important leads to the therapeutic potential of non-NMDA antagonists.

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12. The close collaboration with Squibb Institute for Medical Research, Princeton, NJ, is highly appreciated.

9 March 1988; accepted 25 May 1988

An *E. coli* Promoter That Regulates Transcription by DNA Superhelix-Induced Cruciform Extrusion

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DNA can form structures other than the Watson-Crick double helix. The potential contributions to gene regulation from one such structure have been investigated by assembling a promoter capable of adopting cruciform base-pairing. Transcription from this promoter by RNA polymerase in vitro was repressed as the cruciform was extruded by increasing negative DNA supercoiling. Transcription in vivo was induced as supercoiling was relaxed by growth in conditions that inhibit DNA gyrase. A DNA conformational change is therefore capable of regulating the initiation of transcription.

THE OCCASIONAL DEPARTURE OF DNA from the Watson-Crick double helix suggests a mechanism for the control of gene expression. A variety of secondary structures, including left-handed helices (*1*), heteronomous DNA (*2*), anisomorphic DNA (*3*), loops (*4*), and cruciforms (*5*), have been postulated to regulate transcription through conformational changes in the DNA template. To survey the extent to which DNA secondary structure may influence transcription initiation, we have characterized the interaction of *Escherichia coli* RNA polymerase with a promoter that contains a DNA sequence with the potential to fold into a cruciform structure.

The plasmid pX (Fig. 1A) contains a 50-bp inverted repeat, composed mostly of A-T base pairs, spanning from −23 to +27, with respect to the transcription start site, +1. Each repeated unit contains a "−10" consensus sequence. The most upstream of the units is spaced to align with a "−35" sequence to complete the elements of a promoter (*6*), the start site of which is located near the center of the inverted repeat. The promoter directs transcription of the gene *tet*, which confers resistance to tetracycline. The control plasmid pO (Fig. 1B) contains a 50-bp direct repeat composed of the same units as in pX. The two plasmids differ by an inversion from +6 to +21. Since the sequence of the repeated unit (Fig. 1C) nearly approximates an inverted repeat, the net sequence difference between pX and pO is merely eight A-T transversions. Cruciform extrusion could occur in pX when intrastrand DNA base-pairing replaces interstrand base-pairing within the inverted repeat. Because cruciform extrusion relieves superhelical stress, the cruciform is expected to become the stable species at high negative superhelical densities (*7*).

Two-dimensional gel electrophoresis (*7*) was used to confirm the ability of pX DNA to adopt a cruciform structure in vitro (Fig. 2A). A distribution of topoisomers was prepared by partial relaxation of physiologically supercoiled plasmid DNA (*8*). In the first dimension, topoisomers that had a negative superhelical density sufficient to extrude cruciforms were retarded in mobility by a change in linking difference equal to the change in twist accompanying the helical unwinding of cruciform formation. The addition of an intercalator to the second dimension positively supercoiled the DNA, which removed the cruciform and revealed an arc of spots corresponding to topoisomers that extruded the cruciform during electrophoresis in the first dimension. Topoisomers of pX with a linking difference of −14 or less (superhelical density, σ, ≤ -0.03) were retarded by 5.2 turns (equal to the anticipated length of the cruciform, 50 bp, assuming 10.5 bp per turn). This cruciform was further mapped to the expected location at the *tet* promoter by sensitivity to the single-strand–specific S1 nuclease (*9*). The pO DNA does not form such secondary structure as determined by electrophoresis (Fig. 2B), and by insensitivity to S1 nuclease (*9*).

We assessed the influence of the cruciform on the promotion of in vitro transcription by *E. coli* RNA polymerase. For some promoters negative supercoiling facilitates the helical unwinding of open complex formation (*10, 11*). Reactions performed with plasmid DNA templates of different superhelical densities (Fig. 3A) indicate that transcription from the promoter in pX did initially increase with supercoiling, but was then repressed at the negative superhelical densities at which the cruciform is extruded. By contrast, transcription from the promoter in pO increased fairly uniformly with negative supercoiling.

