Resistance to rifampicin (µg/ml)	Frequency of isolation ^c
(a) MG1655 parent strains				
N4304	<i>ΔrelA ΔspoT</i>	0.184	0	
N4278	<i>recB268</i>	0.00265	0	
N4305	<i>ΔrelA ΔspoT ΔruvAC65</i>	0.000016	0	
N4315	<i>ΔrelA ΔspoT recB268</i>	0.0000448	0	
(b) N4315 derivatives				
BT121	<i>rpoB</i> _{T563P} (= <i>rpo</i> *563)	0.029	>100	3
BT122	<i>rpo</i>	0.00777	>100	
BT123	<i>rpo</i>	0.0033	20	
BT124	<i>rpoB</i> _{H447R}	0.00567	0	1
BT125	<i>rpoB</i> _{V550E}	0.022	>20	1
BT126	<i>rpoC</i> _{E1146D}	0.00417	n/a	1
BT128	<i>rpo</i>	0.008	20	
BT129	<i>rpoB</i> _{G536V}	0.0063	50	1
BT130	<i>rpoB</i> _{L571Q}	0.1	50	1
BT131	<i>rpo</i>	0.016	20	
BT132	<i>rpoB</i> _{H551P} (= <i>rpo</i> *551)	0.00858	20	7
BT133	<i>rpoC</i> _{K215E}	0.000775	n/a	1
BT134	<i>rpoB</i> _{Q148P} (= <i>rpo</i> *148)	0.00797	>100	2
BT135	<i>rpo</i>	0.00493	0	
BT137	<i>rpo</i>	0.005	20	
BT141	<i>rpo</i>	0.00086	0	
BT142	<i>rpoB</i> _{H447P}	0.00883	<20	2
BT143	<i>rpoB</i> _{S788F}	0.0000297	20	1
BT145	<i>rpo</i>	0.00325	0	
BT146	<i>rpoB</i> _{G1260D} (= <i>rpo</i> *1260)	0.026	20	1
BT147	<i>rpo</i>	0.0045	0	
BT149	<i>rpo</i>	0.00348	0	
BT150	<i>rpo</i>	0.00022	0	
BT151	<i>rpoB</i> _{L533P}	0.048	>100	1
BT152	<i>rpoB</i> _{L420R}	0.02	20	1
BT153	<i>rpoB</i> _{A532E}	0.045	>100	1
N4281	<i>rpoB</i> _{H1244Q} (= <i>rpo</i> *35)	0.00437	<10	2
TF2	<i>rpoB</i> _{L448I}	0.019	20	1
(c) N4305 derivatives				
N4576	<i>rpoB</i> _{I572S}		>100	1
(d) AB1157 parent strains				
N4293	<i>ΔrelA ΔspoT ΔruvAC65</i>	0.0000083	0	
BT174	<i>ΔrelA ΔspoT recB268</i>	0.00011	0	
(e) BT174 derivatives				
BT184	<i>rpoB</i> _{Y395D}	0.0027	10	2
BT185	<i>rpoB</i> _{R151S}	0.004	0	1
BT186	<i>rpoB</i> _{P153L}	0.01	10	2
BT190	<i>rpoB</i> _{G181V}	0.0035	10	1
BT195	<i>rpoC</i> _{Δ312–314}	0.0057	n/a	1
(f) N4293 derivatives				
BT199	<i>rpoB</i> _{G537D}	0.0056	50	1
BT200	<i>rpoC</i> _{R1330S}	0.0085	n/a	1
BT205	<i>rpoC</i> _{R1148H}	0.035	n/a	1
BT208	<i>rpoC</i> _{K789Q}	0.064	n/a	1

^aThe *recB*, *ΔrelA* and *ΔspoT* alleles are described more fully in Table II. N4305 and its derivatives, and N4293 are also *eda-51::Tn10*. All strains in category (b–f) grow on minimal medium and therefore in all probability carry a stringent mutation in one of the genes encoding RNAP. Where the mutation has not been identified, the mutation is referred to as *rpo*. Where located to *rpoB* or *rpoC*, the effect on the gene product is indicated by the amino acid change and residue number. Apart from the deletion shown, all identified mutations are the result of single bp substitutions.

^bSurvival is given as a fraction of colony forming cells relative to unirradiated controls. Data are means of at least two independent experiments.

^cOnly one strain number is listed for multiple isolates.

Fig. 2. Phase-contrast microscopy and DAPI staining of *E. coli* cells showing the effect of stringent factors on cell morphology and DNA segregation in *recB* and *ruv* strains. Within each panel, the image on the left shows a wide field of view to illustrate the range of cell sizes; to the right is a representative sample of cells at higher magnification under phase contrast (bottom) or showing DAPI staining (top). Panels (A–D) on the left show *rpo* wild-type cells and (E–H) on the right *rpo**148 mutant cells encoding RpoB Q148P. The strains used are identified by genotype and are (A) MG1655, (B) N4278, (C) N4305, (D) N4315, (E) BT325, (F) BT236, (G) BT259 and (H) BT134.

in all the *rpo** isolates obtained from a *relA spoT recB* strain (data not shown), indicating that it is a property of *rpo** mutations in general.

