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Regulation of Transcription of *SerA* in *Escherichia coli* K-12

Li Yang

A Thesis

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Abstract

Regulation of Transcription of *SerA* in *Escherichia coli* K-12

Li Yang

The *serA* gene in *Escherichia coli* encodes 3-phosphoglycerate dehydrogenase, which is the first enzyme in the serine biosynthesis pathway. Previous studies have determined two different transcription initiation sites for the *serA* gene, corresponding to the promoters of *serA* P1 and P2, and suggested that P1 and P2 were regulated by leucine responsive regulatory protein (Lrp) by different mechanisms. However, very little is known about how the two promoters function in different cell growth conditions, and whether other regulator(s) are involved in expression and regulation of *serA*. In this study, I confirm that Lrp activates P1 but represses P2 in glucose minimal medium by studying native and mutant promoters as *serA::lacZ* operon fusions. Transcription can take place from both P1 and P2 promoters when Lrp is absent. Furthermore, my study demonstrates that the cyclic AMP (cAMP) receptor protein-cAMP (cAMP-CRP) complex may positively regulate *serA* by activating P2 and is mainly functional in the absence of Lrp.

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Introduction

Living cells are self-regulating chemical engines, tuned to operate on the principle of maximum economy. Regulation is essential for the cell to conserve energy and material and to maintain metabolic balance. The task of the regulatory machinery is exceptionally complex and difficult. Pathways must be regulated and coordinated so effectively that all cell components are present in precisely the correct amounts. Furthermore, a microbial cell such as *Escherichia coli* must be able to respond effectively to environmental changes by using those nutrients present at the moment and by switching on new catabolic pathways when different nutrients become available. The flow of carbon through a pathway may be regulated in three major ways. 1. The localization of metabolites and enzymes in different parts of a cell, a phenomenon called metabolic channeling, influences pathway activity. 2. Critical enzymes often are directly stimulated or inhibited to alter pathway activity rapidly. 3. The number of enzyme molecules also may be controlled. In bacteria regulation is usually exerted at the level of transcription. Control of mRNA synthesis is slower than direct regulation of enzyme activity but does result in the saving of much energy and raw material because enzymes are not synthesized when not required. Therefore, regulation at the transcriptional level is the most studied, and perhaps the most direct way to selectively control gene expression.

The leucine responsive regulatory protein (Lrp), recognized as a global transcriptional regulator in *E. coli*, governs expression of a group of genes, known as the leucine/Lrp regulon, by interacting with leucine. *SerA* encoding 3-phosphoglycerate dehydrogenase, the first enzyme in the serine biosynthesis pathway, is one of the leucine/Lrp regulons and is positively regulated by Lrp. Two different transcription initiation sites for the *serA* gene determined by primer extension correspond to the two promoters of *serA*, P1 and P2. They are regulated by Lrp via different mechanisms. Gel retardation assay shows that Lrp binds at least two sites in the *serA* upstream region. DNase footprinting shows that Lrp protects −151 to −81 nucleotides upstream of *serA* (Lin, 1992c). L-leucine, known as a coeffector of the Lrp/leucine regulon, represses the overall expression of *serA*. Lrp has been suggested to activate P1 and repress P2, which is 92 bp upstream of P1. However, no direct evidence has shown that Lrp activates P1 or represses P2, and very little is known about how the two *serA* promoters function in different growth conditions. Do they function separately or correlatively? Does Lrp act directly on the *serA* promoter(s) or indirectly through cooperating with other factors in addition to L-leucine or by affecting the expression of other regulatory genes which in turn affect the *serA* gene? Is any other regulator(s) involved in the regulation of transcription of *serA*?

In this thesis, I present a further study of the regulation of *serA*: determination of the relative importance of these two sites for the transcriptional activation of *serA*, further definition of the nature of the *lrp-serA* interaction *in vivo* and *in vitro*, and demonstration that another global regulator, the cyclic AMP (cAMP) receptor protein-cAMP (cAMP-CRP) complex is also involved in the regulation of *serA* at transcriptional level. The work began with the site-directed mutagenesis of the *serA* promoters. First, the two promoters

were mutated respectively by altering their –10 conserved region without grossly changing DNA structure. I could therefore study the two promoters independently. Second, the mutant and wild type promoters were subcloned into pRS415, a *lac* operon fusion vector. All the subsequent studies were based on these three types of *serA* promoters fused to the *lacZYA* reporter gene. The expression of *serA::lacZ* was studied in both wild type and *lrp* deficient strains *in vivo* and *in vitro*. The effects of these mutations on transcription were determined by RNA primer extension and by the activity of the β-galactosidase. Furthermore, the Lrp protein was partially purified, and gel retardation was performed to investigate the Lrp protein and *serA* promoter interactions *in vitro*. In this study, work has mainly addressed two types of question: physiological (what are the physiological consequences of Lrp action and are other regulators involved in the regulation?) and biochemical (how does Lrp interact with DNA of *serA*?).

In Part I, I will survey global regulatory systems in general and discuss the possible mechanisms of these regulatory systems while discussing in Part II, the details of molecular mechanisms controlling the leucine/Lrp regulon. Since my work focuses on the *serA* gene, Part III of this introduction will describe the genetic and environmental factors that affect the synthesis of serine in *E. coli* and review the previous experimental evidence of regulation of expression of *serA*.

Part 1. A survey of global regulatory systems

The expression of most bacterial genes is regulated at the initiation of transcription. This regulation results from transcription factors binding at or near

promoters, activating or repressing transcription initiation in response to extracellular signals.

1-1. Positive activation of gene expression in *E. coli*

The holoenzyme RNA polymerase (RNAP) in *Escherichia coli* is defined as the 1:1 complex of the core polymerase (subunit composition, 2α, 1β, $1\beta'$) with the appropriate σ (specificity) subunit. RNA synthesis is catalyzed by core enzyme lacking σ subunit, but a σ subunit is essential at least for promoter recognition and thus absolutely required for specific initiation of transcription. At present six or seven different molecular species of σ subunit (σ^{24}, σ^{28}, σ^{32}, σ^{38}, σ^{54}, σ^{70}) are known to exist in *E. coli*. σ^{70}, with a molecular mass of 70 kilodaltons, is necessary for transcription of the majority of genes expressed in exponentially growing *E. coli* cells.

E. coli promoters contain different elements that are recognized by RNAP. The -10 and -35 hexamer elements, which are TATAAT and TTGACA centering approximately 10 and 35 bp upstream from the transcription start point respectively, are contacted by specific surfaces in region 2 and region 4 of the RNAP σ^{70}, respectively. Upstream (UP) elements, which are located just upstream of the -35 region at many promoters, are contacted by the RNA polymerase α subunit carboxy-terminal domain (αCTD). Additionally, at some promoters, an upstream extension of the -10 is contacted by an extension of region 2 of σ^{70}. There are two kinds of promoters: activator-independent and activator-dependent. For the former, recognition of the promoter

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elements by RNAP is sufficient to permit promoter activity in the absence of activator, whereas for the latter, an activator is needed.

Two models about how activators function have been proposed: one is that the activators function by directly contacting with RNAP, and the second supposes that there is no direct contact between the activator and RNAP, but that the activator alters the conformation of promoter DNA to facilitate transcription initiation by RNAP. Most bacterial transcription activators function by making direct contact with RNAP at target promoters. Some activators contact the αCTD of the RNAP α subunit, some contact region 4 of the σ^{70} subunit, while others interact with other surfaces of RNAP which are outside of the αCTD and region 4 of σ^{70} (Rhodius, 1998). A number of activators are ambidextrous and can, apparently simultaneously, contact more than one target site on RNAP. Expression from many promoters is co-dependent on two or more activators. There are several different mechanisms for coupling promoter activity to more than one activator: in one such mechanism, the different activators make independent contacts with different target sites on RNAP.

1-2. The negative control of gene expression in *E. coli*.

Genes are now known to be turned off or down in bacteria and bacteriophages at a variety of steps for regulatory purposes. In this section, I mainly discuss the negative control of gene expression as the inhibition of transcription initiation by binding of a regulatory protein, called repressor. The potential mechanisms of repression are classified as follows: 1. Steric hindrance. This is the simplest way to inhibit transcription initiation

by blocking RNA polymerase interaction with a promoter by repressor binding to an overlapping operator. 2. Protein-protein interaction. By direct contact with RNAP, the repressor may allosterically inhibit any one of the conformational changes of RNAP-promoter closed complex associated with the subsequent steps of transcription initiation. 3. Effect of DNA. Since the structure of the promoter itself plays an active role in transcription initiation (i.e. activator–independent promoter), a regulatory protein may also act by influencing the DNA and hinder RNAP function. The promoter structure can be altered not only by repressor binding immediately adjacent to the promoter but also by repressor binding to a remote site. 4. Repression by antiactivation. Besides interfering with RNA polymerase-promoter interaction or the activity of such a complex, negative control can also be achieved by a repressor interfering with the DNA binding or activity of an activator protein. Although such molecular mechanisms have been found frequently in eukaryotic transcriptional repression, examples of an antiactivtor role of repressor are also known to occur in bacteria.

In general, with the advent of negative and positive controls, it is intuitive that the regulatory proteins are dedicated to their corresponding roles: repression and activation. Many regulatory proteins are bifunctional in that they can both activate and repress in different circumstances, for example, cAMP-CRP and Lrp (see Part 2).

1-3. Global regulation

A factor that mediates transcriptional regulation in a global response is called a regulatory transcriptional factor. The term "regulon" is coined to describe a group of genes from one or several metabolic pathways under the control of a common regulatory factor (global regulator) (Gottesman, 1984). More than 20 global regulatory systems have been recognized in enteric bacteria. Different global response systems can function in different ways. As mentioned above, most of regulators interfere with $E\sigma^{70}$. Among these regulators, some of them are repressors such as LexA that is the repressor of the SOS regulon; other are activators like PhoB, the phosphate regulatory activator. Many are dual regulators, e.g. CRP, HU, H-NS, IHF, Lrp, OxyR, and TyrR. In the following section, I focus on the CRP system.

CRP (cyclic AMP receptor protein), also known as catabolite gene activator protein (CAP), is a cAMP-binding protein and is involved in the regulation of expression of a vast number of *E. coli* genes. Each CRP subunit consists of two domains: the larger N-terminal domain containing the cAMP-binding site and the smaller C-terminal domain carrying a helix-turn-helix motif. The simple outline of the steps involved in gene activation by CRP is as follows. First, cAMP binds to CRP causing a conformational change. Second, the cAMP-CRP complex binds to specific sites located at target promoters. Third, bound cAMP-CRP activates transcription. Four factors appear to determine the efficacy of liganded CRP to activate transcription: (i) the degree of approximation to a CRP-binding sequence; (ii) the spacing of the two halves of the palindrome (6 bp versus 8 bp); (iii) the positioning of that sequence within the promoter

relative to the −10 and −35 RNA polymerase-binding regions, and (iv) the occurrence of cooperative or antagonistic protein-protein interactions. A 22-bp palindromic consensus sequence, AAATGTGATCTAGATCACATTT (the most conserved bases are underlined), is recognized as a high-affinity site for liganded CRP. However, there are more complex patterns of gene regulation that are controlled by CRP, which can either be an activator or a repressor.

1-3-a. Activation by CRP at "simple" promoters

CRP alone is sufficient to activate transcription initiation by RNA polymerase. The paradigm for this is the *lac* operon. The cAMP-induced binding of one CRP dimer centers between base pairs −61 and −62 (-61.5) and promotes the initial binding of RNA polymerase to the promoter. CRP contacts a site located in the C-terminal part of the α subunit of RNAP. The simplest model suggests that, at the *lac* promoter, CRP recruits the α subunit of RNAP to bind just downstream, and this contact guides the RNAP into place such that correct contacts with both the −10 and −35 regions of the promoter can then be made.

1-3-b. Activation by CRP at complex promoters

Many CRP-dependent promoters are also regulated by a second transcription activator. Usually the operon-specific activator binds close to the RNAP binding site with CRP binding further upstream. There is great variety in the organization of such

promoters and diversity in the position of the CRP-binding site. The best studied case is the *malK* promoter which requires maltose-induced MalT binding to both proximal and distal sites and CRP binding to a number of sites in between. In the absence of CRP, maltotriose-MalT binds to three upstream promoter sites of *malK* but the position of the sites is such that transcription activation can not take place. CRP binding triggers a re-positioning of MalT, which, in turn, triggers transcription initiation and ensures that the *malK* expression is co-regulated by CRP and MalT. In this situation, CRP makes no direct contact with RNAP.

Two simple principles explain the phenomenon of coregulation. In some cases activator 1 binds in a nonproductive mode (e.g, the *malK* and *araBAD* promoter). In other cases, there is an apparent repositioning of (or cooperative binding between) activators, but RNAP needs to make at least two contacts with activators (e.g., the *araFG*).

1-3-c. CRP as a repressor and a co-repressor

CRP is responsible for turning off as many genes as it activates. In the most cases liganded CRP directly blocks the access of RNAP to promoter elements (e.g. *cyaP2*). In other cases bound CRP promotes occupation of a secondary promoter and it is this occupation that represses the target promoter (e.g. *crp*). In the most complicated cases, CRP acts as a corepressor. A further scenario is found at the *spf* promoter: in this case, the CRP binding site overlaps the site for a gene-specific activator, and thus CRP binding prevents the activation process by interfering with the activator binding (Polayes, 1988).

Generally, when cytoplasmic cAMP concentrations are high, this cyclic nucleotide associates with CRP to form a liganded complex with a conformation different from that observed for the unliganded form. It can then bind to specific DNA sequences that normally occur near or within the promoters of operons included within the cAMP-CRP modulon. Binding of the liganded CRP complex to the DNA is accompanied by bending of the DNA strand as well as by the binding of other transcriptional catalytic and /or regulatory proteins such as RNA polymerase. Consequently, activation of transcriptional initiation at the target promoter occurs.

Part 2. The leucine/Lrp regulon

The leucine/Lrp regulon is a described global response governed by a transcriptional regulator called the leucine-responsive regulatory protein, or Lrp. Lrp is composed of 163 amino acid residues and has a monomeric molecular mass of 18,800 Daltons. It exists as a dimer in solution (Platko *et al.*, 1993). Lrp, specified by the *lrp* gene at 20 min of *E. coli* chromosome, affects the transcription of a large number of genes, increasing the expression of some and decreasing that of others. Table 1 lists all the genes/operons so far identified to be regulated by Lrp (Newman *et al.*, 1996).

Lrp activates the expression of genes whose products are involved in the biosynthesis of amino acids (*ginA, gltD, gltB, ilvIH, leu,* and *serA*), in ammonia assimilation when cells are grown in nitrogen-limiting conditions (*gltD,* and *glnA*), in C1 metabolism (*gcv*), and in the adaptation of the cells to grow in conditions of low osmolarity and low temperature (*ompF*). On the other hand, Lrp represses expression of

Table 1. *E. coli* operons regulated by Lrp

Operon	Change due to Lrp	Change due to leucine
Activated		
IlvIH	30 ↑	20 ↓
SerA	6 ↑	2 ↓
LeuABCD	11 ↑	N.D.[a]
GltBDF	44 ↑	50 ↓
GcvTHP	20 ↑	None
PntAB	5 ↑	44 ↓
MalT	1.8 ↑	None
LacZYA	1.5 ↑	None
PapBA	35 or 430 ↑	None
FanABC	76 ↑	10 ↓
SfaA	9 ↑	3 ↑
DaaABCDE	9 or 60 ↑	None
Repressed		
SdaA	8 ↓	5 ↑
GlyA	4[b] ↓	N.D.[a]
kbl-tdh	20 ↓	8 ↑
op-	2.5 ↓	N.D.[a]
LysU	22 ↓	4 ↑
LivJ	85 ↓	105 ↓
LivKHMG	9 ↓	N.D.[a]
Lrp	2 ↓	None
Fae	3 ↓	None
OsmY	5 ↓	N.D.[a]

a. ND, not determined.

b. Unpublished data.

