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The RepA repressor can act as a transcriptional activator by inducing DNA bends

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We have shown that a transcriptional repressor protein can regulate promoter activity via DNA bending by using the pLS1 plasmid promoter $\underline{\mathrm{P}}_{\mathrm{II}}$ (which has intrinsic curvature upstream of its $-\underline{35}$ box) and the plasmid-encoded repressor protein RepA (which strongly bends DNA). Substitution of the curved region for a straight DNA fragment containing the RepA target resulted in increased (or decreased) gene expression when RepA was supplied *in trans*: enhanced gene expression was evident when the target of RepA and the promoter were on the same face of the DNA helix; repression was found when they were on opposite faces of the DNA. *In vitro* activation of transcription from $\underline{\mathrm{P}}_{\mathrm{II}}$ was observed when supercoiled DNA was used as template, but not with linear molecules. We propose that promoter activity can be regulated by the proper positioning (in or out of phase) of an induced DNA bend with the RNA polymerase recognition sites.

Key words: DNA–protein interactions/transcriptional activation/RepA repressor/DNA binding

Introduction

Intrinsic curvatures in DNA seem to be a target for a variety of proteins which act by activation or repression of replication, recombination or transcription (reviewed by Travers, 1989). In prokaryotic promoters, regions upstream (up to −130) of the start-point of transcription are important for RNA synthesis (Lamond and Travers, 1983; Bossi and Smith, 1984; Bracco *et al.*, 1989; Tsung *et al.*, 1990), and curving of those upstream DNA regions seems to be a general feature (Plaskon and Wartell, 1987). [According to Trifonov (1985), 'curving' is the sequence-directed distortions in a DNA tract, and 'bending' the induced deformations in DNA.] In addition to the role of DNA curvature, transcriptional control can be mediated by proteins which bind to and bend DNA sequences upstream of promoters. It was early suggested that DNA bends induced by proteins could play a central role in the formation and activity of the transcription initiation complex (Crothers and Fried, 1983), apparently by facilitating the wrapping of the RNA polymerase around the DNA (Amouyal and Buc, 1987). Generation of such high order structures may be facilitated if the DNA is intrinsically curved and/or bent by a protein (Trifonov and Ulanowski, 1987). In fact, it has been shown that both eukaryotic (Schinkel *et al.*, 1988) and prokaryotic (Kuhnke *et al.*, 1989) RNA polymerases are able to bend the DNA upon its binding.

DNA deformations induced by eukaryotic and prokaryotic transcriptional repressors or activators may take place in the vicinity of the promoters (Gartenberg and Crothers, 1988; Gustafson *et al.*, 1989; Pérez-Martín *et al.*, 1989; Schroth *et al.*, 1989; also earlier references therein). However, in many instances, transcriptional activation can be exerted by the binding of proteins to *cis*-acting DNA sites located upstream of promoters (Guarente and Hoar, 1984; Nilsson *et al.*, 1990), causing the DNA to bend (Vignais and Sentenac, 1989). Within many of these upstream regions, there are specific sites recognized by regulatory proteins that act at a distance by the formation of a DNA loop (Majumdar and Adhya, 1984; Hendrikson and Schleif, 1984; reviewed by Adhya, 1989). Another category of DNA–protein interaction includes the histone-like (HU) proteins that bend or wrap the DNA, generating a long-range effect on DNA promoters beyond their contact sites [such as the *Escherichia coli* integration host factor (IHF); reviewed by Friedman, 1988; Nash, 1990]. It thus appears that control of transcription may involve the formation of specific complexes between DNA, RNA polymerase and one or more regulatory proteins.

Several experiments have been designed to show the feasibility of functional replacement of DNA modules if they are curved or subjected to bending by a protein (Bracco *et al.*, 1989; Collins *et al.*, 1989; Goodman and Nash, 1989). By specific changes of certain amino acids, the negative 'regulator of transcription, lambda Cro can be converted into an activator through the 'acidic activating patch' (Bushman and Ptashne, 1988; Ptashne, 1988). These approaches imply either the use of transcription activators acting on promoters different from those usually recognized by them, or the manipulation of a repressor protein. Nevertheless, a recent report has shown that the phage ϕ29 p4 activator protein is able to activate transcription from the phage P_{A3} promoter by binding to, and bending at specific regions located 50–100 nucleotides upstream of the transcription start-point (Rojo *et al.*, 1990). In an attempt to develop a unified model for the regulation of the initiation of transcription, we show here that a natural transcriptional repressor, the pLS1 plasmid-encoded RepA protein (del Solar *et al.*, 1989, 1990), can regulate *in vitro* and *in vivo* the transcription from a promoter. This was achieved by substitution of curved regions upsteam of the promoter by a straight DNA fragment containing the target of the repressor: depending upon the helix phasing of the RepA target relative to the promoter, activation of repression was observed.

Results

Construction of the system

Our rationale was that if curving of DNA *per se* were enough for promoter activation, induction of DNA bends by any protein should also result in enhanced gene expression.

A clear demonstration of our reasoning would arise if the selected protein, in addition to bending DNA, were a trancriptional repressor. We selected RepA, a pLS1 plasmid-encoded *trans*-complementing protein of 45 amino acids (del Solar *et al.*, 1989), because it is a transcriptional repressor which bends pLS1 DNA (del Solar *et al.*, 1990; Pérez-Martín *et al.*, 1989). As the target for the presumed RepA-mediated activation, we selected promoter $\underline{P}_{II}$ from the copy-number control antisense RNA II of plasmid pLS1 (Lacks *et al.*, 1986; del Solar *et al.*, 1990) because: (i) the promoter exhibits curving upstream of the −35 region (Pérez-Martín *et al.*, 1988); (ii) the presence of the curved regions stimulates transcription from $\underline{P}_{II}$ (J.Pérez-Martín and M.Espinosa, in preparation), and (iii) RNA II might be expressed constitutively and it should have a high transcription rate because of its inhibitory function (Novick, 1987). Two curved regions upstream of the $\underline{P}_{II}$ promoter were detected (Pérez-Martín *et al.*, 1988). Their centres of curvature were located at about 30 and 180 bp upstream of the −35 box of $\underline{P}_{II}$, as determined by circular permutation tests (Wu and Crothers, 1984). Binding of the *E.coli* RNA polymerase to DNA fragments containing $\underline{P}_{II}$ resulted in only one strong bend with its centre displaced towards this promoter (not shown).

