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Transcription of the *cam* Operon and *camR* Genes in *Pseudomonas putida* PpG1

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In *Pseudomonas putida* carrying the CAM plasmid, the operon (*camDCAB*) encoding enzymes involved in the degradation pathway of D-camphor is negatively regulated by the CamR protein, and *camR* is autorepressed. S1 nuclease mapping revealed that *camDCAB* and *camR* were divergently transcribed from overlapping promoters, the transcription start sites were separated by 11 bp, and transcriptions of the *cam* operon (*camDCAB*) and *camR* increased about 10- and 4-fold, respectively, immediately after addition of camphor. The transcriptions of *camDCAB* and *camR* were negatively regulated through the interaction of the CamR protein with the one operator located in the overlapping promoter region. In vitro transcription experiments were performed to characterize the regulation of *cam* genes. The *camR* promoter was initiated by *P. putida* RNA polymerase containing σ^{70}, but transcription from the *camDCAB* promoter by σ^{70} holoenzyme was not observed. The purified CamR protein repressed in vitro transcription from the *camR* promoter. This repression was suppressed by camphor. The RNA polymerase binding region of the *camR* promoter was identified by using DNase I footprinting. In addition, footprinting studies revealed that the CamR protein and RNA polymerase coexisted on the promoter region in a joint nonproductive complex.

Pseudomonas putida PpG1 (ATCC 17453) was originally isolated by enrichment culture with D-camphor as the carbon source (5) and shown to carry a plasmid termed CAM which encodes enzymes involved in the pathway for catabolism of D- or L-camphor (20). The reaction pathway for catabolism of camphor has been established (5, 6, 9). Camphor is first converted to 5-exo-hydroxy camphor by a monooxygenase system with coupled redox components. This system for 5-exo-hydroxylation of camphor is composed of three enzymes: NADH-putidaredoxin reductase, a flavine adenine dinucleotide protein of 45 kDa encoded by gene *camA*, putidaredoxin, an iron-sulfide redox protein of 12 kDa encoded by gene *camB*, and cytochrome P-450$_{cam}$, a terminal hydroxylase component of 47 kDa encoded by gene *camC* (10, 11). The second step is the dehydrogenation of 5-exo-hydroxy camphor to form 2,5-diketo camphane by F-dehydrogenase encoded by gene *camD* (9). Genes *camA* to *camD* have been cloned (12, 13) and shown to constitute the *cam* operon (*camDCAB*) (13). Their expression is regulated negatively by the regulatory gene, *camR* (12, 13), which is located immediately upstream of the *camD* gene and transcribed divergently from the *cam* operon (13). Recently, *camR* was sequenced and its gene product was shown to be a repressor (3). The expression of *camDCAB* is negatively regulated through the interaction of CamR with the operator, located between *camR* and *camDCAB*, and a marked induction of *camDCAB* is mediated through inactivation of CamR by the inducer camphor.

In this study, we determined the transcription initiation sites of *camR* and *camDCAB* and describe the transcriptional regulations of the genes by camphor.

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MATERIALS AND METHODS

Bacterial strains and media. *P. putida* PpG1 was grown in phosphate-ammonium salts medium (22) supplemented with 20 mM monosodium glutamate (PASG). *Escherichia coli* JM83 [F′ *ara*(*lac-proAB*) *rpsL*(ø80*lacZ*Δ*M15*)] harboring pHA37-1 was grown in LB medium containing 50 μg of ampicillin per ml. Plasmid pHA37-1 is a recombinant of pUC19, carrying a 314-bp *Sma*I-*Sph*I DNA fragment (Fig. 1).

Preparation of probe DNA. For preparation of probe 1 (Fig. 1), pHA37-1 was digested with *Sma*I and *Sph*I, and the 314-bp fragment was purified by 5% polyacrylamide gel electrophoresis. This fragment was denatured by boiling and labeled with [γ-^{32}P]ATP by using polynucleotide kinase after dephosphorylation with bacterial alkaline phosphatase. For preparation of probe 2 (Fig. 1), pHA37-1 was digested with *Sma*I, and the 5′ ends of the linear plasmid were labeled as described above without denaturation. Next, the end-labeled DNA was digested with *Sph*I, and the 314-bp *Sma*I-*Sph*I fragment was purified. Probe 3 (Fig. 1) was obtained by digesting pHA37-1 with *Xho*I and labeling the 5′ ends of the linear plasmid as described above without denaturation. Then, the end-labeled DNA was digested with *Sph*I, and the 231-bp *Xho*I-*Sph*I fragment obtained was purified.

