Elucidation of Regulatory Elements That Control Damage Induction and Competence Induction of the *Bacillus subtilis* SOS System

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A novel consensus sequence (GAAC-N4-GTTC) has been identified within the promoter regions of DNA damage-inducible (*din*) genes from *Bacillus subtilis*. This sequence has been proposed to function as an operator site that is required for regulation of the SOS system of *B. subtilis*. To test this hypothesis, a deletion analysis of the *dinA* and *recA* promoter regions was utilized. A single consensus sequence is sufficient and necessary for damage-inducible regulation of the *dinA* and *recA* promoters. Deletion of the consensus sequences upstream of these promoters derepressed their expression under uninduced conditions. In addition, this deletion analysis has further defined sequences upstream of the *recA* promoter that are required for expression of the *recA* gene in cells that have differentiated to the state of natural competence. Northern (RNA) hybridization and S1 nuclease protection experiments have demonstrated that the damage-inducible and competence-inducible *recA*-specific transcripts initiate from a single promoter. Mutations within the *comA*, *srfA*, and *degU* loci each completely abolish the competence-inducible expression of the *recA* gene.

The inducible response to DNA damage (the SOS response) has been extensively studied in *Escherichia coli* and other closely related gram-negative bacteria (13, 28). These organisms share a high degree of similarity in the regulation and function of their SOS systems (12, 26, 30, 31). In *E. coli*, the presence of DNA damage generates a signal which activates the RecA protein, which then facilitates the inactivation of the LexA repressor. These events result in the coordinate derepression of a global regulatory network of loci that are responsible for expression of the phenomena that are associated with the SOS response. Examination of the SOS systems of other organisms has led to the realization that different organisms have adapted their SOS systems to suit their own individual requirements. A striking example of this adaptation can be found in the gram-positive bacterium *Bacillus subtilis*. Characterization of the SOS system of *B. subtilis* began following the initial observation that certain prophages were induced in cells that had differentiated to the state of natural competence (35). From this observation it was hypothesized that the SOS system of *B. subtilis* was induced, not only as a consequence of the presence of DNA damage, but also spontaneously within cells that had differentiated to the state of natural competence. This hypothesis was subsequently confirmed following an extensive characterization of the SOS system of *B. subtilis* (14, 15, 32, 34).

Natural competence in *B. subtilis* is a physiological state that enables the cell to bind and internalize exogenous DNA (6). The development of natural competence is influenced by many regulatory genes, which include *spoOA*, *comP*, *comA*, *srfA*, and *degU* (2, 19, 20, 29). These genes as well as other regulatory genes are required for the proper expression of the late-competence genes, which are subject to nutritional, growth stage-specific, and cell type-specific mechanisms of gene regulation (6). Induction of the SOS system in competent cells occurs following the initial induction of the *recA*

gene by a RecA-independent mechanism (15, 34). Thus, the competence-specific mechanism of *recA* induction occurs by a mechanism completely different from the RecA-dependent mechanism that induces expression of *recA* and other damage-inducible genes as a consequence of DNA damage. An increased level of single-stranded chromosomal DNA within competent cells (10) and/or the presence of single-stranded transforming DNA is then believed to activate the RecA protein, which subsequently induces the SOS system.

To study the regulation of the SOS response in *B. subtilis*, Tn917-*lacZ* transposon insertions (operon fusions to the *lacZ* reporter gene) were isolated within three different DNA damage-inducible transcription units (*dinA*, *dinB*, and *dinC*) (9, 14). DNA damage-inducible promoter regions from these three loci were subsequently cloned, and their expression was characterized (3). Characterization of the *B. subtilis* SOS system also led to the cloning (17) and sequencing (27) of the *recA* gene (formerly called the *recE* gene). Within the promoter regions of DNA damage-inducible genes, a novel consensus sequence element (GAAC-N4-GTTC) was identified. This consensus sequence (Cheo Box [4]) has been proposed to function as an operator site that controls DNA damage-inducible regulation in *B. subtilis*. To test this hypothesis, 5' deletions were constructed in the *recA* promoter region (4). Deletion of the consensus sequences upstream of these promoters derepressed expression and abolished damage-inducible regulation. These studies have also begun to identify sequences required for the competence-specific induction of the *recA* gene. This paper describes additional molecular and genetic characterization of the regulatory elements that control damage-inducible and competence-inducible expression of the SOS system of *B. subtilis*.