Figure 1 shows the construction of plasmids used throughout. Removal of the curved regions upstream of $\underline{P}_{II}$ (plasmid pLSM1ΔX), and their substitution for the pLS1 region containing the RepA recognition site yielded plasmid pUCOPR. By footprinting assays, we have shown that the region protected by purified RepA protein contains a 13 bp symmetric element (most likely the operator; del Solar *et al.*, 1990). Cloning a promoterless *E.coli lacZ* gene on pUCOPR gave pLSMpOPR, in which the region containing the target of RepA is placed upstream of the $\underline{P}_{II}$ promoter-*lacZ* fusion without the curved loci. Plasmids lacking $\underline{P}_{II}$ (pLSMpOP), the RepA target (pLSMpR), or a 4 bp insertion within this region (pLSMpOPRΩ4) were constructed from pLSMpOPR. Plasmid pLSMP, carrying the promoterless *lacZ* gene was also employed as control. Computer-generated predictions of the DNA structure (Figure 2A) showed a curvature upstream of $\underline{P}_{II}$, which should be abolished by the substitution of this curved region by the RepA recognition target (Figure 2B). These predictions were confirmed by gel electrophoresis assays performed at different temperatures (not shown). The $(dA)_6$ tract placed between the RepA operator and the −35 box of $\underline{P}_{II}$ (Figure 2C) might create a local DNA deformation (Wu and Crothers, 1984). Two helical turns downstream of the $(dA)_6$ tract, and within the −35 box of $\underline{P}_{II}$, a $(dT)_5$ stretch is located. Both tracts (also present in the wild type $\underline{P}_{II}$ promoter) may not be sufficient to generate a strong DNA curvature (Travers, 1989), but it could facilitate the opening of the strands (Ramstein and Lavery, 1988). Cylindrical projection of the nucleotide sequence around the region containing the RepA target−$\underline{P}_{II}$ promoter fusion, showed that the sequence 5′-GAGTG-3′ was in phase with the −35 and the −10 boxes of $\underline{P}_{II}$, being separated from them by five and seven helical turns, respectively (not shown). This sequence is protected from hydroxyl radical cleavage by purified RepA protein and is included within the RepA operator (del Solar *et al.*, 1990). As a consequence of the 4 bp insertion at the single *Xba*I site (Figure 2C), plasmid pLSMpOPRΩ4 has the RepA target and the $\underline{P}_{II}$ promoter out of phase. The operator−promoter fusion in pLSMpOPR did not alter the start site of transcription from $\underline{P}_{II}$ (compared with pLSMpR), as demonstrated by endonuclease S1 mapping of the 5′-end of the RNA synthesized from this promoter (Figure 2D).

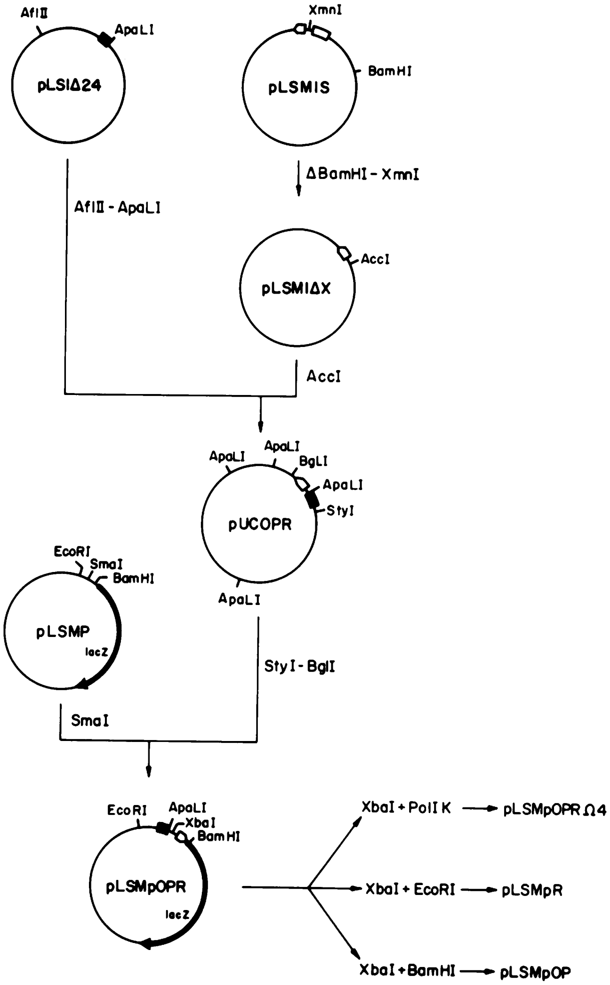

Fig. 1. Construction of the fusion between the RepA target (■) and $\underline{P}_{II}$ promoter (▷). Curved regions upstream of $\underline{P}_{II}$ (□) are also indicated. Plasmid pLSMpOPR and a scheme with the construction of its derivatives are shown at the bottom. Only relevant restriction sites are indicated.