Preparation of RNA. An overnight culture of *P. putida* PpG1 at 30°C in PASG medium was diluted 100-fold with the same medium and allowed to grow to the mid-log phase (A_{660} = 0.35). The culture was divided into two portions: for induced culture, a solution of 1 M camphor in dimethylformamide was added to one portion to give a final concentration of 2.5 mM; for noninduced culture, the same volume of solvent was added to the other portion. Incubation was continued, samples of 40 ml were removed at intervals, and the cells were harvested. Total RNAs were prepared from the cells by the hot-phenol method (2).

S1 nuclease mapping. S1 nuclease mapping was carried out by the method of Aiba et al. (2). RNA (100 μg) was hybridized

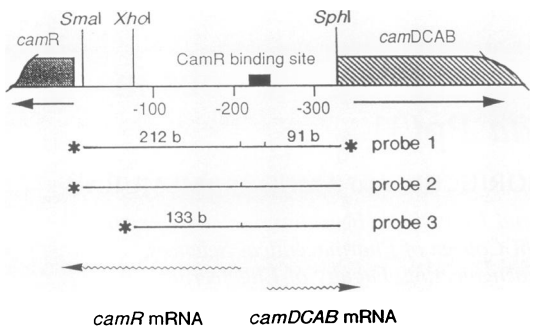

FIG. 1. Locations of probes used for S1 nuclease mapping shown relative to the *cam* genes. The control region of the *cam* genes is shown at the top. Coordinates are in base pairs; +1 refers to the translation initiation site of the *camR* gene. Parts of the genes for *camR* and *camDCAB* and their directions are indicated. The CamR binding site and restriction sites are indicated. The DNA fragments used as probes are shown along with their lengths (in bases [b]) protected by S1 nuclease mapping (labeled at the 5′ end [*]). The wavy arrows indicate the start sites and directions of mRNAs.

with the probe (10,000 cpm) and treated with S1 nuclease. The S1 nuclease-protected DNA fragments were analyzed by 8 M urea–6% polyacrylamide gel electrophoresis.

Purification of RNA polymerase. The purification of RNA polymerase from *P. putida* was based on procedures described previously (7, 8), with following modifications. Polymin P (polyethyleneimine) was added to a final concentration of 0.8% (wt/vol). Further purification was carried out on Bio-Rex 70 ion-exchange chromatography and heparin (Econo-Pac heparin cartridge) chromatography. The properties of purified RNA polymerase as σ^{70} RNA polymerase were determined to be as follows. The molecular mass of σ subunit of purified RNA polymerase was the same as that of *E. coli*. Moreover, purified RNA polymerase was able to initiate transcription from the *tac* promoter in in vitro transcription (data not shown). Therefore, we concluded that the purified RNA polymerase is σ^{70} RNA polymerase.

Purification of CamR. CamR protein was overproduced under the control of p_L promoter in *E. coli* (4). CamR overproducer *E. coli* was suspended in TGED buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.3 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 10% [vol/vol] glycerol) containing 0.1 M KCl and disrupted by sonic oscillation. The extract was centrifuged, and the supernatant was collected. The supernatant was treated with 50% saturation of $(NH_4)_2SO_4$, and the precipitate obtained was dissolved in TGED buffer containing 0.1 M KCl and dialyzed against the same buffer. The dialysate was applied to a DE52 column and eluted with a 0.1 to 0.5 M KCl gradient in TGED buffer. The fractions containing CamR were applied to a Toyopeal HW-55F column. The fractions containing CamR were pooled, dialyzed against storage buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.6 mM dithiothreitol, 50% [vol/vol] glycerol, 0.1 M KCl), and stored at −80°C. We confirmed that the sequence of the NH_2-terminal amino acid residues is identical with that deduced from the nucleotide sequence of the *camR* gene (3).

In vitro transcription. The reaction was done under the single-round reaction condition. In brief, reaction mixtures of 35 μl containing 0.1 pmol of DNA template and 10-fold molar excess of RNA polymerase were incubated for 10 min at 30°C to form open promoter complexes. Transcription was initiated by the addition of 15 μl of a substrate-heparin mixture, and RNA synthesis was allowed to proceed for another 10 min. The reaction was terminated, and RNA products were analyzed by gel electrophoresis as described previously (7, 8). To test repression of *camR* transcription in vitro, CamR was added to the first incubation mixture. Amounts of the CamR protein added to the reaction mixtures are in the legend to Fig. 5.

