Instituto Juan March
de Estudios e Investigaciones

7

CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Transcription Initiation in Prokaryotes

Organized by
M. Salas and L. B. Rothman-Denes

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H. Bujard
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I N D E X

P R O G R A M M E

TRANSCRIPTION INITIATION IN PROKARYOTES

Monday, June 15th

Opening remarks: M. Salas.

Chairperson: C.A. Gross.

M.J.Chamberlin- Structural studies of RNA polymerase complexes formed during RNA chain initiation and elongation.

W. Chul Suh - Kinetic and structural evidence for Mg^{2+}-driven conversion of open complexes ($RP_{OI} + \sim 3Mg^{2+} \rightarrow RP_{OI}$) on the pathway to transcription initiation by $E\sigma^{70}$ RNA polymerase.

H. Bujard - Sequences involved in the activation of E. coli promoters.

K. Schnetz - Why is the promoter of the bgl operon cryptic in the wild type?

R. Díaz Orejas- Transcriptional regulation of repA, the gene of the Pseudomonas plasmid pPS10 replication protein.

Poster Session.

Chairperson: H. Bujard.

C.A. Gross - Polypeptides containing highly conserved regions of transcription initiation factor σ^{70} exhibit specificity of binding to promoter DNA.

K.L. Brown - Isolation and characterization of the major vegetative RNA polymerase of Streptomyces coelicolor A3(2); renaturation of a sigma subunit using GroEL.

P. Stragier - Regulation of gene expression during sporulation in Bacillus subtilis.

M.S. Thomas - The role of the α subunit of E. coli RNA polymerase in transcription - a genetic analysis.

Tuesday, June 16th

Chairperson: S. Busby.

R.L. Gourse - Upstream activation of ribosomal RNA transcription in E. coli.

L.B. Rothman-Denes - The pathway of N4 virion RNA polymerase-promoter recognition.

M. Salas - Control of bacteriophage ϕ29 DNA transcription by the regulatory protein p4.

R. Schleif - Contortions, distortions and positions of AraC.

Poster Session.

Chairperson: R. Schleif.

S. Kustu - Prokaryotic enhancers and enhancer-binding proteins.

V. de Lorenzo - Concerted regulation of the σ^{54}-dependent Pu promoter of TOL plasmid through upstream sequences responsive to multiple signals.

E.P.Geiduschek- Action at a peculiar prokaryotic enhancer.

T. Owen-Hughes- The role of the nucleoid associated protein H-NS in osmotic induction at the proU promoter of Escherichia coli and Salmonella typhimurium.

Wednesday, June 17th

Chairperson: L.B. Rothman-Denes.

R.H. Ebright - Transcription activation by CAP: Identification of the activation surface and analysis of interaction with RNA polymerase.

S. Busby - Molecular interactions during transcription activation by the E. coli cyclic AMP receptor protein and FNR.

S. Adhya - Positive control of transcription initiation by cAMP-CRP in lac promoter of E. coli.

A. Kolb - Interactions between RNA polymerase and CRP in open complexes at the lacP1 and galP1 promoters: Use of mutated proteins.

P. Valentin-Hansen - Heterologous cooperativity: The cAMP-CRP/CYTR nucleoprotein complex in E. coli.

Poster Session.

Chairperson: S. Kustu.

M. Espinosa - Features of the transcriptional repressor RepA, encoded by the promiscuous plasmid pLS1.

P. Ostrovsky de Spicer - Regulation of proline utilization in Salmonella typhimurium: repression requires a nucleoprotein complex formed by rhe repressor - a membrane associated dehydrogenase - bound to two operator sites, curved DNA and IHF.

J. Roberts - Modification of RNA polymerase by the phage Lambda gene Q transcription antiterminator at the phage late gene promoter.

Concluding comments.

MONDAY, JUNE 15th

STRUCTURAL STUDIES OF RNA POLYMERASE COMPLEXES FORMED DURING RNA CHAIN INITIATION AND ELONGATION

Michael J. Chamberlin, Curtis Altmann, Sandra Milan and David Solow-Cordero

Division of Biochemistry and Molecular Biology; University of California; Berkeley, CA 94720 USA

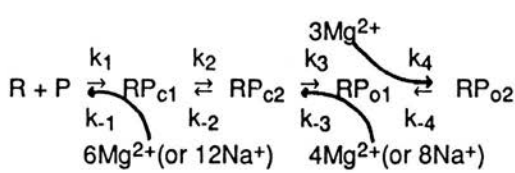
Ternary complexes of RNA polymerase are intermediates in three major steps in transcription, initiation, elongation and termination, and each of these steps is a target of regulatory pathways. We have approached the study of the mechanism of these steps by developing methods to isolate intermediate complexes of the *E. coli* RNA polymerase, followed by biochemical studies of their structure and properties (Levin *et al.*, 1987; Krummel and Chamberlin, 1991; Krummel and Chamberlin, 1992ab). These studies have revealed an unexpected complexity of the initiation and elongation process. Translocation of the polymerase along the DNA is not synchronized with addition of each nucleotide residue to the RNA chain, but appears to be periodic, occurring after addition of 8-12 residues. The structure of different complexes along a transcription unit, determined by DNA and RNA footprinting varies dramatically. The sensitivity of the RNA transcript in the region near the growing point to single strand specific RNases, together with the sensitivity of the template strand to permanganate cleavage, suggests that there is little or no formation of DNA-RNA hybrid in ternary complexes. Instead, RNA binding to the polymerase protein appears to play a major role in complex stability.

Complexes halted during transcription can undergo two unexpected and novel reactions. Some complexes rapidly lose the ability to resume elongation, entering a "dead end" state (Arndt and Chamberlin, 1990; Krummel and Chamberlin, 1992ab). Some complexes undergo transcript cleavage at sites 2-10nt from the 3' terminus of the transcript, and rapidly release the resulting 3' terminal fragment (Surratt *et al.*, 1991). The resulting 5'-terminal fragment remains bound in the ternary complex, and can then be elongated normally. Recent studies from the laboratories of Asis Das and Alex Goldfarb suggest that both dead end complexes and the transcript cleavage reaction are physiologically significant reactions in *E. coli*.

KINETIC AND STRUCTURAL EVIDENCE FOR Mg^{2+}-DRIVEN CONVERSION OF OPEN COMPLEXES (RP_{O1} +~$3Mg^{2+}$ → RP_{O2}) ON THE PATHWAY TO TRANSCRIPTION INITIATION BY $E\sigma^{70}$ RNA POLYMERASE

M. Thomas Record, Jr., Won Chul Suh, Sigrid Leirmo, and Paula J. Richey,
Departments of Chemistry and Biochemistry, University of Wisconsin-Madison
Madison, WI 53706

In vitro structural studies of open complexes involving *Escherichia coli* RNA polymerase and the λP_R promoter, using potassium permanganate, demonstrate the existence of two types of open complexes, RP_{O1} and RP_{O2}, in which strand opening in RP_{O1} (~10-12 base pairs) is less complete at both extremes than in RP_{O2} (~14 base pairs). The conversion of RP_{O1} to RP_{O2} appears rapid under the conditions investigated. This conversion is strongly dependent on the presence of Mg^{2+} but weakly dependent on temperature, indicating that uptake of Mg^{2+} is the primary requirement for the conformational transition of RP_{O1} to form RP_{O2}. Analogous behavior is observed for the *lac*UV5 promoter. Kinetic studies on the λP_R promoter provide further evidence for the existence of at least two mechanistically-significant forms of open complexes. In this proposed mechanism the intermediate open complex, RP_{O1}, takes up approximately 3 Mg^{2+} to form RP_{O2}. The overall mechanism, including the various steps at which ions are taken up to form closed or open complexes, is therefore

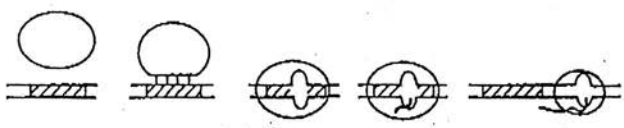


Sequences Involved in the Activation of *E.coli* Promoters

R. Knaus, M. Berlin, R. Lutz and H. Bujard
ZMBH, University of Heidelberg

The *in vivo* activity among unregulated *E.coli* promoters can differ by several orders of magnitude. In addition the activity of individual regulated promoters may be modulated over a wide range by negatively and/or positively acting regulatory proteins. What are the principles which determine promoter activity and how can this activity be controlled by regulatory systems?

Promoter activity or promoter strength is defined by the rate at which productively transcribing RNA polymerases leave the promoter region in their "elongation state". Consequently each step preceeding this event can in principle be rate limiting and thereby determine the strength of a promoter (Fig.)



$R+P \rightleftharpoons RP_c \rightleftharpoons [RP_I] \rightarrow RP_o \longrightarrow RP_i \longrightarrow RDNA_el$

Pathway of RNA-polymerase promoter interaction. Upon encountering a promoter sequence RNA polymerase forms a complex in which the DNA is still in a "closed" state (RP_c). By passing through intermediate states (RP_I) the "open" complex (RP_o) is established in which a stretch of 10 to 15 bp is melted. RP_o is assumed to readily initiate RNA synthesis (RP_i). Upon a drastic conformational change the enzyme clears the promoter engaged in the elongation complex ($RDNA_el$)

We have identified promoters which are limited in their function at different steps within the pathway of polymerase promoter interaction such as promoter recognition and formation of a first stable complex, various isomerisation steps leading to the open complex and clearance of the promoter by an elongation complex. In a number of promoters sequence elements can be correlated with partial functions of the overall process of promoter RNA polymerase interaction and it turns out that sequences upstream as well as downstream of the core promoter (spanning from +1, the transcriptional start site, to -35) can play a decisive role in promoter function (for review see ref. 1). The functional program of a promoter can be optimized in different ways suggesting an adaption of a promoter to the mode by which its activity is regulated. Three prototype promoters were investigated in more detail:

- the unregulated promoter P_{N25} of virulent coliphage T5

- the negatively controlled promoter P_L of coliphage λ
- the positively and negatively regulated P_{lac} of the *E.coli* lac operon.

P_{N25} is a strong promoter *in vivo*. It is recognized by RNA polymerase at an extremely high rate ($k_1 \approx 3x10^8$ M^{-1} s^{-1}) and has a high homology score i.e. it resembles closely the consensus sequence derived from a collection of more than 100 promoter sequences.
By constrast P_L has a low homology score and is recognized about 30 times less efficiently by RNA polymerase since its core region is highly deoptimized for early steps of the promoter/RNA polymerase interaction. Thus, P_L appears as a paradigme for promoters which are tightly repressed in the "classical" mode i.e. RNA polymerase and repressor compete for overlapping binding sites. Interestingly, when derepressed P_L is an even stronger promoter *in vivo* than P_{N25}. This is largely due to upstream sequences in which the ATAAAT motive plays a major role. This sequence motive though located around position -53 can drastically influence the rate of RNA polymerase binding.

P_{lac} is recognized extremely inefficiently by RNA polymerase and can therefore be tightly repressed by the *lac* repressor. In presence of the cAMP/CRP activator it is converted into an efficient promoter which now cannot be as tightly repressed anymore as in its non-activated form. We have varied the P_{lacwt}-sequence in several ways in particular by changing the site for activator binding and measured the activity of these promoter variants *in vivo* in the presence and absence of cAMP/CRP as well as of AraC/arabinose (2). Whereas both activators strongly stimulate transcription from P_{lacwt} the different variants are activated to various extends. To examine whether these activators contribute to promoter recognition we have monitored their effect *in vivo* also in the repressed state, since the repression factor is indirectly proportional to k_{ON}, the rate of complex formation between RNA polymerase and promoter (3). Our data suggest that both activators can increase the rate of promoter recognition *in vivo*. In the context of P_{lac} this appears to be the major effect of activation. However, results with other promoters indicate that also late steps such as promoter clearance may be facilitated by the two activators studied.

1. Knaus, R. and Bujard, H. (1990)
Principles Governing the Activity of *E.coli* Promoters.
In: Nucleic Acids and Molecular Biology **4**; Eds. Eckstein/Lilley,
Springer Verlag, 110-122.

2. Schleif, R. (1987)
"The L-Arabinose Operon" in "*E.coli* and*S.typhjimurium*"
ed. Neidhardt, F., ASM-Press, Washington, USA., pp. 1473-148.

3. Lanzer, M. and Bujard, H. (1988)
Promoters largely determine the efficiency of repressor action.
Proc.Natl.Sci. USA, **85**, 8973-8977.

Why is the promoter of the bgl operon cryptic in the wild type?

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The promoter of the bgl operon of E.coli is very weak causing crypticity of the operon in the wild type. It is, however, activated by various types of spontaneous mutations (see Rak and Schnetz (1992)). These are (i) transposition of mobile elements IS1 and IS5 into the vicinity of the promoter, (ii) point mutations improving the CAP binding site, and (iii) deletion of an upstream AT-rich region. (iv) The operon is also activate in gyrase or hns (bglY, osmZ) host mutants.

While trying to study the mechanisms of activation of the promoter by the various types of mutations we recognized that it would be crucial to try to first understand the reason for crypticity of the promoter in the wild type state. Some relevant data to this end: (i) Substitution experiments revealed that the lac promoter is inactivated in the bgl context, too. (ii) The lac promoter was also inactive in this context when the entire promoter region including the CAP binding site was substituted for the lac promoter region including its CAP site. (iii) Substitution of the bgl promoter together with its CAP binding site for the CAP-independent lacUV5 promoter again resulted in inactivity of the substituted promoter. Taken together these data indicate that inactivation is context-specific rather than promoter or CAP-site specific.

Additional experiments revealed that inactivation of promoter activity is brought about by the concerted interaction of upstream and downstream bgl sequences: Promoter-silencing is releaved when an AT-rich sequence located upstream of the CAP binding site is deleted. It is also releaved when the whole promoter region (with the bgl promoter or its various substitutions) including the upstream AT-rich region is placed in front of the lac operon instead of the bgl operon.

A model on the mechanisms of silencing as well as of promoter activation by the various mutations will be presented on the poster.

Schnetz, K. and B. Rak (1992) IS5: a mobile enhancer of transcription in Escherichia coli" Proc. Natl. Acad. Sci. USA 89, 1244-1248.

Transcriptional regulation of *repA*, the gene of the Pseudomonas plasmid pPS10 replication protein.

Dario García de Viedma, María Jesús Ruiz-Echevarría, Rafael Giraldo Suárez, Rudy Lurz* and Ramón Díaz Orejas

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The RepA protein of plasmid pPS10 has a dual function: it promotes replication from specific region of the plasmid, the origin of replication, and it is a repressor of its own synthesis (Nieto et al. J. Mol. Biol. 223, 415-426, 1992). It is thought that RepA interacts with the origin of replication in a region containing four direct repeats of 22 bp (iterons). In this communication we analyze transcription of *repA* and evaluate the RepA-DNA interactions that autoregulate expression of this gene.

We have determined the 5´and 3´ ends of the *repA* transcript. The promoter of *repA* has been defined to a 90 bp region. This region is adjacent to the origin of replication, can promote transcription of a *lacZ* reporter gene and contains a preferential RNA-Polymerase binding site. Sequence analysis identified in this region, and at the appropriate distance of the transcription initiation point, the -10 and -35 boxes of a typical σ^{70} promoter.

Transcription of the *lacZ* reporter gene driven by the *repA* promoter can be inhibited >80% by the RepA protein *in trans*. Mutations in a sequence that shares homology with the iterons of the origin, and that is located between the -10 and -35 boxes of the promoter, prevent autoregulation mediated by RepA. Possible interactions of the RepA protein with the iterons of the origin or with a 11 bp sequence that precede the *repA* gene and that shares homology with the iterons, do not have an effect in autoregulation. This indicates that the RepA protein autoregulates its own synthesis at the transcription initiation level and shows that the mutated sequence is either the main target for autoregulation or an essential part of it.

