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Rhodobacter sphaeroides LexA has dual activity: optimising and repressing *rec*A gene transcription

Angels Tapias, Silvia Fernández[1], Juan C. Alonso[1] and Jordi Barbé*

Departamento de Genética y Microbiología, Universitat Autónoma de Barcelona, Bellaterra, 08193 Barcelona, Spain and [1]Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

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ABSTRACT

Transcription of the *Rhodobacter sphaeroides rec*A promoter (P_{recA}) is induced upon DNA damage in a *lex*A-dependent manner. *In vivo* experiments demonstrate that LexA protein represses and might also activate transcription of P_{recA}. Purified *R.sphaeroides* LexA protein specifically binds the SOS boxes located within the P_{recA} region. *In vitro* transcription analysis, using *Escherichia coli* RNA polymerase (RNAP), indicated that the presence of LexA may stimulate and repress transcription of P_{recA}. EMSA and DNase I footprinting experiments show that LexA and RNAP can bind simultaneously to P_{recA}. At low LexA concentrations it enhances RNAP binding to P_{recA}, stimulates open complex formation and strand separation beyond the transcription start site. At high LexA concentrations, however, RNAP-promoted strand separation is not observed beyond the +5 region. LexA might repress transcription by interfering with the clearance process instead of blocking the access of RNAP to the promoter region. Based on these findings we propose that the *R.sphaeroides* LexA protein performs fine tuning of the SOS response, which might provide a physiological advantage by enhancing transcription of SOS genes and delaying full activation of the response.

INTRODUCTION

DNA damage induces a set of cellular functions directly or indirectly involved in the repair of lesions and damage tolerance in bacteria (1,2). This network of damage-induced (*din*) genes is known as the SOS system, which is under the control of the *lex*A and *rec*A gene products. LexA (also termed DinR in *Bacillus subtilis*) is a repressor which binds to an imperfect palindrome [the SOS box is 16 bp in *Escherichia coli*, $CTGT(TA)_4ACAG$, and 13 bp in *B.subtilis*, $CGAAC(AT)_2GTTC$] located near the promoters of the SOS genes (3–5). The *E.coli* and *B.subtilis* LexA repressors inhibit transcription by precluding RNA polymerase (RNAP) binding to the promoter region (6–8). Recently, Hamoen *et al.* (5) suggested that *B.subtilis* LexA blocks the interaction of the C-terminal domain of the α subunit (α-CTD) of RNAP with a putative UP element predicted as being between positions –46 to –58 upstream of the *B.subtilis rec*A promoter (P_{recA}), and therefore precluding active RNAP binding to the promoter region.

RecA protein binds to single-stranded (ss)DNA regions, produced following DNA damage-mediated inhibition of replication or by enzymatic processing of broken DNA ends, and filaments on ssDNA (9,10). The RecA–ssDNA nucleofilament facilitates autocatalytic cleavage of LexA (11,12). The decrease in the cellular pool of LexA results in transcriptional derepression of the SOS genes (2,13). LexA repressor inactivation also leads to the synthesis of the rapidly hydrolysed SfiA (also termed SulA) checkpoint protein, which inhibits cell division (14). Once the DNA has been repaired, in *E.coli* cells shut-down of the SOS machinery occurs in a DinI-dependent manner (15,16). The level of LexA increases, with a subsequent repression of the SOS network, the synthesised SfiA protein is degraded by the Lon protease and cell division proceeds (17).

Recently, the SOS box in the Alpha group of Proteobacteria (e.g. *Rhizobium etli* and *Rhodobacter sphaeroides*, among others) has been established (18–21). LexA recognises two copies of the GAAC or GTTC motif which are separated from each other by 7 bp [SOS box, $GAAC(N)_7GAAC$ or $GTTC(N)_7GTTC$], and a different number of copies of the box make up the operator of the SOS system (Fig. 1). The recognition of two direct repeats by a specific transcriptional regulator, instead of a dimer or a tetramer binding to the two halves of a palindromic sequence, is rare in bacteria.

A *R.sphaeroides lex*A null allele [*lex*A(Def)], which presents constitutive expression of several *din* genes (e.g. *rec*A, *uvr*A and *lex*A), does not show any decrease in cell viability (22). In contrast, *E.coli lex*A(Def) mutants are only viable when a mutation in the *sfi*A gene is also present (2). Likewise, *B.subtilis lex*A(Def) mutants seem to require an unidentified secondary compensatory mutation (23). Unlike *E.coli* and *B.subtilis*, constitutive expression of the SOS system in *R.sphaeroides lex*A(Def) cells does not increase cellular

*To whom correspondence should be addressed. Tel: +34 93 581 18 37; Fax: +34 93 581 23 87; Email: jordi.barbe@uab.es
Correspondence may also be addressed to Juan C. Alonso. Tel: +34 91 585 45 46; Fax: +34 91 585 45 06; Email: jcalonso@cnb.uam.es
Present address:
Angels Tapias, Institute de Genetique et de Biologie Moleculaire et Cellulaire, IGBMC 1, Rue Laurent Fris, BP163, 67400 Illkirch, France