(Adapted from Newman; Lin, 1996)

genes whose products are involved in transport of small moleuclues into the cell (*livJ*, *livKHMGF*, and *oppABCDF*), in the degradation of amino acids (*sdaA*, *tdh*, and *kbl*), and in the adaptation of cells to grow in high osmolarity and high temperature conditions (*ompC* and *lysU*). Additionally, Lrp also is involved in the formation of Pili (*fim* and *pap*).

The Lrp molecule has been suggested to consist of three domains: a DNA-binding domain in the N-terminal 40% of the protein, a transcription activation domain in the center constituting 40 to 80% of the protein, and overlapping this domain, a leucine response domain in the C-terminus. A sequence centered at position 40 may represent a helix-turn-helix motif (Willins, *et al.*, 1991). Purified Lrp binds very well to double-stranded DNA containing an appropriate promoter sequence. A possible concensus sequence for Lrp binding has been proposed: YAGHAWARRWTDCTR (Y=C/T, H=not G, W=A/T, D=not C, R=A/G) (Rex *et al.*, 1991; Wang *et al.*, 1993; Cui *et al.*, 1996). This sequence is found upstream of some *lrp*-regulated genes (e.g, *ilvIH*, *lysU*, and *tdh*). However, it is not found upstream of the *serA*, *sdaA*, and *livJ/k* operons. Indeed, this sequence is rather common and can be found in any region of a gene, upstream, downstream, or with in the coding region. It is still on debate whether Lrp binds to a specific consensus sequence of DNA or just AT rich regions (Newman *et al.*, 1996). *lrp*-binding bends DNA. This binding, in some cases, has been shown to help RNA polymerase bind to the DNA, and thus activate gene transcription (Wang *et al.*, 1993). However, in other cases, Lrp's binding to the promoters of some genes, such as *lysU and lrp*, prevents subsequent RNA polymerase binding, and thus represses gene expression (Lin *et al.*, 1992a, Wang *et al.*, 1994). Lrp has been suggested as a chromosome organizer

because there are the large number of Lrp molecules per cell (approximately 3,000) and Lrp is a small basic DNA –bending protein binding to DNA with no apparent site specificity (Newman *et al.*, 1996).

L-leucine is a coeffector of the regulon. It affects the expression of many, but not all, of the Lrp regulon genes (Newman *et al.*, 1995,1996). Among the operons activated or repressed by Lrp, in some cases, L-leucine abolishes the effect, in some cases L-leucine is required for the effect, and in yet other cases L-leucine has no effect. Hence, there are totally six patterns of Lrp/leucine regulon. Lrp is an L-leucine-binding protein. Leucine's binding to Lrp does not prevent the binding of Lrp to its target gene (Ernsting *et al.*, 1993), but may alter the conformation of Lrp, reducing the efficiency of Lrp action and thus leading to weaker activation or repression (Newman *et al.*, 1995).

It is instructive to compare Lrp with CRP, another, more thoroughly studied, *E. coli* global regulatory protein. Like Lrp, CRP activates transcription of some operons and represses transcription of others. However, in the case of CRP, both activation and repression require that CRP interact with cyclic AMP (cAMP). Lrp also interacts with a ligand (leucine), but the ligand has effects that can not be explained solely by a single mode of action, such as reducing the DNA-binding ability of Lrp. In addition, both of CRP and Lrp can have dual control on a specific operon, for example, *gal* and *ilvH*.

The *gal* operon is transcribed from two promoters P1 and P2 separated by 5 bp. cAMP-CRP activates P1 but represses P2. The site to which cAMP-CRP binds to exert such dual action is located at position –41.5. In the absence of CRP, RNAP binds mostly to P2 (Adhya and Miller, 1979). CRP then switches RNA polymerase from P2 to P1 and thus activates P1.

The *ilvIH* operon, which specifies acetohydroxy acid synthase III (AHAS III), also has two promoters P1 and P2. P2 is 60 bp upstream of P1. *In vitro*, P1 is activated but P2 is repressed by Lrp. *In vivo*, the *lrp* parental strain grown in glucose minimal medium transcribes *ilvIH* from P1 only. No *in vivo* transcription from P2 has been reported. Adding leucine decreases transcription. Gel retardation and DNase I footprinting experiments show Lrp binds cooperatively to several sites of *ilvIH* upstream region and thereby activates transcription (Wang and Calvo, 1993).

In many cases, one promoter is regulated by several regulators. It has been reported that some operons are coregulated by Lrp and cAMP-CRP. These operons include *serC-aroA* multifuntional operon (*serC* encodes phosphoserine aminotransferase and *aroA* encodes enolpyruvolylshikimate 3-phosphate synthase [Man *et al.*, 1997]), *dad* (degradative D-amino acid dehydrogenase [Mathew *et al.*, 1996]), *daa* (F1845 fimbrial adhesin [Bilge *et al.*, 1993]), *osmY* (osmotically induced periplasmic protein [Lange *et al.*, 1993]), and *pap* (Pap fimbrae [Van der Woude *et al.*, 1995 and Feutrier *et al.*, 1992]). However, they have different cooperation patterns. *SerC-aroA* expression is regulated by Lrp activation and cAMP-CRP repression. Lrp acts both as a repressor and as an activator of *dad*, which is directly activated by cAMP-CRP. *Daa* expression is activated by both Lrp and cAMP-CRP, and *osmY* expression is repressed by both Lrp and cAMP-CRP. Lrp can be a repressor or activator of *pap* expression and cAMP-CRP may activate *pap* directly and/or indirectly.

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Part 3. The *serA* gene in *E. coli*.

1-1. The *serA* gene and L-serine biosynthesis in *E. coli*

The *serA* gene in *E. coli* encodes 3-phosphoglycerate dehydrogenase, which is the first enzyme in the L-serine biosynthesis pathway. The L-serine biosynthesis pathway of *E. coli* is shown in Figure 1.

The combination of serine, glycine, and C1 biosynthesis constitutes a major metabolic pathway that plays a central role in cell physiology in *E. coli*. During growth on glucose, 15% of the carbon assimilated in *E. coli* involves serine or its metabolites (Pizer *et al.*, 1964). In addition, serine is a three-carbon precursor in the synthesis of cysteine, methionine and tryptophan, and a two-carbon unit for the formation of glycine and purine (Newman and Magasanik, 1963; Kredich and Tomkins, 1966; Trane *et al.*, 1983; Yanofsky, 1960). Serine and 3-phosphoserine are involved in the biosynthesis of other biomolecules such a cysteine, tryptophan, and pyridoxine (Stauffer, 1996).

As shown in figure 1, the genes *serA*, *serB*, and *serC* encode the three enzymes required for serine biosynthesis. The glycolytic intermediate 3-phosphoglycerate is converted to serine in three steps. 3-Phosphoglycerate dehydrogenase (the *serA* gene product) oxidizes 3-phosphoglycerate to 3-phosphohydroxypyruvate, the first committed step in the pathway. 3-phosphoserine aminotransferase (the *serC* gene product) converts 3-phosphohydroxypyruvate to 3-phosphoserine. 3-phosphoserine is dephosphorylated to L-serine by 3-phosphoserine phosphatase (the *serB* gene product). Unlike most genes

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involved in amino acid biosynthesis, these three genes are not organized as an operon. On the genetic map, *serA* is located at 65.8 min, *serB* at 99.6 min, and *serC* at 20.6 min.

3-2. Regulation of *serA* in *E. coli*

L-serine, the end-product of the serine biosynthesis pathway, does not affect expression of the *serA* gene. However, it feedback inhibits the enzymatic activity of 3-phosphoglycerate dehydrogenase (50% inhibition at 4×10^{-5} M) through a conformational change in the enzyme. Inhibition of 3-phosphoglycerate dehydrogenase by serine is the major form of control of serine biosynthesis in *E. coli*, and occurs by an allosteric process. The inhibition of 3-phosphoglycerate dehydrogenase activity is an effective form of control of the metabolic flow of carbon through the serine-glycine pathway (Stauffer, 1996).

Several factors have been found to affect the 3-phosphoglycerate dehydrogenase levels inside the cells. 3-phosphoglycerate dehydrogenase levels are reduced 10 fold in cells grown in minimal medium with lactate as the carbon source and supplemented with amino acids not directly related to serine biosynthesis (threonine, methionine, leucine, isoleucine).

Lrp is a global regulator involved in the control of transcription of numerous genes relating to amino acid metabolism. The *serA* gene is activated six-fold by Lrp, and leucine reduces expression two-fold (Newman and Lin, 1995). Primer extension experiments (Lin, 1992c) have shown that *serA* has two promoters, P1 and P2. It is suggested that P1 is activated by Lrp, while P2 is repressed. However, there is no direct

evidence. Figure 6 shows the position of P1 and P2. Gel retardation assay also showed that there are at least two *lrp*-binding sites in the *serA* promoter region, one related to P1, and the other related to P2. DNaseI footprinting shows that Lrp protects a 70-bp upstream region in the *serA* promoter covering the P2 −10 region. We wonder how these two promoters are mediated in different conditions; whether Lrp directly regulates the expression of *serA*, and in addition to Lrp, whether any other regulator is involved in the expression and regulation of the *serA* gene. Especially, compared with other operons, the Lrp/leucine regulon has less effect on *serA* (Table 1).

3-phosphoglycerate

$\downarrow$ 3-phosphoglycerate dehydrogenase (*serA*, 65.8 min)

3-phosphohydroxypyruvate

$\downarrow$ phosphoserine aminotransferase (*serC*, 20.6 min)

3- phophoserine

$\downarrow$ phosphoserine phosphatase (*serB*, 99.6 min)

NH3 (nitrogen source)

$\uparrow$ Serine deaminase

→ L-Cysteine

or cystathionine β-lyase ———— L-serine → L-Methionine

$\downarrow$

Pyruvate   L-Tryptophan

(carbon source)

$\updownarrow$ Serine hydroxymethyltransferase (*glyA* 57.6min)

Glycine

$\updownarrow$ Glycine cleavage system

$CO_2 + NH_3$

Figure 1. The metabolic pathway of L-serine in *E. coli*

(Major pathway is adapted from Stauffer, 1996)

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Materials and Methods

1. Strains, and plasmids

The strains, and plasmids used in this study are listed in Table 2.

Table 2. Strains, and plasmids

E. coli K-12 Strains		
CU1008	*E .coli* K-12 *ilvA*	L.S. Williams
MEW1	CU1008 *ΔlacZ*	Newman *et al.*, 1985b
MEW26	MEW1 *lrp*::Tn10	Lin *et al.*, 1990
CT4A	MEW26 *ara-*	
Plasmids		
pGT17	pBR325 carrying the *serA* gene cm^R	G.A. Grant
pGU2	pBluescript KS^+ carrying 1.3kb *Hind*III-to-*Bam*HI	
	fragment from pGT17	Lin, 1992c
pRS415	*lac* operon fusion vector, Amp^R	Simons, 1987
p415P1P2	pRS415 carrying the 400 bp fragment of the	
	serA promoter	This study
p415P1	pRS415 carrying the 400 bp fragment of the	
	serA promoter in which only P1 is functional	This study
p415P2	pRS415 carrying the 400 bp fragment of the	
	serA promoter in which only P2 is function	This study
pBAD22 *lrp*+	cm^R	Chen *et al.*, 1997

2. Cultures, media, buffers and solutions

2-1. Minimal Medium:

Liquid minimal medium (+N)

0.54% K_2HPO_4, 1.26% KH_2PO_4, 0.2% $(NH4)_2SO_4$, 0.2% $MgSO_4.7H_2O$, and 0.01% $CaCl_2$, pH 7.0.

Solid minimal medium (+N)

Minimal medium with 2% Bactoagar.

Since MEW1 (*ilvA*) and all its derivatives require isoleucine and valine for growth, both isoleucine and valine are added to final concentration of 50 µg/ml.

Carbon sources were added to the minimal media at the concentration of 0.2%.

2-2. Luria Broth (LB)

1% Bactotryptone, 0.5% yeast extract, and 0.5% NaCl.

For making plates, 2% Bactoagar was added to the medium before autoclaving.

2-3. Other additions to the Medium

Antibiotics were used at the following concentrations: ampicillin (Amp) 200 µg/ml, kanamycin (Kan) 10 µg/ml, chloramphenicol (Chl) 25 µg/ml.

2-4. Buffers and solutions

Gel retardation binding buffer (1x):

20 mM Tris.HAc, 0.1 mM EDTA, 0.1 mM DTT, 50 mM NaCl, 4 mM MgAc,

15% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol.

Lrp elution buffer

TG$_{10}$ ED: 10 mM Tris.HCl (pH 8.0), 0.1 mM EDTA, 0.1 mM DTT, and 10%

glycerol.

TG$_{50}$ED: 10 mM Tris.HCl (pH 8.0), 0.1 mM EDTA, 0.1 mM DTT, and 50%

glycerol.

SOC buffer for electro-transformation

2% Bactotryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM

MgCl$_2$, 10 mM MgSO$_4$, and 20 mM gulcose.

TBE (This-borate and EDTA) buffer for DNA agarose gel electrophoration

Concentration of stock solution (5x)

0.45 M Tris-borate

0.01 M EDTA (pH 8.0).

TE (Tris and EDTA) buffer for dissolving DNA

10 mM Tris·HCl (pH 8.0)

1 mM EDTA (pH 8.0)

X-gal solution for selecting lac^+ colonies

5-bromo-4-chloro-3-indolyl-β-D-galactoside is dissolved in N-N-

dimethylformamide at a concentration of 20 mg/ml.

Z-buffer for β-galactosidase activity assay

1.61% $Na_2HPO_4.H_2O$ or 0.852% Na_2HPO_4, 0.53% $NaH_2PO_4.H2O$, 0.075% KCl, 0.0264% $MgSO_4.7H_2O$, 0.27% (v/v) β-mercaptoethanol, pH 7.0.

3. Enzyme Assays

3-1. β-galactosidase Assay

Cells were grown to early log-phase in the test medium. β-Galactosidase activity was assayed in whole cells according to the method described by Miller (Miller, 1972) and expressed in Miller units. One unit of β-galatosidase is the amount of enzyme that produces 1 MU-mol/ml o-nitrophenol/min in standard assay conducted at 28°C, pH 7.0.

3-2. Protein Assay

Protein concentration was determined with the protein assay reagent (Bio-Rad) according to the manufacturer's protocol. Bovine serum albumin was used as the standards.

4. Transformation

Transformation was performed via electro-transformation by using Gene Pulser (Bio-Rad) and following the instruction manual that comes together with the apparatus.

5. Plasmids isolation and restriction enzyme digestion

5-1. Plasmids Isolation

Plasmids were isolated and purified either by QIAprep Spin Minipreparation Kit or Midipreparation Kit (large amounts) from QIAGEN according to the protocol recommended by the manufacturer.