Partially purified preparations of the RepA protein are able to form specific complexes with a fragment that includes the origin of replication and the promoter of *repA*. Protection experiments indicate that the RepA protein binds to a 45 bp region that extends from the -10 box of the promoter to the dnaA box of the origin of replication. This region contains two out-of-phase inverted repetitions of 6 bp. The sequence of these repetions is found in the iterons of the origin. Further analysis of RepA-DNA complexes suggests that RepA enters in this region in two stages: the first stage implies protein-DNA interactions and the second one protein-protein interactions. The region protected by RepA after this second entrance is similar in lenght to the one protected by the first entrance but the pattern of protection is altered and the protection seems to be stronger. The RepA protein preparation interacts much more efficiently with the promoter of repA that with the iterons of the origin. The implications of these findings for regulation of the pPS10 replicon will be discussed.

This work has been founded by Projects from the Spanish CICYT, BIO88- 049 and BIO91-1055.

POLYPEPTIDES CONTAINING HIGHLY CONSERVED REGIONS OF TRANSCRIPTION INITIATION FACTOR σ^{70} EXHIBIT SPECIFICITY OF BINDING TO PROMOTER DNA

Carol A. Gross, M. Thomas Record, Jr., Deborah A. Siegele, William A. Walter and Alicia J. Dombroski, Department of Bacteriology, University of Wisconsin, Madison, WI 53706

The σ^{70} transcription initiation factor of *E. coli* is required for sequence-specific recognition of promoter DNA. Genetic studies and sequence analysis has indicated that σ^{70} possesses two regions, referred to as regions 2 and 4, that recognize the two conserved portions of the prokaryotic promoter, the -10 and -35 hexamers. Purified intact σ^{70} however does not bind DNA. We hypothesize that assembly of intact σ^{70}, with core RNA polymerase, is necessary to induce a conformational change that reveals its DNA binding domains. We have examined the question of whether σ^{70}, in the absence of core RNA polymerase, is able to recognize and bind to promoter DNA by constructing a series of in-frame fusions between glutathione-S-transferase (GST) and various partial polypeptides of σ^{70}. The purified fusions were characterized *in vitro* using nitrocellulose filter binding assays. A fusion carrying conserved region 4 binds preferentially to ptac promoter DNA and shows specificity for the -35 consensus hexamer. A fusion carrying conserved region 2, also prefers promoter over non-promoter DNA, and shows specificity for the -10 consensus sequence. A fusion combining the predicted DNA binding portions of regions 2 through 4 demonstrates specificity for both the -10 and -35 promoter sequences. Mutations in σ^{70}, which confer altered promoter recognition phenotypes upon holoenzyme *in vivo*, were incorporated into a GST fusion and analyzed for binding specificity *in vitro*. Mutations RC584 (Arg to Cys at 584) and RF588 (Arg to Phe at 588) displayed the same promoter preferences *in vitro* as determined for holoenzyme carrying the mutant σ^{70} *in vivo*. Longer fusions, containing progressively more of the amino terminal residues, became increasingly less capable of binding both promoter and non-promoter DNA. These results therefore support the genetic evidence for the participation of σ^{70} in promoter recognition and support an allosteric model whereby DNA binding ability is only conferrred when the amino-terminal segment of σ^{70} (amino acids 1-360) is deleted or assumes a conformation that unmasks the DNA binding domains.

ISOLATION AND CHARACTERIZATION OF THE MAJOR VEGETATIVE RNA POLYMERASE OF *STREPTOMYCES COELICOLOR* A3(2); RENATURATION OF A SIGMA SUBUNIT USING GroEL, Mask Buttner, [1]Kelly L. Brown, [2]Steven Wood, [1]John Innes Institute, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK and [2]Department of Crystallography, Birkbeck College, Malet Street, London WC1E 7HX, UK

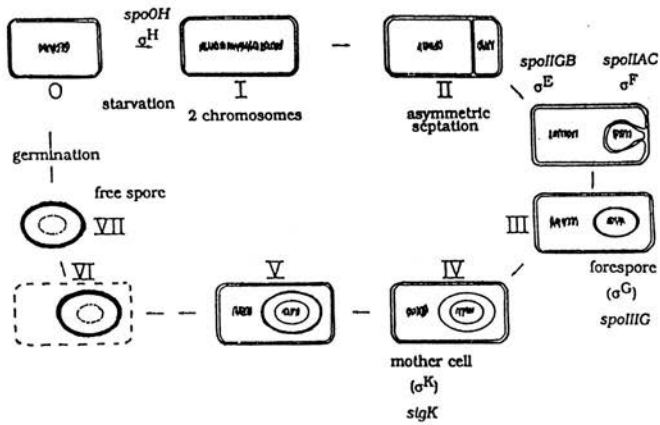
The promoter region of the *dagA* gene is complex; it consists of four distinct promoters with different -10 and -35 regions. We report the isolation of a form of RNA polymerase that mediates transcription *in vitro* from the *dagA*p4 promoter. The core components of this RNA polymerase in association with a polypeptide of ca. 66-kDa direct transcription from the *dagA*p4 promoter; holoenzyme reconstitution experiments show that the 66-kDa polypeptide functions as a sigma factor to direct transcription from both the *dagA*p4 and *veg* promoters *in vitro*. Alignment of the DNA sequences of these two promoters shows they have bases in common at the -10 and -35 regions and these sequences are similar to those observed for the major RNA polymerases of other bacteria. N-terminal amino acid sequence analysis of the 66-kDa polypeptide revealed it to be the product of the *hrdB* gene. Previous experiments showed that the predicted amino acid sequence of the *hrdB* product is very similar to major sigma subunits of other bacteria and that disruption of the *hrdB* gene is lethal. These observations together lead to the conclusion that we have isolated the major RNA polymerase of *Streptomyces coelicolor* A3(2).

We have developed an improved protocol for the renaturation of sigma factors that have been isolated by preparative SDS-polyacrylamide gel electrophoresis. This method involves renaturing the polypeptide in the presence of the bacterial chaperonin GroEL. We expect this protocol to find general application for renaturing other polypeptides that have been subjected to SDS-polyacrylamide gel electrophoresis.

Regulation of gene expression during sporulation in *Bacillus subtilis*

Patrick Stragier
Institut de Biologie Physico-Chimique, Paris, France

In response to nutrient starvation the soil bacterium *Bacillus subtilis* is able to start a developmental process which culminates with the release of a dormant spore. Sporulation involves the formation of two cell types, the forespore and the mother cell, that are created by an asymmetric septation which divides the bacterium in two unequal compartments. Differential gene expression in the two cells is governed by the successive appearance of new sigma factors that are active only in one cell type. The overall process is schematized below.



The four cell-specific sigma factors, σ^F, σ^E, σ^G, and σ^K, are controlled at the level of their activity. Intricate mechanisms delay the appearance of these sigma factors in active form and coordinate gene expression between the two cells. The data currently available indicate that the two cells "talk" to each other and send signals that induce in the other cell the switch to the next developmental stage. The whole sporulation program appears now to depend on four signal transduction pathways that activate the successive sigma factors once specific morphological structures have been achieved.

The role of the α subunit of E.coli RNA polymerase in transcription - a genetic analysis

M.S.Thomas, G.Wegrzyn and R.E.Glass

Until recently, the role of the RNA polymerase α subunit in transcription has remained obscure. However, the isolation of several α mutants which exhibit impaired transcription of certain genes subject to positive control has provided evidence for a functional interaction between this subunit and specific activator proteins. We have been studying one such mutant, rpoA341 (formerly phs), which results in decreased expression of the positively-regulated mel, ara and cys operons, and is characterized by the substitution of a glutamate residue for a lysine at position 271 in the 329 amino acid polypeptide.

Extending our studies further with this interesting mutant we provide evidence for a functional interaction between the bacteriophage λ regulatory proteins cI and cII and the α subunit during transcription activation. Unlike the MelR, AraC and CysB activators, however, the binding sites for cI and cII overlap the -35 consensus hexamer at their target promoters. Therefore the contact site on RNA polymerase for at least some activators of this type (the so-called Contact Site II) is also presumably located on the α subunit. Furthermore, we have utilized the rpoA341 mutant as part of a genetic screen for the isolation of novel α mutants. Our results suggest that replacement of the extreme C-terminal 47 amino acids of the α subunit, comprising part of the so-called Contact Site I for transcriptional activators, by a missense sequence of equivalent length does not prevent the resultant RNA polymerase from supporting cell growth.

TUESDAY, JUNE 16th

Upstream activation of ribosomal RNA transcription in E. coli.

Wilma Ross, Janet T. Newlands, Lin Rao, Khoosheh Gosink, Alex Appleman, A.J. Bokal, Michael Bartlett, Julia Salomon, Cathleen A. Josaitis, Irene Castano, and Richard L. Gourse.
Department of Bacteriology, University of Wisconsin, 1550 Linden Drive, Madison, WI 53706 USA.

Ribosomal RNA transcription is the rate-limiting step in ribosome synthesis. Under conditions of rapid growth, more than half of the cell's RNA synthesis is rRNA. That is, at these times, the seven rrn P1 promoters transcribe more RNA than the rest of the promoters in the cell combined. rRNA transcription is subject to at least two regulatory systems, growth rate dependent control and stringent control. The subject of this presentation is the mechanism that accounts for the extremely high activity of the rRNA promoters.

The -10 and -35 hexamers of the rrn P1 promoters are close matches to the consensus for E. coli promoters recognized by $E\sigma^{70}$, accounting in part for their high activity. For example, rrnB P1 has a perfect -10 sequence, TAATAT, and the -35 sequence, TTGTCA, deviates from consensus at only one position. Spacing between the two hexamers is 16 base pairs, compared to the consensus, 17 bp. Mutations that create a consensus -35 hexamer or 17 bp spacing increase the core promoter activity, but destroy growth rate regulation of the promoter, indicating that the core promoter sequence evolved for regulation purposes rather than for maximum strength (Gaal et al., 1989, Dickson et al., 1989).

Sequences upstream of the -35 hexamer (the upstream activating region, or UAR) play a critical role in determining rrnB P1 promoter strength. An rrnB core promoter lacking rrn sequence upstream of -41 has 300-fold reduced activity in vivo and in vitro compared to a promoter containing rrnB sequences extending 150 bp upstream of the start site, but retains the capacity for growth rate-dependent control (Gourse et al., 1986; Ross et al., 1990; Rao et al., in preparation; Bartlett and Gourse, unpublished data).

There are two functionally distinct components of the rrnB P1 UAR. The sequences between -150 and -60 bind the 11.2 kd Fis protein and increase promoter activity approximately 10-fold. The sequences between -60 and -40 increase promoter activity in vivo and in vitro approximately 30-fold independent of other protein factors. We have termed the latter effect Factor-Independent Activation or FIA. We can view FIA as resulting from an "extended promoter" region.

Fis-Dependent Activation

In vitro and in vivo footprints indicate that Fis protein binds to three sites in the rrnB UAR (centered at -71,-102 and -143; Ross et al, 1990; Ross et al. unpublished). Fis activates transcription in vitro and in vivo in a manner dependent upon binding to Fis Site I (Ross et al, 1990; Josaitis et al., 1990; Bokal and Gourse, unpublished). Fis Sites II and III exert only a very small effect (approximately a 15-25% increase) on transcriptional activity in vivo, either in the presence or the absence of a functional Fis Site I. There is no apparent cooperativity in Fis binding to the three UAR sites (Bokal and Gourse, unpublished).

Several lines of evidence are consistent with a mechanism of Fis activation involving Fis-RNAP interactions. We have identified Fis mutants from a collection isolated and purified in Reid Johnson's laboratory (Osuna et al., 1991) that fail to support

transcriptional activation of rrnB P1 (Gosink et al., submitted). A particularly informative class of mutants retains the ability to bind to Fis Site I and bend this site upon binding, yet fails to activate transcription. Thus, functions in addition to DNA binding and bending are required for activation.

Other evidence is consistent with the presence of Fis-RNAP interactions. Fis-dependent activation can occur on restriction fragments lacking DNA upstream of Fis Site I. Thus, Fis does not function solely to bring sequences upstream of its binding site into the proximity of RNAP. In addition, Fis-dependent activation is face-of-the-helix dependent both in vivo and in vitro, with complete Fis-dependent activation retained when Fis Site I is displaced by 11 bp (Newlands et al., 1992). Furthermore, footprints with a variety of chemical reagents indicate that Fis and RNAP occupy the same side of the helix. Finally, the presence of both RNAP and Fis in the footprinting reaction alters the pattern of Fis protection (Ross et al., unpublished).

We have recently shown that Fis can also activate non-rRNA promoters. We created a hybrid promoter containing the wild type lac core region with rrnB sequences substituted for lac sequences upstream of the -35 hexamer. This promoter was stimulated by Fis both in vitro and in vivo (Appleman and Gourse, unpublished).

Factor-Independent Activation - An Extended Promoter Region

The A+T-rich rrnB P1 sequence from -40 to -60 (between the "core" promoter and Fis Site I) contributes approximately 30-fold to promoter strength in vivo in a fis-independent manner (Rao et al. in preparation). Kinetic analysis of the rate of association of RNAP with a set of rrnB P1 promoters with different upstream endpoints indicates that the -40 to -60 region increases transcription 30-fold in the absence of added protein factors. The -40 to -50 region primarily affects a kinetic step after initial promoter binding (k_i) (Rao et al. unpublished), while the -50 to -60 region increases the bimolecular step, K_B (Leirmo and Gourse, 1991).

The -40 to -60 region is protected by RNAP in DNAseI or hydroxyl radical footprint experiments (Gourse, 1988; Newlands et al., 1991). Protection of sequences in the -50 to -55 region from hydroxyl radical attack requires that UAR sequences be present; it is not observed on rrn promoters with non-rrn sequence substituted upstream of -46 and is thus not directed solely by binding to the core promoter region. A promoter mutant with an 11 bp insertion at -46 retains the specific protection of the -50 to -55 rrn sequence, even though this sequence is now displaced upstream by 1 turn of the DNA helix (Newlands et al., 1992). This further suggests that specific properties of this sequence are required for the interaction.

Utilization of the FIA region by RNAP requires the carboxy-terminal portion of the α subunit of RNAP (Ross et al., in preparation). It has been shown previously that this region of RNAP is required for positive control of certain promoters (e.g. lac by cAMP-CAP; Igarashi and Ishihama, 1991). As indicated above, wild type RNAP transcribes rrnB P1 promoters containing the FIA region with greatly increased efficiency compared to promoters lacking this region. However, reconstituted mutant holoenzymes containing only the 235 or 256 amino-terminal amino acids of α (provided by A. Ishihama) transcribe the rrnB P1 promoter poorly in vitro and do not distinguish between rrnB P1 promoters containing or lacking the FIA region. Consistent with this finding, footprint protection of rrnB P1 by the mutant holoenzymes extends only to approximately -40. The protections further upstream that are characteristic of wild type RNAP-FIA region interactions are missing with the mutant enzymes, as if the mutant enzymes cannot recognize this sequence information.

We have also found that two other stable RNA promoters, previously identified as having upstream activation regions, rrnB P2 and leuV, are not efficiently utilized by the mutant holoenzymes. In addition, we have identified a non-stable RNA promoter that also requires the C-terminal region of α for maximal activity.

As a result, we have identified a promoter element separable from the core region that is a major contributor to E. coli promoter strength. The C-terminal region of the α subunit of RNAP is required for utilization of this element and for contacts with RNAP between -40 and -60. A simple model is that alpha makes contacts to this upstream element and thereby increases promoter activity.

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THE PATHWAY OF N4 VIRION RNA POLYMETASE-PROMOTER RECOGNITION

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Coliphage N4 virions contain a DNA-dependent RNA polymerase (N4 virion RNA polymerase) which is injected into the host cell upon infection and synthesizes the phage early RNAs. The enzyme is unique in that, in vitro, it is inactive on double-stranded N4 DNA; however, denatured, promoter-containing templates are efficiently and accurately transcribed. We have shown that all determinants of promoter recognition are present in the template strand indicating that the N4 virion RNA polymerase is a site-specific, single-stranded DNA binding protein.

