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Determination of DNA Sequences Required for Regulated *Mycobacterium tuberculosis* RecA Expression in Response to DNA-Damaging Agents Suggests that Two Modes of Regulation Exist

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The *recA* gene of *Mycobacterium tuberculosis* has previously been cloned and sequenced (E. O. Davis, S. G. Sedgwick, and M. J. Colston, J. Bacteriol. 173:5653–5662, 1991). In this study, the expression of this gene was shown to be inducible in response to various DNA-damaging agents by using a transcriptional fusion to the reporter gene encoding chloramphenicol acetyltransferase. A segment of DNA around 300 bp upstream of the coding region was shown to be required for expression. However, primer extension analysis indicated that the transcriptional start sites were 47 and 93 bp upstream of the translation initiation codon. Sequence motifs with homology to two families of *Escherichia coli* promoters but also with significant differences were located near these proposed transcription start sites. The differences from the *E. coli* consensus patterns would explain the previously described lack of expression of the *M. tuberculosis recA* gene from its own promoter in *E. coli*. In addition, the *M. tuberculosis* LexA protein was shown to bind specifically to a sequence, GAAC-N4-GTTC, overlapping one of these putative promoters and homologous to the *Bacillus subtilis* Cheo box involved in the regulation of SOS genes. The region of DNA 300 bp upstream of the *recA* gene was shown not to contain a promoter, suggesting that it functions as an upstream activator sequence.

The RecA protein plays a central role in recombination and DNA repair and is highly conserved among all bacteria examined so far (37). Its expression is induced in response to DNA damage as part of the SOS response, the coordinate induction of over 20 genes involved in DNA repair and synthesis, recombination, and cell division, which leads to increased cell survival (56). In combination with repressor protein LexA, RecA controls the induction of the SOS response (31). With DNA damage, RecA becomes activated, probably as a result of its interaction with regions of single-stranded DNA formed by the damage, and activated RecA triggers the autocatalytic cleavage of LexA (29, 30), the cleaved form of which does not bind efficiently to DNA (3). LexA binds to a specific sequence upstream of the genes it regulates, repressing transcription (4, 31, 32); therefore, on activation of RecA and cleavage of LexA, these genes become expressed. In *Escherichia coli* and many other gram-negative bacteria, the consensus binding site for LexA, termed the SOS box, is CTGT-N8-ACAG (56), whereas in *Bacillus subtilis* a different consensus sequence, GAAC-N4-GTTC, termed the Cheo box (7, 8), is found upstream of DNA damage-inducible genes and has been shown to bind LexA from *B. subtilis* (20, 34). Similar sequences have also been identified upstream of the *recA* genes of other gram-positive bacteria, *Staphylococcus aureus* (2) and *Corynebacterium pseudotuberculosis* (41).

The *recA* genes of the gram-positive bacteria *Mycobacterium tuberculosis* and *M. leprae*, the etiological agents of tuberculosis and leprosy, respectively, have previously been cloned and sequenced (12, 14). Although each of these two RecAs possesses an intein (13, 14), an intervening sequence which is removed by protein splicing (10, 40), the mature product is homologous to other RecAs, and the *M. tuberculosis recA* gene has been shown to complement *E. coli recA* mutants for most RecA functions (12). During infection, *M. tuberculosis* resides in host macrophages, part of the normal defense mechanism where conditions might be expected to lead to DNA damage; so the regulation of DNA damage-inducible genes such as *recA* may be important in pathogenesis. In addition, studies of gene expression in mycobacteria are important because many mycobacterial genes, including *recA*, are not expressed in *E. coli* (9, 11, 12) and so presumably have promoters which are not recognized by *E. coli* RNA polymerase. The structure of such mycobacterial promoters and their interaction with mycobacterial RNA polymerase are poorly defined.

Several questions of interest arise. (i) Is *M. tuberculosis recA* expression inducible? (ii) If this is the case, what upstream sequences are required? (iii) Is a LexA homolog involved in regulation of *M. tuberculosis recA* expression? (iv) If so, what is the recognition site for LexA in *M. tuberculosis*? Upstream of both the *M. tuberculosis* and *M. leprae recA* genes, there is a sequence motif with homology to the Cheo box LexA regulatory site of *B. subtilis*, while there is no similarity to the SOS box of *E. coli* (12, 14). We have recently cloned and characterized the *lexA* gene of *M. tuberculosis* and shown that it also has a Cheo box-like sequence which is bound by purified LexA (38).

A two-pronged approach was taken to investigate these points. A chloramphenicol acetyltransferase (CAT) reporter gene was transcriptionally fused to the *M. tuberculosis recA* gene to investigate gene expression and regulation in vivo and to determine the amount of upstream DNA required. Coupled with this, primer extension analysis was used to indicate the transcription start site(s). Additionally, purified *M. tuberculosis*

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TABLE 1. Plasmids used in this study

Plasmid	Construction, source, or reference
pEJ108	CAT promoter probe vector for use in mycobacteria based on pKK232-8 plus a 2.6-kb *Eco*RV-*Hpa*I fragment (myco-origin[a]) of pYUB12 and the kanamycin resistance gene from pUC4-KIXX
pEJ126	12
pEJ135	12
pEJ161	0.4-kb *Bam*HI fragment of pEJ135 (upstream DNA and part of *M. tuberculosis recA* gene) in *Bam*HI site of pEJ108
pEJ257	0.3-kb *Hin*dIII-*Bam*HI superlinker fragment of pSL1180 in *Hin*dIII-*Bam*HI fragment of pEJ108; CAT promoter probe vector
pEJ258	4.2-kb *Nru*I fragment of pEJ126 (upstream DNA and part of *M. tuberculosis recA* gene) in *Eco*RV site of pEJ257
pEJ352	60-bp double-stranded oligonucleotide from −310 to −250 from start of *recA* in *Eco*RV site of pEJ257 in the same orientation as when it precedes *recA*
pEJ353	Same as pEJ352 but with the insert in the opposite orientation
pFM3	1.3-kb *Xho*I fragment of pEJ258 in *Eco*RV site of pEJ257
pFM6	1.3-kb *Xho*I fragment of pEJ258 in *Sal*I site of pUC19
pFM7	0.7-kb *Hin*dIII-*Not*I fragment of pFM6 in *Hin*dIII-*Not*I site of pEJ257
pFM17	0.35-kb *Pvu*II-*Hin*dIII fragment of pFM6 in *Eco*RV-*Hin*dIII site of pEJ257
pYUB12	49
pKK232-8	5
pUC4-KIXX	Pharmacia
pSL1180	6
pUC19	59

[a] myco-origin, mycobacterial origin of replication.

LexA protein was used in in vitro assays (gel retardation and footprinting) to determine its binding site.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* DH5α (47) was used for all plasmid constructions. The plasmids used in this study are listed in Table 1. *E. coli* cultures were grown at 37°C in Luria-Bertani medium (47) with the addition of ampicillin and methicillin, where appropriate, at 50 μg ml^{-1} (each). *M. smegmatis* mc^2155 and *M. tuberculosis* H37Rv were grown in modified Dubos medium (Difco) at 37°C with the addition of 25 μg of kanamycin per ml for strains containing plasmids.