Location of *rpo** mutations in *rpoB* and *rpoC*

The *rpo** mutation was identified by DNA sequencing in 35 of the mutant strains selected for analysis. Most mutations were in *rpoB*, but six were in *rpoC* (Table I; Figure 3). Several were obtained repeatedly, despite the independent origin of the mutant strains. Apart from the

rpoC deletion of nine nucleotides in strain BT195, resulting in removal of R312, G313 and R314, we found only substitutions leading to single amino acid changes. All the mutations affect conserved residues or lie in conserved regions of the protein. In RpoB they form four clusters, far removed from each other, the central one in conserved region D overlapping with Rif regions I and II. In RpoC they are spread over nearly the whole protein. One stringent mutation that does not have *rpo** properties has also been identified by sequencing. It

Fig. 3. Location of stringent mutations on linear maps of RpoB and RpoC. Conserved regions (A–I) in RpoB and (A–H) in RpoC are marked, as are the sites and the nature of the substitutions or deletions caused by *rpo** mutations. The non-*rpo** stringent mutation S788F is in bold. Rifampicin-sensitive changes in RpoB are underlined.

confers resistance to rifampicin, but affects RpoB S788, which is well isolated from the *rpo** clusters.

Relationship between *rpo and resistance to rifampicin**

The majority of the *rpo** mutations studied confer some degree of resistance to rifampicin (19 of 27 different alleles identified by sequencing; Table I). However, among derivatives of a *recB relA spoT* strain selected directly for resistance to rifampicin, we found only 46% had a stringent phenotype, allowing growth in minimal medium, and not all of these had the characteristics of *rpo** clones. Furthermore, as we have shown, some of the *rpo** mutations are alleles of *rpoC*. Rifampicin resistance has never been associated with mutations in this gene (Jin and Gross, 1988). Thus, resistance to rifampicin does not go hand in hand with either stringency or *rpo**. However, some association of *rpo** and rifampicin resistance cannot be dismissed. Of the *rpo** mutations we analysed in *ruv* and *recB* strains, the ones with higher resistance to rifampicin tend to be also better at promoting survival after UV irradiation (data not shown). However, the correlation is by no means absolute, as demonstrated by *rpo**35, which is a very good suppressor of *ruv*, but only weakly resistant to rifampicin (Table I). Furthermore, several derivatives of a wild-type strain selected for resistance to a high level of rifampicin (100 µg/ml) were found to have *rpoB* mutations that hardly suppress *ruv* at all (data not shown).

With the exception of L533P and T563P, all the alleles that confer resistance to rifampicin represent new mutations. These include A532E and I572S, although other changes at these positions have been reported previously to confer resistance (Jin and Gross, 1988; Severinov *et al.*, 1993). Some confer rather low levels of resistance, which may explain why they were not obtained in previous studies. Most lie in or near the previously defined Rif clusters I and II, and around the previously reported V146

location. H1244Q and G1260D are notable exceptions, though they confer only low resistance.

***rpo** mutations affect possible substrate contacts**

The atomic structure of *Thermus aquaticus* RNAP has been solved at 3.3 Å (Zhang *et al.*, 1999), which has led to structural models of transcription elongation (Korzheva *et al.*, 2000). The similarity of *Taq* and *E.coli* enzymes allowed us to map the residues affected by *rpo** mutations on this structure and to consider the potential implications of changing these residues. Almost all the residues affected lie along the path of the DNA through the enzyme, although not all are in a position to make direct contact with the DNA, or the RNA transcript for that matter. However, the buried ones are mostly in key positions where a mutation might be expected to influence enzyme substrate interactions. Figure 4 is a view on the nucleotide entry channel right into the active centre. β'K789 is clearly visible opposite the active centre Mg²⁺. A 90° turn gives a frontal view of RNAP (Figure 4B). Cuts through the structure were made as indicated to expose features within the enzyme where most *rpo** mutations are located, and provide views on the inside surfaces of RpoB and RpoC (Figure 4C and D).