Specific DNA binding and DNA bending mediated by RepA

To know whether placing the RepA target upstream of $\underline{P}_{II}$ resulted in specific DNA bending, we determined the electrophoretic migration of DNA molecules (Zinkel and Crothers, 1987) complexed or not with purified RepA protein. The analyses were performed in agarose−acrylamide composite gels since their high sensitivity allows the detection of a major bend in a relatively large DNA molecule (Mukherjee *et al.*, 1985; Pérez-Martín *et al.*, 1989). The results (Figure 3A) showed that RepA specifically induced a major bend in the DNA of plasmids pUCOPR and pUCOPRΩ4, the bend being located around the region that includes the target of RepA. Furthermore, RepA did specifically bind to its target, since the addition of increasing amounts of purified protein to plasmid pUCOPR resulted in the progressive protection of the *Apa*LI site (but not of

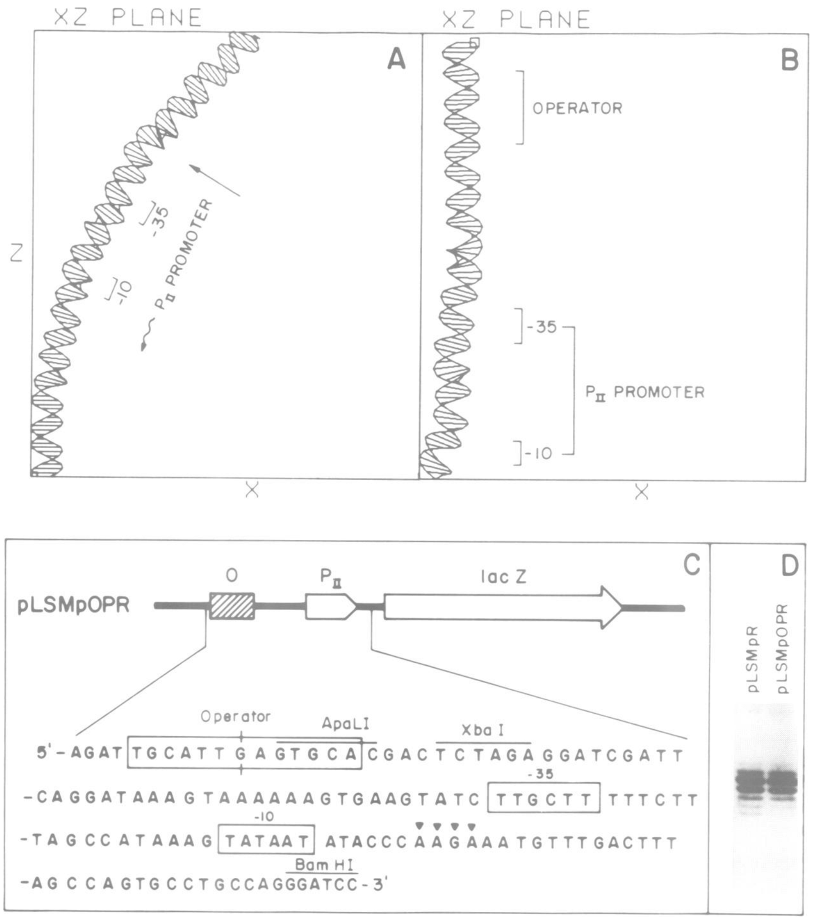

Fig. 2. Features of the $\underline{P}_{II}$ promoter–RepA target fusion. Prediction of the DNA structure in the regions surrounding $\underline{P}_{II}$ in plasmids: pLS1 (**A**; curved centre indicated by the arrow), and pLSMpOPR (**B**). Panel **C**: schematic map of pLSMpOPR and nucleotide sequence of the $\underline{P}_{II}$ promoter–RepA target fusion; relevant positions and site(s) of initiation of transcription (black triangles) are indicated. Filling in at the *Xba*I site results in the 4 bp insertion: 5'-CTAG-3' present in plasmid pUCOPRΩ4. Panel **D**: nuclease S1 mapping of the 5'-end of the mRNA from pLSMpR and pLSMpOPR. The 109 bp *Xba*I–*Bam*HI fragment (see panel C) was used as a probe.

the other *Apa*LI sites) located within the RepA operator (Figure 3B; see Figure 2C). Similar results were obtained when the same experiment was performed with pUCOPRΩ4 (not shown). Taking all the above results together, we may conclude that the fusion between the promoter $\underline{P}_{II}$ and the target of RepA in pLSMpOPR is functionally 'normal' with respect to the initiation of transcription, to the specific binding of RepA to its target, and to the generation of a bend around the region including the target of RepA upon the binding of the protein to the plasmid DNA.

Phasing of the RepA target and $\underline{P}_{II}$ promoter

It has been shown that the rate of intramolecular ligation of DNA fragments can be influenced by protein-induced bends, and that this effect depends upon the location of the bend within the DNA molecule (Kotlarz *et al.*, 1986). To ascertain whether the bends induced by RepA and by RNA polymerase promoted ring closures in the above mentioned constructions, *Sty*I DNA fragments generated from pUCOPR (410 bp) and from pUCOPRΩ4 (414 bp) were used. The fusion between promoter $\underline{P}_{II}$ and the target of RepA is placed around the centre of these fragments. The *Sty*I-generated fragments were subjected to cyclization by T4 DNA ligase in the presence or absence of RepA, RNA polymerase or both. The results (Figure 4) showed that: (i) naked DNA exhibited low (if any) ligation products, which agrees with the DNA fragment being straight; (ii) RepA or RNA polymerase increased the ligation rate of both fragments when added separately, which supports bending generated by RepA and by RNA polymerase; (iii) when the RepA target and $\underline{P}_{II}$ were in phase both proteins cooperated to increase the rate of ring closure, and (iv) when the target of RepA and the promoter were out of phase, a reduction in the rate of ring closure was observed upon addition of both RepA and RNA polymerase. These findings indicate that the bends induced by RepA and by RNA polymerase are in the same direction in the in-phase construction and in opposite directions when 4 bp are inserted between the target of RepA and $\underline{P}_{II}$.

