RecA-Independent DNA Damage Induction of Mycobacterium tuberculosis ruvC Despite an Appropriately Located SOS Box

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Mycobacterium tuberculosis ruvC was induced by DNA damage in a ΔrecA strain despite having an appropriately positioned SOS box to which LexA binds in vitro. An inducible transcript start mapped within the SOS box, and transcriptional fusions identified the promoter. Disruption of the SOS box did not prevent induction, indicating that an alternative mechanism plays a significant role in the control of ruvC expression.

M. tuberculosis is an intracellular human pathogen for which the ability to repair damaged DNA is important to overcome host protective mechanisms (9). A key mechanism for maintaining genomic integrity is recombination repair, a complex multistage process in which RuvC acts in the final resolution step via its activity as a structure-specific endonuclease that cleaves Holliday junctions, acting in concert with the RuvAB branch migration complex (22). Controlled expression of DNA repair systems facilitates repair without affecting undamaged DNA. In Escherichia coli, the primary mechanism of transcriptional regulation in response to DNA damage is the RecA/LexA system (5). LexA is a repressor which binds to the SOS boxes of promoters to inhibit transcription. Derepression is achieved by activation of RecA, a coprotease, which induces the autocatalytic cleavage of LexA, rendering it unable to bind to SOS boxes (16).

This mechanism is conserved in a number of species (10) although the sequence of the SOS box is variable (11). However, some genomes lack a lexA ortholog and even those bacteria that possess LexA can have some genes induced in response to DNA damage independently of LexA and/or RecA (13, 17). In M. tuberculosis, recA is transcribed from two promoters, both of which are DNA damage inducible, but only one of these is regulated by the RecA/LexA system (7) and it is the RecA/LexA independent system that is responsible for controlling expression of the majority of damage-inducible DNA repair genes in this organism (19).

In M. tuberculosis ruvC is induced following DNA damage (2, 19) although it is not in E. coli (21). In vitro mycobacterial LexA bound to an SOS box located 37 bases upstream of the predicted translational start site of ruvC (2), suggesting that LexA was involved in regulation. However, microarray analysis indicated that ruvC was also induced by DNA damage, although to a lower level, in a recA deletion strain of M. tuberculosis (19), suggesting that the RecA/LexA system is not solely responsible for its transcriptional regulation. In this study, quantitative reverse transcription-PCR (RT-PCR) and transcriptional analyses confirmed that ruvC remained inducible in the absence of RecA following mitomycin C stress. Transcript start site mapping revealed a single DNA damage-inducible promoter for ruvC, which shares a high degree of similarity with the recA PI promoter, suggesting a similar mode of regulation.

E. coli DH5α was used for cloning; standard DNA manipulation and bacterial growth conditions were used (20). Primers and plasmids used are listed in Tables 1 and 2. Transcriptional fusions to lacZ were constructed using annealed double-stranded oligonucleotides, covering the promoter regions cloned into vector pEJ414 (18), and were sequence confirmed.

M. tuberculosis H37Rv and ΔrecA (7) strains were grown under category 3 containment conditions as described previously (7). To induce DNA damage, cultures were divided into two aliquots at an optical density at 600 nm (OD600) of 0.3 to 0.4; one sample was induced with mitomycin C (0.02 µg/ml) for 24 h at 37°C, while the other sample was incubated in parallel without treatment to provide an uninduced control. Cell extracts were produced and used for protein and β-galactosidase assays as described previously (6). RNA was extracted from 100- to 200-ml cultures and purified as described previously (6) and then quantified using a spectrophotometer or bioanalyzer (Agilent Technologies).

cDNA synthesis was performed using Superscript II (Invitrogen) and random hexamer primers according to the manufacturer’s guidelines. Real-time quantitative PCR was carried out using HotStarTaq master mix (Qiagen) and TaqMan probes on an Applied Biosystems 7500 fast instrument. The test gene (ruvC) was assayed simultaneously with the normalizing gene (sig4), along with standard curves for each gene and RT-negative controls.

RNase protection assays were performed using 40 µg RNA and 5 × 10⁶ cpm purified 32P-labeled probe, prepared from NotI-linearized pLDRNAse2 using a MAXiScript in vitro transcription kit (Ambion) with T3 polymerase and NucAway columns (Ambion) in accordance with the manufacturer’s guide-

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Following hybridization at 42°C overnight and digestion using a 1:100 ratio of RNase A/T1, protected RNA was resolved on a 5% denaturing polyacrylamide gel, alongside the century marker (Ambion), and visualized using a phosphorimager. Contrast of the image was adjusted using Adobe Photoshop.

