Mutations in the \textit{occQ} Operator That Decrease OccR-induced DNA Bending Do Not Cause Constitutive Promoter Activity\textsuperscript{*}

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OccR is a LysR-type transcriptional regulator of \textit{Agrobacterium tumefaciens} that positively regulates the octopine catabolism operon of the Ti plasmid. Positive control of the \textit{occ} genes occurs in response to octopine, a metabolite released from plant tumors. Octopine causes DNA-bound OccR to undergo a conformational change from an inactive to an active state; this change is marked by a decrease in footprint length from 55 to 45 nucleotides as well as a relaxation of a high angle DNA bend. In this study, we first used gel filtration chromatography to show that OccR is dimeric in solution, and we used gel shift assays to show that OccR is tetrameric when bound to DNA. We then created a series of site-directed mutations in the OccR-binding site. Some mutations were designed to lock OccR-DNA complexes into a conformation resembling the inactive conformation, whereas other mutations were designed to lock complexes into the active conformation. These mutations altered the conformation of OccR-DNA complexes and their responses to octopine in ways that we had predicted. As expected, operator mutations that locked complexes into a conformation having a long footprint and a high angle DNA bend blocked activation by octopine \textit{in vivo}. Surprisingly, however, mutations that locked OccR into a short footprint and low angle DNA bend failed to cause the protein to function constitutively. Furthermore, some of the latter mutations interfered with activation by octopine. We conclude that locking OccR into a conformation having a short footprint is not sufficient to cause constitutive activation, and octopine must cause at least one additional conformational change in the protein.

The \textit{Agrobacterium tumefaciens} OccR protein is a transcriptional regulator that activates the \textit{occQ} operon of the Ti plasmid in response to octopine, a plant tumor-derived arginine derivative (1). This operon encodes proteins required for the uptake and catabolism of octopine (2), which serves as a source of carbon, nitrogen, and energy for tumor-colonizing bacteria. The \textit{occ} operon also encodes the TraR protein, which is a quorum-sensing transcriptional regulator of the Ti plasmid \textit{tra} regulon (3). OccR has a molecular mass of 32.7 kDa and is a member of the LysR family of DNA-binding transcriptional regulators (4), the largest known family of DNA-binding regulators in Proteobacteria (5). Sequence comparisons among LysR proteins indicate that they share a highly conserved amino-terminal domain that contains a helix-turn-helix DNA-binding motif and a far less conserved carboxyl-terminal domain that generally binds low molecular weight inducing ligands. LysR proteins are often encoded by genes that are transcribed divergently from their target genes, and these proteins often activate a target operon and simultaneously repress their own expression by binding to a single intergenic region (6).

OccR binds specific DNA sequences that lie upstream of the promoter it activates, \textit{PoccQ}. In the absence of octopine, OccR protects a region from −80 to −28 nucleotides upstream of its target promoter. Octopine has little effect on binding affinity and does not alter the oligomeric state of bound OccR. However, octopine shortens the DNase I footprint by one helical turn such that only the region from −80 to −38 is protected. Furthermore, apo-OccR incites a high angle DNA bend at this binding site, and this bend angle is relaxed by the addition of octopine (7). Both OccR conformations repress the divergent \textit{occR} promoter, but activation of the \textit{occ} operon occurs only when OccR is in the latter conformation.

DNA bending has been suggested to be an important event in transcriptional activation for a number of LysR family proteins (8–10). Several have been shown to incite bends in their target promoters. For example, CysB of \textit{Salmonella typhimurium} induces a bend at two promoters, and these bends are partially relieved by the inducer, N-acetyl-L-serine (8). Similarly, the OxyR protein of \textit{Escherichia coli} causes a high angle bend at the \textit{oxyR-oxyS} intergenic region that is relieved by reactive oxygen species such as hydrogen peroxide (9). CatR of \textit{Pseudomonas putida} also causes a high angle DNA bend at the \textit{catBC} promoter, whereas the addition of the inducer cis,cis-muconate relaxes this bend (10). Although in the case of OccR and the above proteins, the inducing ligands cause a relaxation of the DNA bend, this is not a general rule, and DNA bending patterns are dependent on the specific regulator. For instance, apo-CatR causes little if any bend at another promoter (the \textit{pheBA} promoter), but actually introduces a bend in the presence of the inducer, due to the binding of additional CatR protomers (10).