In vivo regulation of transcription by RepA

To test our hypothesis of the RepA repressor acting as an activator of $\underline{P}_{II}$ promoter, the above mentioned plasmids (see Figure 1) were transferred to *E.coli* hosts harbouring a compatible replicon which carried the *repA* gene cloned under the control of the *tac* promoter (plasmid pLSMtacA, Figure 5A). The induction of the *repA* gene in the heteroplasmid strains should result in the synthesis of the repressor which, supplied *in trans*, would bind to and bend

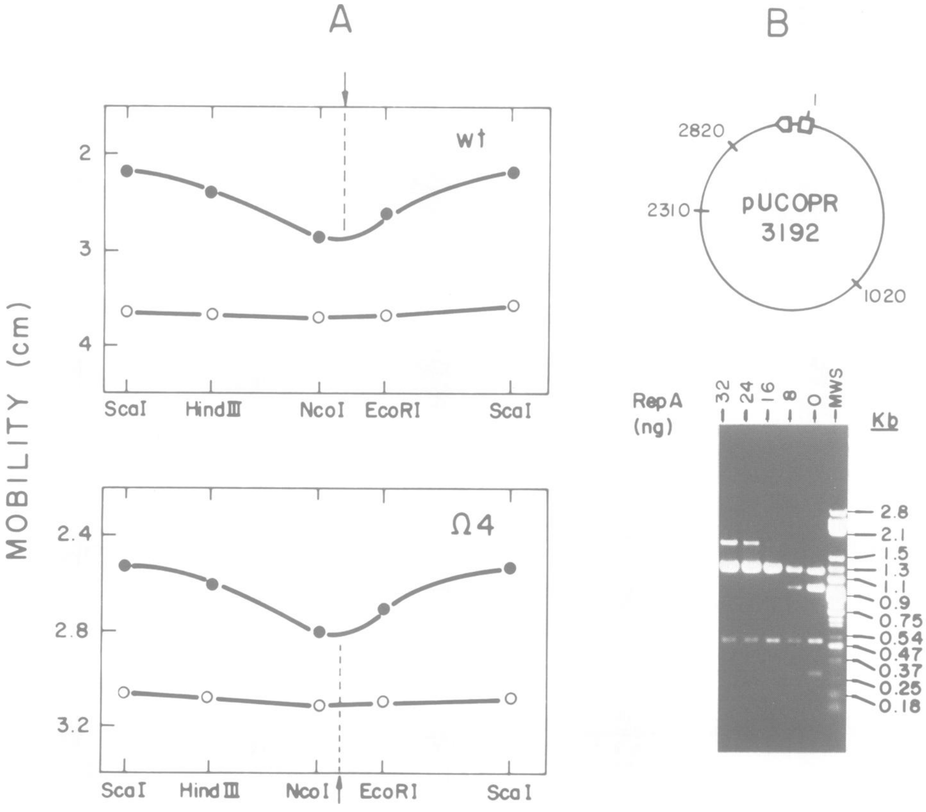

Fig. 3. DNA bending by and DNA binding of RepA. (**A**) Induced bending by RepA protein on plasmids pUCOPR and pUCOPRΩ4 linearized at the indicated restriction sites. Arrows point to the bend centre, which is located in the RepA target region. (**B**) Specific protection by RepA protein of the *Apa*LI site located within the RepA target (open box, coordinate 1) in pUCOPR. In the map, the coordinates of cleavage by *Apa*LI are indicated.

its target. The levels of β-galactosidase would thus be a measure of the *in vivo* activation (or repression) of promoter $\underline{P}_{\mathrm{II}}$ by the bend induced by RepA upon binding to its target. The results (Figure 5B) demonstrated that activation of $\underline{P}_{\mathrm{II}}$ occurred after the induction of RepA synthesis, but only when the promoter and the RepA target were in phase. The 2-fold increase in the synthesis of β-galactosidase, albeit modest, is significant since the amount of enzyme being synthesized by the uninduced strain is already high. This might reflect a high level of transcription from $\underline{P}_{\mathrm{II}}$. Placing the target of RepA out of phase with respect to $\underline{P}_{\mathrm{II}}$ resulted in repression mediated by RepA, since a 5-fold decrease in the β-galactosidase levels were observed. No activation of $\underline{P}_{\mathrm{II}}$ was observed when the RepA target was deleted, and only residual enzymatic activities were found when $\underline{P}_{\mathrm{II}}$ was absent. At the transcriptional level, it could be observed that the initiation of RNA synthesis from $\underline{P}_{\mathrm{II}}$ (in pLSMpOPR) was the same in the presence as in the absence of the *trans*-acting *repA* gene product. Promoter $\underline{P}_{\mathrm{II}}$ activation mediated by RepA could also be observed by quantitation of the amount of mRNA synthesized from it, being 1.7 times higher in the induced than in the non-induced cultures (Figure 5C).