Many of the affected RpoB residues cluster within the RNAP structure to form a fairly well-defined '*rpo** pocket'. Several more border this pocket (Figure 4D). Four residues within the pocket lie also within the previously defined Rif pocket, namely A532, L533, G534 and T563. The Rif pocket (Figure 4D, residues in yellow) outlines the binding site for rifampicin. When rifampicin is bound in the pocket, it blocks the path of the elongating RNA (Campbell *et al.*, 2001). The overlap between the Rif pocket and the *rpo** pocket is narrow and, in contrast to the residues forming the Rif pocket, those in the *rpo** pocket cover most of the DNA entry path, some exposed on the surface, some inside the protein. The most distal residue from the active centre is L420, nearly at the tip of the

Fig. 4. Structural analysis of RpoB and RpoC mutations. Shown are four views of *T.aquaticus* RNAP. The $\alpha 1$ and ω components are represented in white, $\alpha 2$ in gold, β in teal and β' in pink. Residues changed by *rpo** mutations are indicated in red (when in RpoB) or orange (when also part of the Rif pocket in RpoB), or green (when in RpoC). Residues that form the Rif pocket are in yellow. The DNA path through the polymerase is indicated by a semi-transparent arrow, but for clarity of presentation is drawn at ~30% of the diameter of the DNA. The blue-green arrows in panels (A), (C) and (D) point to the nucleotide entry channel. Dashed lines in (A) and (B) represent cutting planes for viewing internal residues. (A) View of intact enzyme showing the nucleotide entry channel, through which the active site Mg^{2+} ion (blue) and the mutated K789 residue are visible. (B) View of the intact molecule turned 90° relative to (A). Four Rpo* residues that are in the path of the DNA are visible. (C) View from a cut through the enzyme along plane I and turning 90° round the x-axis to show the inside surface of RpoC. All residues affected by *rpo** mutations in *rpoC* and H1244 affected by *rpoB**35 lie in a position that is likely to make DNA contacts. (D) View from a cut through the enzyme along plane II and turning 90° to reveal the inside surface of RpoB. Many residues affected by *rpo** lie exposed to the channel through which DNA passes. Q148 affected by *rpo**148 lies just behind R151. Several residues that are part of the *rpo** pocket lie just behind A523 and are not directly exposed to the channel.

bigger RpoB lobe and, though solvent accessible, is unlikely to interact directly with the DNA. Other residues, like R151 and G536 (Figure 4D), lie accessible in the path of the entering DNA and might well contribute to interactions. Several residues are buried within the structure, though still underneath the DNA entry path. Two RpoB mutations, H1244Q and G1260D, are well removed from the *rpo** pocket (Figure 4B and C). They affect residues in a region of RNAP formed predominantly by RpoC and facing the Rif and *rpo** pockets. Exposed on the surface in the path of the exiting DNA, they are well placed to make DNA contacts.

The mutations in RpoC do not cluster. However, they lie on the path of the DNA and all but one is surface exposed. K215 lies on the β'_B rudder, helping to steer the entering DNA. RpoC $\Delta 312-314$ affects the β'_C -rudder steering the exiting DNA (Figure 4B and C). R1330 lies at the base of a

helix that participates in forming the entrance channel for the DNA. The residue is also accessible (Figure 4C). K789 lies neatly in the active site chamber, facing the Mg^{2+} ion (Figure 4A–D). R1148 is surface exposed in the DNA entry channel, opposite the β'_B rudder. Together with E1146 hidden right behind it, it forms part of the wall that bifurcates the main channel into the primary channel, used by the incoming DNA, and the secondary channel, presumably used by the incoming nucleotides (Zhang *et al.*, 1999) (Figure 4A, C and D).

The one stringent but non-*rpo** mutation sequenced encodes RpoB S788F. This mutation confers resistance to rifampicin but lies in a very different region from the other mutations analysed, both on the linear map and in the three-dimensional structure. It is buried deeply in the enzyme, on the opposite side of the Rif region from the *rpo** pocket. Though it is far removed from *rpo** residues, the single

Fig. 5. Effect of *rpo*148* (RpoB Q148P) and UV irradiation of template DNA on formation of RNAP open promoter complexes and stalled elongation complexes. (A) Open-complex (OC) formation between λ cro DNA and wild-type (lane 2) or mutant (lane 6) RNAP. Lanes 3 and 7 are run-off transcription assays. Stalled elongation complexes (sEC) formed in the absence or presence of competitor DNA are in lanes 4 and 8, and 5 and 9, respectively. (B) Effect of UV-irradiated DNA on formation of complexes. Lanes 1 and 3 show irradiated template DNA without RNAP. Lanes 2 and 4 show formation of stalled complexes by wild-type (lane 2) and mutant (lane 4) RNAP under conditions of run-off transcription.

example is not sufficient to explain how *rpo** mutations differ from other stringent mutations that do not promote resistance to UV light in *ruv* and *recB* strains.

RpoB Q148P destabilizes open complexes

Stringent mutations in RNAP have been shown to destabilize open complexes (Bartlett *et al.*, 1998; Zhou and Jin, 1998; Barker *et al.*, 2001a,b). To see whether the *rpo** class has a similar effect, we purified holoenzyme containing RpoB Q148P, an *rpo** substitution identified originally in *rpo** strain BT134 (Table I), and tested its ability to form transcription complexes at the λ cro promoter (Nowatzke and Richardson, 1996), using a simple band-shift assay. Reactions designed to reveal open complex formation produced a clear signal with the wild-type polymerase (Figure 5, lane 2), but very little with the mutant enzyme (lane 6). As might be expected, much less open complex was detected with the wild-type enzyme under conditions allowing run-off transcription (lane 3). No change was seen in similar reactions containing the mutant enzyme (lane 7). Judging from the RNA products generated, the wild-type enzyme stalls on the template very infrequently under the conditions used (data not shown).

