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Leucine/Lrp regulon: Regulation of *serA* gene by Lrp

Jia Zhang

A Thesis

in

The Department

of

Chemistry and Biochemistry

Presented in Partial Fulfilment of the Requirements

for the Degree of Master of Science at

Concordia University

Montréal, Québec,, Canada

May 1994

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ABSTRACT

Leucine/Lrp regulon: Regulation of th serA gene by Lrp

Jia Zhang
Concordia University, 1994.

The in vivo effect of varying the concentration of Lrp (leucine-responsive regulatory protein), via a pBAD-lrp fusion plasmid was studied. It was particularly interested in the effect of Lrp on serA regulation. A direct proportionality between Lrp concentration and serA expression was shown.

Point mutations were introduced into the serA promoter region. The Lrp-mediated regulation of the mutated serA promoter was different from that of the wild-type. Some of the mutated serA fragments also showed changes in Lrp affinity when compared with the wild-type. Two mutants, UP1-2 and UP2-1, which were mutated in the P1 and P2 promoter regions respectively, caused a severe decrease in serA expression and reduced the binding affinity of Lrp to the serA promoter.

serA P1 and P2 promoters were subcloned separately into a lacZ-based operon fusion vector, pRS415. The regulatory studies revealed that the P1 promoter was activated by Lrp, while L-leucine partially reversed the activation. In contrast, Lrp repressed the P2 promoter and L-leucine partially reversed the repression. The Lrp binding sites in the serA promoter region are required for promoter regulation.

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INTRODUCTION

Escherichia coli responds rapidly to environmental changes, inducing some genes, repressing others, and even inactivating some existing proteins. Each of those responses is governed by a specific regulator. A regulator can usually respond immediately to environmental changes by altering its own level of expression, and then regulating other genes in the response group. This rapid response was named the global response phenomenon.

Global response operates by means of various mechanisms. Regulation can occur via a transcriptional repressor (LexA), a transcriptional activator (PhoB), a combined repressor /activator (Crp), or even by changing the RNA polymerase σ factor. The genes in each global response system usually share some common features. In the catabolite repression regulon, for example, almost all of the operons are involved in carbon utilization, and the promoters of the genes share a consensus binding sequence for the Crp regulator.

The Leucine/Lrp regulon is a recently discovered global response system in *E. coli* (Lin, 1990). It is regulated by the leucine responsive regulatory protein (Lrp). Exogenous L-leucine often, but not always, affects the expression of the genes that are regulated by the Lrp protein. The leucine/Lrp regulon is different from other regulons. First, the genes in

this regulon are from disparate sources or families, and it is difficult to determine the relationship between them. Secondly, the mechanism of this system is complex. *In vitro* studies of some genes in this regulon showed that the promoter regions of those genes can bind specifically to Lrp protein. However, there is no convincing consensus sequence for the Lrp protein.

As a conservative estimate, comparing wild-type and *lrp* mutant extracts, there are approximately 30 proteins that have quantitatively detectable differences on two-dimensional gels (Ernsting, 1992). Several Lrp regulated genes are known: *serA* (encoding phosphoglycerate dehydrogenase, the first enzyme in the serine and glycine biosynthesis pathway); *glnA* (encoding glutamine synthetase); *gltBD* (encoding the small subunit of glutamate synthase); *lysU* (encoding lysyl-tRNA synthetase II); *tdh* (threonine dehydrogenase) and *kbl* (2-amino-3-ketobutyrate coenzyme A ligase), the enzymes of the pathway converting threonine to glycine; *ilvIH* (encoding acetohydroxyacid synthase III, an isozyme involved in branched-chain amino acid biosynthesis); *livJ/K* (proteins involved in transport of branched-chain amino acids); *ompC* and *ompF* (encoding outer membrane porins); *oppABCDF* (encoding proteins involved in oligopeptide transport); *sdaA* (encoding L-serine deaminase); *gcv* (encoding glycine cleavage protein); *malEFG, malK, lamB, malM,* and *malT* (genes for maltose metabolism); *lacZYA* (genes for lactose metabolism); *papBA*, *fan, daa, sfa, fae,* and *cis-5;*

the operon involved in serine transport; the gene coding for W protein; and *lrp* itself.

In this thesis, I present a study of the regulation of *serA* by the Lrp protein together with L-leucine, both *in vitro* and *in vivo*. The work presented in this thesis originated from the previous work of Dr. R.T. Lin. His *in vivo* studies show that the deficiency in *lrp* function in an insertion mutation decreases the expression of the *serA* operon. The *in vitro* studies show that *serA* has two transcriptional start sites. Each of them is regulated by Lrp via different mechanisms, and the Lrp protein must therefore bind to at least two sites in the upstream regions of *serA* genes (Lin, 1992c).

This work began with a study of molecular aspects of the *serA* gene. First, the *serA* promoters were subcloned into the vector, pRS415 to study promoter activity. The insertions were confirmed as *serA* by sequencing. Then the expression of *serA::lacZ* was assayed in both wild-type and *lrp*-deficient strain.

In vitro DNA mutagenesis was performed on the *serA* promoter region. The mutations were screened by double-stranded DNA sequencing and the changes in promoter activity of those mutations were studied. Furthermore, the Lrp protein was partially purified, and gel retardation was performed to show the affinity changes of the mutated *serA* fragments.

The expression of *serA* was also studied as a function of Lrp concentration. pBAD-*lrp* fusion plasmids were created that

enabled us to control the level of Lrp expression, by varying the concentration of arabinose given to the cells. By *lrp::lacZ* fusions on these plasmids, the expression of Lrp in the presence of arabinose could then be studied. The expression of the *serA* gene in the presence of the pBAD-*lrp* fusion plasmid was then studied. This work shows that the expression of *serA* is directly proportional to the expression of *lrp*. The goal of this work is to understand Lrp regulation mechanism on the *serA* gene.

In order to provide a background to this study, I will first survey global regulatory systems in general and discuss the possible mechanisms of those regulatory systems. Part II of this introduction will describe the L-leucine/Lrp regulon. In the final section of the introduction, part III, I will review the experimental evidence related to the *serA* gene regulation.

Part 1. A survey of global regulatory system

Under different environmental conditions, there is a great variation in the number of different molecules in bacterial cells. To make this possible, there must be devices to ensure the selective synthesis of the proteins that are required under certain conditions; and there must be ways of rapidly changing the relative numbers of protein molecules in response to the nutritional or other environmental changes.

A factor that mediates transcriptional regulation in a global response is called a regulatory transcriptional factor, it can be either a small molecule such as cyclic-AMP, or a protein (CRP). The regulation of genes involved in a single metabolic pathway (such as the lactose operon) were previously noted and studied. Later on, it was discovered that a single regulatory factor, responding to certain environmental changes, can control the expressions of a group of genes from several pathways. The term "regulon" was 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 recognized in enteric bacteria.

Different global response systems can function in different ways. In the following sections, we will briefly review the different types of global response systems.

1-1 Control by a transcriptional repressor

The first known transcriptional regulators were isolated in 1967: the *lac* and λ repressors. Like most transcriptional repressors that have been studied, both of them selectively bind at specific sites of their respective genes or operon and block the initiation of transcription. A repressor may control a single operon or a global regulon.

1-1a. LexA, the repressor of the SOS Regulon

The expression of the genes in the SOS regulon is controlled by complex circuitry involving protein LexA and RecA. In an uninduced cell (i.e., not heat shocked), the LexA protein acts as a repressor of SOS regulon. A great number of unlinked genes in the SOS regulon including *recA* and *lexA* are repressed by the *lexA* gene product. *In vitro* studies showed that LexA protein represses the transcriptional initiation of the SOS genes.

LexA represses a group of genes by interacting with operators in very different positions relative to the promoter (from 35 bases upstream to downstream of the transcriptional start site). The consensus sequence for a LexA-binding site is TACTNATATA-A-ACAGTA (Walker, 1984). It was suggested that LexA binding at the upstream -35 region might preclude the binding of an unknown activator (Brandsma, 1985) and binding

downstream of the transcriptional start site might not affect the open complex formation, but rather interfere with promoter clearance (Easton, 1983).

1-2 Control by transcriptional activator

Similar to the transcriptional repressor, a transcriptional activator can also selectively bind at specific sites of certain genes or operons, but instead of blocking the transcriptional initiation, it may change the DNA conformation and make the DNA more amenable for RNA polymerase binding and transcription initiation. Like a repressor, an activator can control a single operon or a global regulon.

1-2a. PhoB, phosphate regulatory activator

Phosphorus compounds are essential constituents in biological organisms and phosphate is a abundant element in nature. However, for many organisms including *E. coli*, phosphate is a growth limiting factor as much of the organism's natural supplies are present as insoluble salts, and thus cannot be utilized. To overcome this, *Escherichia coli* and many other bacteria have evolved complex regulatory systems to assimilate phosphorus very efficiently. The regulatory system includes more than 20 phosphate regulated promoters, over 10 regulatory genes and two phosphate

transport systems. One of the regulatory activators in this system is PhoB protein.

Unlike the repressors we have just discussed, PhoB regulates the phosphate regulated promoters in a more complicated way. PhoB is a transcriptional activator at the promoters of *phoA/B/S* and several other genes. In most cases PhoB needs to combine with some other factor(s) to produce an effect. For example, PhoB along with either PhoR or PhoM acts as a transcriptional activator for a group of genes (*phoA, psiB/C/D*) (Shinagawa, 1983 and Wanner, 1981). However, *psiE/H* promoters show PhoB- and PhoR-dependent transcription but it is not PhoM independent (Wanner, 1982 and 1983) This suggests that PhoB may recognize different a promoter along with PhoR or PhoM. In addition, the fact that the phosphate regulation system regulates individual promoters differently may imply that related genes may be arranged in a regulon, instead of an operon; this allows the independent control of their expressions.

1-3 Control by a combined activator/repressor

Recently, more regulatory proteins have been found that were not simply activators or repressors, but that had both functions. For instance, Crp and Fnr which were thought of only as regulatory activators, are in fact bifunctional regulators.

1-3a. Catabolite gene activator protein of carbon utilization system

E. coli is able to grow in minimum medium using one of a large variety of organic compounds as sole carbon source. Judging from the rates of growth in such minimal media, glucose is the most preferred carbon source. Due to the special position of glucose, the enzymes necessary to obtain energy and carbon building blocks from glucose are constitutively synthesized even when there is no glucose present in the media. On the other hand, the presence of glucose in the medium prevents or reduces the expression of the enzyme and uptake systems for the utilization of other compounds as a carbon source. This phenomenon was named catabolite repression. Catabolite repression is under the control of a global regulatory system, the cAMP-CAP regulon. In this regulon, cAMP serves as the metabolic signal and CAP serves as the regulator responsible for catabolite repression. The operons involved in the carbon utilization regulon share a DNA recognition sequence for CRP.

As a metabolic signal, cAMP can be quickly synthesized and degraded inside the cell, or secreted out of the cell. Its level is very low during growth on glucose and the changing of cAMP level affects the level of cAMP-CRP, as binding of cAMP to CRP is a reversible reaction (Ullmann, 1983 and Botsford, 1981).

Upon binding to cAMP, the CRP homodimer undergoes a conformational change, which allows binding of the protein to CRP specific DNA binding sites. The distance between the CRP binding site and the transcriptional starting site is variable from one operon to another. However, for each specific promoter, this distance may be critical, for the angular orientation of the bound CRP to the bound RNA polymerase may be a deciding factor (Straney, 1989 and Gaston, 1990). CRP may activate transcription by inducing a bend in the DNA and/or interacting with RNA polymerase directly (Adhya, 1990).

Almost all operons regulated by CRP or subjected to catabolite repression are positively regulated by CRP. The *galA* operon, in addition, is both positively and negatively controlled by CRP (*gal* P1 is positively controlled by CRP, whereas *gal* P2 is negatively regulated) (Herbert, 1986). Other operons, such as those encoding the five enzymes that interconvert glutamate and glutamine as well as some outer membrane proteins, are negatively regulated by CRP. The cAMP-CRP complex also negatively regulates the transcription of the *cya* and *crp* genes, thus the expression of both genes is autogenously regulated.

1-4. Regulation by alternating the regulator

Another kind of regulation mechanism involves changing the regulators in response to changes in environmental conditions. The use of alternative σ factors is one example of this type of regulation.

RNA polymerase has been purified *in vitro.* In most of the cases it contains five subunits. But for proper function, the sixth subunit, σ factor, is needed. σ70 was the first known σ factor in *E. coli*. Most genes expressed in exponentially growing cell are transcripted by Eσ70. Since then 5 other σ factors have been found (Helmann, 1988, Lonetto, 1992, and Tanaka, 1993).

The product of the *rpoH* gene, σ32, is required for the expression of a class of genes in responding to heat shock by combination with core RNA polymerase ($\alpha_2\beta\beta'$). The product of *rpoH* binds quite tightly with the core enzyme and it was copurified with core. At extremely high temperature, another σ subunit, σE is synthesized to respond to the environmental changes.

The σ factor controlling the expression of genes involved in the fixation and assimilation of nitrogen is the product of *rpoN* gene. This σ subunit does not form a stable holoenzyme complex, but it can bind to some cognate promoters in the absence of core enzyme.

The *rpoS* product, σ38, is a member of the σ70 family

(Mulvey, 1989). Genes expressed only in stationary growth phase can be recognized only by Eσ38 and it is essential for the expression of at least some stationary phase specific genes.

The *rpoF* gene coding for another **σ** subunit, **σ**28, is needed for transcription of a set of genes involved in formation of flagella.

It was suggested, that each of these different **σ** factors can bind interchangeably to the core RNA polymerase, and confer upon it abilities to recognize a different set of promoter sequences; this constitutes a system of positive control.

Part 2. L-leucine/Lrp regulon

It took a long time to recognize the Leucine/Lrp regulon. The earliest studies on this regulon were mediated by L-leucine. Changes in the level of expression of a number of genes were found in the presence of L-leucine in the growth medium (Fraser, 1976). This first suggested that the branched-chain amino acid L-leucine might be the effector of a global response system in *E. coli*. Recently, a mutant named *rbl* was isolated, and it was shown that the expression of several leucine-regulated genes in this mutant is no longer affected by exogenous L-leucine. (Lin, 1990). Several other genes were identified separately as regulators of L-leucine-sensitive operons and have been shown to be identical to *rbl*. These include *ihb* (Ricca, 1990), *livR* (Anderson, 1976), *oppI* (Andrews, 1986 and Andrews, 1985). There was finally agreement that the designation *lrp* (Leucine response regulatory protein) is to be used in the future.

2-1 Classification of the operons in the L-leucine/Lrp regulon

In part I, we have surveyed transcriptional regulatory systems. Needless to say, a transcriptional regulatory system is regulated by a transcriptional regulator. The regulator can be a repressor, an activator, or can have a combination of these two functions. The L-leucine/Lrp regulon is apparently more complicated than the systems we just mentioned. First, it has two regulatory factors: the Lrp protein and exogenous L-leucine. But unlike cAMP and CRP, Lrp and leucine can function together or separately; for some genes they regulate in a similar manner, and for others the two factors may function in an opposing manner. In addition, the rationale behind the constituent genes in this regulon is obscure. As mentioned in the early sections, the genes in this regulon come from quite different operons and have various functions.

In order to place the genes of the leucine/Lrp region in some kind of order, I will try to classify the operons and put the genes into different classes. In the following section, the operons of Lrp regulon will be classified in two ways: by the manner of regulation and by the functions of the genes in the regulon.

2-1a Classification by system of regulation

According to their means of regulation, the Lrp/L-leucine regulon can be divided into the following broad categories.

I. Lrp acts negatively

A. L-leucine reduces the effect

B. L-leucine has no effect

II. Lrp acts positively

A. L-leucine reduces the effect

B. L-leucine has no effect

III. Lrp requires L-leucine to function

A. negatively

B. positively

Table I1. Classification of the Lrp regulon by system of regulation

Lrp	+ L-leucine	regulated genes	function of the genes
repressor	reverses	*oppABCDF*	(oligopeptide transportation)
		sdaA	(L-serine biodegradation)
		tdh/kbl	(L-threonine biodegradation for glycine biosynthesis)
		lysU	(for lysyl-tRNA biosynthesis)
	no effect	*ompC/F*	(structure gene, function of the omsolarity of the growth medium)
		lrp	(regulatory gene for Lrp regulon)

		fae (phase variation gene)
activator	reverses	*ilvIH* (acetolactate synthase)
		serA (first enzyme in L-serine and glycine biosynthesis pathway)
		gltB (for glutamate biosynthesis)
		leu (biosynthesis enzymes for branched chain amino acid metabolism)
		glnA (encodes glutamine synthetase)
		fanABC (K99 pili)
		malEFG (transport)
		malK lamB malM (transport)
	no effect	*papBA* (Pyelonephritis-associate pili)
		gcv (Glycine cleavage)
		W protein
		papBA (phase variation gene)
		daa, sfa (phase variation gene)
		malT (regulatory gene)
		fim DNA inversion
together with leu	represses	*livJ, livKHMGF* (isoleucine, valine, and leucine uptake)
	activates	*sdaB* regulatory gene

2-1b Classification by the function of the genes in the regulon

Another means of classification is by the function of the genes in the regulon. The genes in this regulon can divided into 4 classes.

I. genes related to branched chain amino acid metabolism

II. genes involved in biosynthesis

III. genes involved in biodegradation

IV. genes involved in transport

Table I2. Classification of the genes in the Lrp regulon by the function of the genes

function of the gene product	gene name	Lrp	+L-leucine
Branched chain amino acid metabolism	*ilvIH*	activator	reverses
	leuABCD	activator	reverses
genes involved in amino acid biosynthesis	*serA*	activator	reverses
	gcv	activator	no effect
	glnA	activator	reverses

	gltB/D	activator	reverses
carbon source utilization	*malEFG*(transport)	activator	reverses
	malK lamB malM	activator	reverses
	malT (regulatory gene)	activator	no effect
	lacZYA	activator	reverses
phase variation regulation	*fanABC*	activator	reverses
	papBA	activator	no effect
	daa,sfa	activator	no effect
	fim (switch)	activator	ND
	fae	repressor	no effect
	cis-5	repressor	ND
biodegradation	*sdaA*	repressor	reverses
	tdh/kbl	repressor	reverses
transport	*oppABCD/I*	repressor	reverses
	livJ/K	no effect	repressor
	ompC/F	repressor	no effect
	lysU	repressor	reverses
regulation	*lrp*	repressor	no effect

sdaB (regulatory gene)	no effect	activator
gene coding for W protein	activator	no effect

The genes for biodegradation, transport and the *lrp* gene itself are negatively regulated by Lrp. Others, such as genes related to phase variation and some regulatory genes are more difficult to put into specific classes. Of course, the regulon must be much more complicated than our classification indicates; further study and understanding are needed for this regulon.