Preparation of DNA template. To prepare DNA fragments carrying the promoters for *camR* and *camDCAB*, plasmid pHA37-1 was digested with *Eco*RI and *Hin*dIII, and the products were separated by gel electrophoresis. A 337-bp *Eco*RI-*Hin*dIII fragment that carried the promoters for *camR* and *camDCAB* was eluted from the gel and used as a template.

DNase I footprinting. DNase I footprinting was done essentially by the procedure of Aiba (1). A uniquely end-labeled DNA fragment carrying the promoters for *camR* and *camDCAB* was prepared as follows. pHA37-1, containing the promoters for *camR* and *camDCAB*, was cut at the *Hin*dIII site. Then pHA37-1 was 3′ end labeled with [α-^{32}P]dCTP by using the Klenow fragment of DNA polymerase I, and the labeled DNA was recut with *Eco*RI. The products were separated by gel electrophoresis. The labeled DNA was eluted from the gel and used for DNase I footprinting. In Fig. 7, the rightward end of the upper strand corresponds to the labeled 3′ end. A DNA fragment (0.1 pmol) was mixed with RNA polymerase (1 pmol) or CamR (25 pmol) for 15 min at 30°C in 100 μl of reaction mixture (7). RNA polymerase and CamR were added to the reaction mixture as follows. One of these proteins was added first, and the binding reaction was done for 15 min at 30°C. Then another protein was added, and the second binding reaction was done for another 15 min at 30°C. Camphor (5 mM) was added to some of the reaction mixtures as indicated above the lanes in Fig. 6.

Enzymes and chemicals. [γ-^{32}P]ATP (185 TBq/mmol) and [α-^{32}P]dCTP were purchased from ICN. Restriction enzymes were from Takara Shuzo and Toyobo. Alkaline phosphatase and T4 polynucleotide kinase were from Toyobo.

RESULTS

Transcriptional regulations of *camR* and *camDCAB* in the presence or absence of camphor. The *camDCAB* is repressed by CamR, and *camR* is autorepressed (3, 4). We demonstrate here that the expressions of these elements are regulated at the level of transcription. The transcriptions of *camR* and *camDCAB* were monitored by S1 nuclease mapping. We measured the rates of syntheses of *camR* and *camDCAB* mRNAs in PASG medium with or without camphor. To detect *camR* mRNA, we performed S1 nuclease mapping by using probes 2 and 3 (Fig. 1). As shown in Fig. 2, synthesis of *camR* mRNA was induced by camphor. The sizes of S1-resistant DNAs of probes 2 and 3 were about 210 and 130 bases, respectively. To detect *camDCAB* mRNA, we tried to make a probe with a specifically 5′-end-labeled *Sph*I site. However, the incorporation of ^{32}P into the 5′ end of the *Sph*I site was too low to allow use of the probe for S1 nuclease mapping, since the *Sph*I site has a protruding 3′ terminus. Therefore, we made probe 1 as described in Materials and Methods for this purpose. Probe 1 has ^{32}P-labeled 5′ ends of both DNA strands, and the efficiencies of ^{32}P labeling of these two 5′ ends are expected to be the same, since the denatured single-stranded DNAs were used for the labeling reaction. As shown in Fig. 3A, the syntheses of *camR* and *camDCAB* mRNAs were promptly induced by the addition of camphor. The sizes of S1-resistant DNAs of probe 1 were about 210 and 90 bases for *camR* and *camDCAB*, respectively. Some bands were also detectable below the larger ones. We do not know whether these smaller transcripts are