5-2. Restriction Enzymes Digestion

All restriction enzymes were purchased from MBI and the conditions for digestions followed the protocol of the manufacturer.

6. Gel-electrophoresis

DNA agarose gel electrophoresis and polyacrylamide gel electrophoresis were carried out as the method described by Sambrook (Sambrook *et al.*, 1989).

7. DNA Sequencing

Double-stranded DNA sequencing was performed with Gibco BRL's dsDNA Cycle Sequencing System (Life Technologies), which is based on the method of Sanger *et al.* (Sanger *et al.*, 1978). γ-ATP-^{33}P [specific activity: 3000 ci/mmol, (Amersham)] was used to end-label the primer P1 or primer A.

8. Site-directed Mutagenesis

Site-Directed Mutagenesis was performed by the PCR-based technique of overlap extension ((Figure 2; Z.Q Shao personal communication; Higuchi, 1990). The strategy for this technique is shown on Figure 2. There are two PCR products that overlap in sequence; both contain the same mutation introduced as part of the PCR primers. These overlapping, primary products can be denatured and allowed to reanneal together, producing two possible heteroduplex products. The heteroduplexes that have recessed 3' ends can be extended by Pfu DNA polymerase to produce a fragment that is the sum of the two overlapping products. A subsequent reamplification of this fragment with only the right- and left- most primers ("outside" primers) results in the enrichment of the full-length, secondary product (Higuchi, 1990).

The primers used for site-directed mutagensis were synthesized by Biocorp. and are listed in Figure 3. Primers A, B, E, F were used to create the mutation in P1 and the generated product termed as serAP2; and A, B, C, D were used to create the mutation in P2 yielding product serAP1. In each case, the product was named according to the promoter which remains active. Primers A, B were used to amplify the wild type serA promoter named serAP1P2.
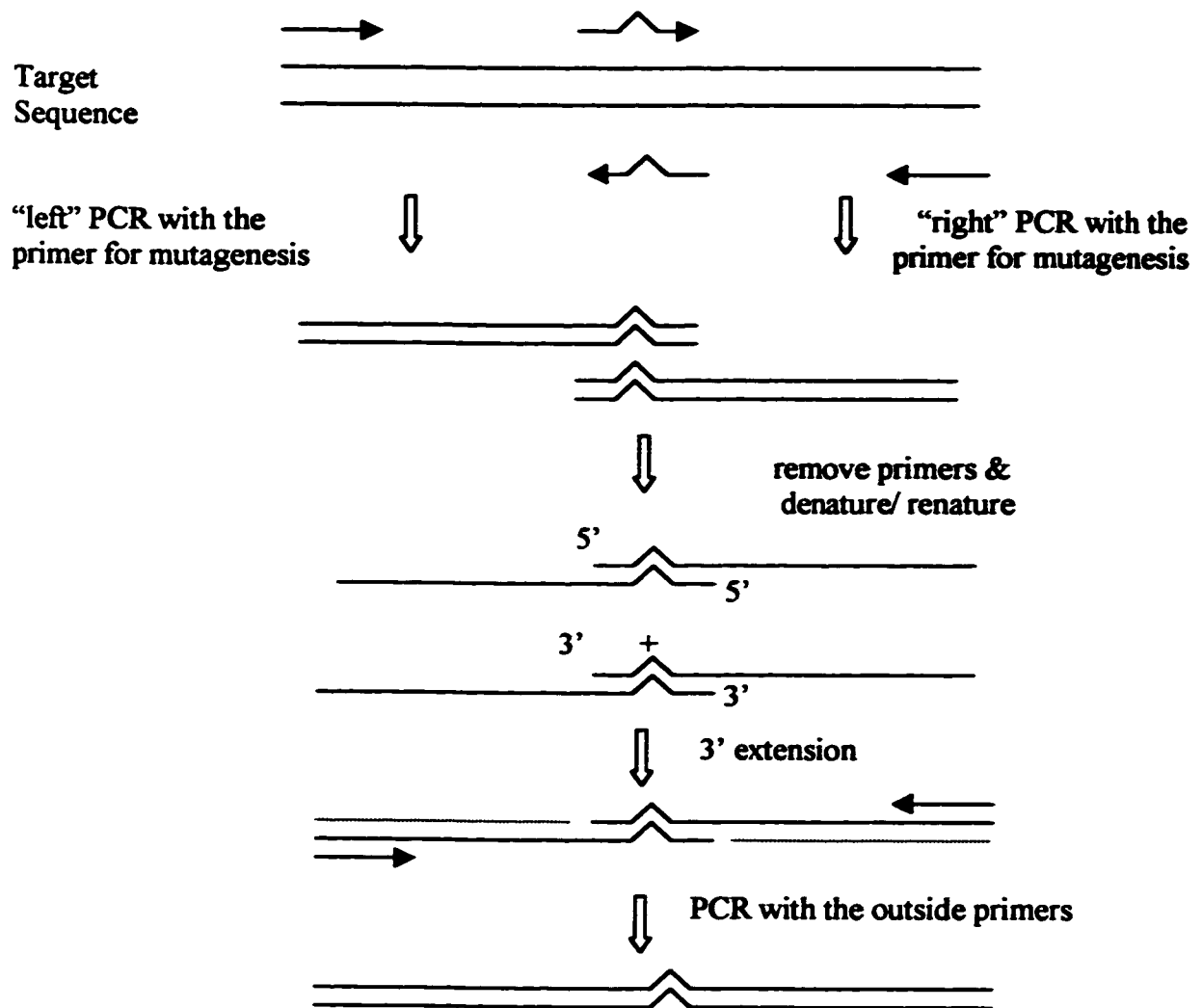
Figure 2. Strategy for the PCR-based site-directed mutagenesis technique.

Combining two separate PCR products with overlapping sequence into one longer product. The two overlapping ("inside") primers are shown containing several mismatched bases to the target sequence.

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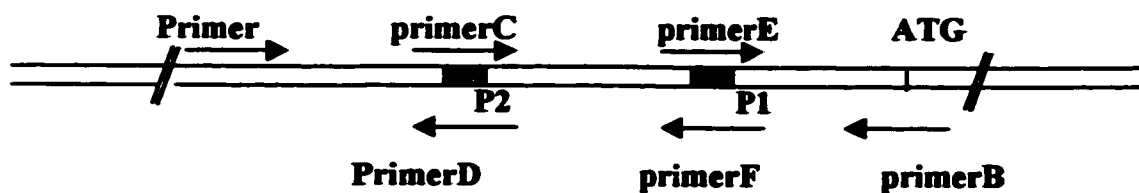
Primer A: 5'-CATG<u>GATAT</u>a<u>C</u>CTTGACCCGATAGC
           EcoRV

Primer B: 5'-CCAG<u>G</u>c<u>GATCC</u>a<u>C</u>CTTTGCCATTTACC
           BamHI

Primer C: 5'-GCTCTAAATAAATCC<u>TTATTT</u>ctaaa CCAGCATATTCATCC
           P2-10

Primer D: 5'-GGATGAATATGCTGG<u>AAATAA</u>tttac GGATTTATTTAGAGC
           P2-10

Primer E: 5'-GCAATATTATTTTTTGA<u>CGCGTT</u>tat GAAAGGCGGATG
           P1-10

Primer F: 5'-CATCCGCCTTTC<u>AACGCG</u>ata TCAAAAAATAATATTGC
           P1-10

Figure 3. Primers used for site-directed mutagenesis of the *serA* promoter.

Figure 3. Primers used for site-directed mutagenesis of the *serA* promoter.

Primers A and B flank the *serA* promoter region and are located at –390 to –366 and at –5 to +19 counted from the "A" base of the *serA* ATG start codon individually. *Eco*RV and *Bam*HI endonuclease cleavage site are introduced into primers A and B respectively. Primers C and D are complementary and include five bases mismatching around the P2 –10 region. Primers E and F are complementary primers containing three bases mismatching around the P1 –10 region. Therefore, Primers A, B, E, F are used to create the mutation in P1; and A, B, C, D are used to create the mutation in P2; and A, B are used to amplify the *serA* wild type promoter. The –10 region sequences are underlined. Lower case letters indicate the mismatching bases.

DNA fragments containing mutations in the upstream region of *serA* were generated in two steps. In the first step of PCRs were done in 40 μl volume mixtures containing 0.01 pmol template pGU2 DNA and 0.08 μM of each of the two primers. To generate mutated P1, primers A and F were used for the left PCR and primers B and E were used for right PCR. To generate mutated P2, primers A and D were used for the left and primers B and C were used for the right PCR. Other ingredients in the reaction were 0.2 mM each of the four deoxynucleoside triphosphates (dNTPs), 200 mM Tris-HCl (pH 8.8), 20 mM MgSO$_4$, 100 mM KCl, 100 mM (NH$_4$)$_2$SO$_4$, 1% Triton X-100, and 1 mg/ml nuclease-free BSA.Finally, 2 units Pfu polymerase (Stratagene) were added and overlaid with mineral oil. The reaction mixtures were run as the following PCR cycles in the DNA thermocycler (Interscience).

95°C 2 min to denature DNA

First 2 cycles:	94°C 30 sec
	57°C 40 sec
	72°C 50 sec
Second 26 cycles:	94°C 30 sec
	58°C 40 sec
	72°C 50 sec
Third 1 cycle:	94°C 30 sec
	59°C 40 sec
	72°C 50 sec

The resulting fragments from each of the two reactions were then purified from agarose gel (QIAEX II Gel Extraction Kit, QIAGEN, Cat. No. 20021) to remove

contaminating template unincorporated primer DNA and the non-specific PCR products. In the second step, the two purified fragments, template pGU2, dNTP, and Pfu polymerase were mixed as described above to a final volume of 40 μl. After an initial denaturation at 94°C for 2 min, the mixture was run 8 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 40 sec, and extension at 72°C for 1 min. Then 0.08 μM each of primers A and B were added to the reactions. The PCR was continued to the following cycles:

<div align="center">

First 20 cycles: 94°C 45 sec

57°C 40 sec

72°C 1 min 10 sec

second 8 cycles: 94°C 45 sec

58° C 40 sec

72°C 1 min 10 sec

last 1 cycles: 72°C 10 min

</div>

The resulting fragments, spanning the region bounded by the outside primers, were digested with *Eco*RV and *Bam*HI restriction endonucleases and ligated to the vector pRS415 cut with *Sma*I and *Bam*HI. The cloned PCR fragments were then verified by dsDNA sequencing.

8. Plasmid Constructions

The *lac* operon fusion plasmid vector pRS415 was used for all plasmid constructions in this work. The plasmid backbone consists of the *ori*, *bla* and the distal portion of the *tet* (*tet'*) genes of pBR322. Between *bla* and *tet'* are several elements in the following order: (i) four tandem copies of the strong transcriptional terminator *T1* from the *E. coli rrnB* operon to block transcription from upstream plasmid promoters; (ii) unique restriction sites for introduction of cloned segments; (iii) a suitable *lacZ* gene to which fusions are made, and (iv) the wild type *lacY* and *lacA* genes, and the *lac* operon transcriptional terminator (Simons *et al.*, 1987).

The gel-purified PCR products *serA*P1P2, *serA*P1 and *serA*P2 were digested by *Eco*RV and *Bam*HI and then the reaction buffer was changed by QIAquick PCR purification kit. The digested fragments were then ligated into vector pRS415 that was cut with the restriction endonuclease *Sma*I and *Bam*HI. The ligation mixtures were transformed into strain MEW1 and plated on LB plates with X-gal and ampicillin. Plasmids from blue colonies on X-gal plates were isolated and checked by restriction enzyme digestion. The wild type *serA* promoter and mutants were confirmed by dsDNA sequencing. All three types of recombinants carried 400 bp insertions of the *serA* upstream region. The recombinants were named as p415P1P2 including both functional P1 and P2 promoters; p415P1 containing only functional P1 and mutated P2; and p415P2 carrying only functional P2 and mutated P1 (figure 4).
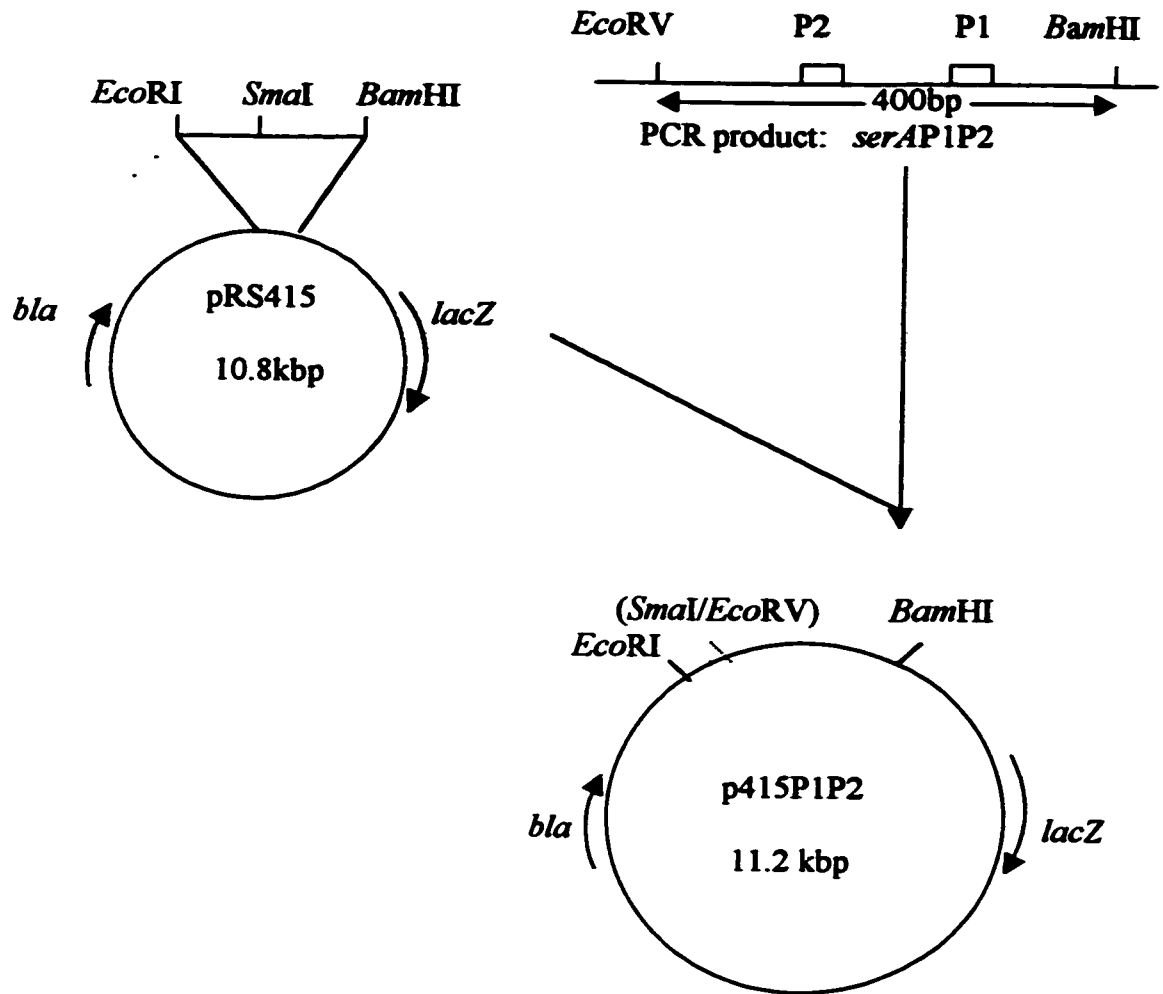
Figure 4. Construction of the p415P1P2 containing both functional P1 and P2

The 400 bp PCR product serAP1P2 was digested with EcoRV and BamHI and inserted into SmaI and BamHI digested pRS415, forming p415P1P2. The strategies for constructing p415P1 and p415P2 were the same as that for p415P1P2, except that PCR fragments serAP1 and serAP2 were used respectively.