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

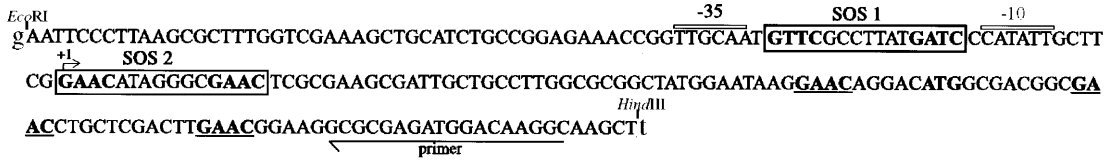

Figure 1. Nucleotide sequence of the non-transcribed strand of the P_{recA} region. The P_{recA} promoter is indicated by empty lines and the transcription start site of P_{recA} is denoted by a bent arrow. The translation start site, the SOS boxes (SOS1 and SOS2, framed) and the putative SOS-like boxes (underlined) are in bold. The position of the primer used in the primer extension assay is also indicated.

resistance against DNA-damaging agents (22). Furthermore, the DNA damage-mediated expression of SOS genes in both *E.coli* and *B.subtilis* wild-type cells is dramatically lower than under conditions of constitutive expression in *lex*A(Def) cells (23,24). However, in *R.sphaeroides* the expression of SOS genes in DNA-damaged wild-type cells is similar to or even higher than that shown in the *lex*A(Def) strain (22). It is likely that the organisation and control of the SOS network in the Alpha Proteobacteria might be quite distinct from the *E.coli* and *B.subtilis* SOS networks.

In this work we report that *R.sphaeroides* LexA protein, both *in vivo* and *in vitro*, represses transcription of the *rec*A promoter (P_{recA}) and also works as an activator. *In vitro* the LexA protein binds to sites which overlap promoter sequences. LexA bound to DNA enhances binding of a heterologous RNAP to DNA without affecting open complex formation. In the presence of an excess of LexA, RNAP is stalled around the +5 region. The results presented provide the first evidence that a LexA repressor can play a dual regulatory function, derepressing SOS genes and delaying maximal activation before the SOS response is fully functional.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Escherichia coli strains DH5α, BL21(DE3), JM109 and S17 λpir (25–27) and *R.sphaeroides* strains UA8000 (wild-type) and UA8165 [*lex*A(Def)] were grown as reported previously (22). Plasmids pBSK, pET3b, pGEM-T, pHP45Km and pHRP309 were used (26,28,29). Plasmid-borne P_{recA} (pUA840) (19) and plasmid-borne P_{lacUV5} (pUC18) (25) were employed for promoter analysis. pET3b-borne *lex*A was used for over-expression. The pHRP309-borne P_{recA}:*lac*Z fusion was introduced into *R.sphaeroides* cells by biparental mating as described (22).

β-Galactosidase (β-Gal) assays

Mitomycin C (MMC) or UV radiation (at 30 J m^{-2}) was applied at time zero and β-Gal experiments were carried out as previously described (19). All data are the averages of three independent assays. β-Gal levels were determined by the standard procedure (30).

DNA, enzymes and reagents

Nucleotides, DNA modification enzymes, RNase A and poly(dG·dC) were purchased from Boehringer-Mannheim and Promega. PCR amplifications were performed using *Taq* Expand DNA polymerase. Oligonucleotides were purchased from Gen Set Oligos and Boehringer-Mannheim. Gel-purified DNA fragments were end-labelled as described (25). The concentration of DNA was determined using molar extinction coefficients of 6500 M^{-1} cm^{-1} at 260 nm and expressed as mol DNA.

Escherichia coli RNAP was purchased from US Biochemical. The soluble 25 kDa LexA protein was purified in three steps by conventional column chromatography (phosphocellulose, S-Sepharose and Superose 12). The N-terminal amino acid sequence of the LexA protein was determined by automated Edman degradation. LexA protein concentration was determined using a molar extinction coefficient of 6400 M^{-1} cm^{-1} at 280 nm, as described previously (31). LexA is expressed in mol protein dimers.

Molecular mass determination

Gel filtration chromatography was carried out in buffer A (50 mM Tris–HCl, pH 7, 5% glycerol) containing 500 mM NaCl at 4°C at a flow rate of 0.5 ml min^{-1} and the A_{280} was measured. About 50–60 µg LexA protein was applied to the Superose 12 column. A standard curve of K_{av} versus the log of molecular mass was determined as recommended by Pharmacia.

Primer extension assays

Transcription assays were performed in a 25 µl reaction in buffer B [25 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 50 mM KCl, 5 mM $(NH_4)_2SO_4$, 2% glycerol, 25 mM NaCl and 0.04 mM DTT] containing ATP, CTP, GTP and UTP (100 µM each), 2 nM linear plasmid (P_{recA} and P_{lacUV5} control) DNA and 40 nM RNAP. The reactions were incubated for 15 min at 30°C with and without increasing amounts of LexA protein, and the reactions were stopped by the addition of 50 µl of a solution containing 2% (w/v) SDS, 10 µg tRNA and 100 mM EDTA. The annealing and primer extension reactions were performed as described (32). The primers used hybridised downstream of P_{lacUV5} (5′-GTTTTCCCAGTCACGAC-3′ at coordinates 87–104, where 1 is the *in vivo* transcription start site) and P_{recA} (5′-GCCT-TGTCCATCTCGCG-3′, Fig. 1). The reactions were stopped and precipitated. The cDNA was analysed by 6% denaturing urea–PAGE (dPAGE) and detected by autoradiography. The relative amounts of transcripts were estimated by laser scanning densitometry of the autoradiographs and the data presented are averages of three independent experiments.