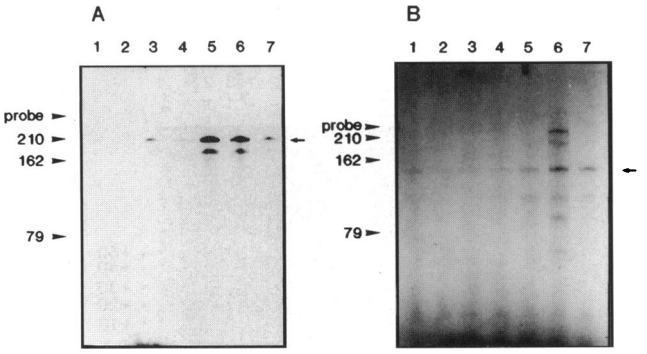

FIG. 2. S1 nuclease mapping of *camR*. The conditions for RNA extraction and S1 nuclease mapping are described in Materials and Methods. (A) Probe 2 was used. RNA was extracted from cultures without camphor (lanes 1 to 4) and with camphor (lanes 5 to 7). Samples are from cultures 0 min (lane 1), 15 min (lanes 2 and 5), 1 h (lanes 3 and 6), and 2 h (lanes 4 and 7) after the addition of camphor or solvent only. (B) Probe 3 was used. Numbers of lanes correspond to those in panel A. The DNA fragments protected are indicated on the right. The positions of probes and DNA size markers (in bases) are indicated.

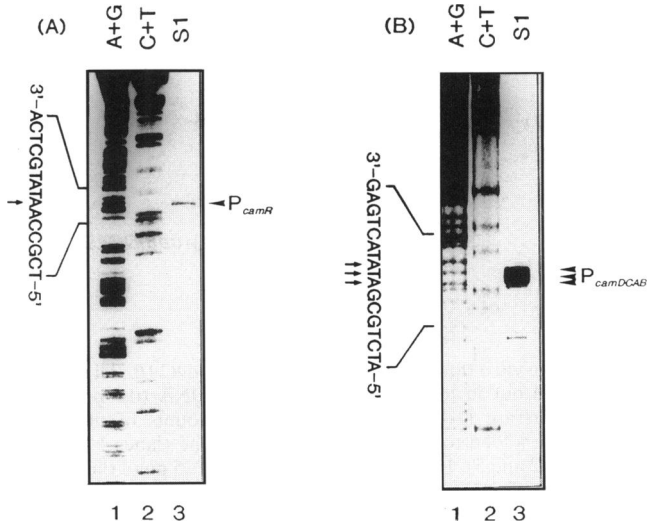

FIG. 4. Determination of transcription initiation sites of *camR* mRNA and *camDCAB* mRNA by S1 nuclease mapping. (A) *camR* mRNA initiation site. Lanes: 1, A+G base-specific chemical cleavage (16); 2, C+T cleavage; 3, DNA fragment protected by *camR* mRNA from S1 nuclease digestion. Probe 2 was used. (B) *camDCAB* mRNA initiation site. Lanes: 1 and 2, as in panel A; 3, DNA fragment protected by *camDCAB* mRNA from S1 nuclease digestion. Probe 1 was used. The 5′ end of *Sph*I site-specific labeled DNA was used for base-specific chemical cleavage (lanes 1 and 2). The S1 nuclease-protected DNA fragments by *camR* and *camDCAB* mRNAs (P_{camR} and $P_{camDCAB}$) are indicated to the right of each panel. The 5′ ends of the transcripts are indicated by arrows to the left of each panel.

processed forms of the larger ones or whether they represent starts from independent promoters. For quantitative comparison, the intensities of the bands of transcripts in the autoradiogram were measured with a densitometer; the results are shown in Fig. 3B. The amount of *camR* mRNA was about 40% of that of *camDCAB* mRNA. The data also show that the derepression levels of *camR* and *camDCAB* transcription in the presence of D-camphor were about 4- and 10-fold, respectively, the levels in the absence of camphor (Fig. 3).

Locations of 5′ ends of *camR* and *camDCAB* mRNAs. The precise locations of the 5′ ends of *camR* and *camDCAB* mRNAs were determined by high-resolution S1 nuclease mapping. As shown in Fig. 4, the 5′ end of *camR* mRNA was located 219 bases upstream from the initiation codon (GTG) of *camR*. The 5′ end of *camDCAB* mRNA was located at three positions, 90, 91, and 92 bases upstream from the initiation codon of *camDCAB*. The DNA sequences preceding the transcription start sites were compared with the consensus promoter sequence (−35, TTGACA; −10, TATAAT [separated by 17 bases]; the start point of transcription is designated +1) recognized by σ^{70} RNA polymerase (19). In the upstream region of the 5′ end of *camR* mRNA, the sequences TTGTTC and TATACT were found as −35 and −10 potential promoter sequences. However, the 22-base spacing between −35 and −10 had poor homology to the σ^{70} consensus sequence (see Fig. 7). In the upstream region of the 5′ end of *camDCAB* mRNA, the sequences TTGACC and TATGCT, separated by 17 bases, were found as −35 and −10 potential promoter sequences. Thus, we concluded that the 5′ ends of mRNAs are the transcription initiation sites.