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9. RNA-Primer Extension

9-1. Isolation of Total Cellullar RNA

The wild type strain and *lrp-* strain carrying p415P1P2, p415P1, and p415P2 were grown overnight in LB and glucose minimal medium with or without 100 µg/ml leucine. Then the cells were subcultured in 5 ml of same medium until O.D 600 around 0.5. Total cellular RNA was isolated with Rneasy Mini Kit (QIAGEN) according to the protocol provided by the manufacturer.

9-2. 5'-End-Labeled Oligonucleotides

Primer P1 5'-GTGAATCCGTAATCATGGTCAT matches to *lacZ* gene +22 to +1, where the first base of the *lacZ* coding region is designated as +1. This primer was used to carry out the primer extension. One pmol primer P1 was 5'-end labeled with 1.7 pmol γ-^{32}P-ATP (6000 ci/mmol, Amersham) using 20 units T4 polynucleotide kinase (MBI).

9-3. RNA Primer Extension

Primer extension was performed according to the method of Lin (1992c) with slight modification. One tenth of pmol γ-ATP-^{32}P labeled primer P1 was mixed with 1 to 5 µg of RNA in a volume of 9 µl that contained 3 µl DEPC treated dH$_2$O. Primer was annealed to RNA by heating at 80°C for 5 min, and cooling on ice. Then 10 µl of reverse transcriptase buffer was added, which contained 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl$_2$, 10 mM dithiothreitol, 0.5 mM spermidine, 1 mM each dATP, dCTP,

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dTTP and dGTP, and 20 units of AMV reverse transcriptase (Promega). The reaction mixture was incubated at 42°C for 1hr. The reaction was stopped by adding 20 μl stop solution [95% (v/v) formamide, 10 mM EDTA (pH 8.0), 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol]. Six μl of this sample was loaded onto a 6% polyacrylamide-8M urea sequencing gel. A sequencing ladder labeled with γ-ATP-^{33}P and primed with the same primer P1 was run in adjacent lanes.

10. Partial Purification of the Lrp Protein

10-1. Crude Protein Extraction

The Strain CT$_4$A/pBAD22 *lrp*+ cm^R was grown overnight in liquid minimal medium with 0.5% glycerol, 25 μg/ml chloramphenicol, and 1:100 subcultured into minimal medium with 0.5% glycerol, 25 μg/ml chloramphenicol and 15 μg/ml arabinose until O.D.600 between 0.4~1. Cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C and were resuspended in TG$_{10}$ED 0.2 M NaCl buffer, 3.5 ml per gram of wet weight of cells. The cells were sonicated and then clarifed by centrifugation at 33,000-x *g* 20 min. The supernatant was the crude extract of the Lrp protein.

10-2. Partial Purification of the Lrp Protein

A 15 ml bed volume of Bio-Rex 70 sodium form column (Bio-Rad) was equilibrated with KPO$_4$ buffer (50 mM, pH 7.5) and then TG$_{10}$ED 0.2 M NaCl (pH 8.0) buffer overnight. The column was connected with a pump, and a fraction collector. The parameters were set up as followed: flow rate 0.5 ml/min; fraction collector 10 min/tube.

After 10 ml of the cell extract was loaded, the column was washed with $TG_{10}ED$ 0.2M NaCl buffer. Proteins were eluted with $TG_{10}ED$ buffer with a NaCl concentration gradient (from 0.2 M to 1.0 M). The elution fractions containing the expected band were dialyzed against $TG_{50}ED$ 0.2mM NaCl buffer and concentrated via Centricon concentrators (from Amicon Company, No.4205) and stored in at $-86°C$.

11. SDS -PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a Bio-Rad Mini Protein II dual slab cell according to the instructions of the manufacturer.

12. Gel Retardation Assay

The DNA fragments used for gel retardation assays were recovered and purified from agarose gels. The purified DNA fragments were 5'-end labeled with γ-ATP-^{32}P by T4 polynucleotide kinase (MBI) and then passed through MicroSpin™ S-200 HR columns (Pharmacia Biotech) to remove the unincorporated radiolabelled nucleotides. The procedures were carried out as described by the manufacturer.

The Lrp protein used in these studies was partially purified by Bio-Rex 70 (sodium form) column chromatography as described above.

Binding of Lrp protein to the upstream region of the wild type *serA* fragment and *serA* mutated fragments was determined by the gel retardation assay described by Ricca

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et al (Ricca *et al.*, 1989) with slight modification. One to five ng (5000 cpm) of 5'-end-labeled DNA fragment was incubated at room temperature for 10 min with 2μg sonicated herring sperm DNA and 0-1.8 pmol Lrp protein in 20μl binding buffer. Samples were resolved by electrophoresis through a 5% polyacrylamide gel pre-electrophoresed at 10 v/cm 1 hr and eletrophoresis of the samples was performed in the same conditions. The gel was cast and run in 1x TBE buffer (Sambrook *et al.*, 1989). The gels were dried at 80°C in gel drier and subjected to radioautography.

Results

In this study, I wished to determine the relative importance of the two promoters P1 and P2 for the transcriptional activation of *serA* and to define further the nature of the *lrp-serA* interaction. I therefore mutated *serA* P1 and *serA* P2 separately by site-directed mutagenesis and studied their activity *in vivo* and *in vitro* independently. The effects of these mutations on the start-site used for transcription were determined by RNA primer extension while their effects on transcription *in vivo* were examined by the activity of the β-galactosidase reporter gene. Finally, gel mobility shift assays were carried out to investigate the interaction of the *serA* promoters and the Lrp protein.

Part 1. Site-directed mutagenesis of the *serA* promoter region and construction of *serA::lacZ* fusion

1-1. Site-directed mutagenesis of the *serA* promoter region

The region of −370 bp to +10 bp from the *serA* ATG start codon carries both P1 and P2 promoters of the *serA* gene and contains all signals needed for the known regulation of the *serA* gene (Zhang, 1994). In this study, I focused on a 400-bp fragment, which included all the information necessary for the expression and regulation of *serA*. This fragment runs from −390 bp to +10 bp as counted from the *serA* ATG start codon.

Zhang used large deletions to change the *serA* promoter, resulting in considerable and unknown changes in the DNA structure, making interpretation of results difficult. To

avoid such change in DNA structure, instead of making deletion mutations, I wished to inactivate one of the promoters by changing its conserved −10 promoter sequence. Thereby RNA polymerase would not be able to bind this site, whereas the other promoter was kept unchanged. The site-directed mutagenesis was performed to obtain such mutants via the PCR based technique of overlap extension (Figure 2; Z.Q Shao personal communication; Higuchi, 1990).

In this study, primers A, B, E, F were used to mutate the P1 −10 promoter and the PCR product was named *serA*P2 while primers A, B, C, D were used to mutate the P2 and the PCR product named *serA* P1. In each case, the product was named according to the promoter which remains active. Primers A, B were also used to amplify the wild type *serA* promoter named *serA*P1P2 (Figure 3). Since the *Eco*RV and *Bam*HI restriction endonuclease cleavage sites were introduced into primers A and B respectively, all the PCR products were digested with these two enzymes and subcloned into the pRS415 vector (Figure 4).

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1-2. *In vitro* construction of *serA::lacZ* operon fusions.

The native and modified *serA* promoter fragments, *serA*P1P2, *serA*P1 and *serA*P2, were inserted into the vector pRS415 separately, resulting in their fusion to the *lacZYA* reporter gene. The resulting plasmids were designated as p415P1P2, p415P1, and p415P2 respectively (Figure 4). That the correct constructs were confirmed by DNA double-strand sequencing and no spontaneous mutation was detected. The vector pRS415 is a multicopy vector designed by Simons to create fusions of regulatory regions to a reporter *lac* operon (Simons *et al.*, 1987). It carries several transcriptional terminators on the upstream of its multiple cloning sites, and the *lacZ* coding region on the downstream of the cloning sites. The insertion of *serA* promoter fragments into one of the multiple cloning sites of pRS415 leads to the formation of an operon fusion with the *lacZ* gene coding region, so that the *serA* promoters direct and regulate the expression of *lacZ*. Expression from the *serA* recombinant plasmids can be studied by measuring β-galactosidase activities of *lacZ* host cells. The pRS415 is constructed in such a way that transcription initiation by any other promoters upstream of *serA* would be stopped by the transcription terminators and therefore would not affect the *lacZ* expression.

p415P1P2 serA::lacZ

(smalI/EcoRV)

400bp

BamHI

-108   -92   -13   +1 P1

TCTAAA ——— TTC ┐→ P2   TATGTT—CG┐→ATG ———— ATG
P2-10   P1-10   serA   lacZ
                                          Primer P1

p415P2 serA::lacZ

                        TAT
                        :::
TCTAAA ——————— cgcGTT—— ATG ———— ATG
P2-10   P1-10   serA   lacZ
                                 Primer P1

p415P1 serA::lacZ

CTAAA
:::::
Ttattt ——————— TATGTT—— ATG ———— ATG
P2-10   P1-10   serA   lacZ
                                 Primer P1
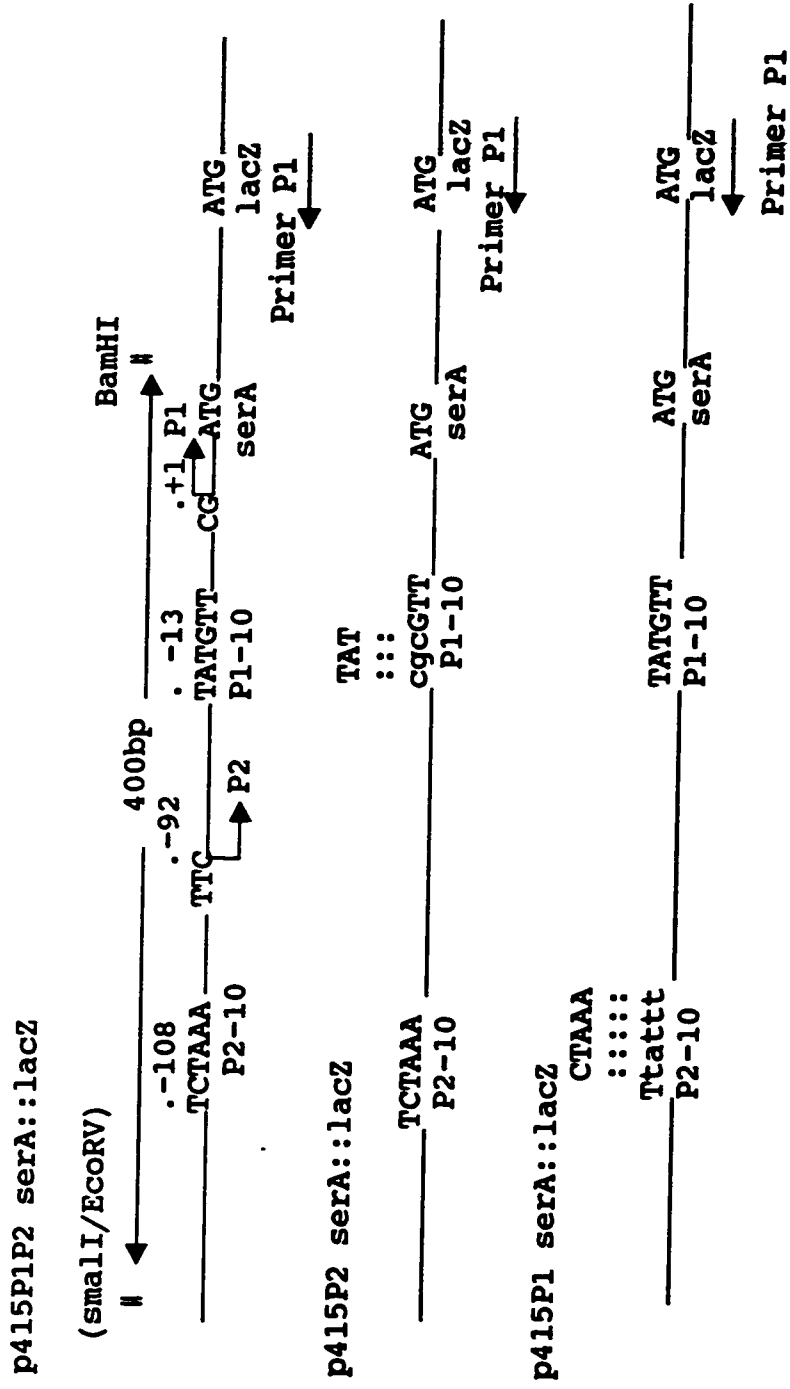
Figure 5. The structure of the *serA* promoters

Figure 5. The structure of the *serA* promoters

PCR products *serAP1P2*, *serAP1*, *serAP2* were purified from agarose gels, digested by *EcoRV* and *BamHI* and inserted into the *smaI* and *BamHI* sites of the vector pRS415 and finally transformed into MEW1 strains. Transformants were selected on LB plates with ampicillin and X-gal. The blue colonies were collected. The plasmids were isolated and checked by digestion with restriction enzymes *EcoRI* and *BamHI*. Plasmids carrying insertions of expected sizes were sequenced and the expected mutants were used for further study.

Primer P1, *5'*-GTG AAT CCG TAA TCA TGG TCA T, which was complementary to the coding strand and matched to *lacZ* gene +22 to +1, where the first base of the *lacZ* coding region was designated as +1, was used for the RNA primer extension assay.

EcoRI (smaI/EcoRV)

GAATTCCAATCCTTGACCCGATAGCGATGGAAAACGCCATAAATGCGATTCCTGGCGTGG

TGACTGTTGGCTTGTTTGCTAACCGTGGCGCGGACGTTGCGCTGATTGGCACACCTGACG

                                           .-200   .-180
GTGTCAAAACCATTGTGAAATGATCTGACGGGGGAACCTCCCCCGTTAAAAAAATTCTCT

                .-160   .-140   .-120
TCATTAAATTTGGTGACATGTGTCAGGCTTTTACCAGGCAATGTCGATTGCTCTAAATA
                                                          P2 -35

                .-100   →P2(-92)   .-80   .-60
AATCCTCTAAACCAGCATATTCATCCAAGAATACCTTTGCGTGATATTTCCTCAACATC
     P2 -10

    .-50   .-30   .-10   →P1(+1)
GCGACGCAAACGTTCATATTGCCGCAATATTATTTTTTGATATGTTGAAAGGCGGATGCA
 ↑   P1 -35   P1 -10
 NruI

AATCCGCACACAACATTTCAAAAGACAGGATTGGGTAAATGGCAAAGGGATCCGGACAC
                                                ↑
                                               BamHI

Figure 6. The sequence of the wild type *serA* promoter p415P1P2 *serA::lacZ* fusion (coding strand)

Figure 6. The sequence of the wild type *serA* promoter p415P1P2 *serA::lacZ* fusion (coding strand). *Sma*I and *Bam*HI sites were used to clone the PCR product *serA*P1P2 into vector pRS415. *SerA* has two transcription initiation sites corresponding to two promoters P1 and P2. The G residue of the P1 initiation site is designated as +1. Boundaries of DNase I protection by Lrp on the *serA* sequence are from –155 to –81 nucleotides covering the P2-10 sequence (Lin, 1992c). A putative cyclic AMP (cAMP) receptor protein-cAMP complex (cAMP-CRP) binding site is recognized at positions –166 to –144, which overlaps Lrp protected sites. The underlined nucleotides are the conserved sequence of the *serA* promoters P1 and P2. The Lrp protected nucleotides are indicated by lines through them and a double-line is drawn under the putative cAMP-CRP binding site.