The three promoters for N4 virion RNA polymerase present in the N4 genome contain blocks of homology from position -18 to +1 with a conserved GC-rich heptamer centered at position -12 (Fig.1). The promoters also contain two sets of short inverted repeats. The upstream inverted repeats, which are centered at the heptamer, are of the same length in all promoters and contain both conserved and non-conserved bases. In contrast, the length and distance between the downstream inverted repeats, which include the site of transcription initiation, vary. Analysis of the transcriptional activity of a large collection of promoter mutants indicates that conserved sequences present between -18 and +1 and the integrity of the inverted repeats centered at -12 are essential for template binding and transcriptional activity. This striking requirement for promoter recognition suggests the necessity for a specific secondary structure. The patterns of T7 endonuclease I and T4 endonuclease VII cleavage of single-stranded DNAs containing N4 promoters indicate the existence of a "hairpin" structure centered at -12.

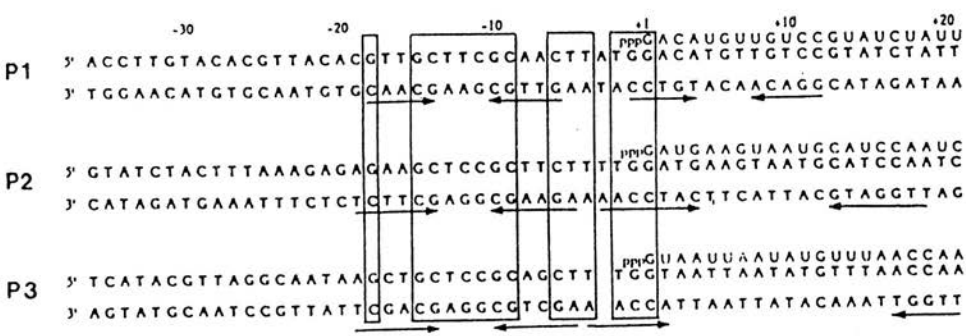
Transcription of the early genes of bacteriophage N4 requires, in addition to the N4 virion RNA polymerase, the activities of two host factors: E. coli DNA gyrase and E. coli single-stranded DNA binding (SSB) protein. In vitro, linear or relaxed templates cannot be activated by SSB; however, supercoiled template and SSB allow the virion polymerase to recognize its promoters on duplex DNA and activate transcription. Activation is E. coli SSB specific, other single-stranded DNA binding proteins cannot substitute. We propose a model for virion RNA polymerase-promoter utilization where gyrase-induced supercoiling elicits single-strandedness at the promoter region (Fig. 2A). Under these conditions, the inverted repeats flanking the conserved GC rich heptamer form the hairpin structure which, in conjunction with conserved sequences present between -18 and +1, is required for RNA polymerase binding. Subsequently, binding of E. coli SSB to the promoter region stabilizes a structure to yield an "activated promoter" (Fig. 2B) which can now be recognized by virion RNA polymerase (Fig.2C). After N4 virion RNA polymerase binding, the stem-loop structure at the

promoter must be melted and initial contacts broken in order to carry out formation of the first phosphodiester bond (Fig. 2D).

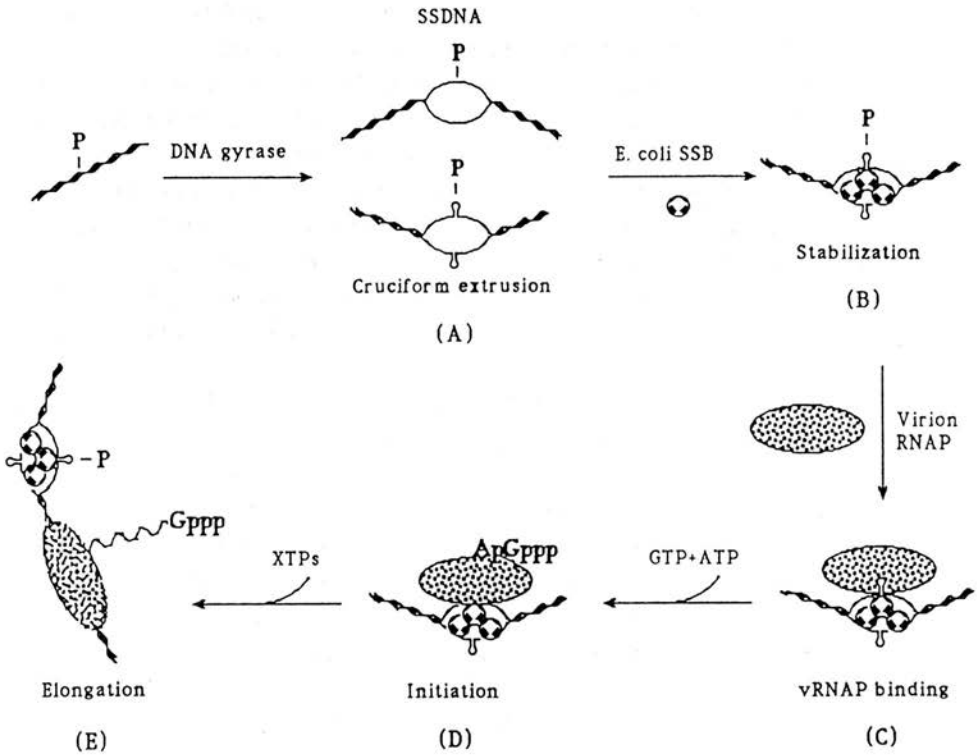
To define the role of supercoiling in transcriptional activation of virion RNA polymerase promoters, we have cloned N4 early promoters in a vector which allows for the generation of minicircles constituted primarily of N4 sequences. Incubation in the presence of varying concentrations of Ethidium Bromide and topoisomerase I allows for the isolation of circles with different superhelical densities. Chloroacetaldehyde (CAA), which reacts with unpaired Cytosines and Adenines to form the etheno derivatives, is used to probe forchanges in promoter structure. Reacted bases are mapped by primer extension. Results of these experiments indicate that sequences at the promoters are insensitive to CAA in relaxed DNA. Upon increase in superhelical density, sequences at the loop of the hairpin in the complementary strand become CAA-reactive. Surprisingly, sequences on the template strand remain unreactive. These results indicate that supercoiling leads to cruciform extrusion and that the template and complementary strands adopt different structures.

To define the basis for E. coli SSB's specificity of activation of N4 early promoters, we have studied the interaction of virion RNA polymerase and SSB with promoter-containing, single-stranded templates. E. coli SSB, although not absolutely required, activates N4 virion RNA polymerase transcription on single-stranded templates. The results of footprinting experiments in the presence of E. coli SSB and virion RNA polymerase, using both wild-type and mutant promoters indicate that the specificity of E. coli SSB activation resides in its unique mode of interacting with promoter-containing templates. We conclude that E. coli SSB acts as an activator of transcription by providing the correct DNA structure for RNA polymerase binding.

Figure 1



N4 virion RNApolymerase-promoter utilization pathway



Control of bacteriophage ϕ29 DNA transcription by the regulatory protein p4.

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Late genes of *B. subtilis* phage ϕ29 are transcribted from the PA3 promoter, which is located close to the divergently transcribed main early promoter, PA2b. The viral early protein p4 is a transcriptional regulator that activates the late promoter and represses the early PA2b promoter (1, 2). Protein p4 binds to a region located between nucleotides -56 and -102 relative to the PA3 transcription start site (-16 and -62 relative to PA2b), recognizing an 8 bp long inverted repeat (3). This DNA region has a sequence-directed curvature that greatly increases by protein p4 binding (1, 4). The induction of the curvature is relevant both for the activation of the late promoter PA3 and for the repression of the early promoter PA2b.

Protein p4 mutants that produce only a partial bending of the promoter do not activate transcription (4). Curvature could provide a DNA conformation that either stabilizes binding of the RNA polymerase to the promoter, orients protein p4 towards the RNA polymerase, enabling adequate contacts between the two proteins, or both. Indeed, several data indicate that protein p4 and RNA polymerase bound to PA3 contact each other. a) The two proteins bind to the promoter cooperatively, stabilizing each other in their binding sites (3). b) When protein p4 binding site was separated from that of the RNA polymerase at PA3, activation only occurred if the two binding sites were separated by 1, 2 or 3 complete helical turns of bent DNA (5). These results also suggest that protein p4 has to be stereospecifically oriented relative to the RNA polymerase for a correct interaction, and that activation at a distance requires the formation of a DNA loop. c) When fragments containing either the RNA polymerase or the protein p4 binding regions were used, the two proteins were able to bind simultaneously to each of those fragments, again indicating direct protein-protein contacts.

Activation of the late PA3 promoter is paralelled by repression of the divergently transcribed early A2b promoter, which partially overlaps with the protein p4 binding site. Binding of protein p4 displaces the RNA polymerase

from the early PA2b promoter to the late PA3 promoter, suggesting that steric hindrance may be involved in the repression of PA2b by protein p4. However, the protein p4-induced DNA bending also seems to participate in the repression process. When the DNA curvature of the protein p4 binding site was increased by site-directed mutagenesis in the same direction as that produced by protein p4, it was shown that DNA bending by itself could inhibit transcription from PA2b (2). This bending affected both the rate of formation of transcriptionally active open complex and the affinity of the RNA polymerase for the PA2b promoter. Therefore, protein p4-induced DNA bending could participate in PA2b repression by producing a DNA structure that prevents the RNA polymerase from recognizing the promoter. This effect could complement the steric hindrance increasing the repression efficiency, since competition between protein p4 and RNA polymerase for their binding sites could be diminished.

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Contortions, Distortions, and Positions of AraC

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In the absence of arabinose or AraC protein, the p_{BAD} promoter of the arabinose operon of *Escherichia coli* is only weakly active. The presence of both arabinose and AraC protein stimulates the activity of the promoter more than 200-fold. In the absence of arabinose, the dimeric AraC protein contacts the more upstream of the two half-sites of *araI* that are adjacent to the RNA polymerase binding site. In this state the protein also contacts the $araO_2$ half-site which is located 210 base pairs away, thereby forming a loop in the DNA. When arabinose is added, AraC protein ceases to contact the $araO_2$ half-site and instead contacts both half-sites at *araI*. The work to be reported addresses two basic questions about this system. First, since the *araI* site appears by inspection to contain elements both of direct repeat symmetry and of inverted repeat symmetry in the half-sites, and previous experiments have suggested that the protein might bind to a directly repeated half-site, what, in fact, is the symmetry of the AraC binding site and the AraC protein? If it is indeed a direct repeat, how is this accomplished without the protein's polymerizing indefinitely? The second question is whether the AraC protein must be located in a particular position with respect to the p_{BAD} promoter in order to activate transcription.

The question of the symmetry of the AraC protein has been addressed by constructing a binding site that contains a direct repeat of the consensus half-site. AraC binds 100-fold more tightly to this site than it does to any naturally occurring site. The pattern of protected and enhanced dimethylsulfate-reactive guanines and DNAse-labile phosphodiester bonds in this site possesses a direct repeat pattern. These suggest that the protein binds to a direct repeat of half-sites.

If a dimer of AraC binds to a direct repeat of half-sites, can a trimer bind to three repeated sites? Experimentally the answer is no. Several schemes can be envisioned which could limit polymerization. The most simple is that AraC possesses a dimerization domain and a DNA binding domain that are only loosely connected. Dimerization could be via the normal isotropic,

face-to-face, interactions, and by virtue of the loose connection between the dimerization and DNA binding domains, the protein could bind to directly repeated half-sites. Indeed, this seems to be the case. AraC binds to directly repeated half-sites, inverted repeat half-sites, and half-sites that are separated by 10 or 21 base pairs. The mechanism by which arabinose decreases the propensity of AraC to engage in looping interactions and instead to bind to directly adjacent sites has also be addressed with these various sites. In summary, the presence of arabinose makes AraC prefer to bind to local sites.

From what positions can AraC activate transcription? In the wild type situation the second half-site at *araI* overlaps the -35 region of the RNA polymerase binding site by four bases. We explored a wide set of positions for the activation by AraC by placing a pair of half-sites far from the p_{BAD} promoter, removing random amounts of DNA from between the half-sites and the promoter, and selecting molecules which expressed the p_{BAD} promoter at high levels. Substantial activation was seen only when the binding sites overlap the polymerase -35 region by two, three, or four bases. An auxiliary finding was that the exact sequence of the p_{BAD} promoter need not be maintained. AraC will activate transcription from a variety of promoters if it is properly positioned.

One might imagine that only the half-site closest to the RNA polymerase is relevant to activation of transcription. This turned out not to be the case. When the more upstream of the half-sites was moved around, induction was inevitably reduced by dramatic amounts. The conclusion from these experiments is that the overal location and shape of AraC protein is relevant to induction of transcription by the protein.

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PROKARYOTIC ENHANCERS AND ENHANCER-BINDING PROTEINS, D. S. Weiss, K. Klose, S. Porter, A. North, A. Wedel, and S. Kustu, Departments of Plant Pathology and Molecular and Cell Biology, University of California, Berkeley.

A number of prokaryotic enhancer-binding proteins activate transcription by the alternative holoenzyme form of RNA polymerase, σ^{54}- holoenzyme, allowing this form of polymerase to transcribe in response to different physiological signals. The best-characterized of these enhancer-binding proteins is the NTRC protein (nitrogen regulatory protein C) of enteric bacteria. The function of NTRC has been studied intensively at the major promoter for the *glnA* gene, which encodes glutamine synthetase. NTRC stimulates the isomerization of closed complexes between σ^{54}-holoenzyme and the *glnA* promoter to open complexes in an ATP-dependent reaction. To do so NTRC binds to its enhancer-like sites, which are normally located between 100 and 150 bp upstream of the transcriptional startsite, and contacts the polymerase by means of DNA loop formation. One function of the enhancer is to tether NTRC in high local concentration near the promoter. To stimulate open complex formation NTRC must hydrolyze ATP. ATP hydrolysis depends upon phosphorylation of an aspartate residue located in a regulatory domain of NTRC that is distinct from the domain responsible for ATP hydrolysis and transcriptional activation. Phosphorylation of NTRC is increased under conditions of nitrogen limitation, conditions under which transcription of *glnA* is increased. It is our working hypothesis that ATP hydrolysis is not needed for specific contact between NTRC and polymerase per se, but rather functions to allow a change in configuration of polymerase once contact has occurred.

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Concerted regulation of the σ^{54}-dependent Pu promoter of TOL plasmid through upstream sequences responsive to multiple signals.

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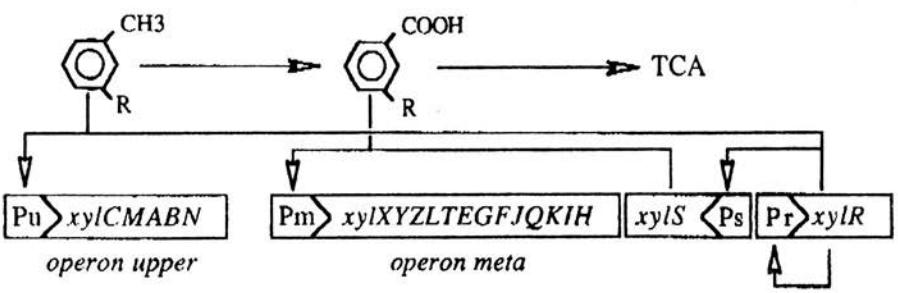
The TOL plasmid of *Pseudomonas putida* affords the growth of harboring bacteria on toluene and *m*-xylene as the only carbon source. Biodegradation occurs in two major steps, involving the biotransformation of toluene/xylene into benzoate/toluate (mediated by the gene products of the*upper* operon) and the subsequent meta-cleavage of the aromatic ring and metabolisation down to TCA intermediates. Transcription of the corresponding catabolic pathways when cells are exposed to pathway substrates or structural analogs is effected by a complex regulatory cascade summarized as follows :



In the presence of toluene, the Pu promoter, which belongs to the σ^{54}-dependent class, is activated by the XylR protein, which binds to at least two upstream binding sites (UBS). Besides the action of XylR, Pu activity requires IHF, which binds between the positions -52/-79 and supposedly helps to bring the UBS/XylR complex into close contact with the σ^{54}-RNApol.