Many LysR proteins bind unusually long regions of DNA upstream of their target genes. Such long binding sites suggest that LysR proteins are functionally multimeric, and several members have been reported to be either dimers or tetramers in solution, yet few data are available about their oligomeric state when DNA bound (see below). Based on mutational studies of several LysR proteins, oligomerization appears to be mediated by the carboxyl-terminal region of the protein (11–13). Although binding to DNA is independent of the presence of an inducer, inducing ligands often cause a conformational change in these complexes. For some proteins, such as CatR (14), TrpI (15), and NahR (16), the inducing ligand appears to...
increase the number of bound protein monomers, whereas in other LysR proteins, such as OccR (7) and OxyR (9), the inducer does not alter the number of bound monomers but alters the sites of DNA contact.

OccR contacts the major groove of its operator at five sites, designated sites 1–5 (Fig. 1, see Refs. 17 and 18). Of these, sites 4 and 5 are sufficient and essential for high affinity DNA binding, as long as additional nonspecific sequences are provided to the left. Sites 4 and 5 together are therefore designated the “high affinity subsite.” Sites 1–3 make little if any contribution to binding affinity but are required for ligand-responsive DNA bending (17). The high affinity binding site contains a dyad symmetrical sequence (ATAAN7TTAT) that resembles the TN11A motif to which many LysR family members bind (19). This sequence lies in the major groove of the region contacted by OccR and is similar to the 2-fold dyad symmetrical sequence (ATAGN−CTATN−ATAGN−CTAT) recognized by the homologous OxyR protein (9).

Subsites 1 and 2 (ATTCN−TTCA) share some sequence similarity with the high affinity subsite (ATAAN−TTAT), whereas subsite 3 (CCGG) shares no apparent similarity. It is thought that apo-OccR makes sequence-specific contacts with sites 1, 2, 4, and 5. OccR-octopine complexes do not contact site 1 and instead contact the remaining four contiguous sites. We postulate that OccR-octopine complexes binding at site 2 might recognize the sequence ATTC rather than the overlapping site TTCA (Fig. 1). The sequence ATTC is therefore designated site 2’. Mutations disrupting site 1 locked OccR into a conformation with a low angle DNA bend, whereas mutations disrupting sites 2 and 2’ prevented the relaxation of the DNA bend in the presence of octopine (17), although the effect of octopine was tested at only one concentration.

In the present study, we used site-directed mutagenesis of the DNA-binding site to identify the requirements for transcriptional activation at the occQ promoter.

**EXPERIMENTAL PROCEDURES**

**Protein Overexpression and Purification—**The strains and plasmids used in this study are described in Table I. For overproduction of wild-type OccR protein, the occR coding sequence was fused to the β10 promoter of bacteriophage T7 by PCR amplification, using pKY125 (20) as a template and oligonucleotides 5’-GGTCTAGACATATGACATTTCCAGGCAGGTC-3’ and 5’-GTAATACGACTTATAGGGC-3’ as primers. The resulting PCR product was digested with NdeI and KpnI and was ligated into pRSETA (Invitrogen) digested with the same enzymes, resulting in plasmid pRA304. To construct an OccR-MBP fusion protein, the occr gene was PCR-amplified using plasmid pKY125 as a template and oligonucleotides 5’-GGTCTAGACATATGACATTTCCAGGCAGGTC-3’ and 5’-GCGGTCCGACATATGACATTTCCAGGCAGGTC-3’ as primers. The resulting DNA fragment was digested with NdeI and cloned into pMal-c2x (New England Biolabs) digested with the same enzyme. This enzyme digests pMal-c2x at the start codon of maltE, such that the resulting fusion protein has native OccR at its amino terminus and MBP at its carboxyl terminus. The lacZα-peptide at the end of the maltE sequence was removed by a fill-in reaction at the BamHI site of the polyclinker using T4 DNA polymerase (New England Biolabs). The resulting plasmid is pRA346.

