Instituto Juan March
de Estudios e Investigaciones

49 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

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EMBO EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Workshop on

Transcriptional Regulation at a Distance

Organized by

W. Schaffner, V. de Lorenzo and J. Pérez-Martín

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INDEX

INTRODUCTION

Walter Schaffner, José Pérez-Martín and
Víctor de Lorenzo

INTRODUCTION

The discovery of enhancer elements about one dozen years ago has been one of the major landmarks in modern Molecular Biology. The notion that discrete DNA sequences and cognate DNA-binding proteins can control the activity of promoters located at considerable distances has provided the key to understand the molecular basis of major biological phenomena such as tissue-related gene expression as well as the organization of complex regulatory cascades in developmental systems. The last few years have witnessed an amazing progress in the analysis of the various components which account for activation at distance, in particular the nature of the regulatory proteins and auxiliary factors involved.

From the information available at the moment, we know that *cis*-acting regulatory elements of eukaryotic genes include promoter sequences located around the transcription initiation site and enhancer sequences located farther away. A promoter often consists of an initiator sequence, a TATA box and one or more upstream sequences where regulatory proteins can bind. Transcription initiation by RNA polymerase II involves the assembly of a multifactor complex at the TATA box and initiation site. While the promoter is the assembly site of the preinitiation complex, enhancers confer additional regulatory information (i.e. cell-type specificity) to the promoter. Eukaryotic transcription factors are divided, in one hand, into the *general transcription factors,* which bind to common motifs (TATA box, initiator motif) and constitute the preinitiation complex, and the *sequence-specific transcription factors* which bind to upstream promoter or enhancer elements. On the other hand, sequence-specific transcription factors are thought to influence the rate of transcription initiation by interacting with the general transcription factors, RNA polymerase II and chromatin components. The main features of a sequence-specific transcription factor include the DNA-binding domain, the nuclear localization signal and the transactivation domain. A common (and somewhat naïve) view of the process assumes that once the initiation complex is assembled, the collection of transcription factors create a constellation of protein-protein interactions that, through a largely undisclosed mechanism, enables the polymerase to initiate transcription. On top of this, the pivotal role of chromatin and chromatin-associated proteins in transcriptional control is becoming an emerging (and expanding) issue in nearly every system where its role has been examined.

Needless to say that these views are under permanent challenge, since new factors and mechanisms are coming into play, mostly from research on transcriptional regulation in yeasts and *Drosophila*. These two experimental systems are the best beneficiaries at the power of the genetics that can be applied to solve otherwise intractable questions. Complex issues on the mechanism of transcription initiation by RNA polymerase II (for example, recruitment of the

holoenzyme to the promoter mediated by transcriptional factors) are amenable to experimental scrutiny, to this day, almost exclusively through genetic means. In addition to the *reverse genetics* with mammalian cell cultures and the increasing availability of transgenic animals, yeast genetics has become the major driving force to raise a wealth of opportunities for fundamental explorations into the mechanisms of transcription in eukaryotes. One example is the control of the mating types of *S. cerevisiae,* one of the most complex -and most fascinating, paradigms of regulation of gene expression in the biological world. Similarly, the study of novel elements such as the chromatin insulators of *Drosophila* that inhibit the function of enhancers, is greatly facilitated in systems with a good repertoire of tools for genetic analysis.

What was believed to be distinct of eukaryotic promoters happens to occur also in prokaryotic systems. A number of observations made in the mid-80s in the Laboratory of B. Magasanik on regulation of nitrogen-starvation systems of *Escherichia coli,* notably the *glnAp2* promoter and its cognate regulator, the protein NRI (widely know by its alternative name, NtrC), indicated that remote transcriptional control was not a privilege of higher cells. *glnAp2* turned out to be the prototype of a novel class of promoters depending on the alternative σ^{54} factor. These are unique in that they are activated at a distance by specific regulators bound to upstream, enhancer-like sequences (UAS). These unusual properties are to be explained by the eukaryotic-like structure of the σ^{54} factor itself and that of the cognate regulatory proteins. In a subset of σ^{54}-dependent promoters, a binding site for the histone-like protein IHF site is found between the binding sites of the RNAP-σ^{54} holoenzyme and the UAS. The major (but perhaps not the sole) role of IHF as co-activator in the σ^{54}-promoters is believed to assist formation of a DNA loop or even a nucleoprotein complex to stabilize contacts between the RNAP and the activator protein bound to the UAS. There is also an increasing evidence that other prokaryotic histone-like proteins (such as HU) play a role in the assembly of the transcription initiation complex.

Although σ^{54}-dependent promoters are the most extensively studied case of activation at distance in prokaryots, other systems are subjected also to transcriptional control by regulatory devices placed at distant sites. A notable case is that of the T4 enhancer, in which tracking (and not looping) of the replication protein accounts for the effect of distant sites in transcription initiation. Repression at a distance is also a well known phenomenon in prokaryotic systems : early observations can be traced back to the late 70s in the work by Bob Schleif on the arabinose (*ara*) operon of *E. coli.* In spite of having been studied for over two decades, the *ara* system seems to be a permanent source of surprises to this day -and surely the best documented case of remote negative control in bacteria.

What lessons can the prokaryotic systems learn from the more complex eukaryotic promoters

and *vice versa*? Perhaps activation at a distance is a general evolutionary strategy to integrate multiple signals for the control of a single promoter. The architecture of some bacterial promoters subjected to distant control may therefore be better understood in light of its evolutionary history and not on the basis of an strict necessity for such a complex setup. An interesting lesson that comes from the prokaryotic side is that DNA structures play an active role in such *signal integration,* instead of being just docking sites for transcription factors. It seems to be true also for some prokaryotic systems (those dependent of σ^{54}) that the assembly of an upstream nucleoprotein complex gives rise to an enzymatic activity that is not present in the non-assembled components of the complex, an issue rarely examined in eukaryotic promoters.

In summary, it appears that, in spite of the intrinsic differences in the mechanism of activation of prokaryotic and eukaryotic systems subjected to remote control, various common themes will help to gain a better insight in the general biological problem of transcriptional control.

Walter Schaffner
José Pérez-Martín
Víctor de Lorenzo

FIRST SESSION:
GENERAL MECHANISMS (I)

Chairperson: Margarita Salas

THE ROLE OF DNA STRUCTURE IN TRANSCRIPTION ACTIVATION OF NR_I-PHOSPHATE

BORIS MAGASANIK

Activation of transcription at the σ^{54}-dependent promoters *glnAp2* and *glnHp2* of *Escherichia coli* is greatly enhanced by NR_I-phosphate bound to two enhancers located more than 100 bp upstream from the transcriptional start site. Binding to these sites facilitates the oligomerization of the dimeric NR_I-phosphate that enables it to serve as transcriptional activator. These sites can be replaced by sequence-dependent superhelical inserts lacking any homology to the nucleotide sequence of enhancers. Apparently, the three-dimensional structure of the DNA can determine its affinity for the activator protein.

In contrast to *glnAp2*, *glnHp2* contains a binding site for IHF located between the enhancers and the promoter. Experiments with linear DNA revealed that transcription can be initiated at *glnAp2*, but not at *glnHp2*. Initiation of transcription at *glnHp2* on linear, but not on supercoiled DNA requires the presence of IHF or HU, proteins without effect on transcription initiation at *glnAp2*. Additional experiments presented in a poster by Carmona and Magasanik identify the DNA region between enhancers and promoters at the critical determinant of transcription initiation at σ^{54}-dependent promoters.

Mark Ptashne

"Molecular Mechanisms of Gene Regulation"

In the first part of my talk I will address two questions relevant to our understanding of the mechanism by which transcriptional activators work. These questions are: what is/are the targets of transcriptional activating regions; and, how does the activating region-target interaction trigger gene activation? I will begin with the second of these questions.

In 1990, we (Himmelfarb *et al.*, 1990) described a mutant strain of yeast in which, as we later showed, a fragment of the yeast activator Gal4 lacking any classical activating region nevertheless works as a strong transcriptional activator (Barberis *et al.*, 1995). In this mutant strain the dimerization region of Gal4 (residues 58-97) works as a strong activator when tethered to DNA by fusion to a DNA binding domain. The mutant yeast strain (called Gal11P-potentiator) bears a single amino acid change (Asn Ile) at position 342 of the protein Gal11. Using surface plasmon resonance we showed that fragments of Gal4 bearing residues 58-97 bind to Gal11P, and that these peptides have no detectable affinity for wild type Gal11. Substituting other hydrophobic residues at position 342 of Gal11 (thr or val) produces two additional Gal11P alleles, and the relative strength of the interaction with Gal4 (58-97) predicts the relative transcriptional activating activity of DNA-tethered Gal4 (58-97) in these three strains. Mutants of Gal4 (58-97) that interact more or less avidly with Gal11P activate more or less well *in vivo*, respectively.

These and other results indicate that we have identified a specific protein-protein interaction that triggers gene activation. The additional fact (Barberis *et al.*, 1995) that Gal11 is found exclusively as part of the RNA polymerase II holoenzyme suggests that recruitment of the holoenzyme to DNA suffices to trigger gene activation. I will discuss additional results that reinforce that conclusion, including: a) Fusion of LexA to Gal11 creates a particularly powerful activator ; b) Fusion of LexA to another holoenzyme component (SRB2) also creates an activator; in this case, the mutant form of SRB2 described by Koleske *et al.* (1992) activates more efficiently than does the wild type (fused to LexA) when tested in a strain bearing a partial deletion of the carboxyl terminal tail of polymerase; c) a fragment of Gal11P encompassing residue 342 fused to a DNA binding domain, works as a strong activator in a strain bearing the carboxyl part of Gal11 (that which inserts into the holoenzyme) fused to Gal4(58-97); in this case no activation is observed if the Gal11 fragment bears the wild type residue (Asn) at position 342; d) the activation described in (c) is "squelched" by overproduction of residues (58-97) in the yeast cells, but activation by a classical yeast activator (GCN4) is unaffected.

Various experiments indicate that Gal11 is not the target of any natural activating region. The mutation creating the interaction with Gal4(58-97) is evidently entirely fortuitous. These considerations suggest that contact between any site on the holoenzyme surface and a DNA-bound molecule would trigger gene activation.

I now turn to the first of the two questions posed above concerning the natural targets of activating regions. A plethora of activating region-target interactions has been described (Triezenberg, 1995), so many in fact that it has been difficult to determine which, if any, is relevant *in vivo*. We (Wu *et al.*, 1996) have analyzed a 41 amino acid fragment (residues 840-

881) taken from the predominant activating region of Gal4 (i.e., Region II). We tested this fragment and each of a variety of deletion and point mutants for their "activating potentials" as measured *in vivo* and *in vitro*: and, using SPR, we measured the affinity of each variant for a variety of putative targets. In brief, we find a remarkable correlation between the affinity (K_A) for TBP and TFIIB, and the activating potential; the affinity of Gal4(840-881) for other proteins tested (includingTAF40, lysozyme, SRB2, SRB5, TFIIE and hPC4) is at least ten fold lower than that for TBP and TFIIB. Moreover, the length of the activating region fragment correlates well with the affinity for the targets as well as with the activating potential. We also find an unexpected species specificity: yeast TBP binds about equally to a human (VP16) and a yeast activating region, whereas human TBP interacts more avidly with the human activating region. This difference predicts the relative efficiency with which the activators work when presented with yeastTBP or humanTBP in yeast as assayed with the altered specificity system of Strubin and Struhl (1992). These and other results argue strongly that TBP and TFIIB are bona fide targets of activating regions. We will discuss possible explanations for the linear relationship between the length of the activating region and the strength of the interaction with the identified targets.

In collaboration with the laboratory of W. Hörz of Munich, Germany, we have performed a series of experiments designed to ask whether activating regions are required for nucleosome removal (remodeling) at the Pho5 promoter of yeast. We find that fusion of Gal11 to the DNA building domain of Pho4, the natural activator of Pho5, creates an activator that efficiently activates transcription and remove nucleosomes at the Pho5 promoter. Moreover, nucleosome removal (but not transcription) is observed even if the promoter bears a deletion of the TATA sequence. Pho4-Gal11 bears no traditional activating region, and so we conclude that recruitment of the holoenzyme, even to a promoter that cannot support transcription by virtue of a TATA deletion, suffices to remove nucleosomes. Further experiments probe the role of the swi/snf complex in this example of nucleosome removal.

