DNA sequence determinants of LexA-induced DNA bending

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ABSTRACT

The LexA repressor from Escherichia coli induces DNA bending upon interaction with the two overlapping operators which regulate the transcription of the colicin A encoding gene cee. Both cee operators harbor T-tracts adjacent to their recognition motifs. These tracts have been suggested to be especially favorable for the promotion of LexA-induced DNA bending. Here we show that this is indeed the case, since disruption of the TTTT-tract adjacent to operator O1 by the replacement of the two central thymin bases by AA, GA or CG markedly reduces LexA-induced DNA bending. Simple A-T-richness in this position is thus not sufficient to promote full LexA-induced bending, albeit a TAAT sequence is always more efficient to promote bending than those sequences containing one or two C/G base pairs.

INTRODUCTION

The LexA repressor from Escherichia coli is a sequence-specific DNA binding protein which negatively regulates the initiation of transcription of about 20 so-called SOS genes which are induced upon DNA damage (for recent reviews see 1—3). The regulatory strategy differs from one gene to the other in that LexA binds to either one, two, or three adjacent operators, and in that the LexA binding sites are located in variable positions within the different promoters. In those cases where a LexA binding site overlaps one of the two conserved hexameric promoter elements the protein should act as a competitive inhibitor of RNA polymerase binding as shown in the case of the uvrA gene (4).

Each operator interacts with a LexA dimer such that the two centrally located monomers will not adopt exactly the same binding geometry as the two external monomers (7,8). Electrophoretic mobility shift assays revealed the presence of two complexes for a low degree of DNA saturation. One species corresponds to a single occupied operator, the other to a complex where both operators are occupied. Both complexes show LexA-induced DNA bending, and the presence of a small intrinsic bent of the free DNA centered on the operator region suggested to us that a T4- and an A4-stretch adjacent respectively to the left and to the right side of the operator region might be involved in LexA-induced DNA bending (9).

Using single-operator mutations, which confine LexA to one or the other of the two operators, we show here that DNA flexure upon LexA binding to the left operator (O1) is more pronounced than upon binding to the right operator (O2). This difference is most likely due to the nature of the DNA sequence flanking the operator region as shown by symmetrizing the internal elements of the two operators. A mutational study of the TTTT-stretch situated 5' to the CTGT-recognition motif of operator O1 showed that this intrinsically bent sequence is indeed especially favorable to promote LexA-induced DNA-bending. Simple A-T-richness is not sufficient to promote full LexA-induced bending, albeit a sequence like TAAT is always more efficient to promote LexA-induced bending than sequences like TGAT and TCGT containing one or two C/G base pairs.

MATERIALS AND METHODS

Plasmid and mutant operator construction

Bending mutant operators within the T4-stretch 5' to the CTGT-motif of operator O1 (see figure 1) were constructed by site-directed mutagenesis (10) in the context of a O1+O2- operator in which binding to operator O2 is suppressed by a double-mutation of the external CTGT-motif of operator O2 (CTGT—CCAT). We used further a O1—O2+ operator (for which binding to O1 is abolished) to construct a new O2 operator identical to O1 called O1—O2+(sym) (see figure 1). The matrix for oligonucleotide-directed mutagenesis was a M13mp19 phage harboring the cee regulatory region (8).
In order to facilitate a bending analysis by circular permutation a special vector (pHF) was constructed as follows: a pUC12 vector was linearized with PstI, dephosphorylated, and recut with ScaI. Two purified DNA fragments were simultaneously inserted into Smal site of pHF to give pHL, pHM and pHN plasmids respectively. These plasmids were selected by DNA sequencing into the Smal site of pHF for bending analysis.

RESULTS

DNA curvature is more pronounced upon LexA-binding to operator O1 than to operator O2

In order to elucidate the DNA sequence requirements for LexA-induced DNA bending of the caa regulatory region, we have compared in a first step the relative degree of DNA deformation upon LexA-binding to operators O1 and O2. The two operators are generally occupied in a cooperative manner, but mutations within one or the other operator allow to study LexA binding to each operator independently (8).