MATERIALS AND METHODS

Bacteria and plasmids. *B. subtilis* strains used in this study are isogenic derivatives of the prophage-cured strain YB886

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TABLE 1. *B. subtilis* strains used in this study

Strain	Relevant genotype	Source or reference
YB886	<i>metB5 trpC2 xin-1 SP^β- amyE sigB</i>	33
YBA886	<i>amy⁺</i> transformant of YB886 <i>sigB</i>	B. Freidman
YB5876	YBA886 <i>amyE</i> ::pDCA120	3
YB5976	YBA886 <i>amyE</i> ::pDCAΔ89	This work
YB6076	YBA886 <i>amyE</i> ::pDCAΔ72	This work
YB3001	YBA886 <i>amyE</i> ::pDCRHindIII	4
YB3003	YBA886 <i>amyE</i> ::pDCRSspI	4
YB3007	YBA886 <i>amyE</i> ::pDCRΔ85	4
YB3009	YBA886 <i>amyE</i> ::pDCRΔ-35	4
YB3011	YBA886 <i>amyE</i> ::pDCRDdeI	This work
YB3013	YBA886 <i>amyE</i> ::pDCRHinfI	This work
YB3020	YB886 <i>comA</i> ::ery ^r	D. Dubnau
YB3021	YBA886 <i>amyE</i> ::pDCRHindIII <i>comA</i> ::ery ^r	This work
YB3030	YB886 <i>srfA</i> ::phleo ^r	M. Nakano
YB3031	YBA886 <i>amyE</i> ::pDCRHindIII <i>srfA</i> ::phl ^r	This work
YB3040	YB886 <i>degSU</i> ::kan ^r	T. Msadek
YB3041	YBA886 <i>amyE</i> ::pDCRHindIII <i>degSU</i> ::kan ^r	This work

(33) and are described in Table 1. Plasmids used in this study are described in Table 2. *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) and GB180 (a derivative of DH5α that maintains low copy numbers of pUC plasmids; kindly provided by G. Barcek) were used for cloning procedures as described below.

Media and growth conditions. *B. subtilis* strains were maintained on tryptose-blood-agar base (TBAB) medium, and liquid cultures were grown in antibiotic medium 3 (Difco Laboratories, Detroit, Mich.) or nutrient broth (Oxoid Ltd., Basingstoke, United Kingdom) with aeration at 37°C unless otherwise stated. When appropriate, selective media contained 0.5 μg of phleomycin per ml (Cayla, Toulouse, France), 2.5 to 20 μg of chloramphenicol per ml, 5 μg of kanamycin per ml, 1 μg of erythromycin per ml, 25 μg of lincomycin per ml, 50 to 500 ng of mitomycin per ml, and 20 μg of 4-methylumbelliferyl-β-D-galactoside per ml (Sigma Chemical Co., St. Louis, Mo.). *E. coli* strains were grown on

Luria-Bertani agar or in Luria-Bertani broth. Ampicillin (50 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (25 to 50 μg/ml) (Bethesda Research Laboratories) were added as required.

DNA manipulations. Isolation of chromosomal DNA and transformation of *B. subtilis* were performed as previously described (35). Plasmid DNA was isolated by the alkaline lysis method (16), and DNA was sequenced by the dideoxy chain termination method (25). *E. coli* was made competent and transformed by the simple and efficient method (11). Restriction endonucleases and T4 DNA ligase (Promega, Madison, Wis.) were used according to the manufacturer's instructions.

***dinA* and *recA* promoter constructs.** Operon fusions were constructed with various DNA fragments that place the *lacZ* reporter gene of plasmid pAF1 (7) under the transcriptional control of the *dinA* and *recA* promoters of *B. subtilis*. Construction of plasmids pUCA120 (containing a 120-bp *dinA* promoter fragment) as well as pUCRHindIII (containing a 202-bp *recA* promoter fragment, formerly designated pUCR202) was described previously (3, 4). Sequences upstream of the *dinA* promoter were deleted by digesting pUCA120 with *EcoRI* and treating the linearized plasmid with *Bal* 31 nuclease (Bethesda Research Laboratories). Deleted plasmid DNA was treated with the Klenow fragment of *E. coli* DNA polymerase I in the presence of deoxynucleoside triphosphates to fill in single-stranded ends. The DNA was then digested with *HindIII*, and fragments with lengths of between 100 and 140 bp were isolated and ligated between the *HincII* and *HindIII* sites of pUC18. The ligation mixtures were then used to transform *E. coli* DH5α. The precise position of the 5' endpoints of two *dinA* deletion constructs designated pUCAΔ89 and pUCAΔ72 were determined by DNA sequencing and are indicated in Fig. 1 (nucleotides 34 and 41, respectively).

The construction of deletions of the *recA* promoter (pUCRΔ85 and pUCRΔ-35) has been previously described (4). The 5' deletion endpoint of an additional deletion construct (pUCRHinfIΔG) was determined by sequencing and is identical to that of pUCRHinfI (described below). Sequenc-