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Unlooping Potentiates Three Activation Mechanisms by AraC

Robert Schleif

The arabinose responsive regulatory protein AraC, in conjunction with the catabolite activator protein CAP, control expression from four promoters in *Escherichia coli*. In the absence of L-arabinose, transcription from the four promoters is low, and in the presence of arabinose, transcription is stimulated 100- to 300-fold. At the *ara* p_{BAD} promoter in the absence of arabinose, one DNA binding domain of the dimeric AraC protein binds to the polymerase-distal half of an AraC binding site. The second DNA binding domain binds to a half-site 200 base pairs away. Upon the appearance of arabinose, the DNA looping which had been generated by AraC ceases, and one DNA binding domain relocates so that the protein now binds to the two half-sites alongside and partially overlapping the RNA polymerase binding site. In this binding state AraC protein stimulates transcription by increasing the affinity of RNA polymerase for the promoter, by accelerating the rate of conversion of a closed to open complex, and (more controversially), by preventing polymerase from binding to the promoter in an inactive and long-lived complex.

Despite the fact that the arrangement of the CAP and AraC binding sites at the *ara* p_{BAD} and *ara FGH* promoters differs, the basic transcription activation mechanisms at the two promoters are the same.

TRANSCRIPTIONAL ACTIVATION AND REPRESSION AT A DISTANCE

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Some 15 years ago a phenomenon was described as the enhancer effect whereby transcription in mammalian cells was activated over large distances of thousands of basepairs, and independent of the orientation of the activating "enhancer" DNA segment. (Banerji et al., 1981; Moreau et al., 1981). Originally described for viral control regions, an enhancer was subsequently identified also in cell type-specific gene, namely in the second intron of immunoglobulin heavy chain genes (Banerji et al., 1983, Gillies et al., 1983). Many enhancers were isolated by means of "enhancer trap" selection (Weber et al., 1984; Bellen et al., 1989). In the mean time, enhancers have been identified in all classes of organisms, notably in mammals and insects (Drosophila), where they are crucial for the control of development and cell differentiation. One and the same gene can be under control of several enhancers that confer specific expression patterns in different stages and tissues. An enhancer is often some 100-300 bp long and represents an array of binding sites for DNA-binding transcription factors which synergyze to activate transcription. While the exact mechanism of activation over large distances is not yet understood, a major contribution seems to come from DNA looping, bringing remote activating sequences to promoter proximity (Müller et al., 1989, Wijngerde et al., 1995).

In analogy to the positive effect on transcription activation over a distance there is also a silencing effect, originally described by Nasmyth and colleagues in the yeast mating type locus (Brand et al., 1985). "Silencer" DNA segments have also been identified in mammals and insects, but often these DNAs and associated factors were only active in the original context. Recently a negatively acting conserved domain (KRAB domain, related to leucine zipper) was identified in a number of Cys_2 His_2 zinc finger factors (Margolin et al., 1994, Pengue et al., 1994). When fused to the DNA binding domains of LexA and GAL4, we have found this domain to specifically silence an associated gene even over large distances.

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The bacterial enhancer-binding protein NtrC (nitrogen-regulatory protein C) and sensing of nitrogen limitation in enteric bacteria

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**Institute for Molecular Biology, University of Oregon

The enhancer-binding protein NtrC activates transcription by catalyzing the isomerization of closed complexes between σ^{54}-holoenzyme and a promoter to open complexes in a reaction that depends upon hydrolysis of ATP. We will present structural and functional evidence that the active form of NtrC is a phosphorylated oligomer which contains two dimers that are bound directly to the two binding sites that constitute the enhancer and additional dimers that are bound to these by protein-protein interaction. Such oligomeric structures, which are not stable in a gel mobility shift assay, have been visualized by scanning force microscopy. That they are required functionally is indicated by two lines of evidence: 1) They can be formed jointly by DNA-binding forms of NtrC and forms that cannot bind DNA. The former are used at concentrations sufficient to occupy the enhancer and serve as a tether but not high enough to yield good activation of transcription. The latter, which carry 3 alanine substitutions in the helix-turn-helix DNA-binding motif of NtrC, activate poorly from solution and form active structures with DNA-bound forms by protein-protein interaction; 2) Active structures can be formed jointly by DNA-binding and non-binding forms of NtrC, neither of which is capable of activating transcription by itself. The DNA-binding forms we have used for this purpose are the $NtrC^{D54A}$ and $NtrC^{D54N}$ mutant proteins, which cannot be phosphorylated (D54 is the site of phosphorylation). The non-binding form is $NtrC^{A216V"3ala"}$, which carries a substitution in the central domain of NtrC that reduces transcriptional activation by at least 1000-fold but does not affect the ATPase activity of the protein.

Phosphorylation of NtrC is controlled by the availability of combined nitrogen. As a first step in studying metabolic control of this phosphorylation in vivo, we have studied control of the growth of *Salmonella typhimurium* in response to nitrogen availability. We have obtained evidence that external nitrogen limitation is perceived as internal glutamine limitation in both batch and continuous cultures. Studies with mutant strains indicate that the drop in the glutamine pool in the parental strain under nitrogen-limiting conditions is probably sufficient to account for slow growth and that it is responsible for slow growth. Glutamate, the only other intermediate in nitrogen assimilation, is not used to sense nitrogen availability, but serves as the counter-ion for K^+. Mutant strains that cannot maintain a normal glutamate pool are unable to maintain a normal steady-state K^+ pool and have growth defects, particularly at high external osmolality.

SECOND SESSION:
GENERAL MECHANISMS (II)

Chairperson: Rudi J. Planta

Transcriptional noise : How promoters evolve to respond to novel environmental signals

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Various features of the regulation of pathways for biodegradation of recalcitrant compounds by *Pseudomonas* provide interesting hints on the mechanisms by which promoters evolve to respond to novel environmental stimuli. The *regulatory noise hypothesis* proposes that transcriptional control systems develop responsiveness to new signals due to the leakiness and lack of specificity of pre-existing promoters and regulators. When needed, these may become more specific through suppression of undesirable signals and further fine-tuning of the recruited proteins to interact with distinct effectors. This hypothesis is supported by the sophisticated regulation of σ^{54}-dependent promoters of the TOL (toluene biodegradation) operons, which can be activated to various degrees by heterologous proteins. Such 'illegitimate' activation is suppressed by bent DNA structures, either static or protein-induced, between promoter core elements. One central concept to understand promoter evolution is that not only the regulatory proteins but also the structural properties of the promoter DNA participate in the generation of repertoires of transcriptional control available for recrutiment by evolving genes and operons. Once a suboptimal regulatory device has been recruited, selective pressure seems to push the evolving system towards suppression of the elements that cause non-specificity. The *Pu* promoter of the TOL plasmid seems to harbor some molecular remnants of this process. These have permited still more refined tuning of its physiological activity in response to specific and less specific signals. Like all σ^{54}-dependent promoters, *Pu* is activated at a distance by its cognate regulator XylR (de Lorenzo *et al.*, 1991; Abril *et al.*, 1991; Marqués and Ramos, 1993). The intervening DNA segment between the upstream activating sequences (UAS) and those for RNA polymerase binding at *Pu* contains an integration host factor (IHF) attachment site that is required for full transcriptional activity. IHF is a small protein (20 kdal) that causes a sharp bend (~140°) upon binding to its target DNA sequence. Although the predominant role of IHF in *Pu* and other σ^{54}-RNAP-promoters is to provide an structural aid to improve contacts between the σ^{54}-RNAP and the activator protein attached to the UAS, (Pérez-Martín *et al.*, 1994), it helps also to maintain low the basal activity of *Pu* in the absence of XylR-mediated induction. This occurs by preventing the RNA polymerase from interacting with other regulators that act in concert with σ^{54} (Pérez-Martín and de Lorenzo, 1995b). Although IHF provides a scaffold for a loop which brings the two proteins into close proximity (thus increasing transcription efficiency), such an effect may, at the same time, restrict the flexibility of the region. The rigid conformation that DNA adopts upon IHF binding may increase activation specificity by preventing access to heterologous activators other than that properly bound and positioned in the UAS. It seems, therefore, that the role of IHF in *Pu* is not only as a co-activator but also as a suppressor of cross-regulation. We have coined the term *restrictor* as an operational definition for this specificity-enhancing function assigned to protein-induced or static bends in σ^{54}-dependent promoters. At least in the TOL system, the presence or absence of a *restrictor element* seems to displace the responsiveness of the promoter either towards specificity or towards promiscuity (Pérez-Martín and de Lorenzo, 1995a, 1995b).

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HOW TRANSCRIPTION FACTORS CONTEND WITH A REPRESSIVE CHROMATIN STRUCTURE

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Our current knowledge of gene regulation rests predominantly on the concept of transcription factors interacting with specific DNA binding sites thereby increasing the frequency of the initiation of transcription by the basal transcription apparatus. This picture ignores, however, the fact that DNA is not free in the nucleus but instead organized in chromatin. Over the past years it has become apparent that the chromatin structure not only serves to package DNA but is also involved in gene regulation.

We are investigating the regulation of the *PHO5* gene which encodes a strongly regulated acid phosphatase in *S. cerevisiae*. In these studies we are focussing on the contribution of histone-DNA interactions to gene regulation and the interplay between transcription factors and nucleosome structure (1-3).

The chromatin structure of the *PHO5* promoter undergoes a massive transition upon induction of the gene in the course of which four nucleosomes are disrupted. A transactivating basic-helix-loop-helix protein, Pho4, and a homeobox protein, Pho2, are primarily responsible for the activation of *PHO5* and the chromatin transition. There are two upstream activating sequences (UAS) in the promoter, one located in an accessible chromatin region and one contained within a nucleosome in the repressed promoter. They serve as target sites for Pho4.

We have shown that Pho4 cannot interact with a binding site when it is contained in a nucleosome. Binding to such a site can only occur with the simultaneous disruption of the nucleosome. The domain required for this function is contained within the transactivation domain of Pho4. A detailed deletion analysis of the domains required for transactivation and those required for chromatin disruption will be presented.

In addition we have used this system to assay for the ability to disrupt chromatin in other proteins from yeast, Drosophila and man. We have also investigated the role of several proteins of the Snf/Swi and Sin/Spt families (4) in chromatin organization and the regulation of the *PHO5* promoter. These proteins were identified in yeast as pleiotropic regulators of transcription, and there is evidence that they exert their effects via chromatin. Our results demonstrate that some of these proteins are required for setting up a repressive chromatin structure at the *PHO5* promoter and that the disruption of the corresponding genes leads to partially constitutive *PHO5* expression.

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DNA Tracking Proteins and Transcriptional Activation.

E. Peter Geiduschek, Department of Biology, University of California at San Diego, La Jolla, California 92093. (Reporting on research by T.-J. Fu, G. A. Kassavetis, G. M. Sanders and R. L. Tinker)

The ~40 bacteriophage T4 late promoters, which generate transcription of ~40% of the viral genome, are extremely simple, consisting merely of a TATA box placed ~10 bp upstream of the transcription start site. The T4 gene 55 protein (gp55) a member of the σ family, confers recognition of these promoters on the *E. coli* RNA polymerase core enzyme (E): E.gp55 accurately initiates late transcription *in vitro* on negatively supercoiled DNA. T4 DNA is not, on average, supercoiled and complex additional requirements – for the RNA polymerase-binding gene 33 protein (gp33) and for concurrent DNA replication – prevail *in vivo*.

An *in vitro* analysis shows how the T4 gene 45 protein (gp45), a replication protein that tracks along DNA, activates transcription. The analysis also suggests a plausible mechanism for coupling transcriptional initiation to concurrent replication, and suggests the existence of dual pathways for assembly of transcriptional initiation complexes at T4 late promoters.

A list of T4-encoded replication and transcription proteins, which may be useful for following the presentation of this work, is attached (Table I).