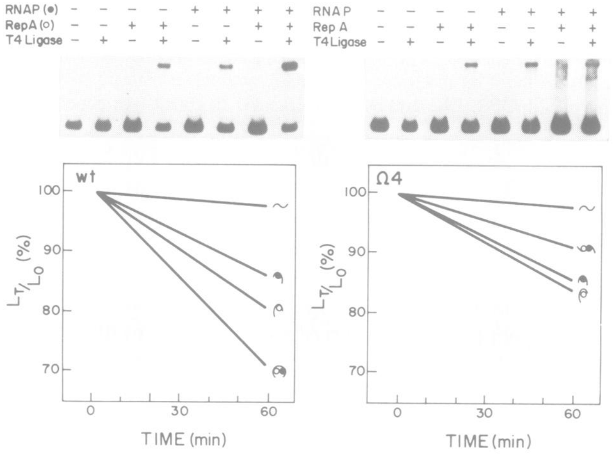

Fig. 4. Intramolecular ligation of the small *Sty*I fragment from plasmids pUCOPR (wt) and pUCOPRΩ4 (Ω4), untreated (−) or treated (+) with the indicated proteins. Samples with RepA (○), with RNA polymerase (●) and with both were subjected to cyclization with T4 DNA ligase at 0 and 60 min, in the conditions indicated in Materials and methods. Bands (upper part) were excised, their radioactivity quantitated and represented as described by Kotlarz *et al.* (1986).

In vitro regulation of transcription by RepA

It has been shown that *gal* promoters can be activated *in vivo* by substitution of the catabolite activator protein (CAP) upstream binding site for naturally curved sequences (Bracco *et al.*, 1989). However, no activation was observed by *in vitro* transcription of linear or supercoiled DNA. To check if our *in vivo* results were reproducible *in vitro*, we assayed transcription with linear or supercoiled DNA templates in the presence or absence of purified RepA protein (Figure 6). When the targets of RepA and $\underline{P}_{\mathrm{II}}$ were in phase, no variation in the synthesis of transcripts was found with linear DNA fragments (Figure 6A). However, an increase in RNA synthesis, dependent on RepA, was observed when super-

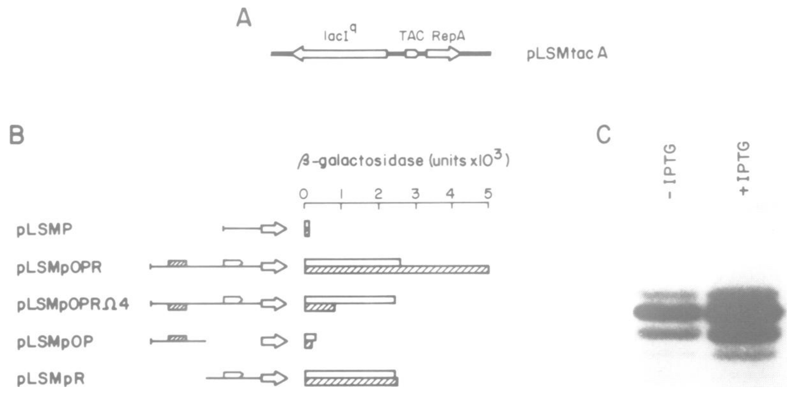

Fig. 5. *In vivo* activation of $\underline{P}_{II}$ promoter by *trans*-complementation with the repressor protein RepA. The plasmids indicated in (B) were transferred to *E.coli* cells harbouring the compatible plasmid pLSMtacA (schematized in A), which contains the *repA* gene under the inducible *tac* promoter. (**B**) Levels of β-galactosidase (Miller units) from uninduced (open bars) or induced (shaded bars) cell extracts. (**C**) Identification of the initiation of transcription of the mRNA synthesized from $\underline{P}_{II}$ in induced and uninduced cells harbouring pLSMtacA and pLSMpOPR.

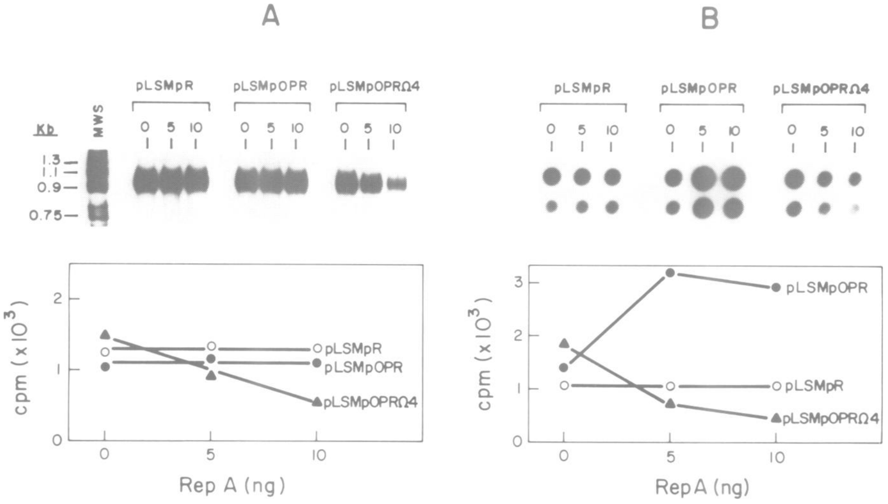

Fig. 6. *In vitro* activation of transcription from promoter $\underline{P}_{II}$ in the presence of purified RepA protein. (**A**) Transcripts were synthesized from linear DNA fragments (isolated from the indicated plasmids; 20 ng per sample) which were treated with 0.15 units of RNA polymerase and with purified RepA protein at 0, 5 and 10 ng. Reaction mixtures were incubated at 37°C, 10 min. The band shown represents ~90% of the total RNA synthesized. (**B**) Transcription from supercoiled plasmid DNA, treated as above, assayed by dot-blot hybridization. Quantitation (lower part) was done by counting the radioactivity in parallel samples.

coiled DNA was used as template (Figure 6B). RepA-mediated repression was found when the target of RepA and $\underline{P}_{II}$ were out of phase, independently of the configuration of the DNA template. RNA synthesis from templates lacking the operator was insensitive to the addition of RepA. The amount of RNA synthesized from supercoiled pLSMOPR increased with the amount of RepA protein added, until a plateau was reached. The highest level of *in vitro* activation of $\underline{P}_{II}$ was 2- to 3-fold the control values (no RepA), which is in good agreement with the *in vivo* results. The requirement of negative DNA supercoiling for RepA-mediated activation could reflect an opening of the DNA helix upon binding of RepA to its target. However, we cannot discard that the intrinsic strength of the $\underline{P}_{II}$ promoter could be different in linear from that in supercoiled DNA. Single-round transcription experiments with supercoiled DNA showed that activation by RepA increased with increasing amounts of RNA polymerase. However, saturation at low levels of enzyme in the RepA-treated samples was not observed (Figure 7B), suggesting that the affinity of the RNA polymerase for $\underline{P}_{II}$ was not changed by the presence of RepA. This could indicate that RepA activation may occur after the binding of the RNA polymerase to $\underline{P}_{II}$, although a direct contact between the two proteins, during the formation of the transcription complex cannot be discarded.