Primer extension analysis was performed with avian myeloblastosis virus (AMV) reverse transcriptase (Promega) at 42°C for 30 min as described by the manufacturer, using 80 μg total RNA annealed to the 32P-labeled primer (180 fmol). The products were analyzed on an 8% polyacrylamide-urea gel, alongside sequencing reactions performed with the same primer on plasmid pLDruvC by the use of a Sequenase V2.0 kit (Amer sham), and then they were visualized by autoradiography.

Confirmation of DNA damage induction of ruvC in the absence of RecA. To confirm that ruvC remained DNA damage inducible in the absence of RecA, its mRNA levels with and without DNA damage in wild-type *M. tuberculosis* and *ΔrecA* strains were compared by quantitative RT-PCR (Fig. 1). The expression levels under uninduced conditions did not differ significantly between the two strains. Following DNA damage, expression of ruvC increased in both strains, but the induced expression level was significantly lower in the *ΔrecA* mutant than in the wild type (*P* < 0.05, Student’s *t* test). The resulting induction ratios of 6.2 for the wild type and 1.8 for the *ΔrecA* strain were very similar to those determined previously by microarray analysis, 5.3 and 2.1, respectively (19). These data suggest that the expression of ruvC is partially controlled by the RecA/LexA system but that another mechanism also plays a role.

Identification of the transcription start sites for ruvC. To obtain an indication of the number and location of promoters for ruvC, transcript start site mapping was performed by RNase protection using RNA extracted from uninduced and induced cultures of both the wild-type and *ΔrecA* strains. In uninduced cultures, two weak signals were observed; following mitomycin

### TABLE 1. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript SK−</td>
<td>General cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pCR4-Blunt</td>
<td>General cloning vector with multiple cloning sites flanked by T7 and T3 promoters</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pEJ414</td>
<td>lacZ transcriptional reporter; integrates into chromosome at L5 <em>attB</em> site</td>
<td>18</td>
</tr>
<tr>
<td>pLDruvC</td>
<td>322 bp ruvC, including 242 bp upstream of coding region, in pBluescript SK−</td>
<td>This study</td>
</tr>
<tr>
<td>pLDNRNase2</td>
<td>322 bp ruvC, including 242 bp upstream of coding region, in pCR4-Blunt</td>
<td>This study</td>
</tr>
<tr>
<td>ruvCP1wt</td>
<td>Positions −42 to +18 relative to transcription start site 1 in pEJ414; annealed oligonucleotides</td>
<td>This study</td>
</tr>
<tr>
<td>ruvCP1-10mut</td>
<td>A-to-C mutation at position −10 in ruvCP1wt; annealed oligonucleotides ruvC 7f and ruvC 7r</td>
<td>This study</td>
</tr>
<tr>
<td>ruvCP1SOShalf</td>
<td>Positions −42 to +4 relative to transcription start site 1 in pEJ414; annealed oligonucleotides ruvC 3f and ruvC 3r</td>
<td>This study</td>
</tr>
<tr>
<td>ruvCP1SOSG+7A</td>
<td>G-to-A mutation at position +7 relative to center SOS box in ruvCP1wt; annealed oligonucleotides ruvC 8f and ruvC 8r</td>
<td>This study</td>
</tr>
</tbody>
</table>