To purify OccR, strain BL21(DE3)pSW213(pRA304) was cultured at 28°C in 1 liter of Luria Bertani (LB) medium containing 10 μg/ml tetracycline and 1 mg/ml ampicillin to mid-log phase, treated with isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM, and incubated for an additional 4 h. pSW213 was provided to ensure a high concentration of Lac repressor (21). Cells were resuspended in 5 ml of TEDG buffer (50 mM Tris−HCl, pH 7.9, 0.5 mM EDTA, 1 mM DTT, 5% glycerol) plus 25 mM NaCl and lysed with a French pressure cell (20,000 pounds/square inch). Cellular debris was removed by ultracentrifugation (150,000 × g for 15 min), and the soluble fraction was chromatographed using 30 ml of heparin-agarose equilibrated with TEDG buffer and 25 mM NaCl. Bound proteins were eluted using a 90-ml linear gradient of NaCl from 25 to 600 mM. Peak fractions of OccR were pooled and applied to a 3 ml α-cyclodextrin column (Amersham Biosciences) equilibrated with a buffer containing 50 mM sodium phosphate, (pH 6.8), 0.5 mM EDTA, 1 mM DTT, 5% glycerol, and 100 mM NaCl. Proteins were eluted using a 24-ml linear gradient of NaCl from 100 to 600 mM.

To purify OccR-MBP, strain DH5α(pRA346) was cultured in LB broth containing 1 mg/ml ampicillin and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside as above. Cells were concentrated and lysed with a French pressure cell and ultracentrifuged. The resulting supernatant was chromatographed with 2 ml of amylose affinity resin (New England Biolabs). Bound OccR-MBP was step eluted with TEDG buffer plus 200 mM NaCl supplemented with 10 mM maltose.

**Gel Filtration Chromatography—**Gel filtration chromatography was performed using a fast protein liquid chromatography apparatus and a Superdex-75 gel filtration column (Amersham Biosciences). Pure OccR protein (1.4 mg/ml) was applied to a 250-μl volume to the column equilibrated with TEDG buffer supplemented with 150 mM NaCl, and eluted using a flow rate of 0.5 ml/min. The eluate was monitored by absorbance at 280 nm and by SDS-PAGE. Molecular weight standard proteins (Sigma) were size-fractionated by the same method.

**Site-directed Mutagenesis—**Site-directed mutants of the occR operator were made using the Altered Sites II mutagenesis systems (Promega). The OccR-binding site was cloned into pALTER-1 as a XhoI-SalI fragment derived from pLW131 (17), resulting in pRA201. Single-stran-
Only vector, hosts, strains, and basic plasmids are shown. Construction of plasmid derivatives containing mutant operators and translational fusions is described in detail under “Experimental Procedures.”

| Strain or plasmid | Relevant genotype | Ref. |
|-------------------|------------------|-----|
| JM109             | E. coli K12 F′traD36 proAB′ lacI′ΔlacI98 lacZΔM15 Δ(lac-proAB) glnV44 e14 gyrA96 recA1 relA2 endA1 thi1 hsdRI7 22 |
| BL21(DE3)         | E. coli B F−gene 1 of bacteriophage T7 | 38 |
| KYC55             | A. tumefaciens R10, no Ti plasmid, KmR | 28 |
| pSW213            | IncP cloning vector, lacZm, lacI−, TcR | 21 |
| pALTER-1         | Phagemid for site-directed mutagenesis, TeR | Promega 24 |
| pBend3            | Derivative of pBlueScript for assays of DNA bending, ApR | Invitrogen |
| pRSETA            | T7 promoter cloning vector, ApR | New England Biolabs |
| pMAL-C2x          | ptna-lacF fusion cloning vector, ApR | This study |
| pRA302            | Broad host range lacZ reporter plasmid, SmR | This study |
| pKY125            | occR cloned into p15A ori plasmid pSW208, CmR | 20 |
| pKY144            | occR cloned into broad host range plasmid pSW213 | K. Cho |
| pLW132            | occQ-occR intergenic region cloned into pBend3 | 17 |
| pRA201            | occQ-occR region cloned into pALTER-1 | This study |
| pRA304            | occR cloned into pRSETA | This study |
| pRA346            | occR cloned into pMal-C2x | This study |

Results that Alter OccR-induced DNA Bending

Mutations That Alter OccR-induced DNA Bending

The resulting protein-DNA complexes were size-fractionated at 4 °C, and radioactive bands were detected using a Storm PhosphorImager (Molecular Dynamics).
buffer containing octopine. The resulting elution profile was identical to that seen in the absence of octopine (data not shown), indicating that octopine does not detectably influence oligomerization of the OccR protein in solution.