Transcription from the Pu promoter is subjected to a very tight control so that it becomes indetectable in non-induced conditions -provided that the whole system is analysed in monocopy. This is explained in part by the transcriptional termination activity of the UBSs, which is manifested *in vivo* regardles of its binding to XylR protein. At least one more control is overimposed to the actions of XylR and IHF : Full Pu activity requires the cells to be in stationary phase, although the mechanism operating for such control is presently unknown. The effect of IHF, DNA curvatures and supercoiling on such responsiveness to growth phase will be discussed.

Action at a Peculiar Prokaryotic Enhancer

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(Reporting on research by: Daniel R. Herendeen, Kelly P. Williams, Rachel L. Tinker, Jeffrey W. Winkelman, Glenn M. Sanders, George A. Kassavetis and E. Peter Geiduschek)

BACKGROUND

Positive transcriptional control in certain prokaryotic systems is exerted from distant sites that can properly be called enhancers. The work that is described in my talk concerns one of these systems, the replication-connected regulation of the late genes of bacteriophage T4.

The T4 late genes occupy approximately 40% of the ~165 kbp viral genome and are accessible to transcription from approximately forty late promoters, which consist of a simple consensus sequence element, TATAAATA, centered 10 bp upstream of the transcriptional start site. Transcription initiates at T4 late promoters during normal phage infection only after DNA has begun to replicate, and it is conditional upon DNA replication. Functions of two proteins encoded by T4 genes 33 and 55 (gp33 and gp55), which bind to the (*E. coli*) host cell's RNA polymerase core, are also required *in vivo* for initiation of transcription at late promoters. Other modifications of the RNA polymerase that occur as a result of T4 infection, including the ADP-ribosylation of its α subunit and attachment of the T4-encoded RpbA protein, do not change the essential characteristics of late transcription.

Phage T4 encodes an entire independent replication machinery, consisting of a DNA polymerase (gp43), a single-stranded DNA-binding protein (gp32), a DNA helicase (gp41) and primase (gp61), and three DNA polymerase accessory proteins (gp44, gp62 and gp45). The gp44 and gp62 form a tightly associated complex with a DNA-dependent ATPase activity that is stimulated by gp45. The preferred DNA substrate of this ATPase is a primer-template junction. The three accessory proteins drastically change the processivity of DNA chain elongation by the gp43 DNA polymerase in an ATP hydrolysis-requiring reaction.

The 188-amino acid T4 gene-55 protein is a member of the σ family of RNA polymerase-binding proteins and is required for T4 late promoter recognition by the *E. coli* RNA polymerase core (E). The E.gp55 RNA polymerase holoenzyme forms specific complexes with T4 late promoters, and specifically initiates transcription at late promoters in negatively supercoiled DNA. This transcription is, however, greatly diminished at higher ionic strength, at lower temperature and in relaxed DNA. T4 DNA is not globally supercoiled *in vivo* at times of the infection at which T4 late transcription takes place (reviewed in, and abstracted from, 1).

AN *IN VITRO* SYSTEM

An *in vitro* system for analyzing the functions of replication proteins in T4 late transcription has been constructed. The DNA templates of this transcription system are plasmids containing the following components: 1) a site that can be nicked by filamentous phage gpII, generating a start site for, and specifying the polarity of, leading strand DNA replication by T4 replication proteins; 2) one or more T4 late transcription units whose transcriptional polarity is identical with, or opposed to, the polarity of leading strand replication. Transcription by gp55-containing

RNA polymerase in this *in vitro* system is greatly stimulated by the three T4 DNA polymerase accessory proteins gp44, gp62 and gp45, even in the absence of DNA replication, and is even further stimulated by replication (with gp43, gp32 and deoxyribonucleoside triphosphates also present), particularly when gp44, gp62 and gp45 are limiting. Transcriptional activation requires a nicked DNA template, and hydrolyzable ATP (or dATP). The nick, acting as a binding site for the three DNA polymerase accessory proteins, must be located in cis to the activable T4 late promoter, but can be placed upstream or downstream, and at large distances, from it. Thus, the nick functions as an enhancer that is peculiar in two respects: 1) it is a DNA structure, rather than a sequence; 2) it is the simplest structural singularity imaginable. Transcriptional activation also requires RNA polymerase-bound gp33. Unenhanced transcription (in covalently closed negatively supercoiled DNA) is not stimulated by gp44, gp62 and gp45, and is, at higher ionic strengths, diminished by gp33. Thus, gp33 functions as a transcriptional co-activator protein, functioning only in enhanced, but not in basal transcription (references 2, 3).

Transcriptional enhancement is due to an ATP hydrolysis-dependent acceleration of the rate of promoter opening. Once a T4 late promoter has been opened, it stays open even if further ATP hydrolysis is blocked. Thus, transcriptional enhancement appears to function by overcoming a kinetic barrier to promoter opening.

COMMUNICATION BETWEEN THE ENHANCER AND THE PROMOTER

Experiments intended to provide an understanding of the function of the enhancer in T4 late transcription contribute surprising answers. The DNA nick that serves as enhancer must have a specific polarity relative to the direction of transcription: it is only effective when placed in the nontranscribed strand, regardless of whether it is located upstream or downstream of the promoter, and regardless of the distance separating the nick from the promoter. The ability of a nick-as-enhancer placed in one ring of a singly linked DNA catenane to activate initiation of transcription at a T4 late promoter placed in the linked (relaxed and covalently closed) DNA circle has also been examined: the DNA polymerase accessory proteins, gp44, 62 and 45, only activate transcription in the DNA ring that contains the nick. The absolute cis-action requirement of transcriptional activation at the T4 late promoter implies a role for DNA as the conduit of transcriptional activation. In contrast, when the enhancer and promoter communicate through space by direct protein-protein interaction, as they are generally thought to do, the connecting DNA functions primarily as a tether that confines the interacting components to a common space. In such systems, when enhancers and promoters are placed on the separate linked DNA rings of catenanes, they communicate effectively (4, 5).

A direct demonstration that tracking along DNA is, instead, absolutely required for transcriptional activation at T4 late promoters has been demonstrated by further experiments in which the DNA path between the nick-as-enhancer and the promoter is blocked with a protein that binds very tightly to DNA. In circular DNA, transcriptional activation is only abolished when both paths between the enhancer and promoter are blocked by the DNA-binding protein. (This is further proof that transcriptional activation can take place from either side of the promoter).

Transcriptional enhancement at a T4 late promoter fails in linear DNA, but is retained in circular DNA, at high electrolyte concentrations. That this is a reflection of the role of proteins that track along DNA in transcriptional activation is demonstrated by an experiment showing that, when the tightly DNA-binding protein is interposed between an enhancer—T4 late promoter unit and the ends of a linear DNA molecule, it confers electrolyte concentration-resistance

upon transcriptional enhancement in linear DNA.

STRUCTURAL BASIS OF TRANSCRIPTIONAL ENHANCEMENT

Aspects of the structure of enhanced and unenhanced transcription complexes at a late promoter are under current investigation. The physical structures of enhanced and unenhanced transcription complexes differ at the upstream end of the RNA polymerase binding site. In contrast, enhanced and unenhanced open promoter complexes are indistinguishable with regard to the formation of the DNA "bubble" at the transcriptional start site. The implications of these findings for the mechanism of transcriptional activation are discussed.

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The role of the nucleoid assosiated protein H-NS in osmotic induction at the *proU* promoter of *Escherichia coli* and *Salmonella typhimurium*.

Tom Owen-Hughes, Graham Pavitt, Diogenes Santos, Chris Hulton, Julie Sidebotham, Jay Hinton, and Chris Higgins.

The *proU* promoter is induced approximately 70-fold by increased osmolarity of the growth medium. Mutations disturbing this regulation have been found in the genes encoding DNA gyrase and Topoismerase. We and other workers have found that DNA supercoiling *in vivo* changes in response to osmolarity. This gave rise to a model in which DNA topology played an key role in the osmoregulation of the *proU* promoter. Mutations at *osmZ(hns)* were also found to derepress *proU* expression. This gene was subsequently found to encode the protein H-NS.

H-NS is a major component of bacterial chromatin present at approximately 1 copy per 400bp of chromosomal DNA. Mutations in *hns* are highly plieotropic affecting the expression of many different genes in an allele specific manor. In addition we have found that mutations in *hns* also affect DNA topology.

We have isolated sites downstream of the *proU* promoter that are important for normal osmoregulation *in vivo*. This region includes curved DNA and normal osmoregulation can be restored if it is replaced with heterologous curved DNA. In addition this downstream DNA binds H-NS *in vitro* and a functional *hns* gene is required for it to confer osmoregulation on the *proU* promoter *in vivo*. There does not appear to be a simple recognition sequence involved in H-NS binding, it does, however, show a preference for curved DNA.

It has previously been demonstrated that plasmid linking number is altered upon osmotic shock. We have now found that the presence of curved DNA affects this linking number change only in the presence of a functional copy of the *hns* gene. This suggests a mechanism whereby H-NS interacts with curved DNA downstream of the *proU* promoter causing a change in the local topology of the DNA which derepresses the promoter. These data provide insights into the relationship between chromatin structure and the regulation of DNA topology and promoter activity.

TRANSCRIPTION ACTIVATION BY CAP: IDENTIFICATION OF THE ACTIVATION SURFACE AND ANALYSIS OF INTERACTION WITH RNA POLYMERASE

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A. Identification of the activation surface of CAP: isolation and characterization of mutants of CAP specifically defective in transcription activation at the lac promoter. We have used random mutagenesis of *crp*, the gene that encodes CAP, followed by application of an *in vivo* genetic screen, to isolate positive-control-defective mutants of CAP ("*crp*^{pc} mutants"). We have isolated and characterized twenty-one independent *crp*^{pc} mutants. The twenty-one *crp*^{pc} mutants are defective in transcription activation at the *lac* promoter, but are not defective in DNA binding. Remarkably, the amino acid substitutions in the twenty-one *crp*^{pc} mutants map to the same seven-amino-acid region of CAP: amino acids 156 to 162 of CAP (Ala156→Asp, Thr158→Ala, Thr158→Ile, His159→Arg, His159→Leu, Gly162→Asp, Gly162→Cys, and Gly162→Ser).
We have purified and have characterized *in vitro* five of the substituted CAP variants: [Ala158]CAP, [Ile158]CAP, [Arg159]CAP, [Leu159]CAP, and [Cys162]CAP. *In vitro* transcription experiments indicate that the substituted CAP variants are completely, or nearly completely, defective in transcription activation at the *lac* promoter (abortive initiation assays). *In vitro* DNA binding experiments indicate that the substituted CAP variants bind to DNA with the same affinity as does wild-type CAP (nitrocellulose filter assays). *In vitro* DNA bending experiments indicate that the substituted CAP variants bend DNA to the same extent as does wild-type CAP (electrophoretic mobility assays, four circularly permuted DNA fragments).
Our results establish that amino acids 156 to 162 of CAP are critical for stimulation of transcription activation at the *lac* promoter, but are not critical for DNA binding or DNA bending. In the structure of CAP, amino acids 156 to 162 form a surface loop. We propose that this surface loop makes a direct protein-protein contact with RNA polymerase at the *lac* promoter (cf. *Nucl. Acids Res.* 18:7243, 1990; *Nucl. Acids Res.* 19:6705, 1991; *J. Bact.* 173:5024, 1991).

B. Detailed characterization of the activation surface of CAP: saturation mutagenesis of the activation surface of CAP. The results described in section *A* establish that the surface loop consisting of amino acids 156 to 162 of CAP is the activation surface of CAP. To further characterize the transcription activation surface of CAP, we have performed saturation mutagenesis of codons 152 to 166 of *crp* (using a "doped" oligonucleotide primer). We have isolated and sequenced fifty independent *crp*^{pc} mutants after saturation mutagenesis. The fifty *crp*^{pc} mutants are defective in transcription activation at the *lac* promoter, but are not defective in DNA binding.
We find that twenty-eight of the fifty mutants are single-amino-acid-substitution mutants. The amino acid substitutions map to amino acids 156, 158, 159, 162, 163 and 164 (Ala156→Asp, Thr158→Ala, Thr158→Ile, His159→Arg, His159→Leu, His159→Gln, His159→Pro, His159→Tyr, Gly162→Cys, Gly162→Ser, Gly162→Ala, Gly162→Arg, Met163→Lys, Gln164→Arg, Gln164→His, and Gln164→Lys).

C. Detailed characterization of the activation surface of CAP: alanine-scanning mutagenesis of the activation surface of CAP. The results in section *A* establish that the surface loop consisting of amino acids 156 to 162 of CAP is the activation surface of CAP. To further characterize the transcription activation surface of CAP, we have performed alanine-scanning mutagenesis of codons 152 to 166 of *crp*. We have substituted each amino acid from 152 to 166, one-by-one, by Ala. For each of the resulting CAP derivatives, we have assessed (*i*) transcription activation at the *lac* promoter, and (*ii*) DNA binding. We find that Ala substitution of amino acid 158 results in a significant specific defect in transcription activation at the *lac* promoter. We find that Ala substitution of no other amino acid tested results in a significant specific defect in transcription activation at the *lac* promoter. These results indicate that amino acid 158 is the only amino acid of the activation surface of CAP for which side-chain atoms other than Cβ are essential for transcription activation at the *lac* promoter.

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D. Detailed characterization of the activation surface of CAP: determination whether transcription activation at the lac promoter involves the activation surface of the promoter-proximal subunit of CAP, the activation surface of the promoter-distal subunit of CAP, or the activation surfaces of both subunits of CAP. We have constructed heterodimers consisting of one CAP subunit having wild-type DNA binding specificity and having a non-functional activation surface, and one CAP subunit having non-wild-type DNA binding specificity and having a functional activation surface. We have oriented the resulting heterodimers on promoter DNA by use of promoter derivatives having DNA sites for CAP consisting of one wild-type DNA half site and one non-wild-type DNA half site, and we have measured the ability to activate transcription.

For the CAP subunit having wild-type DNA binding specificity and having a non-functional activation surface, we have used [Ala158]CAP (see sections *A-C*). For the CAP subunit having non-wild-type DNA binding specificity and having a functional activation surface, we have used [Val181]CAP (*Nature* 311:232, 1984; *Proc. Natl. Acad. Sci. USA* 84:6083, 1987). [Val181]CAP recognizes the DNA half site 5'-AAATGT*N*ATCT-3' (*vs.* 5'-AAATGT*G*ATCT-3' for CAP and [Ala158]CAP); [Val181]CAP binds with equal, high affinities to the consensus DNA half site and to derivatives of the consensus DNA half site having A:T at position 7.

To orient the [Ala158]CAP/[Val181]CAP heterodimers at the *lac* promoter, we have used two derivatives of the *lac* promoter: *lacPL29*, which has a DNA site for CAP in which the promoter-proximal half site has A:T at position 7, and *lacPL8*, which has a DNA site for CAP in which the promoter-distal half site has A:T at position 7. [Ala158]CAP/[Val181]CAP heterodimers bind to *lacPL29* and *lacPL8* in an oriented fashion--having a functional activation surface only in the promoter-proximal subunit at *lacPL29*, and having a functional activation surface only in the promoter-distal subunit at *lacPL8*.