TABLE 2. Plasmids used in this study

Plasmid	Description	Reference
pUCA120	pUC18 with 120-bp <i>PvuII</i> - <i>Sau3AI</i> <i>dinA</i> promoter fragment	3
pUCAΔ89	pUC18 with 89-bp <i>dinA</i> deletion fragment	This work
pUCAΔ72	pUC18 with 72-bp <i>dinA</i> deletion fragment	This work
pUCR694	pUC18 with 694-bp <i>SspI</i> - <i>HindIII</i> <i>recA</i> promoter fragment from pMS1	This work
pUCRHindIII	pUC18 with 202-bp <i>HindIII</i> - <i>Sau3AI</i> <i>recA</i> promoter fragment	4
pUCRSspI	pUC18 with 183-bp <i>SspI</i> - <i>Sau3AI</i> <i>recA</i> promoter fragment	4
pUCRDdeI	pUC18 with <i>DdeI</i> - <i>Sau3AI</i> <i>recA</i> promoter fragment	This work
pUCRHinfI	pUC18 with <i>HinfI</i> - <i>Sau3AI</i> <i>recA</i> promoter fragment	This work
pUCRHinfIΔG	pUC18 with <i>HinfI</i> - <i>Sau3AI</i> <i>recA</i> promoter fragment containing a mutation within the -50 site	This work
pUCRΔ85	pUC18 with 85-bp deletion <i>recA</i> deletion fragment	4
pUCRΔ-35	pUC18 with the -35 <i>recA</i> deletion fragment	4
pAF1	<i>lacZ</i> reporter gene, <i>amyE</i> integration vector	3
pDCA120	pAF1 with <i>EcoRI</i> - <i>HindIII</i> <i>dinA</i> promoter fragment from pUCA120	3
pDCAΔ89	pAF1 with <i>EcoRI</i> - <i>HindIII</i> <i>dinA</i> promoter fragment from pUCAΔ89	This work
pDCAΔ72	pAF1 with <i>EcoRI</i> - <i>HindIII</i> <i>dinA</i> promoter fragment from pUCAΔ72	This work
pDCRHindIII	pAF1 with <i>EcoRI</i> - <i>HindIII</i> <i>recA</i> promoter fragment from pUCRHindIII	4
pDCRSspI	pAF1 with <i>EcoRI</i> - <i>HindIII</i> <i>recA</i> promoter fragment from pUCRSspI	4
pDCRDdeI	pAF1 with <i>EcoRI</i> - <i>HindIII</i> <i>recA</i> promoter fragment from pUCRDdeI	This work
pDCRHinfI	pAF1 with <i>EcoRI</i> - <i>HindIII</i> <i>recA</i> promoter fragment from pUCRHinfI	This work
pDCRHinfIΔG	pAF1 with <i>EcoRI</i> - <i>HindIII</i> <i>recA</i> promoter fragment from pUCRHinfIΔG	This work
pDCRΔ85	pAF1 with <i>EcoRI</i> - <i>HindIII</i> <i>recA</i> promoter fragment from pUCRΔ85	4
pDCRΔ-35	pAF1 with <i>EcoRI</i> - <i>HindIII</i> <i>recA</i> promoter fragment from pUCRΔ-35	4

FIG. 1. Nucleotide sequence of the *dinA* promoter region (non-transcribed strand). The consensus sequence is indicated by bold-face type. A region of dyad symmetry is marked by arrows. The sigma A-dependent promoter elements, ribosome binding site, and relevant restriction sites are marked and underlined. The precise positions of the 5' endpoints of the *dinA-lacZ* fusion constructs are indicated as the nucleotide base pair directly under the first letter of the construct designation.

ing of pUCRHinfIΔG revealed that this construct contained a single base pair deletion that resulted in the loss of the first nucleotide of the consensus sequence positioned 50 nucleotides upstream of the site of transcription initiation (the -50 site). The position of this deleted base is nucleotide 109 in Fig. 3. An *SspI-HindIII* restriction fragment from pUCRHindIII containing the *recA* promoter was isolated and ligated between the *HincII* and *HindIII* sites of pUC18, generating the plasmid pUCRSspI. Similarly, pUCRHindIII was digested with either *DdeI* or *HinfI*. The digested plasmids were treated with Klenow enzyme in the presence of deoxynucleoside triphosphates to fill in the single-stranded ends and then digested with *HindIII*. The resulting fragments containing the *recA* promoter were isolated and ligated between the *HincII* and *HindIII* sites of pUC18, generating pUCRDdeI and pUCRHinfI, respectively. The precise position of the 5' endpoints of each *recA* deletion construct are indicated in Fig. 3. All pUC18-derived clones containing the *recA* promoter were isolated from *E. coli* GB180 since they were unstable in DH5α.

Promoter fragments from pUCA120, pUCAΔ89, pUCAΔ72, pUCRHindIII, pUCRSspI, pUCRDdeI, pUCRHinfI, and pUCRHinfIΔG were isolated from agarose gels following digestion with *EcoRI* and *HindIII*. The isolated fragments were then ligated between the *EcoRI* and *HindIII* sites of pAF1, resulting in pDCA120, pDCAΔ89, pDCAΔ72, pDCRHindIII, pDCRSspI, pDCRDdeI, pDCRHinfI, pDCRHinfIΔG, pDCRΔ85, and pDCRΔ-35, respectively. These constructs are subsequently referred to as A120, AΔ89, AΔ72, RHindIII, RSpI, RDdeI, RHinfI, RHinfIΔG, R85, and RΔ-35, respectively. Each pDC derivative (recombinant pAF1 plasmids) was linearized with *NruI* and used to transform YBA886 to chloramphenicol resistance as described previously (3). Chloramphenicol-resistant transformants that had lost the ability to hydrolyze starch (because of disruption of the *amyE* gene) were selected for further analysis. The resultant *B. subtilis* strains generated from transformations with the pDC constructs are listed in Table 1.