Electrophoretic mobility shift assay (EMSA) experiments

For EMSA experiments, the 210 bp α-^{32}P-labelled *Eco*RI–*Hin*dIII P_{recA} (2 nM) DNA fragment was incubated, in the presence of 1 µg poly(dG·dC) as a non-specific competitor DNA, in buffer C (25 mM Tris–HCl pH 7, 2 mM $MgCl_2$, 50 mM KCl, 5% glycerol) with different amounts of LexA and/or RNAP for 10 min at

30°C in a 20 µl final volume. The reaction mixture was then immediately loaded onto the gel (25). The samples were separated by 5% non-denaturing (nd)PAGE (80:1 acrylamide/bis-acrylamide) gel. Gels were run with 1× Tris–glycine or 0.5× TAE at 150 V at room temperature and dried prior to autoradiography (32).

DNase I and $KMnO_4$ footprinting

The 228 bp α-^{32}P-labelled *Eco*RI–*Pst*I P_{recA} DNA fragment (2 nM) was incubated, in the presence of 1 µg poly(dG·dC) as a non-specific competitor DNA, with different amounts of LexA and/or RNAP in buffer C for 10 min at 30°C in a 20 µl final volume. When indicated, the samples were preincubated with the appropriate NTPs (ATP, CTP and GTP) at 100 µM at 30°C for 10 min. After DNase I treatment (50 ng for 5 min at 37°C) the DNA was precipitated in the presence of 1 µg tRNA. $KMnO_4$ footprinting was performed as indicated with DNase I. The samples were treated with 1 mM $KMnO_4$ for 30 s at 37°C as previously described (33). The DNA was cleaved with piperidine and the samples were separated by 6% dPAGE, dried and analysed by autoradiography.

RESULTS

The *R.sphaeroides recA* gene is under negative and positive control

It is assumed that the maximal expression level of a LexA-regulated gene in DNA-damaged cells should be similar to that shown under conditions in which the SOS genes are being constitutively transcribed [e.g. *lex* null mutant cells, *lex*A(Def)]. The pHRP309-borne P_{recA}:*lacZ* fusion was introduced into both wild-type and *lex*A(Def) *R.sphaeroides* cells to address their kinetics of expression upon DNA damage. The *rec*A gene is constitutively expressed in *lex*A(Def) mutant cells (22). Surprisingly, upon MMC treatment P_{recA} utilisation, measured as β-Gal accumulation, is up to three times greater in wild-type than in *lex*A(Def) cells (Fig. 2). The same results were found when UV radiation (at 30 J m^{-2}) was used as the inducing agent (data not shown).

The data obtained cannot be explained by assuming solely a negative role for the LexA protein because the expression ratio of P_{recA} between the wild-type and *lex*A(Def) would increase until it reaches a plateau which would represent maximal promoter utilisation (derepression). Hence, we hypothesised that the kinetics of *rec*A gene induction might be due to a combination of two overlapping processes: (i) transcriptional derepression of P_{recA}, as a consequence of LexA cleavage, which will enable cells to achieve the maximal level of expression obtained in *lex*A(Def) cells; (ii) activation of P_{recA} (direct effect) upon *de novo* synthesis of LexA. Alternatively, an uncharacterised activator, which only works upon LexA cleavage, could regulate both promoters. We have to assume that this hypothetical and uncharacterised activator is non-functional or not synthesised in the absence of LexA protein [e.g. in the *lex*A(Def) background] and the presence of cleaved LexA is required for its activity (indirect effect).

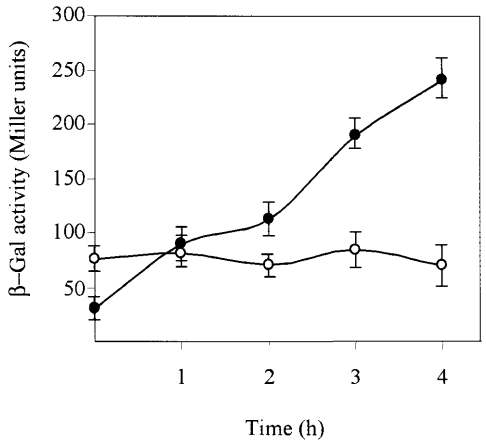

Figure 2. Expression of the P_{recA}:*lacZ* fusion in *R.sphaeroides* wild-type and *lexA*(Def) cells under SOS-induced conditions. *Rhodobacter sphaeroides* wild-type and *lexA*(Def) cells (closed and open symbols, respectively) were treated with MMC at 0.4 µg ml^{-1} and the level of expression of the P_{recA}:*lacZ* fusion measured. All determinations are the means of at least three experiments (each in triplicate) and a single standard error of any value was never >10%.

Purification and properties of the *R.sphaeroides* LexA protein

The 25 kDa LexA protein (with a predicted mass of 24 928 Da) was purified to >99% as assayed by SDS–PAGE and quantitative analysis of the N-terminal amino acid. The sequence of the first 10 N-terminal amino acids of the purified protein is in agreement with the predicted amino acid sequence derived from the *lex*A gene (data not shown).

