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Analysis of the Ros Repressor of *Agrobacterium virC* and *virD* Operons: Molecular Intercommunication between Plasmid and Chromosomal Genes

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The virulence genes of the *Agrobacterium tumefaciens* Ti plasmid are regulated both positively and negatively. The products of the genes of the *virC* and *virD* operons play an important role in host specificity and T-DNA processing. These operons are transcribed in opposite directions and therefore bear diametrically oriented promoters. These promoters are positively regulated by the VirG protein, which is believed to be activated through phosphorylation by a histidine kinase encoded by the *virA* gene. The *virC* and *virD* operons are also regulated by a 15.5-kDa repressor protein encoded by the *ros* chromosomal gene. A mutation in *ros* causes the constitutive expression of *virC* and *virD* in the complete absence of the VirG protein. It appears, therefore, that the Ros repressor interacts with the regulatory region of these operons. The Ros repressor is shown here to bind to an upstream sequence (Ros box) comprising 40 bp bearing a 9-bp inverted repeat, TATATTTCA/TGTAATATA, in the promoter region of these operons. The affinity for this sequence is specific and tenacious, since the addition of at least a 20,000-fold excess of competitor DNA failed to remove the Ros protein coding sequence from the Ros box. DNase I footprint analysis showed that the Ros box overlaps the binding site of VirG (Vir box). This result suggests that *virC* and *virD* transcription is modulated by Ros and VirG proteins.

Agrobacterium tumefaciens mediates the transfer of part of its genetic material in the form of a 25-kb sector (T-DNA) from a large Ti plasmid to plant host cells. Although the mechanism of this DNA transfer is unknown, the genes required for T-DNA processing and transfer have been identified. These genes reside in six major operons, *virA*, *virB*, *virG*, *virC*, *virD*, and *virE* (12, 21, 25), that are clustered in a 28.6-kb sector of the Ti plasmid. The *vir* genes are induced by phenolic compounds, such as acetosyringone (17, 24) and sinapic acid (20), and are potentiated by simple sugars released by wounded cells of the host (2, 23). The recognition of these signal molecules through a two-component signal transduction system comprising VirA and VirG proteins (10, 11, 14, 17, 18, 23, 26) activates a histidine kinase associated with VirA; this enzyme phosphorylates an aspartyl residue in VirG (10, 11). Efficient transcription of the *vir* operons is thereby mediated by phosphorylated VirG. In addition to this positive regulatory pathway, there is the negative regulation of *vir* genes. Specifically, the genes of the *virC* and *virD* operons, which encode functions involved in host range specificity (8) and T-DNA processing and transfer (8, 30), are regulated by the product of *ros*, a chromosomal gene found in *Agrobacterium* and *Rhizobium* strains (3–6). Mutations in *ros* cause the constitutive expression of the *virC* and *virD* operons. Transcription of these operons is switched off when these mutants are complemented by a cloned *ros* gene. The promoters of *virC* and *virD* are oriented to transcribe in opposite directions (29). The precise stretch of nucleotides in the promoter region to which the Ros protein binds has not been determined. Thus, we report here the identification of the specific Ros binding sequence in the promoter region of the *virC* and *virD* operons.

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MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* JM109 [*endA thi hsdR17 recA1 supE relA1* (*lac-proAB*) *gyrA96* (F′ *proAB traD36 lacI*^{q} *ZM13*)] was obtained from Stratagene, La Jolla, Calif. Strain HB101 (*proA leuB thi lacY rpsL hsdS20 recA*) (22) and strain BL21(DE3) [*hsdS gal* (kc*Its*857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7 gene *1*)] (28) were described previously. The latter strain was used for high-level expression of the *ros* gene cloned into expression vector pET3a containing the bacteriophage T7 promoter (28). The plasmids used in these strains are listed in Table 1. The strains were grown in Luria-Bertani (LB) medium (22) at 37°C. Antibiotic concentrations used were as follows: ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; gentamicin, 20 μg/ml; tetracycline, 5 μg/ml; spectinomycin, 20 μg/ml; and streptomycin, 20 μg/ml. Strain BL21(DE3) was grown in LB medium with the addition of 5 g of glucose per liter and 200 μg of ampicillin per ml. Strains were transformed by electroporation or by $CaCl_2$ treatment as described previously (5, 15). Standard cloning techniques were those of Sambrook et al. (22).