OccR was shown previously (7) to bind DNA with a high degree of cooperativity, showing a Hill coefficient of 2.0. We interpreted this to mean that the oligomeric state of bound OccR is 2-fold higher than that in solution. The length of the DNase I footprint also suggests that OccR may bind DNA as a tetramer (7). To test this we constructed a fusion between OccR and the maltose-binding protein (MBP). This OccR-MBP fusion was active in a gel mobility shift of the occQ promoter (Fig. 2B, lane 7). As expected, the mobility of these complexes was considerably less than that of OccR-DNA complexes (Fig. 2B, lane 2), due to the larger mass of the fusion protein. We then combined OccR and OccR-MBP in varying ratios on ice and quickly added DNA for gel mobility shift assays. Three types of complexes were detected (Fig. 2B, lanes 3–6), including one having a mobility intermediate between that of OccR-DNA complexes and OccR-MBP-DNA complexes (marked with an asterisk). We interpret this new band as containing one dimer of OccR and one dimer of OccR-MBP, supporting our previous conclusion that the oligomeric state of OccR increases 2-fold upon DNA binding. An alternative interpretation of these data that we do not favor is that these two protein species rapidly exchange subunits, forming mixtures of soluble homodimers and heterodimers that, upon DNA addition, caused the three shifted fragments. When these proteins were combined and allowed to equilibrate overnight at 28 °C, additional complexes were detected, although the number and mobilities of these fragments were difficult to interpret (data not shown). This suggests that a considerable time interval is required for formation of heterodimers and that they did not form in the experiment shown in Fig. 2B.

To provide independent evidence that bound OccR is tetrameric, we compared the gel mobility of these complexes to that of a control protein known to bind DNA as a dimer. The TraR protein of A. tumefaciens is a LuxR-type quorum sensing protein that binds as a dimer to specific DNA sequences called tra boxes (29).

A fragment of the Ti plasmid containing two tra boxes was isolated as a 188-nucleotide fragment and used as a control for gel mobility shift assays using purified TraR (obtained from T. Pappas). As observed previously,3 TraR-DNA complexes formed two species, one bound at just one tra box and one bound at both sites (Fig. 2C, lanes 6–8). The faster complex therefore contained two TraR protomers, whereas the slower complex contained four TraR protomers. These gel mobilities were compared with that of OccR bound to a 192-nucleotide fragment. As expected, the mobilities of the two unbound DNA fragments are extremely similar (Fig. 2C, lanes 1 and 5). All OccR-DNA complexes migrate as a single species, as seen previously (Fig. 2C, lanes 2–4). These complexes migrate slightly more slowly than the TraR-DNA complexes containing four TraR protomers. This was expected because OccR has slightly greater mass than TraR (32.7 versus 26.6 kDa). DNA bending does not contribute significantly to these mobilities, because TraR causes only a slight DNA bend, and the TraR-binding site lies near one end of this fragment (39), where DNA bending does not significantly affect gel mobility. We conclude that OccR binds DNA as a tetramer.

OccR Binding and Bending of Mutant Operators—We identified previously (17) a 55-nucleotide region containing five adjacent sites to which OccR binds (Fig. 1). Of these, sites 4 and 5 contribute virtually all binding affinity, whereas sites 1–3 play only minor roles in affinity but are required for octopine-induced conformational changes in bound OccR. To determine in more detail which sequences may be important in the ligand-responsive changes in conformation, mutations were created in these sites. We attempted to "improve" sites 1–3, that is, to make them more closely resemble sites 4 and 5. We hypothesized that altering sites 1 and 2 to match sites 4 and 5 would cause OccR to bind all four of these sites, resulting in a locked high angle DNA bend and a long DNase I footprint. Conversely, 1 R.-g. Zhang, T. Pappas, J. L. Brace, P. C. Miller, T. Oulmassov, J. M. Molyneaux, J. C. Anderson, J. K. Bashkin, S. C. Winans, and A. Joachimiak, submitted for publication.