The degree of supercoiling at which this cruciform is extruded in vitro is within the

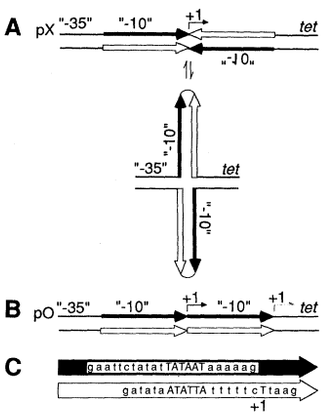

Fig. 1. Promoters studied. (**A**) The inverted repeat of the promoter in pX, illustrating potential cruciform base-pairing. (**B**) The direct repeat of the promoter in pO. A secondary start site is represented in stippled print. (**C**) The sequence of each repeat, contained on an Eco RI restriction fragment obtained from pAT12 by the technique of random selection (*22*). The "−10" sequence and starting base (template strand) are in uppercase. The promoters were constructed by ligation of this fragment into the Eco RI site of pBdEC, a derivative of pBR322 modified by deletion of the *tet* promoter from Eco RI to Cla I (*22*). Both pX and pO contain a β-lactamase gene.

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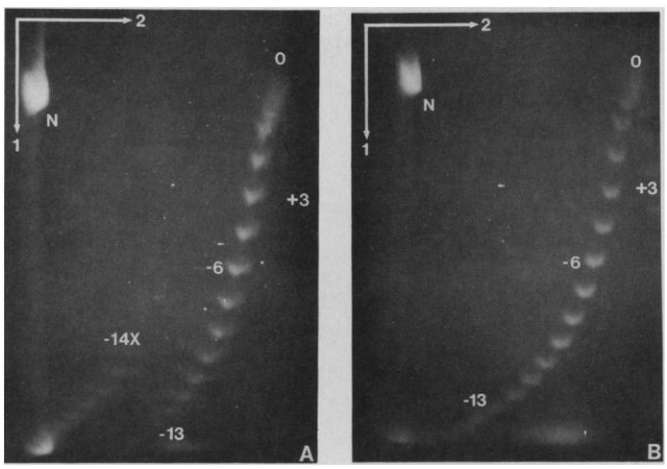

Fig. 2. Two-dimensional gel electrophoresis. (**A**) pX DNA. (**B**) pO DNA. The linking difference of some topoisomers is noted; X, cruciform conformation; N, nicked circular DNA; dimensions marked by arrows. The DNA was separated by electrophoresis (*7*) in 1% agarose in tris-borate (TBE) buffer in the first dimension and TBE plus 0.85 μ*M* chloroquine in the second dimension. The gel was stained with ethidium bromide.

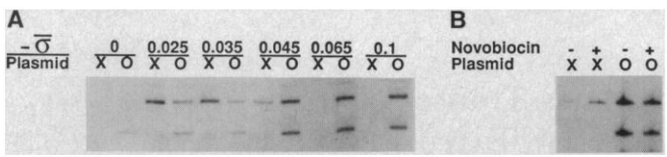

Fig. 3. Quantitative analysis of transcription initiation sites by primer extension. RNA synthesized in vitro (**A**) or in vivo (**B**) was used to generate cDNA products by reverse transcriptase–catalized primer extension (*23*). The cDNA products, which were resolved in sequencing gels and revealed by autoradiography, correspond to transcripts that have 5′ ends positioned at the junction of the two repeats (upper band). A second start site is unique to pO (lower band). The primer was a 5′ ^{32}P-labeled 22-mer complementary to *tet* and located 125 bases from the pX start site. (**A**) RNA synthesized in vitro as a function of DNA superhelicity. DNA templates of the indicated mean negative superhelical density ($-\bar{\sigma}$ were prepared by relaxation of negatively supercoiled plasmid DNA in the presence of various concentrations of ethidium bromide (*24*). The ethidium bromide was extracted, and the DNA was then transcribed in vitro (*25*) by *E. coli* σ^{70} RNA polymerase holoenzyme (*26*). (**B**) Cultures were grown in Luria-Bertani (LB) medium at 37°C to a density of 3×10^7 cells per milliliter, divided, and then grown for one more generation (30 min) in the absence or presence of novobiocin (250 μg/ml) before RNA was extracted (*27*). The quantity of RNA used in each lane (30 μg) was normalized to viable cell count and β-lactamase transcript, neither of which is significantly affected by novobiocin treatment.