Elongation complexes were detected using transcription reactions to which only three nucleotides were added. CTP was omitted, halting elongation at position +10. The wild-type and mutant enzymes produced similar levels of the stalled complex (Figure 5, lanes 4 and 8). Clearly, the

mutant polymerase is able to bind the promoter and initiate transcription with reasonable efficiency, which is not surprising as it functions well *in vivo*. The failure to detect an open complex therefore suggests that this complex is unstable. To test stability directly, (stalled) elongation reactions were repeated, except competitor DNA was added at the same time as the three nucleotides. Competitor had little effect on the wild-type polymerase (lane 5), but (stalled) elongation complexes all but disappeared with the mutant enzyme (lane 9). Taken together, these data demonstrate that RpoB Q148P reduces the half-life of RNAP open complexes.

Given *rpo*148* *ruv* cells show much increased resistance to UV light, we investigated complex formation on templates irradiated with UV, which generates lesions known to block progression of RNAP *in vitro* (Selby and Sancar, 1993). Run-off transcription assays with wild-type RNAP showed signals at the position of both open complex and stalled elongation complex (Figure 5B, lane 2). We assume some RNAP molecules are stalled at or very near the promoter, before dissociation of the σ factor, as UV damage is introduced randomly. Similar bands were obtained with the mutant enzyme, but the signals were much weaker (lane 4). Given the enzyme is effectively and stably stalled by nucleotide starvation, the lack of a signal with the UV-damaged substrate could be due either to a diminished rate of escape from damaged promoters or to a shorter half-life of complexes stalled at sites of UV damage.

We have also purified RNAP holoenzyme with the RpoB H1244Q substitution encoded by *rpo*35*, the RpoB L420R substitution from strain BT152, and the RpoC R1330S from BT200 (Table I). All three of these enzymes also form open complexes that have reduced stability (data not shown).

Effect of *rpoB*35* on *greA greB* and *mfd* mutants

GreA and GreB proteins are transcript cleavage factors, promoting the endogenous transcript cleavage activity of RNAP (Orlova *et al.*, 1995), reviving backtracked RNAP (Toulme *et al.*, 2000; Park *et al.*, 2002) and acting at regulatory pause sites (Marr and Roberts, 2000). Although GreB has the stronger effect *in vitro* it is outweighed in the cell by the abundance of GreA, making *greA* strains mildly temperature sensitive. This phenotype is exacerbated by *greB* in that *greA greB* double mutants do not form colonies on Luria-Bertani (LB) agar at 42°C (Sparkowski and Das, 1990). This is thought to reflect the exceedingly long half-life of backtracked RNAP complexes. Since stringent mutations appear to destabilize RNAP open complexes, we considered the possibility they might similarly destabilize backtracked complexes that have become stuck on the DNA, and thereby allow a *greA greB* strain to grow at 42°C. Four *rpo** mutations, *rpoB*35*, 148, 420 and 563, and the one stringent *rpoB* mutation that is not an *rpo** (encoding the RpoB S788F substitution), were introduced into a *greA greB* double mutant. The *rpoB*35* derivative showed much improved growth at 42°C (Figure 6A). None of the other mutations caused any improvement (data not shown). This observation provides an indication that some *rpo** mutations may destabilize backtracked complexes or prevent their formation.

Fig. 6. Suppression of *greA greB* and *mfd* phenotypes by *rpo*35*. (A) Effect of *rpo*35* on growth of a *greA greB* double mutant at 42°C. The strains were streaked onto LB agar plates from fresh overnight cultures grown at 32°C and incubated either at 32 or 42°C as indicated, before photographing. The strains used are identified by genotype and are MG1655, N5301, N5302, N5306 and N5308. (B) Effect of *rpo*35* on survival of an UV-irradiated *mfd* mutant. The strains used are identified by genotype and are MG1655, N4879 and N4880.

We next investigated the effect of *rpo*35* on a strain lacking Mfd protein to see whether we could gain further evidence that it directly affects the stability of transcription complexes. Mfd protein dislodges elongation complexes stalled at UV-induced lesions in the template DNA and subsequently recruits UvrA to the site of damage, thereby facilitating excision repair of the transcribed strand (Selby and Sancar, 1993; Park *et al.*, 2002). Cells lacking Mfd are consequently somewhat sensitive to UV light, although it is not clear whether this is because they have difficulty removing stalled RNAP or in recruiting excision enzymes, or both. Figure 6B shows that *rpo** effectively eliminates the UV-sensitivity of an *mfd* strain. Given *rpo*35* alone has no effect on survival (data not shown) (McGlynn and Lloyd, 2000), this finding suggests that removal of stalled RNAP molecules is no longer a limiting factor for repair, despite the absence of Mfd. Since the mutant polymerase transcribes DNA and

presumably still stalls at lesions, the data therefore also suggest that the stalled transcription complex is less stable.