2-2. Molecular studies of L-leucine/Lrp regulon

In this part, I will review what is known about the mechanisms of the L-leucine/Lrp regulon and the molecular level studies for the individual genes in the regulon.

2-2a Lrp binds to the promoter region of its regulated genes

Several genes in this regulon have been tested with regard to their binding affinities for the Lrp protein. Lrp was reported to bind to the promoter regions of those genes with various affinities. In the following part, I will give a brief review about these studies.

2a-1 Studies on the *ilvH* regulatory region

The *ilvH* operon is one of the most extensively studied operons in the leucine/Lrp regulon. It codes for one of two acetohydroxyacid synthases (AHAS isoenzyme) which catalyze the first step of branched chain amino acid biosynthesis. The transcription of the *ilvH* operon is activated by Lrp, and the activation is released by exogenous L-leucine.

Lrp has been shown to bind specifically to the upstream region of *ilvH* operon *in vitro*. There are three binding regions for Lrp on the *ilvH* upstream region, -260 to -210 , -140 to -90 and -80 to -40. Binding of Lrp to *ilvH* upstream region was reduced by adding L-leucine. This result supports the *in vivo* regulation results (Ricca, 1989).

2a-2. Other genes

There are other genes in the regulon which have also been tested for binding activity with Lrp.

The *lysU* gene which codes Lysyl-tRNA synthetase (LRS) isozyme II was shown to be negatively regulated by Lrp. Gel retardation and DNase I digestion protection results showed that the Lrp protein binds to the upstream region of *lysU*, from -60 to -160.

The *sdaA* gene which codes L-serine deaminase was shown to be negatively regulated by Lrp, and it was also shown to

interact with Lrp by both gel retardation and DNase I digestion protection; these experiments show that the binding activities were reversed by adding the L-leucine into the reaction mix. The footprint shows that there are two high affinity binding regions of Lrp on *sdaA* and they are 78 and 40 basepairs respectively.

Deletion analysis showed the Lrp binding site of *tdh* to be located at the promoter region, from -44 to -69 (Rex, 1991).

In addition, the *serA* gene was also shown to interact with the Lrp protein, this will be discussed in detail in a later section.

2-2b. Mutation studies on Lrp

Lrp protein may consist of different functional domains. This hypothesis is supported by the results of randomly mutating Lrp. (Platko, 1993 and Willins, 1991). Three groups of mutants were isolated on the basis of their effects on the *ilvIH* operon; the first group (the leucine response mutants) were resistant to leucine repression. These mutants had changes in the Lrp amino acid sequence from amino acid resides 108 to 149. Another group of mutants (DNA-binding mutants) reduced the affinities of Lrp for the *ilvIH* DNA, and reduced the expression of *ilvIH*. The amino acid changes of this group of mutants were from 16 to 70. The third group of mutants also

reduced the expression of *ilvIH*, but the affinity between the DNA and the protein did not change, this group of mutants were named activation mutants, as they changed the activating ability of Lrp. The amino acid changes in this group of mutants were from 76 to 125.

Another study on Lrp (Wang, 1993b) showed that Lrp can induce DNA bending, but the function of this bending still remains unknown. Does this bending activate or repress gene expression by changing the conformation, and possibly improving the interaction between the regulatory factors and RNA polymerase, or does it store elastic energy and activate the RNA polymerase directly?

These data improve our understanding of Lrp, but further studies are still needed.

Part 3 The *serA* gene and its association with Lrp

The *serA* gene encodes phosphoglycerate dehydrogenase, the first enzyme in the biosynthetic pathway of serine and glycine. It is one of the genes in the L-leucine/Lrp regulon.

3-1 Physiological studies of the *serA* gene

The regulation of serine biosynthesis differs from the regulation of other amino acids. The enzyme level is not reduced by growth in a medium that contains the end-products of the pathway, nor is it elevated when the growth rate is limited by the supply of serine. In fact, the end-product inhibits the phosphoglycerate dehydrogenase activity rather than the amount of the biosynthetic enzyme.

However, phosphoglycerate dehydrogenase levels are subject to regulation. Lower enzyme levels were found in cells when certain amino acids including L-leucine were present in the growth medium. In addition, the *serA* gene was found to be activated by Lrp protein.

3-2. Regulation of *serA* by Lrp

serA is one of the genes in the L-leucine/Lrp regulon. It is positively regulated by Lrp and the effect of Lrp on *serA* is reduced by L-leucine.

3-2a. *serA* has two transcriptional start sites

Primer extension analysis shows that the *serA* gene has two transcriptional start sites, and the types of control that occurs at these two sites are quite different. According to the primer extension results, the P1 promoter was highly expressed in the wild-type strain, and in minimal medium, where *lrp* is highly expressed, and in these conditions the P2 promoter had almost no expression. In *lrp*-mutated strain, or LB medium, the expression of the P1 promoter was considerably reduced and the P2 promoter was expressed (Lin, 1992c).

3-2b. Lrp binds at least two sites on the *serA* promoter region

According to the gel retardation experiment, Lrp binds at least two sites on the *serA* promoter region with different affinity. The high affinity region covers the P2 RNA polymerase binding site. The low affinity one is downstream of the P1 promoter.

I began my studies by subcloning the *serA* promoters into the operon fusion vector and examining their regulation. Mutations on the promoter region were constructed later on, and the effect of those mutations were studied both *in vivo* and *in vitro*. The studies are intended to provide further data for better understanding the mechanism of the *serA* regulation by Lrp.

MATERIALS AND METHODS

Part 1. Strains and Plasmids

The strains and plasmids used in this study are described in Table M1.

Part 2. Media and Growth Conditions

2-1. Glucose Minimal Medium

0.527% KH_2PO_4, 1.500% K_2HPO_4, 0.020% $MgSO_4$, 0.001% $CaCl_2$. For solid medium, 0.8% gelrite was added.

0.2% sterile D-glucose was added after autoclaving (+NGlu). 50μg/ml L-isoleucine and L-valine were added to all media used to grow strain MEW1 and all its derivatives to compensate for the deletion in *ilvA* carried by these strains (NIVGlu).

2-2. SGL Medium

Medium with a combination of L-serine, glycine and L-leucine as the only carbon sources other than L-isoleucine and L-valine is called SGL medium. L-Serine, glycine and L-leucine were usually provided at 2,000, 300, and 300 μg/ml, respectively.

Table M1. Bacterial Strains, Phages, and Plasmids

Strains	Genotype and/or relevant characteristics	Source or reference
MEW1	Cu1008 *lac*	Newman, 1985
MEW26	MEW1 *lrp*::Tn10	Lin, 1990
P90C	F⁻ *ara* Δ(*lac, proB*) *thi*	C. Cupples
CP54	MEW1 *ilvA::lacZ*	Lin, 1992a
XL-1	*recA⁻ lac⁻ hsdR⁻* (F' *proAB* *lacIQ lacZ* M15, Tn10)	Stratagene Co.
MEWS2	MEW1 *serA::lacZ*	Z.Q. Shao
MEW22	MEW1 *sdaA::lacZ*	Su, 1989
MEW1A	MEW1 *ara⁻*	This study
CA	MEW1A *lrp*::Tn10	This study
CAS2	CA *serA::lacZ*	This study
CAP22	CA *sdaA::lacZ*	This study
Plasmids		
Bluescript KS+ and KS-,		Stratagene Co.
pRS415		Simon, 1987
pBAD18		Guzman, 1992
pGU2	Bluescript⁺ carrying 1.3 Kb *Hind*III-to-*Bam*HI fragment from pGT17	Lin, 1992c
WSA1	pRS415 carrying 1.3 Kb *Hind*III -	

	to-*Bam*HI fragment from pGU2	This study
SAP2	pRS415 carrying 0.9 kbp *Hind*III to *Ssp*I fragment from pGU2	This study
BSWSA2	pBluescript carrying 380 bp *serA* promoter region	This study
BSSA1	pBluescript carrying *serA*P1 region	This study
BSSA2	pBluescript carrying *serA*P2 region	This study
WSA2	pRS415 carrying *Eco*RI-to-*Bam*HI region from BSWSA2	
SA1	pRS415 carrying *Eco*RI-to-*Bam*HI region from BSSA1	
SA2	pRS415 carrying *Eco*RI-to-*Bam*HI region from BSSA2	
BSMUT*[a]	pBluescript carrying 380 bp *serA* promoter region with mutation on the *serA* region	This study
MUT*[a]	pRS415 carrying *Eco*RI -to-*Bam*HI region from BSMUT*	This study
MT1	pBAD18 carrying *Eco*RI to *Hind*III fragment from pLR3	R.T. Lin
MT3	pBAD18 carrying *lrp* coding region and 16 bp upstream of the ATG site which contain three bases change	Z.Q. Shao
MTΔ	pBAD18 carrying *lrp* coding region	Z.Q. Shao
MT1*lrp::lacZ*	pBAD18 carrying in frame fusion of the *lrp::lacZ* gene from MT1	Z.Q. Shao

MT3*lrp::lacZ*	pBAD18 carrying in frame fusion of the *lrp::lacZ* gene from MT3	Z.Q. Shao
MTΔ*lrp::lacZ*	pBAD18 carrying in frame fusion of the *lrp::lacZ* gene from MTΔ	This study

a: MUT* stands for: UATG1, UATG2, DP1-1, UP1-1, UP1-2, UP1-3, UP2-1, and UP2-2

2-3. NSIV Medium

Medium with 0.2% of L-serine to replace D-glucose as sole carbon source.

2-4. Luria Broth

1% bactotryptone, 0.5% yeast extract, and 0.5% sodium chloride. For solid medium, 1.8% bactoagar was added.

2-5. Medium for Plasmid Isolation

Minimal medium as described above; 0.2% glycerol instead of D-glucose; 0.5% bactotryptone; 0.25% yeast extract; 0.5% sodium chloride.

2-6. Medium for growth of P1 phage

1% Bacto-trytone, 1% Bacto-yeast extract, 0.8% NaCl.

1.7% Bacto-agar for plate and 0.6% Bacto-agar for top agar. $CaCl_2$ and glucose were added to the top agar to the concentration of 2mM and 0.1% respectively before use.

2-7. Other Additions to the Medium

Antibiotics were used at the following concentrations, in µg/ml: ampicillin (Amp) 100, tetracycline (Tet) 15, kanamycin (Kan) 80.

Part 3. Enzyme Assays

3-1. β-Galactosidase Assay

Cells were growth to the log-phase in the test medium. β-galactosidase activity was assayed in whole cells according to the method described by Miller and expressed in Miller units (Miller, 1972).

3-2. Protein Assay

Protein concentration was determined by the method of the Bradford assay (Bradford, 1976).

Part 4. Transformation and Transduction

Transformation was performed according to the methods described by Maniatis (Maniatis, 1982).

P1-mediated transduction was carried out by the method described by Miller (Miller, 1972).

Part 5. DNA Isolation and Restriction Digestion

5-1. Plasmid DNA Isolation

"miniprep" of plasmids was carried out by the method of Maniatis (Maniatis, 1982).

Large amounts of high-quality plasmid, which are used in subcloning, sequencing, are isolated by the method used by Su (Su, 1991).

5-2. Restriction Digestion

Restriction enzyme diggestions were carried out as described by Maniatis (Maniatis, 1982).

5-3. DNA Recovery from Agarose Gels

The DNA fragment over 1 kbp was isolated by the protocol indicated in the Geneclean kit (BioCan Scientific); the one less than 1 kbp was isolated by the protocol indicated in the MERmaid kit (BioCan Scientific).

Part 6. Gel-electrophoresis

DNA agarose gel electrophoresis was carried out by the method described by Maniatis, (Maniatis, 1982).

Part 7. DNA Sequencing

7-1. Double-stranded DNA Sequencing

Double-stranded DNA was sequenced by the method of R.T. Lin (personal communication). 3-5 μg purified DNA was treated with 2 μl 2M NaOH in a final volume 20 μl at room temperature for 10 minute.

Denatured DNA was recovered by ethanol precipitation with 0.3M sodium acetate and rinsed with 70% ethanol.

The sequencing reaction was carried out by the protocol indicated in the sequenase™ kit from United States Biochemical Corporation, Cleveland, Ohio. DNA were sequenced with dGTP and with dITP.

7-2 DNA Sequencing Gel

The sequencing gel system used in this study was developed by Lang. (Lang, 1990).

Part 8. Mutagenesis

The primers, which were designed for the PCR amplification on the *serA* promoter region, correspond to the wild-type *serA* sequence, except at one base pair in primer I and two in primer II. The amplified DNA therefore carries mutations at either end, which created new restriction sites, *Eco*RI and *Bam*HI.

The 380 bp of upstream *serA* DNA containing both P1 and P2 was amplified using Taq DNA polymerase which routinely makes about 0.1% mistaken pairing in 30 cycles of amplification. Mutations in this region were identified by double-stranded DNA sequencing (Higuchi, 1989).

Part 9. Strain Constructions

Strains were constructed by transduction as follows.

MEW1A construction: The P1 phage of strain P90C was made and transferred into strain CP54, a λ*placμ* insertion mutant (*ilvA*$^-$, L-leucine auxotroph), then spread on an +NGlu plate to select for *ilvA*$^+$. As *ilvA* and *ara* are quite close according to the genetic map of *E. coli*, there is a chance that *ara*$^-$ gene will cotransduct with *ilvA*. The *ilvA*$^+$ colonies were streaked on LB, LB kanamycin, and on arabinose and glucose minimal media. One which is *ara*$^-$, *ilvA*$^+$ and kanamycin sensitive was kept for the further studies.

lrp from strain MEW26 was transferred by selecting for tetracycline resistance and verifying that the transductants grew with L-serine as carbon source, except for derivatives of MEW22, in which this verification could not be made.

serA from strain MEWS2 was transferred by selecting for

kanamycin resistance and the transductants were verified to require serine.

sdaA from strain MEW22 was transferred by selecting for kanamycin resistance and verifying that the transductants could not grow on SGL plates.

Part 10. Plasmid Constructions

10-1. Subcloning of the *serA* Promoter Region from GU2

The plasmid GU2 was constructed by R.T. Lin. (Lin, 1992c) To further subclone the *serA* promoter for regulatory studies, plasmid GU2 was cut with *Hind*III, treated with Klenow enzyme and dNTP mix to make blunt ends and then cut with *Bam*HI. A 1.26 kbp fragment containing the *serA* promoter fragment was isolated from an agarose gel with Geneclean. The fragment was then ligated into pRS415 cut with *Sma*I and *Bam*HI (Fig. M1). The ligation mix was transformed into strain NEWI and plated on LB plate with X-gal and ampicillin. Plasmids from blue colonies on X-gal plates were isolated and checked by restriction enzyme digestion. One of the resulting plasmids was named as SWA1.

The 1.26 kbp fragment was further digested with *ssp*I, and the 0.9 kbp fragment from *Hind*III to *Ssp*I containing the *serA*P2 promoter was isolated from an agarose gel. The fragment

was then ligated into pBluescript cut with *Sma*I forming SAP2(Fig. M2). The selection and checking are the same as above.

10-2. Subcloning of the *serA* Promoter Region from PCR Amplification Fragments

The primers used for the PCR amplification on the *serA* promoter region are described in Fig. M3.

The *serA* fragment amplified from SWA1 with primer I and II was digested with *Eco*RV and *Bam*HI and then isolated from a low-melting-point gel with MERmaid kid. The isolated fragment was ligated into pBluescript KS+ cut with the *Sma*I and *Bam*HI forming BSWSA2 which contains 380bp *serA* upstream region with both P1 and P2 promoters (Fig. M4).

The *serA* insertion was confirmed by sequencing. The plasmid with wild-type insertion was named as SWA2. The amplifications which contain mutation inside were further named and kept for later studies. They are BSUATG1, BSUATG2, BSDP1-1, BSUP1-1, BSUP1-2, BSUP1-3, BSUP2-1 and BSUP2-2.

The *serA* fragment amplified from WSA1 with primer II and III was treated with Klenow enzyme and dNTP mix to make blunt ends. The fragment was then ligated into pBluscript cut with *Sma*I forming BSSAI (Fig. M5).