A comparison of lanes 1 and 3 in figure 3A shows that a DNA fragment harboring the caa operators O1+O2-, O1+O2+ and O1+O2+ were cut with EcoRI, 5' end-labelled and recircularized with ligase. After ligation, dephosphorylation was performed to remove the 5' end-labels from the linear fragments. The 5' labelled circular plasmids were then recut with the following restriction enzymes: ScaI, NcoI, PvuII, BstNI and PstI giving 291 bp DNA fragments.

For competition binding analysis two or three different operators were used in the same band shift assay. The different operator DNA fragments were: EcoRI-HincE (224bp) for O1+O2+; BstNI-HincE (177bp) for O1+O2+; SacI-HincE (218bp) for O1+O2-. These plasmids were selected by DNA sequencing into the Smal site of pHF to give pHH1, pHM and pHN plasmids respectively. In the first case double-stranded M13mp19 DNAs harboring the caa operators O1+O2-, O1+O2+ and O1+O2+ were cut with EcoRI, 5' end-labelled with 32P, and recut with PvuII giving 364 bp DNA fragments with centrally located operators (see figure 1) which were purified by polyacrylamide gel electrophoresis and electroelution.

For the circular permutation analysis plasmids pHL, pHM and pHN were linearized with XbaI, 5'end-labelled and recircularized with ligase. These assays were done as previously described using 5% polyacrylamide gels (8) with DNA fragments derived either from double-stranded phage DNA or from plasmids pHL, pHM, and pHN (see above). In the first case double-stranded M13mp19 DNAs harboring the caa operators O1+O2-, O1+O2+ and O1+O2+ were cut with EcoRI, 5'end-labelled with 32P, and recut with PvuII giving 364 bp DNA fragments with centrally located operators (see figure 1) which were purified by polyacrylamide gel electrophoresis and electroelution.

In order to insert different caa operator constructions into pHF, double-stranded DNAs from M13mp19 phages harboring respectively the caa operators O1+O2-, O1+O2- and O1+O2+ were purified, 80 bp HincII-HgiAI fragments (see figure 1) were isolated, subjected to Klenow polymerase treatment and inserted into the Smal site of pHF to give pHL, pHM and pHN plasmids respectively. These plasmids were selected by DNA sequencing such that all three operator constructions were cloned in the same orientation.

Electrophoretic mobility shift assays

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A comparison of lanes 1 and 3 in figure 3A shows that a DNA fragment harboring the LexA binding sites roughly in the center of the fragment is more strongly retarded if LexA binds to operator O1 (mutant operator O1+O2-) than upon binding to operator O2 (mutant operator O1+O2*). This difference in migration upon O1 or O2 occupancy depends on the location of the LexA binding site(s) within the DNA fragment, since the two insertion of a synthetic DNA fragment (sequence comprised between base pairs 185 and 211 in figure 2) and a SacI-PstI DNA fragment of pColA9 (161 bp) into a BamHI-PstI linearized pTTQ19 vector (12). The pHF plasmid for bending analysis contains thus a tandem sequence with 7 restriction sites present in duplicate and a central multiple cloning site (see figure 2).

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We may conclude from these experiments that the difference in LexA-induced curvature of the DNA fragments for which the LexA binding site is close to the bending loci of the two operators, then the mobility difference observed in figure 3A between complexes formed at O1 and O2 might in principle be simply due to the fact that the bending loci of the two operators would be potentially displaced by as much as 35 base pairs. The operator O2 bending locus would be closer to the EcoRI end of the DNA fragment than the O1 bending locus (see figure 1) giving potentially rise to a position-dependent increased gel mobility of the LexA-O2 complex as compared to the O1 complex.

In order to address this possibility the O1*O2- and O1~O2~ mutant operators have been subcloned into a plasmid (pHF) suitable for a convenient application of the 'circular permutation assay', which consists in creating a family of DNA fragments of identical length but variable position of the protein binding site (13). Figure 3B shows the expected bell-shaped migration potential of the DNA fragment than the O binding site within the Lac promoter and the left half-site of the CAP binding she dyad axis (•).

A comparison of lanes 3 and 4 in figure 3A shows that LexA-induced curvature upon binding to O2 does not increase if the internal sequence of O2 between the two bold-faced recognition-motifs is identical to the internal sequence of O1. Similar results were respectively obtained with the O1*O2- and O1~O2~ operators.