Competence regulatory mutations. Chromosomal DNA from the *comA*, *srfA*, and *degU* mutants was isolated and used to transform YB886 to erythromycin, phleomycin, and kanamycin resistance, respectively. Chromosomal DNA from a representative transformant of each mutant was isolated and used to transform YB3001 (YBA886 carrying the RHindIII *recA-lacZ* fusion construct) to their respective antibiotic resistances.

DNA damage induction and competence induction. DNA damage-inducible promoter activity from the *dinA-lacZ* and *recA-lacZ* fusion constructs was determined as described previously (3, 4). Similarly, competence-specific promoter activity from the *recA-lacZ* fusion constructs was determined as described previously (4).

mRNA isolation. Total RNA was isolated from uninduced and mitomycin-induced cultures of YB886 as well as from cultures of YB886 and YB1015 grown to natural competence by the method of Qi and Doi (21) as described below. Cells were harvested from 35 ml of culture by centrifugation at $8,000 \times g$ for 10 min at 4°C. The cells were washed in 0.5 ml of SET buffer (20% sucrose, 50 mM EDTA, 50 mM Tris-HCl [pH 7.6]) and recentrifuged. The cell pellets were then suspended in 1.5 ml of SET buffer containing 2 mg of lysozyme per ml. The cell suspension was incubated for 5 min at 37°C and centrifuged in a microfuge for 5 min at 4°C. The cell pellet was suspended in 0.5 ml of hot (60°C) denaturation buffer (4.0 M guanidinium thiocyanate, 50 mM Tris-HCl [pH 7.6], 10 mM EDTA, 2% sodium dodecyl sulfate [SDS], 140 mM β-mercaptoethanol), and 0.5 ml of hot phenol (at 60°C) was added. The samples were vortexed for a few seconds and returned to a 60°C water bath. Then, 0.2 ml (each) of TEN buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA, 10 mM NaCl) and chloroform was added, and the samples were vortexed for 30 s, placed on ice for 30 s, and centrifuged for 3 min at 4°C. The samples were extracted four times with phenol-chloroform until the solution became clear. An equal volume of isopropanol was added, and the nucleic acids were precipitated at -20°C overnight. The nucleic acids were centrifuged for 15 min at 4°C, washed with 70% ethanol, air dried, suspended in 200 μl of TEN buffer containing 0.5 mg of proteinase K per ml, and incubated at 37°C for 30 min. The mixture was extracted twice with phenol-chloroform, and the nucleic acids were precipitated with ethanol at -20°C for 2 h. The pellet was suspended in 100 μl of DNase I solution (40 mM Tris-HCl [pH 8.0], 6 mM MgCl₂, 10 mM NaCl, 10 U of RNase-free DNase I [Promega]), incubated at 37°C for 1 h, and extracted twice with phenol-chloroform. The RNA was precipitated with ethanol, washed, air dried as before, suspended in 250 μl of RNase-free water, and quantitated by determining the optical density at 260 nm.

Northern analysis. RNA samples for Northern (RNA) analysis consisted of 10 μg of total RNA, 2.5 μl of formaldehyde, 7.5 μl of formamide, 1.5 μl of 10× MOPS-EDTA buffer (0.2 M MOPS [3-(*N*-morpholino)propanesulfonic acid], 50 mM sodium acetate, 10 mM EDTA [adjusted to pH 7.0 and autoclaved]) in a total volume of 15 μl (8). The samples were prepared and incubated for 15 min at 65°C. Before electrophoresis, 1 μl of 1% ethidium bromide and 1.5 μl of 10× tracking dye (50% glycerol, 0.25% xylene cyanol, 0.25% bromophenol blue) was added to each sample. A 3-μg aliquot of RNA markers (Bethesda Research Laboratories) was prepared similarly. The RNA samples were electrophoresed within a 1.2% formaldehyde-agarose gel (8). The gel was rinsed in water, and the RNA was transferred to nitrocellulose filters by capillary blotting (16). The filter was baked at 80°C for 3 h and prehybridized in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% SDS, 0.1% bovine serum albumin, 200 μg of sheared and denatured salmon sperm DNA per ml) at 65°C with gentle shaking for 2 h. A 714-bp *HindIII* fragment (containing sequences specific for the *recA* gene) from pMS1 (4) was isolated from an agarose gel and labeled with [³²P]dATP by random

FIG. 2. Diagram of the *dinA-lacZ* fusion constructs and β -galactosidase activity generated in *B. subtilis* from cultures grown to mid-exponential phase and either treated (solid bars) or left untreated (hatched bars) with mitomycin (MC). Black boxes represent the consensus sequences, and open boxes represent the N terminus of the *dinA* structural gene. The positions of the putative sigma A-dependent promoter elements (-10 and -35) and transcription initiation site (arrow) are indicated. Pv, *PvuII*; Sa, *Sau3AI*.

priming (16). The probe was then added directly to the prehybridization reaction mixture, and the incubation was continued at 65°C overnight. The filter was washed in 2× SSC–0.1% SDS for 2 h and then in 0.5× SSC for 1 h at room temperature, with three changes of each wash solution. Hybridizing transcripts were visualized by autoradiography.