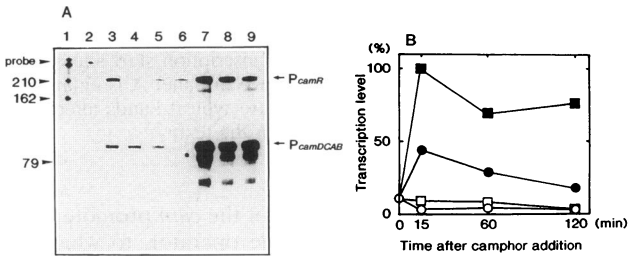

FIG. 3. S1 nuclease mapping of *camDCAB* and *camR*. (A) RNA was extracted from cultures without camphor (lanes 3 to 6) or with camphor (lanes 7 to 9). Samples are from cultures 0 min (lane 3), 15 min (lanes 4 and 7), 1 h (lanes 5 and 8), and 2 h (lanes 6 and 9) after the addition of camphor or solvent only. Lane 1 contained DNA size markers (in bases), and lane 2 contained probe 1 without S1 nuclease treatment. The positions of protected DNAs (P_{camR} and $P_{camDCAB}$) are indicated on the right. (B) Quantitation of relative synthesis rates. The intensities of the bands of transcripts in the autoradiogram were measured with a densitometer. The transcriptional levels were normalized to the maximum level and expressed as percentages. Symbols: ○, *camR* mRNA without camphor; ●, *camR* mRNA with camphor; □, *camDCAB* mRNA without camphor; ■, *camDCAB* mRNA with camphor.

In vitro transcription of *camR* and *camDCAB*. The principal form of RNA polymerase holoenzyme was isolated from exponential-phase *P. putida* PpG1 cells, and in vitro transcription was carried out. A DNA fragment carrying the promoters for *camR* and *camDCAB* was used as a template. If transcription of the truncated template is initiated from the position corresponding to the 5′ ends of *camR* and *camDCAB* mRNAs and terminated at the ends of template, each promoter will give the following transcript: 97-base RNA for *camDCAB* and 226-base RNA for *camR*. Synthesis of transcript analyzed by gel electrophoresis is shown in Fig. 5. One major transcript of about 220 bases in length was found, whereas transcript of 90 bases in length was not observed. These results indicates that in vitro transcription by σ^{70} RNA polymerase from *P. putida* PpG1 is initiated from the *camR* promoter and not from the *camDCAB* promoter. To determine whether the CamR protein actually functions as a repressor for transcription from the *camR* promoter, we added the CamR protein to an in vitro transcription system. Synthesis of a 220-base transcript was

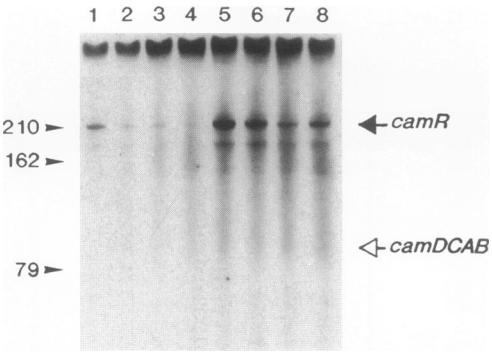

FIG. 5. In vitro transcription of *camR* and *camDCAB*. All reactions were carried out in the presence of template DNA (0.1 pmol) and RNA polymerase (1 pmol). The following amounts of the CamR protein were added to the reaction mixtures: none (lanes 1 and 8), 5 pmol (lanes 2 and 5), 10 pmol (lanes 3 and 6), and 25 pmol (lanes 4 and 7). Camphor (5 mM) was added to the mixtures in lanes 5 to 8. The runoff transcript of *camR* is indicated by the closed arrow; the expected position of the *camDCAB* transcript is indicated by the open arrow. Positions of DNA size markers (in bases) are indicated on the left.

repressed by the CamR protein (Fig. 5, lanes 2 to 4). The addition of camphor suppressed the repression of mRNA synthesis (lanes 5 to 7).