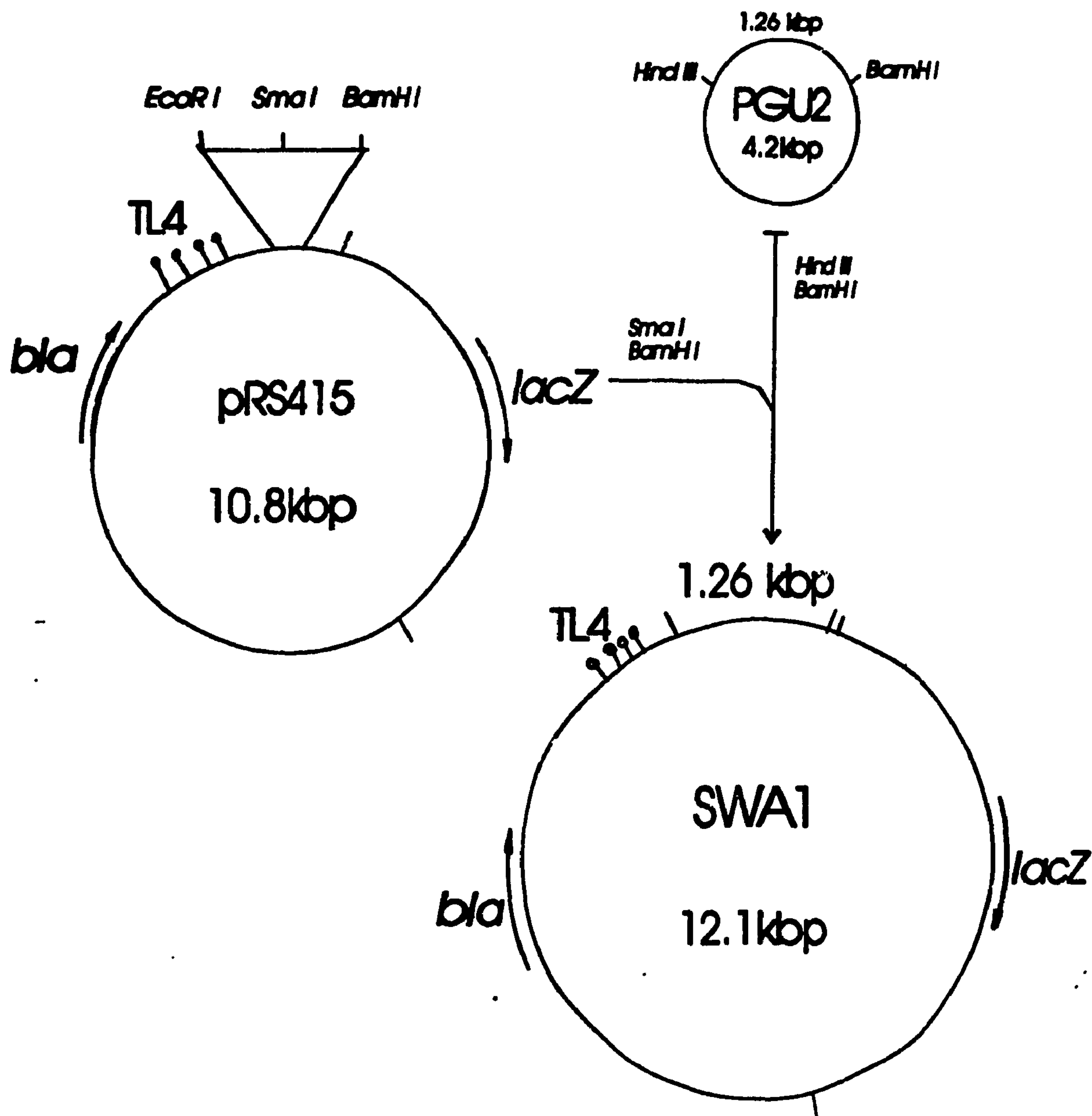


Fig. M1. Subcloning of the wildtype *serA* promoter from pGU2

Fig. M1. Subcloning of the *serA* promoter

Plasmid GU2 was cut with *Hind*III, the resulting linear DNA fragment was treated with Klenow enzyme and dNTP mix. This DNA fragment was then cut with *Bam*HI. A 1.26 kbp *Hind*III to *Bam*HI fragment was isolated and inserted into pRS415, forming SWA1.

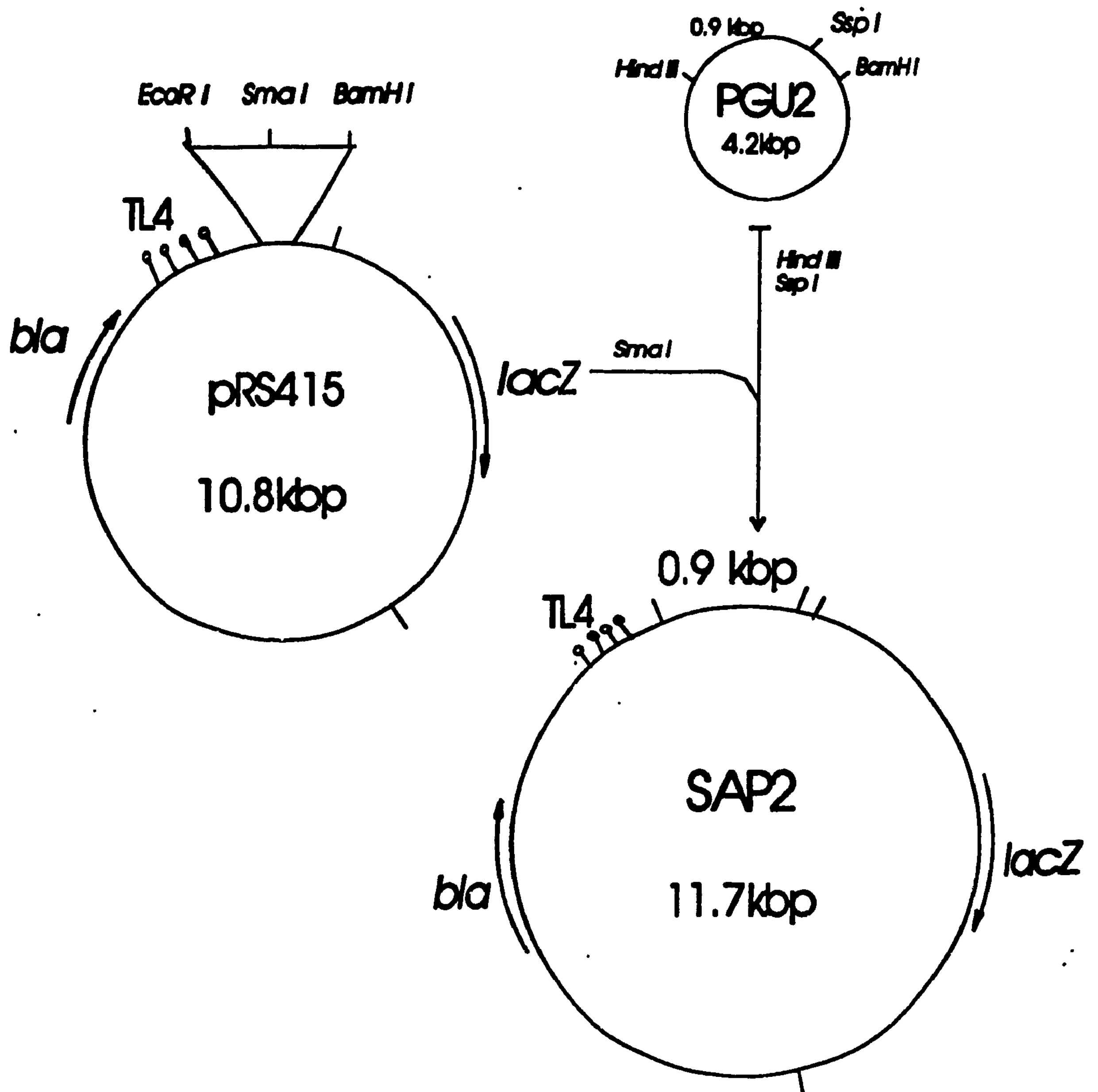


Fig. M2. Subcloning of the *serAP2* promoter from pGU2

Fig. M2. Subcloning of the *serA*P2 promoter from pGU2

A 0.9 kbp *Hind*III to *Ssp*I fragment was isolated from GU2. The fragment was treated with Klenow enzyme and dNTP mix before inserted into the *Sma*I site of pRS415, forming SAP2.

The *serA* fragment amplified from SAP2 with primer I and IV was cut with *Eco*RV and *Bam*HI. A 300 bp fragment was isolated from a low-melting-point gel and ligated into pBluescript cut with *Sma*I and *Bam*HI forming BSAII (Fig. M6).

All the PCR subcloned plasmids were confirmed by DNA sequencing and cut with *Eco*RI and *Bam*HI. The *serA* insertions of them were isolated and ligated into pRS415 cut with the same two enzymes resulting in plasmids WSA2, SA1, SA2, UATG1, UATG2, DP1-1, UP1-1, UP1-2, UP1-3, UP2-1 and UP2-2 respectively.

All BS-series plasmids were cut with *Eco*RI and *Bam*HI. The *serA* insertions were isolated and ligated into pRS415 cut with the same two enzymes, forming WSA2, SAI, SAII and the mutated plasmids as well.

10-3. Subcloning of the *lrp* Coding Region into pBAD18 Plasmid

The plasmid pLR3 was constructed by R.T. Lin.

pLR3 was cut with *Eco*RI and *Hind*III. The fragment contain *lrp* gene was isolated and ligated into pBAD18 cut with the same two enzymes. One of the resulting plasmids was known as MT1 (Fig. M7).

This work was done by R.T.Lin.

Fig. M3. Primers used for amplifying *serA* promoter fragment by PCR

Primer I: ATCCT TGACC CGATA TCGAT GC

*Eco*RV

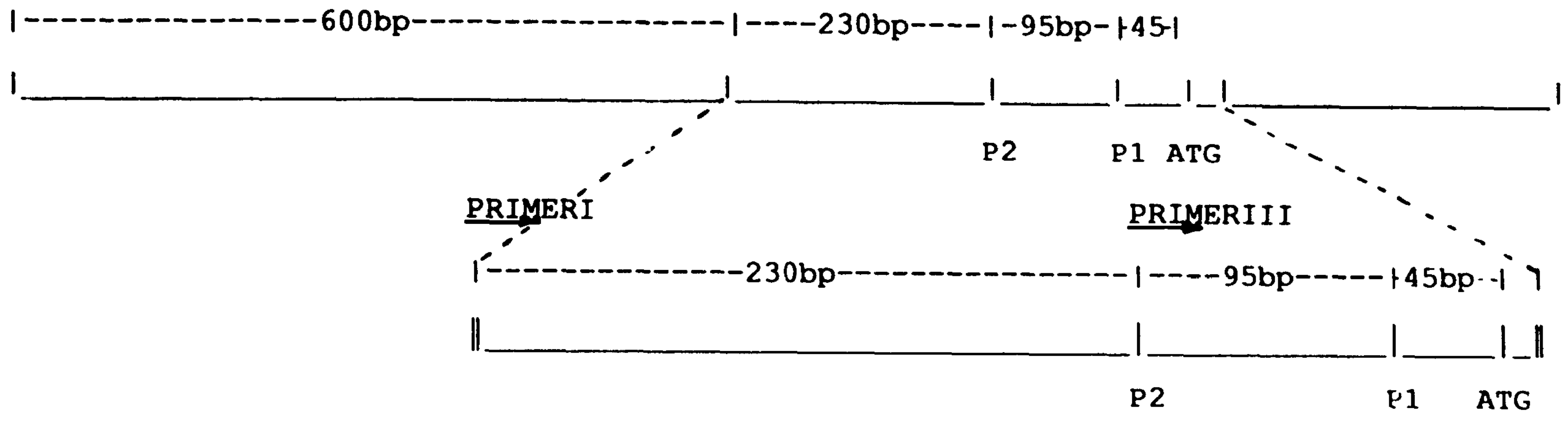
Primer II: CTCCA GGGAT CCCTT TGCCA TTTACC

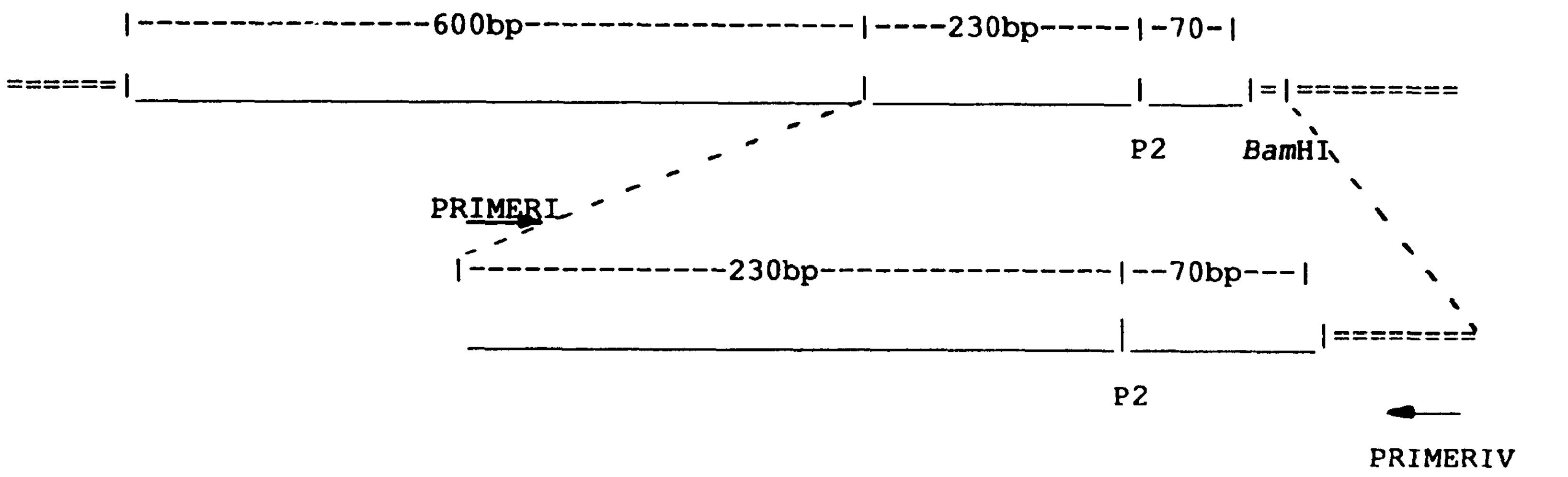
*Bam*HI

Primer III: CTCTA AACCA GAATA TTCAT

*Ssp*I

Primer IV: TAGCT GTTTC CTGTG TG


600bp
230bp
95bp
45
P2
P1 ATG
PRIMERI
PRIMERIII
230bp
95bp
45bp
P2
P1
ATG


600bp
230bp
70
P2
BamHI
PRIMERI
230bp
70bp
P2
PRIMERIV

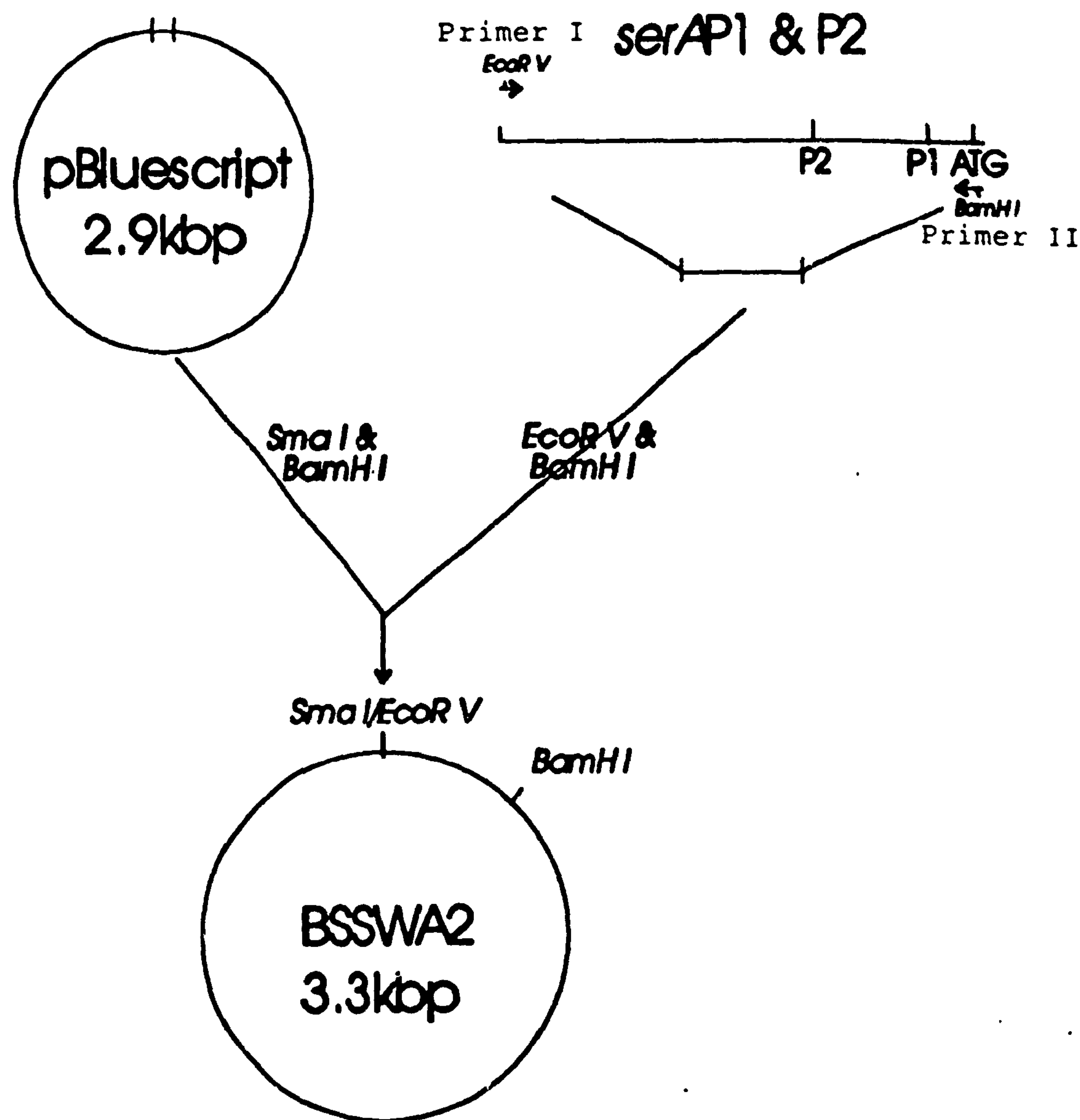


Fig. M4. Subcloning of *serA* promoter from PCR amplified fragment

Fig. M4. Subcloning of the *serA* promoter from PCR amplified fragment

The *serA* promoter region was amplified by PCR using primer I and II. The amplified fragment was digested with *Eco*RV and *Bam*HI. A 380 bp fragment was isolated and inserted into pBluescript.

The insertion was confirmed by sequencing. The plasmid containing the wild-type *serA* sequence was named as BSSWA2.

Others which contain mutation inside *serA* fragment were selected and named according to the location of the mutations.