S1 nuclease protection. Total RNA from uninduced and mitomycin-induced cultures of YB886 as well as from cultures of YB886 and YB1015 grown to natural competence was isolated (as described above), and an S1 nuclease protection experiment was performed (22). The plasmid pUCR694 (Table 2) was digested with *AccI* and then treated with alkaline phosphatase to remove 5' phosphates. The plasmid DNA was then digested with *SacI*, and a 369-bp *SacI-AccI* DNA fragment (that spanned the *recA* promoter region) was isolated from an agarose gel. The fragment was treated with T4 polynucleotide kinase in the presence of [32 P]dATP to label the 5' end at the *AccI* site, and 10 pmol was mixed with 30 μ g of total RNA. The nucleic acids were precipitated with ethanol, washed, air dried, and suspended in 20 μ l of hybridization buffer (70% formamide, 0.4 M NaCl, 0.04 M PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid; pH 6.4)] with NaOH, 1 mM EDTA). The samples were then sealed within 25- μ l capillary tubes, denatured at 95°C for 5 min, and immediately transferred to a 45°C water bath in which hybridization was performed overnight. The samples were then diluted into 250 μ l of S1 nuclease buffer (50 mM sodium acetate [pH 4.6], 0.28 M NaCl, 4.5 mM ZnSO₄, 20 μ g of denatured salmon sperm DNA per ml) containing 250 U of S1 nuclease (Boehringer Mannheim) and incubated at 37°C for 1 h. Samples were extracted once with phenol-chloroform, ethanol precipitated, washed, air dried, and suspended in 10 μ l of RNA loading buffer (70% formamide, 0.1% bromophenol blue). Samples were then denatured for 5 min at 95°C and fractionated on a 6% sequencing gel. The gel was vacuum dried, and 32 P-labeled products were detected by autoradiography. Fragment sizes were estimated by comparison to a dideoxy sequencing ladder.

RESULTS

Sequences required for DNA damage-inducible and competence-inducible regulation of the *B. subtilis* *dinA* and *recA* genes were examined by constructing a set of 5' deletions

that enter their respective operator-promoter regions. The deletion constructs were coupled to a *lacZ* reporter gene, integrated into the *amyE* locus of *B. subtilis* YBA886 (*recA*⁺), and assayed for β -galactosidase activity.

DNA damage-inducible expression of the *dinA* promoter. The A120 *dinA-lacZ* fusion construct had previously been shown to be capable of generating damage-inducible β -galactosidase activity in *B. subtilis* (3). This result demonstrated that the 120-bp *PvuII-Sau3AI* *dinA* promoter construct contained all the sequences necessary for DNA damage-inducible expression (Fig. 1 and 2). Within these sequences (positioned at -50 relative to the putative site of transcription initiation) is the consensus sequence GAAC-N4-GTTC which has been identified in the promoter regions of other DNA damage-inducible genes of *B. subtilis*. Deletion of the consensus sequence upstream of the *dinA* promoter (AΔ89) derepressed expression approximately two-fold over the expression of A120 from uninduced cells. In addition, the expression of AΔ89 was not significantly different in mitomycin-induced uninduced cells (Fig. 2). When sequences containing the putative sigma A-dependent -35 promoter element were deleted (AΔ72), expression was similar to the expression of A120 from uninduced cells and demonstrated no significant difference in expression in mitomycin-induced or uninduced cells (Fig. 2).

Damage-inducible expression of the *recA* promoter. In the *recA* promoter region, three sequences similar to the consensus sequence were found at -110 , -50 , and $+80$ relative to the site of transcription initiation (3). A *recA-lacZ* fusion construct that removed the $+80$ site leaving the -110 and -50 sites intact (RHindIII) preserved damage-inducible regulation (4). However, a deletion construct that removed the -110 and -50 sites upstream of the *recA* promoter derepressed expression under noninduced conditions and abolished damage-inducible regulation (4). To determine whether both the -110 and -50 sites are required for damage-inducible regulation, the expression of additional *recA-lacZ* fusion constructs was examined. The RHindIII, RSspI, RDdeI, and RHinfI *recA-lacZ* fusion constructs all generated damage-inducible β -galactosidase activity, whereas the RΔ85 fusion construct derepressed expression and abolished damage-inducible regulation (Fig. 3 and 4). These results have demonstrated that deletion of the -110 site (RHinfI) has no effect on damage-inducible regulation of the *recA*

FIG. 3. Nucleotide sequence of the *recA* promoter region (non-transcribed strand) including the upstream regulatory regions and the N terminus of the structural gene. Putative regulatory elements are indicated by boldface type. Regions of dyad symmetry are marked by arrows. Potential sigma A-dependent promoter elements (-10 and -35), the ribosome binding site (RBS), and relevant restriction sites are marked and underlined. The precise positions of the 5' endpoints of the *recA-lacZ* fusion constructs are indicated as the nucleotide base pair directly under the first letter of the construct designation.