CAT and protein assays. *M. smegmatis* mc^2155 cells were grown in Lemco broth containing kanamycin (25 μg/ml) for about 36 h and then subcultured 1/1,000 in Dubos A + B–0.2% glycerol–kanamycin (25 μg/ml) for about 24 h. When the optical density (OD) reached 0.6 to 0.7, the culture was divided into 10-ml aliquots to be induced in various ways and incubated with shaking at 37°C for 5 to 6 h (see Results for the optimization of induction conditions). Bacteria were harvested, washed in 5 ml of phosphate-buffered saline, resuspended in 1 ml of phosphate-buffered saline, lysed with a minibead beater for 1 min, and spun for 3 min in a microcentrifuge. The supernatant was taken for protein and CAT assays. The protein assay was done with a bicinchoninic acid protein assay kit (Pierce), and the CAT assay was done with a CAT enzyme-linked immunosorbent assay kit (Boehringer Mannheim).

Recombinant DNA techniques. Standard molecular and recombinant DNA techniques were used (47). Plasmid minipreps were prepared by the boiling method (22) or with a QIAGEN miniprep kit. Maxipreps were prepared with a QIAGEN plasmid and/or cosmid purification kit. DNA was digested with restriction enzymes (New England Biolabs and Life Technologies) according to the manufacturers' instructions. DNA fragments were purified from agarose gels with Geneclean (Bio 101).

Preparation of *M. tuberculosis* RNA. RNA was isolated by the method of Gonzalez-y-Merchand et al. (19).

Primer extension reactions. Oligonucleotide primers were end labelled with [γ-^{32}P]ATP at their 5′ termini by means of T4 polynucleotide kinase as described in the primer extension kit (Promega). One picomole of labelled primer was annealed to 40 μg of total RNA at 42 to 65°C (based on the melting temperature of the primer being used) for 1 h. Extension was carried out with avian myeloblastosis virus reverse transcriptase (Promega) according to the manufacturer's instructions at 42°C for 1 h. The products were ethanol precipitated, resuspended in 5 μl of Tris-EDTA (pH 7.5) plus 5 μl of loading dye (98% [vol/vol] formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue), and denatured at 90°C for 10 min before being separated on an 8% (wt/vol) polyacrylamide–urea gel.

RT-PCR analysis. RNAs prepared from three different uninduced and induced *M. tuberculosis* cultures were treated with 10 U of RNase-free DNase I according to the manufacturer's (Boehringer Mannheim) instructions, phenol extracted, and precipitated by standard protocols (47). The reverse transcription (RT) reaction was carried out in a 20-μl volume in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 400 μM (each) deoxynucleoside triphosphates, 29 U of RNase inhibitor (Pharmacia), 1 μg of template RNA, 2.5 μg of random hexamer oligonucleotide primers (Boehringer Mannheim), and 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL). In the negative control, reverse transcriptase was omitted. The RT reaction mixture was incubated at 37°C for 60 min and then heat inactivated at 70°C for 15 min.

In PCRs, a 1-μl aliquot of the RT reaction product was used as the template in *Taq* buffer containing 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates, 300 nM (each) upstream and downstream primers, and 1.25 U of AmpliTaq DNA polymerase (Perkin-Elmer). cDNA was amplified in a Techne Progene temperature cycler with cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min. The oligonucleotide primers used were AGGCGCTGCGGAAAATGA and CACCGACGCGTAGAACTT for *recA* and GCGGGCGATACGGGCAGACTA and CTCCCCACGCTTTCGCTCCT CAG for the *rrn* control. Preliminary reactions with *M. tuberculosis* DNA for 30 cycles confirmed that these primer pairs worked in PCR.

To determine the number of cycles where the PCR was in the exponential phase and thus reflected the amount of template added, [α-^{32}P]dCTP was incorporated in the reaction mixtures and reactions were run for various numbers of cycles. After electrophoresis of the product through 6% denaturing polyacrylamide gels, the dried gels were analyzed with a PhosphorImager and the log of the quantification of the signal was plotted against the cycle number. A cycle number within the linear phase of this plot was chosen for subsequent comparisons of uninduced and induced samples; 22 cycles were used for *recA*, and 10 cycles were used for *rrn*. Three PCRs were performed in the same way for each sample in the final analysis of uninduced and induced samples, and the amount of product formed was quantified by volume in a PhosphorImager.

Purification of *M. tuberculosis* LexA protein. LexA from *M. tuberculosis* was expressed in *E. coli* with a His tag by cloning in vector pET-15b and purified by Ni-nitrilotriacetic acid chromatography as described previously (38).

Gel retardation assays. Gel shift assays were carried out with a DIG gel shift kit (Boehringer Mannheim). The double-stranded oligonucleotide or restriction fragment (3 to 4 pmol μl^{-1}) was labelled according to the manufacturer's instructions and then diluted to a concentration of 15 to 30 fmol μl^{-1} in TEN buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl [pH 8]). The gel shift reaction was set up according to the manufacturer's instructions with 0.1 μg of LexA protein and poly(dI-dC) competitor DNA. After incubation at room temperature for 15 min, the sample was applied immediately to a preelectrophoresed native 8% (wt/vol) polyacrylamide gel. The gel system used was a maxigel (Biometra) with dimensions of 20 cm by 20 cm by 1 mm. Electrophoresis was carried out in 0.5× Tris-borate-EDTA buffer (pH 8) at 160 V and 4°C in a cold room. One lane was loaded with sample buffer containing bromophenol blue to monitor the extent of electrophoresis; the dye was run three-fourths of the way to the bottom of the gel. The gel was blotted onto a positively charged nylon membrane (Boehringer Mannheim) with a semidry electroblotter (Ancos) at 400 mA for 1 h. Then the membrane was soaked in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 s and cross-linked for 1 min at 300 nm with a transilluminator. Chemiluminescent detection was performed according to the manufacturer's instructions. The membrane was exposed to X-ray film (Hyper film-MP; Amersham) for 15 to 40 min at room temperature.

Footprinting. Footprinting was carried out by a method (28) based on PCR and the dideoxy DNA sequence reaction. A pair of primers (ACGATCGGTG GTGAGGTTG and AGGCGCATCACCGAACCTTTG) were designed for producing the target DNA by PCR with a label at the 5′ end of each strand in separate reactions. Oligonucleotide primers were end labelled with [γ-^{32}P]ATP at their 5′ termini by means of T4 polynucleotide kinase as described in the primer extension kit (Promega). PCR was performed with an Expand high-fidelity PCR system kit (Boehringer Mannheim). Ten nanograms of template DNA (pEJ258) containing the upstream sequence of the *recA* gene was used. Two PCRs were performed. In one reaction, the downstream primer was end labelled, and in the other reaction, the upstream primer was end labelled with [γ-^{32}P]ATP. The PCR product was purified by filtration through a Probind spin column (Millipore Corporation). An optimal ratio of end-labelled DNA (10 ng of PCR product) and LexA protein (0.3 μg) was prepared in the same buffer as was used for the gel shift assay. Due to a requirement for cations in the subse-

quent DNase I cleavage reaction, $MgSO_4$ was added to a final concentration of 5 mM (1 μl of 100 mM $MgSO_4$). A concentration of 0.1 U of DNase I (10 U/μl; Boehringer Mannheim) per μl was prepared on ice; 1 μl was added to the DNA-protein mixture, mixed carefully, and left for 2 min at room temperature. Twenty microliters of prewarmed (37°C) stop solution (200 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate, and 50 ng of tRNA per μl) was added and vortexed. To inactivate DNase I, the cleaved DNA fragments were heated at 90°C for 1 min, purified by phenol-chloroform extraction and ethanol precipitation, washed with 70% ethanol (twice), and resuspended in 5 μl of sequencing gel loading buffer (95% [vol/vol] formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The samples were heated for 10 min at 90°C and electrophoresed on a 6% polyacrylamide DNA sequencing gel. The primer that was labelled in the protection assay was also used to perform the sequencing reactions for double-stranded DNA (Sequenase kit; U.S. Biochemical Corporation) which were run alongside the footprinting reaction.