Part 2. Expression of *serA::lacZ* fusion carrying wild type *serA* promoter P1P2, only the P1 promoter and only the P2 promoter

To more precisely determine the contribution of each of the two promoters to Lrp regulation and to transcriptional activation, I mutated each promoter (P1 or P2) without changing their spacing with respect to one another or to the transcriptional start sites. The fusion plasmids p415P1P2 *serA::lacZ*, p415P1 *serA::lacZ*, and p415P2 *serA::lacZ* were introduced into parental strain (MEW1) and into the corresponding *lrp-* strains (MEW26). The resulting strains were grown in the presence or absence of L-leucine. In this way, I was able to study the regulation of expression of the *serA* gene at transcriptional level by primer extension and by measuring β-galactosidase activity in different growth conditions.

2–1. Determination of transcription initiation sites for the wild type *serA* promoter and mutants

The *serA* transcription initiation sites were previously mapped by primer extension by Lin (Lin 1992c). He showed that two different promoters could initiate the transcription of *serA*. The initiation site of transcription by P1 promoter was located at 45 bp and by P2 promoter at 138 bp upstream of the *serA* translation start codon AUG (Figure 6). He suggested that Lrp activated P1 but repressed P2.

In this work, I determined separately the transcription activities of the *serA* P1 and P2 promoter by primer extension using multicopy plasmids p415P1P2 carrying the wild type *serA* promoter P1P2, p415P2 which only P2 was functional, and p415P1 which only P1

was functional. These plasmids were transformed into *lrp+* parental strain (MEW1), and isogenic *lrp-* (MEW26) strains, both of which were *lacZ*. The Cells were grown in minimal medium without or with 100 μg/ml L-leucine and LB rich medium. RNAs from the cells under different conditions were extracted. A 22-mer ^{32}P 5'-end-labelled primer P1 was used to hybridize the 5'terminus of the *lacZ* coding strand sequence (Figure 5). The reverse transcriptase was used to extend the primer. The extension products were analyzed by polyacrylamide gel electrophoresis, in parallel with the products of a dideoxy sequencing reaction performed with the same oligonucleotide primer 5'-end-labelled by γ-ATP-^{33}P.

Two groups of extension products were detected. The first 5'-end transcripts which were 154 bp corresponding to 45 bp upstream from the *serA* start codon AUG. This extension product indicated the initiation site of was located 45 bp upstream of the *serA* start codon AUG. The G residue of T1 initiation site was designated +1 (Figure 7). This result was consistent with the previous study (Lin, 1992c). The DNA sequence upstream from this start site (Figure 6 and 7) revealed a −10 region showing 4 of 6 bp homology with the consensus Pribnow box sequence and a −35 region showing 4 of 6 bp homology with the −35 box consensus sequence for other *E. coli* promoters (Hawley and McClue, 1983). The other 5'-end transcripts were 246 bp in length located 138 bp upstream from *serA* translation start codon (Figure 7).

These results indicated that transcription initiation can take place at two sites, P1 at −45 and P2 at −138 upstream from the translation start site of *serA*, which were identified to previous study (Lin, 1992c). Both sites showed −10 and −35 region homology with the expected consensus sequence. A further minor background band could be seen (about 165 bp in length). This may be due to the RNA secondary structure that blocked the cDNA

elongation or to the formation of immature products or to the degradation of the transcription products from P2. In addition, the non-specific products might also account for the background bands.

Which promoter was used varied greatly with the growth conditions and the particular host used. From the wild type serA promoter, only transcripts 1 which were derived from P1 were detectable in the presence of Lrp either without or with exogenous L-leucine (Figure 7, lane 4 and 7). Transcripts from P2 appeared in cells carrying the wild type promoter p415P1P2 grown in LB, a condition in which Lrp is expressed at a much lower level than in minimal medium (Newman *et al.*, 1996), and in the *lrp*- strain grown in both minimal medium and LB. However, the intensity of transcript from P2 was much lighter than that from P1 (Figure 7, lane1, 11, 14, and 18). These results demonstrated that transcription from the *serA* promoter may come from two sites P1 and P2; P1 is a strong promoter but P2 is a weak promoter, which is repressed by Lrp.

These results do not indicate whether Lrp activated P1. The detection of extension products from P1 both in the presence or absence of Lrp (Figure 7, lane 4 and 14) could be due to either the activation of expression of P1 by Lrp or constitutive expression of P1. P2 might be activated either by counteracting the repression due to Lrp, or directly by activation due to another as yet unidentified factor, perhaps acting only in the absence of Lrp. Even in the *lrp*- host strains, there were still some transcripts from the P1 promoter. These could result from weak recognition of −10 and −35 hexamer elements of the *serA* P1 promoter by RNA polymerase thereby permitting promoter activity in the absence of an activator (if Lrp was the only activator). Additionally, P1 might be also regulated by other factors when Lrp was not present.

With mutant p415P2, only transcripts from P2 were detectable under the different condition tested, whether Lrp or leucine was present or not. This verified that the P1 function was completely abolished as intended (Figure 7, lane 2, 5, 8, 12, 15, and 17). P2 was the only promoter directing the transcription of p415P2 *serA::lacZ*. The comparison of the P2 and the P1P2 transcripts in *lrp*+ host showed that changes in the −10 region of P1 which prevented the use of P1 also decreased the repression of P2 by Lrp (Figure 7, compare lanes 4 and 5), either directly or indirectly. It could be that the presence of a functional P1 allowed Lrp to repress P2 or that when P1 could not function, other regulatory systems might bring about the activation at P2. Similarly, the fact that only transcripts from P1 were observed from the p415P1 *serA::lacZ* construct verified that P2 function was completely inactivated in this case and that the expression of p415P1 *serA::lacZ* was directed by P1 (Figure 7, lanes 3, 6, 9, 13, 16, and 19).

The addition of L-leucine did not change transcription initiation site of the wild type *serA* promoter and mutants whether they were in an *lrp*+ or *lrp-* background (Figure 7, lanes 7, 8, 9, 17, 18, and 19).
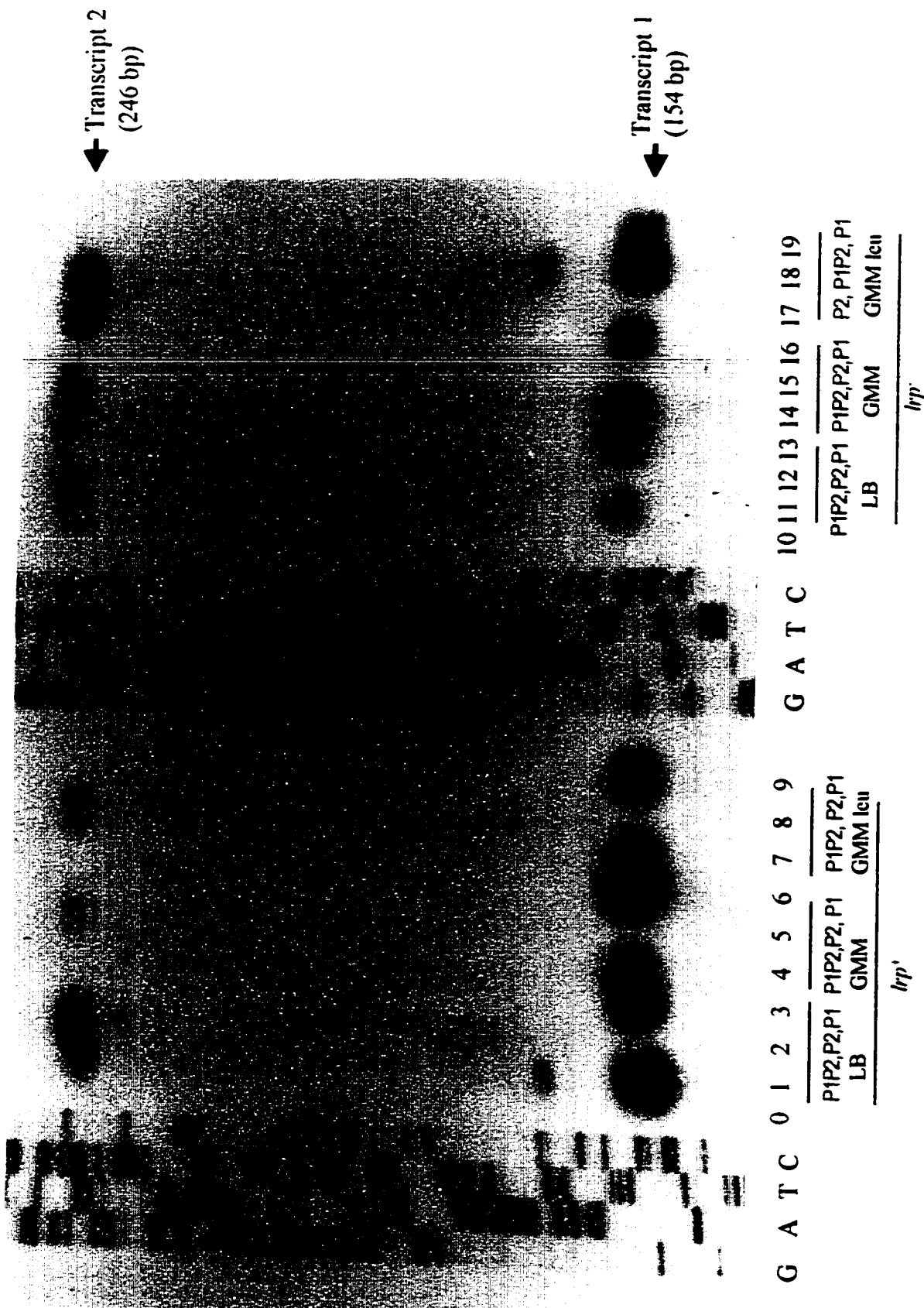
Transcript 2
(246 bp)

Transcript 1
(154 bp)

G A T C 10 11 12 13 14 15 16 17 18 19

P1P2,P2,P1   P1P2,P2,P1   P2, P1P2, P1
    LB   GMM   GMM leu

lrp'

G A T C 0 1 2 3 4 5 6 7 8 9

P1P2,P2,P1   P1P2, P2,P1
    LB   GMM leu
          GMM

lrp'

Figure 7. Determination of transcription initiation sites of the wild type *serA* promoter and mutants. Total cellular RNA was extracted from *lrp*+ strains MEW1/p415P1P2, MEW1/p415P2, MEW1/p415P1 and *lrp-* strains MEW26/p415P1P2, MEW26/p415P2, and MEW26/p415P1 which were grown in LB, and in minimal medium with 0.2% glucose without or with 100 µg/ml leucine. The RNA was hybridized with a 22-mer 5'-end-labeled single strand oligonucleotide primer complementary to the *lacZ* gene from the *lacZ* translation start site to 22 bases downstream. After extension of the primer with AMV reverse transcriptase, the products were resolved by electrophoresis on a 6% polyacrylamide 8M urea gel. The size of the transcripts was determined by comparison with the products of a sequencing reaction performed with the same oligonucleotide primer. Lanes G, A, T, C correspond to the sequencing pattern of *serA* obtained through the dideoxy chain termination method with DNA extracted from p415P1P2. Lanes 1 to 9 were *lrp*+ strains (MEW1) containing p415P1P2, p415P2, and p415P1 *serA::lacZ* fusion respectively which were grown in different medium, and lanes 11 to 19 were *lrp-* (MEW26) strains containing the above plasmids. Lanes 1 to 3: the cells were grown in LB rich medium. Lanes 4 to 6: the cells were grown in glucose minimal medium; lanes 7 to 9: the cells were grown in the glucose minimal medium with 100 µg/ml leucine. Lanes 11 to 16 were in the same order as lanes 1 to 6 except that all three types of plasmids were transformed into *lrp-* strains. Lane 17 was the same as lane 8 and lane 18 was the same as lane 7 except p415P2 and p415P1 were transformed into *lrp-* strains respectively. Lane 19 was the same as lane 9 except *lrp-* strain was used. Lane 0 and lane 10 were the same as lane 1 except that reverse transcriptase was not added.

2–2. Expression of the *serA::lacZ* fusion *in vivo*

In order to study regulation of the *serA* gene in vivo, wild type and *lrp*‾ deficient strains containing p415P1P2, p415P1, and p415P2 *serA::lacZ* fusion plasmids were grown in minimal medium with or without exogenous L-leucine , or in LB for β-galactosidase activity assays (Table 3, Figure 7) .

2–2-1. Activation of the overall expression of *serA* by Lrp

The β–galactosidase activity of *serA* wild type promoters carried on the multicopy plasmids as *serA::lacZ* fusions was increased 5 folds by the presence of Lrp. The induction was reduced almost 2 folds by exogenous L-leucine in the presence of Lrp but L-leucine had little effect on the expression of *serA* in the absence of Lrp (Table 3). These expression patterns were similar to those of *serA::lacZ* from a chromosomal fusion (Lin, 1992c). These results demonstrated that the overall effect of Lrp on the regulation of the wild type *serA* promoter was positive and that leucine reduced the activation when Lrp was present. In LB, in which Lrp protein is expressed at a much lower level than in the minimal medium (Newman *et al.*, 1996), the β–galactosidase activities of all three fusions p415P1P2, p415P2, p415P1 were much lower than the activities in minimal medium.

2-2-2. Repression of the *serA* P2 promoter by Lrp

In plasmid p415P2, in which P1 was inactivated, the β–galactosidase activity was drastically decreased and was only 1/50 of that seen from the wild type promoter p415P1P2 in minimal medium in presence of Lrp (Table 3). This supports the conclusion that P1 is a major promoter and is activated by Lrp. Additionally, it is clear that Lrp repressed P2 promoter since the activity of P2 was increased by absence of Lrp in minimal medium (3 fold). L-leucine potentiated the repression of P2 about 2 folds in the presence of Lrp but had little effect on it in the absence of Lrp (Table 3).

2-2-3. Activation of the *serA* P1 promoter by Lrp

The β–galactosidase activity of p415P1, in which P2 was mutated, was increased 7-fold by the presence of Lrp. This demonstrated that Lrp activated the *serA* P1 promoter. There was no obvious difference between the activity of P1 alone and the wild type promoter P1P2 in cells in minimal medium in the *lrp*[+] host strain. This result supported the conclusion that the overall expression of *serA* was the combination of the activation of P1 and repression of P2 in the presence of Lrp. It also implied that P2 was repressed by Lrp while P1 was functional. In minimal medium, exogenous L-leucine reduced the activity of P1 by almost two folds in the presence of Lrp (Table 3) while a similar decrease was observed from the wild type promoter P1P2 and P2 alone.