Buffers. Extraction buffer contained 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 5% glycerol. Binding buffer contained 1 mM EDTA, 100 mM KCl, and 50 μg of bovine serum albumin per ml. Lysis buffer contained 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM KCl, and 10 mg of lysozyme per ml. Wash buffer contained 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM KCl, and 4 mg of deoxycholate. Dialysis buffer (HEGD) contained 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 1 mM EDTA, 5% glycerol, and 1 mM dithiothreitol. TE buffer contained 50 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

TABLE 1. Plasmid used in this study

Plasmid	Resistance	Description	Source or reference
pUCD4401	Ap	pET3a derivative with the *ros* gene cloned in the *NdeI-BamHI* site	This study
pUCD4402	Ap	pTZ18R derivative with fragment CD25 subcloned in the *SalI* site	This study
pCR8	Gm, Sp	*ros* clone from a plasmid bank	5
pCR8-D14	Gm, Sp, Tc	TnCAT inserted in *ros*	5
pUCD612	Ap, Sp, Km	Cloning vector of *ros*	5
pTZ7R	Ap	*BamHI-BglII* subclone of pCR7	5
pTZ18R	Ap	Expression vector with T7 RNA polymerase promoter	U.S. Biochemicals (Cleveland, Ohio)
pTZ19R	Ap	Expression vector with T7 RNA polymerase promoter	U.S. Biochemicals (Cleveland, Ohio)
pET3a	Ap	Derivative of pET3; expression vector	28

Preparation of protein extracts. Proteins were extracted from 50 ml of *E. coli* cells grown to the early exponential phase in LB broth in a 250-ml flask at 37°C with vigorous shaking (250 rpm). The cells were harvested by centrifugation at 4°C, resuspended, and washed twice with 20 ml of TE buffer containing 10 mM β-mercaptoethanol at 4°C. The pellet was frozen in liquid nitrogen and thawed by being left in crushed ice for 10 to 20 min. This freeze-thaw step was repeated two more times. The cells were resuspended in 1.0 ml of extraction buffer and sonicated for four pulses of 30 s each at setting 50 with a Microson Ultrasonic cell disrupter (Heat Systems Ultrasonics, Inc., Farmingdale, N.Y.). The cell debris was removed by centrifugation at 17,210 × *g* for 10 min at 4°C. The supernatant contained the crude Ros protein preparation. Glycerol was added to this preparation to a final concentration of 30%, and the mixture was stored at −20°C.

PCR. The polymerase chain reaction (PCR) was done with a GeneAmp kit in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). Primers were 25-mer oligonucleotides synthesized from sequences corresponding to the ends of a 210-bp *SalI-SalI* fragment (5). This fragment, containing the promoter region of *virC* and *virD* was subcloned in pTZ18R to yield hybrid plasmid pUCD4402. An *NdeI* restriction site (CATATG) was introduced 18 bp from the start of the coding sequence, and a *BamHI* restriction site (GGATCC) was placed 95 bp downstream of the stop codon. This procedure facilitated cloning of the *ros* gene into pET3a. PCR was carried out for 25 cycles at 92°C for melting, 42°C for annealing, and 72°C for extension, for 1 min each. The synthesized *ros* open reading frame was verified by sequencing.

Purification of the Ros protein. The 539-bp *NdeI-BamHI* fragment bearing *ros* cloned in frame in pET3a yielded hybrid plasmid pUCD4401 maintained in *E. coli* JM109. Restriction analysis with *NruI* generated fragments of 1.023 and 4.015 kb, a result that correlates with the map of *ros* (5). Plasmid pUCD4401 was transformed into *E. coli* BL21 (DE3), which was then grown to an optical density at 600 nm of 1.0 in LB medium containing 5% glucose and 100 μg of ampicillin per ml. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM to the culture, which was then incubated for 2.5 h at 37°C with vigorous shaking (250 rpm). The cells were collected by centrifugation, and the pellet was resuspended at 1/10 its volume in lysis buffer and left in crushed ice for 30 min. The mixture was sonicated for 1 min (setting 40) and centrifuged at 26,890 × *g* for 15 min. The pellet (containing the Ros protein in inclusion bodies) was washed twice with wash buffer and then dissolved in HEGD containing 6 M guanidine HCl. The solution was dialyzed three times at 4°C: first, for 18 h against 200 volumes of HEGD containing 1 M guanidine HCl and 0.5 M potassium chloride; second, for 12 h against the same buffer with stirring; and third, for 12 h against HEGD containing 0.5 M potassium chloride. The last dialysis step caused the Ros protein to precipitate from the solution. The precipitate was collected by centrifugation and solubilized by the dropwise addition of 2 M potassium chloride until the precipitated protein dissolved. The solubilized protein was adjusted to a final potassium chloride concentration of 0.2 M and stored at −20°C. This method yielded a substantial amount of partially purified protein (about 90% of total protein) that was functional in both gel retardation and footprinting assays.