RESULTS

Confirmation that *M. tuberculosis recA* expression is inducible by DNA damage. The expression of *recA* in *E. coli* and *B. subtilis* is inducible by agents which cause DNA damage (18, 58). This inducibility is controlled by the combined action of the repressor LexA, which binds to a specific sequence upstream of the *recA* gene to prevent transcription under noninducing conditions (4, 20, 32, 34), and the RecA protein itself, which becomes activated under inducing conditions, resulting in the autocatalytic cleavage of LexA and thus preventing its binding to DNA (31). The binding sites for LexA in *E. coli* (CTGT-N8-ACAG) (31) and *B. subtilis* (GAAC-N4-GTTC) (34) are completely different. A sequence homologous to the LexA binding site of *B. subtilis* (termed a Cheo box) is located 121 bp upstream of the *M. tuberculosis recA* gene (12); therefore, it might be expected that *M. tuberculosis recA* expression is also induced by DNA damage-inducing agents.

To investigate whether this is indeed the case, construct pEJ161 was made by transcriptionally fusing the amino-terminal region of *recA* to the CAT reporter gene in a mycobacterial shuttle vector; it contained 269 bp of upstream DNA, including the putative Cheo box. However, preliminary CAT assays with this construct in *M. smegmatis* showed no sign of induction by ofloxacin or mitomycin (data not shown). The *Bam*HI site used to make this construct cut within a sequence with more limited similarity to a Cheo box, having six matches among eight conserved bases, so it was possible that this motif or additional Cheo boxes further upstream were required for inducible expression. Therefore, a construct containing a large amount of upstream DNA (approximately 3 kb), pEJ258, was made. In this case, expression of the CAT reporter gene was induced two- to threefold by ofloxacin and the basal level of expression under uninduced conditions (55 ng of CAT/mg of protein) was also higher than that found for pEJ161 (4 ng of CAT/mg of protein), which was not significantly different from the value obtained with the vector (2.3 ng of CAT/mg of protein). As it seemed unlikely that this much upstream DNA would be required for regulated expression of the *recA* gene, a construct of intermediate size containing approximately 1.3 kb of upstream DNA, pFM3, was tried next; this exhibited inducible expression with almost identical properties (data not shown).

Optimization of the conditions required for *M. tuberculosis recA* induction in *M. smegmatis*. After it had been established that expression of the *M. tuberculosis recA* gene is inducible by DNA-damaging agents in *M. smegmatis*, the next step was to define the growth conditions for maximum induction. The assays were performed with *M. smegmatis* because of its more rapid growth rate, which facilitated the assessment of a number of conditions, both environmental and genetic, required for induction. The optimum growth conditions were studied by using construct pFM3. First, the effect of the growth stage of culture at the point of induction was assessed. An arbitrarily chosen concentration of the DNA-damaging agent ofloxacin (1 μg/ml) was added when the culture reached an OD at 600 nm (OD_{600}) of 0.47, 0.6, or 0.74, and after a further 6 h of incubation at 37°C, cell extracts were made and assayed for CAT and protein. The results showed that the highest level of induction (3-fold) occurred when inducer was added to culture at an OD_{600} of 0.6, with induction at an OD_{600} of 0.74 only slightly less effective (2.5-fold). Surprisingly, at an OD_{600} of 0.47 essentially no induction was seen, despite the growth being logarithmic between an OD_{600} of 0.15 and an OD_{600} of 1.0 before finally reaching a plateau around an OD_{600} of 2.

Then the optimal concentrations of various DNA damage-inducing agents were analyzed. Various concentrations of the relevant agent were added to cultures at an OD_{600} of 0.6 to 0.7 for 6 h. Mitomycin, which cross-links complementary DNA strands by alkylation of guanines (23, 24), was used over a concentration range of 0.2 to 30 μg/ml. Concentrations of 5 μg/ml and above showed toxicity to cultures during the incubation period in the presence of inducer and resulted in an apparent decrease in expression at the highest concentration used. There was no significant difference in the level of induction (2.5-fold) observed within the range of 0.2 to 2.5 μg/ml (Fig. 1a).

Ofloxacin is a quinolone inhibitor of DNA gyrase (54) which exhibits higher toxicity to mycobacteria (45) than does nalidixic acid, the more frequently used gyrase inhibitor (53). The ability of each of these compounds to induce the *M. tuberculosis recA* gene was examined. Ofloxacin was tested at concentrations ranging from 0.05 to 1.5 μg/ml, with the greatest degree of induction (twofold) apparent at 1 μg/ml (Fig. 1b). Nalidixic acid was examined at concentrations ranging from 10 to 200 μg/ml and gave the highest level of induction (2.5-fold) when used at 150 μg/ml (Fig. 1c).

The ability of UV irradiation, which primarily causes the formation of pyrimidine dimers (21), to induce the *M. tuberculosis recA* gene was also investigated. *M. smegmatis* cultures carrying the cloned *M. tuberculosis recA*-CAT transcriptional fusion were grown to an OD_{600} of 0.6 to 0.7, irradiated with UV (0.1 J m^{-2} s^{-1}) for times ranging from 10 to 150 s, and then incubated at 37°C for 6 h before being assayed. Induction was apparent, with the greatest effect (1.7-fold) after irradiation for 70 to 90 s (Fig. 1d).

Finally, the effect of the time of induction was assessed. For this experiment, clone pFM7, which contained an even smaller amount of upstream DNA (see below), was used. Cultures grown to an OD_{600} of 0.6 to 0.7 were incubated with 1 μg of ofloxacin per ml for 1, 3, 5, or 7 h before being assayed. Induction was seen from 3 h (twofold) and was maximal (threefold) after incubation for 5 h before decreasing with continuing incubation for 7 h (twofold).

From this series of experiments, the optimal conditions for induction chosen for further experiments with different DNA fragments were growth to an OD_{600} of 0.6 to 0.7 and the addition of 1 μg of ofloxacin per ml for 5 h. In general, ofloxacin rather than mitomycin was used because it appeared to have less toxicity, as assessed by the growth of cultures, although for some experiments the results obtained by induction with ofloxacin were confirmed with mitomycin.