Discussion

We have shown how RNAP mutations selected for their ability to promote survival of UV-irradiated *recB* or *ruv* strains change amino acid residues flanking the path of DNA through the enzyme complex. The residues affected are generally well placed to contact the DNA or lie within features that wrap around the DNA or guide it through the enzyme. Changes at these positions are likely to disrupt interactions critical to the stability of transcription complexes. They may enable complexes trapped at non-pairing lesions in the template DNA to dissociate spontaneously or to backtrack from the lesion, thus facilitating lesion repair and removing obstacles that might otherwise block replication fork progression and create a need for the RecBCD and RuvABC proteins to promote restart.

In the four cases where we examined the mutant RNA polymerase *in vitro* (*rpo*35*, 148, 563 and 1330) we found evidence that the substitutions reduce the stability of open complexes. The substitution associated with the *rpo*148* mutant protein also reduces the stability of elongation complexes stalled on an UV-irradiated template, which may be highly significant in terms of the suppression of UV sensitivity. In addition, *rpo*35* suppresses the thermo-sensitive growth phenotype of a *greA greB* strain and the UV sensitivity of an *mfd* mutant, indicating that it also destabilizes elongation complexes. Furthermore, the *rpo*533* and *rpo*563* alleles encode RpoB substitutions (L533P and T563P, respectively) shown previously in studies of rifampicin resistance to reduce RNAP-promoter half-life (Zhou and Jin, 1998). We sequenced only one stringent mutation that does not display *rpo** characteristics. Significantly, it affects an amino acid in RpoB (S788) well removed from the path of DNA through the enzyme. Taken together, these findings indicate that *rpo** mutations achieve their effect by disrupting interactions essential for stable DNA binding.

Transcription complexes are 'massively stable' (von Hippel, 1998) and therefore remain bound when trapped by non-pairing lesions in the template strand. Unless removed they prevent lesion repair and block DNA synthesis (Selby and Sancar, 1993, 1994; Selby *et al.*, 1997; van den Boom *et al.*, 2002). Even if displaced by the replication machinery, DNA synthesis would be blocked by the unrepaired lesion, which in the case of a leading-strand block also disrupts replication fork progression, and may lead to fork collapse or breakage (McGlynn and Lloyd, 2002). Exactly why stalled complexes are so stable is not clear. They may backtrack, displacing the 3' end of the growing transcript from the active site, further trapping the complex. Such complexes formed at natural pause sites or generated artificially *in vitro* can be revived by GreA/GreB or nudged into action by Mfd (Marr and Roberts, 2000; Toulme *et al.*, 2000; Park *et al.*, 2002). Neither GreA/GreB nor Mfd could reactivate complexes trapped at lesions. However, Mfd can displace such complexes, and remains in place to recruit excision enzymes that repair the lesion (Selby and Sancar, 1993; Park *et al.*, 2002). *rpo*35* may therefore function by preventing backtracking, thus facilitating spontaneous dissociation of stalled complexes

Table II. Additional *E.coli* K-12 strains used

Strain ^a	Relevant genotype
MG1655	wild-type (wt)
N4235	$\Delta relA::kan \Delta spoT207::cat rpoB_{H1244Q}$ (= rpo^*35)
N4305	$\Delta relA::kan \Delta spoT207::cat \Delta ruvAC65 eda-51::Tn10$
N4583	$\Delta ruvABC::cat$
N4879	$mfd::kan$
N4880	$rpoB_{H1244Q} mfd::kan$
N5034	$spoT1 recB268::Tn10$
N5301	$greA::cat$
N5302	$greB::kan$
N5306	$greA::cat greB::kan$
N5308	$greA::cat greB::kan rpoB_{H1244Q}$
BT163	$\Delta ruvABC::cat rpoB_{L571Q}$
BT164	$\Delta ruvABC::cat rpoB_{H551P}$
BT175	$recB268::Tn10 rpoB_{H551P}$
BT181	$recB268::Tn10 rpoB_{L571Q}$
BT230	$\Delta ruvABC::cat rpoB_{Q148P}$
BT235	$recB268::Tn10 rpoB_{T563P}$
BT236	$recB268::Tn10 rpoB_{Q148P}$
BT259	$\Delta relA::kan \Delta spoT207::cat \Delta ruvAC65 eda-51::Tn10 rpoB_{Q148P}$
BT325	$rpoB_{Q148P}$
BT321	$\Delta ruvABC::cat rpoB_{T563P}$

^aAll strains are derivatives of MG1655.

and circumventing the need for either GreA/GreB or Mfd. The location of the encoded RpoB H1244Q substitution near the DNA exit point is consistent with this possibility. Significantly, three other mutations tested, rpo^*148 , 420 and 563, do not promote growth of a $greA greB$ strain at 42°C and affect RpoB residues located well away from H1244. Yet, like rpo^*35 , rpo^*148 and 563 are strong suppressors of *ruv*.