The native molecular mass of purified LexA was estimated by size fractionation through a Superose 12 FPLC gel filtration column in a buffer containing 500 mM NaCl. From the elution profile of LexA and of a number of protein standards we estimated that the M_r of LexA is ~50 000, about twice that of a LexA protomer. If we assume that LexA is spherical in shape, it is likely that LexA is a dimer in solution. This is consistent with the observation that *E.coli* LexA exists within the cells exclusively as a dimer (34). We cannot rule out, however, that *R.sphaeroides* LexA is an elongated monomer with a large Stokes radius.

Rhodobacter sphaeroides LexA protein represses transcription

Previously, the *in vivo* transcription start of P_{recA} has been mapped in *R.sphaeroides* upon SOS induction (19) (Fig. 1). A computer-based analysis of P_{recA} has pointed out the existence of two hexameric promoter elements located ~10 and 35 bp upstream of the *in vivo* mapped transcription start site (+1) (19), however, the presence of the third promoter element (UP element; 35,36) in the –40 to –60 position is not obvious (Fig. 1). To test whether a heterologous RNAP (*E.coli* RNAP) recognises P_{recA} *in vitro* and to determine whether the LexA protein regulates promoter utilisation, transcription experiments were performed. The run-off transcripts produced *in vitro* by RNAP (40 nM) using a linear DNA substrate (2 nM), containing the *R.sphaeroides* P_{recA} and the *E.coli* P_{lacUV5} control, were analysed by primer extension using the primer

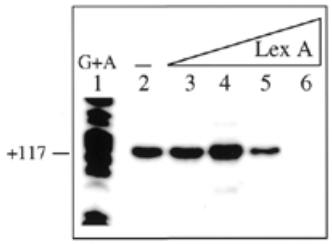

Figure 3. Primer extension assay of P_{recA} in the absence and presence of LexA. Linear plasmid DNA (2 nM) was incubated with 40 nM RNAP in the absence and presence of increasing amounts of LexA (1.7, 3.5, 7 and 14 nM) and subjected to *in vitro* transcription followed by primer extension. The length of the cDNA obtained is indicated. –, absence of LexA. The G + A sequencing reaction is presented. A standard error of any value was never >10%.

denoted in Figure 1 for P_{recA}. A mRNA species of 117 nt in length was detected for P_{recA} (Fig. 3). From the position of the primer we could infer that the 117 nt long transcript started at the position mapped *in vivo* for P_{recA} (19). Furthermore, a mRNA species of 114 nt in length was detected for the P_{lacUV5} control (data not shown). It is likely, therefore, that *E.coli* RNAP recognises the P_{recA} promoter and initiates transcription, as does *R.sphaeroides* RNAP *in vivo*.

As revealed in Figure 3, the addition of 1.7 LexA dimers per DNA molecule increases P_{recA} utilisation, but the presence of 3 LexA dimers reduces promoter utilisation by ~3-fold. Furthermore, 7 LexA dimers repress transcription initiation by >15-fold. Hence, at low LexA concentrations it controls P_{recA} positively, whereas at higher LexA concentrations promoter utilisation by RNAP is repressed.

Concentrations of LexA protein equal to or higher than those required to repress the P_{recA} promoter did not affect utilisation of the unrelated *E.coli* P_{lacUV5} control promoter (data not shown). From this result we can rule out that a contaminant RNase or any other non-specific effect could be responsible for the lack of RNA synthesis at higher LexA concentrations.

Rhodobacter sphaeroides LexA and RNAP interact with P_{recA}

The affinity of the LexA protein for the 210 bp α-^{32}P-labelled *Eco*RI–*Hin*dIII P_{recA} DNA fragments (2 nM) was determined by EMSA following complex formation as a function of LexA concentration. P_{recA} has two SOS boxes spaced 13 bp apart and two half copies (Fig. 1). No binding was detected at LexA concentrations <0.3 nM, whereas at higher protein concentrations the amount of LexA–DNA complex formed is greatly increased until a plateau was observed (Fig. 4A). The exponential increase in complex formation suggests that LexA dimers bind to their cognate sequence in a cooperative manner. Three protein–DNA complexes (L1–L3) are observed (Fig. 4A). The apparent equilibrium constant (K_{app}) of LexA–DNA complex formation is ~3.5 nM at pH 7 and 30°C. Since the apparent binding stoichiometry to saturate the DNA substrate is ~7 P_{recA} per LexA dimers, it is likely that the purified LexA protein is active. Furthermore, the stoichiometry of LexA observed by EMSA is in clear agreement with the amount of LexA required for P_{recA} repression.

Few diffuse complexes are observed at low amounts of RNAP (10 nM). In the presence of 20 nM RNAP one major protein–P_{recA} DNA complex is formed (R1 complex), whereas