Determination of the amount of upstream DNA required for induction. Under the optimal conditions for induction defined above, constructs containing progressively smaller amounts of upstream DNA (Fig. 2a) were investigated for expression level and inducibility. With plasmid pFM3, containing approximately 1.3 kb of upstream DNA, a two- to threefold increase in expression on induction was observed (data not shown). A very similar result (2.5-fold induction) was obtained with pFM7,

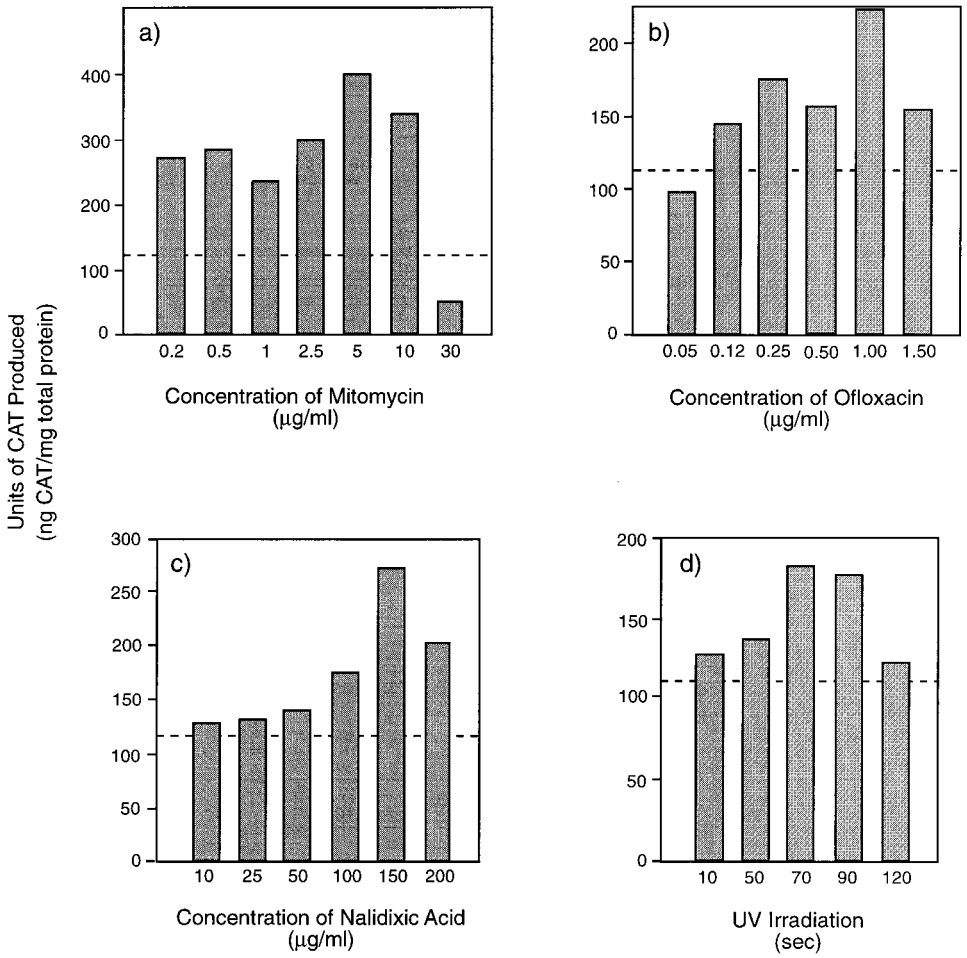

FIG. 1. Effects of different concentrations of the indicated inducing agents on the expression of *M. tuberculosis recA*, as determined by CAT assays of transcriptional fusion plasmid pFM3 in *M. smegmatis* mc^2155. Dashed lines represent uninduced levels of activity.

where the amount of upstream DNA was reduced to 667 bp (Fig. 2b). A further reduction to 310 bp of upstream DNA in pFM17 resulted in lower absolute levels of expression for both uninduced and induced cultures, but this construct retained inducibility and in fact exhibited a higher induction ratio, 4.3-fold (Fig. 2b). Surprisingly, however, the elimination of only 41 bp from this construct, resulting in pEJ161, which contained 269 bp of upstream DNA, resulted in a dramatic reduction in the uninduced level of expression, to a value not significantly different from that obtained for the vector, and the loss of inducibility (Fig. 2b), despite the fact that this smallest fragment retained the Cheo box suspected to be important for induction. This suggested that the promoter or other sequences essential for transcription were located in this region, either within the 41 bp between 269 and 310 bp upstream of the *recA* gene or overlapping the cloning site used to make pEJ161.

Mapping the transcriptional start site(s) of the *M. tuberculosis recA* gene. In order to obtain an indication as to whether the promoter for the *recA* gene lay in the region defined as essential for expression by the deletion analysis described above, the transcription start points were identified by primer extension. Total RNA was isolated from cultures of *M. smegmatis* mc^2155 carrying plasmid pFM7 which were uninduced or induced with either 1 μg of ofloxacin per ml or 2.5 μg of mitomycin per ml for 5 h. Primer extension was then performed with this RNA and a primer complementary to the CAT coding region located downstream of the cloned *M. tuberculosis* DNA containing a functional *recA* promoter. The products of these reactions were run alongside sequencing reactions carried out with the same primer (Fig. 3a).

Two products, one 47 bp and another 93 bp upstream from the translation initiation codon (Fig. 3a and 4), were obtained, suggesting the presence of two promoters for the *recA* gene. No extension products were observed when RNA prepared from *M. smegmatis* containing the vector was used. The same endpoints of RNA were obtained with other primers in this region (Fig. 3b and data not shown), suggesting that the extension products represent genuine RNA termini and are not due to mispriming. In order to assess whether other promoters existed further upstream, primer extension analysis was performed with two primers from different regions of the upstream sequence, but no further products were identified (data not shown).

The intensity of the extension product nearest to the *recA* gene appeared to be greater with RNA from induced cultures than with RNA from uninduced cultures, particularly when the inducing agent was mitomycin. Additional bands within a few bases of the extension product nearest the *recA* gene were seen with RNA from induced cells. Similar patterns have been re-