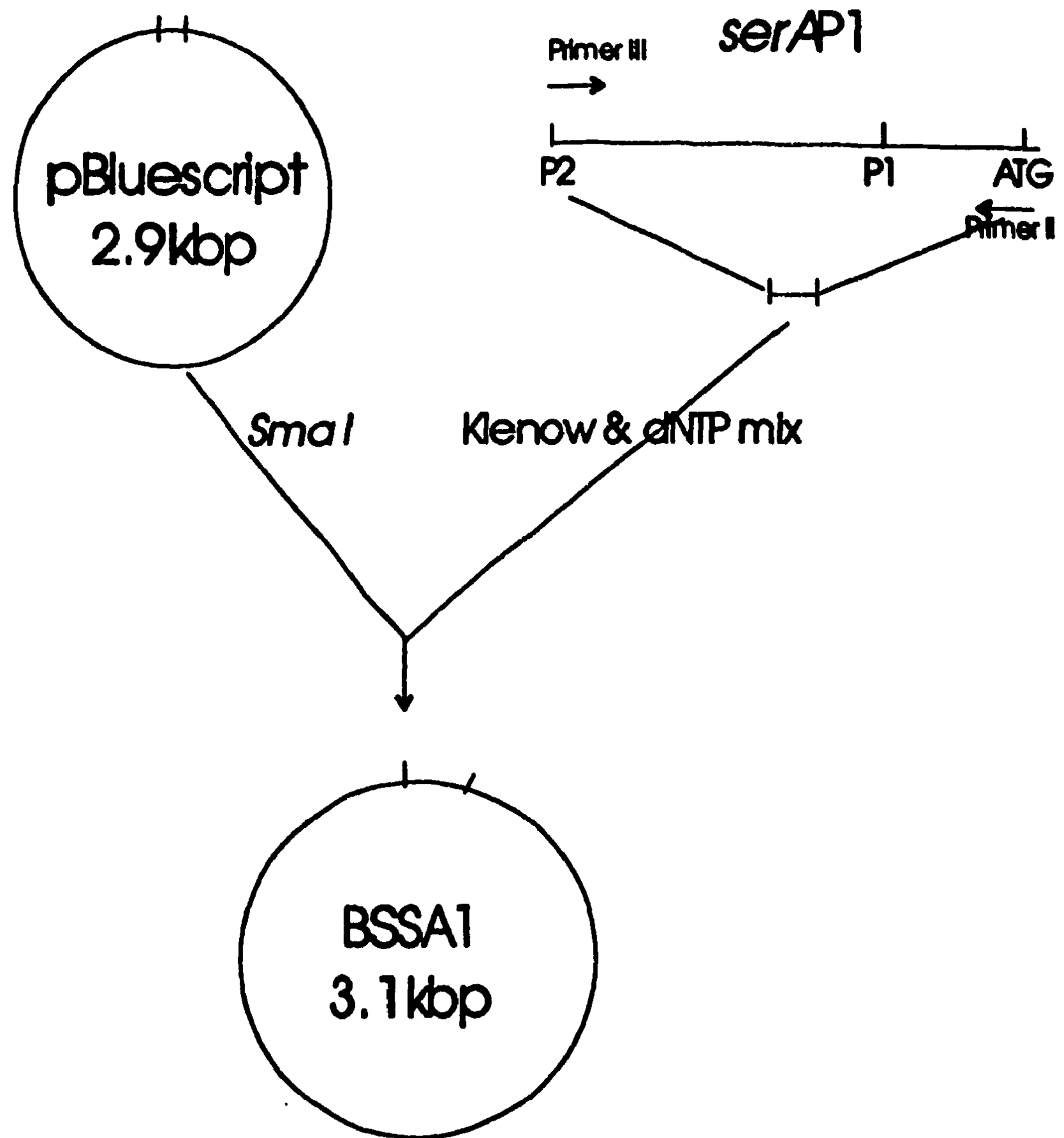


Fig. M5. Subcloning of *serA*P1 promoter from PCR amplified fragment

Fig. M5. Subcloning of the *serA*P1 promoter from PCR amplified fragment

The region containing *serA*P1 promoter was amplified by PCR using primer II and III. The amplified fragment was treated with Klenow enzyme and dNTP mix and inserted into pBluescript, forming BSSAI.

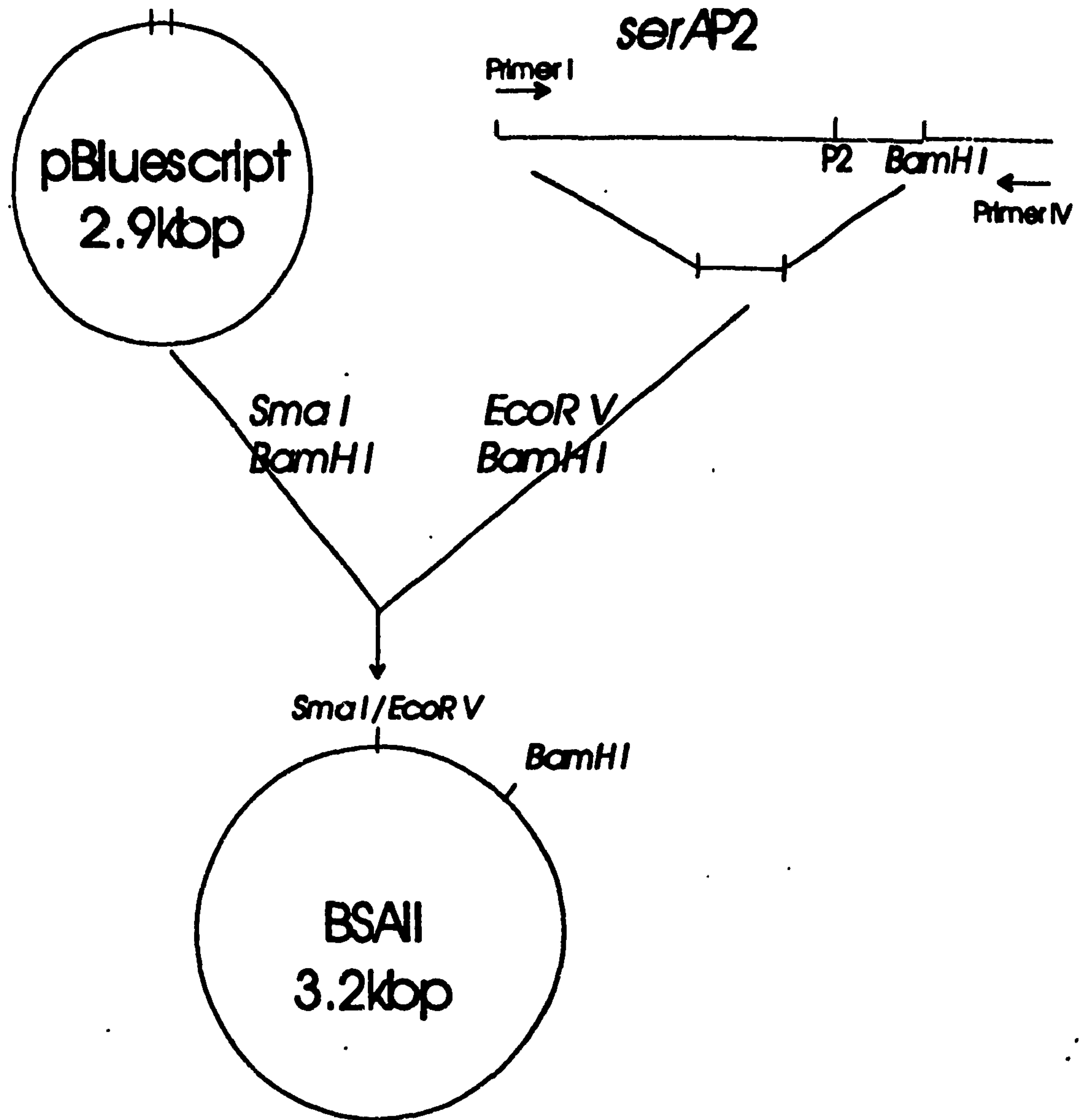


Fig. M6. Subcloning of *serAP2* promoter from PCR amplified fragment

Fig. M6. Subcloning of the *serA*P2 promoter from PCR amplified fragment

The *serA* fragment amplified from SAP2 with primer I and IV was digested with *Eco*RV and *Bam*HI. The isolated fragment was ligated into pBluscript cut with *Sma*I and *Bam*HI, forming BSSAII.

The other two pBAD*lrp* plasmids were constructed from PCR products.

The primers used for the PCR amplification on the *lrp* coding region are described in Fig. M8.

The *lrp* gene was amplified by PCR using primer I and primer II. The amplified fragment was digested with *Eco*RI and *Hand*III. The fragment was isolated from an agarose gel and ligated into pBAD18 cut with the same two enzymes forming MT3, in which *lrp* coding region with 16bp upstream of ATG site is under the promoter of pBAD.

The same amplification was digested with *Ssp*I and *Hand*III, and was ligated into pBAD18 cut with *Sma*I and *Hand*III forming MTΔ, which carries the *lrp* coding region with only 2bp upstream of ATG site (Fig. M9).

Plasmids MT3 and MTΔ were constructed by Z.Q. Shao.

10-4. Construction of MT*lrp::lacZ* Fusion Plasmid

Plasmid pMC1871 was digested with *Bam*HI. A 3.1 kbp *Bam*HI fragment containing truncated lacZ gene was isolated from an agarose gel. This fragment was then ligated into MT series plasmids cut with *bgl*II. This resulted in an in-frame fusion of *lacZ* with *lrp*. The ligation mix was transformed into strain MEW1 and plated on LB plate with arabinose, X-gal and ampicillin. Plasmids from blue colonies on X-gal plates were isolated and checked by restriction enzyme digestion. The

plasmids were known as MT1*lrp::lacZ*, MT3*lrp::lacZ*, and MTΔ*lrp::lacZ*.

Plasmids MT1*lrp::lacZ*, and MT3*lrp::lacZ*, were constructed by Z.Q. Shao.

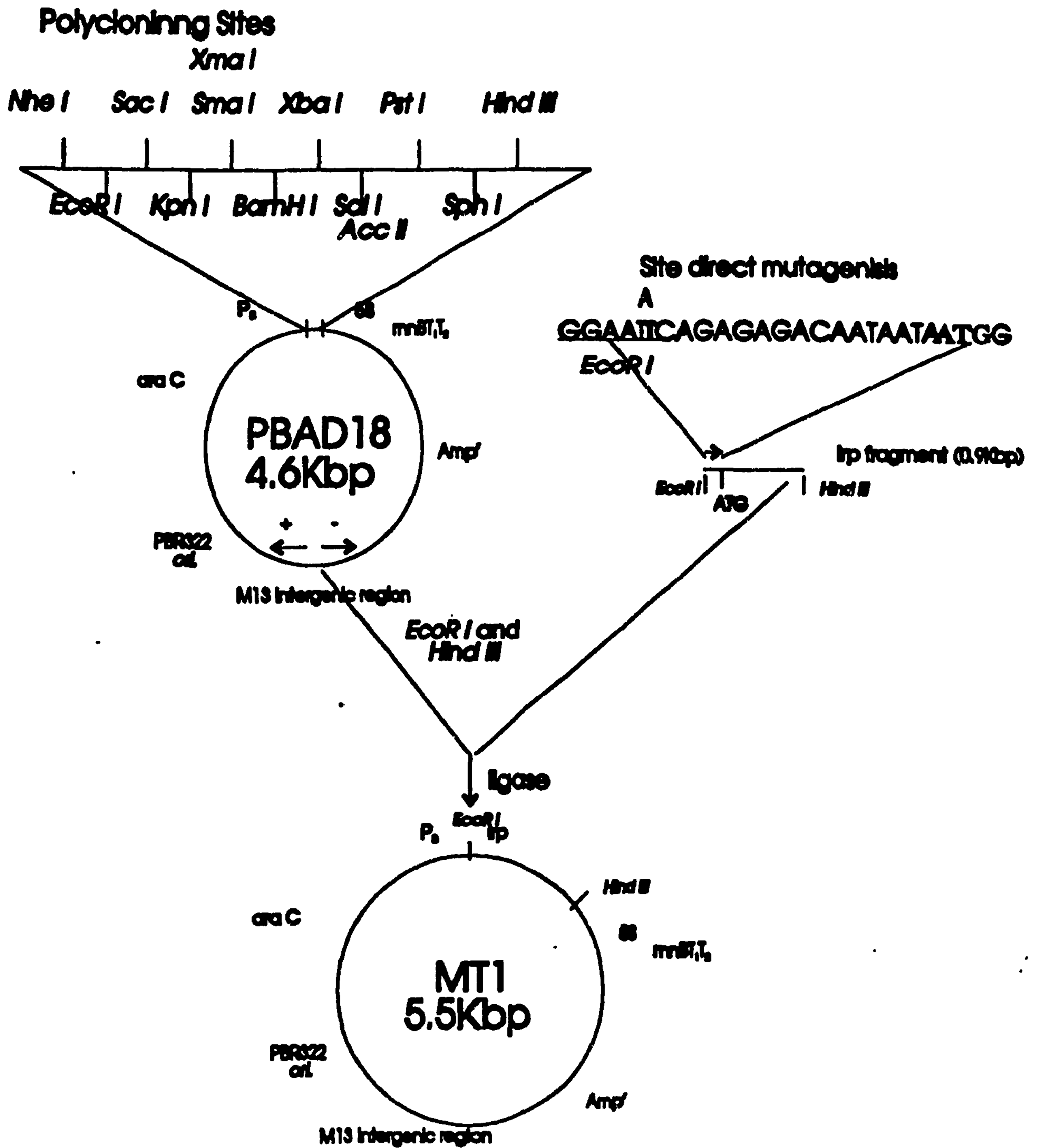


Fig. M7. Construction of plasmid MT1

Fig. M7. Construction of plasmid MT1 (pBAD::*lrp*)

An *Eco*RI to *Hind*III fragment containing *lrp* gene was isolated from pLR3. The isolated fragment was then inserted into pBAD18 cut with the same two enzymes, forming MTI.

This plasmid was constructed by R.T.Lin.

Fig. M8. Primers used for amplified *lrp* gene

PRIMER I: GGAAT TCAAA GAGAC AATAT TAATG G

*Eco*RI *Ssp*I

(translational start site is underlined)

PRIMER II: GGAAA AGCTT GTAAC CTGGA G

*Hind*III

(about 100bp after stop codon)

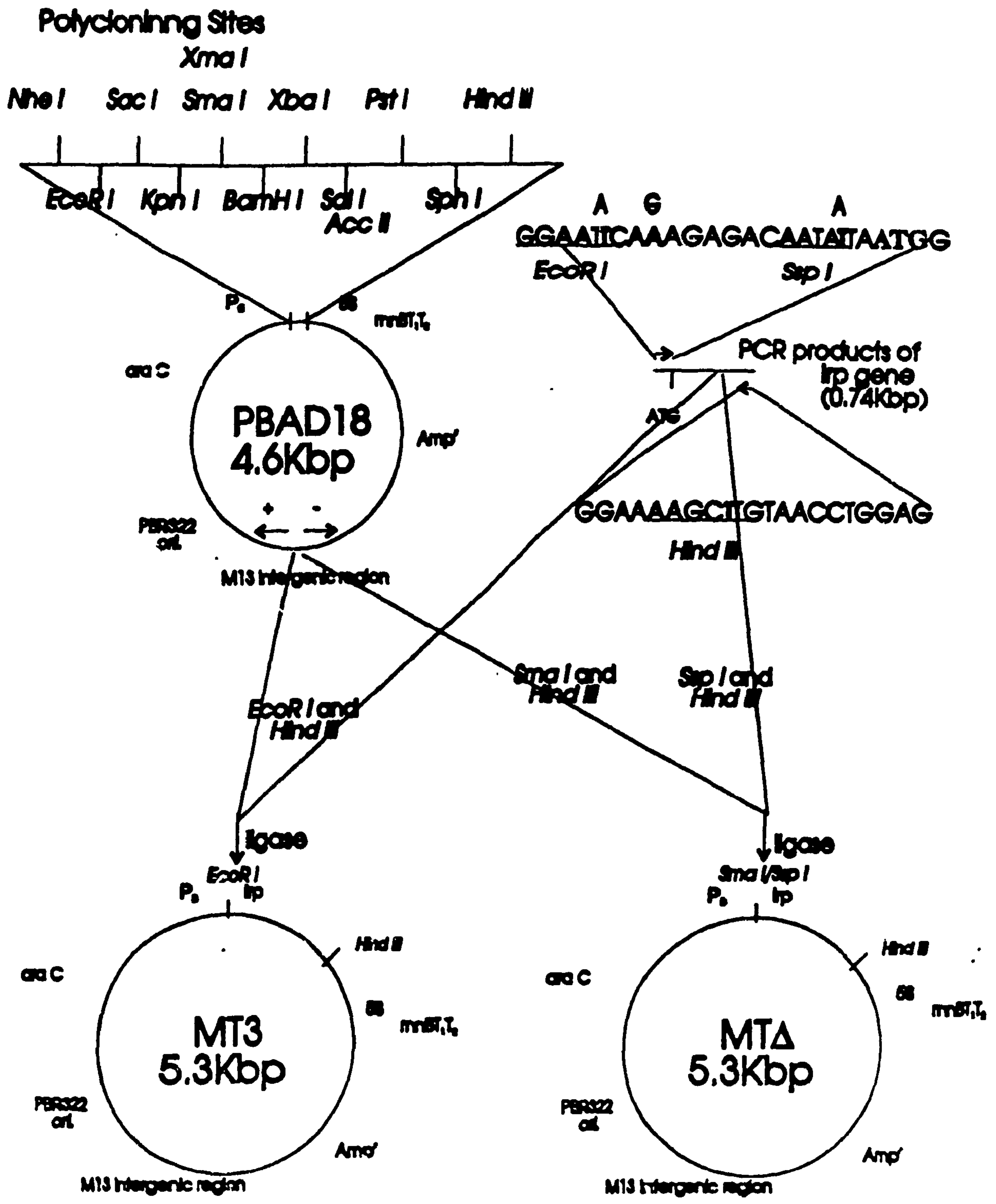


Fig. M9. Construction of plasmids MT3 & MTΔ

Fig.M9. Construction of plasmid MT3 and MTΔ

The *lrp* gene was amplified by PCR using primer I and II. The amplified fragment was digested with *Eco*RI and *Hind*III, and subcloned into pBAD18 cut with the same enzymes forming MT3.

By cutting the same fragment with *Ssp*I and *Hind*III, the *lrp* fragment was isolated and subcloned into pBAD18 cut with *Sma*I and *Hind*III, forming MTΔ.

These two plasmids were constructed by Z.Q. Shao.