Binding of RNA polymerase and CamR to the *cam* regulatory region. To determine the binding region of RNA polymerase and CamR, we performed a DNase I footprinting experiment using a restriction fragment containing the *cam* promoter and operator region. The region protected by CamR (Fig. 6A, lane 4; Fig. 7) corresponds well to the previous results (4). As expected, RNA polymerase protected a longer region corresponding to the *camR* promoter (Fig. 6A, lane 3; Fig. 7). Therefore, the DNA segment protected by CamR extensively overlaps with the RNA polymerase binding site. To examine whether CamR prevents RNA polymerase binding to the *camR* promoter, a DNase I footprinting experiment was performed in the presence of both CamR and RNA polymerase. Lanes 7 in Fig. 6A and B show the results of adding CamR first and then RNA polymerase followed by a DNase I reaction. Protection by both CamR and RNA polymerase was observed. Arrows in lanes 4 and 7 of Fig. 6B indicate the typical pattern of protection by CamR. Arrows in lanes 3 and 6 of Fig. 6B indicate the enhanced band upon binding of RNA polymerase. The enhanced band was not observed upon binding of CamR. This result suggested that CamR and RNA polymerase coexist on the promoter in a joint nonproductive complex. An experiment in the reverse order of adding RNA polymerase first and then CamR was also performed (Fig. 6A and B, lanes 6). Lane 6 showed the same protection pattern as does lane 3: protection by RNA polymerase only was observed. This result indicates that RNA polymerase acts by occluding the operator from CamR binding.

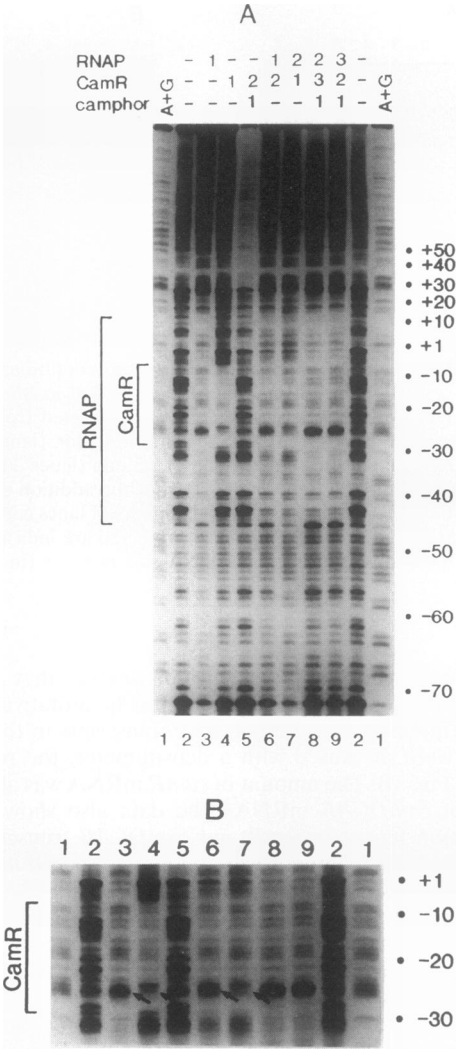

FIG. 6. Binding of RNA polymerase and CamR to the *cam* regulatory region. Numbers above the lanes indicate the order of addition of corresponding factors to the reaction; a minus sign indicates the omission of corresponding factors from the reaction; A+G indicates chemical reaction. The regions protected from DNase I attack by RNA polymerase and CamR are shown on the left. Nucleotide positions are shown on the right (taking the *camR* transcription start site as +1). The area of the CamR binding site shown in panel A is enlarged in panel B. Arrows indicated in panel B are typical bands affected by binding of protein factors as described in the text.

DISCUSSION

In this report, we characterized the transcriptional regulation by camphor of the genes for the *cam* operon and its repressor and determined the transcription start sites of the genes.

The role of CamR as a negative regulator of the *cam* operon has been described elsewhere (13). The increased expression of β-galactosidase from a heterologous *camR*::*lacZ* fusion gene in the presence of camphor is observed in *P. putida*. Purified CamR can protect a specific region of the *cam* promoter from DNase I digestion (4) (Fig. 6). One operator, to which the CamR protein binds, is present between *camR* and *camDCAB*.

We analyzed the syntheses of *camR* and *camDCAB* mRNAs of *P. putida* PpG1 under induction and noninduction conditions and found that these mRNAs are transcribed divergently from overlapping promoters, the two transcription start sites being separated by only 11 bp.