Clearly, rpo^* mutations are not a homogeneous group and may alleviate the phenotypes of *recB* and *ruv* strains by different mechanisms. This is reinforced by the considerable variation in their ability to promote survival of UV-irradiated cells. Some, like rpo^*551 , do so rather poorly in both *recB* and *ruv* strains (Figure 1C). Such mutations may act simply by generally reducing the rate of growth, providing more time to repair DNA ahead of the advancing replication fork. Others, notably $rpoB^*35$, 148, 563 and 1260, enhance survival much more strongly in *ruv* strains than in *recB* strains, indicating a much more specific effect, as we have suggested.

We did not find a single mutation strongly suppressing UV sensitivity in both *recB* and *ruv* strains. The much greater effect of the rpo^*35 , 148, 563 and 1260 in *ruv* strains is therefore highly significant from a second viewpoint. Assuming these mutations do act to reduce the incidence of stalled replication forks, it indicates that RuvABC acts upstream of RecBCD to promote fork rescue, at least in UV-irradiated cells. It is consistent with the ability of RuvABC resolvase to break forks that have regressed to Holliday junctions, thereby creating a substrate for RecBCD. In a *recB* mutant, RuvABC may break forks, and possibly at a higher frequency than in *rec*⁺ cells (Seigneur *et al.*, 1998), but the broken forks can no longer be rescued by RecBCD-mediated recombination.

Stringent mutations mimic the effect of (p)ppGpp on RNAP in that they reduce the affinity of the enzyme for all promoters tested, thus decreasing transcription of *rrn* operons and increasing transcription of amino acid

biosynthetic operons (Bartlett *et al.*, 1998; Zhou and Jin, 1998; Barker *et al.*, 2001a,b). The results presented here, demonstrate that the rpo^* class of stringent mutations also mimic the ability of (p)ppGpp to promote survival of UV-irradiated *recB* and *ruv* strains. Given that not all stringent mutations have this ability, they reinforce the idea that (p)ppGpp affects the stability not only of promoter complexes but also of elongation complexes and may therefore play an important part in releasing complexes stalled on the template DNA, particularly at lesions in the template strand, as suggested previously (McGlynn and Lloyd, 2000).

Materials and methods

Bacterial strains

Escherichia coli K-12 strains are listed in Tables I and II. All are derivatives of MG1655 or AB1157 (Bachmann, 1996), as indicated. rpo^* mutations in *rpoB* or *rpoC* are identified by the amino acid change they encode. The altered DNA sequence is available on request. Except for rpo^*35 , which encodes RpoB H1244Q, the rpo^* allele number used corresponds to the number of the affected amino acid residue. The *argE::Tn10*, *recB268::Tn10*, $\Delta ruvABC::cat$, $\Delta relA::kan$ and $\Delta spoT207::cat$ mutations were introduced by P1 transduction and selecting for antibiotic resistance. $\Delta ruvAC65$ was introduced by cotransduction with *eda-51::Tn10*. rpo^* mutations were transferred by cotransduction with *argE*⁺ into strains carrying *argE::Tn10*. Full strain derivations are available on request.

Media and general methods

LB broth and agar, and 56/2 salts media were used for bacterial culture. Media recipes and procedures for strain construction and measuring radiation survival have been cited previously (Al-Deib *et al.*, 1996). UV survival values are means of at least two, often three to four, independent experiments. Cell viability was measured in cultures grown in LB broth to an A_{650} of 0.4 and is expressed as the fraction of the cells observed microscopically that are able to form colonies on LB agar. Stringent rpo mutations were obtained by selecting for derivatives of $\Delta relA spoT$ strains carrying *ruv* or *recB* that formed colonies on 56/2 minimal agar (McGlynn and Lloyd, 2000).

Microscopy

Cultures were grown in broth to an A_{650} of 0.4 before concentrating 150-fold in PBS. Concentrated cells (5 µl) were mixed with 5 µl DAPI solution (50 µg/ml in PBS) and dropped on poly-L-lysine slides. Phase-contrast and fluorescence digital images were collected using an Olympus BX51 microscope.

Enzymes

Wild-type and mutant RNAP holoenzyme complexes were purified using pREII-NHα essentially as described previously (Niu *et al.*, 1996), except polymyxin P precipitation was omitted. Protein concentrations are expressed as moles of the holoenzyme.

DNA substrates

λ cro DNA was prepared by PCR from pCBC1 (Nowatzke and Richardson, 1996). The PCR product was ethanol precipitated, gel purified, dephosphorylated and 5' end labelled with [γ^{32} P]ATP using T4 polynucleotide kinase. Where indicated, the DNA substrate was irradiated prior to use with 10 J/m² UV light.