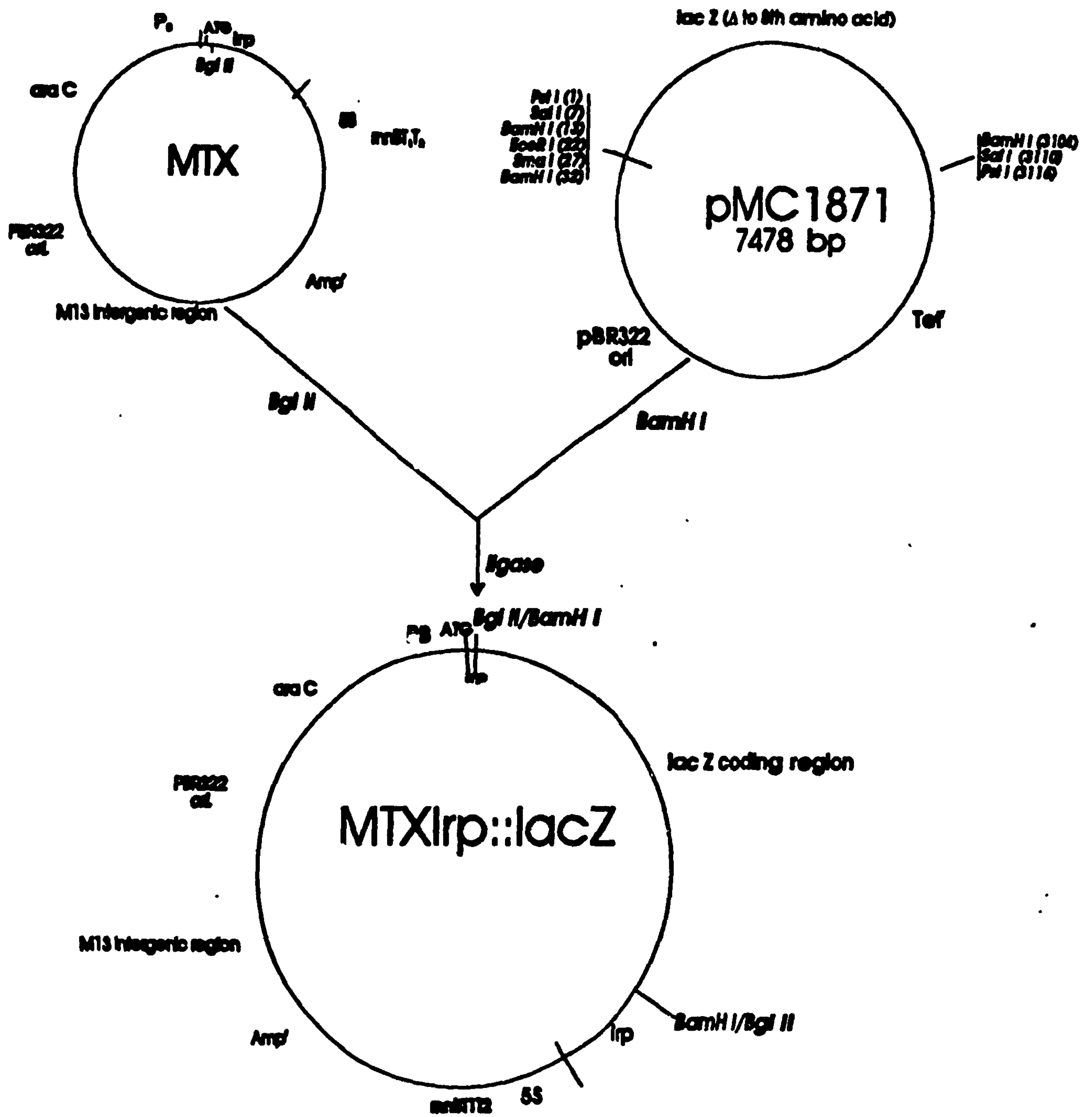


Fig. M10. Construction of *lrp::lacZ* fusion plasmids

Fig. M10. Construction of *lrp::lacZ* fusion on MT1, MT3, and MTΔ

pMC1871 was cut with *Bam*HI. A 3.1 kbp fragment containing truncated *lacZ* gene was isolated and ligated into MT1, MT3 and MTΔ cut with *Bgl*II forming MT1*lrp::lacZ*, MT3*lrp::lacZ*, and MTΔ*lrp::lacZ*.

Plasmids MT1*lrp::lacZ*, and MT3*lrp::lacZ* were constructed by Z.Q. Shao.

Part 11. Lrp Protein Partial Purification

Strain MEW1A/pMT1 was grown overnight in LB with arabinose 0.1% and ampicillin 100 ug/ml to ensure plasmid maintenance, and subcultured in the same medium in a 1-L flask with 400 ml culture medium. One hour prior to harvesting, more arabinose was added to a final concentration 0.5%. Cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C and disrupted by sonication in TGED 0.2M NaCl buffer (10mM Tris-Cl, pH8.0, 10% glycerol, 0.1mM EDTA, 0.5mM DTT). The crude extract was clarified by centrifugation at 33,000 x g, and the supernatant was loaded on a 15ml bed volume Bio-Rex 70 column which had been incubated overnight with KPB buffer (KPO_4 pH 7.5, 50 mM) and then washed with TGED 0.2M NaCl buffer. The column was connected with a UV monitor (280 nm) and a recorder. After the extract was loaded, the column was washed with TGED 0.2M NaCl buffer, until the recorder reached the base line of the buffer alone, then eluted with increasing concentrations of NaCl in TGED buffer (from 0.2 to 1 M). The fractions were collected with a fraction collector. The eluate was dialyzed with 0.2M NaCl TGED buffer for at least 24 hrs, at 4°C. The buffer was changed three times during this period to remove the NaCl. The eluate was then concentrated by the "speed vac" and kept in TGED50 buffer with 0.2M NaCl at -20°C.

Part 12. SDS Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Bio-Rad Mini Protein II dual slab cell according to the instructions of the manufacturer. Coomassie staining was performed as described in the cloning manual (Maniatis, 1982).

Part 13. Gel Retardation Assay

The DNA fragments used for gel retardation studies were isolated from agarose gels using the MERmaidTM kit (Bio-101 Inc) by the procedure indicated in the kit, and 3'-end labelled with α-^{32}P-dATP using Klenow enzyme by the method described by Maniatis (Maniatis, 1982).

The Lrp protein used in these studies was purified by Bio-Rex 70 column.

Binding of Lrp protein to the upstream region of wild-type *serA* fragment and *serA* mutated fragments was determined by the gel shift assay described by Ricca *et al.* (Ricca, 1989) with slight modifications. From 1-5 ng (5000 cpm) of 3'-end-labelled DNA fragment was incubated at room temperature for 10 min with 2 µg sonicated herring sperm DNA and 0-50 ng Lrp protein in 20 µl binding buffer. Samples were resolved by electrophoresis through a 5% polyacrylamide gel pre-electrophoresed at 10 v/cm and electrophoresis of the samples

was performed in the same conditions. The gel was cast and run in 1x TBE buffer (Maniatis, 1982). The gels were dried at 60°C in gel drier and subjected to radioautography.

RESULTS

In vivo Regulation of *serA* by Lrp

Leucine-responsive regulatory protein (Lrp) has been demonstrated to regulate, either positively or negatively, the transcription of several *Escherichia coli* genes. The transcription of these genes is usually but not always affected by exogenously provided L-leucine.

Lrp has very clearly been shown to affect *serA* transcription *in vivo*. This has been done by comparing transcription in the parent cell with that in *lrp*-deficient mutants. In this study, I first repeated the regulation experiment of *serA* in strain MEWS2 (MEW1 with *serA::lacZ*), and its derivative, MEWS26 (MEWS2 with *lrp*::Tn10). The results agreed with previous data. The next step was to perform similar studies, varying the amount of Lrp *in vivo*. This was done with a plasmid carrying the *lrp* gene under the control of *ara* promoter, which allows expression to be varied over a very wide range (Guzman, 1992). At the same time, the regulation of *lrp* from the same promoter was studied by *lrp::lacZ* fusion.

Part 1 Strains and Plasmids Construction

1-1 Variable Expression Plasmids Carrying Functional *lrp* Genes

Using a vector with a multiple-cloning site downstream of the *ara* promoter, constructed and kindly provided by Beckwith and Guzman, three pBAD-*lrp* fusion plasmids were constructed by R.T.Lin and Z.Q.Shao. In the first fusion construct, MT1, the *Eco*RI and *Hind*III sites of the vector were ligated to the same sites of the *lrp* gene including the coding region and 16bp upstream of the ATG start site, thus putting the *lrp* coding region and ribosome binding region under the control of the *ara* promoter. Site-directed mutagenesis was used to alter the upstream sequence making plasmid MT3. In the last construct, MTΔ, almost all the upstream region originally derived from the *lrp* gene was deleted. Sequencing of the relevant region showed that the construct MT1 had one basepair changed, and construct MT3 had three changes. In the third plasmid used here, MTΔ, the entire sequence of the *lrp* upstream region was deleted, leaving only two basepair upstream and the *lrp* coding region (details in Materials and Methods). With these plasmids, I could hope to vary the cell's Lrp concentration over a wide range.

1-2 Variable Expression Plasmids Carrying *lacZ* Fused to the *lrp* Coding Region.

In order to assess how much Lrp each construct would make under various growth conditions, Z.Q. Shao and I made a corresponding series of plasmids in which the *lacZ* coding region was fused into the coding region of the *lrp* gene on each of these plasmids, thus fusing an easily detectable reporter gene to *lrp*. The new plasmids were named MT1*lrp::lacZ*, MT3*lrp::lacZ* and MTΔ*lrp::lacZ* respectively.

1-3 Construction of Target Strains Needed for *in vivo* Regulatory Studies Using the MT pBAD Plasmids.

I wanted to vary the Lrp concentration by varying the arabinose concentration, and determine the effect on *serA* and on another target gene *sdaA*. However, arabinose can be degraded by *E. coli*. It was therefore necessary to make arabinose-nondegrading strains, particularly for experiments at very low arabinose concentrations. Then the initial arabinose concentration in the medium will not vary during the experiments. I therefore constructed *ara*$^-$ derivatives: strain CA, which is MEW1 *lrp*::Tn10 *ara*$^-$, and strains CAS2, and CAp22 which also carry *serA*::*lacZ*, and *sdaA*::*lacZ* respectively (see Materials and Methods).

In these strains, we assured that the chromosomal *lrp*

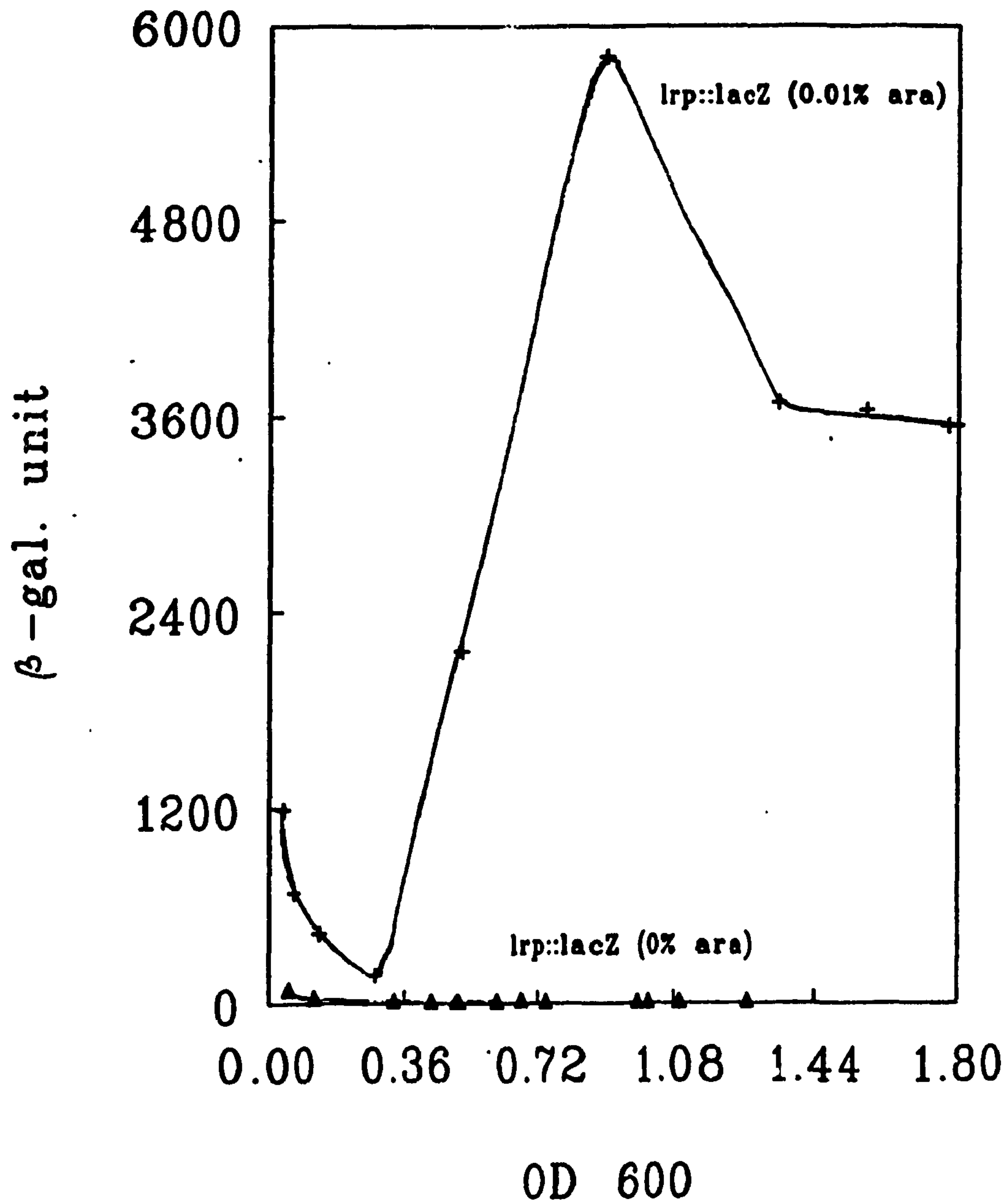
gene did not function by transducing *lrp*::Tn10 into the host chromosome. In this case, the host strain itself will not produce any Lrp protein from the chromosomal gene, and any Lrp made will be made from the plasmid-carried gene. This construct provides the possibility of regulating *lrp* expression from zero (in the case where the plasmid does not produce Lrp protein because the pBAD promoter is shut down) to different levels by varying the amount of arabinose in the medium. By studying transcription from *serA::lacZ*, and *sdaA*::*lacZ*, at various arabinose concentrations, one could determine the sensitivity of the *serA*, and *sdaA* promoter to Lrp. Measuring the amount of β-galactosidase made from the *lrp::lacZ* constructs in the same conditions, would indicate how much Lrp was being made in each particular condition.

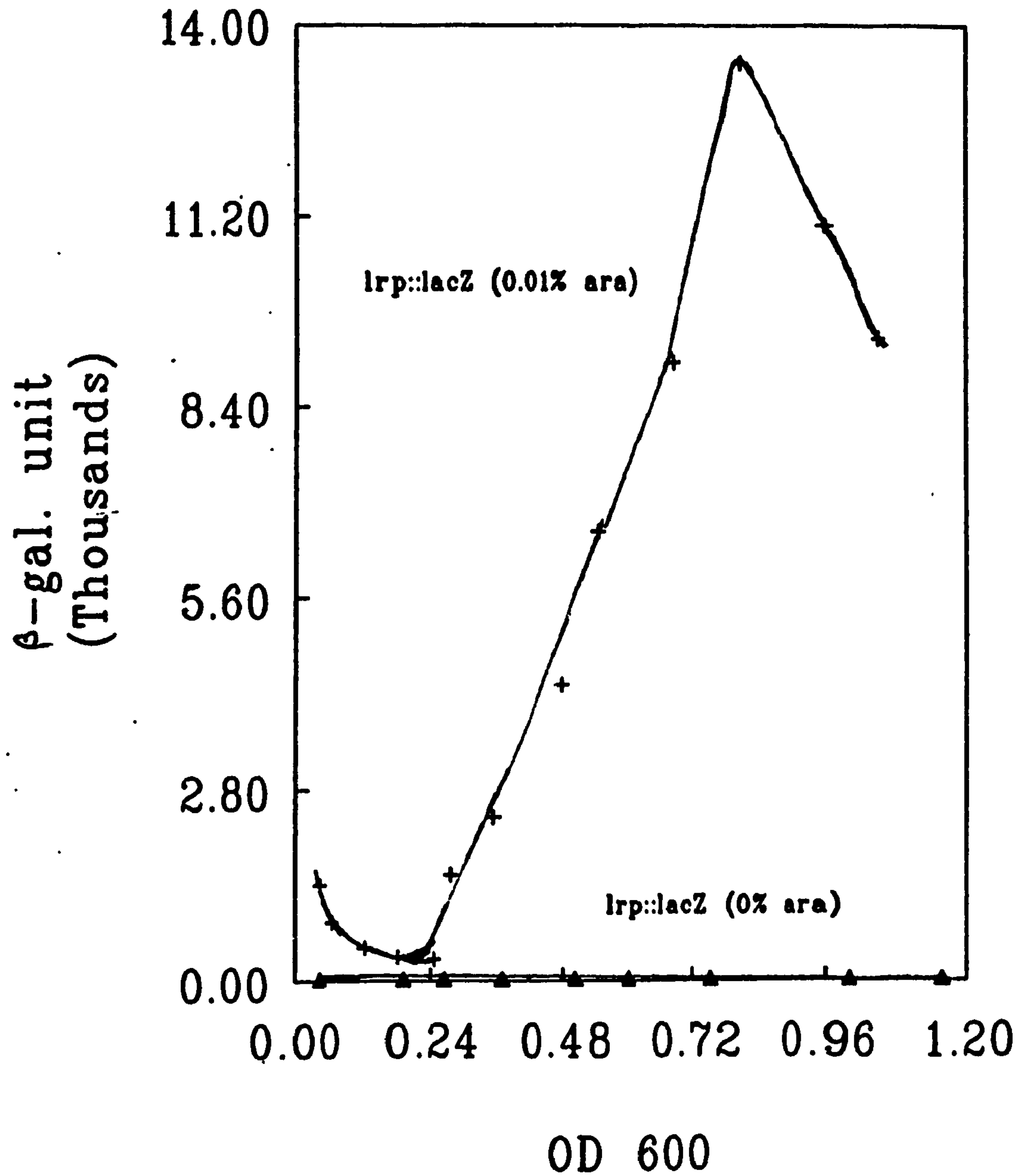
Part 2. Regulatory Studies with the Variable Expression Plasmid