2-2-4. Additional regulator(s) involved in the expression of serA

It was clear that Lrp activates expression of serA by inducing transcription of P1. In cells grown in LB, some Lrp is made, less than in glucose minimal medium, but more than in an lrp mutant. One might therefore expect more expression from P1P2 in the lrp⁺ strain grown in LB than in the lrp mutant. In fact, the reverse was true: activity directed from P1P2 in the lrp deficient strain in minimal medium being higher (3595 vs.1538 units, Table 3). This was also true for the P2 and P1 constructs (1470 vs. 566 units; 2714 vs. 1127 units, Table 3). This suggested that Lrp was not the only factor governing serA expression. This was also indicated by the fact that in the lrp- background, expression was higher in glucose minimal medium than in LB (3595 vs. 734 for P1P2; 1470 vs. 582 for P2; 2714 vs. 399 for P1).

The existence of other potential regulator(s) had not been reported before. I therefore wished to identify the potential regulator and study its mechanism. A putative cyclic AMP (cAMP) receptor protein-cAMP complex (cAMP-CRP) binding site (TTTCGTGACATGTGTCACGCTT) was located upstream at positions −74 to −52 relative to the P2 transcription at +1 (Figure 6). The possible CRP box showed a 74% match to the consensus sequence, and an 80% match to the most conserved two CRP binding sequences TGTGA and TCACA which were correctly spaced for optimal CRP binding.

If the serA promoter was regulated by cAMP-CRP, one would expect its expression to be regulated according to the carbon source used for growth. Many genes are indeed controlled in this way, a phenomenon known as catabolite repression. When glucose is

present in the medium, the amount of intracellular cAMP is greatly reduced, and genes requiring cAMP-CRP for expression are correspondingly repressed.

To determine whether the expression of *serA* was subject to catabolite repression, the β-galactosidase synthesized from *serA::lacZ* fusions was examined in *lrp*⁺ or *lrp*⁻ host strain grown in minimal medium containing different carbon sources (Table 4). To be certain that catabolite repression was involved, I also determined β-galactosidase activity of cells grown in glucose minimal medium with various concentrations of cAMP (Table 5).

When the *lrp*+ cells were grown in glycerol minimal media, the β–galactosidase activities of *serA* promoter P2 alone was elevated 5 fold. No change in expression of P1, nor yet P1P2 was observed. The use of glycerol had an even stronger effect in the *lrp*- host. In this case, induction was seen in glycerol for all three constructs: the expression of *serA* P1P2, P2 alone, and P1 alone were induced 2-, 9-, 2-fold respectively in glycerol minimal media (Table 4). The fact that expression increased dramatically from P2 in glycerol minimal medium strongly suggests a role of cAMP-CRP in its regulation.

In fact, the addition of cAMP had the corresponding effect (Table 5). In the *lrp*⁺ host, the addition of 3 mM cAMP to the growth medium doubled expression from the P2 construct, but did not change the expression from the P1P2 and P1 constructs. As was the case in the glycerol experiment, the *lrp* deficient host was even more strongly affected, expression from both P2 and P1P2 being 3-fold and 2-fold increased respectively at 3 mM cAMP (Table 5). Expression from P1 was not affected. These results led to the hypothesis that cAMP-CRP was involved in the expression and regulation of the *serA* gene via activation of P2, at least in the absence of Lrp. It seemed like that cAMP-CRP competed with Lrp and it was functional mainly in the absence of Lrp.

In summary, both *serA* promoters P1 and P2 can direct the transcription of the *serA* gene but are regulated differently under various cell environments. Lrp is a main regulator for *serA*. The overall expression of *serA* under the regulation by Lrp is the combination of the activation of P1 and repression of P2. L-leucine reduced the activation of *serA* by reversing the activation of P1 and enhancing the repression of P2. Moreover, cAMP-CRP activates expression of *serA* by inducing P2, at least, in the absence of Lrp but has less effect on P1.

Table 3. The influence of Lrp on the expression of *serA::lacZ* operon fusion

Medium	Plasmids[d]	β-galactosidase activity [a]	
		lrp+	*lrp-*
LB	p415P1P2	1538	734
	p415P2	566	582
	p415P1	1127	399
Minimal medium, glu[b,c]	p415P1P2	19413	3595
	p415P2	482	1470
	p415P1	19194	2714
Minimal medium, glu leu[b,c]	p415P1P2	10865	3483
	p415P2	268	1632
	p415P1	13510	2694

a) β-galactosidase values were in Miller units and were the means of three or more independent experiments.

b) Abbreviation : glu- glucose; leu- leucine.

c). Glucose and L-Leucine were added to 0.2% and 100 μg/ml respectively.

d). The extent of plasmid loss was tested by plating the cells on LB and LB ampicillin plates respectively after each experiment. The cells that grow on LB but can not grow on LB ampicillin were determined as plasmid loss. No significant difference in the number of colonies on LB or LB ampicillin was observed.

Table 4. Expression of *serA::lacZ* fusions in minimal medium with different carbon source

Strain	Plasmids[c]	β–galactosidase activity[a]	
		Glucose[b]	Glycerol[b]
Irp[+]	p415P1P2	19413	20956
	p415P2	482	2172
	p415P1	19194	21058
Irp[−]	p415P1P2	3595	8070
	p415P2	1470	10488
	p415P1	2714	6192

a) β–galactosidase values were in Miller units and were the means of three or more independent experiments.

b). Glucose, and glycerol were added to 0.2% as sole carbon source in the media.

c). The extent of plasmid loss was tested by plating the cells on LB and LB ampicillin plates respectively after each experiment. The cells that grow on LB but can not grow on LB ampicillin were determined as plasmid loss. No significant difference in the number of colonies on LB or LB ampicillin was observed.

Table 5. The effect of exogenous cAMP on the expression of serA::lacZ fusions

Strain	Plasmids[c]	β-galactosidase[a,b] Concentration of cAMP (mM)			
		0	1.0	2.0	3.0
lrp+	p415P1P2	19413	17771	18310	19919
	p415P2	482	613	779	1002
	p415P1	19194	17425	23883	20229
lrp-	p415P1P2	3595	4071	4030	6407
	p415P2	1470	3179	2907	3416
	p415P1	2714	3030	3300	2952

a) β–galactosidase values were in Miller units and were the means of three or more independent experiments.
b) All cells were grown in the minimal media with 0.2% glucose as sole carbon source.
c) The extent of plasmid loss was tested by plating the cells on LB and LB ampicillin plates respectively after each experiment. The cells that grow on LB but can not grow on LB ampicillin were determined as plasmid loss. No significant difference in the number of colonies on LB or LB ampicillin was observed.

Part 3. *In vitro* binding studies of Lrp protein to the *serA* promoters

To investigate whether the regulation of *serA* by Lrp was mediated by a direct interaction between Lrp and the *serA* promoter region, I performed the gel mobility shift assay to determine the binding affinity of Lrp in the mutants and the *serA* wild type promoter.

1-1. Partial Purification of Lrp

Partially purified Lrp was used for studying the binding affinity of Lrp protein and mutated *serA* promoters. Lrp purification was performed as described in Zhang's thesis with slight modification. *E. coli* strain CT₄A (MEW1 *ara-*) carrying the pBAD-*lrp* operon fusion plasmid (Chen *et al.*, 1997) was used to overexpress Lrp protein. This plasmid carries the Lrp coding region under the control of the arabinose promoter (pBAD). Therefore, arabinose was used to induce the overexpression of the *lrp* gene.

Cells of strain CT₄A/pBAD22 *lrp*+ cm^R were grown overnight in minimal medium with 0.5% glycerol as carbon source, and subcultured in the same medium with 15 μg/ml arabinose. Cells were harvested and resuspended in TG₁₀ED buffer (0.2 M NaCl, pH 8.0), then sonicated and then centrifuged to prepare a crude extract. The crude extract was loaded on to a 15ml bed volume Bio-Rex 70 ion-exchange column which had been incubated overnight with KPO₄ buffer (pH 7.5) and then equilibrated with TG₁₀ED 0.2 M NaCl (pH 7.5). The column was washed with TG₁₀ED 0.2 M NaCl buffer, and eluted with a gradient of NaCl concentrations in TG₁₀ED buffer (from 0.2 M to 1.0 M).

Since the *lrp* gene product does not have any easily identifiable characteristics, for example, catalytic properties, it is difficult to demonstrate explicitly that a certain fraction contains Lrp. I therefore studied a preparation of cells grown without arabinose for comparison. The proteins from the fractions with high OD_{280} values were examined by SDS-PAGE. A strong band corresponding to 20 kDa and not present in uninduced cells was detected both in crude extract and in purified fractions of induced culture (Figure 8). The size of the protein was identical to that of Lrp polypeptide which was determined as 20-21.5 kDa by SDS-PAGE *versus* 18.8 kDa predicted from the nucleic acid sequence (Willins *et al.*, 1991). The elution fraction containing the expected band was dialyzed and concentrated via Centricon concentrators (from Amicon Company, No.4205), and then eluted in $TG_{50}ED$ buffer (0.2 M NaCl, pH 7.5), and stored at $-86°C$. The protein was used to examine the binding affinity of Lrp to the *serA* promoters.
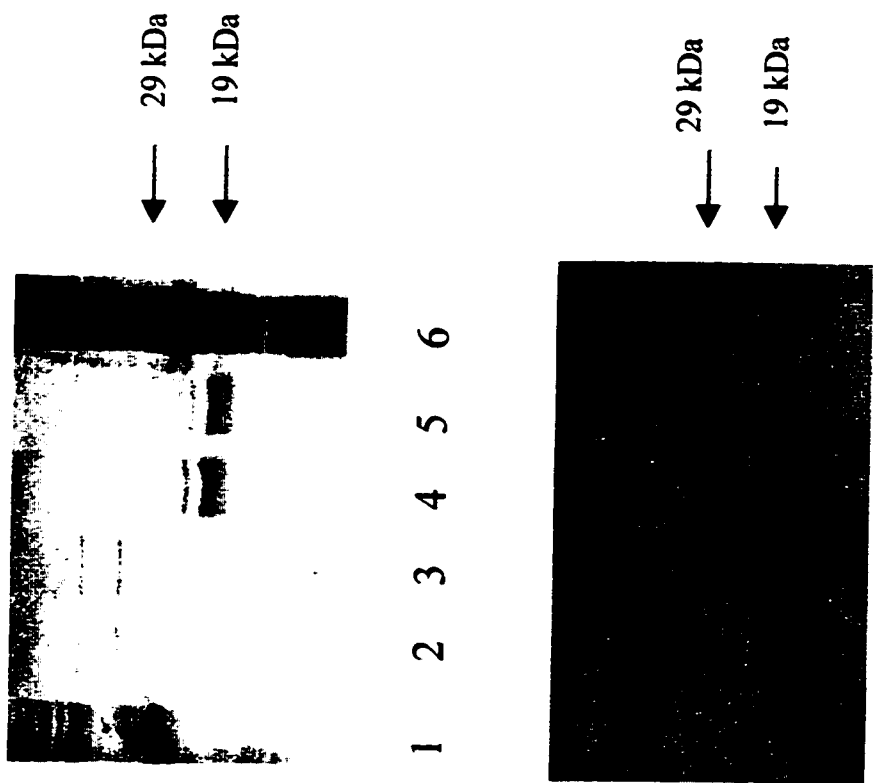
Figure 8. Partial purification of the Lrp protein using ion exchange column. Lane 1: Crude cell extract from CT$_4$/pBAD22 *lrp*$^+$ grown without arabinose induction. Lanes 2 to 5 and 7 to 10: the protein from different purified fractions. Lanes 6 and lane 11: Crude cell extract from CT$_4$/pBAD22 *lrp*$^+$ induced by arabinose. The fraction of lane 10 was used for further study.

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3-2. *In vitro* study of interaction between the Lrp protein and the *serA* gene

In the preceding sections, I have attributed the changes in gene expression to the differences in the promoter being used. These changes could be caused, as expected, by changes in polymerase binding. However, they might also be caused by changes in Lrp binding. I therefore investigated whether there was a change in binding affinity of Lrp for the mutant promoters.

To do this, the wild type *serA* plasmid p415P1P2 and its mutants p415P1 and p415P2 were digested with *Eco*RI and *Bam*HI yielding 407 bp fragments which contained both the P1 and P2 regions. The fragments were purified from an agarose gel and further digested with *Nru*I yielding two kinds of small fragments (Figure 6), one of 300 bp carrying the P2 promoter region, and the other of 107 bp carrying the P1 promoter region. All DNA fragments were 5'-end-labeled and incubated with various concentrations of the Lrp protein, followed by polyacrylamide gel electrophoresis.

Two bound complexes were observed for the 407 bp fragment of the P1P2 promoter, protein-DNA complex I and a second complex II which migrated somewhat more slowly. The same two complexes were observed with each mutant promoter, indicating that there was little if any change in either binding affinity or complex pattern in Lrp binding as a result of the –10 region mutations (Figure 9).

This was further confirmed by the Lrp binding to two small fragments digested from the 407 bp fragment: the 300 bp fragment, which only carried the P2 promoter region, and the 107 bp fragment, which only carried the P1 promoter region. Lrp bound to the 300 bp fragment and formed two bound complexes, a strong retarded band and a less retarded one,

with wild type promoter and each mutant (Figure 10). No binding affinity and complex pattern change was detected between wild type and mutants. Neither did the 107 bp fragment, which also formed two complexes. However, it required much more Lrp to show binding to the 107 bp fragments. It seemed then that Lrp bound with lower affinity to promoter P1 which it activated strongly than to promoter P2 which it repressed (Figure 10 and 11).