promoter. Regulation was not affected until sequences at the -50 site were deleted (RA85). Fusion constructs that were identical except for the presence (RHinfI) or absence (RHinfI Δ G) of the first nucleotide base pair in the -50 site were also examined for damage-inducible expression in *B.*

subtilis. The RHinfI construct demonstrated normal damage-inducible regulation, whereas both repression and damage-inducible regulation were abolished from the RHinfI Δ G construct (Fig. 4). Finally, the RA-35 *recA-lacZ* fusion (a deletion of the putative -35 element of the *recA* promoter) abolished almost all promoter activity (Fig. 4).

mRNA analysis. The accumulation of transcripts generated from the *recA* gene was detected on a Northern blot by using a *recA*-specific hybridization probe (Fig. 5). A *recA*-specific transcript with a length of 1.2 kb was detected from RNA isolated from mitomycin-induced YB886 (*recA*⁺). This transcript was detectable by using an equivalent amount of RNA isolated from uninduced cells. The *recA*-specific transcript was also detectable from total RNA isolated from mitomycin-induced and uninduced YB1015 (*recA*⁺). However, a very large accumulation of a 1.2-kb *recA*-specific transcript was detected from RNA isolated from competence grown cultures of both YB886 and YB1015 (Fig. 5).

Transcription initiation of the *recA* promoter. S1 nuclease protection was used to establish whether damage-inducible transcripts and competence-inducible transcripts from the *recA* gene initiate at the same or different sites (Fig. 6). Total RNA from uninduced and mitomycin-induced cultures of YB886 as well as RNA from competence grown cultures of YB886 and YB1015 were hybridized to a 5'-end-labeled probe that spanned the *recA* promoter region (*SacI*-*AccI* fragment from pUCR694 5'-end-labeled at the *AccI* site within the *recA* structural gene). The hybridized nucleic acids were then treated with S1 nuclease as described in Materials and Methods. A fragment with a length of 217 to 218 bp (estimated by comparison to a dideoxy sequencing ladder) was protected from digestion with S1 nuclease fol-

FIG. 4. Diagram of the RHindIII, RSspI, RDdeI, RHinfI, RHinfI Δ G, RA85, and RA-35 *recA-lacZ* fusion constructs and β -galactosidase activity generated in *B. subtilis* following 1 h of growth in the presence (solid bars) or absence (hatched bars) of mitomycin (MC). Black boxes represent consensus sequences, and open boxes represent the *recA* structural gene. The positions of the putative sigma A-dependent promoter elements (-10 and -35) and transcription initiation site (arrow) are indicated. H, HindIII; Ss, SspI; Dd, DdeI; Hf, HinfI and Sa, Sau3AI.

FIG. 5. Total mRNA was isolated from cultures of YB886 (*recA*⁺) and YB1015 (*recA4*) that were either untreated (–) or treated (+) with mitomycin (MC) or grown to the competent state (T₃). The RNA was fractionated on a 1.2% formaldehyde-agarose gel in the presence of ethidium bromide (left panel). The gel was transferred to a nitrocellulose filter that was hybridized to a *recA*-specific ³²P-labeled probe and used to expose X-ray film (right panel).

lowing hybridization with each of these RNA samples (Fig. 6). Both the mitomycin-inducible transcripts and the competence-inducible transcripts initiated at precisely the same site (nucleotides 162 and 163, Fig. 3). These results map the site of transcription initiation of the *recA* gene in competent cells and in damage-induced cells to a promoter that closely resembles a sigma A-dependent promoter (Fig. 6).

Competence-inducible expression of the *recA* promoter. Sequences required for induction of *recA* expression within

competent cells have been identified within a 20-bp region between the *Hind*III and *Ssp*I sites upstream of the *recA* promoter (4). However, deletion of these sequences did not completely abolish all of the competence-specific induction of the *recA* promoter. To further define sequences involved in the induction of *recA* during competence, competence-specific expression of the *RHind*III and *RSsp*I fusion constructs was compared with the competence-specific expression of the *RDde*I and *RHinf*I fusion constructs (Fig. 7). Deletion of sequences upstream of *Dde*I (*RDde*I) reduced expression approximately twofold from the level of expression of *RSsp*I. Similarly, deletion of sequences upstream of *Hinf*I (*RHinf*I) reduced expression twofold from the level of expression of *RDde*I. Thus, as additional sequences upstream of the *recA* promoter were deleted, competence-specific expression was further reduced.