Using an in vitro transcription assay, we have shown that the *camR* is transcribed by σ^{70} RNA polymerase. However, transcription from the *camDCAB* promoter by σ^{70} RNA polymerase was not observed. These results suggested that the *camDCAB* is transcribed by alternative RNA polymerase. Another possibility is that the transcription of *camDCAB* may need not only RNA polymerase but also some positive fac-

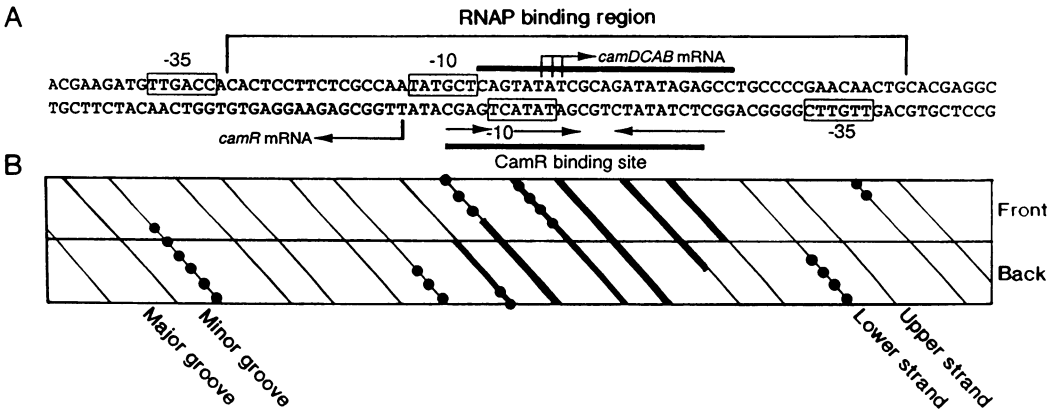

FIG. 7. Nucleotide sequence of the *cam* control region (A) and its cylindrical projection (B). The nucleotide sequence was determined previously (3, 12). The promoters are boxed, and transcription start sites are indicated by arrows. The horizontal arrows indicate palindromic sequences. Bracket indicates the region protected by RNA polymerase from DNase I attack. Thick bars covering the lower (4) and upper (this study) strands indicate the region protected by CamR from DNase I attack. The diagonal lines in the cylinder represent the positions of the phosphate backbone of each strand of DNA. Symbols: ▬, region protected by CamR from DNase I digestion; ●–●–●, hexanucleotides of the −35 and −10 promoter regions. The front and back of the DNA helix are arbitrarily designated.

tor(s) interacting with the σ^{70} RNA polymerase. The DNase I footprinting experiment revealed that σ^{70} RNA polymerase recognized two conserved hexamers, −10 and −35, relative to the 5′ end of *camR* mRNA. Some researchers have postulated that *lac* repressor prevents the initial binding of RNA polymerase to the promoter (15). This is taken as a generality in the mechanisms for other repressors, such as the λ and *cro* repressors (17, 18). However, Schmitz and Galas (21) showed through footprinting studies that although the binding sites for *lac* repressor and RNA polymerase overlap, there was some evidence for concurrent binding. Since this assay was done in solution, though, the results could also be interpreted as the sum of two or more separate interactions on different DNA molecules. Recently, Lee and Goldfarb (14) reported that RNA polymerase engaged in the joint complex with the *lac* repressor at the *lacUV5* promoter cannot escape into elongation but generates abortive RNA oligomers. The data shown in Fig. 6 imply that the *lac* repression system reported by Lee and Goldfarb (14) is also applicable to the *camR* repression system.

To examine the spatial positionings of RNA polymerases at the promoters and repressor at the operator, we represented these relative locations on a cylindrical projection of the DNA helix (Fig. 7). σ^{70} RNA polymerase protected a region corresponding to the *camR* promoter. However, RNA polymerase could not bind a region corresponding to the *camDCAB* promoter (Fig. 7). The strength of transcription initiation depends on the affinity between RNA polymerase and promoter. Unlike the *camDCAB* promoter, the *camR* promoter shows low homology to the σ^{70} consensus sequence. In vivo, the amount of *camR* mRNA was about 40% of that of *camDCAB* mRNA in the presence of camphor (Fig. 3). However, the *camDCAB* promoter could not be initiated by σ^{70} RNA polymerase. At present, we do not know whether transcription of *camDCAB* needs an alternative RNA polymerase or some positive factor(s) interacting with the σ^{70} RNA polymerase.

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