### TABLE 2. Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>sigA f</td>
<td>TCGGTTCGCGCCTACCT</td>
<td>TaqMan primer</td>
</tr>
<tr>
<td>sigA p</td>
<td>TTGACCGACGCTACCTGCGCGG</td>
<td>TaqMan probe</td>
</tr>
<tr>
<td>sigA r</td>
<td>TGTCCTAGCTCAGCTTCCTCCT</td>
<td>TaqMan primer</td>
</tr>
<tr>
<td>ruvC f</td>
<td>GCTCAAGGTCAACCCGAGG</td>
<td>TaqMan primer</td>
</tr>
<tr>
<td>ruvC p</td>
<td>CACCAAAAATCCTTTTGCGCTGGAACGCGTCAG</td>
<td>TaqMan probe</td>
</tr>
<tr>
<td>ruvC r</td>
<td>CGGCGGTTGTCGAGT</td>
<td>TaqMan primer</td>
</tr>
<tr>
<td>ruvC small f</td>
<td>AATCTAGAGGTTCTGGGCGGCGGCTCAATCT</td>
<td>Cloning</td>
</tr>
<tr>
<td>ruvC small r</td>
<td>CGAAGCTTTAGACTGCTGCGCCACCAGCGCCACTCT</td>
<td>Cloning and primer extension</td>
</tr>
<tr>
<td>ruvC 1f</td>
<td>CTAGGTGTTGTCGCGGATGTCGGAATGTTCCAGGTCGTACGATCGTACGTCGTA</td>
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<tr>
<td>ruvC 1r</td>
<td>GAACGATTTGTTGATGCCCAAATCGTGTTGATCGATCGTACGATCGTACGTCGTA</td>
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<tr>
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<td>AGCTCGTACGATCGGATGTCGGAATGTTCCAGGTCGTACGATCGTACGTCGTA</td>
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<tr>
<td>ruvC 7f</td>
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<tr>
<td>ruvC 7r</td>
<td>AGCTAGTTACGCTGCTGCGGATGTCGGAATGTTCCAGGTCGTACGATCGTACGTCGTA</td>
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</tr>
<tr>
<td>ruvC 8f</td>
<td>CTAGGTGTTGTCGCGGATGTCGGAATGTTCCAGGTCGTACGATCGTACGTCGTA</td>
<td>Cloning</td>
</tr>
<tr>
<td>ruvC 8r</td>
<td>AGCTACGATTTGTTGATGCCCAAATCGTGTTGATCGATCGTACGATCGTACGTCGTA</td>
<td>Cloning</td>
</tr>
</tbody>
</table>

* The underlined bases indicate bases added to the native sequence to form overhangs compatible with restriction sites on annealing of appropriate pairs of oligonucleotides.
C exposure, the signal nearer to the coding sequence (P1) was strongly enhanced, while the signal distal to ruvC (P2) was not (Fig. 2a). Elevated expression from P1 was also apparent in the ΔrecA strain, but this appeared to be at a lower level, consistent with the observations made by microarray and quantitative RT-PCR analyses of the transcript coding region.

To define the precise positions of the ruvC transcript start sites, primer extensions were performed using RNA extracted from uninduced and induced cultures of wild-type M. tuberculosis and the products run alongside sequencing reactions carried out with the same primer. As with the RNase protection analysis, a clearly inducible transcript (P1) was observed near the coding sequence, and a weak signal that did not change in response to mitomycin C (P2) was observed at a greater distance from it (Fig. 2b). It is not clear whether this weak signal that maps to an A 33 bases upstream of the coding region represents a true transcript start or an artifact in the analyses; the activity of a transcriptional fusion containing 48 bp, including the potential promoter region and extending to 5 bp beyond the putative transcription start site, was not significantly above background (data not shown), so this potential second promoter was not analyzed further.

The transcript start site for P1 mapped to an A 33 bases upstream of the predicted translation initiation codon and was located within the SOS box (Fig. 2c). Therefore, P1 could be repressed by LexA bound to the SOS box interfering with transcription initiation.

Assessment of promoter activity. Promoter motifs that exhibited a high degree of homology with the recA P1 promoter and matched the proposed consensus sequence for this family of promoters (12) with the appropriate spacing were identified upstream of the proximal transcription start site of ruvC (Fig. 3a). Although this spacing between promoter elements is unusual, it has been demonstrated for recA P1 that the sequences at these locations are important for promoter activity, whereas the sequence in the −35 region is not (14). At present, it is not clear whether both these elements are contacted by a sigma factor; M. tuberculosis lacks sigma-54 family sigma factors (3), but it is possible that a regulatory protein could modify the spacing recognized by a sigma-70 family sigma factor, for example, by interacting with the β-flap domain of RNA polymerase (15).

To assess whether the P1 promoter for ruvC had been correctly identified, a transcriptional fusion to lacZ encompassing the promoter region and the SOS box within a 60-bp fragment was constructed, and an A-to-C mutation was introduced into the predicted −10 motif. This base change has been shown to ablate the activity of the recA P1 promoter in M. tuberculosis (14). β-Galactosidase assays of these reporter clones in wild-type M. tuberculosis revealed that the cloned DNA containing P1 exhibited promoter activity well above the background from the vector control that was induced approximately 3-fold following DNA damage and that the mutation in the −10 motif eliminated promoter activity (Fig. 3b). Thus, the P1 promoter had been correctly identified.