We find that [Ala158]CAP/[Val181]CAP heterodimers are functional in transcription activation at *lacPL29* but are not functional in transcription activation at *lacPL8*. We conclude that transcription activation at the *lac* promoter involves only the activation surface of the promoter-proximal subunit of CAP.

E. Detailed characterization of the activation surface of CAP: determination whether transcription activation at other CAP-dependent promoters involves the same surface of CAP. CC, CC+20, and CC+31 are artificial CAP-dependent promoters having spacings between the center of the DNA site for CAP and the transcription start point of, respectively, 41.5 base pairs (as in the *galP1* promoter), 61.5 base pairs (as in the *lac* promoter), and 72.5 base pairs (essentially as in the *malT* promoter) (*Cell* 62:733, 1990). We have measured the effects on transcription activation at CC, at CC+20, and at CC+31 of each of eight CAP derivatives specifically defective in transcription activation at the *lac* promoter: [Asp156]CAP, [Ala158]CAP, [Ile158]CAP, [Arg159]CAP, [Leu159]CAP, [Asp162]CAP, [Cys162]CAP, and [Ser162]CAP (see section *A*).

We find that each of the eight CAP derivatives specifically defective in transcription activation at the lac promoter also is specifically defective in transcription activation at CC, at CC+20, and at CC+31. We conclude that the surface loop consisting of amino acids 156 to 162 of CAP is critical for transcription activation at promoters having spacings of 41.5 base pairs, 61.5 base pairs, or 72.5 base pairs.

In further experiments, we have measured the effects on transcription activation at CC, at CC+20, and at CC+31 of each of the fourteen CAP derivatives constructed in alanine-scanning mutagenesis of amino acids 152 to 166 of CAP (section *C*): i.e., [Ala152]CAP, [Ala153]CAP, [Ala154]CAP, [Ala155]CAP, [Ala157]CAP, [Ala158]CAP, [Ala159]CAP, [Ala160]CAP, [Ala161]CAP, [Ala162]CAP, [Ala163]CAP, [Ala164]CAP, [Ala165]CAP, and [Ala166]CAP.

We find that Ala substitution of amino acid 158, amino acid 160, amino acid 162, or amino acid 163 results in a significant specific defect in transcription activation at CC, substitution of amino acid 162 having the largest effect. We find that Ala substitution of amino acid 158 or amino acid 162 results in a significant specific defect in transcription activation at CC+20 and at CC+31, substitution of amino acid 158 having the largest effect. We conclude that: (*i*) the surface loop consisting of amino acids 156 to 162 of CAP is critical for transcription activation at promoters having spacings of 41.5 base pairs, 61.5 base pairs, or 72.5 base pairs, and (*ii*) the interactions made by the surface loop consisting of amino acids 156 to 162 of CAP differ at promoters having spacings of 41.5 base pairs (DNA site for CAP overlaps the -35 site; amino acids 158, 160, 162, and 163 sensitive to Ala substitution) *vs.* at promoters

having spacings of 61.5 base pairs or 72.5 base pairs (DNA site for CAP does not overlap the -35 site; amino acids 158 and 162 sensitive to Ala substitution).

F. Analysis of CAP-RNA polymerase interaction in solution: development of a fluorescence polarization method to analyze CAP-RNA polymerase interaction in solution. In collaboration with Drs. T. Heyduk and J. Lee, of the Dept. of Human Biological Chemistry and Genetics, University of Texas Medical Branch, we have used fluorescence polarization experiments to analyze CAP-RNA polymerase interaction in solution in the absence of promoter DNA. We have utilized the following components: (*i*) ICAP42FL, a fluorophore-derivatized 42 base pair DNA fragment containing the consensus DNA site for CAP and containing no known specific determinants for binding of RNA polymerase, (*ii*) CAP, and (*iii*) RNA polymerase holoenzyme. We have measured fluorescence anisotropy of the CAP-ICAP42FL complex in the absence of RNA polymerase holoenzyme, and in the presence of RNA polymerase holoenzyme.

Our experimental design was premised on the large difference in size, and thus the large difference in rotation rates and in fluorescence anisotropy, of the CAP-ICAP42FL complex (≈73,000 D) vs. the CAP-ICAP42FL-RNA polymerase holoenzyme ternary complex (≈520,000 D).

We find that addition of RNA polymerase holoenzyme to the CAP-ICAP42FL complex results in a large increase in fluorescence anisotropy. Control experiments indicate that addition of RNA polymerase holoenzyme to the [Ala158]CAP-ICAP42FL complex (see sections *A-C*) or to ICAP42FL alone does not result in a large increase in fluorescence anisotropy. Additional control experiments, performed in collaboration with Dr. A. Ishihama of the National Institute of Genetics, Japan, indicate that addition of α(1-256) RNA polymerase holoenzyme (*Cell* 65:1015, 1991) does not result in a large increase in fluorescence anisotropy.

We conclude that CAP interacts with RNA polymerase in solution in the absence of promoter DNA. The equilibrium constant for the interaction is 2×10^{-7} M, an equilibrium constant comparable to the concentration of RNA polymerase holoenzyme *in vivo*. The interaction requires the activation surface of CAP and amino acids 257-329 of RNA polymerase α subunit. We speculate that the interaction involves a direct protein-protein contact between the activation surface of CAP and RNA polymerase.

G. Analysis of the activation target of RNA polymerase: isolation and characterization of mutants of RNA polymerase α subunit specifically defective in response to transcription activation by CAP. We have used random mutagenesis of the *rpoA* structural gene, which encodes the RNA polymerase α subunit, followed by application of an *in vivo* genetic screen, to isolate mutants of α specifically defective in response to transcription activation by CAP--i.e., mutants of α defective in CAP-dependent transcription, but not defective in CAP-independent transcription. We designate such mutants "pc,cap,d" (for positive control, CAP, defective).

Thus far, we have isolated and characterized six independent *rpoA*^{pc,cap,d} mutants. The six *rpoA*^{pc,cap,d} mutants are defective in CAP-dependent transcription at the *lac* promoter, but are not defective in CAP-independent transcription at the *lacPL8UV5* promoter. Three of the six independent *rpoA*^{pc,cap,d} mutants have strong phenotypes; the amino acid substitutions in these mutants map to amino acid 261 of α (Glu261→Lys, Glu261→Gly). Three of the six independent *rpoA*^{pc,cap,d} mutants have weak phenotypes; the amino acid substitutions in these mutants map to amino acids 295 and 298 of α (Gly295→Ser, Ser298→Pro). We conclude that amino acids 261, 295, and 298 of α are critical for response to CAP at the *lac* promoter, and we speculate that amino acid 261 of α may participate in a direct protein-protein contact with CAP at the *lac* promoter.

MOLECULAR INTERACTIONS DURING TRANSCRIPTION ACTIVATION BY THE *E. COLI* CYCLIC AMP RECEPTOR PROTEIN AND FNR

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Introduction

The role of the *E. coli* cyclic AMP receptor protein (CRP) is the induction of the expression of certain genes during conditions of glucose starvation. In response to increases in intracellular cyclic AMP, CRP activates transcription initiation at a number of different targets. The basis of the DNA binding specificity of CRP is reasonably well understood but the exact mechanism of the activation is less clear. A number of other transcription factors are related to CRP, including *E. coli* FNR which is responsible for induction of gene expression in response to oxygen starvation. FNR (the protein required for expression of fumarate and nitrate reductase) appears to be activated directly or indirectly by Fe^{++} ions, which trigger binding to a 22 base pair sequence very similar to the CRP-binding target. Both FNR and CRP bind as dimers to an inverted repeat sequence: CRP also causes a sharp bend at the target (it is likely that FNR also triggers a bend but there are no data on this at present).

Activation of Transcription by CRP

CRP action has been intensively studied at a number of promoters. In most cases, CRP makes a direct contact with RNA polymerase, likely involving the α subunit. This contact results in either a tightening of the initial binding of RNA polymerase to the promoter or in an acceleration of the transition from the closed to the open complex. In a number of cases CRP also directly prevents RNA polymerase from being captured at alternative promoters.

One of the puzzles of CRP action is that, at different promoters, the 22 base pair CRP-binding site is located at different distances upstream from the transcription start. In cases such as the *lac* promoter, where the centre of the site is at -61½ (with respect to the start site) RNA polymerase contacts the closer CRP subunit resulting primarily in a tightening of polymerase binding to the promoter.

At other promoters, where the CRP site is centred at -41½, the CRP-induced DNA bending promotes contacts between polymerase and DNA sequences upstream of the CRP site, resulting in an acceleration of the closed to open complex. In open complexes at these promoters, RNA polymerase can contact both subunits of the CRP dimer.

Independent evidence from three different laboratories shows that a surface-exposed loop in CRP around T158 and H159 is directly involved in important contacts with RNA polymerase: mutations in this loop prevent CRP from activating transcription. We have shown that changes in a second surface-exposed loop, including K52, can compensate for the effects of mutations in the "158/159 loop", but only at promoters where CRP binds at -41½. Thus changes in the "52 loop" create a second "patch" on CRP that may interact with RNA polymerase. In the dimer, the 158/159 and 52 loops are located adjacent to each other but on different subunits. At promoters where dimeric CRP binds at -41½, transcription

activation is prevented by changes in the 158/159 loop in the upstream subunit but can be restored by mutation in the 52 loop of the downstream subunit.

At promoters where CRP binds at -41½, the CRP-site can be replaced by bending sequences. These constructions resemble a small class of *E. coli* promoters at which specific -35 region sequences are not required. In these cases, RNA polymerase compensates for the lack of -35 region contacts by making an extended interaction with the -10 hexamer and by binding further upstream than at "consensus" promoters. This binding results in distortion and wrapping of the upstream sequences around RNA polymerase. When bound at -41½, CRP appears to emulate this type of promoter.

Activation of Transcription by FNR

Analysis of a number of FNR-dependent promoters shows that, in most cases, FNR binds to sites centred around -41½. A consensus FNR-binding site can be deduced: transplanting this sequence into other promoters confers FNR-dependence. Comparison of the amino acid sequences of FNR and CRP suggest that FNR does not contain the equivalent of the CRP 158/159 loop but that an equivalent of the 52 loop is present. Mutations in this loop of FNR lead to a reduction in FNR activity whilst not affecting binding to target sites. We suggest that this loop is responsible for transcription activation by FNR. Evidence that this involves interaction with the α subunit of RNA polymerase will be discussed.

Dimeric transcription activators that bind to inverted repeat sequences display two different faces on adjacent subunits and, when bound at -41½, it is possible for both subunits to contact RNA polymerase. We suggest that all members of the CRP/FNR class of proteins can activate transcription by either one or other of two different surface exposed loops displayed on adjacent subunits in the dimer.

Dual Control of Transcription

CRP and FNR are "global" regulators in that they convey a major metabolic signal (i.e. lack of glucose/oxygen) to a large number of targets. Most of these target promoters are also controlled by specific regulators that "interpret" the availability of specific metabolites. Direct and indirect interactions of CRP with AraC, MalT, MelR, GalR, CytR etc. are well known. Similarly, FNR works in concert with a second factor at a number of promoters. For example, the *E. coli* nitrite reductase (*nirB*) promoter is controlled by nitrite as well as oxygen starvation. Nitrite is signalled by the NarL protein that appears to bind at the *nirB* promoter just upstream of the FNR-binding site, and may affect the interaction of upstream sequences with RNA polymerase.

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POSITIVE CONTROL OF TRANSCRIPTION INITIATION BY cAMP•CRP IN lac PROMOTER OF *E. COLI*

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cAMP receptor protein (CRP) is a dimer that binds to DNA nonspecifically. Upon cyclic AMP addition, the dimer undergoes a conformational change such that it binds to specific sites at or near the promoters of many genes in *E. coli* with high affinity. Binding occurs through a helix-turn-helix motif in the protein. The allosteric change in CRP induced by cAMP and DNA binding makes it an activator of transcription initiation for many of these promoters. The biochemical steps of transcription initiation by σ70- RNA polymerase have been grossly divided into four steps: (i) Binding of RNA polymerase to the promoter to form a closed complex; (ii) Isomerization of the closed to an open complex; (iii) Transcription initiation and idling; and (iv) Promoter clearance. In principle, cAMP•CRP could stimulate transcription initiation at any of these steps. The DNA binding site of cAMP•CRP can vary from a location very close to RNA polymerase to further upstream regions from the start site of transcription. It has been shown that the step at which cAMP•CRP influences transcription may depend on the location of the cAMP•CRP on DNA with respect to the position of RNA polymerase binding. For example, cAMP•CRP influences RNA polymerase binding in *lacP1* promoter from a binding site at -60 region, whereas it stimulates both binding and isomerization in *galP1* from -40 position. However, the mechanism(s) by which cAMP affects these steps remains to be investigated. Two models of action are generally considered: (a) The DNA-bound cAMP•CRP complex influences RNA polymerase by a direct contact with it; and (b) cAMP•CRP contorts the DNA to facilitate RNA polymerase action at the promoter. Current genetic and biochemical evidence suggests a potential contact between the activator complex and RNA polymerase. cAMP•CRP complex is also known to induce different amounts of bends in DNA in different promoters. We have investigated whether any structural change in DNA induced by cAMP•CRP influences transcription initiation in the *lacP1* promoter of *E. coli*. Results will be discussed that suggest that structural changes induced in DNA by cAMP•CRP may affect RNA polymerase activity. (i) cAMP•CRP complexed with DNA must be located in *cis* to the *lacP1* promoter. (ii) We have found a certain structural integrity of the short stretch of DNA between the cAMP•CRP binding site at -60 and RNA polymerase binding at -35 is essential for transcription activation.

Interactions between RNA polymerase and CRP in open complexes at the *lac*P1 and *gal*P1 promoters : Use of mutated proteins.

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The cAMP-CRP complex regulates transcription initiation at a number of different promoters. At the *lac*P1 and *gal*P1 promoters, it activates open complex formation by increasing initial binding of RNA polymerase and/or accelerating the isomerisation rate from closed to open complex. Protein-protein contacts occur between the enzyme and the cAMP-CRP complex which therefore bind cooperatively to their targets on both promoters. However, the CRP site is centrered at -61.5 from the *lac*P1 transcription start whilst CRP binds 41.5 bp upsteam of the *gal*P1 start ; interactions between RNA polymerase and the CRP protein are hence different at the *lac* and *gal* promoters. This observation is strengthened by the existence of CRP mutants [1,2,3] or RNA polymerase [4,5] mutants which are deficient in *lac* activation but can still stimulate *gal* transcription initiation.

Recently K. Igarashi and A. Ishihama have shown that holoenzyme, reconstituted with truncated α-subunits (containing the first 235 or 256 residues of α) was able to support CRP activated transcription at the *gal* promoter, but not at the *lac* promoter [4].

Using two up promoter mutants of the *lac* and *gal* promoters (*lac* UV5 and *gal* 9A16C), we first compare the binding of CRP and cAMP to the promoter alone and to the open complex formed with wild-type RNA polymerase ; CRP binds with a much higher affinity to the open complex and reciprocally RNA polymerases binds better to both promoters in the presence of CRP and cAMP. However holoenzyme, reconstituted with truncated α-subunits behaves differently at the *lac* and *gal* promoters. Cooperative binding only occurs with the *gal* promoter. Footprinting data confirm these results and suggest a plausible explanation for the loss of cooperativity in the case of the *lac* promoter.

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HETEROLOGOUS COOPERATIVITY: THE cAMP-CRP/ CYTR NUCLEOPROTEIN COMPLEX IN E.coli.

