point mutations with oligonucleotides prepared to contain defined mismatches and create additional unique restriction enzyme sites in pCAM (altered residues are underlined below): Bam HI (GTGAGTTTTCCTAGGGTCTGG, positions 167 to 187); Pvu II (GGAGGTCGACTTCCCATTT, positions 187 to 205); and Xba I (ACAAAGGGAGATCTTTCTTAGGG, positions 160 to 182). To generate the compensatory changes, we used the following oligonucleotides: A^{202} replaced with a C (CAAAGGGGAAATGGGC, positions 195 to 210); A^{206} replaced with a C (AGGGTAAAGGGGCCCAAA, positions 198 to 215); and A^{156} and C^{157} replaced with G and U, respectively (GAAAACCCAGCUGUUU, positions 149 to 164). To combine all mutations in this study with the 22-bp deletion, we digested p2CSdI22 (pPM2-2-47M+ with a deletion of positions 57 to 78) and the various constructs with Pst I and Nco I (Pst I digests at a single site about 1 kb upstream from the cDNA insert and Nco I digests at a single site 22 bp downstream from the Sna BI site in the sat–RNA C cDNA), the fragments were gel purified, and the larger fragment (containing alterations) was ligated to the shorter fragment (containing the *E. coli* RNA polymerase promoter and the 22-bp deletion).

15. Plasmid pCAMB (Table 2) was digested with Apa I and Bam HI, the ends were made flush with the Klenow fragment, and the large plasmid fragment was ligated to a 31-bp segment that had been generated by digestion of pUC8 with Sma I and Hind III, making the ends flush with the Klenow fragment, and gel purifying the small fragment. The Sma I end of the 31-bp fragment was nearest to the corresponding 5′ end of the sat-RNA.
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17. Nucleotide reversion to the wild-type residue was found in nearly all sat-RNAs derived from constructs containing the alteration of U to C at position 196. Furthermore, sat–RNA C species from plants inoculated with HVI and transcripts of pCAM55, in which A^{179} had been changed to a G, revealed that in samples from some plants the G residue had reverted to the original A residue, whereas in other samples G^{182} was converted to an A residue.
18. Insertions and deletions at the Apa I, Mlu I, and Bam HI sites were generated by digestion of either pCAM (alterations at the Apa I and Mlu I sites) or of pCAMB (alterations at the Bam HI site) with the appropriate enzyme, followed by incubation with the Klenow fragment and deoxynucleotides and then by intramolecular religation.
19. It is not clear what caused the addition of U residues. The RNA-dependent RNA polymerase of TCV has been shown to insert additional U residues in a region containing five consecutive U residues during the replication of certain altered sat–RNA C species [C. D. Carpenter, P. J. Cascone, A. E. Simon, *Virology* **183**, 595 (1991)]. This type of phenomenon has also been reported for other systems; for example, *E. coli* RNA polymerase can add extra nucleotides when replicating on poly(U) tracts [L. A. Wagner, R. B. Weiss, R. Driscoll, D. S. Dunn, R. F. Gesteland, *Nucleic Acids Res.* **18**, 3529 (1990)], and in paramyxovirus, additional G residues can be added into a series of G residues [J. A. Southern, B. Precious, P. E. Randall, *Virology* **177**, 388 (1990); S. Vidal, J. Curran, D. Kolakofsky, *J. Virol.* **64**, 239 (1990)].
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21. Sequencing the sat-RNAs derived from infection of these transcripts without the 22-base deletion revealed that the two new compensatory changes were maintained; however, as before, A^{195} was preserved, yet C^{196} reverted to the wild-type U residue. Although the generation of C^{196} would have had no effect on the ability of the two loops to interact, C^{196} could base-pair with G^{191}, thereby disrupting the structure of the six-base loop. This could affect the viability of the molecule in an as yet unknown fashion.
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Protein-Induced Bending as a Transcriptional Switch

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The question of whether protein-induced DNA bending can act as a switch factor when placed upstream of an array of promoters located in tandem was investigated in vivo. The catabolite activating protein binding site of the *fur* operon was replaced by the binding site of the RepA repressor protein, which is able to bend DNA immediately after binding. Appropriately phased induced bending could act as a transcriptional switch factor in vivo.