Previously published work shows that the three accessory proteins of the phage T4 DNA polymerase, gp45, 44 and 62, activate transcription by a novel mechanism, requiring the ATPase activity of these proteins and a DNA entry site, such as a nick, gap or primer-template junction, at which they can be assembled onto DNA. The assembly/entry site has the formal properties of an enhancer, but with two peculiarities: 1) the enhancer and the late promoter must be connected by a continuous and unobstructed path along DNA; 2) certain enhancers impose a polarity constraint upon transcriptional activation.

The direct activator of transcription is gp45; the gp44/62 complex serves as the DNA-loading enzyme of gp45. Gp45 tracks from its entry site to the promoter and ultimately forms part of the stable open promoter complex.

Recent experiments suggest how coupling between concurrent replication and late transcription is generated: 1) The loading sites for gp45 are transient, continuously repaired and removed by the processes – replication and recombination – that generate them. 2) The DNA-tracking state of gp45 is transient. Experiments that follow the dynamics of gp45 tracking by DNase I footprinting will be presented.

A direct interaction of gp45 as it tracks along DNA with gp55 has been demonstrated in two ways: 1) co-photocrosslinking of gp55 with DNA-tracking gp45; 2) stabilization by gp55 of the DNA-tracking state of gp45. The direct interaction of the DNA-tracking protein and the promoter-recognition protein implies the existence of an alternative pathway for assembly of transcription complexes at T4 late promoters.

Table I
The Cast of Characters:
Phage T4 Transcription and Replication Proteins

Gene	Protein	Function
→ 55	RNApol-binding; σ-family	Promoter recognition
→ 33	RNApol-binding	Transcriptional co-activator
43	DNA polymerase	Viral DNA replication
→ 45	accessory protein	Sliding clamp of the DNA polymerase and transcriptional activator
→ 62	accessory protein	Loading enzyme of the
→ 44	accessory protein	sliding clamp
32	single-stranded DNA-binding (ssb)	DNA replication
61	primase	DNA replication
41	helicase	DNA replication
59	anchor of the primase/helicase	DNA replication

The Molecular Basis of Gene Transcription

Silencers, Origins, and DNA Replication in Regulating the Expression of a Eukaryotic Genome. Jasper D. Rine, Division of Genetics, Department of Molecular and Cellular Biology, 401 Barker Hall, University of California, Berkeley 94720 (510-642-7047).

The genetic control of mating types of *Saccharomyces cerevisiae* provides a wealth of opportunities for fundamental explorations into the mechanisms controlling gene expression in eukaryotes. These studies have led to many important discoveries including the role played by DNA transposition in gene expression, the first eukaryotic operator and DNA binding protein, and a recognition that regulatory pathways are best described as networks of interacting genes and proteins. The focus of the work described in this talk is yet another dimension to gene expression, namely the influence of the position of a gene in the genome on the expression of that gene. Such influences of physical location on gene expression are referred to generically as position effects.

Mating type in Saccharomyces is controlled by the alleles of the mating type locus (*MAT*). Cells of the a mating type have the *MAT*a allele and cells of the α mating type have the MATα allele. The expression of these alleles of *MAT* control the set of genes that are responsible collectively for the host of differences between a cells and α cells. Cryptic copies of the mating type genes are also found at two additional loci known as *HML* and *HMR*, located on opposite sides of and distal to the *MAT* locus on chromosome III. *HML* contains an unexpressed copy o f the *MAT*α genes and *HMR* contains an unexpressed copy of the *MAT*a genes. The genes at *HML* and *HMR* contain all the promoter and enhancer sequences needed for their expression. Their lack of expression is blocked by the combined action of regulatory sites and proteins. A pair of regulatory sites known as silencers flank both *HML* and *HMR* and are required for the position-effect blocking the expression of these loci. In addition to these sites, the four proteins encoded by the four *SIR* genes (Silent Information Regulators) and several silencer binding proteins are required to maintain these loci in an inactive state.

The *HMR*-E silencer, on the centromere side of the *HMR* locus is the best studied of the silencers. Three different proteins that are required for silencing bind this silencer. These proteins include ORC, which is the DNA replication initiator protein of yeast, as well as RAP1 and ABF1, two well studied proteins that function as transcriptional activators at other sites in the genome. Mutations affecting any of these proteins result in a loss of silencing.

A key insight into the mechanism of silencing came from work from Grunstein and colleagues who discovered that mutations in the amino terminus of histone H4 cause a loss of silencing, establishing that silencing involves formation of a specialized structure of chromatin. Following this clue we developed methods for studying silenced chromatin in vitro. We learned that the mechanism of silencing has nothing to do with the transcriptional apparatus per se. Rather, silencing involves the formation of a chromatin structure that can block essentially any protein-nucleic acid interaction in the vicinity, regardless of whether it is the recognition of a promoter by RNA polymerase or a restriction site by a bacterial restriction endonuclease. The repressed structure includes the entire *HMR* locus. Importantly, the silencers flanking *HMR* do not define the boundaries of the silenced domain, rather there may be boundary-determining sequence elements that limit the silenced domain.

It has been known for some time that passage of cells through the S-phase of the cell cycle is critical for silencing of *HMR* and *HML*. The *HMR*-E silencer is a chromosomal origin of DNA replication, leading to the suggestion that initiation from

the silencer may be part of the mechanism of silencing. Some early experiments lent temporary support to this notion. For example, mutations in the ARS sequence of the silencer block the ability of the origin to function either as a silencer or as an origin of replication. Moreover, certain mutations in the genes encoding at least two different subunits of the ORC complex result in both temperature-sensitive growth due to a defect in replication initiation and defect in silencing. This result indicates that ORC plays a key role in both processes. However, multiple lines of evidence indicate that the role of ORC in replication initiation is separable from its role in silencing. These include the ability to recover mutations in genes encoding ORC subunits that are specifically defective in replication initiation or in silencing, and the ability to bypass the requirement for ORC at a silencer by tethering the Sir1p to the silencer. Indeed, the S phase is still required for silencing even in cells in which ORC itself is no longer required bound to the silencer for silencing to work. Thus we now think of the role of the silencer binding proteins as being responsible for providing a protein surface that recruits SIR proteins to the silencer, which in turn are responsible for producing silenced chromatin. This hypothesis has recently been strongly supported by the recovery of mutations that block the ability of the Sir1 protein from being recruited to the silencer but do not block the silencing function of Sir1 protein once it is tethered to the silencer.

In summary, studies of silencing in Saccharomyces are providing a view of how the mechanisms that replicate the genome are integrated with the mechanisms that control its expression. We are beginning to uncover how specific structural domains of chromatin are specified and assembled, and how these domains establish and maintain certain regions in the genome in a transcriptionally inert state.

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Designing TBP/zinc finger fusion transcription factors to control gene expression

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Many transcription activators and repressors exert their effects through interaction with the TATA-box binding protein (TBP). Fusion transcription factors in which the DNA-binding domains of activators or repressors are directly connected to TBP may have important applications in molecular biology and biotechnology. The structure-based design strategy developed by Pomerantz, Sharp, and Pabo (*Science* **267**, 93 (1995)) allowed us to construct a fusion protein in which a zinc finger protein, Zif268, is linked to the *C*-terminus of yeast TBP. Gel shift experiments show that this fusion protein forms an extraordinarily stable complex (half time of dissociation, $t_{1/2}$=410h), when the TATA box and Zif binding sites are juxtaposed in the appropriate arrangement. *In vitro* transcription experiments are in progress to study the regulatory properties of this remarkable fusion protein. Since the DNA-binding specificities of zinc finger proteins can be altered by phage display systems (Rebar and Pabo, *Science* **263**, 671 (1994)), related TBP/zinc finger hybrids may allow sequence-specific regulation of target genes, and this may eventually lead to a novel method of gene therapy.

COORDINATED SYNTHESIS OF NIFL AND NIFA IN *Klebsiella pneumoniae*.

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The regulatory operon *nifLA* is responsable for nitrogen- and oxygen-dependent regulation of nitrogen fixation genes in *K. pneumoniae*. Correct regulation of the system requires interaction between the nitrogen and oxygen sensor protein, NIFL, and its counterpart, the transcriptional activator NIFA. By integrating an extra copy of the wild type operon and a *ΔnifL* derivative in the chromosome of *K. pneumoniae* we demonstrate that a correct stoichiometry between both proteins is required for full represion of the *nifH* promoter in the presence of oxygen.

Polarity and translational coupling at the *nifLA* operon provide a tight coordination of the synthesis of both proteins, by conditioning the synthesis of NIFA to the previous synthesis of NIFL. Sequences showing RHO-dependent transcription termination activity are present in both *nifL* and *nifA* coding sequences. Frameshift *nifL* mutants show a high decrease of the expresion of *nifA::lac* protein fusions even in the absence of polarity. We propose that translational coupling is responsable for this effect. The involvement of a stable stem-loop structure in the stablishment of translational coupling and the mechanisms proposed will be discussed.

THIRD SESSION:
PROTEIN-PROTEIN INTERPLAY

Chairperson: Jay D. Gralla

Genetic and molecular dissection of a chromatin insulator. Victor G. Corces, Department of Biology, The Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218, USA.

The *suppressor of Hairy-wing* [*su(Hw)*] protein inhibits the function of transcriptional enhancers located distally from the promoter with respect to the location of *su(Hw)* binding sites. The polarity in the repressive effect of this protein is due to the ability of the *su(Hw)* binding region to form a chromatin insulator. Mutations in a second gene, *modifier of mdg4* [*mod(mdg4)*], enhance the effect of *su(Hw)* by inhibiting the function of enhancers located on both sides of the *su(Hw)* binding region. This inhibition is not complete, but rather it results in a variegated expression pattern. Mutations in *mod(mdg4)* act as classical enhancers of position-effect variegation and molecular studies indicate this gene is identical to the previously identified *E(var)3-93D*. The *mod(mdg4)* and *su(Hw)* proteins interact with each other. As a consequence of this interaction, the *mod(mdg4)* protein controls the nature of the repressive effect of *su(Hw)*: in the absence of *mod(mdg4)* protein, *su(Hw)* exerts a bi-directional silencing effect, whereas in the presence of *mod(mdg4)* the silencing effect is transformed into uni-directional repression. These results suggest that the *mod(mdg4)* protein plays a central role in transforming a silencer into a chromatin insulator. Analyses of the chromatin in the region surrounding the su(Hw) insulator indicate that these effects are accompanied by changes in chromatin structure as judged by alterations in patterns of hypersensitivity to DNase I and nucleosome positioning.

Mutations in the *mod(mdg4)* gene enhance the phenotype of *Ubx* mutations, causing transformations of posterior abdominal segments into more anterior fates. In addition, *mod(mdg4)* mutants enhance the phenotype of *trithorax* group mutations and suppress the phenotype of *Polycomb* group mutants. These results suggest that *mod(mdg4)* is a new member of the *trithorax* group family. Furthermore, mutations in *trithorax* group genes enhance the effect of *mod(mdg4)* mutations on gypsy-induced alleles, causing an increase in the bi-

directional silencing effects observed in the presence of su(Hw) protein. These results suggest that silencing effects caused by su(Hw) are mediated by changes in chromatin structure maintained by Polycomb and trithorax group proteins.

Regulation of bacteriophage ø29 DNA transcription. Margarita Salas[1], Mario Mencía[1], María Monsalve[1] and Fernando Rojo[2]. [1]Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), and [2]Centro Nacional de Biotecnología (CSIC), Universidad Autónoma. Canto Blanco. 28049 Madrid. Spain.

Bacteriophage ø29 early promoters are recognized by the *Bacillus subtilis* RNA polymerase (RNAP) containing the major σ^A subunit, while transcription from the late promoter, PA3, requires, in addition to the σ^A RNA polymerase, the product of the viral gene 4, p4. Protein p4 binds to a region located between nucleotides –58 to –104 relative to the PA3 transcription start site, recognizing an 8 bp long inverted repeat. This DNA region has a sequence-directed curvature that greatly increases by protein p4 binding. Protein p4 activates late transcription by stabilizing the binding of the RNAP to the promoter as a closed complex. Several evidences indicate that activation of the late promoter requires a specific interaction between p4 and RNAP: 1) both proteins bind to the same face of the DNA helix, and adjacent to each other; 2) binding of p4 and RNAP is strongly cooperative; 3) activation requires a precise stereospecific alignment between the two proteins; 4) protein p4 can form complexes with RNAP in the presence of DNA fragments containing only either the binding site for p4 or the one for RNAP; and 5) protein p4 mutants at residue Arg 120 have been isolated which bind DNA efficiently but are unable to interact with RNAP and to activate transcription. Indeed, we have found that protein p4 can induce the binding of purified *B. subtilis* RNA polymerase α subunit to the late promoter. This binding does not occur with p4 mutants at Arg 120 or when a deletion mutant at the α-subunit, lacking the 15 C-terminal amino acids, are used. The use of reconstituted RNAPs with deletion-containing α subunits indicated that the last 15 residues of the latter are required for p4-dependent transcription activation. In addition, by site-directed mutagenesis at the C-end of p4, several basic residues have been identified, including Arg 120, that contribute to maintain the DNA bending.