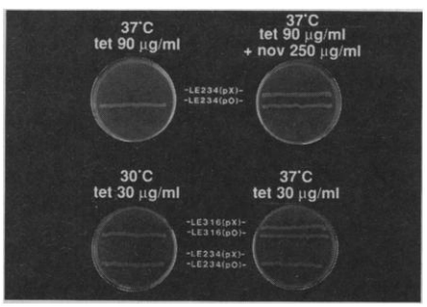

Fig. 4. Phenotypic analysis of *E. coli* strains hosting plasmids pX and pO. Plates contain LB agar. Abbreviations: tet, tetracycline; nov, novobiocin.

range of recent estimates of in vivo superhelical densities of −0.025 to −0.048 (*11, 12*). For some inverted repeat sequences cruciform extrusion is not kinetically favored in vitro and is thus unlikely to occur (or is undetectable) in vivo (*13*). Inverted repeats rich in A-T, which include the one in pX (*9*), however, readily extrude cruciforms in vitro (*5, 14*) and are perhaps more likely to adopt this structure in vivo. In fact, cruciform extrusion in vivo has recently been demonstrated in *E. coli* by sensitivity of an inverted repeat to a single-strand–specific nuclease (*15*). Cruciform extrusion may be further facilitated intracellularly by helicases and other DNA binding proteins, including RNA polymerase.

It thus becomes of interest to determine whether the promoters in pX and pO are differentially active in vivo, and, if so, whether transcription can be manipulated by growth under conditions that alter DNA topology. Analysis of in vivo mRNA levels (Fig. 3B) in *E. coli* strain LE234 (*16*) revealed less transcription from the *tet* promoter of pX than from pO. Inhibition by novobiocin of the enzyme responsible for maintaining negative DNA supercoiling, DNA gyrase, increased the *tet* mRNA level in cells carrying pX, but decreased the level of *tet* mRNA in cells carrying pO. The most direct explanation is that the cruciform is extruded within the cell and is responsible for the repression of transcription in pX; inhibition of gyrase by novobiocin relaxes supercoiling (*17*) and thus induces activity in pX by disfavoring cruciform formation.

Cells carrying pX displayed a phenotype consistent with supercoil-dependent transcriptional regulation of *tet* (Fig. 4). *Escherichia coli* LE234 was tetracycline-sensitive when harboring pX, but resistant when harboring pO. Growth in the presence of novobiocin, however, induced pX to confer tetracycline resistance, whereas the level of resistance conferred by pO (*9*) was reduced. In a second experiment we used the isogenic strain LE316 (*16*), containing a temperature-sensitive gyrase allele. Gyrase is active at the permissive temperature of 30°C. At the restrictive temperature of 42°C gyrase is inactive, and negative supercoiling of DNA is relaxed (*17*). At 30°C, pX did not confer tetracycline resistance. At 37°C, a temperature presumably inhibitory to gyrase activity, pX did confer tetracycline resistance. The change in phenotype resulted from the gyrase mutation, as demonstrated by the absence of an increase in tetracycline resistance upon temperature shift in the strain LE234 containing the wild-type gyrase allele.

In summary, we have shown that a cruciform may regulate transcription initiation. The cruciform may obstruct recognition of the promoter by RNA polymerase, or it may disrupt a later step in the kinetic pathway. In particular, the transition between abortive cycling and the formation of an elongating complex has been proposed as a point of control for the *E. coli* DNA gyrase promoters, which are stimulated by supercoil relaxation (*18*). The abundance of potential cruciform sequences within genetic regulatory regions (*19*) highlights the biological significance of this DNA secondary structure. However, since many secondary structures are stabilized by negative supercoiling (*20*), DNA conformational transitions may constitute one mechanism for the regulation of genes that are repressed by supercoiling (*21*). The regulation of transcription by supercoiling in other promoters could further depend on the participation of transcription factors and other DNA-binding proteins.

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28. We thank A. Blank and R. Burgess for providing RNA polymerase, D. Morris for strains, and G. Smith and H. Weintruab for comments on the manuscript. Supported by NIH and the University of Washington Medical Scientist Training Program.