2 T. Pappas and S. C. Winans, submitted for publication.

FIG. 2. Oligomeric state of soluble and DNA-bound OccR. A, OccR is a dimer in solution. Purified OccR was size-fractionated using a Superdex-75 gel filtration column at a flow rate of 0.5 ml/min. Molecular mass standards were bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12 kDa). The dashed line represents the calculated native mass of OccR. B, increase in oligomeric state upon DNA binding. Lane 1, no protein; lanes 2–7, OccR and OccR-MBP combined at 0 °C in ratios of 1:0, 1:0.3, 1:1, 1:3, 1:10, and 0:1, respectively. The total protein concentration was 100 nM in all assays. The presence of both proteins resulted in a novel complex in all assays. The presence of both proteins resulted in a novel complex in all assays.

C, comparison of gel mobility rates to a control protein of known stoichiometry. Mobility of complexes between a 192-bp DNA fragment (100 ng, lane 1) and OccR added at 0.12, 0.5, and 2 μM (lanes 2–4, respectively) are compared with mobility of complexes between a 188-bp DNA fragment containing two TraR-binding sites (100 ng, lane 5) and TraR added at 1.8, 5.4, and 16.2 μM (lanes 6–8, respectively).
changing sites 2' and 3 (site 2' overlaps site 2 but is shifted by 1 nucleotide) to match sites 4 and 5 would cause OccR to bind these sites, resulting in a locked low angle DNA bend and a shorter DNase I footprint. Alteration of just one site rather than two was predicted to cause a more subtle phenotype than alteration of both sites.

We constructed a total of six such altered operators, three of which were predicted to favor a high angle DNA bend (Fig. 1, mutants A1, A2, and A3) and three of which were predicted to favor the low angle bend (mutants B1, B2, and B3). These sequences were introduced into plasmid pBend3, which is designed to measure DNA bending, and the mobilities of the resulting OccR-mutant operator complexes were observed using native polyacrylamide gels. OccR-DNA complexes with high angle DNA bends migrate slowly in these gels, whereas complexes with low angle DNA bends migrate more quickly (7). Gel mobility was monitored over a range of octopine concentrations. The wild-type OccR-DNA complex migrates slowly on the gel when octopine is present at 10 μM or less but relaxes its DNA bend and migrates faster in the gel when supplied with 30 μM octopine or more (Fig. 3A, lanes 1). Gel mobility of these complexes are graphed in Fig. 3B (diamonds), where a low mobility indicates a high angle DNA bend. In this and all assays described below, we detected many different gel mobilities under different conditions, and in all cases we detected only single bands. This could be interpreted to mean that complexes can take many different static conformations, each with a different bend angle. However, we strongly prefer the alternative hypothesis that OccR has only a small number of possible conformations (probably two) and that intermediate migration rates are due to a dynamic equilibrium between these conformations during electrophoresis.

All the mutations in the operator region affected OccR-induced DNA bending (Fig. 3), although none resulted in a fully locked conformation. Instead, these complexes favored one or the other conformation, but were still somewhat influenced by octopine. The mutation that alters sites 1 and 2 to resemble sites 4 and 5 (mutant A1) caused OccR to migrate predominantly with a high angle DNA bend conformation, even in the presence of octopine (Fig. 3A, lanes 2). Although complexes containing this mutation migrated somewhat more quickly at high octopine concentrations than at low concentrations, the gel mobility is far less than that of the wild type even at the highest concentrations of inducer (Fig. 3B). Mutating just site 1 or just site 2 to resemble sites 4 or 5 (mutants A2 and A3, respectively) also caused the same effect, although less severely than mutating both sites together (Fig. 3A, lanes 3 and 4; Fig. 3B). These results suggest that OccR can bind with high affinity to sequences resembling sites 4 and 5, even when they are placed at sites 1 and 2. However, OccR is not completely locked into one conformation by these mutations but still retains a limited response to high concentrations of the inducer.