Since p4 is a dimer in solution and binds to DNA as a tetramer we propose a model in which two of the p4 subunits would interact with the RNAP while the other two would be used for DNA binding and bending.

The regulatory protein p4 also represses transcription from the early promoters A2b and A2c, that are divergently transcribed from the late A3 promoter. Binding of p4 displaces the RNAP from the A2b promoter, both by steric hindrance and by the bending induced upon binding. Protein p4 represses the A2c promoter by binding to a DNA site immediately upstream from RNAP in a way that does not hinder RNAP binding. In the presence of p4, RNAP can form an initiated complex at PA2c that generates short abortive transcripts, but cannot leave the promoter. Mutation of p4 residue Arg 120, which relieves the contact between the two proteins, leads to a loss of repression. In addition, deletion of the 15 C-terminal amino acids of the RNAP α subunit also leads to a loss of repression. Therefore, the contact of p4 and RNAP through the same regions can activate or repress transcription depending on the promoter.

Polycomb Group complexes, chromatin states and the regulation of homeotic genes in Drosophila.

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The expression domains of Drosophila homeotic genes are established in the early embryo by the segmentation genes, ultimately depending on maternal antero-posterior cues. When these early genes cease functioning, the correct expression domain is maintained by a generalized chromatin-based repressive mechanism used by all homeotic genes and by a large set of other genes, including many responsible for the development of the nervous system. This is the Polycomb Group (Pc-G) repressive system, which establishes a complex initiating at specific targets, the PREs. and spreading over a large chromatin region. In the Ultrabithorax gene, this complex maintains repression in cells in which Ubx was initially repressed but allows expression in cells in which it was originally active. The repressed state persists over many cell divisions, providing a cellular memory of transient developmental cues that were present at some earlier stage of development.

The Ultrabithorax gene contains a principal PRE and a number of weaker response elements that contribute to the establishment of Pc-G repression over a large region. In vivo, transposons containing the PRE can cause a partial (variegation) or total repression of neighboring genes strongly resembling the repressive effects caused by the vicinity of heterochromatin. The formation of Pc-G complexes can be detected on polytene chromosomes at the site of insertion of the transposon. The PRE generates a set of strong in vivo DNase hypersensitive sites mapping within the PRE sequence that can be reproduced in vitro with nuclear extracts. The PRE itself appears to be composite of multiple sequence elements which interact with different subsets of Pc-G proteins.

Our current model is that complex formation requires the cooperative interactions of a number of Pc-G proteins, some of which may interact individually with DNA sequences at the PRE but too weakly to result in stable binding by themselves. We suppose that once a nucleating complex is formed, the cooperative interactions extend the complex over an larger region region and are prevented from doing so when a gene is active but not when the gene is inactive at the time of complex formation. The Pc-G proteins are a heterogeneous lot and include some with functional homologies to Drosophila heterochromatin proteins (Suvar products) which are thought to form extended chromatin complexes in much the same manner. Several Pc-G proteins have mammalian homologues and experiments by others have shown that the mammalian Pc homologue is functionally equivalent to the Drosophila Pc while Drosophila Pc-G proteins can be recognized by a silencing mechanism in mammalian cells.

Insertion of a transposon carrying a PRE can often silence not only genes contained in the transposon but also genes flanking the insertion site in the Drosophila genome. Genetic evidence suggests that the genome contains boundary sequences that normally prevent silencing complexes from extending to inappropriate regions. We have shown that boundary elements such as the scs, scs' and su(Hw) binding sites contained in the gypsy transposon not only block interactions between enhancer and promoter but prevent the spreading of Pc-G complexes. These experiments have also demonstrated that the formation of an effective silencing complex requires interactions of a PRE with other cooperating sites in flanking sequences or in homologously paired sequences on the homologous chromosome.

Molecular and genetic similarities with Pc-G silencing suggest a relationship with mechanisms such as heterochromatic and telomeric silencing, X chromosome inactivation and genetic imprinting in mammals. It may be possible to interpret these phenomena as initiated as chromatin complexes related to those formed by Pc-G proteins which then induce changes in histone acetylation and CpG methylation. The latter may be necessary to insure continued silencing in organisms that, unlike Drosophila, have long lives and a large number of cell divisions intervening between embryo and adult.

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CREM, NUCLEAR PACEMAKER OF THE cAMP RESPONSE

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The CREM gene encodes both repressors and activators of cAMP-dependent transcription (1). Differently from the other members of the CRE-binding protein family, CREM expression is tissue-specific and developmentally regulated (2). In addition, multiple and cooperative phosphorylation events regulate the function of the CREM proteins (3).

CREM appears to play a key physiological and developmental role within the hypothalamic-pituitary-gonadal axis. There is a functional switch in CREM expression during the development of male germ cells which is directed by the pituitary hormone FSH (4). The hypothalamic-pituitary axis is modulated by the pineal hormone melatonin, whose production is in turn controlled by the endogenous clock. A CREM product, ICER, is rhythmically expressed in the pineal gland, being at high levels during the night (5). ICER functions as a powerful repressor of cAMP-induced transcription. Rhythmic adrenergic signals originated by the clock direct ICER expression (5).

CREM is inducible by activation of the cAMP signalling pathway with the kinetic of an early response gene (6). The induction is transient, cell-specific, does not involve increased transcript stability and does not require protein synthesis. The subsequent decline in CREM expression requires *de novo* protein synthesis. The induced transcript encodes ICER and is generated from an alternative, intronic promoter. A cluster of four CREs in this promoter directs cAMP inducibility. ICER represses the activity of its own promoter, thus constituting a negative autoregulatory loop (6). Recent results also indicate that CREM plays a pivotal role in the regulation of proliferation and cell cycle in neuroendocrine cells.

We have also shown that CREM expression undergoes a dramatic developmental switch during the spermatogenesis (2, 4, 7). This is a complex process by which undifferentiated male germ cells develop into mature spermatozoa. This process involves remarkable structural and biochemical changes which include nuclear DNA compaction and acrosome formation. We have previously shown that the transcriptional activator CREM is highly expressed in postmeiotic cells. We have also suggested that CREM may be responsible for the activation of several haploid germ cell-specific genes involved in the structuring of the spermatozoon. We have recently addressed the specific role of CREM in spermiogenesis using CREM-mutant mice generated by homologous recombination (8). Analysis of the seminiferous epithelium in mutant male mice reveals postmeiotic arrest at the first step of spermiogenesis. Late spermatids are completely absent and

there is a significant increase in apoptotic germ cells. We show that CREM deficiency results in the lack of postmeiotic cell-specific gene expression. The complete lack of spermatozoa in the mutant mice is reminiscent of cases of human infertility.

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Allosterism in σ^{54}-dependent activators : ATP binding to XylR triggers a protein multimerization cycle catalyzed by UAS DNA

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The events that take place at the prokaryotic enhancer of the *Pu* promoter of *Pseudomonas putida* prior to the engagement of the σ^{54}-RNA polymerase in transcription initiation have been studied *in vitro*. Occupation of the two upstream binding sites of the enhancer by active XylR protein, the cognate regulator of the system, showed cooperativity dependent on ATP binding, but not on ATP hydrolysis. In the presence of either ATP or ATPγS, the UAS catalyzed formation of an XylR multimer, as detected with cross-linking agents under various conditions. Limited proteolysis of activated XylR in the presence of ATP indicated also that XylR undergoes a major conformational change upon ATP binding. ATP hydrolysis is, therefore, preceeded by the multimerization of XylR at the enhancer, that is itself triggered by the sole allosteric effect of ATP binding to the protein. Since ADP is unable to support such a multimerized state, we argue that ATP hydrolysis is followed by a return to the non-multimerized state. This notion is further supported by the properties of mutant proteins that seem to be *frozen*, respectively, in either the non-multimerized or the multimerized state. Taken together, our results support a cyclic mechanism of ATP-dependent association /dissociation of XylR at the *Pu* enhancer that preceeds any engagement of the polymerase in transcription initiation. The protein surfaces that determine multimerization lie on the central domain of the activator, that is homologous to two unrelated proteins. i. e., TyrR, the repressor/activator of various operons for biosynthesis of aromatic amino acids in *E. coli*, and the ornithine decarboxylase antizyme (ODC-Az) of *E. coli*, that down-regulates post-translationally the activity of ODC when cells face polyamines. The presence of homologous domains in these otherwise unrelated proteins suggests that a basic module endowing ATP-dependent multimerization may have been recruited for different purposes, not necessarily linked to transcription activation. This raises some questions on the precise mechanism by which ATP hydrolysis causes formation of the open complex in σ^{54}-promoters.

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The NagC protein of *Escherichia coli* is a transcriptional activator for the *glmU* gene.

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The *glmUS* operon of *Escherichia coli* encodes proteins for the synthesis of glucosamine and UDP-N-acetyl-glucosamine (UDP-GlcNAc), early cytoplasmic precursors in the biosynthesis of peptidoglycan and lipopolysaccharide. The operon is expressed from two promoters of which the *glmU* proximal promoter, *glmU*p1, is only active in the presence of the NagC protein. Two binding sites for NagC are located upstream of the *glmU*p 1 promoter. They are centred at -47 and -200 relative the transcription start site and both sites are necessary for activation (Plumbridge, EMBO. J. 14 3958-3965 (1995)). The NagC protein is the repressor for the *nagE-BACD* operons involved in the utilisation of GlcNAc as carbon and nitrogen source. The NagC protein binds to two operators overlapping the divergent *nagE* and *nagB* promoters forming a repression loop. Repression of the *nagE-B* operons requires that the two operators which are separated by 94 bp i.e. by 9 turns of B-form DNA, be in phase on the DNA helix. Insertion of DNA, corresponding to a non-integral number of turns, results in loss of repression (Plumbridge and Kolb, Mol. Microbiol. 10 973-981 (1993)). In the case of *glmU*, where NagC is now acting as an activator, the centre to centre distance between the two operators corresponds to 14.7 turns of B-form helix. Deletions or insertions within this region show that there is some DNA phase dependence but that it is weaker than in the case of *nagE-B*. Gel retardation analysis of the *glmU* regulatory region, using a crude extract of a strain overproducing NagC, produces a very slowly migrating band which might be expected to contain some additional protein components besides NagC. The upstream operator behaves as an upstream activating sequence (UAS) but it is unusual in the sense that it is binding the same protein, which bound at -47, is expected to make direct contacts with RNA polymerase to stimulate transcription. We are attempting to isolate mutations in the NagC protein to define the different functional domains.

FOURTH SESSION:
PROTEIN AND DNA STRUCTURES

Chairperson: Sydney Kustu

Modulation of the activity of the transcriptional activator NIFA by the NIFL regulatory protein

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The NIFL regulatory protein controls *nif* transcription in *Azotobacter vinelandii* by direct interaction with the enhancer binding protein NIFA, a member of the σ^{54} (σ^{N})-dependent family of transcriptional activators. Stimulation of open promoter complex formation by NIFA requires nucleoside triphosphate hydrolysis catalysed by the central domain of this activator. Modulation of NIFA activity by NIFL *in vivo* occurs in response to the external oxygen concentration or the level of fixed nitrogen.