Discussion

We have demonstrated that a transcriptional repressor is capable of promoter activation or repression, provided that the target of the repressor and the promoter are placed in the same or in different faces of the DNA helix, respectively. Modulation of promoter activity can be observed both *in vivo* and *in vitro*. To our knowledge, this is the first example of a natural repressor turned into an activator by appropriate positioning of its DNA target. Previous reports have used specific amino acid changes (Bushman and Ptashne, 1988) or chimeric repressors having activation domains (Brent and Ptashne, 1985; Labow *et al.*, 1990).

RepA is capable of *in vivo* and *in vitro* transcriptional repression from its own promoter, but not from $\underline{P}_{II}$ (del Solar *et al.*, 1989, 1990). Due to the small size of the protein (45 amino acids) and to its amino acid composition (del Solar *et al.*, 1989), it seems unlikely that the RepA-dependent promoter activation can be due to any acidic region (Bushman and Ptashne, 1988), or to any other 'activating' domain, such as a glutamine-rich motif (Courey and Tjian, 1988) or a proline-rich region (Mermod *et al.*, 1989). Functional replacement of DNA molecules and promoter activation by 'swapping' between curved regions and targets for DNA bending proteins has been reported for CAP (Bracco *et al.*, 1989). However, activation was observed *in vivo* but not *in vitro*, which we found was not the case with supercoiled templates (Figures 6B and 7). Several factors may explain this difference. First, the presence of two overlapping −10 boxes in the *gal* promoter and only one in $\underline{P}_{II}$, may require a more specific range of superhelical density of the DNA (and hence, a more delicate positioning of the RNA polymerase) in the former than in the latter. Such a need for superhelicity would also explain our failure to detect activation with linear DNA molecules, although RepA does bend these molecules (Figure 3A; Pérez-Martín *et al.*, 1989). Second, the differences in size between RepA (5.1 kd) and CAP (9 and 11 kd per subunit) and the differences between their targets (symmetric versus asymmetric) can strongly influence the steps affected by activation. Third, the degree of DNA bending introduced by the proteins may be different, affecting the positioning of the RNA polymerase. And fourth, the DNA sequence between the protein binding site and the promoter may influence the helix opening. A relationship between helix phasing and enzymatic activity has been reported for the Alu156 promoter from the *Bacillus subtilis* bacteriophage SP82 (McAllister and Achberger, 1989) and for CAP (Gaston *et al.*, 1990). Both observations, together with our results, suggest that promoter activation depends upon phasing of the promoter with upstream DNA curvatures. In our case, the RepA-mediated activation seems to be exerted by appropriate positioning of its target with the promoter. Repression was found *in vivo* (Figure 5) and *in vitro* (Figure 6) when the RepA target and $\underline{P}_{II}$ promoter were placed out of phase. Since no other transcription start-point was found, we may conclude that specific repression of $\underline{P}_{II}$ by RepA occurred in this fusion. This repression could be explained because the DNA seems to be bent by RepA and by RNA polymerase in opposite directions (Figure 4). hindering the correct positioning (but not the binding) of the RNA polymerase with the promoter. This could account for the *in vivo* observations and for the *in vitro* results with supercoiled DNA, and it could also explain the results with linear DNA in which repression was found, but not activation. DNA bends (in addition to the steric hindrance generated by the binding of the repressor to its target) have been proposed to play a role in repression (Pérez-Martín *et al.*, 1989; Zwieb *et al.*, 1989). Transcrip-

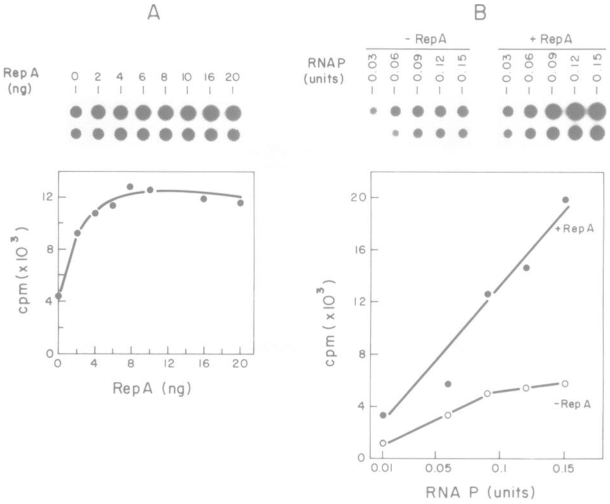

Fig. 7. Activation of transcription in supercoiled DNA from plasmid pLSMpOPR. (**A**) Dependence on the amount of purified RepA protein. (**B**) Single round of transcription in RepA-treated or untreated samples in the presence of increasing amounts of RNA polymerase.