Competence regulatory loci. The competence-specific induction of the *recA* gene was previously demonstrated to be dependent upon *spoOA* and *spoOH* (regulators of stationary-phase-specific gene expression such as the development of natural competence and the initiation of sporulation). To establish whether the competence-specific expression of the *recA* gene is regulated by the same genes that are required for the development of natural competence, mutations within competence-regulatory loci (*comA*, *srfA*, and *degU*) were tested for their effect on the competence-specific induction of the *recA* promoter. Mutations within the *comA*, *srfA*, and *degU* loci were introduced into *B. subtilis* carrying the *RHind*III *recA-lacZ* fusion, and expression of β -galactosidase activity was assayed throughout growth to natural competence. All three competence-regulatory mutations completely abolished the competence-specific expression of the *recA* promoter (Fig. 8). However, these same mutations

FIG. 6. Nucleotide sequence of the *recA* promoter region (nontranscribed strand). The promoter elements (–10 and –35) and ribosome binding site (RBS) are marked and underlined; the consensus sequence is indicated by boldface type. Also indicated is the position of transcription initiation (asterisks above the sequence). The mRNA transcript is depicted as a wavy line. Below the sequence is a diagram of the 369-bp *Sac*I–*Acc*I DNA fragment from pUCR694 (Table 2) that was 5'-end-labeled with [³²P]dATP at the *Acc*I site and used as a probe in an S1 nuclease protection experiment. The DNA fragment protected from S1 nuclease digestion is indicated as a shaded box. Total RNA was isolated from uninduced (–), mitomycin-induced (+), or competent (T₃) cell cultures of YB886 (*recA*⁺) as well as from competent cell cultures (T₃) of YB1015 (*recA4*). The RNA was hybridized to the probe, treated with S1 nuclease, and fractionated within a 6% sequencing gel along with a sample of untreated probe (P) and a DNA sequencing ladder (not shown). The sizes (in nucleotide base pairs) of the labeled DNA fragments are indicated and marked with arrows along the autoradiogram.

FIG. 7. Diagram of the RHindIII, RSspI, RDdeI and RHinfI *recA-lacZ* fusion constructs and β -galactosidase activity generated in cultures of *B. subtilis* grown to natural competence. Aliquots of each culture were taken 3 h past exponential phase and assayed for β -galactosidase activity. The black boxes represent the -50 operator site, and the open boxes represent the *recA* structural gene. The positions of the putative sigma A-dependent promoter elements (-10 and -35) and transcription initiation site (arrows) are indicated. The hatched boxes and shaded boxes represent possible regulatory elements involved in competence induction of the *recA* promoter. H, *Hind*III; Ss, *Ssp*I; Dd, *Dde*I; Hf, *Hinf*I; Sa, *Sau*3A1.

had no effect on the damage-inducible expression of the RHindIII fusion construct (data not shown).

DISCUSSION

Identification of the consensus sequence GAAC-N4-GTTC within the promoter regions of DNA damage-inducible genes of *B. subtilis* has led to the hypothesis that this sequence functions as an operator site that is required for damage-inducible regulation (3). A single consensus sequence was identified upstream of the *dinA* promoter positioned at 50 bp upstream from the putative site of transcription initiation (3). A deletion that removed sequences at the

-50 site (A Δ 89) derepressed expression approximately two-fold and abolished damage-inducible regulation of the *dinA* promoter (Fig. 2). These results demonstrate that these sequences which contain the consensus sequence are required for repression of the *dinA* promoter. Interestingly, the level of expression of A Δ 89 was only partially derepressed over the uninduced level of expression of A120. An explanation for this result is that the deletion of sequences between the 5' endpoints of these two *dinA* promoter constructs has eliminated a positive component of the regulation. Alternatively, these sequences may be required for efficient RNA polymerase-promoter interactions.

Sequences similar to the consensus sequence were previously identified at -110 , -50 , and $+80$ relative to the site of transcription initiation of the *recA* gene (3). The RHindIII fusion construct, which removed the $+80$ site, still demonstrated damage-inducible regulation. Similarly, a deletion construct that removed the -110 site upstream of the *recA* promoter (RHinfI) also demonstrated damage-inducible regulation. These data have demonstrated that the $+80$ and -110 sites are not required for damage-inducible regulation of the *recA* promoter (Fig. 3 and 4). However, deletion of sequences at the -50 site (R Δ 85) derepressed expression and abolished damage-inducible regulation. These results demonstrate that, as was the case for *dinA*, sequences which contain a single consensus sequence (the -50 site) are required for repression and damage-inducible regulation of the *recA* promoter.

Previous analysis of the *recA* promoter region suggested that the -110 and -50 consensus sequences might interact to repress transcription of the *recA* gene (4, 32). This conclusion was based on the constitutive phenotype of a deletion construct (R Δ 109) that removed the -110 site, leaving the -50 site upstream of the *recA* promoter. However, the R Δ 109 promoter construct was subsequently shown to be missing the first G-C base pair in the consensus sequence at the -50 site. In this study, we have demonstrated that the presence of this mutation generated a constitutive phenotype (RHinfI Δ G) while deletion of the -110

FIG. 8. β -Galactosidase activity from competence-grown cultures of *B. subtilis* strains carrying the RHindIII *recA-lacZ* fusion construct in the presence of the *comA*, *srfA*, and *degU* mutations. Time (in hours) past the exponential phase of growth is indicated.

site did not affect damage-inducible regulation (RHinfI) as long as the -50 site was intact (Fig. 4). Interestingly, the level of expression from RHinfI Δ G was greater than the level of expression from R Δ 85, suggesting that sequences within or adjacent to the -50 site are also required for some positive regulatory component or are required for optimal promoter-RNA polymerase interactions. This result was very similar to the results obtained with the *dinA* promoter deletions.