In order to address the question if the difference in mobilities comigrate if the binding sites are close to one end of the DNA fragment (see figure 2 in ref. 8).

We may conclude from this experiment that the observed mobility difference between the complexes formed at O1 and O2 most likely arises from the nature of the DNA sequences situated 5' to the conserved CTGT motif (the sequence 3' to the conserved ACA motif is the same for both operators), i.e. the immediately adjacent TTTTTA and AAAAA sequences and possibly sequence elements even further away from the center of the two operators.
A T₄-stretch adjacent to the LexA binding site is particularly favorable for protein-induced bending

In the following we have subjected the TTTT-stretch preceding operator O₁ to a mutational study, first, because this sequence seems to support LexA-induced curvature more efficiently than the A₅-stretch adjacent to O₂, albeit an influence of sequence elements even farther apart from the dyad axis may not be excluded. Second, the T₄-stretch is localized just in front of the transcriptional start point at the following adenine base and LexA-induced DNA bending in this region might contribute to transcriptional repression, since RNA polymerase has been reported to induce DNA bending and/or increased flexibility in base pairs upstream of the initiation point of RNA synthesis in the case of the A₁ promoter of bacteriophage T₇ (19), i.e. in a position which would be situated in the case of the cco promoter within the TTTT motif.

Three different O₁⁺O₂⁻ mutant operators have been constructed which contain respectively TAAT, TGAT and TCGT- motifs (instead of the wild-type TTTT-motif) in front of operator O₁. The choice of the mutated dinucleotides in the center of the T₄-stretch was based on the dinucleotide ranking deduced from CAP-induced DNA bending (see table 2 in reference 20). According to this ranking an AA-step is as favorable for DNA bendability towards the minor groove as a TT-step, whereas GA- and CG-steps are increasingly unfavorable for this type of DNA bending.

Figure 4A shows however that all three mutant motifs are less efficient in supporting LexA-induced DNA bending than the original TTTT-motif. The order of bendability is such that:

$\text{TTTT} > \text{TAAT} > \text{TGAT} \equiv \text{TCGT}$,

i.e. simple A-T-richness of this sequence is not sufficient to support full LexA-induced bending. However an A-T-rich sequence in this position is still more favorable than a sequence containing one or two G/C-base pairs. This latter finding is in agreement with the DNA sequence determinants of bending induced by the CAP protein, whereas the preference of LexA for an intrinsic bent sequence over simple A-T-richness seems to be a particular feature of the LexA complex.

DNA binding affinity

We asked further if mutations within the T₄-stretch have a measurable influence on the DNA binding affinity of LexA. Since the differences in binding affinity are expected to be small, because these mutations are located outside the recognition sequence, we have used a competition gel retardation assay. DNA fragments containing mutant binding sites were mixed with a DNA fragment of different size containing the wild-type cca regulatory region and incubated with variable amounts of the LexA repressor. The different species were resolved by gel electrophoresis and the relative amounts of free and bound DNA were determined by densitometry of the autoradiographs. The relative amount of LexA, with respect to the wild-type sequence, necessary to bind half of the DNA was 0.9 for TAAT, 1.5 for TGAT and 2.2 for TCGT. Those DNA fragments which support DNA bending poorly (TGAT and TCGT) have thus an about two-fold smaller binding affinity for the LexA repressor. However as in the case of the CAP protein (17, 20) the correlation between binding strength and bending is not perfect in that TGAT and TCGT show essentially the same degree of bending but different binding strength, whereas TTTT and TAAT show a different degree of bending but similar binding affinity.

DISCUSSION

Protein-induced DNA bending has been observed for numerous sequence specific DNA binding proteins including transcriptional activators and repressors (for reviews see 15, 17). Only in very few cases the DNA sequence determinants for protein-induced DNA bending have been addressed, the most thoroughly studied example being the CAP protein (17, 20). In this case A-T-richness in a sequence element flanking the recognition element is necessary and sufficient to support DNA bending. An earlier study on LexA-induced bending of the colicin A regulatory region suggested that in this case a simple A-T-rich sequence flanking the recognized base pairs might not be sufficient to support full bending activity, but that a segment of intrinsically bent DNA might be required. Here we show that this hypothesis was essentially correct, since the disruption of a T₄-tract flanking the cca operator O₁ increases the gel mobility of the LexA-DNA complex.