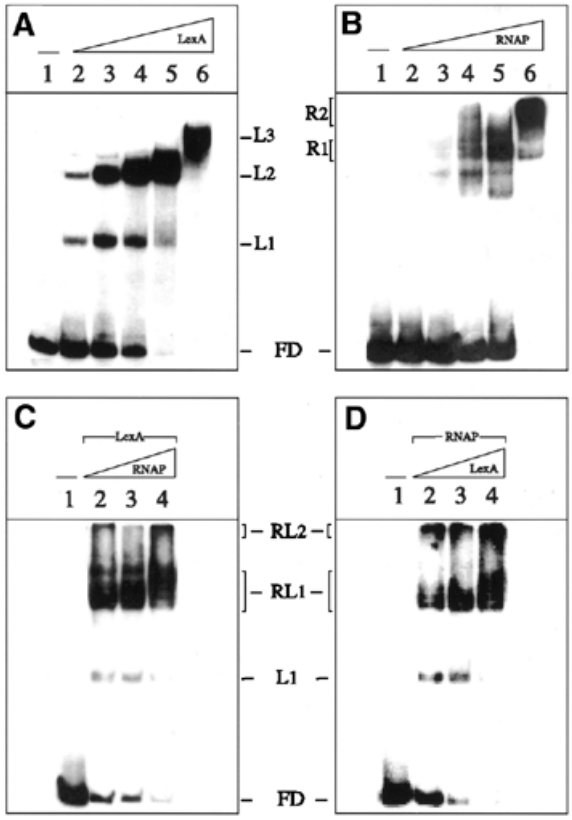

Figure 4. LexA protein does not displace RNAP from P_{recA} DNA. The 210 bp α-^{32}P-labelled *Eco*RI–*Hin*dIII P_{recA} DNA fragment (2 nM) was incubated with increasing concentrations of LexA (1.7–28 nM) (**A**) or RNAP (2.5–40 nM) (**B**) in buffer C for 10 min at 30°C. Complexes were analysed by ndPAGE with a suboptimal amount of LexA (1.7 nM) and then with varying amounts of RNAP (1.2–5 nM) (**C**) or with a suboptimal amount of RNAP (1.2 nM) and then with varying amounts of LexA (1.7–7 nM) (**D**) in buffer C for 10 min at 30°C. The free DNA (FD), LexA complexes (L1, L2 and L3), RNAP complexes (R1 and R2) and RNAP–LexA complexes (RL1 and RL2) are indicated. –, absence of the denoted protein.

in the presence of 40 nM a discrete complex (R2) is the major observed form (Fig. 4B). In quantitative terms, the K_{app} of the RNAP–P_{recA} DNA complexes is ~25 nM at pH 7 and 30°C.

The amount of heterologous RNAP required to form an RNAP–DNA complex was reduced at least 15-fold when LexA (1.7 nM) was preincubated with P_{recA} DNA (Fig. 4C, lanes 2–4). Enhanced binding of LexA was also observed when it was added to preformed RNAP–P_{recA} DNA complex (Fig. 4D, lanes 2–4). It is likely, therefore, that LexA and RNAP are present in the RL1 and RL2 ternary complexes, and this suggest that steric hindrance is not the mechanism of LexA repression (see below).

Since LexA helps loading of heterologous RNAP to the P_{recA} substrate DNA, we consider the above mentioned hypothesis of transcription activation by an uncharacterised LexA-dependent effector (indirect effector) unlikely.

RNAP and LexA protein form a ternary complex on P_{recA}

To localise the sequences recognised by both proteins and to address whether both proteins form a ternary complex with