Band shift assays

The buffer used in the reactions was 150 mM KCl, 40 mM Tris-HCl pH 7.9, 4 mM MgCl₂, 1 mM DTT, 0.02% IGEPAL, 0.002% acetylated BSA and 6% (v/v) glycerol. Reactions (final volume 20 µl) contained 10 nM of RNAP holoenzyme and 0.5 nM DNA substrate. RNAP was added to the reaction mixture containing the DNA and incubated for 3 min at 37°C for open complex formation. Samples were then kept on ice during the addition of nucleotides to 2 µM and competitor to 35 nM, where indicated. The reactions were then incubated for a further 20 min at 37°C. After addition of heparin to 2.5 µg/µl, samples were analysed by electrophoresis through 0.8% agarose gels in TG buffer (0.05 M Tris-HCl

pH 7.9, 0.05 M glycine) at 100 V for 90 min. Gels were dried and analysed by autoradiography and phosphorimaging.

Structural analysis of RNA polymerase

The atomic structure of *T.aquaticus* RNAP resolved at 3.3 Å (MMDB 15252; PDB 1HQM) was analysed using PyMol (www.pymol.org).

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References

- Al-Deib,A.A., Mahdi,A.A. and Lloyd,R.G. (1996) Modulation of recombination and DNA repair by the RecG and PriA helicases of *Escherichia coli* K-12. *J. Bacteriol.*, **178**, 6782–6789.
- Bachmann,B.J. *et al.* (1996) Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12. In Neidhardt,F.C. *et al.* (eds), *Escherichia coli and Salmonella Cellular and Molecular Biology*, 2nd edn. ASM Press, Washington, DC, pp. 2460–2488.
- Barker,M.M., Gaal,T. and Gourse,R.L. (2001a) Mechanism of regulation of transcription initiation by ppGpp. II. Models for positive control based on properties of RNAP mutants and competition for RNAP. *J. Mol. Biol.*, **305**, 689–702.
- Barker,M.M., Gaal,T., Josaitis,C.A. and Gourse,R.L. (2001b) Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation *in vivo* and *in vitro*. *J. Mol. Biol.*, **305**, 673–688.
- Bartlett,M.S., Gaal,T., Ross,W. and Gourse,R.L. (1998) RNA polymerase mutants that destabilize RNA polymerase-promoter complexes alter NTP-sensing by *rrm* P1 promoters. *J. Mol. Biol.*, **279**, 331–345.
- Campbell,E.A., Korzheva,N., Mustaev,A., Murakami,K., Nair,S., Goldfarb,A. and Darst,S.A. (2001) Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell*, **104**, 901–912.
- Capaldo-Kimball,F. and Barbour,S.D. (1971) Involvement of recombination genes in growth and viability of *Escherichia coli* K-12. *J. Bacteriol.*, **106**, 204–212.
- Cashel,M., Gentry,D.R., Hernandez,V.J. and Vinella,D. (1996) The stringent response. In Neidhardt,F.C. *et al.* (eds), *Escherichia coli and Salmonella Cellular and Molecular Biology*, 2nd edn. ASM Press, Washington, DC, pp. 1458–1496.
- Cox,M.M. (2001) Historical overview: searching for replication help in all of the rec places. *Proc. Natl Acad. Sci. USA*, **85**, 8173–8180.
- Flores,M.J., Bierne,H., Ehrlich,S.D. and Michel,B. (2001) Impairment of lagging strand synthesis triggers the formation of a RuvABC substrate at replication forks. *EMBO J.*, **20**, 619–629.
- Hanawalt,P.C. (1966) The U.V. sensitivity of bacteria: its relation to the DNA replication cycle. *Photochem. Photobiol.*, **5**, 1–12.
- Henge-Aronis,R. (1996) Regulation of gene expression during entry into stationary phase. In Neidhardt,F.C. *et al.* (eds), *Escherichia coli and Salmonella Cellular and Molecular Biology*, 2nd edn. ASM Press, Washington, DC, pp. 1497–1512.
- Ishioka,K., Fukuoh,A., Iwasaki,H., Nakata,A. and Shinagawa,H. (1998) Abortive recombination in *Escherichia coli* *ruv* mutants blocks chromosome partitioning. *Genes Cells*, **3**, 209–220.
- Jin,D.J. and Gross,C.A. (1988) Mapping and sequencing of mutations in the *Escherichia coli* *rpoB* gene that lead to rifampicin resistance. *J. Mol. Biol.*, **202**, 45–58.
- Kogoma,T. (1997) Stable DNA replication: interplay between DNA replication, homologous recombination, and transcription. *Microbiol. Mol. Biol. Rev.*, **61**, 212–238.
- Korzheva,N., Mustaev,A., Kozlov,M., Malhotra,A., Nikiforov,V., Goldfarb,A. and Darst,S.A. (2000) A structural model of transcription elongation. *Science*, **289**, 619–625.