Sequence analysis of NIFL indicates that this protein is comprised of two domains separated by a glutamine rich flexible linker. The amino terminal domain shows homology to the *bat* gene product from *Halobacterium halobium*, which potentially has an oxygen sensing function and also to the rhizobial FixL family of heme-based oxygen sensors, although the significance of these homologies is at present unknown (1). The carboxy terminal domain of NIFL shares characteristic features with the histidine protein kinase family of two-component regulatory proteins and in the case of the *A.vinelandii* protein possesses all five of the conserved regions found in other transmitter domains. However although *A.vinelandii* NIFL contains a conserved histidine residue known to be the site of autophosphorylation in other members of this family, a number of substitutions of this residue do not impair function, implying that sensory transduction by NIFL does not involve phosphorylation of this residue (2). Moreover, neither autophosphorylation of NIFL nor phosphotransfer to NIFA have so far been detected *in vitro* (3,4). Inhibition of NIFA activity by NIFL apparently requires stoichiometric amounts of the two proteins implying direct protein:protein interaction rather than catalytic modification of NIFA activity. Since the nucleoside triphosphatase activity of *A.vinelandii* NIFA decreases when the inhibitory complex between NIFL and NIFA is formed, NIFL may block NIFA activity by inhibiting its catalytic function. Moreover inhibition by *A.vinelandii* NIFL is stimulated by the presence of adenosine nucleotides, particularly ADP, suggesting that formation of the inhibitory complex might be regulated by the ATP/ADP ratio (5).

Spectral features of purified NIFL and chromatographic analysis indicate that it is a flavoprotein with FAD as prosthetic group, which undergoes reduction in the presence of sodium dithionite. Under anaerobic conditions, the oxidised form of NIFL inhibits transcriptional activation by NIFA *in vitro* and this inhibition is reversed when NIFL is in the reduced form. Hence NIFL is a redox sensitive regulatory protein and may represent a novel type of flavoprotein in which oxidation and reduction of the flavin acts as a molecular switch to control gene expression (6).

The response to adenosine nucleotides overrides the influence of redox status on

NIFL and is also observed with refolded NIFL apoprotein, lacking the flavin moeity. These observations suggest that both energy and redox status are important determinants of *nif* gene regulation *in vivo*.

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Protein-DNA interactions in the control of fimbrial adhesin genes in *E. coli*.

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The ability to adhere to host cell surfaces is an important property of many bacterial pathogens, and enterobacteria often express specific adhesins in the form of fimbrial structures, also referred to as pili, on their cell surface. Uropathogenic isolates of *Escherichia coli* commonly have the potential to express different fimbrial adhesins that are encoded by separate polycistronic determinants. Transcription of those genes is regulated in response to environmental growth conditions. The *pap* genes encoding pyelonephitis-associated pili in *E. coli* strain J96 are transcribed from a major promoter which is subject to regulation by upstream activating sequences (UAS) where several DNA binding proteins interact. Characterization of the regulatory region by genetic analysis and *in vitro studies* has established that the CRP-cAMP complex, the LRP protein, and at least two *pap* encoded proteins (PapB and PapI) all interact with this UAS region (1). Two other determinants for fimbrial adhesins in strain J96, the *prs* and *foc* genes, have a similar genetic and transcriptional organization, and regulatory cross-talk is possible through homologs to PapB and PapI of the different gene systems. The PapB protein binds DNA in a multimeric fashion, and the target is an AT-rich region in which the number of copies of a repeated sequence may vary among *pap* determinants from different bacterial isolates. The binding sequence for the CRP-cAMP complex is located at a relatively long distance from either of the *pap* promoters, and the protein interaction results in bending of the DNA according to assays *in vitro*. In addition to this protein-induced bending our analyses suggest that the 0.4kb UAS region has features of strong intrinsic curvature with a major bend center in the middle. The protein LRP binds to the curved DNA region, and together with the other proteins it forms a multi-nucleoprotein complex that stimulates the transcription. The *pap* transcription is negatively influenced by the nucleoid-associated protein H-NS, and in *hns* mutant *E. coli* the transcription of the *pap* genes becomes activator-independent and can occur in the absence of the UAS (2). Introduction of mutations in the UAS region that seemed to alter the DNA curvature and thereby distorted the normal DNA conformation led to reduced expression of the *pap* operon (3). We suggest that the DNA conformation and the architectual role of proteins here are important features for the normal expression of fimbrial adhesins.

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Regulation of gene expression by ambient pH in *Aspergillus nidulans*.

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Extremes of pH represent an occupational hazard for many microorganisms. The filamentous ascomycete *Aspergillus nidulans* is able to grow over a wide pH range (between 2.5 and 10.5 pH units). This versatility demands the existence of a regulatory mechanism tailoring the synthesis of gene products (such an extracellular enzymes and permeases) which are not protected by the intracellular homeostatic system only at ambient pH values close to their corresponding optima.

Despite its possible ubiquity in the microbial world, this pH regulation has only been characterised in detail at the molecular and genetic level in *A. nidulans*. In this fungus, pH regulation is mediated by PacC, a tri-dactyl zinc-finger transcription factor. PacC is synthesised as an inactive version and is converted to an active form in response to a signal mediated by the *pal* gene pathway at alkaline ambient pH. This active form has a dual function, simultaneously activating transcription of alkaline-expressed genes (such as that encoding an alkaline phosphatase) and repressing transcription of acid-expressed genes (such as that encoding an acid phosphatase). Under acidic conditions (or with acidity-mimicking *pal*$^-$ mutations interrupting the signal transduction pathway) PacC remains inactive, with the consequent derepression of "acidic" genes and lack of activation of "alkaline " genes. Conversion of PacC from the 678-residue full-length, inactive form into an active form requires the proteolytic removal of the approx. 60% C-terminal residues of the protein. The resulting, active PacC version (approx. residues 1-270) is competent both in activation and repression of structural genes. Under acidic conditions, the C-terminal region of PacC prevents its own proteolysis and alkalinity-mimicking *pacC*^{c} gain-of-function mutations, which represent truncations of this region, result in constitutive proteolytic activation and are epistatic to *pal*$^-$ mutations (thereby bypassing the requirement of the *pal* pathway). In addition, this negatively-acting C-terminal region appears to prevent DNA binding *in vitro*.

The zinc-finger region of PacC, which is sufficient for *in vitro* binding to its cognate, physiologically functional sites at an alkaline gene promoter, recognises an hexanucleotide with core sequence 5'-GCCARG-3' and, somehow heretically, appears to bind DNA in a parallel mode. No acidic gene promoter has yet been characterised, although the factors specifically required for the repressing function of PacC are starting to be identified.

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Interactions between the SV40 capsid proteins and cellular transcription in gene regulation in vivo and in DNA binding in vitro

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Our model for SV40 packaging suggests that the viral capsid proteins interact with cellular transcription factors at the SV40 packaging signal *ses* (which includes the GC-boxes and part of the enhancer) in the initiation of viral assembly. The model predicts that the capsid proteins function in the turning off of the viral promoters. This has been tested by cotransfection experiments, which have shown that the early and late SV40 promoters are significantly inhibited by the SV40 capsid proteins, supplied in trans. In contrast, two mouse ribosomal protein promoters, rpS16 and rpL30, were strongly enhanced. The human γ–globin promoter remained unaffected. These results indicate that the capsid proteins interact with transcriptional regulators affecting promoter activity in vivo.

The in vitro interactions between the viral VP3 and cellular transcription factors were investigated. Gel retardation assays demonstrated that purified GST-VP3 fusion protein produced in *E. coli*, inhibited binding of crude nuclear extracts to the SV40 *ses* element and also to a cellular SP1 binding site. Further experiments with purified SP1 and GST-VP3 demonstrated, unexpectedly, strong cooperativity between the two in DNA-binding. We found that the cooperativity between VP3 and SP1 did not depend on the number of SP1 binding elements. We further observed that the DNA binding domain of VP3 was required for this interaction. Furthermore, GST-VP3ΔC13, a truncated GST-VP3 fusion protein lacking the DNA-binding domain, was found to compete with the full length GST-VP3 in its cooperativity with SP1 in a dominant negative manner. These results suggest protein-protein interaction between SP1 and VP3.

VSG expression site regulation in bloodstream form *Trypanosoma brucei*

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African trypanosomes such as *Trypanosoma brucei* are capable of varying their major cell surface protein (Variant Surface Glycoprotein or VSG) during the course of an infection. The active VSG gene is invariably located in a discrete telomeric location known as an expression site. Switching expression of the active VSG gene normally involves either gene conversion of a silent VSG gene into the active expression site or an *in situ* switch whereby the active expression site is silenced, and a new one is activated without DNA rearrangements. We have inserted drug resistance genes behind the active expression site promoter, and replaced the active expression site promoter by an rDNA promoter. These transgenic trypanosomes were allowed to undergo switching in mice immunized against the old VSG coat. The total switching frequency was unaffected in the transgenic trypanosomes (between 10^{-6} and 10^{-7}). Active expression sites where the endogenous promoter was replaced by an rDNA promoter, were as efficiently silenced via *in situ* switches as those transcribed from an expression site promoter. Selecting for activation of the now silenced drug resistance gene, we were able to determine the frequency of reactivation of the last active expression site. Unexpectedly this frequency was quite high (at least 1 in 10^{-5}) and comparable using transgenic trypanosomes with expression sites driven either by the endogenous or the rDNA promoter. This could indicate that the switching frequency *in vivo* is composed of the frequency of activation of a new expression site, plus the frequency of inactivation of the last active expression site with a selection pressure against double expressors.

FIFTH SESSION:
REGULATORY PROTEINS (I)

Chairperson: Mark Ptashne

Regulation of σ^L directed transcription in *Bacillus subtilis*

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In bacterial cells, several forms of RNA polymerase holoenzymes are present and differ with respect to the associated sigma factor and the promoter sequences that they recognize. At least, ten different sigma factors have been identified in *Bacillus subtilis*. Most of sigma factors are members of the sigma 70 family of proteins. A clear exception is the sigma factor sigma 54, which is not member of the sigma 70 family and belongs to a second family of sigma factors widely distributed in prokaryotes.

We have characterized in *B. subtilis* a gene called *sigL*, encoding a sigma factor equivalent to sigma 54 from Gram-negative bacteria (1). Strains containing a *sigL* null mutation have a pleiotropic phenotype : the mutants are unable to grow in minimal medium containing arginine, ornithine, valine or isoleucine as sole nitrogen sources. The degradation of polymers of fructose is abolished in *sigL* mutants (see below).

Two operons, called *rocABC* and *rocDEF*, involved in arginine and ornithine catabolism, were characterized in *B. subtilis*. The transcription of these two operons is induced by the presence of arginine or ornithine in the growth medium and depends on the presence of the sigma factor σ^L. The *rocABC* and *rocDEF* promoters contain -12 and -24 regions almost identical to those observed in sigma 54-dependent promoters (2). Two regulatory genes, *rocR* and *ahrC* control the metabolism of arginine in *B. subtilis*. The AhrC protein represses the synthesis of biosynthetic enzymes and activates the two operons involved in arginine catabolism. AhrC is similar to the ArgR repressor and binds *in vitro* to the promoter of the *rocABC* operon. It was proposed that binding of AhrC causes binding and looping of DNA playing a role equivalent to that of IHF in Gram-negative bacteria (3). The product of the regulatory gene *rocR* which activates transcription of *rocABC* and *rocDEF* belongs to the NifA/NtrC family of transcriptional enhancers. It contains an activating central domain believed to interact with the σ^L containing form of RNA polymerase. Two tandemly repeated Upstream Activating Sequences recognized by RocR are involved in the expression of the *rocABC* and *rocDEF* operons (4).

The levanase operon (*levD*, *levE*, *levF*, *levG* and *sacC*) encodes the proteins of a fructose-specific phosphotransferase system (PTS) and a levanase able to hydrolyze fructose polymers (levan-inulin) and sucrose. The expression of this operon is induced by fructose and repressed by glucose.

Transcription of the levanase operon is controlled by LevR, an activator of the NifA/NtrC family which interacts with an upstream activating sequence (UAS)

centered 125 bp upstream from the transcription start site (5). The ability of truncated LevR polypeptides to activate transcription to respond to inducer (fructose) or to interact with the UAS was tested. The results obtained suggest that LevR is a multidomain protein. The amino-terminal part of the protein is required for DNA-binding whereas the central domain A, allows the activation of transcription. The carboxy-terminal region is involved in the modulation of the LevR activity by the PTS. It contains two domains B and C, homologous to members of another family of PTS-dependent regulators such as SacT/SacY from *B. subtilis* or BglG from *E. coli*. In response to the presence of fructose, LevR is probably inactivated by phosphorylation (in the domain C) *via* the fructose specific PTS encoded by the levanase operon. It was also shown that the PTS is not only involved in negative regulation of LevR but also stimulates its activity (probably *via* a second phosphorylation in the domain B).