The expression of *recA-lacZ* fusion constructs as measured by β -galactosidase activity was correlated to the accumulation of *recA*-specific mRNA transcripts. A single damage-inducible *recA*-specific mRNA transcript with a length of approximately 1.2 kb was detected from cultures of YB886 (*recA*⁺) (Fig. 5). This transcript was not inducible by DNA damage within YB1015 (*recA* Δ). However, a very large accumulation of *recA*-specific mRNA was detected from both YB886 and YB1015 after growth to natural competence. It is important to note that within a culture of competent cells only 10 to 20% of the total population of cells ever become competent (36). Thus, this large accumulation of competence-inducible transcripts was generated from the competent subpopulation of the culture and is representative of approximately 1/5 to 1/10 the actual level of *recA*-specific mRNA accumulation per cell. The size of the *recA*-specific mRNA transcript corresponded very well with the size predicted from the nucleotide sequence of the *recA* gene (27). Both damage-inducible and competence-inducible transcripts are initiated from the same position within the *recA* promoter region (Fig. 6). Therefore, the *recA* gene is transcribed from a single promoter that is induced by two different regulatory mechanisms.

Sequences involved in the competence-specific induction of the *recA* promoter have previously been localized to a region within or adjacent to the *Hind*III and *Ssp*I restriction sites (4). However, deletion of these 20 bp did not completely abolish competence expression of the *recA* promoter, suggesting that additional sequences downstream of the *Ssp*I site are involved in this regulation. In this study, competence expression of two additional deletion constructs (RDdeI and RHinfI) was examined. As additional sequences were deleted, the competence-specific expression of the *recA* promoter was further reduced (Fig. 7). Within these upstream regulatory regions are sequences (centered at -126 relative to transcription initiation) that share similarity to regulatory regions of the late-competence genes, *comC* and *comG*, that have been proposed to be required for the activity of a competence-specific transcription activator (1, 4, 18). Interestingly, also within this competence-regulatory region (positioned at -85 relative to transcription initiation) is a sequence that is similar to the consensus sequence for integration host factor binding in *E. coli* (5). Deletion of this site has no effect on DNA damage-induction of the *recA* promoter but does have a subtle effect on competence induction (Fig. 7). To induce *recA* expression in competent cells, repression must be relieved initially by a mechanism that is independent of the function of the RecA protein. This may occur by displacement of the repressor from the -50 site by the binding of an integration-host-factor-like protein at the putative -85 operator site. Assuming that conservation in the activity of this integration-host-factor-like protein exists between *E. coli* and *B. subtilis*, its binding upstream of the *recA* gene would sharply bend the DNA between the promoter and the competence-regulatory elements. This bent DNA complex may then facilitate the interaction of a competence-specific transcription factor with the *recA* pro-

motor. This regulatory loop may form a stable activation complex that is able to initiate transcription of the *recA* gene while at the same time occluding the repressor from its binding site. The level of β -galactosidase activity generated from RHindIII is approximately the same in cells induced by DNA damage and in competence-grown cultures (compare Fig. 4 and 7). However, from the results of mRNA analysis (Fig. 5 and 6), *recA*-specific mRNA transcripts accumulate at much higher levels in competent cells than in cells treated with mitomycin. One explanation for this discrepancy concerns the stability of β -galactosidase at different developmental stages in *B. subtilis*.

The development of natural competence in *B. subtilis* is controlled by a complex cascade of regulatory events (6). This process is dependent upon many different regulatory loci which include *spoOA*, *comA*, *degU*, and *srfA*. In response to nutritional, developmental, and cell-type-specific signals, these regulatory proteins initiate the expression of a set of genes that ultimately are responsible for the development of natural competence. Previously, the competence-inducible expression of *recA* was demonstrated to be dependent on the *spoOA* and *spoOH* genes. In this study, we have shown that mutations within the *comA*, *srfA*, and *degU* loci abolish the competence-specific expression of the *recA* promoter (Fig. 8). Therefore, the regulatory loci required for the competence-specific induction of the *recA* gene share extensive overlap with the regulatory network that controls the development of natural competence in *B. subtilis*.

Interestingly, the product of the *dinR* gene has recently been shown to influence the regulation of *din* genes in *B. subtilis* (23, 24). The DinR protein shares significant homology to the LexA protein of *E. coli*, particularly within the regions of LexA that are involved in RecA-mediated autocatalytic cleavage. DinR therefore is a candidate for a repressor of the SOS regulon. However, it is important to note that a mutation in *dinR* (*dinR1*) reduces the basal level of expression of *din* genes and renders them uninducible. Analogous mutations in the *E. coli* *lexA* gene (super-repressor mutations) are dominant to the wild-type *lexA*⁺ gene. In *B. subtilis*, the *dinR1* mutation is recessive to the wild-type *dinR*⁺ gene. Further studies of SOS regulation in *B. subtilis* will ultimately elucidate these regulatory mechanisms and advance our understanding of how the SOS system interacts with processes of prokaryotic differentiation and development.

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