Radiolabelling of DNA and preparation of a probe. The cloned DNA fragment containing the promoters of *virC* and *virD*, described previously as CD25 (29), was subcloned into pTZ18R at the *SalI* site, such that the *virC* proximal end was near the *PstI* site of pTZ18R. The resulting plasmid was purified by dye-buoyant density gradient centrifugation in CsCl, linearized by *EcoRI* digestion, labelled with [α-^{32}P] dATP at both ends, and treated with the Klenow polymerase to fill in the recessed 3′ termini.

Footprinting assays were carried out with [γ-^{32}P]ATP and bacteriophage T4 polynucleotide kinase (9). The DNA was dephosphorylated with calf intestinal alkaline phosphatase, purified by phenol-chloroform extraction, and then treated with kinase in the presence of [γ-^{32}P]ATP. The DNA was passed through a spin column preequilibrated with restriction enzyme buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.2) for *Hin*dIII digestion.

The linear plasmid DNA was next digested with *Hin*dIII to generate two fragments, one of which was labelled on the 3′ end and the other of which was labelled on the 5′ end. The fragments were separated electrophoretically in a low-melting-point agarose gel, and the labelled CD25 fragment was eluted by melting the gel slice at 55°C and then extracting the solution once with buffered phenol and twice with chloroform. Labelled CD25 DNA was precipitated with ethanol and collected by centrifugation.

Gel retardation assay. Approximately 1 ng of labelled DNA was mixed with 0.2 μg of crude Ros protein extract and 1 μg of sonicated herring sperm DNA in binding buffer and incubated at room temperature for 15 min, after which the samples were immediately loaded onto a 1.5% horizontal agarose gel and subjected to electrophoresis in Tris-acetate–

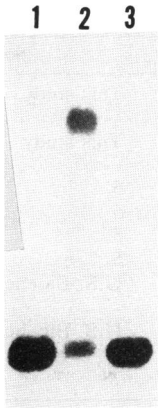

FIG. 1. Gel retardation analysis of DNA fragment CD25, which contained the promoters of the *virC* and *virD* operons and interacted with proteins produced in *E. coli* HB101 containing one of the following plasmids: lane 1, vector pUCD612; lane 2, *ros* containing plasmid pCR8; lane 3, *ros* containing a chloramphenicol acetyltransferase gene insert, cloned in pCR8-D14.

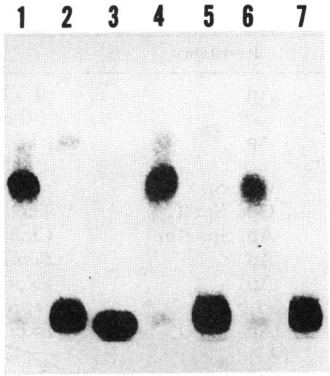

FIG. 2. Gel retardation analysis of CD25 DNA, which interacted with proteins extracted from *E. coli* HB101 containing one of the following plasmids: lanes 1, 4, and 6, pTZ7R; lanes 2, 5, and 7, pTZ19R; lane 3, fragment CD25 alone as a control. In lanes 1 and 2, the reaction contained a 5,000-fold excess of competitor DNA; in lanes 4 and 5, the reaction contained a 10,000-fold excess of competitor DNA; in lanes 6 and 7, the reaction contained a 20,000-fold excess of competitor DNA.

EDTA buffer (pH 8.0) at 5 V/cm. The gel was air dried at 80°C and then exposed to X-ray film with an intensifying screen overnight at −70°C.

DNase I footprinting. The target DNA samples that had been end labelled with [γ-^{32}P]ATP were used in the DNase I footprinting assay described by Galas and Schmitz (7). The binding reaction was carried out with 4 to 5 ng of labelled DNA and 0.1 to 1.0 μg of protein. The reaction mixture was treated with DNase I (5 to 25 ng per reaction) and then extracted with phenol-chloroform (1:1 [vol/vol]). The DNA was precipitated by the addition of 2 volumes of ice-cold ethanol. Each preparation along with appropriate controls was loaded onto a sequencing gel.

DNA sequencing and miscellaneous methods. DNA sequencing reactions were carried out by the chemical degradation method (16). Protein concentrations were determined by a dye-binding method (1). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein samples was performed as described by Laemmli (13).