The levanase operon is also subject to carbon catabolite repression involving the pleiotropic repressor CcpA (catabolite control protein) (6-7).

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Analysis of the mechanism of action of FhlA, the transcriptional activator of the formate regulon from E. coli

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The FHLA protein is the transcriptional activator of the genes of the formate regulon from *Escherichia coli*. FHLA belongs to a subgroup of σ^{54}-dependent regulators which are activated by binding of an effector molecule to some domain presumably located in the N-terminal half of the molecule. There is no experimental evidence that these regulators are phosphorylated by a sensory kinase.

FHLA has been purified recently and shown to be active in a coupled in vitro transcription/translation system (1). Activation requires formate and a certain threshold ratio between FHLA and the upstream activating sequence (UAS) at the target DNA. Purified FHLA also catalyses a nucleoside triphophatase reaction in which the presence of formate significantly improves the apparent affinity for the substrate. Oligodesoxynucleotides lacking or possessing the UAS lead to an equal and moderate stimulation of ATPase activity in the absence of formate. In its presence, however, only the specific DNA, i.e. that containing the UAS, stimulates ATP hydrolysis. Under this condition both the apparent K_m for ATP and K_{cat} of the reaction are improved (2).

An *in vitro* transcription system has been set up for formate- and FhlA-dependent transcription activation at the -12/-24 promoter of the *fdhF* gene from *E. coli* by σ^{54}-RNA polymerase. It requires presence of the upstream activation sequence (UAS) on supercoiled DNA. Transcription is independent from the effector formate at nucleoside triphosphate concentrations of 400 μM and above and completely dependent on the presence of the effector when the concentration is lowered to 300 μM. Inclusion of nucleoside diphosphates into the system raises the nucleoside triphosphate level at which specific induction by formate can take place. The threshold level of FhlA relative to template DNA required for transcription activation in the absence of formate was lowered at high nucleoside triphosphate concentration. On the other hand, transcription activation at the *fdhF* promoter lacking the UAS requires an increased ratio of FhlA to promoter plus the presence of formate; high ATP concentrations cannot by-pass the effect of formate. These results are interpreted in terms of a model which implies that FhlA must undergo a change in its oligomeric state for transcription activation and that this oligomerisation is favoured by high nucleoside triphosphate concentrations, by the effector formate and by the target DNA. In the absence of the target DNA, FhlA can line up at "unspecific" DNA and activate transcription; in this case, however, presence of formate and a higher FhlA concentration are required to stabilise and increase the amount of active oligomer.

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GLOBAL REGULATORS OF GENE EXPRESSION IN YEAST

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Three abundant, ubiquitous DNA-binding protein factors appear to play a major role in the control of ribosome biosynthesis in yeast. Two of these factors mediate the regulation of transcription or ribosomal protein genes (rp-genes) in yeasts: The majority of the yeast rp-genes is under the transcriptional control of Rap1p, while a small subset of rp-genes is activated through Abf1p. The third protein, designated Reb1p, which binds strongly to two sites located upstream of the enhancer and the promoter of the rRNA operon, respectively, appears to play a crucial role in keeping the chromosomal rDNA units in an optimal spatial configuration, possibly by anchoring consecutive enhancers and promoters to the nucleolar matrix (Kulkens *et al.*, EMBO J. 11 (1992), 4665). In addition, it has been suggested by others that Reb1p may participate in termination of rDNA transcription.

All three proteins, however, have many target sites on the yeast genome, in particular in the upstream regions of several Pol II-transcribed genes, suggesting that they play a much more general role than solely in the regulation of ribosome biosynthesis. The specific functional role of these proteins when bound at a given cognate site seems to be determined by neighbouring DNA sequences. Furthermore, some evidence has been obtained suggesting that these factors influence the chromatin structure and create a nucleosome-free region surrounding their binding sites.

In support of the proposed general function of Abf1p, Rap1p and Reb1p is our recent finding that the binding sites for these proteins are always occupied *in vivo*, irrespective of the growth conditions. Also the amount of binding of Rap1p and Abf1p to the promoters of rp-genes did not change significantly when growth conditions varied.

We have investigated the function of these proteins in reconstituted promoters in the chromosomal context. These studies demonstrated that the transcription activation of yeast rp-genes requires additional elements, *viz.* dA/dT-regions, apart from binding sites for Abf1p or Rap1p. In addition, only the proper combination of the various *cis*-acting elements can mediate the characteristic nutritional upshift of rp-gene transcription by adding glucose to a culture growing on a non-fermentable carbon source (Gonçalves *et al.* (1995), Nucl. Acids Res. 9, 1475).

Furthermore, Abf1p- and Rap1p-binding sites can functionally replace each other in the context of rp-gene promoters. In yeast strains, conditionally expressing either the ABF1- or the RAP1-gene, both the Abf1p-Rap1p and the Rap1p-Abf1p chimeric proteins were found to be able to complement the growth defect of the respective strains. These findings strongly suggest that the C-terminal domains of Abf1p and Rap1p have similar functions (Gonçalves *et al.* (1995), Mol. Microbiology, in press). The data taken together suggest strongly that the three factors, Abf1p, Rap1p and Reb1p are primary DNA-binding factors which serve to render adjacent (usually down-stream) *cis*-acting elements accessible to the real, specific *trans*-acting factors. They might help in the recruitment of these factors by protein-protein interactions.

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Converting E. coli RNA Polymerase into an Enhancer Responsive Enzyme: Role of an N-terminal Leucine Patch in Sigma 54

Sigma 54 is known to associate with E. coli core RNA polymerase to form a holoenzyme that can bind DNA but is inactive in the absence of enhancer activation. Mutant polymerases are identified that transcribe in an enhancer-independent manner, having bypassed the need for enhancer protein and ATP. The mutations are in a leucine patch within the N-terminal glutamine-rich domain of sigma 54. Multiple leucine substitutions mimic the effect of enhancer, suggesting that the role of enhancer protein is to disrupt this leucine patch. The data suggests that 2 activities of sigma 54 jointly confer enhancer responsiveness: an inhibitor of polymerase activity and a receptor that interacts with enhancer protein to overcome this inhibition.

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Transcriptional Activation by DctD, a Rhizobial σ54-dependent Activator.

Rhizobium are capable of utilizing dicarboxylates, which are found in soil and are supplied by the host, as carbon and energy sources. Three genes are required for the transport of dicarboxylates; *dctA*, *dctB*, and *dctD*. *DctA* encodes the structural protein responsible for transport. *DctB* and *dctD* gene products are involved in regulating *dctA* transcription and make up a two component regulatory system. DctB, probably a transmembrane protein, has homology to histidine protein kinases. DctD is a σ54 dependent transcriptional activator protein which itself is activated by phosphorylation by DctB.

DctD was predicted to possess three separate functional domains. Prior results described an N-terminal regulatory domain, homologous to two-component regulators, that inhibits transcriptional activation but not sequence-specific DNA binding, and showed that the central domain is sufficient for activation. More recent work shows that: 1) DctD and the N-terminal deletion derivative DctDΔ(1-142) bind the *dctA* UAS cooperatively; 2) additional removal of the HtH motif from DctDΔ(1-142), making DctDΔ(1-142,405-437), retains a nonspecific DNA binding activity; and 3) a subset of 18 point mutants map a surface of the N-terminal domain that is required for inhibition of the central domain. This region extends beyond the DctD two-component receiver module, as defined by homology to other regulators. Furthermore, Lee and Hoover, my collaborators, have recently shown that DctD crosslinks σ54 and the β subunit of RNA polymerase, strongly suggesting a direct contact between the activator and these components of polymerase. Experiments illustrating these points will be presented. If preliminary results are confirmed and extended as expected, it will also be possible to present fluorescence emission spectra showing that DctD and a constitutively active point mutant each interact with ATP, so binding of ATP is not likely to be the activation step regulated by phosphorylation.

A SUBUNIT OF THE HUMAN TRANSCRIPTION FACTOR TFIID IS A COACTIVATOR FOR MEMBERS OF THE NUCLEAR RECEPTOR SUPERFAMILY

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The HeLa (h) cell RNA polymerase II transcription factor TFIID is a multiprotein complex comprising the TATA-binding protein (TBP) and possibly up to 13 TBP-associated factors (hTAF$_{II}$s). hTFIID was immunopurified from HeLa cell extracts and cDNAs encoding 5 hTAF$_{II}$s were isolated.

hTAF$_{II}$28, is a core hTAF$_{II}$ present in both of the previously described hTFIID species, which either lack or contain hTAF$_{II}$30 (hTFIIDα and hTFIIDβ respectively), and is the homologue of *Drosophila* (d)TAF$_{II}$30β. hTAF$_{II}$135 is the homologue of dTAF$_{II}$110 and is also a core hTAF$_{II}$. hTAF$_{II}$18 is a novel hTAF$_{II}$ which shows homology to the N-terminal region of the yeast TAF$_{II}$ SPT3, but has no known *Drosophila* counterpart. In contrast to hTAF$_{II}$28, hTAF$_{II}$18 is a TFIIDβ-specific hTAF$_{II}$. hTAF$_{II}$55 is also a hTAF$_{II}$ with no known *Drosophila* homologue and is preferentially, but not exclusively, associated with TFIIDβ. hTAF$_{II}$20 is the homologue of p22 an alternatively spliced form of dTAF$_{II}$30α (p32). Using a combination of protein affinity chromatography, and cotransfection and immunoprecipitation assays, we have identified a series of *in vitro* and intracellular interactions amongst the novel hTAF$_{II}$s, and between the novel hTAF$_{II}$s and hTAF$_{II}$30, hTAF$_{II}$250 or TBP. The results of these experiments reveal differences, not only in subunit composition, but also in the organisation of dTFIID and hTFIID complexes. The role of these hTAF$_{II}$s in gene regulation by members of the nuclear receptor superfamilly will be discussed.

MUTATIONAL ANALYSIS OF THE N-TERMINAL END OF THE XYLR PROTEIN: IMPLICATIONS FOR THE ACTIVATION OF THE REGULATOR

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The Pseudomonas putida TOL plasmid encodes a catabolic pathway for the metabolism of toluene and related hydrocarbons. The XylR regulator activated by hydrocarbon effectors stimulates transcription from the "upper" pathway operon promoter. This promoter exhibits a modular structure with three domains. The domain at -12/-24 is recognized by the RNA polymerase-sigma 54 complex. In the -44/-66 domain IHF protein acts as a DNA-bending protein; and in the -106/-120 and -140/-160 domain, inverted repeats are recognized by the XylR regulator.

The N-terminal domain of XylR has been implicated in interactions with effectors. The bases for this role are:

i) The substitution of Lysine for glutamic acid 172 resulted in a mutant with altered effector specificity. The regulator acquired the ability to recognize 3-nitrotoluene.

In vivo kinetics studies with different nitro-substituted toluenes indicated that activation of the regulator is substrate concentration-dependent, and requires cooperative interactions involving the activated regulator.

ii) Mutations that led to the substitution of glutamine or asparragine for aspartic acid 135 resulted in mutant regulators that activate transcription in the absence of effectors, suggesting that these mutations mimicked the active form of the regulators. These mutant regulators were able to stimulate transcription from Pu with a single UAS and did not require IHF to stimulate transcription, which suggests that the regulator exhibited increased affinity for target sequences and was able to mediate DNA conformational changes.

These mutant regulators were still able to stimulate 2- to 3-fold higher induction over the already high basal levels in response to toluenes, which suggests that 1) these mutants still allowed further changes for improved transcriptional activity, and 2) that other residues are important for the active form of the regulator.

iii) In connection with this point is the finding that substitution of serine 85 with proline also resulted in a mutant that stimulates transcription from Pu in the absence of effectors, although it required both UASs and needed the IHF protein for activity.