- Kuzminov,A. (2001) Single-strand interruptions in replicating chromosomes cause double-strand breaks. *Proc. Natl Acad. Sci. USA*, **98**, 8241–8246.
- Marr,M.T. and Roberts,J.W. (2000) Function of transcription cleavage factors GreA and GreB at a regulatory pause site. *Mol. Cell*, **6**, 1275–1285.
- McGlynn,P. and Lloyd,R.G. (2000) Modulation of RNA polymerase by (p)ppGpp reveals a RecG-dependent mechanism for replication fork progression. *Cell*, **101**, 35–45.
- McGlynn,P. and Lloyd,R.G. (2002) Recombinational repair and restart of damaged replication forks. *Nat. Rev. Mol. Cell Biol.*, **3**, 859–870.
- Niu,W., Kim,Y., Tau,G., Heyduk,T. and Ebright,R.H. (1996) Transcription activation at class II CAP-dependent promoters: two interactions between CAP and RNA polymerase. *Cell*, **87**, 1123–1134.
- Nowatzke,W.L. and Richardson,J.P. (1996) Characterization of an unusual Rho factor from the high G + C Gram-positive bacterium *Micrococcus luteus*. *J. Biol. Chem.*, **271**, 742–747.
- Orlova,M., Newlands,J., Das,A., Goldfarb,A. and Borukhov,S. (1995) Intrinsic transcript cleavage activity of RNA polymerase. *Proc. Natl Acad. Sci. USA*, **92**, 4596–4600.
- Otsuji,N., Iyehara,H. and Hideshima,Y. (1974) Isolation and characterization of an *Escherichia coli* *ruv* mutant which forms nonseptate filaments after low doses of ultraviolet light irradiation. *J. Bacteriol.*, **117**, 337–344.
- Park,J.S., Marr,M.T. and Roberts,J.W. (2002) *E. coli* transcription repair coupling factor (Mfd protein) rescues arrested complexes by promoting forward translocation. *Cell*, **109**, 757–767.
- Sandler,S.J. and Mariani,K.J. (2000) Role of PriA in replication fork reactivation in *Escherichia coli*. *J. Bacteriol.*, **182**, 9–13.
- Seigneur,M., Bidnenko,V., Ehrlich,S.D. and Michel,B. (1998) RuvAB acts at arrested replication forks. *Cell*, **95**, 419–430.
- Selby,C.P. and Sancar,A. (1993) Molecular mechanism of transcription-repair coupling. *Science*, **260**, 53–58.
- Selby,C.P. and Sancar,A. (1994) Mechanisms of transcription-repair coupling and mutation frequency decline. *Microbiol. Rev.*, **58**, 317–329.
- Selby,C.P., Drapkin,R., Reinberg,D. and Sancar,A. (1997) RNA polymerase II stalled at a thymine dimer: footprint and effect on excision repair. *Nucleic Acids Res.*, **25**, 787–793.
- Severinov,K., Soushko,M., Goldfarb,A. and Nikiforov,V. (1993) Rifampicin region revisited. New rifampicin-resistant and streptolydigin-resistant mutants in the β subunit of *Escherichia coli* RNA polymerase. *J. Biol. Chem.*, **268**, 14820–14825.
- Skalka,A. (1974) A replicator's view of recombination (and repair). In Grell,R.R. (ed.), *Mechanisms in recombination*. Plenum Press, New York, NY, pp. 421–432.
- Sparkowski,J. and Das,A. (1990) The nucleotide sequence of *greA*, a suppressor gene that restores growth of an *Escherichia coli* RNA polymerase mutant at high temperature. *Nucleic Acids Res.*, **18**, 6443.
- Toulme,F., Mosrin-Huaman,C., Sparkowski,J., Das,A., Leng,M. and Rachid Rahmouni,A. (2000) GreA and GreB proteins revive backtracked RNA polymerase *in vivo* by promoting transcript trimming. *EMBO J.*, **19**, 6853–6859.
- van den Boom,V., Jaspers,N.G.J. and Vermeulen,W. (2002) When machines get stuck—obstructed RNA polymerase II: displacement, degradation or suicide. *BioEssays*, **24**, 780–784.
- von Hippel,P.H. (1998) An integrated model of the transcription complex in elongation, termination, and editing. *Science*, **281**, 660–665.
- Xiao,H., Kalman,M., Ikehara,K., Zemel,S., Glaser,G. and Cashel,M. (1991) Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. *J. Biol. Chem.*, **266**, 5980–5990.
- Zhang,G., Campbell,E.A., Minakhin,L., Richter,C., Severinov,K. and Darst,S.A. (1999) Crystal structure of *Thermus aquaticus* core RNA polymerase at 3.3 Å resolution. *Cell*, **98**, 811–824.
- Zhou,Y.N. and Jin,D.J. (1998) The *rpoB* mutants destabilizing initiation complexes at stringently controlled promoters behave like 'stringent' RNA polymerases in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **95**, 2908–2913.

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