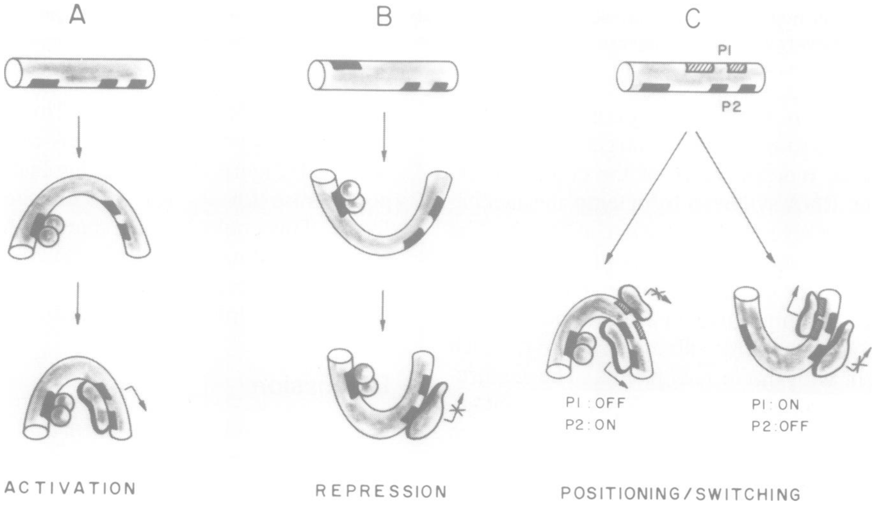

Fig. 8. Model for promoter recognition by RNA polymerase leading to activation, repression or switching on/off after the induction of a bend in DNA by a DNA binding protein (spheres); black regions are the targets for the proteins.

tional repression by IHF on *ompC* has been shown to occur with linear DNA as template (Huang *et al.*, 1990). Interestingly, the phage ϕ29 transcriptional regulator protein p4 activates the late P_{A3} promoter, but it is also able to repress transcription from the overlapping early promoter P_{A2b} (F.Rojo and M.Salas, personal communication).

A naive model (Figure 8) can be derived from previous (Bracco *et al.*, 1989; Salvo and Grindley, 1988; Goodman and Nash, 1989; Zwieb *et al.*, 1989) and our present data. When curved regions or the recognition target of a protein that bends DNA are placed in phase with a promoter, any increase in the DNA curvature would facilitate the RNA polymerase–promoter interaction during the initiation of transcription. When those sequences (curved or straight) are out of phase with the promoter, induced bends would hinder the complex formation. Our model also explains the switch on/off of two tandem promoters when they are placed in different faces of the DNA helix: introduction of a DNA bend by a protein may lead to the proper positioning of one of them, depending upon the direction of the induced bend. Thus, transcriptional enhancers or repressors could regulate gene expression by generating mechanical deformations of DNA sequences upstream of promoters. Some observations are consistent with our model. First, various prokaryotic transcription factors are able to bend the DNA (Friedman, 1988; Travers, 1989), and a role of DNA bends in transcriptionally active regions to facilitate the recognition of the RNA polymerase for its target has been suggested (Buc, 1986; Gustafson *et al.*, 1989). Second, most repressor proteins do bend the DNA at their target regions (Zwieb *et al.*, 1989; Travers, 1989 and references therein). And third, the model can account for the upstream activation or repression of promoters exerted by the *E.coli* IHF protein, because the main role of IHF seems to be the bending of the DNA to facilitate recognition by other proteins (Friedman, 1988; Nash, 1990). Promoter $\underline{P}_{II}$ has a weak −35 region and a consensus −10 region as compared with the canonical *E.coli* promoters, features that might be general for regulated promoters (Raibaud and Schwartz, 1984; Bracco *et al.*, 1989). If our model is correct a new method of regulation of gene expression by DNA binding proteins, in addition to the acidic activation mechanism proposed by Ptashne (1988), could be envisaged: transcriptional activation could be achieved by in-phase DNA deformations upstream of promoters regardless of the causal agent.

Materials and methods

Bacteria, plasmids and DNA preparations

E.coli CC118 (Manoil and Beckwich, 1985) was used for plasmid constructions and for experiments with *lacZ*. Plasmids used were: pLS1Δ24 (Puyet *et al.*, 1988), pLSM1S (Pérez-Martín *et al.*, 1988), and those described in this work (schematized in Figures 1 and 4). Detailed constructions of plasmids pLSM1ΔX, pLSMP and pLSMtacA will be published elsewhere (J.Pérez-Martín and M.Espinosa, in preparation). Plasmid pLSM1ΔX derives from pLSM1S by removal of a 436 bp *Bam*HI–*Xmn*I fragment; in its single *Acc*I site, a 422 bp *Apa*LI–*Afl*II fragment (from pLS1Δ24) was inserted to construct pUCOPR. By filling in at the pUCOPR single *Xba*I site (Figure 2C), pUCOPRΩ4 was also constructed. Plasmid pLSMP is a ColE1-based replicon in which a promoterless *lacZ* gene has been cloned. It was used to construct the plasmids described in Figure 1: pLSMpOPR (by cloning a 344 bp *Sty*I–*Bgl*I fragment from pUCOPR); pLSMpR (by deletion of a 250 bp *Xba*I–*Eco*RI fragment); pLSMpOPRΩ4 (a 4 bp insertion at the single *Xba*I site located between the RepA target and $\underline{P}_{II}$), and pLSMpOP (a 109 bp *Xba*I–*Bam*HI deletion that removed $\underline{P}_{II}$). Plasmid pLSMtacA has the *repA* gene cloned under the inducible *tac* promoter. To prepare riboprobe, plasmid pSPTlacZ was constructed by cloning a 1.5 kb *Bam*HI–*Cla*I fragment from pLSMpOPR in the single site *Hin*dIII of plasmid pSPT19 (Boehringer Mannheim) in front of the T7 promoter of the latter plasmid. With this construction, RNAs synthesized from the T7 promoter and from promoter $\underline{P}_{II}$ are in opposite orientations. Purified plasmid DNAs were prepared by CsCl gradient centrifugation as described (del Solar *et al.*, 1987).