Comparison of the 407 bp fragments binding pattern with the 300 bp or 107 bp fragments (Figure 9, 10, and 11) showed that the 407 bp fragments were much more disproportionally retarded by Lrp. This strongly implied Lrp might bend DNA. It did not seem just a mere side effect of DNA-protein complex conformation, but could be of functional significance in biological processes.

bound complex II

bound complex I

Free DNA
(407 bp)

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |

p415P1P2   p415P2   p415P1

Figure 9. DNA binding assay of Lrp to the 407 bp fragments derived from the *serA* wild type promoter and mutants. Lanes 1 to 5: the 407 bp DNA fragments from p415P1P2 plasmids. Lanes 6 to 10: the DNA fragments were yielded from p415P2 plasmids. Lanes 11 to 15: the DNA fragments were yield from p415P1 plasmids. Lanes 1, 6, and 11: No Lrp was added in the reaction. Lanes 2, 7, and 12: Lrp at 1 pmol was added. Lanes 3, 8, and 13: Lrp at 1.8 pmol was added. Lanes 4, 9, and 14: Lrp at 2.2 pmol was added. Lanes 5, 10, and 15: Lrp at 2.6 pmol was added.
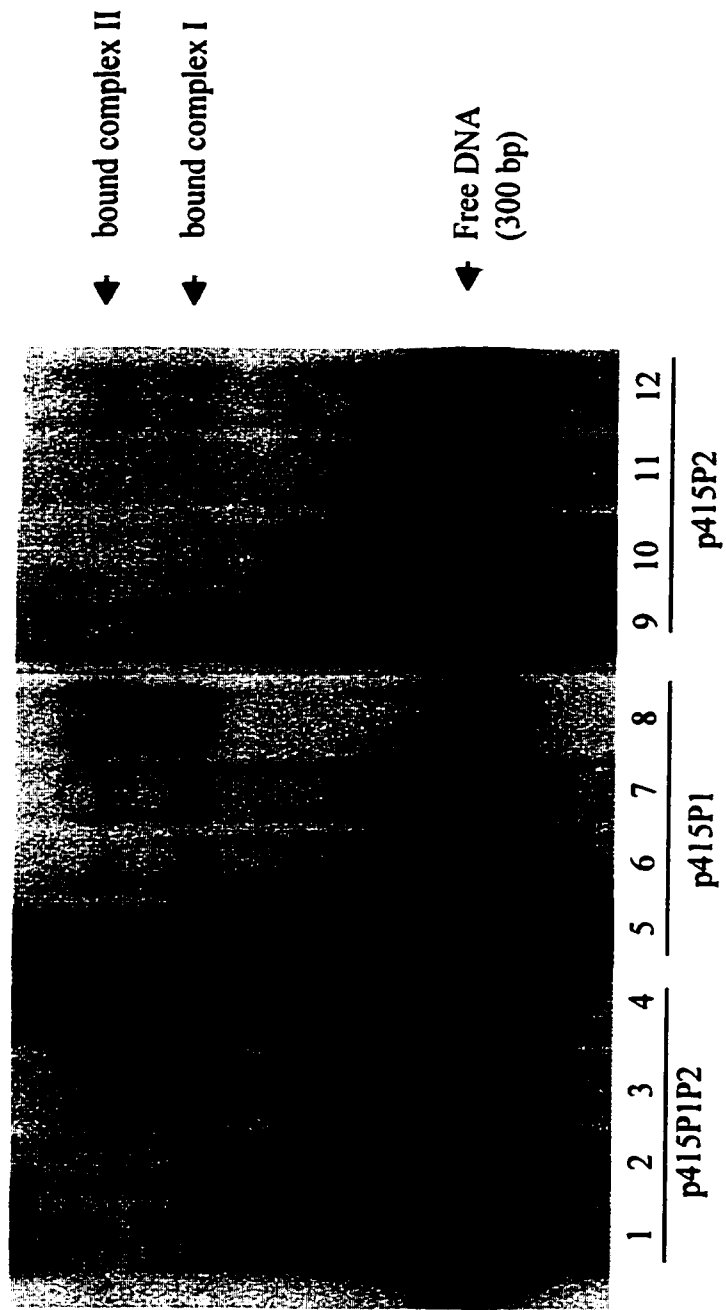
Figure 10. DNA binding assay of Lrp to the 300 bp fragments derived from the *serA* wild type promoter and mutants. Lanes1 to 4: the 300 bp DNA fragments from p415P1P2 plasmids; lanes 5 to 8: the 300 bp DNA fragments were derived from p415P1; lanes 9 to 12: the 300 bp DNA fragments were derived from p415P2. Lanes 1, 5, and 9: No Lrp was added in the reaction. Lanes 2, 6, and 10: Lrp at 1 pmol was added. Lanes 3, 7, and 11 : Lrp at 1.8 pmol was added. Lanes 4, 8, and 12: Lrp at 2.6 pmol was added.
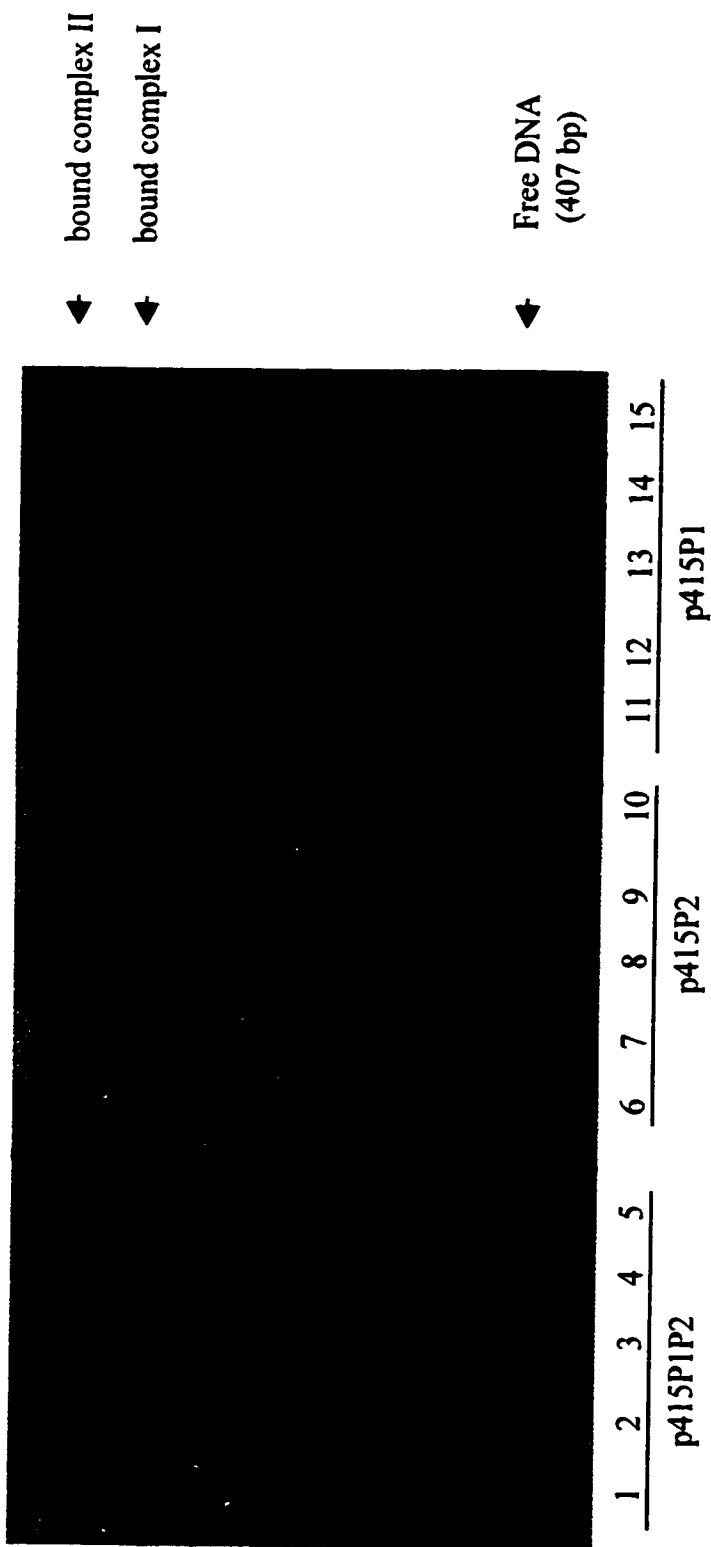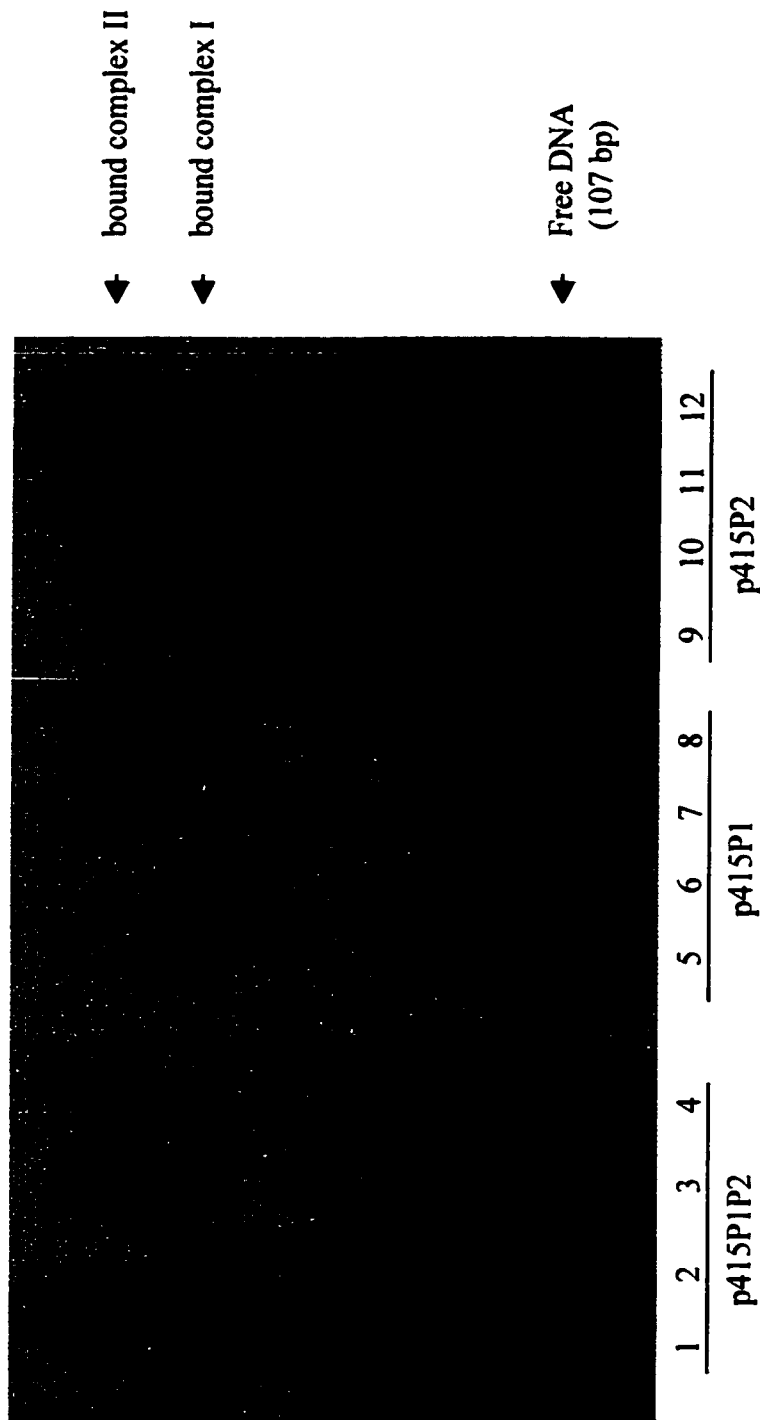
Figure 11. DNA binding assay of Lrp to the 107 bp fragments derived from the *serA* wild type promoter and mutants. Lanes 1 to 4: the DNA fragments were derived from p415P1P2 plasmids. Lanes 5 to 8: DNA fragments were yielded from p415P1. Lanes 9 to 12 : the DNA fragments were yielded from p415P2 plasmids. Lanes 1, 5, and 9 : no Lrp was added in the reaction. Lanes 2, 6, and 10: Lrp was added at 1 pmol. Lanes 3, 7, and 11 : Lrp was added at 1.8 pmol. Lanes 4, 8, and 12 : Lrp was added at 2.6 pmol.

Discussion

The work in this thesis has been devoted to an understanding of regulation of the *serA* gene in *E. coli* K-12. The two promoters of the *serA* gene have been studied independently and the results lead to the hypothesis that in addition to Lrp, cAMP-CRP may be involved in the expression and regulation of the *serA* gene. In this section, I will discuss the possible physiological roles of the two *serA* promoters, and the possible molecular regulatory mechanisms.

Part 1. Physiological role of the two *serA* promoters

1-1. The expression and regulation of *serA*

The *Escherichia coli* metabolism is well regulated. *E. coli* growing in glucose-minimal medium excretes almost nothing into the medium. This is accomplished by a complex of mechanisms which govern the rate of gene expression, the extent to which transcripts are translated, and the activity of enzymes once formed. In this work, I consider control of the transcription of *serA* coding phosphoglycerate dehydrogenase (PGDH), which is the first enzyme in serine biosynthesis. For many amino acids, the most important control to assure an appropriate cellular pool of the amino acid is the feedback by the amino acid on the synthesis and the activity of the first biosynthetic enzyme. The regulation of serine biosynthesis differs from that of other amino acids. L-serine only inhibits the enzyme activity of PGDH, the *serA* gene product (Mckitrick and

Pizer, 1980). Surprisingly, L-serine does not affect *serA* transcription. The amino acids glycine and leucine decrease both transcription and enzyme activity, but serine does not. The work in this thesis has been devoted to describing some factors that do regulate *serA* expression, a question approached here by studying the characteristics of the *serA* promoter.

1–2. The regulation of *serA* by Lrp

The involvement of leucine in the regulation of *serA* transcription suggests a role of Lrp, the usual transducer of leucine effects. Indeed Lrp is a major regulator of *serA* expression: it activates expression, and this activation is reversed by L-leucine. However even without Lrp, the cell is not an auxotroph. The structure of the *serA* gene provides, even in the absence of Lrp, for enough serine biosynthesis to allow the cell to grow, albeit slowly, at temperatures below 37°C.

The presence of Lrp increases the expression of *serA* and removes the stress on serine biosynthesis seen in an *lrp* mutant. In minimal medium with glucose as sole carbon source, almost all *serA* expression originates from the P1 promoter. This is shown by primer extensions in wild-type *E. coli* both here and earlier (Lin, 1992c). In this work, the two promoters were separated and it was possible to show by assaying fused *lacZ* expression that in glucose minimal medium, P1 is transcribed strongly in an *lrp*+ background whereas transcription of P2 is much lower (<15%) (Table 3). In fact, no transcription of P2 could be observed in the primer extension assay (Figure 7).

Thus the major control we have shown is the activation of P1 transcription by Lrp. When Lrp is not present, transcription takes place from both promoters. Expression from P1 is vastly decreased whereas expression from P2 is increased. Total expression (2714 units from P1 and 1470 units from P2, Table 3) is lower than in the *lrp+* synthesizing host, but sufficient to account for the fact that the *lrp-* mutant is not an auxotroph.

P2 is not activated by Lrp, but it in fact is repressed about 3 fold- from 482 units in the wild type to 1470 units in the *lrp* mutant. Thus in the presence of Lrp in glucose minimal medium, P2 is a minor promoter. In the absence of Lrp, P2 accounts for almost 40% of the total transcription, though this total transcription is much lower than in the wild-type.

SerA is not the only biosynthetic gene with two or multiple promoters regulated by Lrp. *IlvIH*, which encodes acetohydroxy acid synthase III which catalyzes the first step in the biosynthesis of branched-chain amino acids, also has two promoters P1 and P2. The structures of the *ilvIH* and *serA* promoters are reasonably similar. *IlvIH* P2 is 60 bp further upstream of P1 and the Lrp binding site centers at −72 overlaps promoter P2. In the absence of Lrp, transcription is initiated *in vitro* at both promoters P1 and P2 of *ilvIH*. The addition of Lrp increased transcription from P1 but decreased transcription from P2 (Willins *et al.*, 1992). The reason for the existence of two or multiple promoters is probably that they are used under different environment conditions such as the expression level of Lrp and allow *E. coli* cells to be able to respond effectively to environmental changes.