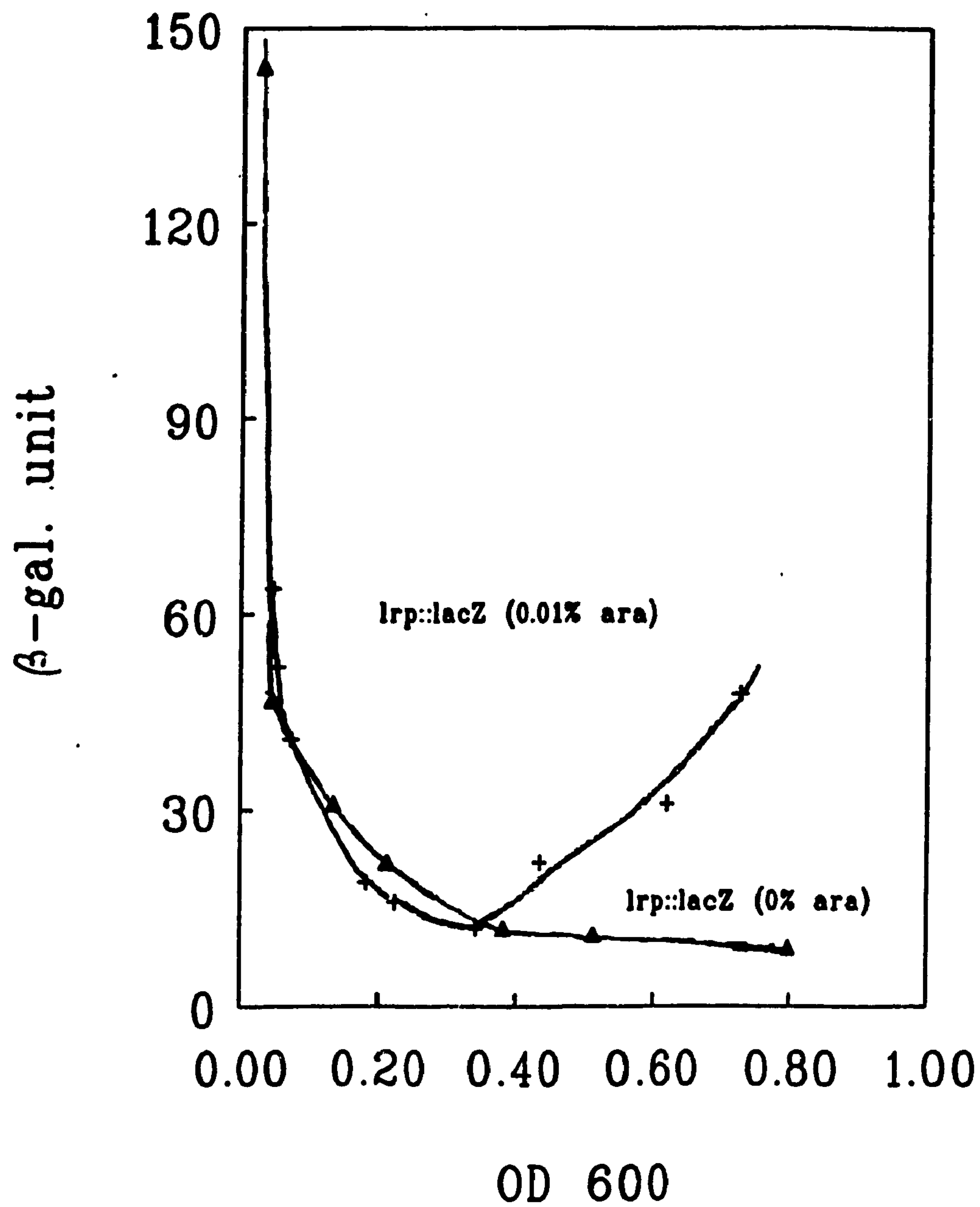
2-1. Expression of *lrp* under the control of pBAD promoter

To study how arabinose regulates expression of the *lrp* gene, the three lacZ fusion plasmids were transformed into strain CA, *lrp*::Tn10 and *ara*⁻. I then measured ß-galactosidase activities of each strain with a variety of amounts of arabinose in different media. I expected that as I increased the arabinose concentration, the expression of *lrp* (i.e. ß-galactosidase activity) would increase. This is of course based on the idea that arabinose is an inducer of the pBAD promoter, and *lrp* expression is under the control of pBAD promoter in these constructs.

It is important to note that these results indicate nothing about the normal synthesis of Lrp from its own promoter. They provide a measure of how much Lrp is present at different times and in different conditions using this particular vector. Because this varied greatly, and in an unexpected way, these experiments allow a second set of experiments which demonstrate that target genes are regulated rapidly according to Lrp concentration.


Expression studies of MT3lrp::lacZ
in LB
6000
4800
3600
2400
1200
0
β-gal. unit
lrp::lacZ (0.01% ara)
lrp::lacZ (0% ara)
0.00
0.36
0.72
1.08
1.44
1.80
OD 600


Expression studies of MT1lrp::lacZ
in LB
14.00
11.20
8.40
5.60
2.80
0.00
β-gal. unit
(Thousands)
lrp::lacZ (0.01% ara)
lrp::lacZ (0% ara)
0.00
0.24
0.48
0.72
0.96
1.20
OD 600


Expression studies of MTΔlrp::lacZ
in LB
150
120
90
60
30
0
β-gal. unit
lrp::lacZ (0.01% ara)
lrp::lacZ (0% ara)
0.00
0.20
0.40
0.60
0.80
1.00
OD 600

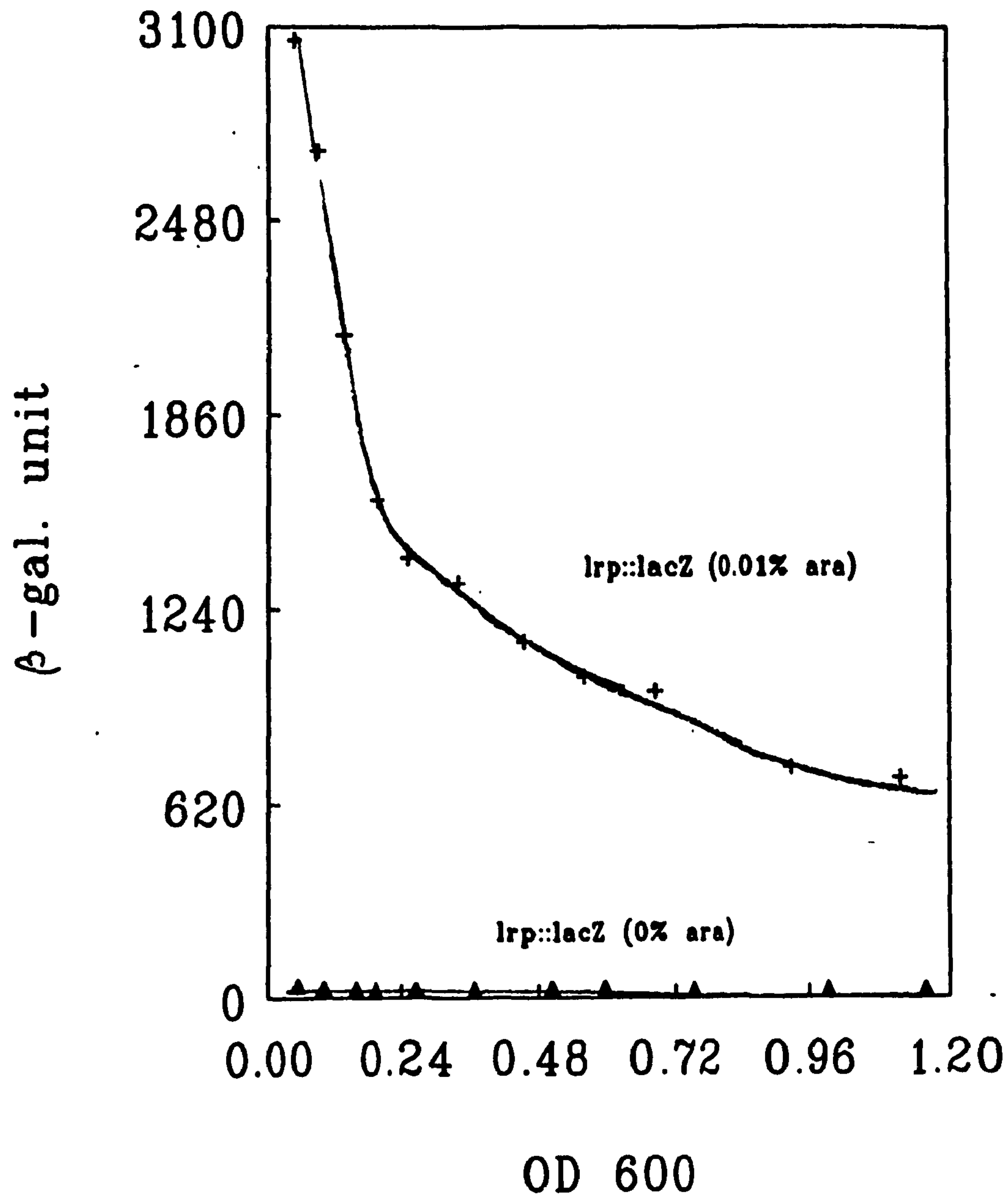

Expression studies of MT3lrp::lacZ
in minimal medium
3100
2480
1860
1240
620
0
β-gal. unit
lrp::lacZ (0.01% ara)
lrp::lacZ (0% ara)
0.00
0.24
0.48
0.72
0.96
1.20
OD 600

Fig. R1. Expression studies of MT*lrp::lacZ* series plasmids, pBAD*lrp::lacZ*

I examined *lacZ* expression from all three pBAD*lrp::lacZ* plasmids, MT3*lrp::lacZ*, MT1*lrp::lacZ* and ΔMT*lrp::lacZ*, all transformed into strain CA. Cells were grown in LB and in glucose-minimal medium, with and without arabinose 0.01%. Overnight cultures were subcultured to mid-exponential phase, harvested, and reinoculated in the same medium. Samples were then taken at various times and assayed for β-galactosidase.

Each figure represents one experiment and is typical of many.

The expression of MT3*lrp::lacZ* and MT1*lrp::lacZ* (pBAD *lrp::lacZ* with 16 bp) in LB with 0.01% arabinose as judged by the ß-galactosidase activities was induced by arabinose and greatly affected by the growth phase. In both cases, the level of *lrp* expression varied directly with the density of the culture. There was a gradual progression from 0.2 units at low O.D. to 0.8 units in a much more dense culture and then gradually decreases thereafter. It should be noticed the variation of the Lrp level with the density of the culture is nothing to do with the *lrp* gene itself, but with the characteristics of the arabinose promoter.

The plasmid without the 16-base pair sequence (MTΔ) had much lower expression compared to the two plasmids containing the 16-base pair sequence from *lrp* (MT1 and MT3, (Fig. R1D). Compared with the highest expression of MT3, the expression of MTΔ plasmid is nearly 100-fold lower (Fig. R1A and R1D, 6000 *vs* 60). This data suggests that the 16 base pair sequence of the *lrp* upstream sequence from ATG start site was necessary for the *lrp* expression. This region probably provides the ribosome binding site necessary for the translation of mRNA of the *lrp* gene. No ribosome binding site is coded by the pBAD vector itself.

The expression of MT3*lrp::lacZ* in minimal medium with 0.01% arabinose was considerably lower than the expression in

LB (Fig.R1B, minimal medium and 6A, LB, 6000 *vs* 600), because of glucose (catabolite) repression on the pBAD promoter and on arabinose uptake. Expression in minimal medium was also affected by the phase of growth, but the expression patterns in minimal medium were different from those in LB (Fig. R1B, and R1A). For the minimal medium, in the earlier growth phase (OD600 less than 0.2), the expression of MT3 in minimal medium decreased with the cell growth, after that the expression was more or less steady.

2-2. *serA* Expression Proportion as Affected by Amount of Lrp Produced

As can be seen from the preceding experiments, *lacZ* expression was greatly affected not only by arabinose as expected, but also by culture growth. The ß-galactosidase activities were quite different at various times after subculture even with the same amount of arabinose in the medium. That is, with a particular amount of arabinose in different growth stages, expression from the *lrp* gene varies greatly. If one did the same experiments using the same promoter fused to a functional *lrp* gene, one could expect that the Lrp concentration in the cell would vary in the same way.

If the Lrp concentration did in fact vary, expression from the Lrp regulated promoters might also be expected to vary. To study this, I transformed the MT3 plasmid into

strain CAS2 (*serA::lacZ ara*⁻), and CAP22 (*sdaA::lacZ ara*⁻), then carried out exactly the same experiments in LB as in the preceding section. The results of these experiments are shown in the following figure R2.

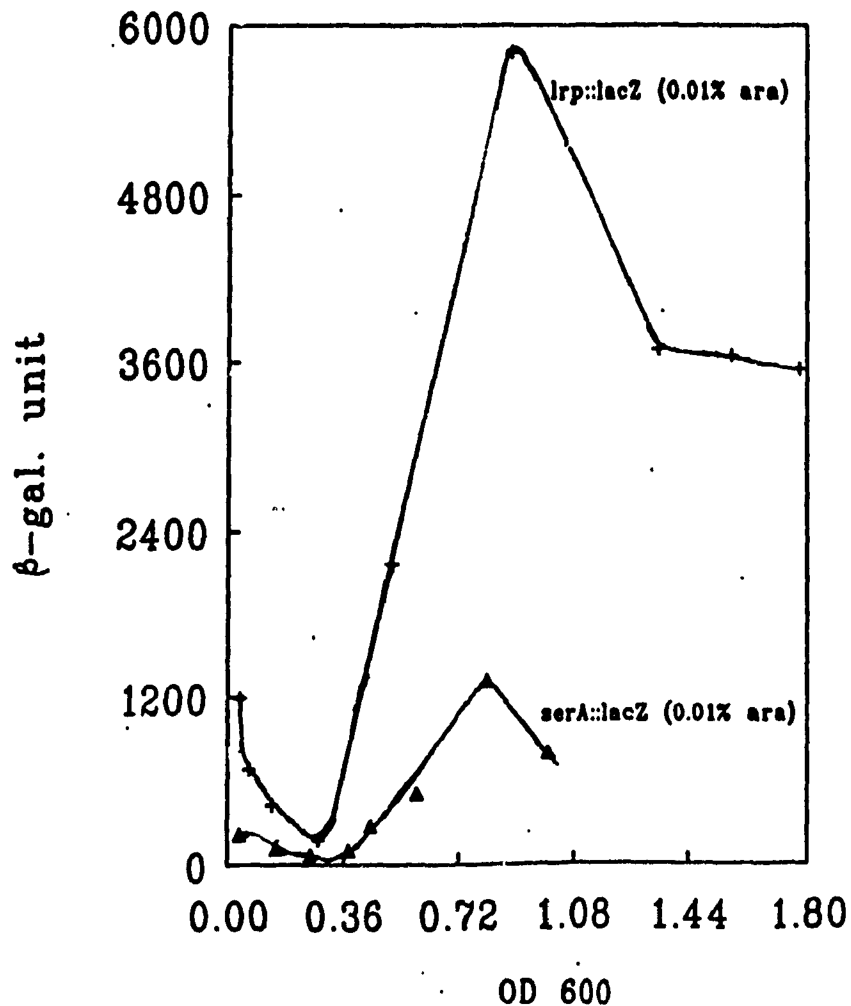


Fig. R2. Expression studies of the *serA* gene in the presence of different amounts of Lrp

Synthesis of ß-galactosidase from strain CAS2 (*lrp*⁻, *ara*⁻ *serA::lacZ*) carrying plasmid MT3 in LB as noted was monitored exactly as in Fig. R1A.

The results showed that the *serA* expression curves governed by the pBAD plasmids in LB medium followed the *lrp* expression curves of the same plasmid perfectly. The expression level of *serA* in LB was relatively low in comparison with the amount of Lrp present in the cell (Fig. R2, MT3, Lrp 6000 unit *vs* 1200 unit *serA*). One reason is that the concentration of Lrp present inside cells may be more than saturating, so that only part of Lrp can be involved in regulation. In addition, in the LB medium, there were many other factors such as L-leucine that may affect the *serA* expression or reverse the Lrp activation on the *serA* gene. Otherwise, the *serA* expression curve, which also varied directly with the growth phase, follow the same kind of *lrp* expression curve perfectly(Fig. R2).

The expression of chromosomal *sdaA::lacZ* with MT3 is repressed by arabinose (Fig. R3.). One should notice that, unlike the expression of *serA*, the *sdaA* expression did not exactly follow inversely the expression curve of *lrp*. Once the expression of *sdaA* is repressed by the highly expressed *lrp* gene product inside cell, it does not regain its expression level even when the expression level of *lrp* is going down. This might due to the accumulation of Lrp in the cell. The repression level in LB by Lrp is relatively low in comparison with the amount of Lrp protein in the cell. In LB, there are many other factors such as leucine, which may reverse the Lrp

repression partially or because the Lrp in the cell is likely in excess than enough, and only part of it may be involved in the regulation.

The control of transcription on *lrp* regulon is more complicated than anticipated but a direct proportionality between Lrp concentration and the gene expression in the regulon is clearly seen.