RESULTS

Ros is a DNA-binding protein. To demonstrate that a specific DNA-binding protein was present, we carried out initial experiments with crude proteins extracted from *E. coli* bearing pCR8, pCR8-D14, or pUCD612, plasmids containing *ros*, *ros* with a chloramphenicol acetyltransferase gene insert, or the cloning vector itself, respectively (5). The DNA-binding property of a protein present in the extract was evident, as seen in Fig. 1. The protein extract from *E. coli* containing pUCD612 alone or containing the interrupted *ros* clone, pCR8-D14, did not have any effect on the mobility of fragment CD25, which contains the promoters of *virC* and *virD*. On the other hand, the mobility of CD25 DNA was retarded appreciably when this DNA was incubated with protein derived from *E. coli* containing the intact *ros* clone, pCR8. The experiment was repeated with a smaller, 4.3-kb DNA fragment containing *ros* cloned in pTZ19R (Fig. 2). The specificity of binding of the Ros protein to CD25 DNA was tested by use of herring sperm DNA as a nonspecific competing target. As shown in Fig. 2, the presence of a 20,000-fold excess of the competitor did not interfere with the binding of the Ros protein.

Overexpression of the *ros* gene and purification of the Ros protein. Our previously determined sequence of *ros* (5) provided the limits of this gene so that useful restriction sites that flanked either side of the gene could be added. Therefore, *Nde*I and *Bam*HI sites flanking the 5′ and 3′ ends of the *ros* gene were generated by PCR and cloned into expression vector pET3a, resulting in the hybrid construct pUCD4401. Synthesis of the Ros protein in *E. coli* BL21(DE3) containing pUCD4401 was confirmed by the appearance of a major electrophoretically separated protein band of 15.5 kDa, a size represented by the 142-amino-acid residues predicted from the sequence of Ros (5). The overexpressed Ros protein was concentrated in inclusion bodies in *E. coli* (Fig. 3); this protein was collected and solubilized in 6 M guanidine HCl in HEGD. The Ros protein in the solubilized

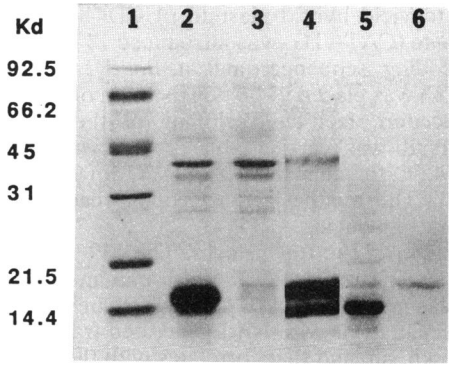

FIG. 3. Overexpression and purification of the Ros protein from *E. coli* BL21(DE3) containing pUCD4401. Soluble proteins were analyzed electrophoretically on a sodium dodecyl sulfate–12% polyacrylamide gel. Lanes: 1, polypeptide molecular mass standards; 2, total cellular protein from *E. coli* BL21(DE3) with pUCD4401; 3, total cellular protein from *E. coli* BL21(DE3) without pUCD4401; 4, solubilized proteins from inclusion bodies of *E. coli* BL21(DE3) containing pUCD4401; 5, proteins from the last fractional dialysis step containing mainly the 15.5-kDa Ros protein and egg white lysozyme as a carrier; 6, sample of the Ros protein preparation obtained from the last dialysis step and diluted in HEGD containing 0.2 M potassium chloride.

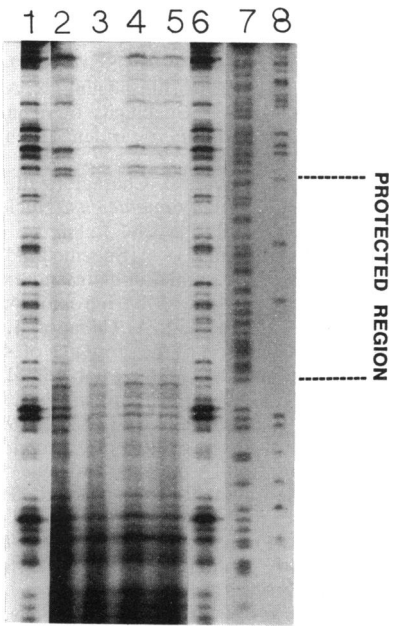

FIG. 4. DNase I footprinting analysis of fragment CD25, which interacted with purified Ros protein. Lanes: 1, CD25 DNA treated with proteins from *E. coli* BL21(DE3); 2 to 5, CD25 DNA interacting with purified Ros protein at increasing concentrations: 0.1, 0.2, 0.5, and 1.0 μg, respectively; 6, fragment CD25 alone; 7 and 8, A+G and G reactions, respectively, of Maxam and Gilbert (16).

material, representing about 90% of the solubilized protein, was subsequently purified by precipitation during dialysis against HEGD containing a low salt concentration as described in Materials and Methods. The protein pellet was dissolved in a small volume of concentrated potassium chloride and subsequently diluted to a final salt concentration of 0.2 M. A sample of the solubilized material showed that it comprised primarily Ros (Fig. 3).