POSTERS

THE TRANSCRIPTIONAL COACTIVATOR GCN5 IS INVOLVED IN THE CHROMATIN ORGANIZATION OF HIS3 PROMOTER.

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We have isolated a gene whose sequence analysis has revealed complete homology to GCN5.This gene has been firstly described by Georgakopoulos and Thireos (EMBO J. 1992 ,11:4145-4152) as a coactivator required to fully induce the transcriptional activation of genes under GCN4 or HAP4 control. Guarente et al. (G.A..Marcus et al., EMBO J 1994,13:4807-4815) demonstrated successively that GCN5 is part of a multiproteic complex and in particular is phisically associated with ADA2. The only structural characteristic of the CGN5 protein is the presence, at the carboxy terminal, of a so called "Bromo" domain (70 amino acids long) previously described in other transcriptional activators, like the human CCG1 protein (TAF250), the *Drosophila* "Brahma" protein and yeast SW1/SNF2 , but always in association with other functional domains (i.e. helicase) . For some of the above mentioned proteins a role in chromatin organization has been demonstrated , nevertheless no specific role has been assigned untill now to the "Bromo" domain.

We have obtained evidences that GCN5 interferes with the nucleosomal positioning over the regulatory sequences of HIS3 , a gene under its regulation, as demonstrated by Thireos et al.
HIS3 is a gene involved in amino acid biosynthesis and its trascription is strongly induced, in starvation conditions (experimentally obtained by 3AT) , by the yeast transcriptional activator GCN4 We have performed *in vivo* chromatin analysis over the promoter region of HIS3 in a *S.cerevisiae* wild type and Δgcn5 isogenic strain . Nucleosomes positioning has been defined by indirect end-labelling hybridization of chromatin samples treated *in vivo* with increasing amounts of micrococcal nuclease. The chromatin analysis performed in conditions of induced (starvation) or constitutive HIS3 transcription has evidentiated a difference of the chromatin organization in the null gcn5 upon gene activation. The pattern of chromatin organization over the promoter region of a not regulated yeast gene, like actin, has been performed in order to evidentiate the gene specific function of GCN5. We interpret the "less organized" mutant nucleosomal pattern as a reduced capability of the HIS3 promoter region to "open" the chromatin structure upon induction without GCN5 coactivator. Nucleosome phasing analysis has also been perfomed over HIS3 promoter and codogenic regions .The results obtained suggest that the phasing of nucleosomes of the Δgcn5 strain in the HIS3 promoter region is loosely fixed while the coding region is unaffected by the mutation. These data substantiate an involvement of GCN5 in the organization of active chromatin of HIS3.

In order to know if the action of GCN5 is strictily related to the presence of a GCN4 activation site in the HIS3 promoter, we disrupted GCN5 in a yeast strain (from K.Struhl), which GCN4 binding site has been sostituted by GAL4. The growth phenotypes of the mutants has been investigated .

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Redundancy of the integration host factor (IHF) and the HU protein in the co-activation by bent DNA of the σ^{54} dependent promoter *Pu* of *Pseudomonas putida*

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The σ^{54} dependent promoter *Pu* of the TOL plasmid of *Pseudomonas putida* is activated at a distance by the prokaryotic enhancer-binding protein XylR (1). The region between the upstream activation sequences (UAS) and the binding site for the σ^{54}-containing RNA polymerase is intrinsically curved and contains a site for the integration host factor (IHF), the binding of which sustains an optimal promoter geometry (2, 3). However, in the absence of IHF, *Pu* still maintains *in vivo* (but not in *vitro*) a considerable degree of XylR-dependent transcription (4). This can be shown to be kdue to the HU protein, that takes over co-regulation of the wild-type *Pu* by bent DNA when IHF is not available *in vivo*. A hybrid promoter in which the IHF site was replaced by an intrinsically curved DNA was fully active regardless of the IHF or HU. The same patterns were observed *in vitro* , where the co-activation effect of IHF could be mimicked not only by HU, but also by the mammalian non-histone chromatin protein HMG-1, and could be bypassed by bent DNA. These result suggest that the functional redundancy of IHF and HU plays a role to backup the co-regulation of *Pu* through structural changes of promoter DNA.

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The transcription initiation in the promoter *glnAp2* and *glnHp2* of *Escherichia coli.*

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The *glnALG* operon of *Escherichia coli* codes for glutamine synthetase (GS) and the nitrogen regulators II (NR_{II}) and I (NR_I). (reviewed in 1). The *glnHPQ* operon of *E.coli* encodes the components of the high-affinity glutamine transport system (1). Both operons are activated by the nitrogen regulator I (NR_I)-phosphate in response to the nitrogen limitation. NR_I-phosphate binds to sites upstream from the σ^{54}-dependent *glnAp2* (*glnALG* operon) or *glnHp2* (*glnHPQ* operon) and activates transcription by catalyzing the isomerization of the closed σ^{54}-RNA polymerase complex to an open complex. On linear DNA, the initiation of *glnHp2* transcription requires in addition to NR_I-phosphate, the presence of integration host factor (IHF), which binds to a site located between the NR_I-binding sites and the promoter (2). On supercoiled DNA, the IHF protein does not play an essential role, but enhances the activation of transcription by NR_I-phosphate. (2). IHF bends the DNA to align the activator with the closed σ^{54}-RNA polymerase promoter complex to facilitate the interactions that result in open complex formation (3). In *glnAp2*, the IHF protein does not play any role in the initiation of the transcription, either in supercoiled or linear DNA. Initial data shows that both, *glnAp2* and *glnHp2* have greater number of open complex when the DNA is supercoiled than when it is linear. Our latest results suggest that the stability of the open complex, once they are formed, is higher when the DNA is supercoiled than when it is linear. Moreover, the *glnAp2* promoter has a greater number of open complexes than *glnHp2*, in the absence of the IHF protein, when both are present in a linear form. We found that moving the NR_I binding sites of the *glnAp2* promoter 5 bp upstream dramatically decreased the formation of open complex. This suggests that both, the promoter and the sites for the activator, have to be located on the same face of the helix. Computer modeling predicts that the *glnAp2* promoter has a bend of 50 degrees in one plane, which may allow the proteins bound at the promoter and the activator binding sites interact if they are located on the same face of the helix. Furthermore, the addition of 200 bp between the NR_I binding site and the *glnHp2* promoter increases greatly the number of open complex formed, on linear DNA in absence of IHF. Our data suggests that the structure of the sequence located between the *glnHp2* promoter and the sites for the activator is in wrong orientation for the initiation of the transcription

and the presence of IHF corrects the orientation making an interaction between the σ^{54}-RNA polymerase and the activator possible. This possibility has been reported for other systems (4).

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Involvement of Sigma Factor σ^{54} in Exponential Silencing of the *Pseudomonas putida* TOL Plasmid *Pu* Promoter. Cases I., de Lorenzo V. and Pérez-Martín J. CIB-CSIC. Velazquez 144, 28006 Madrid. Spain

The σ^{54}-dependent *Pu* promoter of the TOL plasmid pWW0 of *Pseudomonas putida* becomes activated by the prokaryotic enhancer-binding XylR protein when cells encounter *m*-xylene in the medium. However, even in the presence of the aromatic inducer, *Pu* activity is silenced *in vivo* during exponential growth of the cells in rich medium. Various elements known to be involved in the control of the transcriptional activity of the promoter were examined to ascertain the mechanism by which expression of *Pu* is submitted to the growth stage of the cells. A truncated and fully constitutive XylR derivative deleted of its signal-reception N-terminal domain was found to be subjected to the same exponential silencing that the wild-type XylR when exposed to *m*-xylene. This indicated that the phenomenon is unrelated to growth on *m*-xylene as carbon source and is not due to a late activation of XylR by the aromatic effector. A *Pu* variety in which the integration host factor (IHF) binding site had been functionally replaced by an statically curved DNA segment showed an induction pattern dependent also on growth phase, thus ruling out variations in the intracellular levels of IHF changes along growth as the element responsible for the inactivity of *Pu* in growing cells. On the contrary, overproduction of the σ^{54} factor relieved the apparent growth-phase dependence of *Pu* expression. Since σ^{54} protein levels remain approximately constant along growth, the exponential silencing of *Pu* seems to be caused ultimately by changes in the activity of the factor itself and not by variations of its expression. This effect may not be exclusive to *Pu*, but could be a general co-regulation mechanism in σ^{54}-dependent promoters that connects transcription of a specific set of genes with the general physiological status of the cells.

Abstract for the poster

Expression of the *rocDEF* operon involved in arginine catabolism in *Bacillus subtilis*

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Two regulatory genes, *rocR* and *ahrC*, control the metabolism of arginine in *B. subtilis*. We have previously characterized an operon referred to as *rocABC* and which is probably involved in arginine catabolism. The *rocA* gene encodes a pyrroline-5-carboxylate dehydrogenase and the product of *rocC* shares similarity with amino acid permeases. The *rocB* gene is of unknown function. The expression of this operon is controlled by a sigma 54-like sigma factor (SigL) and by RocR which belongs to the NifA/NtrC family of transcriptional activators.

Three new genes, called *rocDEF*, were found near the *rocR* gene. The *rocD* gene encodes an ornithine aminotransferase. The product of *rocE* shares similarity with the product of *rocC* and the *rocF* gene encodes an arginase.

Arginine utilization was abolished in both *rocD* and *rocF* mutants in *B. subtilis* confirming the role of these genes in arginine catabolism. The *rocDEF* genes form an operon transcribed from a -12/-24 promoter almost identical to the -12/-24 promoter of the *rocABC* operon. The expression of the *rocDEF* operon was induced by the presence of arginine, ornithine or proline in the growth medium and depended on the presence of both SigL and RocR. Two tandemly repeated Upstream Activating Sequences (UAS) very similar to those previously identified in the *rocABC* system were found centered at positions -120 and -70, respectively, upstream from the transcription start site of *rocDEF*. Theses sequences are probably the target of RocR. Analysis of a *rocR'-'lacZ* fusion strain showed that the expression of *rocR* is not induced by arginine and is negatively autoregulated.

rocR mutants leading to constitutive expression of the *roc* regulon have been recently isolated. A molecular characterization of the corresponding mutations is in progress.

Mechanism for Facilitated Binding of Glucocorticoid Receptor to a Nucleosome

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A nucleosome contains 146-bp DNA wrapped 1.75 turns around histone octarmer, the three-dimentional folding of the DNA in a nucleosome brings the distant (80-bp) DNA elements into side by side proximity, and sets the more nearby (40-bp) DNA elements apart. In glucocorticoid inducible genes, there are often multiple glucocorticoid receptor response elements (GRE) organized in one positioned nucleosome. We studied the impact of this phenomenen by comparing the binding of glucocortiocid receptor (GR) to reconstituted nucleosomes presenting two GREs with different spatial distribution. DNase I footprinting analysis and mobility retardation assay revealed that the affinity of GR to the nucleosoms depended on the proximity of the GREs in the nucleosomes, or was proportional to the linear distance of the two GREs in the DNA. This was in sharp contrast to the coorperative GR binding observed in free DNA, which was inversely correlated to the distance of the two GREs. However, the contacts of GR to the two GREs organized in one nucleosome were the same as to the single GRE containing nucleosome, or the same as to the free GRE, shown by DMS methylation protection. Our data may support the hypothesis that the function of architectural arrangement of the nucleosome is to construct a infrastructure for the regulartory DNA to regulate the access of the transcription factors during gene expression.

SIGMA-S AND SIGMA-70-DEPENDENT POLYMERASES ARE REQUIRED TO MAINTAIN FULL EXPRESSION FROM THE Pm PROMOTER OF *Pseudomonas putida* ALONG THE GROWTH CURVE.

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Transcription from the TOL plasmid Pm promoter is dependent on the XylS regulator activated by benzoate effectors. In a wild-type *E. coli* background, expression from the Pm promoter determined as ß-gal activity increased with time, reaching maximal values 3 to 4 h later, and was maintained at a relatively high level afterwards. In the ð-S deficient background, high levels of transcription from Pm were observed in the early logarithmic growth phase, but not in the late logarithmic or early stationary phases. When a cloned *rpoS* gene was introduced in the mutant strain, XylS/3mBz-dependent transcription from Pm during the late stages of the growth curve was restored. The transcription initiation point of Pm was the same regardless of the growth phase and the sigma-S background. In addition, mRNA analysis confirmed the induction results determined with ß-gal. The requirement of sigma-S for stimulation of transcription from Pm in the late phases was overcome by using a combination of certain mutant Pm promoters and the constitutive mutant regulator XylSG44S. We suggest that the transcription from Pm involves the use of two sigma factors: sigma-70 during the early logarithmic phase and sigma-S thereafter.