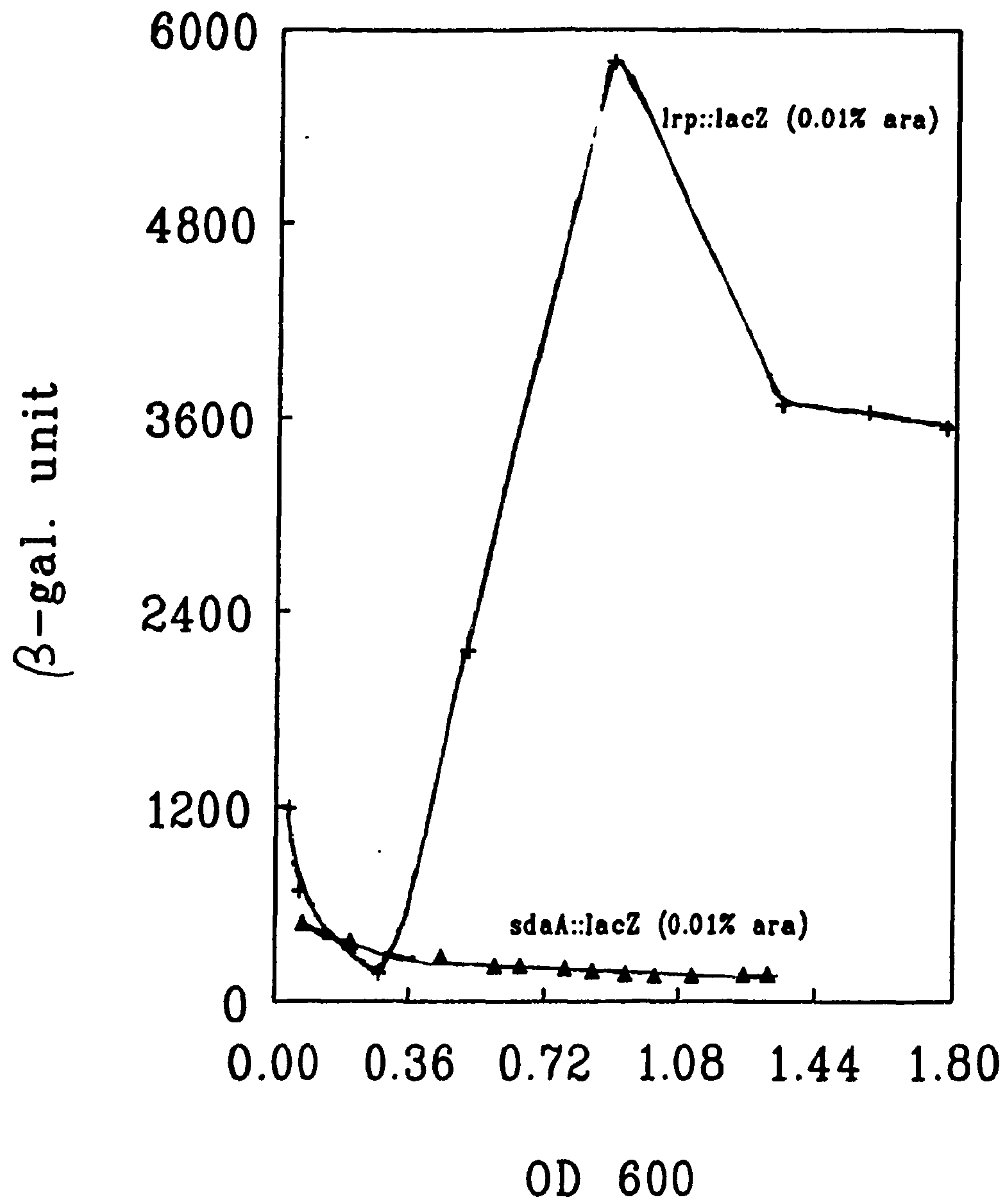


Fig. R3. Expression studies of the *sdaA* gene in the presence of different amounts of Lrp

Synthesis of ß-galactosidase from strain CAP22 (*lrp*⁻, *ara*⁻ *sdaA*::*lacZ*) carrying plasmid MT3 in LB as noted was monitored exactly as in Fig. R1A.

In vitro Study of the *serA* Promoter Region

Earlier work of R.T. Lin in our lab showed that the *serA* gene has two promoters, P1 and P2. P1 and P2 promoters are defined by primer extension experiment. Lrp protein binds to the *serA* P2 promoter with high affinity and protects the *serA* P2 region from DNase I action in the region -155 to -81 bp upstream of the P1 promoter transcriptional starting site. Lrp protein also binds to a second *serA* upstream region closer to P1. The affinity of Lrp for this region is comparatively low and the exact binding region has not been defined precisely. In addition it has been shown that the expression of *serA* is proportional to the amount of Lrp. This suggested that Lrp may activate *serA* transcription initiation through directly binding to its promoter region.

The experiments in this part are devoted to elucidating the mechanism which regulates *serA* expression. To do this, *serA* promoter mutants were isolated and characterized in order to identify which specific bases in the *serA* promoter region cause a significant alternation in Lrp and/or L-leucine effects on *serA* transcription initiation. In addition, the two separated *serA* promoter were subcloned and studied.

Part 1. The Construction of *serA::lacZ* Fusion Plasmids

To start this work, the promoters of the wild-type *serA* gene were first subcloned into an operon fusion vector, pRS415. The process resulted in the formation of a *serA-lacZ* operon fusion. Expression directed by the *serA* promoter could therefore be studied by assaying β-galactosidase activities of the plasmid.

1-1. Subcloning of the *serA* Promoter into a Multicopy Operon Fusion Vector

The whole *serA* promoter (including both the P1 and the P2 promoters) was subcloned to allow mutagenesis studies (Fig. R4). Vector pRS415 is a multicopy operon fusion vector constructed by R. Simon (Simon, 1987). Upstream from its multiple cloning sites, it carries several transcriptional terminators. Downstream from the cloning sites, it carries the *lacZ* coding region. When the *serA* promoter region is subcloned into the multiple cloning sites, the *serA* promoter region and the *lacZ* gene coding region form an operon fusion, so that the *serA* promoter directly regulates *lacZ* expression. The transcription initiation of any other promoters upstream from *serA* will be stopped by the transcription terminators and not affect the *lacZ* expression. Expression from the *serA* plasmid can be studied by measuring β-galactosidase activities of

cells otherwise devoid of *lacZ* (for detail of plasmid constructions, see materials and methods). The subcloned regions of the *serA* promoters are shown in Fig. R4.

Fig. R4. The subcloned regions of the *serA* promoter

the whole *serA* promoter, WSA1 (from pGU2 plasmid 1.26kbp)

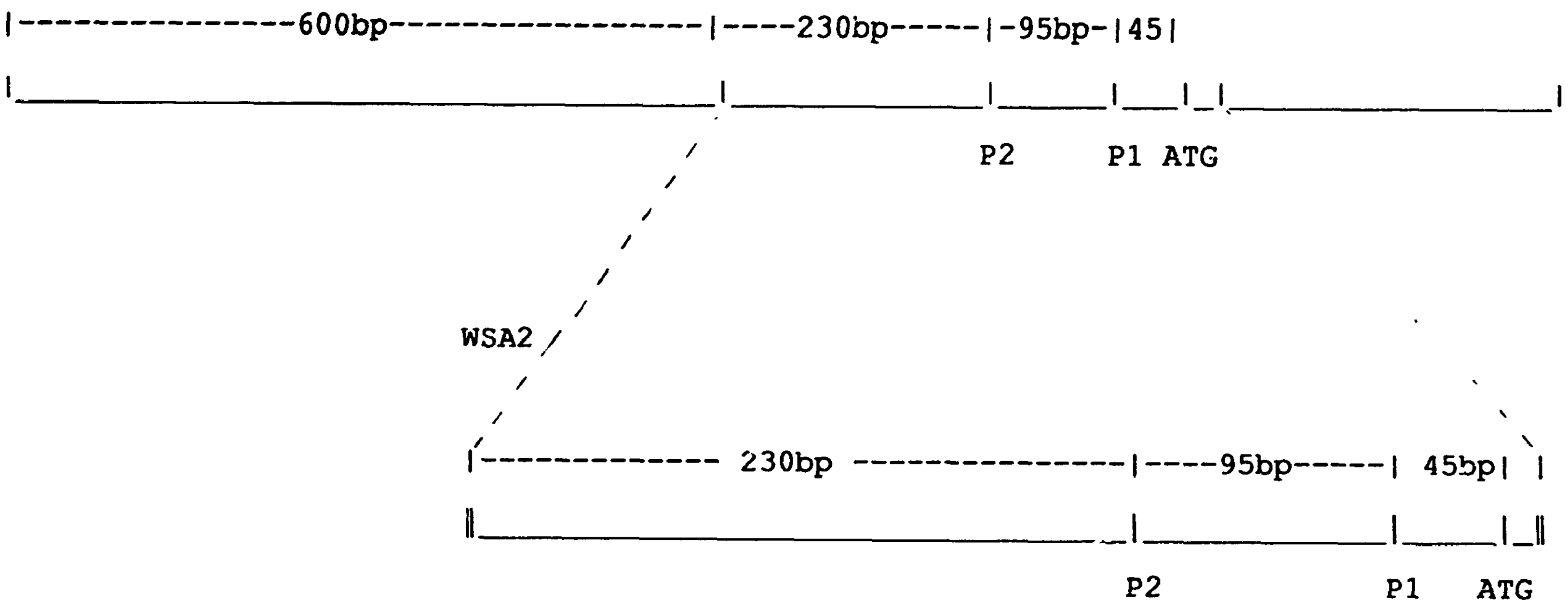


Fig. R4. The subcloned regions of the *serA* promoter

The whole *serA* promoter from pGU2, 1.26 kb carrying both the P1 and the P2 promoters, was subcloned into pRS415 and the resultant plasmid named pWSA1. From WSA1, a 380bp fragment from -370bp to +10 (counting the ATG site, A as +1) was subcloned again into pRS415 and named pWSA2. This plasmid is expected to contain the whole *serA* promoter region. This construction reduces the 5′ upstream region in pGU2 by 600bp and reduces the coding region 3′ of the ATG start codon to 10 bp.

1-2. The Expression and Regulation of the Wild-type *serA* Promoter

In order to study regulation by the *serA* promoter, the plasmids described in Fig. R4 were transformed into both MEW1 (*lrp*$^+$) and MEW26 (*lrp*::Tn10). Plasmid pWSA1 has a long sequence upstream of the *serA* promoters, and plasmid pWSA2 has 600 bp less. To determine whether either of these carries all the determinants for wild-type *serA* expression and its regulation, the β-galactosidase activities of cultures grown in various growth media were measured, and compared with expression of a chromosomal insert of *lacZ* in *serA* (Table R1).

It is clear that *lacZ* is expressed at a much higher level from the plasmid than from the chromosome, which one would expect for a gene carried on a multi-copy plasmid (Table R1 expt. 1, 2, and 3, column A, 24400, and 24300 *vs* 1030). Leucine reduced activity by 30% for the chromosomal gene, and 45% for the plasmid-associated version so that both plasmids clearly retained the leucine effect (Table R1, Expt. 1, 2, and 3, column D, 55% and 54% *vs* 70% chromosomal wild-type expression). In the *lrp*-deficient host, expressions were reduced greatly in all cases (Table R1, expt. 1, 2, and 3, column E, 27% and 28% *vs* 7% chromosomal wild-type expression). However, the effect of leucine was much greater on the chromosomal gene (7% transcription remaining) than on the shorter plasmid-carried versions (27% remaining).

It seems then that the subcloned promoters are regulated very similarly to the chromosomal gene, and that the base pairs removed in shortening pWSA1 to pWSA2 have no effect on regulation of transcription. Further studies were therefore done with pWSA2.

Table R1. Expression of the wild-type *serA::lacZ* carried on chromosome and plasmids

Experiment		ß-galactosidase			D	E
		A	B	C		
Host strain		WT		*lrp*⁻	Effect of leu B/A (%)	Effect of *lrp*⁻ C/A (%)
Addition[a] to medium		0	leu[b]	0		
Expt.	strain/plasmid					
1	MEWS2[c]	1030	720	77	70	7
2	SWA1[d]	24400	13300	6650	55	27
3	SWA2[e]	24300	13100	6740	54	28

a: Assays were done on mid-exponential-phase cultures grown in glucose minimal medium at 37°C. The data in this table is the average of three different experiments and reported in Miller units.

b: L-leucine was added at the concentration of 100 ug/ml

c: Strain MEWS2 carries a chromosomal *serA::lacZ* fusion

d: SWA1, vector pRS415 carries the *serA* promoter region (P1+P2); from -970 to +290[f]

e: SWA2, vector pRS415 carries the *serA* promoter region (P1+P2); from -370 to +10[f]

f: Bases are number from the A of ATG as +1

Part 2. Characterization of the *serA* Promoter

Transcription initiation is controlled by both the regulators and the operator region of a gene's promoter. Changes to the promoter region will therefore affect the regulation and expression. The regulation of the *serA* promoter was studied in two ways: I. Mutations were made in its promoter region and the effect on expressions and binding affinities with Lrp were examined. II. The separated *serA* promoters, P1 and P2, were subcloned and studies were performed on how the deletions of the *serA* promoter affect its expression.

2-1. *In vitro* random mutagenesis of the entire *serA* promoter region

The goal of this study was to isolate and characterize *serA* promoter mutants with an altered response to Lrp. To do this, the *serA* promoter region was amplified by PCR, which routinely makes about 1 error per 1000 bp synthesized (Higuchi, 1989). Thus, when the PCR-amplified DNA was subcloned into pBluescript, an average of 1/3 should contain a single mutation in the 300 bp region upstream of the *serA* translation start site. This is tantamount to using PCR as a mutagenic technique.

The 300 bp upstream of the ATG were sequenced in about 60

subclones. Those containing mutations were subcloned into pRS415, forming a *serA::lacZ* fusion which allowed expression studies to be made. This two-stage method was used because it proved difficult to sequence directly from pRS415.

2-1a PCR Random Mutagenesis

For these experiments, the 380 bp of upstream *serA* DNA containing both P1 and P2 was amplified using Taq DNA polymerase for which this error rate was reported (Higuchi, 1989). The primers used are described in Fig. R5A. They correspond to the wild-type sequence, except at 1 base pair in primer I, and 2 in primer II. The amplified DNA therefore carries mutations at either end, which created new restriction sites, *Eco*RV and *Bam*HI. Amplification was thus accompanied by site-directed mutagenesis.

After amplification, the promoter region was subcloned into pBluescript, taking advantage of the 2 new restriction sites. Plasmids were isolated from about 60 colonies and their structure checked by restriction enzyme digestion. 36 plasmids carrying inserts of the expected size were then sequenced by double-stranded DNA sequencing and the mutated plasmids were selected for further study. The procedure is outlined in Fig. R5B.

Fig. R5A. Primers used for amplification and site-directed mutagenesis.

Primer I

WT SEQ: ATCCT TCACC CTATA TCGAT GC

*

PRIMER: ATCCT TGACC CGATA TCGAT GC

*Eco*RV

Primer II

WT SEQ: CTCCA GCGAT ACCTT TGCCA TTTACC

* *

PRIMER: CTCCA GGGAT CCCTT TGCCA TTTACC

*Bam*HI

Fig. R5A. Primers used for amplification and site-directed mutagenesis

The oligonucleotides were synthesized chemically to allow for creation of new restriction sites by site-directed mutagenesis. In the case of primer I, an *Eco*RI site was created by a one base change from T to G. In the case of primer II, a *Bam*HI site was created by the two base changes indicated: from C to G and A to C. The changed bases are marked with an asterisk *.

Fig. R5B. Strategy for the genetic dissection of a DNA sequence element, the *serA* promoter region of *E. coli*, by "mutagenic" PCR

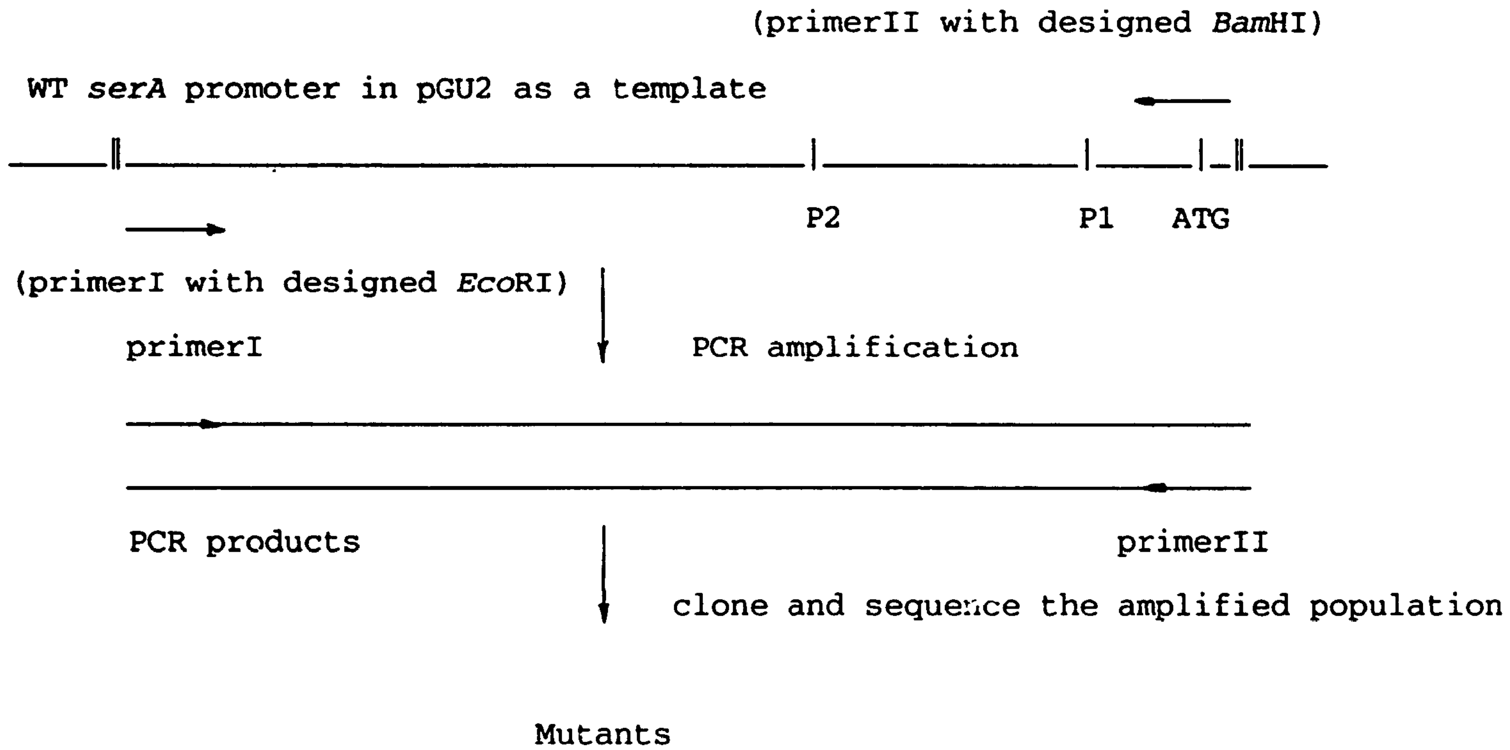


Fig. R5B. Strategy for the genetic dissection of a DNA sequence element, the *serA* promoter region of *E. coli*, by "mutagenic" PCR

1. The *serA* upstream region from -370 to +10, (counting from the translational start site ATG, A as +1) was amplified. Taq DNA polymerase randomly made mismatches during DNA synthesis. 2. The PCR products were cleaned and digested with *Eco*RV and *Bam*HI. The *serA* fragments were isolated with low-melting-temperature agarose and ligated with pBluescript which was digested with the same two enzymes. The ligase-treated preparations were transformed into XL-1 and selected on LB plate with Ampicillin and X-gal. The white colonies which contained inserts in the vector were selected. 4. These transformants were collected and the plasmids were isolated and checked by restriction enzyme digestion with *Eco*RV and *Bam*HI. 5. The plasmids carrying an insertion of the expected size were sequenced and the mutated ones were selected. 6. The relative contribution of individual basepairs to the activity of the *serA* promoter was assessed.