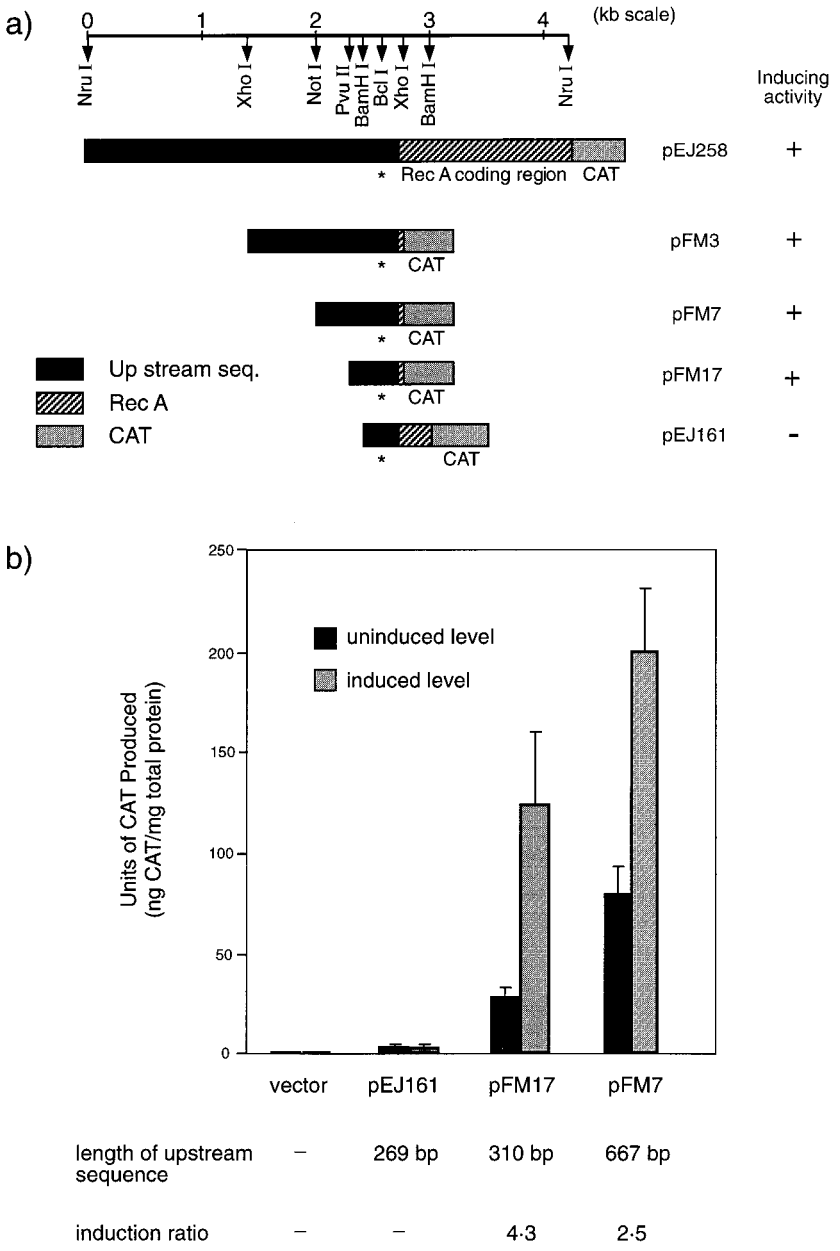

FIG. 2. Deletion analysis of the amount of upstream DNA required for induction and expression of *M. tuberculosis recA* with transcriptional fusions to CAT in *M. smegmatis* mc^{2}155. (a) Constructs used, with the amount of upstream DNA (Up stream seq.) in each construct shown. Inducibility (+) is indicated on the right; −, no induction. An asterisk indicates the position of the Cheo box, as mentioned in the text. (b) Expression levels of the constructs indicated, as determined by the CAT activity generated after incubation in the presence (grey bars) or absence (black bars) of ofloxacin (1 μg/ml for 5 h). The number of bases of upstream DNA and the induction ratio for each construct are indicated below the histogram. Data are the means of duplicate assays from six independent cultures, except that data for the vector control are from three cultures.

ported previously for some other mycobacterial genes, e.g., *hsp60* (51).

Primer extensions were also carried out with RNA isolated from *M. tuberculosis* cultures which had been induced with 1 μg of ofloxacin per ml; this time a primer complementary to the *recA* coding region was used. The extension products obtained were compared directly with those produced from the same primer with RNA from cultures of *M. smegmatis* containing pFM7 (Fig. 3b). The same transcription start sites were identified with RNA expressed from the *M. tuberculosis* chromosome and for the *M. tuberculosis recA* gene expressed from a plasmid in *M. smegmatis*. The same endpoints were found with a second primer in this region, and analysis with primers located further upstream failed to generate any additional extension products (data not shown).

It was clear with *M. tuberculosis* RNA that the extension product nearest the *recA* gene was considerably stronger than the one further away. In addition, the multiple banding at this location seen previously with plasmid-derived RNA in *M. smegmatis* was not apparent with RNA from plasmid- or chromosome-encoded *recA* in *M. tuberculosis* itself. Similar findings have been reported for the *M. tuberculosis purC* gene (25),

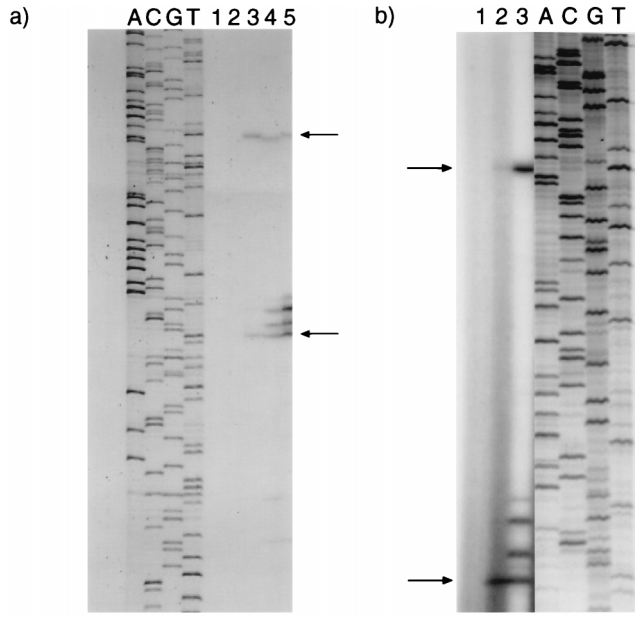

FIG. 3. Mapping the transcriptional start sites of *M. tuberculosis recA* by primer extension. (a) RNA from fusion plasmid pFM7 in *M. smegmatis* mc^{2}155 and a primer complementary to CAT were used. Lane 1, no RNA substrate; lane 2, RNA from cells containing the vector pEJ257; lane 3, RNA from uninduced cells containing pFM7; lane 4, RNA from cells containing pFM7 and induced with ofloxacin (1 μg/ml); and lane 5, RNA from cells containing pFM7 and induced with mitomycin (2.5 μg/ml). (b) Comparison with *M. tuberculosis* RNA by using a primer complementary to *recA*. Lane 1, no RNA substrate; lane 2, RNA from *M. tuberculosis* cells induced with ofloxacin (1 μg/ml); lane 3, RNA from *M. smegmatis* mc^{2}155 cells containing pFM7 and induced with ofloxacin (1 μg/ml); and lanes A, C, G, and T, sequencing reactions of the upstream region of the *recA* gene with pFM7 and the same primer used in the extension reactions shown alongside. Arrows indicate the positions where transcription of the *recA* gene starts.

where primer extension with RNA from a clone in *M. smegmatis* yielded three bands, two of which were just 2 bases apart, whereas with RNA from *M. tuberculosis* itself, only one extension product was obtained. This difference was presumed to be due to differences in the transcriptional machinery of the two species of mycobacteria.

These results, particularly the differential increase in the relative amounts of the two products on induction, suggested that the primer extension products obtained represented genuine transcription start sites, although it remained a possibility that they resulted from processed products from a larger unstable RNA species. The role of specific sequences identified nearby (see Discussion) in functioning as promoters has yet to be addressed experimentally, but it is usual for promoters to be located in the vicinity of the transcription start sites. The region of DNA identified by deletion analysis as essential for expression is considerably further upstream from the *recA* gene than are these putative promoter elements and hence might be expected to play some other role in the regulation of transcription.