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The Escherichia coli CytR regulon includes at least nine operons/genes coding for proteins involved either in uptake or catabolism of nucleosides and deoxynucleosides. A series of recent experiments have unambiguously shown that cAMP-CRP is an authentic component of the CytR repression apparatus and serves as an adaptor that allows the CytR repressor to associate with promoter-DNA. Using purified components we have now analyzed the interactions of the two regulators at all known CytR regulated promoters These analysis show that CytR is a sequence specific DNA binding protein that binds its targets with a relatively low affinity. In the presence of cAMP-CRP, however, DNA binding of CytR can be stimulated more than 1000 fold, and also, the presence of CytR enhances CRP DNA binding. This cooperative binding results in the formation of a nucleoprotein complex in which the regulators are located close to each other on the same side of the DNA helix. Moreover, the combined binding of the two regulators results, in some of the promoters, in a repositioning of the cAMP-CRP complex. Mutational analysis have established that amino acids 13, 17, 18, 108 and 110 of CRP are critical for its action as a co-repressor but not critical for transcriptional activation. In the structure of CRP these amino acids are located in two regions (loops) that are in very close proximity on the surface of the protein. We propose that these surface loops are involved in a direct protein-protein contact with the CytR repressor at the promoters.

FEATURES OF THE TRANSCRIPTIONAL REPRESSOR RepA, ENCODED BY THE PROMISCUOUS PLASMID pLS1

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pLS1 is a 4408-bp long multicopy streptococcal plasmid able of autonomous replication in a variety of bacteria, gram-positive and gram-negative. Three plasmid genes are involved in pLS1 replication and its control: *repB*, *repA* and *malI*. Initiation of replication is mediated by the plasmid-encoded RepB protein, which has sequence-specific endonuclease and topoisomerase activities. RepB synthesis is rate-limited, being controlled by two plasmid-encoded elements belonging to the same regulatory circuit: i) the *repA* gene product, RepA, which is a transcriptional repressor, and ii) the 50-nt long antisense RNA II. The latter probably acts by specific pairing with its complementary region of the *rep* mRNA, blocking the RepB-translation initiation signals. RNA II is transcribed from promoter P_{II}, which shows two in-phase curved DNA regions that acts as upstream activating sequences (UAS).

Genes *repA* and *repB* are co-transcribed from a single promoter, P_{AB}. RepA is a *trans*-acting 5.1-kDa protein which regulates its own synthesis and synthesis of RepB, probably by keeping a constant level of *repAB* mRNA. The RepA protein has been purified to homogeneity in an *E.coli* expression system, the protein being synthesized as a dimer of identical subunits. The amino acid sequence of RepA has been determined, being identical to that derived from the DNA sequence. RepA has 45 amino acids, including the initial Met, so that it is the smallest repressor so far described. Computer-assisted predictions indicate a simple structure for RepA: one β-sheet N-terminal structure, the αhelix-turn-αhelix (HTH) motif typical of many DNA-binding proteins, and a positively charged, random coil C-end.

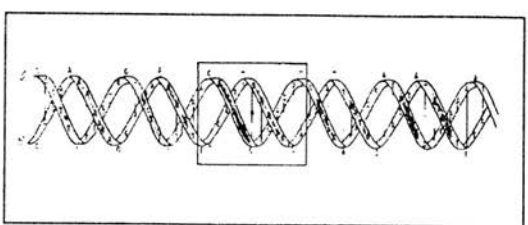


Fig. 1

High-resolution analyses of the contacts between RepA and its DNA target showed that the protein binds to a 13-bp symmetric element, most likely its operator, within which part of the -35 box of P_{AB} promoter is included. The RepA-DNA contacts extend beyond the symmetric element, within a DNA region encompassing 40 bp (Fig. 1). Such a long protected region may be explained because upon binding, RepA introduces a strong DNA bend in a region already curved. The centre of the RepA-induced bend is displaced some 30 nt upstream of the centre of the intrinsically curved DNA.

Substitution of the UAS of P_{II} by a straight DNA region containing the target of RepA showed that the expression of a reporter gene fused to P_{II} could be modulated, both *in vitro* and *in vivo*. RepA behaved as a transcriptional activator when its target was phased with P_{II} (Fig. 2A), whereas placing the RepA target out of phase with P_{II} resulted

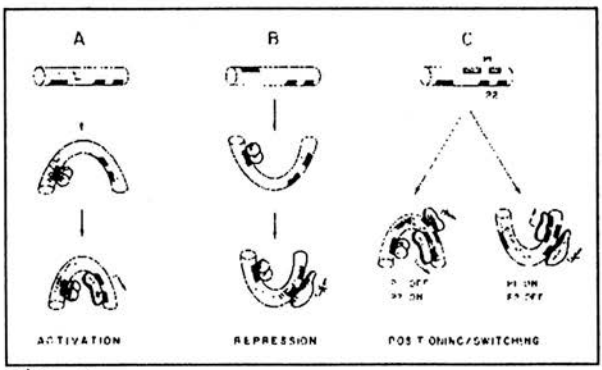


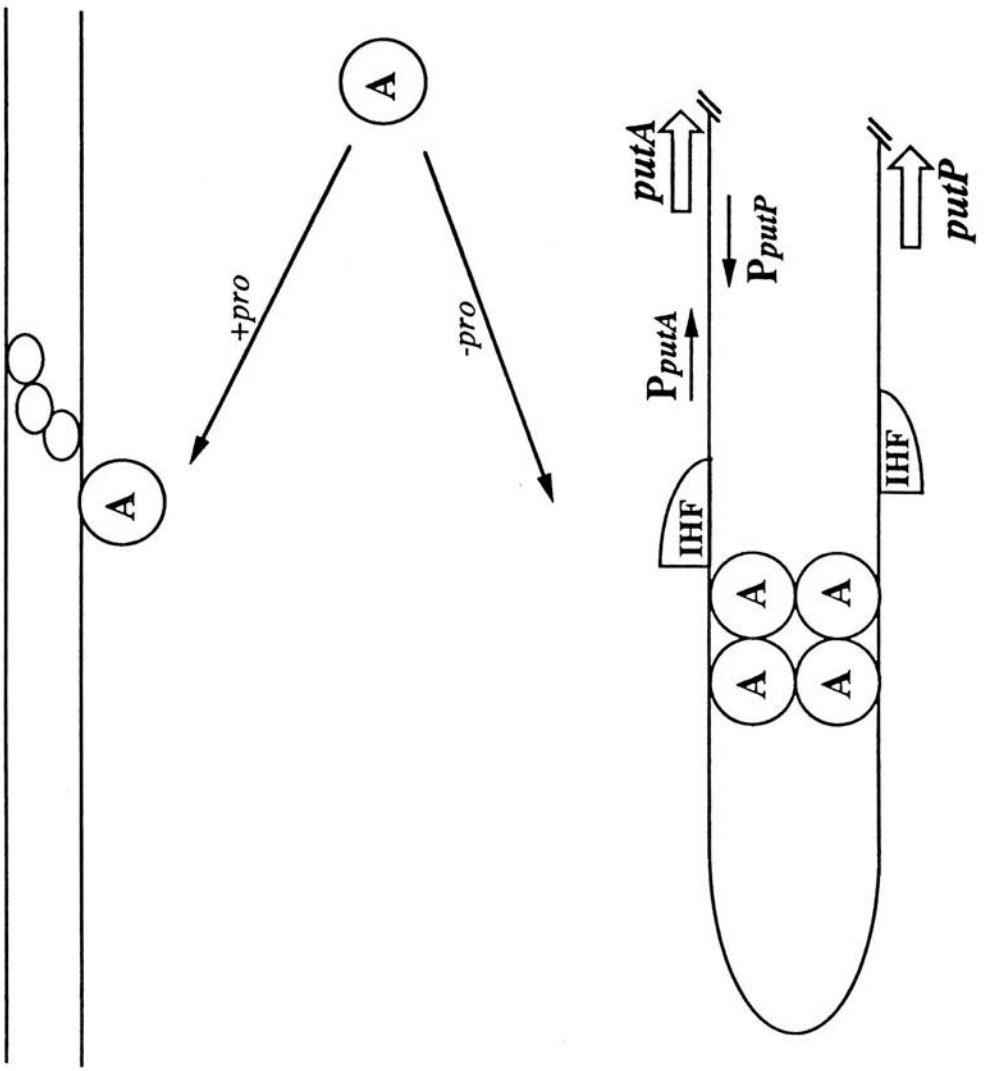
Fig. 2

in RepA-mediated gene repression (Fig. 2B).

On the basis of our results, we have proposed a unified model for the regulation of transcription initiation of regulated genes. The model assumes that the appropriate positioning of a promoter with a repressor (activator) element is a key feature for promoter switching (Fig. 2C). This model has been tested by the use of the *E.coli fur* operon regulatory signals. They are composed by two overlapping promoters, differently phased on the DNA helix. The regulatory signals of the *fur* operon contain a target for the catabolite-repressor protein (CAP), which was substituted by the target of RepA, keeping its distance to the promoters. The *repA* gene was placed under an inducible promoter, and was supplied *in trans*. We have been able to show that, *in vivo*, RepA was able to modulate mRNA synthesis from one or the other promoter, just by in-phase placing the RepA-target with the desired promoter. Our findings support the above model, but do not discard other elements as being involved in the initiation of transcription.

REGULATION OF PROLINE UTILIZATION IN SALMONELLA TYPHIMURIUM: REPRESSION REQUIRES A NUCLEOPROTEIN COMPLEX FORMED BY THE REPRESSOR - A MEMBRANE ASSOCIATED DEHYDROGENASE - BOUND TO TWO OPERATOR SITES, CURVED DNA AND IHF. P. Ostrovsky de Spicer, and S. Maloy. University of Illinois, Urbana, Il.

Salmonella typhimurium can utilize proline as a sole carbon or nitrogen source. Proline utilization requires the expression of the two genes in the *put* operon: the *putP* gene, which encodes proline permease, and the *putA* gene, which encodes a bifunctional dehydrogenase that degrades proline to glutamate. Expression of the *put* operon is induced by proline. Genetic evidence suggests that the PutA protein represses transcription of the *put* operon when the intracellular concentration of proline is low. The conclusions of the genetic studies are confirmed by molecular biological studies. Gel retardation and in vitro footprinting of wild type and *putA* mutants demonstrate that purified PutA protein binds to at least two sites in the *put* regulatory region. In vivo footprinting experiments demonstrate that the PutA protein itself is the repressor. The PutA binding sites are accompanied by two IHF binding sites and separated by a region of curved DNA. IHF enhances the protection against DNAase digestion by purified PutA, suggesting that IHF might promote or stabilize PutA binding to this region. When both PutA binding sites are present on a single DNA fragment, PutA protein binds DNA with a higher affinity. Cooperativity between PutA molecules bound to the two distant sites has been confirmed in vivo using the challenge phage system. These results indicate that regulation of the *put* operon may occur by DNA looping between two operator sites, facilitated by DNA sequences that promote DNA bending. Furthermore, the presence of proline appears to disturb PutA binding to the operator sites in vivo but not in vitro, suggesting that the membrane sites for PutA interaction might be essential to prevent PutA's repressor activity and achieve full induction.



MODIFICATION OF RNA POLYMERASE BY THE PHAGE LAMBDA GENE *Q* TRANSCRIPTION ANTITERMINATOR AT THE PHAGE LATE GENE PROMOTER.

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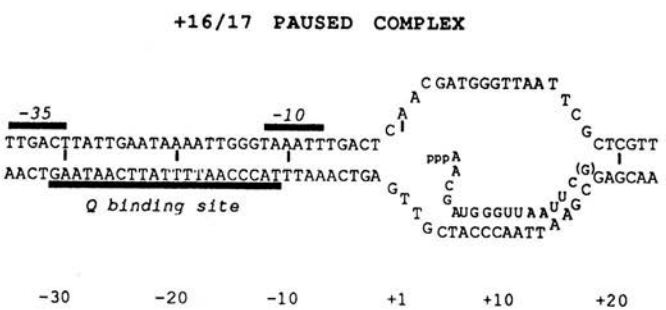
Lambda growth is controlled by two transcription antiterminators, the products of its genes *Q* and *N*. The N protein modifies RNA polymerase as it binds an RNA structure encoded by a DNA site that may be distant from the promoter. In contrast, the Q protein binds DNA between the -35 and -10 elements of the late gene promoter (1). RNA polymerase is modified by Q not in the open complex, but instead in an early elongation complex in which RNA polymerase pauses at nucleotides 16 & 17 (2,3) and exposes the Q binding site in the promoter region (1); interaction of Q both modifies RNAP to an antiterminating form, and greatly reduces the time of the pause.

Although RNA polymerase in the early pause clearly has moved from the open complex and has acquired essential properties of elongation complexes, it also retains some properties of the initiation complex. Thus, footprinting shows interactions of RNA polymerase with DNA around -10 and -20 that are associated with the tendency to pause (1). Furthermore, RNA polymerase initiating at a promoter placed upstream of the late gene promoter does not pause detectably at the early pause site—and Q does not modify this enzyme—even though the enzyme transcribes all sequences required for Q function (4); thus formation of the early paused complex requires that RNA polymerase go through the process of initiation at the associated promoter.

The pause is encoded mostly by nucleotides +2 and +6 (5,6), but is also affected by bases +7, +8, and +9, as well as bases at the downstream edge of the transcription bubble (6; MK, WY & JWR, unpublished). Transcription of heteroduplex templates in vitro shows that bases in the non-template strand at positions +2 and +6 promote the pause (BR and JWR, unpublished). Q protein affects the DNA footprint of the paused complex not only at the Q binding site between -35 and -10, but also at the beginning of the transcription bubble—the segment where mutations also affect the pause, and therefore the region where some structure induces the pause. We suggest that Q has a similar effect on an essential pause at terminators.



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POSTERS

REGULATION OF THE ANTIBIOTIC PRODUCTION IN *Streptomyces coelicolor*

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Streptomyces are gram positive soil bacteria with two remarkable features: (1) undergoing a complex life cycle which involves spore germination, vegetative mycelia formation, its differentiation to aerial mycelia and the formation of new spores and (2) being capable to produce an enormous variety of compounds of human interest such as antibiotics, antitumoral agents, herbicides, hydrolitic enzymes and so on.

Streptomyces coelicolor is the most genetically characterized specie of the genera, it undergoes a typical life cycle and produces five secondary metabolites, four of them with antibiotic activity: actinorhodin, undecylprodigiosin, methylenomycin and CDA. The production of these secondary metabolites are always associated to morphologycal changes in the colony, suggesting a common mechanism of control for both processes.

In the last years, a number of systems of antibiotic production have been characterised from different origins, what has let to come out some conserved features: structural genes are clustered, the mechanisms for resistance to metabolites from the pathway with antibiotic activity are inside the cluster and there is a regulatory gene, close to the structural ones, which controls the expression of all of them. Actinorhodin is a polyketide antibiotic well characterized, which shares all those characteristics. Production of actinorhodin is controlled by a gene (the so called *actII*ORF4) internal to the cluster which determine the transcription of the other *act* genes.

Actinorhodin and undecylprodigiosin, although belonging to different chemical families, have in common to be regulated by a positive effector highly conserved. Similar regulatory gene products have been described in other routes such as the antitumoral agent doxorubicine from *Streptomyces peucetius*. In *S.coelicolor*, *act*IIORF4 has been shown to be the target of a regulatory gene of pleiotropic action: *bld*A. So, the two main processes in *Streptomyces*, morphological and biochemical differentiation, are under the control of the same gene.

At date, several genes with pleiotropic action on antibiotic production in *S.coelicolor* have been characterised : *afs*R, *abs*A,B, and *afs*B. Recently, we have isolated and characterized the locus *aba*A from *S.coelicolor* (although higly conserved in different *Streptomyces* species) which induce the actinorhodin production in *Streptomyces lividans*, which has the structural information but lacks some regulatory signal/s. Analysis of *aba*A revealed two ORFs in which ORFA seems to be involved in biosynthesis of actinorhodin, undecylprodigiosin and CDA, but not in methylenomycin. Moreover, when ORFA is in high copy number on wild type strain, this take a phenotype very similar to *bld*A: lack of antibiotic production and sporulation.