DNA and RNA manipulations

Restriction enzymes were obtained from New England Biolabs, Amersham or Boehringer Mannheim, and used as specified by the suppliers. DNA fragments were purified from agarose gels with the GeneClean kit (Bio101, Inc.). DNA restriction fragments were treated with alkaline intestinal phosphatase and labelled at their 5′-ends with [τ-^{32}P]ATP and phage T4 polynucleotide kinase or filled in with the Klenow fragment of *E.coli* DNA polymerase I (PolIK). Total RNA was isolated from exponentially growing cultures as reported (López *et al.*, 1989). RNA synthesized in the *in vitro* transcription experiments was labelled with [α-^{32}P]UTP and treated as described (del Solar *et al.*, 1990). Riboprobes were synthesized *in vitro* from pSPTlacZ by treatment with T7 RNA polymerase, as recommended by Boehringer Mannheim.

Determination of the 5′-ends of transcription by S1 mapping

The 5′-termini of specific $\underline{P}_{II}$ transcripts were determined by S1 analyses as described (Ballester *et al.*, 1990). The probe used was a 109 bp *Xba*I–*Bam*HI fragment from pLSMpOPR (see Figure 2C), labelled at the 5′-end of the complementary strand of the *lacZ* transcript. This fragment overlaps promoter $\underline{P}_{II}$ and covers the transcription start-point.

RepA protein purification and protein–DNA binding

Purification of RepA protein was done essentially as described (del Solar *et al.*, 1989). The final concentration of the protein in this preparation (a gift of G.del Solar) was ~4 ng/μl. The protein was dialyzed against buffer R [40 mM Tris–HCl pH 7.9, 10 mM $MgCl_2$, 150 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT)], and diluted in the same buffer to a concentration of 1 ng/μl. To measure the bending induced by RepA, gel retardation assays were performed (Pérez-Martín *et al.*, 1989). Plasmid DNAs from pUCOPR or from pUCOPRΩ4 (200 ng per sample) were linearized at the indicated sites (Figure 3) and complexed or not with RepA protein (2.5 ng per sample). After incubation (15 min, 20°C), samples were electrophoresed in composite agarose (0.5%)–acrylamide (1.8%) gels as described (Pérez-Martín *et al.*, 1989). Protection of the *Apa*LI site by RepA (G.del Solar and M.Espinosa, in preparation) was tested by incubating plasmid DNA (200 ng) with increasing amounts of RepA protein, in *Apa*LI buffer (10 mM Tris–HCl pH 7.5, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$). After 60 min at 37°C, 5 units of *Apa*LI enzyme were added; the mixtures were incubated at 37°C for 60 min and electrophoresed in 2% agarose gels.

Intramolecular ligation assays

The assays were performed essentially as described (Kotlarz *et al.*, 1986). DNA from plasmids pUCOPR and pUCOPRΩ4 was digested with *Sty*I, which gave two fragments of 2760 and 410 bp (pUCOPR) and of 2760 and 415 bp (pUCOPRΩ4). The fragments were made blunt and end-labelled with PolIK. DNA concentration was adjusted for the small fragment to 200 ng/ml and reactions were performed at 10°C in 20 mM Tris–HCl pH 7.6, 20 mM $MgCl_2$, 150 mM KCl, 1 mM ATP, 1 mM DTT. In a typical experiment, 40 μl of the above mixture were incubated 10 min with RepA (8 ng), RNA polymerase (1.0 units) or both. Phage T4 DNA ligase (1 unit) was added and at 0 and 60 min, aliquots were withdrawn and quenched by mixing with a solution containing 2% SDS, 50 mM EDTA and 0.25% bromophenol blue. Separation of the ligation products was performed by electrophoresis on 4% non-denaturing polyacrylamide gels. Bands were identified by autoradiography and quantitated by cutting the appropriate gel segments. Due to its size, neither the 2760 bp fragments nor their ligation products entered into the gels.

Transcriptional activity in vivo

Cells (50 μl) from overnight cultures (in LB medium) were employed to inoculate 5 ml of fresh prewarmed medium containing or not 0.2 mM IPTG. Cultures were harvested at OD_{600} = 0.4, and β-galactosidase was determined as described (Miller, 1972).

In vitro transcription

Transcripts synthesized from linear DNA were prepared from purified *Kpn*I–*Cla*I fragments from pLSMpOPR (1.2 kb), pLSMpOPRΩ4 (1.2 kb) and pLSMpR (0.95 kb). The three fragments contain promoter $\underline{P}_{II}$; the length of the transcripts generated is of ~900 nucleotides. Samples were prepared and treated as described (del Solar *et al.*, 1990), except that transcripts were run in composite agarose (0.5%)–acrylamide (2%) gels. When supercoiled plasmid DNAs were used as templates, no labelled nucleotide was added: the RNAs synthesized were fixed to nitrocellulose membranes and hybridized with a labelled riboprobe complementary to the first 1.5 kb of the *lacZ* gene. Single-round transcription was done by preincubation of the template DNA (supercoiled pLSMpOPR DNA, 120 ng per assay), with or without different amounts of RepA protein (15 min, 20°C), followed by the addition of increasing amounts of RNA polymerase (0.01–0.15 units). RNA synthesis was started by adding the four ribonucleotides and heparin (200 μg/ml), and samples were treated as described (Herendeen *et al.*, 1990).

Computer analyses

The structures presented in Figure 2 were generated by the program BEND (DNASTAR, London), which is based on the Trifonov (1985) model. The A–A wedge angle was estimated to be 8.6°. Similar predictions were obtained when the Calladine model was used (Calladine *et al.*, 1988).

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