The replication termination proteins of *Escherichia coli* and *Bacillus subtilis* are polar anti-transcriptases.

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Termination of DNA replication in prokaryotic chromosomes occurs at specific replication termini that are recognized by replication termination proteins. The terminator proteins of *Escherichia coli* and *Bacillus subtilis*, called ter (tus) and RTP respectively, impose polar blocks on replication fork movement at the replication termini. The blockage is mostly or perhaps excusively due to the polar contrahelicase activity of the ter protein (or RTP) that impedes the replicative helicase catalysed DNA unwinding.

We have investigated other biochemical activities of ter protein and of RTP and have discovered that both are polar anti-transcriptases. The anti-transcriptase and contrahelicase activities are isopolar. We have also discovered that passage of an RNA transcript through a replication terminus functionally inactivates the latter *in vivo*. *In vitro*, RNA transcription abrogates the contrahelicase activity of the terminus-terminator protein complex. Thus, we believe that the *raison de'tre* for the anti-transcriptase activity is to protect replication termini from transcriptional inactivation.

Transcription repression mechanism of Phage Φ29 transcription regulator at the early A2c promoter.

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Regulatory protein p4, modulates transcription of phage Φ29 by repressing the two main early promoters A2b and A2c, while simultaneously activating the late A3 promoter (Barthelemy, 1989; Rojo, 1991; Monsalve, 1995). Repression of the A2b promoter is exerted by hindering the binding of the RNA polymerse to the promoter (Rojo, 1991), while activation of the A3 promoter implies the p4-mediated stabilization of the RNA plymerase to the promoter in a process that involves a direct interaction between the two proteins (Nuez, 1992; Mencia, 1993). We have recently studied the mechanism by which protein p4 represses the A2c promoter.

Using various *in vitro* techniques, we have observed that protein p4 and the RNA polymerase, bind simultaneously and cooperatively to the A2c promoter. Therefore, protein p4 should repress transcription at the A2c promoter through the blockage of a step following the formation of the closed complex. By KMnO4 footprinting , we observed that p4 does not prevent open complex formation. Nor does p4 interfere with the incorporation of the first initiating nucleotides, as determined by gel retardation assays. In these conditions the repressed complex showed a different DNaseI footprinting, suggesting a modification of the complex. Short abortive thanscripts were also produced in the absence and presence of protein p4. In the presence of protein p4 and the four NTPs, the RNA polymerase could not leave the promoter, that implies that p4 repression acts at the promoter clearance step.

Protein p4 is known to activate transcription at the late A3 promoter by interacting with the RNA polymerase through a short carboxy terminal domain (Nuez, 1992; Mencía, 1993). In fact, a point mutation in Arg120 renders a protein p4 derivative that binds to DNA normally, but is unable to activate transcription and to interact with RNA polymerase. We have shown that this p4 mutant is also unable to repress the A2c promoter.

Therefore, it is likely that protein p4 represses the A2c promoter by interacting with the RNA polymerase in a way that holds it at the promoter. Under these conditions, the RNA polymerase can make short transcripts but cannot leave the promoter.The interaction is likely to be mediated by protein p4 residue Arg 120.

THE BACTERIOPHAGE λ P_L PROMOTER AND ITS RESPONSE TO ENVIRONMENTAL STRESS

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The P_L promoter is expressed early in bacteriophage λ infection and is regulated by the CI and Cro repressors. The P_L promoter is composed of two tandem promoters, the major P_L promoter and a minor upstream P_L2 promoter. Both promoters are affected by integration host factor (IHF). IHF represses P_L2 and stimulates the major P_L promoter. Furthermore, the P_L promoter responds to DNA supercoiling. We have shown that the C-terminal portion of the α subunit of RNA polymerase plays an essential role in the expression of both promoters.

Experiments with *lacZ* reporter gene fusions demonstrated that the activity of the phage λ P_L promoter is inversely dependent on temperature. It was demonstrated that increase in DNA supercoiling plays an important role in the stimulation of the P_L promoter at low temperature.

RNA polymerase recognizes three promoter regions, the -10, -35 and an AT-rich region centered at -50, the UP element, which can interact with the C-terminus of the α subunit of RNA polymerase.

An UP element was identified in the λ P_L promoter centered at position -90 from the transcription start site. We found that the α subunit of RNA polymerase interacts with the UP element in a region nested within the region protected by IHF in DNase I footprinting.

From these results we suggest that the UP element, located at a distance from the core promoter, is presented by IHF for interaction with the C-terminus of the α subunit of RNA polymerase. According to this model IHF acts at P_L by bending the DNA in a specific way. This mode of action of IHF is similar to its function in the stimulation of λ site specific recombination and in the activation of promoters transcribed by σ54 RNA polymerase. Alternatively, it is possible that, in the stimulation of P_L by IHF, IHF and the α subunit of RNA polymerase interact at the DNA surface. In the absence of IHF, the P_L2 promoter is derepressed, the UP element is utilized to enhance P_L2 promoter activity.

Fine tuning of the level of the protein that initiates and terminates plasmid pUB110 leading strand replication by repression of its promoter from a distant binding site

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Modulation of the activity of a promoter by a regulatory protein bound to a distant site is most frequently achieved by an interaction of the regulator either with other regulatory proteins or with the RNA polymerase; in this latter case the intervening DNA is normally bent to bring both proteins into proximity. We have found that protein RepU, that functions as the initiator and terminator of the leading strand replication in plasmid pUB110, regulates the synthesis of its own gene by binding to a target (the replication origin, *dso*) distant from the RNA polymerase binding site and interfering with promoter utilization by a mechanism that differs from those mentioned above.

Plasmid pUB110, that replicates via a rolling circle mechanism, regulates its copy number by limiting the availability of the RepU protein. The amount of the initiator protein is kept at a low limiting level, since an increase of its concentration leads to an increase in the replication initiation events. The control is exerted at several levels. The expression of the *rep*U gene is regulated by an antisense RNA (IncA) that interferes with *rep*U mRNA translation. In addition, the amounts of "active" RepU protein are also controlled. RepU is a monomer in solution, but binds to DNA as a dimer. An "active" RepU dimer is used for initiation only once per replication event; re-utilization is prevented by a covalent modification of one of the subunits when replication of the leading strand is completed, giving rise to the so called RepU-RepU* heterodimer. RepU* is inactive as initiator. We have observed that RepU levels are limited by a third mechanism, in which the RepU-RepU* protein mixture acts as a repressor of the promoter for the *rep*U gene in an unusual way. Both the unmodified RepU and the RepU-RepU* protein mixture bind to the *dso* region with high affinity, though with different outcomes. At half-saturating protein concentrations, about 2 RepU or 6 RepU-RepU* protomers bind to the *dso* region. At higher protein concentrations, the stoichiometry of the "active" RepU with the *dso* remains unaltered, whereas the RepU-RepU* protein mixture forms an extended complex whose length depends on the RepU-RepU* concentration. We have localized the promoter for the *rep*U gene, which lies downstream of the *dso* region, and shown that it is efficiently repressed by the RepU-RepU* complex. Since the RepU-RepU* protein mixture cannot bind to the promoter, we propose that it

interferes with promoter utilization by initially binding to the *dso*, located about 140 bp upstream from the promoter start site, and reaching the RNA polymerase binding site by polymerizing over the DNA in a concentration dependent way. Therefore, the way of binding of the RepU-RepU* protein mixture to the *dso* DNA offers an elegant and sensitive repression mechanism to finely control the amount of RepU protein in the cell. Since the cooperative polymerization event is very sensitive to the concentration of RepU, small increases in the intracellular RepU concentrations could quickly cover the promoter region and inhibit further RepU synthesis.

EXPRESSION ANALYSIS OF HYDROGENASE STRUCTURAL GENES (*hupSL*) FROM *Rhizobium leguminosarum* bv viciae

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The genetic determinants for the H_2-uptake (*hup*) system of *Rhizobium leguminosarum* bv. viciae UPM791 have been characterized in our laboratory [1-4]. At least 18 genes are necessary for synthesis, assembly, and regulation of a functional uptake hydrogenase, responsible for recycling the H_2 generated in the nitrogenase reaction. The first 12 genes (*hupSLCDEFGHIJK hypA*) are only expressed in symbiosis with the legume host. Hydrogenase structural genes (*hupSL*) are transcribed from a -24/-12 promoter (P1). Within the P1 regulatory region, an IHF-binding consensus sequence is present, and binding of *Escherichia coli* IHF to it was demonstrated. We have followed three approaches to study the regulation of P1 expression: 1.- Study of the spatial/ temporal pattern of expression of P1 in pea nodules by RNA *in situ* hybridization. We were able to show that *hupSL* are exactly co-expressed with *nif* genes, suggesting that they respond to similar environmental stimuli and activating proteins. 2.- Study of the effect of NifA on P1 activation. By using *hupL-lacZ* fusions in the heterologous *Klebsiella pneumoniae* host, NifA-dependent expression of P1 promoter was demonstrated. The activation of *hupL-lacZ* fusion by *K. pneumoniae* NifA was also observed in the genetic background of *E. coli*. The binding of NifA to P1 DNA was required for this activation. 3.- Search for NifA-binding site in the P1 regulatory region. Deletion analysis identified an 85 bp region (positions -88 to -172) that was essential for activation. Within this region a palindromic sequence (ψUAS), TGA N_{10} TCA, reminiscent of known NifA-upstream activating sequence (UAS), TGT N_{10} ACA, was identified. However, a series of *hupL-lacZ* fusions mutated in the ψUAS (G to A, C to T, the first A to T/ the last T to A) showed the same overall pattern of responsiveness to NifA as the wild type P1 promoter. These results strongly suggest that, in *R. leguminosarum*, the expression of *hupSL* genes is under control of NifA, probably through binding to unidentified non-consensus UAS located at the -172/-88 promoter region. Supported by CICYT (BIO93-0046) and DGICYT (PB91-0120). 1.- Hidalgo et. al. (1990). Plant Mol. Biol. **15:** 367-370; 2.- Hidalgo et al. (1992). J. Bacteriol. **171:** 4130-4139; 3.- Rey et al. (1992). J. Mol. Biol. **228:** 998-1002; 4.- Rey et al. (1993). Mol. Microbiol. **8:** 471-481.

MECHANISM OF ACTIVATION OF THE PROKARYOTIC ENHANCER BINDING PROTEIN NTRC

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The response regulator NtrC is the transcriptional regulator for nitrogen regulated promoters and is activated by phosphorylation of its N-terminal receiver module. Active NtrC-P has an ATPase activity which is required for transcriptional activation. NtrC consists of three domains, the N-terminal receiver module, the central output domain carrying the ATP-binding site and the C-terminal dimerization and DNA-binding domain. Interactions between these domains play a critical role in the regulation of activity of NtrC and NtrC-P.

To study these domain interactions we analyzed fusion proteins between various domains of NtrC and the N-terminal DNA-binding domain of λ-repressor. Based on the analysis of these fusion proteins we have previously shown (EMBO J. 14, 3696-3705), that the receiver domain is a potential dimerization domain. In unphosphorylated NtrCreceiver and output domain interact leading to inhibition of the receiver domain. Phosphorylation relieves inhibition and induces oligomerization of NtrC-P via the receiver domain. This oligomerization is essential for activation, dimeric NtrC-P is inactive.

The C-terminal domain is homologous to FIS and involved in DNA-binding, bending and constitutive dimerization. We show, that this domain is also responsible for co-operative binding of unphosphorylated NtrC to DNA. We have isolated NtrC-mutants (S422P and E423G), where this co-operative interaction is abolished. Currently we are analyzing the effect of these mutations on the activation of NtrC in vitro.

List of Invited Speakers

Workshop on

TRANSCRIPTIONAL REGULATION AT A DISTANCE

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