Gene expression in prokaryotes is frequently controlled by DNA binding proteins that attach upstream of the region proximal to the transcription start site (*1*). Many of these regulatory proteins bend cognate DNA sequences after binding (*2*), but whether the resulting DNA curvature forms part of the mechanism for transcription initiation remains uncertain. Intrinsically bent DNA activates several promoters (*3*, *4*), and curved regions can be exchanged with the binding site of a DNA bending protein (*5*). The catabolite activator protein (CAP) from *Escherichia coli*, which is involved in transcription activation of several operons, bends the DNA after binding to its target (*6*). There are instances of CAP-dependent systems, such as *lac* and *gal* operons, in which upstream intrinsic DNA bends have by themselves the capacity to activate transcription, thus functionally replacing the binding site of CAP (*7*). These findings support the model that upstream DNA bending is an important element in transcription activation (*8*). Recent studies have suggested that, however, contacts between CAP and RNA polymerase are more important than CAP-DNA interactions (*9*), thus making it unclear how bending would affect such protein-protein interactions. To study the effects of protein-induced DNA bending in transcription initiation in vivo, we examined the effect of substituting the CAP site of the *E. coli fur* operon with the heterologous binding site of the DNA bending repressor RepA [recently renamed CopG (*10*)]. Our results support a model in which DNA bending could act as a transcriptional switch: one promoter is activated, whereas an adjacent one is repressed.

The product of the *fur* gene is involved in the control of iron transport and metabolism in *E. coli*, and its synthesis is regulated by CAP (*11*). Like other CAP-activated systems, the *fur* gene is expressed from two tandem overlapping promoters having their −35 and −10 regions located on opposite sides of the DNA helix. Primer extension analysis of *fur* RNA isolated in vivo indicated that under catabolite repression conditions, activity of the downstream promoter P_A (placed at the opposite helix side of the CAP binding sequence) is paralleled by a decrease in transcription from the upstream promoter P_B. In contrast, transcription from P_B (located at the same helix side as the CAP binding site) is stimulated during catabolite activation, whereas transcription from the downstream promoter P_A is repressed (*11*). Transcriptional switches have also been observed in systems like the *gal* operon (*12*), although they may be explained by competition between CAP and RNA polymerase for nearby sites within the same DNA region at the basal promoter. In the case of *fur*, however, target sequences of CAP are significantly distant from the −10 and −35 regions of the basal promoter, P_A (−70 with respect to the transcription start site).

We have proposed (*5*) that when curved regions, or the recognition target of a protein that bends DNA, are placed in phase with a promoter, increases in the DNA curvature would facilitate RNA polymerase-promoter interactions during the initiation of transcription. In contrast, when those sequences are not phased with the promoter, static or induced bends would hinder the formation of a transcriptional

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initiation complex. Our hypothesis could explain the on-off switch of two tandem promoters placed on different faces of DNA helix, such as the *fur* operon: introduction of a DNA bend by a DNA binding protein could properly position one of the promoters for transcription.