Unexpectedly the A₅-tract flanking operator O₂ seems to be less efficient than the T₄-tract adjacent to operator O₁ in supporting LexA-induced DNA-bending. This difference is only observed upon inactivation of one of the operators, since the wild-type cca operator does not give rise to two different single-operator complex species (see figure 3A, lane 2 and reference 9). This finding is likely to be due to dissociation and reassociation events during gel electrophoresis, which may lead to the appearance of a single intermediate complex or even a complete disappearance of any intermediate complex as shown in the case of the Tet repressor-operator system (21). In the LexA case the intermediate complex with the wild-type cca operator (O₁⁺O₂⁻) migrates close to the position of the O₁ complex (see figure 3A) in agreement with earlier findings that O₁ binds LexA more tightly than O₂ (8).

The finding that the A₅-tract adjacent to O₂ seems to support bending less efficiently than the T₄-tract adjacent to O₁ is unexpected in view of the observation that the gel mobility of intrinsically bent sequences like (A₅x₅x₅T₄x₅x₅)ₖ is in general similar to that of sequences like (A₅x₅x₅T₄x₅x₅)ₖ suggesting that the direction and magnitude of bending is largely independent of the orientation of the (dA)-(dT) tract (22). However, if the gel mobility of these sequences is examined in the presence of a ligand like Mg²⁺, the gel mobility of these sequences is not anymore identical (23) and one may suppose that a protein ligand like LexA might also disrupt this apparent symmetry.

In the case of the CAP protein, Gartenberg and Crothers (20) have shown that maximal bending occurs when a site situated at about one helical turn from the binding site dyad is A-T rich, whereas minimal bending occurs when this site is G-C rich. The reverse pattern, though less pronounced, is observed at a site centered roughly 16 base pairs from the dyad axis. Alignment of the dyad axes of the CAP and the LexA binding sites (figure 4B) shows that the three dinucleotide steps of the T₄-tract adjacent to the cca operator O₁ (i.e. steps 12, 11 and 10 with respect to the dyad axis) coincide with the three dinucleotide steps of the CAP binding site which are most sensitive to the incorporation of G/C base pairs in terms of reduction of DNA bending (17). Figure 4B summarizes further the bendability values for the three dinucleotide steps as deduced from CAP-induced bending (20). The CAP-based average values show that TTTT and TAAT should support bending with equal efficiency. This is clearly not the case for LexA-induced bending (as shown in figure 4A) suggesting that the DNA segment with the property to form an intrinsic bent is more favorable to support LexA-
induced bending. LexA is likely to enhance the natural tendency of T-tract bending towards the minor groove as suggested earlier (9) in order to form additional, most likely non-specific electrostatic contacts with nucleotides outside the specific recognition sequence.

DNA backbone atoms situated at the 3'-edge of the TTTT-stretch are expected to be in contact with LexA even in the absence of intrinsic DNA bending, since in the case of the recA operator, those phosphates for which ethylation suppresses complex formation (24) extend until a position corresponding to the phosphate between the third and the fourth thymine of the TTTT-tract (i.e. the position corresponding to dinucleotide step 10 in figure 4B). Furthermore the two nucleotides belonging to this step may be crosslinked with a photoreactive agent attached site-specifically to a LexA mutant repressor harboring a cysteine in position 52 (25). It is thus plausible that even minor changes of the DNA path due to intrinsic and/or induced DNA bending might bring DNA segments situated further apart from the dyad axis (most probably: steps 11 and 12) in close contact with the protein.

Once the TTTT-tract (and thus intrinsic bending) has been disrupted, LexA seems to behave similar to the CAP protein in that G/C base pairs in these positions unfavour protein-induced bending with respect to A/T base pairs. In the case of the CAP protein this observation has been linked to the bendability of these sequence elements towards the minor groove allowing the DNA to wrap around the protein (20).

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