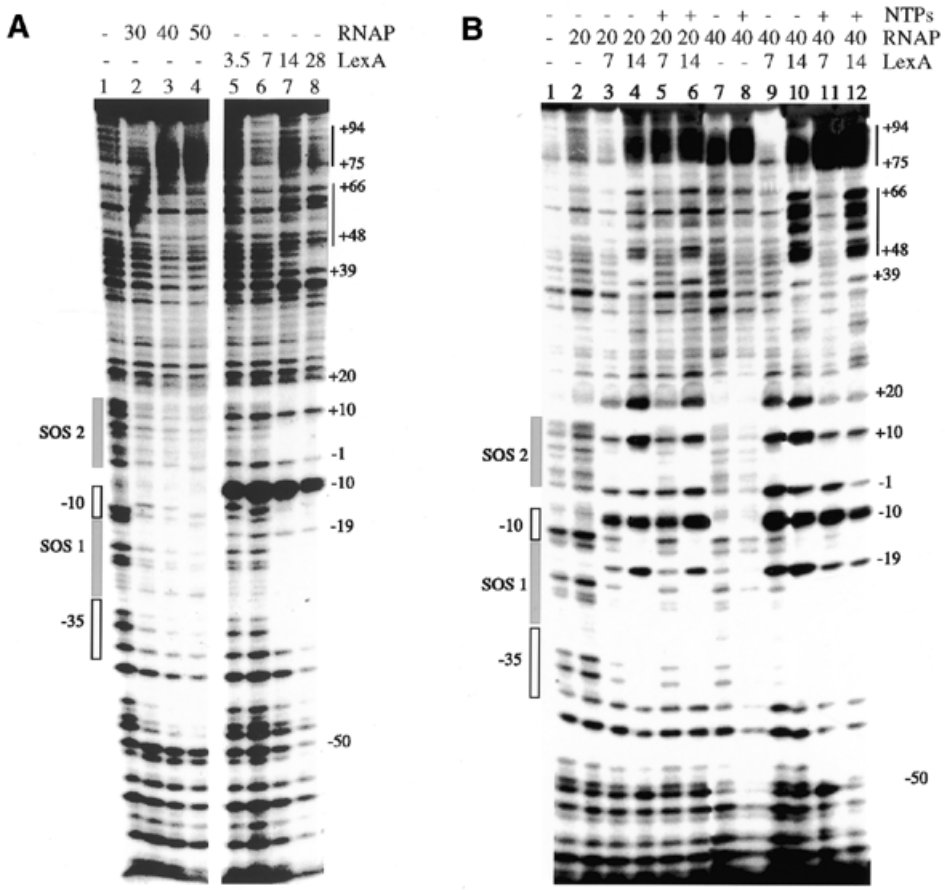

Figure 5. Interaction of RNAP and LexA with P_{recA} DNA. (**A**) The 228 bp α-^{32}P-labelled *Eco*RI–*Pst*I P_{recA} DNA (3 nM) fragment was incubated with RNAP (30–50 nM) or LexA (3.5–28 nM) in buffer C for 10 min at 30°C. (**B**) The 228 bp α-^{32}P-labelled *Eco*RI–*Pst*I P_{recA} DNA (2 nM) fragment was incubated with RNAP (20 and 40 nM) and then with varying amounts of LexA (7–28 nM) in the presence or absence of 100 µm NTPs (ATP, CTP and GTP) in buffer C for 10 min at 30°C. +, presence of the denoted factor; –, absence of the denoted factor. The location of the promoter, SOS boxes and the hypersensitive sites are indicated at the side of the gel. The coordinates, relative to the +1 (transcription initiation) site, are indicated.

P_{recA}, the protein–DNA complexes were analysed by DNase I footprinting. As shown in Figure 5, RNAP (20–50 nM) protected discrete regions on the 228 bp P_{recA} DNA (2 nM) from nuclease attack. In the presence of 20 nM RNAP, sites protected from DNase I attack were not observed (Fig. 5B, lane 2). At higher RNAP concentrations (30–50 nM) protection from nuclease attack matches the location of the RNAP consensus sequences (–10 and –35; see Fig. 1) and it extended from position –48 to +23 (Fig. 5A, lanes 2 and 4, and B, lane 7). Hypersensitive sites spanning positions +75 to +94 were not observed at low RNAP (20 nM), but rather were detected at high concentrations (40–50 nM) (Fig. 5A, lanes 3 and 4, and B, lanes 2 and 7). In the presence of ATP, CTP and GTP (NTPs) or the absence of NTPs, hypersensitive sites spanning positions +75 to +94 were observed (Fig. 5A, lanes 2 and 4, and B, lanes 7 and 8). Only in the presence of 100 µM ATP, CTP and GTP, however, does RNAP protect broader regions from nuclease attack (positions –60 to +25) (Fig. 5B, lanes 7 and 8). It is likely, therefore, that under the experimental conditions used some RNAP molecules might leave the P_{recA} region.

The K_{app} of the LexA–P_{recA} DNA complexes is ~12 nM as determined by DNase I footprinting, slightly lower than that determined by EMSA. In the presence of low LexA (3.5–7 nM) concentrations, sites protected from DNase I attack were not observed, but a phosphodiester bond strongly hypersensitive to DNase I at position –10 was detected (Fig. 5A, lanes 5 and 6). At higher concentrations (14–28 nM), LexA protected discrete regions in the P_{recA} DNA from nuclease attack. The protected sites match the location of the SOS boxes (positions –47 to –20 and –18 to –11, SOS 1, and +1 to +9 and +11 to +19, SOS 2) and upstream sequences which coincide with the half-SOS box (positions +34 to +37, +40 to + 47, +56 to +59 and +76 to 79; see Fig. 1) were observed (Fig. 5A, lanes 7 and 8). A phosphodiester bond hypersensitive to DNase I at position –10 and protected bonds at positions –19, –1 and +10 were also observed. The hypersensitive site (position –10) and protected bonds at the same relative positions within SOS 1 (position –19) and SOS 2 (position +10) and at positions –1 and +20 are separated by 9 ± 1 nt, i.e. ~1 helical turn (assuming 10.5 bp per turn) in double-stranded (ds)DNA. These periodic anomalies in the DNase I cleavage pattern suggest that the protein interacts with one face of the DNA helix.

Mutual binding of RNAP and LexA to P_{recA}, which matches both protein-binding sites, and the formation of ternary complexes which are cooperatively stimulated by the presence

of both proteins were observed at low protein concentrations (Fig. 5B, lanes 3 and 9). In the presence of low RNAP (20 nM) and high LexA (14 nM), however, protection from nuclease attack at position +40 to +47 and hypersensitive sites at position +48 to +66 were also observed (Fig. 5B, lanes 4 and 6). The same general conclusions are reached when 100 µM ATP, CTP and GTP were added. The protection from nuclease attack at positions +40 to +47 and hypersensitive sites at positions +48 to +66 in the presence of both LexA and RNAP could correlate with the presence of imperfect SOS-like boxes or an SOS box having an unusual distance of the repeat spacer [GAAC$(N)_{16}$GAAC$(N)_{11}$GAAC] at positions +56 to +94 (see Fig. 1). The same features were detected in the presence of saturating amounts of RNAP (40 nM), but here the hypersensitive sites within positions +48 to +66 are more evident. It is likely, therefore, that (i) suboptimal LexA concentrations, in the absence of NTPs, enhance binding of a heterologous RNAP to P_{recA} DNA; (ii) the LexA repressor does not prevent RNAP from binding to P_{recA}, hence ruling out repression by steric hindrance; (iii) in the presence of 100 µM ATP, CTP and GTP, saturating amounts of RNAP present an extended footprint; (iv) the repression mechanism used by LexA is at a post-RNAP binding step. It is likely that LexA might bind to the promoter region together with RNAP in a way that hinders formation of either the open complex or promoter clearance by RNAP (37,38).