The small transcriptional repressor RepA protein, encoded by plasmid pLS1, bends DNA sharply at its cognate target sequences (*10*). If the switch observed at the *fur* operon in different catabolite conditions is entirely determined by CAP-induced bending, then RepA-induced bending should be able to substitute for CAP as a switch factor in vivo. To test this hypothesis, we replaced the CAP binding site at *fur* operon with a RepA binding site. In its native configuration, the CAP binding site is located on the same DNA side as the catabolite-responsive P_B promoter and on the opposite face to the −10 and −35 region of the basal P_A promoter. We placed the RepA binding site alternatively in phase with either P_A or P_B, depending on the construction (*13*), and supplied RepA in trans (Fig. 1). Determination of β-galactosidase activity (*14*) of the resulting chimeric operons (Fig. 1C) suggested that such an exchange allowed RepA (which is a natural repressor) to activate transcription from *fur* promoters. Furthermore, the amounts of β-galactosidase detected in cells bearing the pLSMpOTΩ4 construction, in which the RepA binding site is placed in phase with the former catabolite-responsive promoter P_B, are similar to those observed when native CAP-dependent activation was analyzed (*11*). In summary, although the two proteins are different, RepA mimics in vivo the effect of CAP activation. RepA-dependent activation of the hybrid promoters was completely independent of CAP protein because β-galactosidase values obtained using a *crp* mutant as the host for the constructions turned out to be virtually identical (*15*). Nuclease S1 analysis of transcripts produced by the chimeric operons showed that only the promoter phased with the RepA binding site became active in each case, whereas the promoter at the opposite side of the DNA helix simultaneously decreased its transcriptional activity to negligible levels (Fig. 2).

The above data suggest that activator proteins could act as switch factors as a result of their DNA bending properties. To explain the ability of a repressor protein (like CopG) to behave as a switch factor, we propose a model (which may have a more general significance) for the role of induced bending in the regulation of tandem promoters located on opposite faces of the DNA helix. Induction of a curvature in the same DNA face as one of the two promoters would facilitate transcription from the in-phase promoter but not from the other, which would be turned off. The presence of a DNA curvature on the right or the wrong side of the RNA polymerase binding site has been shown to activate or to repress, alternatively, the activity of a corresponding promoter (*16*). In the case of tandemly located natural promoters, such as those present on the *fur* or *gal* operons, RNA polymerase binding to the constitutive basal promoter may contribute to repression of transcription from the catabolite-responsive promoter because binding of the RNA polymerase to the basal promoter generates a bend at the opposite DNA helix side (*3*, *17*). When the activator protein binds to its upstream site, the activator-induced bend counteracts the RNA polymerase–induced bend at the constitutive promoter and may drive the RNA polymerase into the catabolite-responsive promoter. The activator-induced bend, reinforced with the RNA polymerase–induced bend at the activator-responsive promoter, could help to inhibit the binding of RNA polymerase to the basal promoter, located now at the wrong face of the bend.

In summary, intrinsic or protein-induced DNA bends may help or hinder the interaction of RNA polymerases (or other DNA binding proteins) with their binding sites, thus adding another level of regula-