We also altered sites 2' and 3 to resemble sites 4 and 5 (mutant B1). This mutation caused complexes to migrate slightly faster than wild type in the absence of octopine (Fig. 3A, lanes 5) indicating a slight decrease in bend angle. This mutation also caused complexes to respond to octopine in the indicated concentrations and size-fractionated on 5% polyacrylamide gels. Octopine was also supplied in the gel and running buffer. Lane 1, wild-type operator (pLW132); lane 2, mutant A1 (pPJD204); lane 3, mutant A2 (pPJD111); lane 4, mutant A3 (pPJD112); lane 5, mutant B1 (pRA205); lane 6, mutant B2 (pRA208); lane 7, mutant B3 (pRA209); lane 8, mutant C1 (pLW142); lane 9, mutant C2 (pLW143); lane 10, mutant C3 (pLW134). B, effects of mutations A1, A2, and A3 on octopine-responsive gel mobilities. The migration mobilities of the different OccR-DNA complexes were measured from an arbitrary standard reference point and plotted against octopine concentration. wt, wild type. C, effects of mutations B1, B2, and B3 on octopine-responsive gel mobilities. D, effects of mutations C1 and C2 on octopine-responsive gel mobilities.
Mutations That Alter OccR-induced DNA Bending

Wild-type and representative mutant operator fragments from the indicated plasmids were end-labeled on the top strand and incubated in the absence of OccR (lanes 2), with 4 μM OccR (lanes 3), with 4 μM OccR and 10 μM octopine (lanes 4), or with 4 μM OccR and 300 μM octopine (lanes 5). Lanes 1 shows the positions of G and A residues. A, pRA201 (WT); B, pRA202 (mutant A1); C, pRA203 (mutant B1); D, pLW142 (mutant C1); E, pLW143 (mutant C2).

FIG. 4. DNase I footprinting of mutant operators. Wild-type and representative mutant operator fragments from the indicated plasmids were end-labeled on the top strand and incubated in the absence of OccR (lanes 2), with 4 μM OccR (lanes 3), with 4 μM OccR and 10 μM octopine (lanes 4), or with 4 μM OccR and 300 μM octopine (lanes 5). Lanes 1 shows the positions of G and A residues. A, pRA201 (WT); B, pRA202 (mutant A1); C, pRA203 (mutant B1); D, pLW142 (mutant C1); E, pLW143 (mutant C2).

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Wild type at all higher octopine concentrations (Fig. 3C). Site 3 of the wild-type operator shows very little similarity to sites 4 or 5, yet the position of this sequence suggests that it could contact OccR. We conclude that OccR does not recognize the wild-type site 3 but can recognize a high affinity site when one is created at that position.

Mutations that disrupt sites 1 or 2 were described previously (17) but not tested for bending over a range of octopine concentrations and never tested in vivo in A. tumefaciens. We found that a mutation disrupting site 1 almost completely locked the complex into a fast-migrating conformation (Fig. 3A, lanes 8; Fig. 3D). In contrast, a mutation disrupting site 2 and 2’ caused complexes to migrate primarily in the slow conformation (Fig. 3A, lanes 9; Fig. 3D).

There was a formal possibility that the differences in gel mobility that we observed were due to differences in the binding affinity of OccR or to differences in the stoichiometry of OccR-DNA complexes rather than due to a difference in conformation. These bend assays require that the bends be positioned close to the center of the DNA fragment (30), and plasmid pBend3 is designed to allow the permutation of the bend formation close to the center of the DNA fragment (30), and plasmid pBend3 is designed to allow the permutation of the bend. Gel retardation assays were therefore carried out with OccR, and fragments were generated by digestion with a combination of EcoRI and SalI. This causes the DNA bend center to lie close to one end of the fragment. All the OccR-DNA complexes migrated at virtually the same mobility in the presence or absence of octopine (data not shown), indicating that the same number of OccR monomers are bound to the different operator mutants. This indicates that the differing gel mobility rates seen in Fig. 3A are due to differing bend angles.

**DNase I Footprinting of Operator Mutants**—We established previously (7) that slow migrating OccR-DNA complexes have a DNase I footprint of ~60 nt, whereas less bent complexes have a DNase I footprint of about 50 nt (Fig. 4A). We wanted to make sure that the low mobility complexes observed in this study had the characteristic long footprint and that high mobility complexes had the shorter footprint. DNase I footprinting assays were conducted with OccR and the occQ-occR intergenic region containing four of the operator mutations described above.

Octopine was added to the DNA-binding reactions at 0, 10 (a limiting concentration for changes in OccR conformation), and 300 μM (a saturating concentration).