LexA protein enhances RNAP open complex formation

To determine how LexA is able to activate/repress transcription, its interaction with RNAP–P_{recA} DNA was investigated by monitoring $KMnO_4$ modification of stable initiation complexes formed in the presence and absence of LexA. $KMnO_4$ preferentially modifies single-stranded thymines (T) and to a lesser degree cytosines (C), and this will allow the characterisation of the strand-separated DNA in open RNAP transcription complexes.

The 228 bp α-^{32}P-labelled *Eco*RI–*Pst*I P_{recA} DNA fragment (2 nM) was incubated with LexA, RNAP or both proteins in the presence or absence of 100 µM ATP, CTP and GTP. The complexes were treated with $KMnO_4$ and subjected to piperidine cleavage and the reaction mixture visualised by dPAGE. In the presence or absence of ATP, CTP and GTP, RNAP (40 nM) binds to and melts the dsDNA helix over a region which includes two T residues and a potential C in the –10 region, hence a nucleated complex and an incomplete open complex are suggested (Fig. 6, lanes 7 and 9). In the presence of 80 nM RNAP (~3-fold K_{app}) an incomplete open complex, defined as a melting of the dsDNA helix over the –10 region, was observed in the absence of NTPs (Fig. 6, lane 11), whereas RNAP-promoted melting of the dsDNA helix at T residues at positions +2, +3, +5 and beyond +5 was observed only in the presence of 100 µM ATP, CTP and GTP (Fig. 6, lane 13). The strand separation observed beyond +5 (+7 position) in the presence of 100 µM ATP, CTP and GTP may be due to slight contamination of the nucleotides with UTP or deamination of CTP and read-through at position +6 (first U in the mRNA). The activation of a second transcription start site, however, cannot be ruled out (see Fig. 3, lane 4). In the presence of 40 nM RNAP, nevertheless, the formation of a ternary RNA-producing complex was not observed (Fig. 6, lanes 7 and 9).

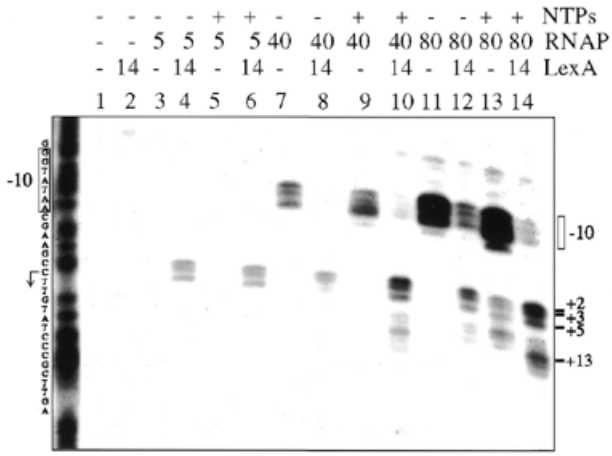

Figure 6. LexA protein enhances RNAP open complex formation. The 228 bp α-^{32}P-labelled *Eco*RI–*Pst*I P_{recA} DNA fragment (2 nM) was incubated with suboptimal amounts of RNAP (5 nM) or saturating amounts of RNAP (40 and 80 nM) and with a saturating concentration of LexA (14 nM) in the presence or absence of 100 µM NTPs (ATP, CTP and GTP). The reaction was then treated with $KMnO_4$ and piperidine and analysed by dPAGE.

The LexA protein (14 nM) increases RNAP-induced melting of the dsDNA helix over a region which includes three T residues in the +1 region at positions +2, +3 and +5 (Fig. 6, lanes 3 and 4). RNAP (5 nM) binding and promotion of strand separation at or near the start site is markedly increased (>16-fold) by LexA protein (14 nM) when compared to similar conditions in the presence of 80 nM RNAP and the absence of LexA (Fig. 6, lanes 4 and 11). The addition of 100 µM ATP, CTP and GTP does not seem to further increase open complex formation beyond the start site at a low ratio of RNAP over LexA (0.3 RNAP/LexA) (Fig. 6, lanes 5 and 6). It is likely that under this condition RNAP cannot leave the +1 to +5 region. It is also likely that under this condition formation of a ternary RNA-producing complex does not take place.

In the presence of increasing amounts of RNAP (40 nM) over LexA (14 nM) the LexA enhances RNAP-promoted melting of the dsDNA helix at T residues at positions +2, +3 and +5, with a subsequent reduction in RNAP-induced melting at the –10 region even in the absence of NTPs when compared to the absence of LexA (Fig. 6, lanes 7 and 8). In the presence of both LexA and 100 µM ATP, CTP and GTP strand separation is near and beyond the start site (positions +2, +3 and +5 and +13 and +14) (Fig. 6, lanes 7–10). Read-through at position +6 was observed, but not at position +16 nor beyond (Fig. 6, lanes 10 and 14). The same general conclusions, strand separation promoted by RNAP at or near the start site in the presence of LexA, were reached when $KMnO_4$ footprinting was performed with the non-template strand and in the presence or absence of heparin (150 µg ml^{-1}). We have shown that formation of open complexes after the initial binding step is enhanced in the presence of LexA. At a high LexA over RNAP ratio (2.8 LexA/RNAP) RNAP cannot leave the +1 to +5 region, whereas at a low LexA/RNAP ratio (0.17) it leaves the +1 to +5 region. It is likely, therefore, that in the presence of an excess of LexA RNAP transcription is repressed by trapping RNAP at P_{recA}, whereas in the presence of low LexA it activates RNAP transcription of P_{recA}.