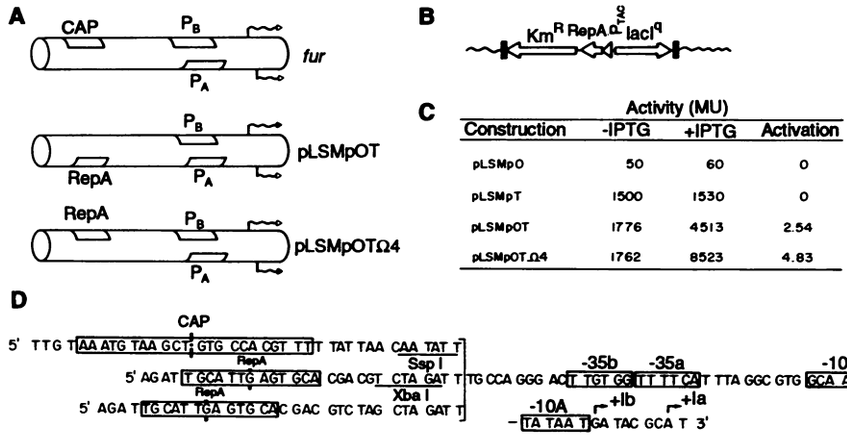

Fig. 1. RepA repressor mimics CAP-mediated activation of the *fur* operon. (**A**) Scheme of the organization of the *fur* operon, with indication (top) of the relative positions of the constitutive promoter P_A, the CAP-activated promoter P_B, and the CAP binding site. The organization of the constructions with the RepA binding site, either in phase with P_A (pLSMpOT) or with P_B (pLSMpOTΩ4), is also shown. (**B**) Scheme of a specialized kanamycin resistance mini-transposon used for chromosomal insertion of RepA and its IPTG-dependent expression. (**C**) Activation by RepA, measured by β-galactosidase levels (Miller units) determined in *E. coli* CC118 cells carrying a chromosomal copy of the *repA*-encoding mini-transposon and bearing either pLSMpOT or pLSMpOTΩ4. Cells bearing the indicated plasmid were grown overnight in LB medium and inoculated on a fresh, prewarmed medium with or without 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested at OD_{600} = 0.4, and β-galactosidase was determined as described (*18*). For the controls, β-galactosidase activity was measured in the same strain bearing control plasmids pLSMpO and pLSMpT. These plasmids carry a *lacZ* reporter gene downstream of the RepA target (pLSMpO) or the tandem promoters of *fur* gene (pLSMpT). (**D**) Nucleotide sequence of *fur* operon (top), pLSMpOT (middle), and pLSMpOTΩ4 (bottom) constructions.

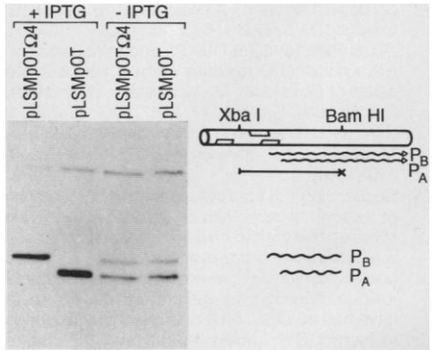

Fig. 2. RepA acts as a transcriptional switch factor in vivo. S1 nuclease assay of transcripts obtained in vivo from *E. coli* strains bearing pLSMpOT or pLSMpOTΩ4 under noninduced (−IPTG) or induced (+0.1 mM IPTG) conditions. The relative positions of P_A, P_B, and the RepA binding site in the plasmid pLSMpOT are schematized on the right. Total RNA was prepared as described (*19*) after 4 hours of induction of RepA synthesis with IPTG. The approximate locations of the 5′ ends of the mRNAs synthesized from each promoter (wavy line) and the radiactive DNA probe (a 104-bp Xba I–Bam HI fragment derived from pLSMpOT) used to form mRNA-DNA hybrids (straight line) are also indicated. Hybridization was done by mixing of DNA probe (20,000 cpm) and total RNA (50 μg) at 46°C. Hybridized samples were digested with 100 U of nuclease S1 for 20 min at 30°C and treated as described (*20*).

tion to transcriptional control circuits. The presence or absence of specific DNA bending proteins bound at their target sites would determine which neighboring binding sites for other proteins are favored.

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13. The parent plasmids pLSMpOPR and pVLN102 have been described previously (*5, 11*). A 1.3-kb Ssp I–Cla I fragment from pVLN102, which carries the tandem promoters without the CAP binding site, was cloned in pLSMpOPR digested with Xba I–Cla I. This construct placed the RepA binding site upstream of the tandem promoters at a distance similar to the CAP binding site in its natural situation (around −70). The Xba I restriction site, regenerated after ligation of Ssp I and Xba I sites treated with Klenow fragment, was used to introduce 4 bp in the pLSMpOPRΩ4 construct. The DNA symmetry centers of targets for RepA and CAP proteins were located with respect to the transcription start-site (+1a) at −77 (CAP binding site in natural operon), −72 (RepA binding site in pLSMpOPT), and −76 (RepA binding site in pLSMpOPTΩ4) (Fig. 1D).
14. The strain used for β-galactosidase assays was *E. coli* JM002 {*araD139, ΔlacX74, galE, galK, phoA20, thy, rpsE, rpoB, argE*(*am*), [*mini Tn5-Km copG*]}, which is derived from *E. coli* CC118 [C. Manoil and J. Beckwith, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8129 (1985)]. It carries gene *copG* under the control of P_{tac} promoter, inserted into the chromosome with the use of a specialized transposon (K. Timmis and V. de Lorenzo, *Methods Enzymol.*, in press).
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Ryanodine Receptor Adaptation: Control Mechanism of Ca^{2+}-Induced Ca^{2+} Release in Heart