The mutant operator A1 had very similar footprints in the absence or presence of octopine (Fig. 4B). These footprints resembled the longer footprint of the wild-type operator (made in the absence of octopine). The promoter proximal end was slightly less protected with high levels of octopine than in its absence. The change in DNase I hypersensitivity seen with the wild-type complex is also absent from this mutant. These results correspond precisely with the patterns observed from the DNA bending assays and establish a clear correlation between slow migrating OccR-DNA complexes observed by native PAGE and long-footprint OccR-DNA complexes seen by DNase I footprinting.

The mutant operator B1 showed the converse pattern (Fig. 4C). It showed the long footprint in the absence of octopine but showed the short footprint in the presence of just 10 μM octopine. The footprint length did not change with the higher level of octopine, although a slight change in hypersensitivity is seen at the higher octopine concentration. Although the DNA sequence at this site has been altered in this mutant, it still displays a strong hypersensitivity comparable with that of the wild-type OccR-DNA complex.

Mutant C1 showed a short footprint in the presence or absence of octopine (Fig. 4D), agreeing with the high gel mobility of this mutant. Addition of 300 μM octopine caused little if any alteration in footprint. Finally, the mutant C2 showed a long footprint at all octopine concentrations, as expected (Fig. 4E). Here too, the high level of octopine caused only slight changes in the footprint.

**Operator Mutants Have Altered Activity in Vivo**—We wanted to test the activity of these mutant promoters in vivo. Fragments containing these promoters were introduced into a broad host range promoterless lacZ reporter plasmid, pRA302, to create occQ-lacZ translational fusions. The resulting plasmids were introduced into A. tumefaciens strains lacking the Ti plasmid and containing or lacking a second plasmid that expresses OccR. We predicted that complexes that are fully or partly locked in a high angle DNA bend conformation may not be inducible by octopine or may require unusually high amounts of octopine for induction, whereas mutations that fully or partially lock OccR into a low angle DNA bend may be constitutive or may require just trace amounts of octopine for induction.

As expected, mutations A1, A2, and A3, which cause OccR to favor a high angle DNA bend, showed far less occQ-lacZ expression than wild type. The wild-type promoter was fully induced by just 1 μM of octopine, whereas these mutants were impaired at β-galactosidase expression at all octopine concentrations (Fig. 5A). A mutation that disrupts site 2 (mutant C2), and therefore caused complexes to favor the high angle bend (Fig. 3D), also blocked transcription (Fig. 5C). These results correlate well with the DNA bending results.

When we tested the activity of OccR binding to mutant operators that lock OccR into the low angle bend conformation (mutants B1, B2, and B3), we obtained some unexpected results. Almost no activity was observed unless very high levels of octopine (300 μM) were provided (Fig. 5B), and expression was impaired even then. We had anticipated the opposite result, because these mutations cause OccR to favor a conformation that resembles the active conformation. Formally, this lack of induction could be caused by an altered recognition of the promoter by RNA polymerase, because these mutations alter...
the operator in a region upstream of the −35 motif of this promoter, where the α-subunit of RNAP is known to bind several promoters (31).

Mutant C1, which disrupts site 1 (Fig. 1), showed especially interesting properties in vivo. This mutant, when bound by apo-OccR, caused a severe decrease in bend angle (Fig. 3D) and in footprint length (Fig. 4D), suggesting that it could be constitutively active in vivo. However, this promoter was inactive in the absence of octopine and showed approximately wild-type activity over a broad range of octopine concentrations (Fig. 5D).

The finding that mutant C1 approximates wild type in its response to octopine indicates that attaining a low angle DNA bend conformation is not sufficient for transcriptional activation. Octopine must therefore cause additional changes in OccR conformation that are required for it to activate transcription.

**DISCUSSION**

In an effort to understand how OccR specifically binds its operator DNA, we first attempted to determine the stoichiometry of these complexes. Our finding that OccR is dimeric in solution and tetrameric when DNA-bound is consistent with the extended footprints at the occQ operator and with earlier studies that OccR binds DNA cooperatively (with a Hill coefficient of 2.0). Several other LysR family proteins were previously shown to be either dimers or tetramers in solution. For example, NodD3 of Sinorhizobium meliloti, MetR of E. coli, CatR of P. putida, and ClcR of P. putida have been reported to be dimers in solution (32, 33, 37), whereas OxyR of E. coli, CysB of S. typhimurium, Trpl of Pseudomonas aeruginosa, and NahR of P. putida have been shown to be tetramers in solution (12, 13, 34, 35). In some cases, gel filtration was carried out at high protein concentration, which might force the protein into a higher oligomeric state than found in vivo. To our knowledge, the only protein whose stoichiometry has been determined when bound to DNA is CysB, which binds as a tetramer (36). Several other LysR proteins have been hypothesized to bind as tetramers on the basis of the lengths of their DNase I footprints.