Regulation of the P1 promoter. The recA P1 promoter is DNA damage inducible independently of RecA (7), so the sequence similarity noted above suggests that ruvC P1 may be
regulated by this uncharacterized alternative mechanism. However, the position of the SOS box, to which LexA has been shown to bind in vitro (2), overlapping the transcription start site, coupled with the reduced extent of induction in the ΔrecA strain, suggests that LexA is at least partially involved in controlling the expression of ruvC following DNA damage.

To explore the role of LexA in the control of ruvC expression, P1 reporter clones which lacked half the SOS box, or in which a point mutation in the SOS box known to prevent LexA binding (6) was introduced, were constructed. Deletion of half the SOS box resulted in a different 3' end for the insert; however, the adjoining vector DNA contained bases matching the missing half-site at three of six positions (Fig. 4a). The clone bearing the point mutation contained 5' and 3' ends identical to those of the wild-type clone.

When either the wild-type or ΔrecA M. tuberculosis strain was assayed (Fig. 4b), the lack of half the SOS box resulted in a small but statistically significant increase in the uninduced expression level (P < 0.05, Student's t test), consistent with interference with LexA repression, but there was no significant increase with the G-to-A mutation at position +7 (numbering is as in reference 6) in the SOS box. Furthermore, in both cases, expression increased following DNA damage. These results are in contrast with those obtained previously for a promoter regulated only by LexA, the recA P2 promoter, where mutation of half the SOS box resulted in constitutive expression at three to four times the induced level (6; E. O. Davis, unpublished data) and the G-to-A mutation at position +7 caused constitutive expression at the induced level of the native sequence (Davis, unpublished). In addition, the induction ratios seen with the reporter clone did not differ significantly between the wild-type and ΔrecA ruvC strains, in contrast to the results obtained by analyzing mRNA levels (Fig. 1) (19). Thus, analysis of the transcriptional fusions suggests that LexA is not important for DNA damage induction of ruvC but that an alternative mechanism is active at P1 and that the 60-bp sequence analyzed contains the regions necessary for this alternative mechanism to function.

It has been established that M. tuberculosis possesses at least two mechanisms controlling gene expression in response to DNA damage (7). One of these is mediated by the repressor protein LexA binding to an SOS box upstream of the regulated gene, with alleviation of this repression requiring RecA. The other is independent of RecA, and the possibility of this being mediated by another protein able to cleave LexA was eliminated by the observation that induction of a promoter regulated only by LexA is blocked by expression of a noncleavable LexA. The alternative mechanism is yet to be defined, but a conserved promoter motif has been found among a number of genes that are induced by DNA damage independently of RecA (12).

ruvC is part of a group of 28 genes identified by genomic analysis as potentially regulated by both LexA/RecA and an
alternative mechanism (19). This group includes recA, which has previously been shown to be expressed from two promoters, one of which is controlled by LexA and the other by a RecA-independent mechanism (7). However, another gene in this group, Rv2719c, has since been found to be expressed primarily, if not exclusively, from a promoter that is DNA damage inducible independently of RecA (1). This may also be the case for other genes that retain a relatively high induction ratio in the recA mutant, particularly in cases where no SOS box has been identified. In this study, transcriptional fusion assays indicated that the primary mode of regulation of ruvC was independent of RecA, although it remains possible that its expression may be modulated by LexA binding to the SOS box that overlaps the transcription start site. The similarity between the promoter motifs of ruvC and recA P1 suggests that they are likely to be controlled by the same mechanism.

RuvC acts to resolve Holliday junctions (4, 8), the final step in recombination repair; control of this DNA cleavage activity is important to facilitate DNA repair while minimizing unwanted activity in the absence of DNA damage. In E. coli, RuvC is reported to be expressed at a relatively low level and is not induced by DNA damage (21), but its activity is stimulated by interaction with the branch migration complex RuvAB (22), the expression of which is induced following DNA damage. Thus, the activity of RuvC is controlled posttranslationally in E. coli but not at the transcriptional level. In contrast, as demonstrated here and in other studies (2, 19), ruvC is transcriptionally regulated in M. tuberculosis, along with ruvAB; it is possible that this obviates the need for posttranslational control, and whether RuvAB stimulation of RuvC activity occurs in M. tuberculosis is a question of interest for future study.

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REFERENCES