21 March 1988; accepted 2 June 1988

Breast Cancer–Associated pS2 Protein: Synthesis and Secretion by Normal Stomach Mucosa

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The human pS2 gene is specifically expressed under estrogen transcriptional control in a subclass of estrogen receptor–containing human breast cancer cells. The pS2 gene encodes an 84–amino acid protein that is secreted after signal peptide cleavage. The distribution of pS2 protein in normal human tissues was studied with antibodies to pS2; pS2 was specifically expressed and secreted by mucosa cells of the normal stomach antrum and body of both female and male individuals. Moreover, no estrogen receptor could be detected in these cells, indicating that pS2 gene expression is estrogen-independent in the stomach. The function of the pS2 protein in the gastrointestinal tract is unknown. However, the pS2 protein is similar in sequence to a porcine pancreatic protein that has been shown to inhibit gastrointestinal motility and gastric secretion.

The human pS2 gene was discovered by differential screening of a cDNA library from breast cancer cells (MCF-7) grown in the presence of estrogen (*1*). Estrogen induction of pS2 mRNA synthesis corresponds to a primary transcriptional event (*2*), and the single-copy pS2 gene (*3*) encodes a cysteine-rich 84–amino acid polypeptide bearing characteristics of a secreted protein (*4*). Secretion of pS2 from MCF-7 cells was demonstrated by means of polyclonal antibodies directed against a synthetic peptide that corresponds to the carboxyl terminal half of the cDNA-deduced amino acid sequence (*5*). These antibodies and RNA blotting analysis have been used to show that the pS2 gene is specifically expressed in a subclass of estrogen receptor (ER)–containing breast cancers, and a preliminary survey of normal and tumoral human tissues and cells indicated that this expression was restricted to some primary breast cancers and their lymph node metastases (*6*).

Paraffin-embedded sections of endoscopic biopsies of normal stomach antrum and body were reacted with rabbit polyclonal antibodies directed against a synthetic peptide corresponding to the last 31 amino acids of pS2 (*5, 6*) and stained with a peroxidase-labeled second antibody. Material cross-reactive to pS2 was detected in mucosa cells of the superficial epithelium and crypts of both antrum (Fig. 1D, left side) and body (not shown) of the stomach. No staining was observed in body glands. Staining was specific for pS2, as it was suppressed by the presence of the synthetic peptide used to raise the antibodies (Fig. 1E). Similar results were obtained with a monoclonal antibody directed against a synthetic peptide corresponding to the last 28 amino acids of the pS2 protein. The corresponding staining of pS2 protein–positive cells of a ductal breast carcinoma is shown for comparison (Fig. 1, A and B). As in breast tumor cells (*6*), pS2 staining in stomach mucosa cells was predominantly cytoplasmic with an uneven distribution and often a perinuclear localization (Fig. 1D, right side), which may correspond to the Golgi apparatus. The pS2-specific staining was observed in both male and female stomach mucosa. However, no significant staining was observed in a variety of normal human specimens (colon, esophagus, gallbladder, pancreas, liver, thyroid and parathyroid glands, skin, lymph nodes, lung, brain, bladder, prostate, kidney, adrenal gland, testis, placenta, endometrium, ovary, and pituitary gland). Weak staining was observed in salivary glands.

When monoclonal antibodies directed against the human ER were used, the presence of nuclear ER was readily revealed in frozen sections of pS2 protein–positive breast carcinoma (*6*) (Fig. 1C). In marked contrast, no ER could be detected in frozen sections of stomach mucosa (Fig. 1F).

These results suggest that pS2 protein is synthesized by stomach mucosa cells and could possibly be secreted into the gastric fluid. Portions of stomach fluid obtained from fasting male and female individuals were acetone-precipitated, separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and reacted with the monoclonal antibody to pS2. In all cases immunoreactive material with an apparent size of approximately 7 kD, similar to that of the breast cancer pS2 protein (*5*), was readily revealed (Fig. 2, lanes 1 and 2). The presence of the synthetic peptide used for generating the monoclonal antibody eliminated staining (Fig. 2, lanes 3 and 4). The migration of the presumptive pS2 protein present in stomach fluid was then directly compared after extensive purification with that of pS2 protein purified from the culture medium of the breast cancer cell line MCF-7. Both proteins migrated identically and reacted similarly with the monoclonal antibody (Fig. 2, lanes 5 and 6, MCF-7 and gastric pS2 proteins, respectively). When pure pS2 protein prepared from MCF-7 cell culture medium was used as a standard, an average of 70 ng of pS2 protein per milliliter

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