Role of the upstream sequence required for *recA* expression. To clarify the role of the upstream sequence identified as essential for *recA* expression, it was important to determine whether it contained promoter activity. The results obtained so far for the expression of the *recA* gene could be interpreted in three ways. (i) The promoter lies 250 to 310 bp upstream of the coding region, and transcription starts a long way from the promoter. (ii) The promoter lies 250 to 310 bp upstream of the gene, transcription starts nearby, and a transient mRNA molecule is rapidly processed. (iii) The promoter(s) lies close to the mapped RNA endpoints, and the DNA 250 to 310 bp upstream of *recA* has another function in activating transcription, such as binding an activating protein. In order to distinguish between the last and the first two of these possibilities, a double-stranded 60-bp oligonucleotide consisting of the sequence between positions −310 and −250, relative to the *recA* coding region, was made, cloned into the CAT fusion vector in both orientations, and then assayed for promoter activity in *M. smegmatis*.

The level of CAT expression obtained was very low and essentially the same whether the cloned DNA was in the same orientation as when it precedes *recA* (6.5 ng of CAT/mg of protein) or in the reverse orientation (7.5 ng of CAT/mg of protein); in either case, expression was not significantly different from those of constructs described previously which did not express. There was no difference in the level of expression under DNA-damaging conditions (6.5 and 6.3 ng of CAT/mg of protein, respectively). Each value given above is the mean of duplicate assays from three or four cultures. Even with the first possibility mentioned above, where transcription starts at around 200 bp from the promoter, CAT expression should have been detected if a promoter was present, as there was sufficient distance from the cloning site to the start of the CAT gene. Thus, this 60-bp region does not contain a promoter. This, coupled with the requirement for this sequence demonstrated by deletion analysis, strongly suggests that there is a binding site for a transcription-activating protein in this region, i.e., an upstream activating sequence.

Identification of the LexA binding site. As well as the motif with a precise match to the Cheo box consensus sequence located 121 bp upstream from the translation initiation codon, another motif with some similarity to a Cheo box was positioned 276 bp upstream of the translation start site (Fig. 4). Although this motif is not as well conserved as the Cheo box closer to the *recA* gene (it has two mismatches), since LexA possibly binds to both of these sequences, the binding site(s) for LexA was determined experimentally.

The binding of LexA to the region of DNA upstream of the *M. tuberculosis recA* gene was confirmed by gel retardation with a large (350-bp) fragment extending from the beginning of the 41-bp sequence essential for expression into the coding region of the gene. A comparison of the migrations of this labelled fragment in the presence and absence of purified *M. tuberculosis* LexA clearly demonstrated that LexA binding did occur (Fig. 5a).

To localize the site(s) of LexA binding, DNase I footprinting analysis was carried out. DNA fragments labelled at the 5′ end of one strand were generated by PCR with one labelled primer and one unlabelled primer as detailed in Materials and Methods. A 480-bp fragment, including both the Cheo box located 121 bp upstream and the Cheo box-like sequence located 276 bp upstream of the *recA* gene, was used. The products of DNase treatment of DNA bound to LexA and of DNA without LexA were separated alongside sequencing reactions generated with the primer which had been labelled to make the fragment. A clear protected region of approximately 24 bp was observed (Fig. 6); it encompassed the highly conserved Cheo box located 121 bp upstream of the *recA* gene, flanked by 5 to 6 bp on each side. There was no evidence of any binding to the weakly conserved Cheo box-like sequence located further upstream, which was included in the target DNA fragment used. An identical result was obtained when the DNA fragment was labelled on the other strand (data not shown). This result demonstrated that LexA did indeed bind around the Cheo box

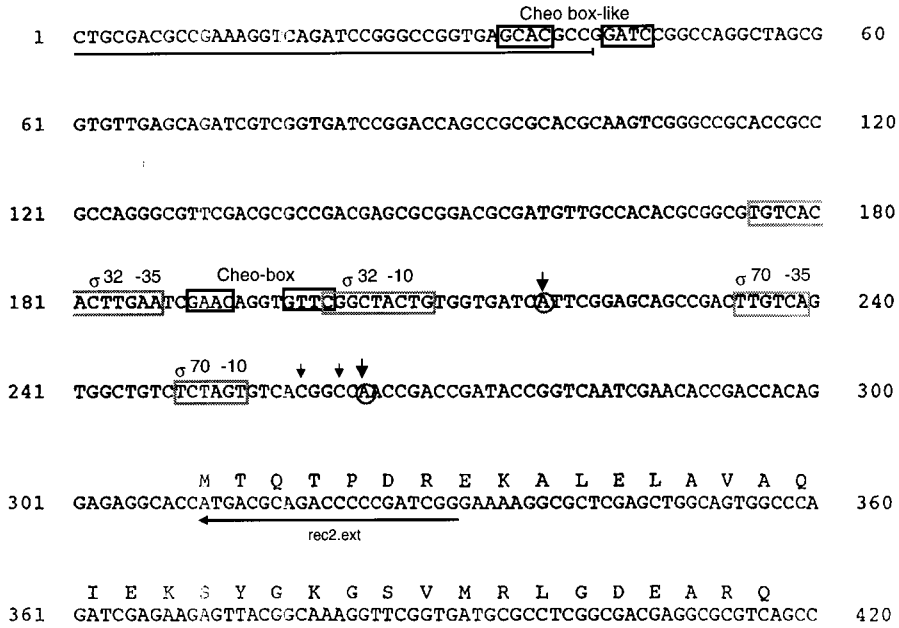

FIG. 4. Regulatory elements upstream of the *M. tuberculosis recA* gene. The DNA sequence upstream of the *M. tuberculosis recA* gene is shown. The transcription start sites identified by primer extension are circled, and the primer (rec2.ext) used for some of these experiments is indicated by a labelled arrow beneath the sequence. The putative promoter elements with homology to *E. coli* promoters identified near these transcription start sites, the Cheo box, and the Cheo box-like sequence are boxed and labelled. The 41-bp sequence shown to be essential for expression by deletion analysis is underlined.

originally identified but did not define which residues in that region were important for the interaction.

Further gel retardation analysis was performed with two 32-bp double-stranded oligonucleotides spanning the Cheo box; one of them was identical to the wild-type sequence, and the other was mutated at each of the eight conserved bases of the Cheo box but was identical to the wild type at all other positions. The results clearly revealed that LexA bound to the wild-type sequence but did not bind at all to the mutant sequence (Fig. 5b), establishing that the conserved bases of the Cheo box are required for *M. tuberculosis* LexA binding. The proportion of labelled DNA retarded by LexA was reduced in the presence of excess unlabelled oligonucleotide of the wild-type sequence. A similar oligonucleotide probe spanning the upstream Cheo box-like sequence did not show any evidence of LexA binding in the gel retardation assay (data not shown), confirming the result obtained by DNase footprinting.

***M. tuberculosis recA* is also inducible in *M. tuberculosis* itself.** As the series of experiments showing DNA damage induction of *recA* described above had been performed with *M. smegmatis* for reasons of convenience, some preliminary studies of *recA* expression in *M. tuberculosis* itself were also undertaken. With pFM7, described above, expression of the CAT reporter gene in *M. tuberculosis* was induced 2-fold by ofloxacin and 2.2-fold by mitomycin but the time required to see this level of induction was 24 h (data not shown). Thus, the *recA* gene in *M. tuberculosis* was inducible to an extent similar to that of the *M. tuberculosis recA* gene in *M. smegmatis*, but the induction was greatly delayed in *M. tuberculosis*. A detailed comparative analysis of the kinetics of *recA* induction in *M. tuberculosis* and *M. smegmatis* is under way but is beyond the scope of this paper.