We previously observed that sites 4 and 5 contain a high affinity OccR-binding site, probably for one OccR dimer, and that the sequences ATAAAN7TTAT, which span this site, are essential for high affinity binding. In this study, we tested the hypothesis that placing similar sequences at sites 1 and 2 (whose native sequences are ATTC and TTCA, respectively) would reduce the ability of OccR to alter its conformation in response to octopine. This hypothesis was largely confirmed in that complexes containing this mutant sequence (mutant A1) required very high levels of octopine to cause a limited conformational change. Changing just site 1 (from ATTC to ATAA, mutant A2) or changing just site 2 (from TTCA to TTAT, mutant A3) had similar but more subtle effects. These data support the hypothesis that the sequence ATAAAN7TTAT plays a fundamental role in the affinity of OccR for its operator and that placing these sequences at sites 1 and 2 can cause OccR to bind at these positions more strongly than it binds the wild-type sequence.

The same hypothesis was further tested by altering sites 2′ and 3 (ATTC and CCGG, respectively) to ATAA and TTAT. This alteration had no effect on conformation in the absence of octopine. However, very low levels of octopine were sufficient to induce a drastic conformational change, supporting our hypothesis. Changing both sites 2′ and 3 caused a slight change in bend angle in the absence of octopine and caused a full shift, with as little as 10 μM octopine. Altering just site 3 (from CCGG to TTAT, mutant B3) gave a somewhat unexpected result, in that this mutant was similar to mutant B1 in the absence of octopine, but showed a lower bend angle than mutant B1 (and even lower than wild type) in the presence of higher concentrations of octopine. We conclude that the native site 3 may not be recognized specifically by OccR, but that when a high affinity site is created at this position, it can readily be decoded by OccR.

Sites 1 and 2 were further studied using mutations that abolish all or part of each sequence. Disruption of site 1 (mutant C1) caused complexes to have a low angle DNA bend at all octopine concentrations and to be little affected by octopine. This indicates that site 1 is essential for the OccR conformation...
that causes a high angle DNA bend and a long footprint. Disruption of site 2 and 2′ (mutant C2) caused a high angle DNA bend at all octopine concentrations. We interpret this to mean, first, that site 2 is not essential for the high angle bend and long footprint and, second, that site 2′ is essential for the low angle DNA bend and short footprint.

We had anticipated that operator mutations that caused OccR to favor the conformation having a low angle bend in vitro would cause it to transcribe the occQ promoter constitutively in vivo. However, this was not the case, as such mutants either were impaired for induction or had properties similar to wild type in vivo. This is most clearly shown with mutant C1, whose conformation in vitro drastically favors the low angle DNA bend, but whose activity in vivo is still dependent on the presence of octopine and is only slightly more sensitive to the inducer than the wild type. Octopine must therefore cause a conformational change in OccR that cannot be mimicked by this operator mutation.

Mutation C1 caused a reproducible but very slight (2–10-fold) increase in responsiveness to octopine concentrations between 1 and 8 nM. However, at octopine concentrations 16 nM or higher, the promoter was slightly impaired in activity. The most straightforward interpretation is that this mutation caused a slight increase in responsiveness to octopine, in accord with our original predictions, but failed to reach wild-type expression levels at high octopine concentrations because of impaired contacts with RNA polymerase.

It remains somewhat puzzling that mutants B1, B2, and B3 are strongly impaired for activity even in the presence of octopine. Because each of these mutations cause OccR to favor the conformation having a low angle bend, we anticipated that these mutants would also cause OccR to act constitutively or to need only low concentrations of octopine. One possibility is that these mutants affect RNA polymerase contact sites, although it is surprising that sequences at both sites would be so critical. An alternative possibility is that this mutant traps OccR in a conformation that resembles the active conformation but actually differs from it in some way that is critical for activation.

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