All this, reveal the presence of a complex net of regulatory interconnected signals where process-specific control genes are depending on several pleiotropic genes with more global action. Its characterization is one of the main subjects of our group.

CONTROL OF THE EXPRESSION OF THE DIVISION OPERON *ftsQAZ* IN *Escherichia coli*.

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The main cell division operon of *Escherichia coli* contains three essential genes that are transcribed in the same direction: *ftsQ*, *ftsA* and *ftsZ*. Expression of the *ftsQ* and *ftsA* genes is driven by both a *gearbox* and a regular promoter located upstream from *ftsQ*. *Gearbox* promoters work in combination with *housekeeper* promoters so that the encoded gene products are produced at constant amounts per cell. The presence of this type of promoter in the regulatory region of the *fts* genes ensures that the amount of gene expression complies with the septal needs of the cell at different growth rates and, hence, at different cell sizes. The *ftsZ* gene is expressed from four promoters located within the open reading frame of *ftsA*, the upstream gene from *ftsZ* in the operon. Although FtsZ is also produced at constant amounts per cell at different growth rates none of the four *ftsZ* promoters showed a *gearbox* mode of expression (Aldea *et al*, 1990 EMBO J. 9: 3787-3794). FtsZ is a key protein in septation, present in 5000 molecules per cell, it stays in the cytoplasm (Pla *et al*, 1991. Mol.Microbiol. 5: 1681-1686) during most of the cell cycle, but forms a ring structure in constricting cells (Bi and Lutkenhaus, 1991. Nature 354: 161-164)

We have investigated the behaviour of a strain in which the expression of *ftsZ* can be triggered at will, independently of the cell cycle. For this purpose a genetic surgery procedure has been devised, so that expression of *ftsZ* is dissociated from its natural regulatory signals, and becomes IPTG-dependent. These cells need levels of FtsZ 40% over the wild type ones to divide as efficiently as the wild type strain.

We have quantitated the celular levels of the *ftsZ* mRNA using PCR amplification. The procedure used is based on the sequential action of reverse transcriptase and *Taq* polymerase. Both enzymatic reactions are simultaneously quantitated by including an RNA standard that uses the same pimers as the messenger of *ftsZ*, and contains an internal deletion what results in a product with a lower molecular weigth. Using this procedure we have found taht each cell contains on the average three molecules of *ftsZ* mRNA.

Transcription analysis of the hydrogen-uptake genes in Rhizobium leguminosarum bv viceae.

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The production of hydrogen by nitrogenase is a major factor affecting energy efficiency of the nitrogen fixation process. In some strains of Rhizobium leguminosarum bv viceae a hydrogenase system is induced which allows the recycling of part of the energy lost as hydrogen.

The DNA involved in H_2 oxidation in R. leguminosarum bv viceae 128C53 is clustered in a 20 Kb region cloned in cosmid pAL618 (1). The analysis of the nucleotide sequence of the whole cluster revealed the presence of the genes encoding the structural subunits of the hydrogenase enzyme (hupS and hupL, 2) along with 15 additional genes. By mutant complementation analysis it was shown that the hup genes are organized in at least four transcriptional units, designated regions hupI/II (hupSLCDEF), hupIII (hupGH), hupIV (hupIJK) and hupV/VI (hypABNCDE) (3). One of these regions, hupV/VI, is expressed in vegetative cells in response to low O_2 tensions in the culture medium, whereas the remaining regions are only expressed in symbiosis with peas (4). In this communication we are presenting data on the characterization of the promoters identified in the hup cluster as well as on the regulation of its transcription.

A transcription initiation site for region hupI/II was identified 57 bp upstream of hupS by primer extension experiments. This site is preceded by sequences highly homologous to the σ^{54}- and IHF-consensus binding sites, suggesting that the transcription of the structural region is positively regulated. Both type of sequences are also present upstream the coding region of hupIII region. In order to investigate the functionality of these two boxes, band-shift and foot-print experiments are currently being carried out in our group.

Two transcription initiation sites for the region hupV/VI were identified in microaerobically grown cells of strain 128C53, while only one of them is used in symbiotic cells. Both sites are located upstream of hypB, within hypA. Using a Rhizobium meliloti mutant strain affected in the symbiotic regulatory gene fixK, it was shown that the two start sites depend on an active FixK in R. meliloti. In addition, one more transcription initiation site was identified upstream of hypA only in symbiotic cells. This is the only start site which allows the transcription of the gene hypA. Although the complementation analysis of region hupV/VI indicates the existence of two separate transcription units, the presence of a transcription start site for hupVI has not been prooved. The actual sizes of the hup RNA transcripts are currently being determined by Northern hybridization experiments.

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THE CONTROL OF TRANSCRIPT ELONGATION BY RNA POLYMERASE II: SII PROTEIN AND THE BYPASS OF BLOCKS TO ELONGATION Caroline M. Kane, H. Carrie Chen, Karen R. Christie, Guadalupe Cipres-Palacin, and Zoe Weaver, Division of Biochemistry and Molecular Biology, University of California at Berkeley, Berkeley CA 94720, USA.

RNA polymerase II recognizes many blocks to elongation within both cellular and viral transcription units. If these blocks were used without relief in cells, then little mature message (and therefore little protein) would be produced for any of these transcription units. However, the use of these sites within cells appears to be regulated, and several different strategies are used for this regulation.

One of these strategies involves a transcript elongation factor, SII, first identified and characterized by Natori and coworkers (1). This purified protein can act in conjunction with purified RNA polymerase II to promote readthrough of several sites which block the elongation of the polymerase *in vitro*. The protein interacts directly with RNA polymerase II, and the mechanism by which this interaction promotes readthrough is the subject of intensive study in several laboratories. It has recently been shown that SII mixed with paused RNA polymerase II elongation ternary complexes results in a cleavage of the 3' end of the nascent transcript (2). The ternary complex can then elongate the 5' end of this cleaved transcript. This reaction is reminiscent of one recently reported for *E. coli* RNA polymerase (3). How such a reaction might promote readthrough of blocks to elongation for RNA polymerase II is not known.

We have begun to ask which subunits of RNA polymerase II might be important for the interaction with SII protein. We have used purified SII and RNA polymerase II from yeast to show that subunits 4 and 7 of the polymerase are not necessary in order for SII to exert its effect on readthrough. In addition, the unusual conserved C-terminal domain heptapeptide repeat region of RNA polymerase II is not necessary for the readthrough promoted by SII.

We have also begun site-directed mutagenesis of the cDNA for the human SII protein, and we have shown that only 170 amino acids at the C-terminal end of the 301 amino acid protein are needed for stimulating RNA polymerase II. Alanine-cassette mutagenesis is being performed on the truncated active SII protein in an attempt to identify residues important both for the interaction with RNA polymerase II and for the readthrough activity.

Finally, we have evidence that there may be a family of genes encoding SII-related proteins in both human and yeast cells. Genomic cloning and genetic selection are being used to identify and characterize additional SII-related proteins in both organisms.

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Genetic analysis of XylS binding to its operator at the *Pm* promoter of the TOL plasmid: transcriptional activation through weak interactions of the regulator with an upstream direct repeat

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Pseudomonas putida strains containing the TOL plasmid pWW0 are able to grow on toluene and xylenes as the sole carbon source *via* initial conversion of these compounds into benzoate/toluates followed by *meta*-cleavage of the aromatic ring. Transcription of the pWWO-borne *upper* operon, which specifies the bioconversion of xylenes into toluates, originates from the *Pu* promoter when this is acted on by the XylR protein activated by pathway substrates and other structural analogs. Transcription of the *meta* operon, which determines the further metabolism of toluates to pyruvate and acetaldehyde, originates from the *Pm* promoter when this is acted on by the XylS protein activated by substituted benzoates or by an excess of XylS protein itself. Transcription of the *xylS* gene is driven by a XylR-dependent promoter, *Ps*. Unlike the complex regulation of the σ^{54}- and IHF-dependent promoters *Pu* and *Ps*, transcription from the *Pm* promoter seems to require only the presence of active XylS protein (along with a benzoate effector) and cognate upstream *cis*-sequences.

The *cis*-acting sequences within the *Pm* promoter/operator region have been identified through deletion analysis and mutagenesis of the region. To ensure a faithful monitoring of the controlling elements *in vivo*, chromosomal integration systems for *E. coli* and *Pseudomonas* were utilized to obtain *Pm-lacZ* fusions and *xylS* in monocopy dosage. Integrity and proper phasing of two homologous tandem sequences 5´-TGCAAPuAAPuPyGGNTA-3´ separated by 6 base pairs and overlapping with the -35 region of the Pm promoter was essential for *m*-toluate activation of a *Pm-lacZ* fusion in *xylS*$^+$ strains. The center of the direct repeats were found on the same DNA helix side as that of the RNA polymerase and slightly bent towards it. *Pm* promoter sequences did not interact *in vitro* with RNA polymerase alone and therefore transcription activation is predicted to occur through XylS-mediated formation of a closed complex, as is the case in the related P_{araBAD}/AraC system. The lack of saturability of *Pm* by overproduced XylS, as well as multicopy inhibition and transcription interference experiments *in vivo*, indicate that formation of the oligomeric XylS form which binds to operator DNA and/or the protein-DNA interactions at the direct repeats is very weak.

THE EFFECT OF *cup-1* ON *lac* OPERON EXPRESSION AND LACTOSE TRANSPORT

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cup-1 is a pleiotropic mutatation of *E.coli* K-12 (Mahajan et al 1988). It blocks the growth of the mutant cells on a variety of carbon sources including PTS as well as non-PTS sugars, and catabolite repressible as well as catabolite non-repressible carbon sources. It prevents the uptake of ^{14}C labelled sugars. This mutation maps near 97 min on the standard *E.coli* map and is transferred as a distal marker by HrfH. In F' merodiploids *cup-1* is dominant over its *cup*$^+$ allele. It grows very poorly in rich media and frequently accumulates revertants. The *cup-1* strains frequently acquire partial suppressors that allow the mutant cells to grow on a specific sugar or a group of sugars (Mahajan et al, 1988).

Cells harbouring the mutant allele *cup-1* on a multicopy plasmid failed to grow on glucose in addition to other carbon sources. This and the observation that *cup-1* was not cotransducible with markers to the left indicated that the growth of the original *cup-1* isolate on glucose was due to the presence of a glucose suppressor mutation, tentatively designated as *gls*. P1 transduction data give evidence for the presence of *gls* near 98 min on the *E.coli* map. A glycerol suppressor mutation was also found to be located nearby. Levels of ß-galactosidase in isogenic *cup*$^+$ and *cup-1* *lacI*$^-$ strains showed that *cup-1* drastically reduces induction of the lactose operon. The induction was partially restored by exogenous cAMP, indicating that one of the effects of *cup-1* is to lower the levels of cAMP in the cell by reducing its synthesis. In addition, there is genetic evidence that *cup-1* blocks lactose transport even when enzymes of the *lac* operon are expressed.

Regulation of Gene Expression in Halophilic Archaebacteria.

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The halophilic archaebacterium *Haloferax mediterranei* is able to grow over a wide range of salt concentrations (from 10% total salts to saturation of NaCl). To be able to grow at such a different salinities the organism has to suffer considerable physiological changes, many of which could be regulated at transcriptional level. A markedly different expression of the gene encoding the gas vesicle protein of *H. mediterranei* depending on the salinity of the medium has been described (1). On the other hand, on the basis of the effect of gyrase inhibitors on gene expression in *Halobacterium halobium* and the presence of putative Z-DNA sequences in certain halobacterial genes, some authors have suggested a possible role of topology of DNA on the regulation of expression in halobacteria (2).

We have studied, by Northern blot hybridization, the expression of different DNA fragments from a genomic library of *H. mediterranei*, all of which presented restriction pattern modifications depending on the salinity of the growth medium (3). Our results show that at least three of these DNA sequences presented markedly different transcript levels depending on the salt concentration of the growth medium. All these sequences showed a marked enhancement of expression at higher salinities (at 30% salts much higher than at 20% salts, which is the optimal salt concentration) and also in some cases a slight enhancement of transcript levels at low salinity (12% salts, aproaching the minimal concentrations for growth). These DNA sequences could be related to structural or regulatory proteins being highly espressed in osmolie stress conditions. These DNA fragments are also being sequenced to determine the corresponding ORFs. In the regions upstream one of these ORFs (ORF 61) we have observed peculiarities such as purine-pyrimidine alternancy and inverted and direct repeats similar to those described in promotor regions of certain halobacterial genes. We have also studied the local supercoiling of sequences upstream ORF 61 by a method based on the preferential intercalation of psoralen into supercoiled versus relaxed DNA (4), and have detected significant changes in supercoiling after cells were shifted from 12% and 20% salts to 30% salts. Further studies are in progress to clarify the mechanisms involved in regulation of expression in halobacteria.

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XYLS, THE POSITIVE REGULATOR OF THE *Pseudomonas* TOL META-CLEAVAGE PATHWAY

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The TOL *meta*-cleavage pathway operon of *Pseudomonas putida* encodes the enzymes required for growth on alkylbenzoates. Transcription from the operon promoter is mediated by the XylS positive regulator in the presence of benzoate effectors, or, when overproduced, in the absence of effectors.
The XylS protein belongs to a family of positive regulators that includes at least eighteen proteins. These proteins activate transcription from a number of operons for sugar metabolism and alkylating agent response, among other functions.
Based on protein alignments, the members of this family exhibit a long stretch of homology at the C-terminal end where a motif involved in DNA-binding has been located.
Chemical and oligonucleotide site-directed mutagenesis at the N-terminal end of XylS have identified residues important for effector binding and activation of this regulator.

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CONTROL OF GENE EXPRESSION OF RHAMNOSE OPERON IN ESCHERICHIA COLI

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L-Rhamnose is a methylpentose sugar that can be used as a sole carbon and energy source by *Escherichia coli*. The genes required for the metabolism of the L-Rhamnose have been mapped at 88.4 min on the *Escherichia coli* chromosome and constitute an operon formed by five closely linked genes: the rhamnose regulatory gene *rhaC*, formed by two overlapping genes (*rhaS* and *rhaR*) and four structural genes; *rhaT* (encoding rhamnose permease), *rhaB* (encoding rhamnulose kinase), *rhaA* (encoding rhamnose isomerase) and *rhaD* (encoding rhamnulose-1-P aldolase). *rhaT* is separated from the three other structural genes by their common regulatory gen *rhaC*. It has been described that *rha* operon is under positive control.

The four structural genes have been cloned and sequenced previously in our laboratory. Two protein-binding domains were found in the 5' region of *rhaB*, constituting a good catabolite repression protein consensus binding site. No clear -10 or -35 consensus sequences are apparent, which is not unusual in genes belonging to an operon under positive control. Some inverted repeat sequences are present in the *rhaA-rhaD* intergenic region and there is a rho-independent transcriptor terminator in the 3' region of *rhaD*.

The "Northern blot" analysis reveals the existence of one transcript of 1.5 Kb for *rhaB*. For *rhaA* and *rhaD* two transcripts of 1.3 and 2.3 Kb can be seen. This is uncommon to Prokaryotes operons which normally are expressed in a polycistronic transcript. The presence of more than one transcript could be due to degradation of a single mRNA or to the presence of several transcriptional start points. To discard the possible existence of intergenic transcriptional start points we prepared *lacZ* gene fusions. The 5' region of the *rhaB*, *rhaA* and *rhaD* genes has been ligated to the *lacZ* gene. Analysis of β-Galactosidase activities of cells with a single copy of the fusion yielded 20,000 β-galactosidase units for *rhaB-lacZ* fusion, 100 β-galactosidase units for *rhaA-lacZ* fusion and 50 β-galactosidase units for *rhaD-lacZ* fusion. In accordance with these results, there seems to be only one promoter, *rhaB* promoter, which would control the three structural genes. These results support the notion of mRNA instability.