The copy number of the replicon used in the reporter constructs is very low, having been reported as 1.7 (50), 3 (43), and 5 (51) by various investigators; therefore, copy number effects on expression are likely to be minimal. Nevertheless, to confirm that induction of the *recA* gene itself, rather than the *recA* promoter on a plasmid, also occurs in *M. tuberculosis*, RNAs from uninduced *M. tuberculosis* cultures and from cultures of *M. tuberculosis* which had been induced with ofloxacin (1 μg/ml) for 24 h were analyzed.

The levels of *recA* RNA were assessed by RT-PCR, using a number of cycles for PCR determined experimentally (see Materials and Methods) to occur during exponential amplification. Under these conditions, the amount of PCR product

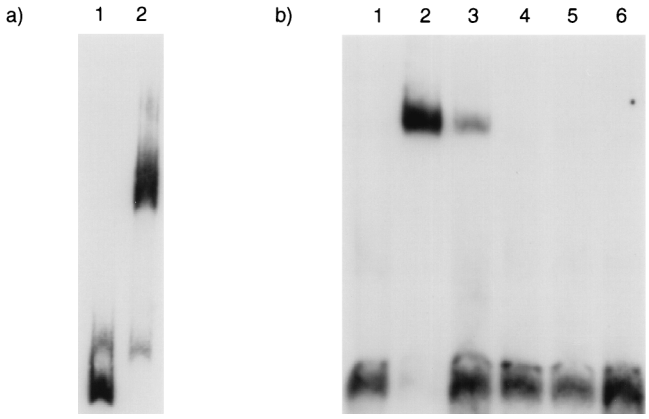

FIG. 5. Gel retardation analysis to show that *M. tuberculosis* LexA binds to a specific sequence upstream of *M. tuberculosis recA*. (a) A 350-bp restriction fragment (30 fmol) of upstream DNA, including all the DNA required for expression, was used with no added protein (lane 1) or with 0.2 μg of LexA added (lane 2). (b) A 32-bp double-stranded oligonucleotide (CAC TTG AAT C**GA AC**A GGT **GTT C**GG CTA CTG TG; 30 fmol) spanning the Cheo box (in bold) (lanes 1 through 3) or an identical oligonucleotide except for mutations (in bold) introduced in the Cheo box (CAC TTG AAT C**TC CA**A GGT **TGG A**GG CTA CTG TG; 30 fmol) (lanes 4 through 6) was used with no added protein (lanes 1 and 4), 0.1 μg of LexA added (lanes 2 and 5), or 0.1 μg of LexA plus 7.6 pmol of unlabelled oligonucleotide of the same sequence added (lanes 3 and 6).

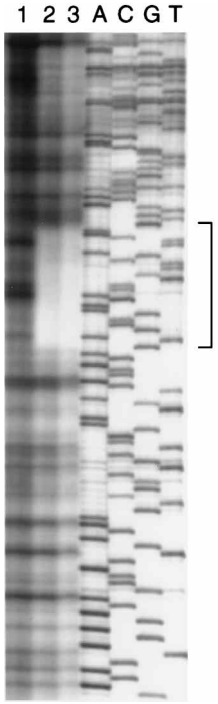

FIG. 6. DNase I footprinting analysis of *M. tuberculosis* LexA binding to the DNA upstream of *M. tuberculosis recA*. DNA labelled at one end was generated by PCR, incubated with or without LexA under conditions which allowed binding, and then treated with DNase I. Lane 1, without protein; lane 2, with protein in the presence of poly(dI-dC); lane 3, with protein in the absence of poly(dI-dC); and lanes A, C, G, and T, sequencing reactions of the cloned DNA upstream of the *recA* gene (pEJ135) with the same primer end labelled for PCR. The region of DNA protected by LexA is indicated.

formed reflects the amount of template, i.e., cDNA, introduced into the reaction mixture. As a control for the amount of RNA in the reaction mixtures, the level of *rrn* RNA, which should not be altered by exposure to a DNA-damaging agent, was measured in the same way. The incorporation of [α-^{32}P]dCTP during PCR allowed the amount of product formed to be quantitated with a PhosphorImager. The results of triplicate assays with RNA pooled from three cultures (Fig. 7) show that while the levels of *rrn* RNA were essentially identical for uninduced and induced samples, the amount of *recA* RNA in the induced preparation was 1.6-fold higher than that in the uninduced one. Controls in which the reverse transcriptase was omitted (Fig. 7, lanes 3 and 4) gave no product, showing that there was no contaminating DNA present. Thus, the degrees to which induction occurs in *M. tuberculosis* for the native *recA* gene and for the *recA* gene on the reporter plasmid are similar.

DISCUSSION

The results described here demonstrate that the *recA* gene of *M. tuberculosis* is inducible by DNA-damaging agents, and we can begin to analyze the requirements for this regulated expression. The maximum level of induction of the *M. tuberculosis recA* gene observed was 4.3-fold with the smallest clone which was functional in the reporter assays, with induction ratios of 2- to 3-fold obtained for larger constructs. These values should be compared with those reported for *B. subtilis recA*, 5.5-fold (44) and 6- to 10-fold (18) with an *xylE* reporter gene, and for *E. coli recA* with gene fusions, 6.8-fold (17) and 5- to 6-fold (58) with a *lacZ* fusion and 8.5-fold (27) with a *galK* fusion.

Deletion analysis of the DNA upstream of the *recA* gene revealed the requirement for expression of a 41-bp region located between 310 and 269 bp upstream of this gene. This result is in contrast to that of a similar deletion analysis of *B. subtilis recA* gene expression (8), where no effect on expression was seen until the Cheo box was altered, resulting in constitutive expression. Thus, either the promoter or other sequences essential for transcription must be located in this DNA region around 300 bp upstream of *recA* in *M. tuberculosis*. However, fusion of 60 bp of this sequence to the CAT reporter gene revealed no evidence of promoter activity within this stretch of DNA. Primer extension analysis also indicated that the transcription start sites were located only 47 and 93 bp upstream of *recA*. Although these primer extension products could be the result of processing of a longer message, no indication of a start site further upstream could be obtained with primers from various locations in the upstream DNA.

An inspection of the sequences near these mapped transcription start sites revealed some interesting similarities and differences from known *E. coli* consensus promoters (Fig. 4). Eight bases away from the start site nearer to the gene, a sequence, TCTAGTG, homologous to the −10 consensus sequence (TATAATg) of *E. coli* σ^{70} promoters was found. In addition, a sequence, TTGTCA, homologous to the −35 consensus sequence (TTGACA) of these promoters was located upstream of the −10-like sequence. However, the spacing between these two elements is very different from that found in *E. coli*, with only 9 bases instead of 17 to 21 bases (46). The *M. tuberculosis recA* gene is not expressed in *E. coli* from its own promoter, so it is possible either that mycobacterial RNA polymerase recognizes the same motifs as does *E. coli* polymerase but at a different spacing or that it binds to a different sequence in the −35 region.