DISCUSSION

In vivo experiments carried out with the P_{recA} promoter in wild-type and *lex*A(Def) strains suggested a dual role of LexA protein in the control of *R.sphaeroides* SOS gene expression. Upon SOS induction P_{recA} utilisation increases 3-fold in wild-type cells when compared to the constitutive level (RNAP full promoter occupancy) of *lexA*(Def) cells. This result was confirmed by an analysis of promoter mutants presenting a reduction in transcription efficiency (data not shown).

We have shown in this study that *R.sphaeroides* LexA is a bona fide transcriptional repressor. Unlike the *E.coli* and *B.subtilis* LexA repressors, which block initial binding of the RNAP (5–8), we have documented that *R.sphaeroides* LexA can bind at the same time as RNAP and that the contacts with DNA in such a ternary complex are different from those made by either protein alone. The mutual binding of both proteins to P_{recA} and formation of the ternary complex is cooperatively stimulated by the presence of LexA and RNAP. The inhibitory effect of LexA on P_{recA} utilisation correlates with the amount of LexA–DNA complex formed, indicating the specificity of the repression system even in the presence of heterologous RNAP.

The contacts with DNA in such a ternary complex are slightly different from those made by either protein alone. The mechanism of LexA-dependent repression at P_{recA} can be explained as a trap of RNAP beyond template position +1 in the initial transcription complex, whereas in the presence of a low LexA/RNAP ratio LexA enhances promoter clearance. In both cases LexA increases the initial binding of RNAP to the promoter region and isomerisation from a closed to an open complex. In the absence of *R.sphaeroides* RNAP to test whether any of these hypotheses are correct, it is herein proposed that either LexA interacts with RNAP using a domain conserved between both *E.coli* and *R.sphaeroides* RNAP or LexA modifies the DNA topology, with subsequent generation of a discrete DNA structure which enhances or inhibits P_{recA} utilisation.

Few repressors have been shown to repress transcription by inhibiting promoter clearance: ϕ29-P4 (39,40), HNS at the *rrn*B P1 promoter (41), FIS at the *gyr*B promoter (42) and P22-Arc under certain conditions (43). Furthermore, sometimes the ϕ29-P4 and P22-Arc transcription factors, among others, show dual regulation of open complex formation and promoter clearance (38,43).

Replication forks are often inactivated under normal growth conditions. The accumulation of ssDNA regions resulting from stalled replication seems to be the induction signal for the SOS system (2). The pathways for fork reactivation involve the homologous recombination system (44). After the DNA lesions are healed, the induction signal disappears and the LexA pool accumulates in a passive manner (1,11). The biological significance of the dual function of *R.sphaeroides* LexA must be understood as an efficient way to adjust the range of SOS response to cell requirements. *Rhodobacter sphaeroides* is habitually present in the soil. Soil represents an ecological niche in which temperature fluctuations, osmotic stress, solar light and biological secondary metabolites excreted by different microbiota components constantly give rise to DNA-damaging stress situations. *Rhodobacter sphaeroides* has developed a finely tuned SOS response which is accomplished by having promoters of different strengths (19,22), by different LexA-binding affinities and by modulating the SOS response at the transcriptional level. Dual LexA regulation might have a buffering effect. On the one hand, 'activated' RecA protein accumulates at a stalled replication fork, RecA-dependent cleavage of LexA occurs and LexA repression of RNAP is relieved, allowing the system to respond rapidly to the release of repression. However, in the sub-induced SOS state (minor damage leading to low levels of 'activated' RecA protein) LexA would activate expression of SOS genes and quickly reach a repressive state, rather than producing the sub-induced SOS state as described for *E.coli* (45). On the other hand, when the damage signal accumulates, SOS cleavage of LexA occurs rapidly, leading to derepression of the SOS regulon. A low level of LexA might activate synthesis of the SOS genes. Concomitant with a reduction in the damage signal, LexA increases the speed of recovery to the repressed state. This DNA damage feedback response strategy is also utilised by mammalian cells (46). Indeed, CRT1 protein, which is the autoregulated key transducer of the transcriptional response to replicational stress and is induced by DNA-damaging agents, acts as a repressor of the transcription of repair genes and as a weak inducer (46–48).

Rhodobacter sphaeroides LexA binds to SOS boxes and represses expression of the SOS genes. LexA bound to its target sequences increases the initial binding of unbound RNAP to P_{recA} and, therefore, traps the RNAP and keeps it at P_{recA}, holding it ready for transcription. We assume that *R.sphaeroides* LexA inhibition of expression through trapping a functional transcription initiation complex at the promoter is of physiological advantage because the arrested RNAP can be relieved without decomposition of the initiation complex. Furthermore, the *R.sphaeroides* LexA feedback loop may bolster SOS induction in the presence of low levels of damage, and when the damage signal disappears few copies of LexA might permit rapid transcriptional repression. It is unlikely, therefore, that at partial occupancy (LexA bound to only one SOS box) LexA is acting as an activator and under full occupancy (LexA bound to both SOS boxes) as a repressor.

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