It is well known that transcription and translation are coupled processes in Prokaryotes. To analyse the influence of translation in mRNA stability we performed some experiments of RNA obtention under different conditions, including time after inducer addition or presence of antibiotics, such as chloramphenicol. Under some specific conditions we have found a 4.3 Kb transcript which would correspond to the full length *rhaBAD* mRNA. Therefore, "Northern blot" products mentioned above would most likely be the result of mRNA degradation.

TRANSCRIPTION OF THE E. COLI HEMOLYSIN OPERON. ROLE OF THE COORDINATE TRANSCRIPTION ACTIVATOR PROTEIN RFAH.

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The hemolysin toxin (HlyA) is secreted by pathogenic *Escherichia coli* and forms membrane pores in cells of the host immune system, causing cell dysfunction and death. Hemolytic determinants can be found both in large plamids (pHly152) or in chromosomal locations (LE2001 and pSF4000). Secretion of HlyA occurs without a periplasmic intermediate and is directed by an uncleaved C-terminal targetting signal and the HlyB and HlyD translocator proteins, the former being a member of a transporter superfamily central to import and export of a wide range of substrates by prokaryotic and eukaryotic cells. The process by which HlyA is targetted to mammalian cell membranes is dependent upon fatty acylation of the precursor, proHlyA, directed by the activator protein HlyC and the cellular acyl carrier protein. The secretion proteins HlyB and HlyD are encoded by genes contiguous and coexpressed with the *hlyC* and *hlyA* genes responsible for synthesis of cytolytically active toxin. HlyB and HlyD are present at very low levels in the cell membrane fraction, primarily due to down regulation of secretion gene expression. This is achieved by uncoupling operon transcription of the synthesis and secretion genes via transcript termination at a *rho*-independent terminator within the operon, an effect probably accentuated by differential stability of the truncated and elongated transcripts. Expression of the specific hemolysin export function is mediated by transcript antitermination at the *hlyA-hlyB* intergenic terminator and this is dependent on structural elements, including the *hlyR* locus, which are positioned upstream of the *hly* operon promoter sequences and which are thought to act as binding sites for regulatory proteins involved in both *hly* transcription activation and antitermination. We have recently identified an activator of *hlyCABD* gene expression as the 18kDa product of the *rfaH* gene which positively regulates transcript initiation and possibly antitermination in the operons encoding synthesis of surface sex pilus and lipopolysaccharide of *E.coli* and *Salmonella*. We are currently studying the role of RfaH in transcription initiation and possibly elongation and the possible interaction of the activator protein with putative transcription sequences directing the coordinate expression of virulence and fertility.

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"Protein p4 stabilizes RNA polymerase closed complex by direct protein-protein contacts". B. Nuez, F. Rojo and M. Salas. Centro de Biología Molecular (CSIC-UAM) Universidad Autonoma, Cantoblanco, 28049 Madrid. Spain.

Trancription of the late genes of *Bacillus subtillis* phage Ø29 occurs from PA3 promoter. This promoter is located adjacent to the divergently transcribed main early promoter (PA2b). Protein p4 binds to a DNA region located between positions -56 and -102, relative to PA3 transcription start site, activating PA3 transcription while repressing PA2b. It has been shown that binding of protein p4 and RNA polymerase to PA3 is highly cooperative and that a correct stereospecific alignment of both proteins is required for activation.

The role of protein p4 in transcription initiation has been analysed kinetically. The protein p4 major activating effect was shown to be in closed complex formation with little or no effect in subsequent steps of the initiation process.

The role of direct protein-protein interactions between protein p4 and RNA polymerase in the initiation process was investigated by band-shift assays. A DNA fragment containing the PA3 promoter was digested at position -56, relative to the PA3 transcription start point, producing two DNA fragments that contained either the protein p4 binding site, upstream from position -56, or the RNA polymerase binding region. Two findings were taken as evidence for direct protein-protein contacts between the regulator and the RNA polymerase. First, the RNA polymerase could retard specifically the DNA fragment containing protein p4 binding site only in the presence of protein p4 and second, its binding to the fragment containing the PA3 transcription start site was greatly enhanced by protein p4. Neither of these effects were obtained if a mutant protein p4, that efficiently recognizes its binding site but does not activate transcription from PA3, was used. This mutant protein only lacks twelve amino acids at its carboxyl-end, suggesting that interactions with the RNA polymerase are maintained through a specific region of protein p4. When *E. coli* RNA polymerase was used instead of *B. subtilis* RNA polymerase, retardation of DNA fragments in the presence of protein p4 was not obtained.

These results strongly suggest that the protein p4 stimulation of closed complex formation could be mediated by specific protein-protein interactions between protein p4 and *B. subtilis* RNA polymerase.

PROTEIN-INDUCED BENDING COULD ACT AS A TRANSCRIPTIONAL SWITCH FACTOR IN VIVO

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Gene expression in prokaryotic organism is controlled by DNA-binding proteins. Many of these regulatory proteins bend cognate DNA sequences upon binding, but whether the resulting DNA curvature forms part of the mechanism for transcription initiation remains uncertain. To study the effects of protein-induced DNA bending in transcription initiation *in vivo*, we have examined the effect of substituting the CAP site of the *E. coli fur* operon by the heterologous binding site of the DNA-bending repressor RepA from the pneumococcal pLS1 plasmid. The *fur* gene is expressed from two tandem overlapping promoters, being their -35 and -10 regions located at opposite sides of the DNA helix. Under catabolite repression conditions, activity of the downstream promoter P_A (placed at the opposite helix side of the CAP-binding sequence) is paralelled by a decrease in transcription from the upstream promoter P_B. On the contrary, transcription from P_B (located at the same helix side than the CAP binding site) is stimulated in conditions of catabolite activation, with simultaneous repression of the downstream promoter P_A.

How is the transcriptional switch effected in the *fur* operon?. The data obtained suggest a model for the transcriptional switch in which bending is a crucial step for alternate promoter activation/repression. The presence of a DNA curvature on the rigth or wrong side of the RNA polymerase binding site has been show to activate or to repress, alternatively, the activity of corresponding promoter. In the case of natural promoters, tandemly-located, such as those presents on the *fur* or *gal* operons, RNA polymerase binding to constitutive basal promoter may contribute to repress transcription from the catabolite-responsive promoter, because the RNA polymerase generate itself a bend at the opposite DNA helix side. Only when the activator protein binds to its upstream site, the activator-induced bend counteracts the 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 transcription of basal promoter, located now at the wrong face of the bend.

Intrinsic or protein-induced DNA bends may increase the interaction of RNA polymerases and other DNA-binding proteins, "poised" at high affinity sites, towards nearby low affinity sites, thus adding another level of complexity to transcriptional regulatory circuits.

In Vivo Thermodynamic Analysis of Looping as a Function of Interoperator Distance in *lac* Constructs: Estimates of Free and Local *lac* Repressor Concentrations and of Physical Properties of the Interoperator Region of Supercoiled Plasmid DNA

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A strong-binding primary (O^+) *lac* operator located 100-200 basepairs upstream of a *lac* promoter control region reduces expression from a lac promoter controlled by a weaker binding (O^c) *lac* operator between three- and twenty-fold on a multicopy plasmid in *E. coli*. We attribute this effect to loop formation in which a thermodynamically stable complex is formed between bidentate *lac* repressor tetramers and the O^+ and O^c operators. We have used this looping assay to characterize the physical properties of the intervening DNA region *in vivo*. The extent of expression is a periodic function of the distance in base pairs between the O^+ and the O^c operators. The periodicity of modulation is 11.28±0.04 basepairs, which we interpret as the helical repeat of this region of supercoiled plasmid DNA *in vivo*. Possible origins of this altered helical repeat include the global linking deficit of the supercoiled DNA and any local linking deficit induced by divergent transcription from promoters bracketing the interoperator region. By application of a thermodynamic model for repression and the analysis developed for DNA cyclization, we calculate that the local concentration of *lac* repressor at the O^c site from looping varies periodically in the range 4-40 μM, with an amplitude which decreases monotonically with increasing interoperator distance in the range examined. (For comparison, we estimate that the thermodynamic activity of *lac* repressor is less than 2 μM in this strain.) The DNA cyclization analysis also yields an apparent *in vivo* persistence length of 63±26 Å (which is ~15% of the *in vivo* result) and an *in vivo* torsional rigidity constant of $(1.1\pm0.1)\times10^{-19}$ erg cm (which is at the lower end of the range of values found *in vitro*) for this DNA segment.

DNA supercoiling stabilizes open complexes formed at the main early and late promoters of *Bacillus subtilis* phage Ø29

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Transcription of phage Ø29 genome takes place in two stages. Early genes are transcribed from several promoters, of which PA2b is the main one. Late genes are expressed from a single promoter named PA3, which is adjacent to PA2b but is transcribed in the opposite direction (1). Transcription from PA3 requires the viral early protein p4, a transcriptional regulator that activates PA3 while simultaneously repressing PA2b (2,3).

The study of a number of strong *B. subtilis* promoters in linear templates has recently led to propose that promoters in this organism fall into two classes: those forming stable open complexes with σ^A-RNA polymerase and those giving rise to unstable complexes, that can be dissociated by a competitor challenge (4). In line with this finding, we have observed that open complexes formed by σ^A-RNA polymerase at the regulated Ø29 PA2b and PA3 promoters in linear templates are unstable, being sensitive to heparin challenge. The presence of the two initiating nucleoside triphosphates could only stabilize these complexes to a low extent. Interestingly, the introduction of moderate or high DNA supercoiling significantly stabilized the open complexes at both promoters. The overall effect of DNA supercoiling on the activity of both promoters was nevertheless different: it considerably stimulated PA2b while it had no effect or was moderately inhibitory for PA3 in the presence of protein p4. The behaviour of this class of *B. subtilis* promoters is therefore analogous to the *Escherichia coli* ribosomal RNA promoters which, unlike the majority of *E. coli* promoters, also form unstable open complexes with σ^{70}-RNA polymerase that are stabilized by DNA supercoiling (5).

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Regulation of *parD*, a stability system of plasmid R1

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parD is a stability system of plasmid R1 that is located in a region adjacent to the basic replicon of the plasmid (1). This system is normaly sylent but its derepression greately increases the maintenance of the R1 replicon in the bacterial populations (1). This stabilizing effect is modulated by the action of the two proteins of the system, a killing protein, Kid , and a protein, Kis, that antagonizes the killer (1,2). Analysis of *parD* sequences (1) and characterization of *parD* transcripts (3) indicates that the *kis* and *kid* genes form an operon in which the *kis* gene is transcribed first.

A detailed analysis of the regulatory role of the Kis and Kid proteins inidicates that they act coordinately to repress at the transcriptional level expression of the system and that the Kis protein alone has a weak regulatory activity (3). Derepression of *parD* originates two transcripts, that occur with similar aboundance and that have similar stability: a polycistronic messanger and a shorter transcript that codes only for the antagonist of the system. The shorter transcript terminates within *kid* and after a sequence that can form an stem-loop structure. However a detailed analysis indicates that this structure does not seems to act as a transcriptional terminator. Polarity and translational coupling prevent synthesis of the killer protein when the antagonist is not synthesized.
These data indicate that synthesis of the first component of *parD* , the antagonist, is favored, both at the transcriptional and postranscriptional levels, over synthesis of the killer.

We have found that the *parD* system is transcriptionaly derepressed when replication of the plasmid is inefficient and that this derepression is associated with a clear stabilization of the plasmid. Further analyses indicate that this stabilization is due at least to two effects: counterselection of segregants and increased efficiency of replication. The recovery in replication seems to occur at the expenses of the autoregulatory potential of the system, indicating a direct role of Kis and/or Kid in this effect. Copy number mutations, that restore the efficiency of plasmid replication, switch-off expression of *parD*. This indicates that gene dosage regulates expression of the system and suggests that derepression of the *parD* system when replication is inefficient, is triggered initially by low gene dosage.
These data underline the interlink between the basic replication functions of R1 and the *parD* stability system (both at the levels of regulation and activity), and suggest that the close assotiation of both regions is functional.

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CONCLUDING COMMENTS

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Concluding Comments

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It is tricky to discharge the task of offering concluding comments to the participants of a workshop; for readers of these abstracts, the task is different, but no easier. For partipants, there are several things to avoid: (1) Offering a conscientious summary of the meeting, pointing out the highlights, or offering parting personal insights is not particularly useful. In the first place, the participants do not need that. Moreover, it is the spontaneous and multilateral exchanges of ideas, in direct response or challenge to just-presented information, that make workshops such as this particularly valuable for the fortunate participants. Sifting what is significant and might stimulate new experiments or thinking is very much an individual task. (2) Telling those assembled what is important to do next is not likely to be appreciated. This is not, after all, the human genome project! People who work in our field have already demonstrated their stubborn tendency to independence of judgment and their preference for making their own decisions about what fascinates and motivates them, the shifting winds of fashion to the contrary notwithstanding.

For the non-participant reader of this booklet, many of the abstracts are sufficiently detailed to serve as capsule reviews. What they lack is the presentation of latest results and substantial changes of emphasis in some of the presentations at the workshop. Nevertheless, the abstracts are superior to a brief summary.

It is, I hope, instead useful to add a more general but widely agreed-upon judgement of the research enterprise of which this workshop is a manifestation: that it is in robust intellectual and creative shape. At the workshop, this was demonstrated in two ways. New insights were offered

into problems that have been studied for two decades or more and have been regarded as understood, at least in respect to what has been conventionally thought to be "important" to know, for much of that time. Nevertheless, the sustained drive to understand what is really going on is currently being rewarded with fundamental new insights into a number of these classical problems, including the mechanism of RNA chain elongation, the modes of interaction of proteins with RNA polymerase that generate positive transcriptional regulation, and regulatory antitermination of transcription by the phage λ gene Q protein. Digging constantly deeper is in the tradition of the "harder" physical sciences; in the field of transcription, it is a tradition that, at least for the time being, is most readily followed by working with some of the classical problems of prokaryotic gene regulation. The reasons for this rest partly with material advantages: the availability of powerful genetic systems, tractable levels of biochemical complexity, large and reliable bodies of relevant information. Equally important is a working milieu that tends to hold to meticulous standards of experimental analysis and to a punctilious regard for the rules of evidence. Also important is an appreciation, on at least a part of the research community that is engaged in this kind of work, for the enormous chemical complexity of these problems, and for the potential benefits of quantitative analysis.

I hope that it is not too trite to point out what the stakes of this kind of deep and detailed analysis are. It is, after all, clear that all transcription apparatus evolves from common historical roots and operates within common chemical constraints. As a direct consequence of this, sufficiently basic insights about transcriptional mechanisms are almost certain to have universal implications.

In a second dimension, there were several presentations of gene regulatory systems that are relatively new to detailed investigation at the molecular level. Just as there is great promise in

the deep analysis of what we already know something about, there is also enormous excitement in realizing how vast is the unexplored territory before us. The greatest part of evolution has happened in the prokaryotes; the animals, plants and fungi together represent ten percent or less (Woese, 1990). It is a foregone conclusion that the largely unexplored biological diversity of prokaryotes will yield genuine novelty in gene regulation.

Many of the presentations at the workshop fit somewhere in between these roughly orthogonal modes of exploration. Several presentations concerned multiprotein-DNA complexes that contort DNA as they generate regulated transcriptional activity. Other talks dealt with transcription-regulatory manifestations of bacterial chromatin structure, still difficult to define clearly and precisely at the molecular and mechanistic level, though now (happily, I think) on the minds of many. The remarkable interplay between cell-cell communication, signal transduction pathways, cell differentiation and serial changes of transcriptional regulation in bacterial sporulation was sketched in one presentation. Enhancer-like systems and the principles on which they operate were also discussed. For the participants of the workshop, this breadth of coverage and the selection of topics was exhilarating and thought-provoking. It is hoped that, for others, these abstracts will provide access to current thinking about some of these problems, and that they will be of some help to those who are interested in watching out for what comes next or, better still, joining the game.

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