Many *E. coli* genes have two or multiple promoters. One of them, the *gal* operon, has two promoters regulated by the extensively studied global regulator cAMP-CRP. The *gal* operon is transcribed from two promoters P1 and P2 separated by 5 bp. cAMP-CRP activates P1 but represses P2. The site to which cAMP-CRP binds to exert such dual action is located at position –41.5. In the absence of CRP, RNA polymerase binds mostly to P2, which is active *in vivo* (Adhya and Miller, 1979). CRP then switches RNA polymerase from P2 to P1 and thus activates P1. Lrp is not likely to work in the same way at *serA* since the separation of the two *serA* promoters (92 bp) is much further than that of the gal operon (5 bp).

L-leucine is a major effector of *serA* transcription, reducing it by 2 folds. However, Leucine reverses the activation of P1 alone but enhances the repression of P2 alone. It is interesting that leucine interact with Lrp in the different pattern on one operon although it is known that leucine can antagonize, potentiate, or has little effect on *lrp*-mediated regulation (Newman *et al.*, 1996). The mechanism by which this interaction takes place is not known. Lrp has been shown to bind L-leucine and this may result in a change in conformation such that its binding site on the DNA changes or that it leaves the DNA altogether. The primer extension experiments reported here show that exogenous L-leucine does not change the transcription start sites of the *serA* gene in any of the growth conditions tested (Figure 7). The effect of leucine might not be to simply remove some Lrp from the DNA by decreasing its binding affinity. This question could be settled by more detailed retardation and footprinting studies.

1–3. cAMP-CRP probably is involved in the regulation of *serA*

This study showed that the absence of Lrp results in the induction of P2 in minimal medium. The expression of *serA* P1P2 and P1 in the total absence of Lrp (i.e. *lrp-* mutant) in minimal media is higher than the expression in the wild type *lrp* strain in LB where some Lrp is made (Table 3). These results suggest that some other factor(s) must activate transcription when Lrp is absent. Some previous data also imply that Lrp is not the only regulator for the *serA* gene. For example, the effect of Lrp on *serA* expression are relatively weak compared to its effects on the other operon (Table 1). The six-fold decrease in an *lrp* mutant does not make the strain auxotrophic for serine in aerobic growth at 37°C (Ambartsoumian *et al.*, 1994). However, no detailed study on the potential regulator(s) has been reported.

Analysis of the nucleotide sequence of the *serA* gene reveals that a putative cyclic AMP (cAMP) receptor protein-cAMP complex (CRP-cAMP) binding consensus sequence is located upstream of *serA*. The match percentage of the consensus sequence (80% matches to the most conserved two CRP binding sequences TGTGA and TCACA), the position of the binding box (located −74 to −52 relative to the P2 transcription start +1 [Figure 7]), and the spacing of two putative consensus CRP binding sites (flanking exactly 6 bp nucleotides which are important for the CRP binding) show that cAMP-CRP may be involved in the regulation of *serA*.

A role of cAMP in the regulation of *serA* is strongly indicated by the increased

transcription from P2 in the Lrp mutant grown in glycerol minimal medium. Glycerol is

known to be one of the least catabolite-repressing of the common carbon sources (Saier *et*

al., 1996). In glycerol-grown *lrp* mutant cells, the expression of *serA* P2 is induced 10-

fold while the expression of P1P2 and P1 alone are elevated relatively slightly. Even in

the *lrp*$^+$ parent, the expression of P2 is elevated in glycerol minimal medium although no

induction of P1P2 and P1 is detectable (Table 4).

This interpretation is supported by the effect of exogenously added cAMP which

increases expression of P2 both in the presence or absence of Lrp even in cells grown in

glucose minimal medium. In the *lrp* deficient host, the expression of P1P2 is also

increased by exogenous cAMP in the glucose minimal medium whereas no change is

observed in the Lrp wild type strain. The addition of exogenous cAMP has little effect on

P1 either in *lrp*$^+$ or *lrp*-host.

The level of *lrp* expression in glycerol is slightly lower than that in glucose (Chen

et al., 1997). Moreover, by comparing the expression of Lrp in cya and crp mutant strains

with that in wild type strains, other investigators suggest that cAMP-CRP complex itself

is not involved in the *lrp* expression (Landgraf *et al.*, 1996). Hence, it can be ruled out

that the induction of *serA* P2 promoter by glycerol and exogenous cAMP is due to the

variation of Lrp itself.

It is clear that under certain circumstances: the absence of Lrp, cAMP-CRP is

involved in the expression and regulation of *serA*. It is no doubt that when Lrp is highly

expressed, Lrp is the main regulator of *serA* expression. However, the expression of *serA*

follows the Lrp level quite closely, decreasing drastically when the Lrp level falls, as

shown by cloning the *lrp* gene on a variable expression vector (Chen *et al.*, 1997). There may then be conditions when Lrp levels fall, and cAMP synthesis increases and assures *serA* expression. The activation of *serA* gene by cAMP-CRP in the absence of Lrp is mainly derived from the activation of *serA* P2.

Earlier investigators showed that exogenous cAMP at 3 mM lowered the phosphoglycerate dehydrogenase (the *serA* gene product) levels about 20% in *E. coli* grown on glucose and thus proposed that cAMP might modulate the *serA* gene products in a negative manner (McKitrick and Pizer, 1980). However, at that time, the existence of Lrp was not known. My data show that the addition of cAMP has little effect on the β–galactosidase activity of *serA* P1P2 and P1 in the presence of Lrp (Table 5). Furthermore studies in the *lrp* deficient strain show that the exogenous cAMP-CRP actually positively controls the activity of the *serA* promoter, especially, P2 alone.

In summary, the physiological significance is that Lrp is a main positive regulator for the *serA* gene while *E. coli* grown in glucose minimal medium. Alternatively, when Lrp is low expressed or absent, cAMP-CRP activates the *serA* gene and thus maximizes the expression of *serA* gene.

It has been reported that both Lrp and cAMP-CRP also play roles in regulating transcription of *serC* by determining differential rates of β-galactosidase synthesis in a merodiploid strain carrying a single-copy *serC::lacZ* operon fusion (Man *et al.*, 1997). *SerC* encodes phosphoserine aminotransferase in *E. coli*, which catalyzes the second steps in the serine biosynthetic pathway (Figure 1). Similar to *serA*, the expression of *serC* is 2-fold induced by Lrp and is reduced 1.7-fold by addition of leucine in glucose minimal medium. Serine has no effect on the transcription of *serC* either. However, the

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repression of *serC* by cAMP-CRP seems not to parallel the activation of *serA* by cAMP-CRP. Unlike *serA*, in which case cAMP-CRP is mainly functional in the absence of Lrp, the activation of *serC* by Lrp and repression by CRP seem to be independent. Studies involving the deletion of putative Lrp and CRP binding box suggested that Lrp regulates *serC* transcription by directly binding to the upstream Lrp box whereas the CRP repression is indirect and involved changes in the amount or activity of a new repressor. Regarding *serA*, Lrp is a direct activator and CRP may also directly activate *serA* P2 although there is no *in vitro* evidence. It is not clear why cAMP-CRP activates *serA* coding the first enzyme in the serine biosynthesis but represses *serC* coding the second enzyme in the serine biosynthesis. It is of physiological significance to learn whether the expression of the third gene *serB* involved in serine biosynthesis is regulated by Lrp and cAMP-CRP together.

More and more genes are reported to be regulated by both Lrp and cAMP-CRP including *dad* (degradative D-amino acid dehydrogenase [Mathew *et al.*, 1996; Zhi *et al.*, 1999]), *daa* (F1845 fimbrial adhesin [Bilge *et al.*,1993]), *osmY* (osmotically induced periplasmic protein [Lange *et al.*,1993]), and *pap* (Pap fimbrae [Van der Woude *et al.*, 1995; Feutrier *et al.*, 1992; Goransson *et al.*, 1989]). However, they have different cooperation pattern. Lrp acts both as a repressor and as an activator of *dad*, which is activated by cAMP-CRP. *Daa* expression is activated by both Lrp and cAMP-CRP, and *osmY* expression is repressed by both Lrp and cAMP-CRP. Lrp can be a repressor or activator of *pap* expression and cAMP-CRP may activates *pap* directly and indirectly. Preliminary *in vitro* experiments suggest that Lrp strongly prevents cAMP-CRP binding to dad promoter DNA (Zhi *et al.*, 1999). However, there is not much detailed study on the

interaction between Lrp and cAMP-CRP. It is still a puzzle whether Lrp and cAMP-CRP are functional cooperatively or independently.

It will be interesting to further study how Lrp and CRP coordinate with each other and maximize the expression of the *serA* gene and the relative contributions of Lrp and cAMP-CRP to the activation of *serA* transcription. It will be helpful to study β-galactosidase activity of *serA::lacZ* in *crp⁻ lrp⁺*, *crp⁻ lrp-*, and *cya⁻* strains.

Part 2. Molecular studies on the mechanism of regulation of *serA*

In the above section, I mainly discussed the physiological roles of the two *serA* promoters. I will discuss the possible molecular regulatory mechanism of *serA* in the following section.

2-1. Molecular studies on the mechanism of regulation of *serA* by Lrp

The overall expression of P1P2 by Lrp is the combination of activation of P1 and repression of P2. To study this in vitro, I destroyed the RNA polymerase (RNAP) binding sites of each promoter separately by site directed mutagenesis, producing constructs in which only P1 or P2 could function. This would not be expected to affect Lrp binding, at least as measured here, in the absence of RNAP, and in fact the affinity with which Lrp bound showed no gross difference in the three constructs (Figure 9, 10, and 11).

The plasmid p415P1 carrying both P1 and mutated P2 does not show any change in binding affinity of Lrp compared to p415P1P2 (Figure 9), the activity of p415P1 is as high as wild type promoter P1P2. However, the deletion of −325 bp to −95 bp relative to P1 transcription at +1, which contains the Lrp high affinity binding site determined by DNaseI footprinting at -155 to -81 (Lin, 1992c), severely reduces the expression of the P1 promoter (Zhang, 1994). These data support that the high affinity binding site is important for Lrp function. This site overlaps the entire P2 RNAP binding site, and the site where P2 transcription initiates. It seems that the most obvious reason that Lrp represses at P2 is that it gets in the way and prevents RNAP binding.

This binding of Lrp, which inhibits P2 expression, results in a large stimulation of transcription at P1. This might come about in several ways. First, Lrp might make contact with RNA polymerase directly, in particular the α carboxy-terminal domain (αCTD). This domain is carried on a flexible linker arm and can contact proteins at a considerable distance. CRP has been shown to interact with αCTD of RNAP when it binds anywhere from -41 to -91 (Zhou *et al.*, 1994a; Zhou *et al.*, 1994b; Zou *et al.*, 1992). Lrp protects a much larger region of DNA at all genes studied (Newman and Lin, 1995), in this case 74 bp, and it takes several dimers of Lrp to do so. It is therefore quite reasonable to think that one of the downstream dimers contacts the αCTD of RNAP, the more so since the binding of Lrp is highly cooperative so that any DNA molecule binding Lrp would have enough Lrp to make that contact.

There are other ways in which Lrp might activate, including DNA bending. Lrp is known to bend *ilvIH* DNA, and there seems some indication in the anomalous migration of a 407 bp fragment compared to 300 bp and 107 bp fragments that Lrp may also bend

serA DNA. DNA may be wrapped around the Lrp molecule in such a way as to bring an upstream sequence in contact with RNAP, or it might result in other downstream conformational changes which activate transcription.

It has been known that Lrp has three domains, which is based on the studies of *ilvIH*: a DNA-binding domain, a transcription activation domain, and a leucine response domain. Recently, a region(s), which is specifically required for Pap phase variation to help define the mechanisms by which Lrp responds to PapI and the *pap* DNA methylation state, has been identified. It will be of interest to determine if other operons in the Lrp regulon, besides the Pap-like family of fimbrial operons, code for regulatory proteins that can interact with Lrp and modify Lrp binding.

2-2. Possible molecular regulatory mechanism of *serA* by CRP

CRP is likely to regulate the *serA* gene directly since a CRP binding box can be recognized at the *serA* upstream although there is no *in vitro* evidence of CRP binding to the *serA* promoter. The CRP binding box locates at −166 to −144 from P1 and -74 to −52 from P2. This suggests that CRP should regulate P2 transcription since CRP sites are normally located at −41.5, -61.5, and −70.5 respectively for *gal, lac, malT* operon (Kolb *et al.*, 1993). Nonetheless, the CRP binding box locates relatively far from P1 promoter. This may also account for the little affect of CRP on P1 since the extent of CRP-dependent promoter activation decreases with increasing CRP-binding site distance from the start site of transcription (Gaston *et al.*, 1990).

The high affinity Lrp binding site on *serA* extends from -155 to −81 relative to the P1 transcription start site +1, covering P2 promoter region. The location of CRP binding

site is located by examination of the sequence at –166 to –144. A competition between the two is made likely by the overlapping of these sites. The affinity of the two regulators is not determined in detail. However, the fact that cAMP effects are observed mainly in the absence of Lrp seems to mean that Lrp binds more strongly, though other factors might intervene in vivo. While intracellular Lrp is low or absent, cAMP-CRP can efficiently bind *serA* upstream, -74 to –52 relative to P2 transcription start site +1, and thereby activates P2 promoter.

To investigate whether the cAMP-CRP complex might regulate *serA* operon transcription by direct interaction with its putative recognition sites in the regulatory region of *serA*, one can alter the CRP binding box on the *serA* gene by site-directed mutagensis. Thereby, one can determine the affect of the mutation on the expression of *serA in vivo* by measuring the β-galactosidase activity. Primer extension can be performed to reveal the regulation of *serA* from the transcription level. DNase footprinting and gel retardation experiments can also be carried out to provide the molecular evidence of cAMP-CRP binding to *serA*. It will be also interesting to compare the binding affinity of Lrp or CRP to the *serA* gene.

Summary

SerA encodes phosphoglycerate dehydrogenase, the first enzyme in the serine biosynthesis in *E. coli*. It has two promoters P1 and P2 which is 92 bp upstream of P1. It has been suggested that the global regulator Lrp activates P1 but represses P2. However, there is no direct evidence for it, and very little is known how the two *serA* promoters function in different growth conditions.

To understand how these two promoters are mediated in different conditions by Lrp, and whether any other regulator is involved in the expression and regulation of the *serA* gene in addition to Lrp, I mutated *serA* P1 and *serA* P2 separately by site-directed mutagenesis and studied their activity independently. The native *serA* promoter and mutants were fused to the *lacZYA* operon. The effects of these mutations on the start-site used for transcription were determined by RNA primer extension while their effects on transcription *in vivo* were examined by the activity of the β-galactosidase reporter gene. Finally, gel mobility shift assays were carried out to investigate the interaction of the *serA* promoters and the Lrp protein.

In this work, I confirm that Lrp activates the overall of expression of *serA* and leucine reverses the activation in glucose minimal medium. *SerA* P1 is a major promoter, which is activated by Lrp, whereas P2 is repressed by Lrp. Leucine reduces the activation of P1 and enhances the repression of P2. In the absence of Lrp, transcription of *serA* takes place from both promoter P1 and P2.

It is no doubt that Lrp is a major regulator of expression of *serA*. A discovery in this study is that in addition to Lrp, another global regulator CRP in *E. coli* is also involved in the regulation of *serA* at transcriptional level. By measuring the β-

galactosidase activity of *serA::lacZ* fusions in different carbon source and with exogenous cAMP, the results suggest that cAMP-CRP is involved in the regulation of transcription of the *serA* gene via activation of P2, at least in the absence of Lrp, while cAMP-CRP has little effect on P1.

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