It has recently been demonstrated that mycobacterial promoters can accommodate a large variety of sequences at the

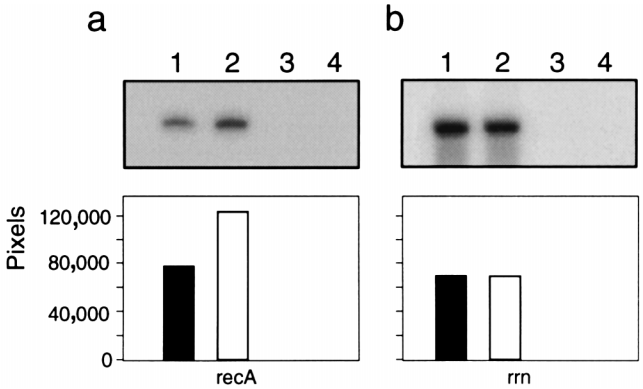

FIG. 7. Induction of *recA* mRNA in *M. tuberculosis*. The levels of *recA* (a) and *rrn* (b) RNA were measured by quantitative RT-PCR from uninduced cultures (lanes 1 and 3) (black bars) and cultures which had been induced with ofloxacin (1 μg/ml) for 24 h (lanes 2 and 4) (white bars). In lanes 3 and 4, the reverse transcriptase was omitted; the lack of a product shows that there was no contamination with DNA. The number of cycles used was determined experimentally to be within the exponential phase of amplification (see Materials and Methods) (22 cycles for *recA* and 10 cycles for *rrn*). The reaction mixtures included [α-^{32}P]dCTP, and the amount of product formed was measured with a PhosphorImager. Representative examples of the products formed, as assessed by autoradiography (exposed for 1 h in each case), are shown at the top, and the means obtained with a PhosphorImager (exposed for 4 h [*recA*] or 30 min [*rrn*]) of triplicate PCRs with RNA pooled from three cultures are shown in the histograms below.

−35 region (1, 26), possibly related to the presence of multiple sigma factors. Additionally, the principal sigma factors (MysAs) from *M. smegmatis* (42) and *M. tuberculosis* and *M. leprae* (15) are not only very highly conserved among themselves but also nearly identical to the principal sigma factors of *Streptomyces aureofaciens* (HrdB) and *E. coli* (RpoD) in the region (region 2.4) responsible for binding to the −10 region of promoters. However, they differ significantly from RpoD in the region (region 4.2) which binds to the −35 promoter region (1) and are more similar to this region of HrdB, the sigma factor from the genus *Streptomyces*, whose species also tolerate a large variety of sequences in the −35 region of their promoters (52).

Upstream of the start site further from the *recA* gene is a sequence, TGTCACACTTGAA, which conforms to the −35 consensus sequence (TNTCNCcCTTGAA) of *E. coli* σ^{32} heat shock promoters, although the sequence, CGGCTACTG, located eight bases away from the transcription start site and corresponding to the −10 region is not well conserved with the −10 consensus sequence (CCCCATtTa) of these promoters. Again, it may be that mycobacterial RNA polymerase recognizes a different sequence in one of its contact sites. This putative promoter overlaps the Cheo box which we have shown here to be the binding site for LexA. Such an arrangement of overlapping promoter and LexA binding site has been described for several gram-negative bacterial *recA* genes (16, 39, 60).

The probability that the primer extension products represent real transcription start sites is supported by this identification of elements with homologies to known *E. coli* promoters. In addition, the demonstration that LexA binds to the Cheo box located in the vicinity of these putative promoters adds weight to this hypothesis, as binding sites for LexA are frequently found to overlap promoter elements in *E. coli* (48). Experiments to determine whether these motifs do indeed function as promoters and to unambiguously locate the *recA* promoter(s) are in progress.

Thus, it appears that there are most likely two promoters for the *M. tuberculosis recA* gene and that only one of these is inducible. Curiously, it was the primer extension product from the putative promoter nearest to the coding region which appeared to increase in intensity on induction and was responsible for the majority of the transcripts in *M. tuberculosis*, not the putative promoter further from the gene which was overlapped by the LexA binding site and might thus be expected to be interfered with by LexA binding. It has been proposed that in *E. coli*, there is a main inducible promoter for *recA* and a weak promoter which is not regulated by the SOS response (57). Such an arrangement of dual promoters may allow differential regulation in response to different environmental signals or alternatively may simply ensure that a basal level of expression is maintained. Alternatively, both promoters may be regulated by LexA, as has been found for the nucleotide excision repair gene, *uvrB*, of *E. coli* (55). This gene has a similar arrangement of two promoters, with the one more distal from the coding sequence overlapping a LexA binding site and the more proximal one being responsible for the main part of transcription. Despite this, both promoters are induced by UV in vivo and mutation of the LexA binding site affects expression from the promoter proximal to the *uvrB* gene.

If this interpretation of the promoter location(s) is correct, it means that there must be a different function for the 41-bp region located approximately 300 bp upstream of the *recA* gene that the deletion experiments revealed to be essential for *recA* gene expression. One interesting possibility is that part of this sequence, maybe extending into the adjacent DNA, forms an upstream activating sequence to which an as-yet-unknown activator protein must bind to permit transcription. The combination of both positive and negative LexA-like regulatory factors in the control of expression of DNA damage-inducible genes has been described for the pectin lyase gene of *Erwinia carotovora* (33, 36) and pyocins of *Pseudomonas aeruginosa* (35). We are investigating this scenario and attempting to identify any proteins that interact with this region of DNA.

This work has also shown that the *M. tuberculosis* LexA protein recognizes the same motif as does the *B. subtilis* LexA protein and that the conserved bases are required for this interaction, as substitutions for all eight conserved bases eliminated LexA binding. Whether all the bases within this sequence are equally important for recognition by *M. tuberculosis* LexA remains to be determined, although it is known that LexA binds to a motif with one mismatch from the consensus sequence (38). However, binding to the related sequence with two mismatches from the consensus sequence found in the *recA* upstream DNA did not occur. No further Cheo box-type motifs were found in an additional 360-bp sequence of upstream DNA (data not shown). The binding of LexA to the Cheo box would be predicted to cause repression of *recA* transcription by analogy with other systems, and experiments to confirm this are in progress.

This analysis of the requirements for induction of the *M. tuberculosis recA* gene has been undertaken with transcriptional fusions in *M. smegmatis*. Although these fusions were on replicating plasmids, the copy number of the replicon used is very low (two to five copies per cell) (43, 50, 51) in mycobacteria. In addition, analysis of RNA isolated from *M. tuberculosis* indicated that induction of *recA* does occur in the native organism, although the time required for this induction was much longer than that in *M. smegmatis*. A detailed analysis of the kinetics of *recA* induction in *M. tuberculosis* and *M. smegmatis* is being undertaken with a view to understanding the very slow induction in *M. tuberculosis*; the results of preliminary experiments suggest that this is due to slow cleavage of LexA. This study has revealed that the DNA upstream of the *M. tuberculosis recA* gene contains the elements required for regulated expression, that LexA binds to a specific sequence in this region, and that there appears to be a second level of control, possibly an upstream activating sequence, considerably further upstream from the promoter(s). Further studies to investigate the role of the SOS response in intracellular survival of pathogenic mycobacteria are in progress.

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