Ros binding site. Although we have shown that the Ros protein has a high affinity for DNA containing the promoter region of *virC* and *virD*, the Ros binding sequence within this region was not identified. Therefore, DNase I footprinting was used to locate the sequence to which Ros specifically binds. As shown in Fig. 4, a 40-bp stretch of the 200-bp DNA fragment containing the promoter region of *virC* and *virD* was protected from DNase I digestion. The protected 40-bp sequence included the inverted repeats TATATTTCA and TGTAATATA (Fig. 5). These repeated sequences were

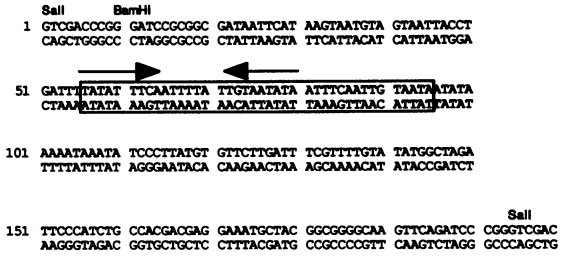

FIG. 5. Sequence of CD25 DNA. The region enclosed in the box represents the Ros protein binding site. The arrows above the sequence show the position of the inverted repeats within this Ros box.

homologous to those in the promoter region of *virC* and *virD* of pTiA6 and pRiA4b and in the promoter region of *ros* itself. In the absence of the Ros protein, this 40-bp sequence was not protected from DNase I digestion (Fig. 4, lanes 1 and 6). These studies suggest that the 40-bp sequence containing the above-described inverted repeats is the binding site of the Ros protein.

DISCUSSION

The regulation of the genes involved in the virulence of *A. tumefaciens* plays an important role in preventing the needless production of gene products specifically used during infection by the bacterium to process and transfer its T-DNA to plant cells. The two-component signal transduction system involving the products of the *virA* and *virG* genes provides an efficient means for initiating the expression of the *vir* genes when the bacterial cells interact with the plant cells. It is clear, however, that genes of the *virC* and *virD* operons are also regulated by the product of the *ros* chromosomal gene. Some insight on how these latter operons are dually regulated is provided by the identification of the binding site for the Ros protein in the promoter region of *virC* and *virD*. The length of this binding site is 40 bp; this 40-bp sequence contains a 9-bp inverted repeat. This 40-bp sequence, termed the Ros box, overlaps the binding site (Vir box) of the transcriptional activator VirG (5). The consensus Vir box sequence is TNCAATTGAAAPy (N = any deoxyribonucleotide; Py = pyrimidine deoxyribonucleotide) (27). Pazour and Das (18) have reported site-specific mutations in this sequence. One such mutation altering a single base in the right inverted repeat of the Ros box caused a sixfold increase in basal expression, potentially because of the loss of the Ros binding site. Other mutations in the left and right inverted repeats failed to change the basal expression of *virC*. The combined information regarding the regulation of *virC* and *virD* suggests that VirG and Ros compete for their respective binding sites, which overlap each other in the promoter region of the *virC* and *virD* operons. During *vir* gene induction, VirG protein binding activity appears to overcome the effect of the Ros repressor. Recent studies, however, suggest that a Vir box-like sequence is situated upstream of the *ros* gene (9a). This observation suggests that VirG may modulate the expression of *ros* by serving as a repressor blocking the transcription of *ros* when VirG is produced at high levels in induced *Agrobacterium* cells. In an in vivo study, VirG was indeed shown to function as a repressor (19). A mutation in *ros* causes the derepression of *virC* and *virD* genes and results in the formation of T-DNA intermediates (3). The concentrations of T-DNA intermediates generated by induction of *ros* mutants with acetosyringone are higher than those generated in the acetosyringone-induced wild-type strain (3). Although the requirement for the *ros* gene in *A. tumefaciens* virulence appears subtle in the laboratory, in nature this gene may indeed be needed for modulating the amounts of T-DNA intermediates formed during the infection process.

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