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Adaptation of single cardiac ryanodine receptor (RyR) channels was demonstrated by application of the caged calcium ion (Ca^{2+}) methodology. In contrast to conventional desensitization found in surface membrane ligand-gated channels, single cardiac RyR channels adapted to maintained Ca^{2+} stimuli, preserving their ability to respond to a second (larger) Ca^{2+} stimulus. RyR adaptation may represent a molecular control mechanism for smoothly graded Ca^{2+}-induced Ca^{2+} release in heart and may be a fundamental feature of channels, including the inositol trisphosphate receptor, that are involved in intracellular Ca^{2+} signaling in many cell types.

In cardiac muscle, Ca^{2+} release from the sarcoplasmic reticulum (SR) is mediated by a Ca^{2+}-activated channel called the ryanodine receptor (RyR) (*1–3*). The cardiac RyR is regulated by Ca^{2+} influx through voltage-gated Ca^{2+} channels in the surface membrane. This process, termed Ca^{2+}-induced Ca^{2+} release (CICR), is fundamental to cardiac excitation-contraction coupling, the mechanism that links surface membrane depolarization to Ca^{2+} activation of the contractile proteins (*4–8*).

In its simplest form, CICR should be an "all or nothing" phenomenon because of its intrinsic positive feedback. In vivo, however, CICR is smoothly graded (*5–7, 9*). To reconcile this paradox, Ca^{2+}-dependent inactivation was proposed as the essential negative control mechanism that counters the inherent positive feedback of CICR (*10*). Studies on intact and permeabilized cells present contradictory results concerning the existence of Ca^{2+}-dependent inactivation (*7, 10–12*). Also, single-channel studies done under steady-state conditions show no signs of inactivation at physiologically relevant Ca^{2+} concentrations (*13, 14*). In these single-channel experiments, however, it is possible that a regulatory subunit was lost during RyR isolation or that steady-state studies were inappropriate to describe a transient phenomenon.

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To directly define how CICR is controlled, we made calibrated changes in the Ca^{2+} concentration ($[Ca^{2+}]$) in the medium around single cardiac RyR channels from dog hearts by photolysis of caged Ca^{2+}. These experiments revealed a mechanism of Ca^{2+}-dependent adaptation of RyR channels that is distinct from conventional desensitization in that the channels adapt to a maintained Ca^{2+} stimulus and are thereby able to respond to subsequent Ca^{2+} stimuli. This adapting mechanism allows single RyRs to respond transiently to a Ca^{2+} stimulus in a dose-dependent manner and may represent a molecular control mechanism that underlies the smoothly graded nature of CICR in vivo.

Activation of a single cardiac RyR channel by flash photolysis of caged Ca^{2+} (*15*) is illustrated in Fig. 1A. To assure precise control of the $[Ca^{2+}]$ in the microenvironment of the channel, we used Cs^+ rather than Ca^{2+} as the conducting ion (*16–18*). The identity, sidedness, and number of the channels in the bilayer were determined under steady-state conditions (10 μM Ca^{2+}) before each experiment (*16–19*). We then added DM-nitrophen (caged Ca^{2+}) to the myoplasmic side of the RyR to buffer the $[Ca^{2+}]$ at 100 nM. At this $[Ca^{2+}]$, the stationary open probability was zero. Liberation of Ca^{2+} in the microenvironment of the channel by an ultraviolet (UV) flash (arrowhead) triggered a transient burst of channel activity (Fig. 1A). We reestablished resting conditions be-