To locate promoters carrying mutations, plasmids of the expected size were sequenced by double-stranded DNA sequencing. Eight mutants were isolated. Among them, six mutants showed changes at only 1 base pair; the other two mutants showed two substitutions. The results are shown in Fig. R6.

The eight mutations screened occurred in several regions of the *serA* promoter. In this small sample, A and T were substituted by G and C much more frequently than the reverse. This might be explained by the binding affinity difference between GC and AT. The GC pair contains three hydrogen bonds, whereas the AT pair contains only two bonds, so that the GC pair is comparatively more stable and less easily substituted.

Fig. R6 The location and identification of the nucleotide changes

The whole PCR amplified region

mutations in the ATG Region

```
WT SEQ TTCAA AAGAC AGGAT TGGGT AAATG
UATG1  ----- ----- C---- ----- -----
UATG2  ----- ---G- ----- ----- -----
```

mutations in the P1 promoter region

```
WT SEQ TTGCC GCAAT ATTAT TTTTT GATAT GTTGA AAGGC GGATG CAAAT CCGCA
          -35                          -10
DP1-1  ----- ----- ----- ----- ----- ----- ----- ----- ----C -----
UP1-1  ----- ----- ----- ----- -G--- ----- ----- ----- ----- -----
UP1-2  ----- ----- ----- -C--- ----- ----- ----- ----- ----- -----
UP1-3  -C--- ----- ----- ----- ----- ----G ----- ----- ----- -----
```

mutations in the P2 promoter region

```
WT SEQ CCAGG CAATT GTCGA TTGCT CTAAA TAAAT CCTCT AAACC AGCAT ATTCA
                 -35                            -10
UP2-1  ----- ----- ----- ----- ----- ----- ----- --G-- ----- -----
UP2-2  --T-- ----- ----- ---T- ----- ----- ----- ----- ----- -----
```

underline: RNA polymerase binding site

strike-out: Lrp protected region by footprint experiment

2-1b. Characterization of the Mutants

The mutants, identified by double-stranded DNA sequencing, were subcloned into an operon fusion vector, PRS415. These subclones were checked by restriction enzyme digestion. Tc determine the effect of the mutations on the transcription of the *serA* promoter, the plasmids were transformed into MEW1 (wild-type *E. coli* strain) and MEW26 (*lrp* mutant) strains. The effect on *serA* expressions of these mutants was then assessed.

2-1b-1. Expression from Promoters with Mutations in the ATG Region

As mentioned, there were two mutants which mutated near the ATG site. The *serA* expression of these two mutants as measured by β-galactosidase activities is shown in Table R2. The UATG1 and UATG2 substitutions reduced overall expression from the *serA* promoter to 68% and 73% of wild-type expression (Table R2, expt. 2, 3; column D). This perhaps indicates that the mutations cause the whole *serA* promoter to be less efficient, since otherwise, regulation was only slightly altered. The decrease in the *lrp*$^-$ host was the same for all three plasmids (Table R2, expt 1, 2, and 3, column F, 28%, 24% and 25%) indicating that this region is not involved in Lrp recognition and binding.

Table R2. Expression from *serA* promoter carrying mutations near ATG

Experiment		ß-galatosidase			D	E	F
		A	B	C			
Host strain		WT		*lrp*⁻	% WT activity A/A1	Effect of leucine B/A (%)	Effect of *lrp*⁻ C/A (%)
Addition to medium		0	leu	0			
Expt.	plasmid						
1	WTSA2	24300	13100	6740	100	54	28
2	UATG1	16600	8700	4050	68	52	24
3	UATG2	17800	14200	4500	73	80	25

Expressed same as in Table R1.

The UATG1 promoter was also unchanged in its response to Lrp with leucine. (Table R2, expt 1 and 2, column E, 52% *vs* 54%). However UATG2 showed much less sensitivity to leucine (Table R2, expt 1 and 3, column E, 80% *vs* 54%).

The results suggested that the mutations in the ATG region did not affect the regulatory pattern of the *serA* promoter and the ATG region was not involved in Lrp recognition and binding, though the response to Lrp complexed to leucine maybe somewhat affected.

2-1b-2. The Expression from Promoters with Mutations in the P1 Promoter Region

Four fragments with mutations in the P1 promoter region were isolated. One of them contains two base substitutions. Another one was in the downstream region of the P1 promoter and the other two mutants were in the upstream region of the P1 promoter. According to the data of Table R3, all mutations in the *serA* P1 region changed the expression pattern of the *serA* promoter.

In comparison with the wild-type *serA* promoter, the mutation in the downstream region of P1 promoter increases overall expression from the *serA* promoter (Table R3, expt. 2, column D 117% wild-type expression). The decrease in the *lrp*⁻ host of DP1-1 was almost the same as the wild-type *serA* promoter (Table R3, expt. 1 and 2, column F, 34% *vs* 28%).

This indicates that the mutation does not affect Lrp recognition and binding significantly. However, the leucine repression of DP1-1 was less effective than that of the wild-type *serA* promoter (Table R3, expt. 1 and 2, column E, 86% *vs* 54%). This suggests that this region alters the response to the leucine/Lrp complex. Indeed DP1-1 with leucine expressed ß-galactosidase to the same extent as WTSA1 without leucine.

UP1-1, carrying a mutation in the upstream region of promoter P1, reduced overall expression from the *serA* promoter (Table R3 expt. 3, column D, 58%). The substitution also seriously affected leucine repression. In this case, Leucine not only did not reverse the Lrp activation as occurred at the wild-type promoter but leucine actually enhanced Lrp activation (Table R3, expt. 1 and 3, column E, 130% *vs* 54%). The level of decrease in *lrp*⁻ host in this substitution was less than the wild-type indicating that the mutation also affect the Lrp activation (Table R3, expt. 1 and 3, column F, 58% *vs* 28%). The UP1-1 promoter was considerably less efficient than the wild-type (Table R3, expt. 1 and 3, column A, 14100 *vs* 24300). However, the residual activity was much less dependent on Lrp (Table R3, expt. 1 and 3, column F, 58% *vs* 28%).

These effects were even more marked with the UP1-2 mutation. In this case, only 16% of promoter "strength" remained, and the activity was not affected by either Lrp or leucine (Table R3, expt. 1 and 4, column A and E, F, overall

expression 3810 *vs* 24300, leucine respond 95% *vs* 54%, in *lrp*⁻ host 90% *vs* 28%). It seems that the mutation not only affected the P1 promoter function, but also somehow affected or blocked the P2 promoter and made the expression of the P2 promoter decrease. The data suggest that this region is important for all aspects of *serA* promoter function.

The sequencing data showed that the two base changes of UP1-3 were near the P1 promoter, one base change near the P1 -10 region, the other one changed inside the P1 -35 region.

In the case of UP1-3, the activation of Lrp on *serA* promoter was largely reduced (Table R3, expt. 1 and 5, column D, 36%) and L-leucine effect in the substitution was also largely reduced (Table R3, expt. 1 and 5, column E, 89% *vs* 54%), same as the *lrp*⁻ host (Table R3, expt. 1 and 5, column F, 80% *vs* 28%). If we compare the expression level in the *lrp*⁻ host of UP1-3 substitution with wild-type promoter, it shows that the expression levels on both cases are almost the same (Table R3, expt. 1 and 5 column C, 7000 *vs* 6740). The effect on the P1 promoter could be explained by a change of RNA polymerase binding region. Such a change may cause a decrease in RNA polymerase binding to the P1 promoter, and this would reduce the expression of the related promoter (Fig. R7). In addition, the mutations may modify leucine effect directly either at the leucine/Lrp complex interaction site on the promoter or by changing the DNA conformation.

Fig. R7. The RNA polymerase binding site changes on *serA* mutated fragment UP1-3

consensus sequences:

```
TTGACA                TATAAT
::: :                 :::  :
TTGCCGCAATATTATTTTTTGATATGTTG   WTserA
TTGACA                TATAAT
: : :                 :::  :
TCGCCGCAATATTATTTTTTGATATGTTG   UP1-3
```

Table R3 Expression from *serA* promoters carrying mutations near the P1 promoter

Expt.		ß-galatosidase			D	E	F
		A	B	C			
Host strain		WT		*lrp*⁻	% WT activity A/A1	Effect of leu B/A (%)	Effect of *lrp*⁻ C/A (%)
Addition to medium		0	leu	0			
Expt.	Plasmid						
1	WTSA2	24300	13100	6740	100	54	28
2	DP1-1	28500	24400	9700	117	86	34
3	UP1-1	14100	18300	8220	58	130	58
4	UP1-2	3810	3610	3440	16	95	90
5	UP1-3	8740	7740	7000	36	89	80

Expressed same as in Table R1.

2-1b-3. Changes in Regulation in the Mutants near the P2 Promoter

Two mutants were found in the P2 promoter region. One of them contained two-base changes. The other one is a point mutation. The expression of these two mutants is shown in Table R4.

The UP2-1 substitution caused an almost total loss of *serA* promoter function (Table R4, expt. 1 and 2, column A, B, and C, 330 *vs* 24300, 1060 *vs* 13100 , 260 *vs* 67400). The mutation of UP2-1 was located at the -10 region of P2 promoter. It could be predicted that this change would affect expression of the P2 promoter (Fig. R8). The earlier footprint analysis on the wild-type *serA* sequence showed that the *lrp* gene product protects this region. One explanation for the dramatically reduced level of *serA* expression of this mutant is that the mutation affects the Lrp-*serA* binding either by affinity or by conformation changes.

The mutant UP2-2 carried two base changes in the P2 region, and both changes were inside the Lrp protected region (Lin, 1992c).

Fig. R8. The RNA polymerase binding site changes on *serA* mutated fragment UP2-1

consensus sequences:

```
TTGACA                  TATAAT
::: :                   : :::
TTGTCGATTGCTCTAAATAAATCCTCTAAAC   WTserA

TTGACA                  TATAAT
::: :                   : :::
TTGTCGATTGCTCTAAATAAATCCTCTAAGC   UP2-1
```

In UP2-2, the overall expression from the *serA* promoter was reduced a little bit (Table R4, expt. 3, column D, 86%). The activity was less dependent on Lrp than wild-type (Table R4, expt. 1 and 3, column F, 50% *vs* 28%). The activation of Lrp on *serA* expression in the UP2-2 substitution was not reversed but enhanced by L-leucine (Table R4, expt. 1 and 3, column E, 125% *vs* 54%).

The combination of two mutations in the wild-type strain had very little effect on expression from UP2-2 (Table R4. expt. 3, column D, 86% wild-type expression), very different from the case with UP2-1 (Table R4. expt. 2, column D, 1% wild-type expression). This activity was less dependent on Lrp than wild-type activity (Table R4, column F, 79% and 50% *vs* 28%), and leucine actually enhanced activity rather than repressing it (Table R4, column E, 320% and 125% *vs* 54%). That is, the UP2-1 mutation formed an almost defective promoter and the UP2-2 mutations left the promoter with considerable activity. However, in both cases, the changes made transcription much less dependent on Lrp and stimulated by the Lrp/leucine complex.

Table R4. Expression from *serA* promoters carrying mutations near the P2 promoter

Experiment		ß-galatosidase			D	E	F
		A	B	C			
Host strain		WT		*lrp*⁻	% WT activity A/A1	Effect of leucine B/A (%)	Effect of *lrp*⁻ C/A (%)
Addition to medium		0	leu	0			
Expt.	plasmid						
1	WTSA2	24300	13100	6740	100	54	28
2	UP2-1	330	1060	260	1	320	79
3	UP2-2	20800	25900	10400	86	125	50

Expression same as in Table R1.

2-1c. Purification of Lrp and its Use in Gel Retardation Studies

Lrp has been shown to bind to the *serA* promoter region in *in vitro* studies, and to stimulate transcription from the *serA* promoter as judged by *in vivo* studies. This clearly suggests that the Lrp protein may regulate *serA* transcription initiation via direct protein-DNA interactions at the *serA* promoter. If this were true, it should be possible to find mutations which affect expression of *serA* and also change binding affinity of Lrp for the mutated *serA* gene. To verify this, I checked whether any of my mutant promoters showed altered binding in retardation studies using Lrp protein and *serA* promoter fragments.

2-1c-1. Purification of Lrp

To study the affinity of Lrp protein for mutated fragments, a supply of purified Lrp was required. Lrp purification was performed by the method of Dr. R. Matthews (personal communication) with slight modification. I used for this work a plasmid containing a p*ara*BAD-*lrp* operon fusion (see Materials and Methods). This plasmid, called MT1, carries the Lrp coding region under the control of the arabinose promoter (pBAD). Adding arabinose to the medium allows

production of Lrp in high amount.

To produce Lrp, I transformed the MT1 plasmid into strain MEW1A (MEW1 *ara*$^-$), and grew the plasmid-containing cells in the presence of arabinose to induce *lrp* production, harvested the cells, made extracts, and purified the Lrp on Bio-Rex 70.

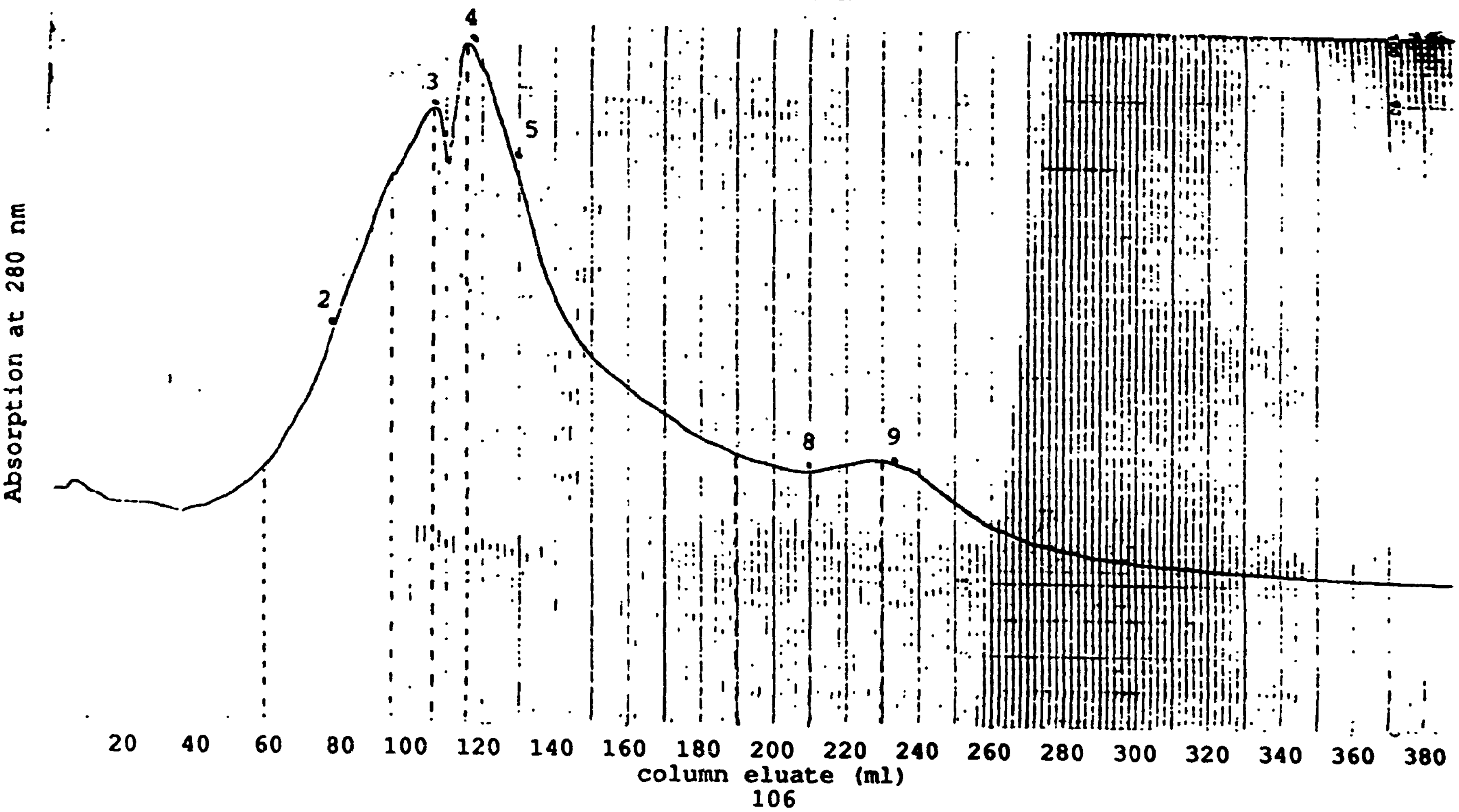
Thus strain MEW1A/MT1 was grown overnight in LB with arabinose 0.1% and ampicillin 100 ug/ml to ensure plasmid maintenance, and subcultured in the same medium in a 1-L flask with 400 ml culture. One hour prior to harvesting, more arabinose was added to a final concentration 0.5%. Cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C.

The cells were resuspended in TGED buffer (0.2M NaCl pH 8) 3.5ml per gram wet weight of cells and sonicated to clarity and centrifuged. 10ml of the extract was loaded onto a 15ml bed volume Bio-Rex 70 column which had been incubated overnight with KPB buffer and then washed with TGED 0.2M NaCl buffer. The column was connected with a UV monitor (280 nm) and a recorder. After the cell extract was loaded, the column was washed with TGED 0.2M NaCl buffer, until the recorder reached the base line of the buffer alone, then eluted with increasing concentrations of NaCl in TGED buffer (from 0.2 to 1 M). The fractions were collected with a fraction collector (Fig. R9)

Since the *lrp* gene product does not have any easily identifiable characteristics (i.e., no catalytic properties),

it is difficult to demonstrate explicitly that a certain fraction contains Lrp. To determine which fractions contained Lrp protein, SDS PAGEs were run with each fraction from the purification, with extracts of strains MEW1A and MEW1A/MT1 as controls (Fig. R10). It is immediately obvious that the density of one band is greatly increased in the Lrp-induced strain (MEW1A/MT1) relative to wild-type (MEW1A). This band is likely to be Lrp, since the size of the protein in this band is the same as that of the Lrp monomer (Willins, 1991). The elution fraction that is enriched for the expected band was dialyzed with 0.2 M NaCl TGED buffer (pH 8.0) for at least 24 hr, at 4°C. The buffer was changed three times during this period to remove the NaCl. The eluate was then concentrated by the "speed vac". The final purified protein was kept in TGED50 buffer with 0.2M sodium chloride at -20°C. This was used to examine binding of Lrp to *serA*.

Fig. R9. Elution of protein from Bio-Rex 70 column



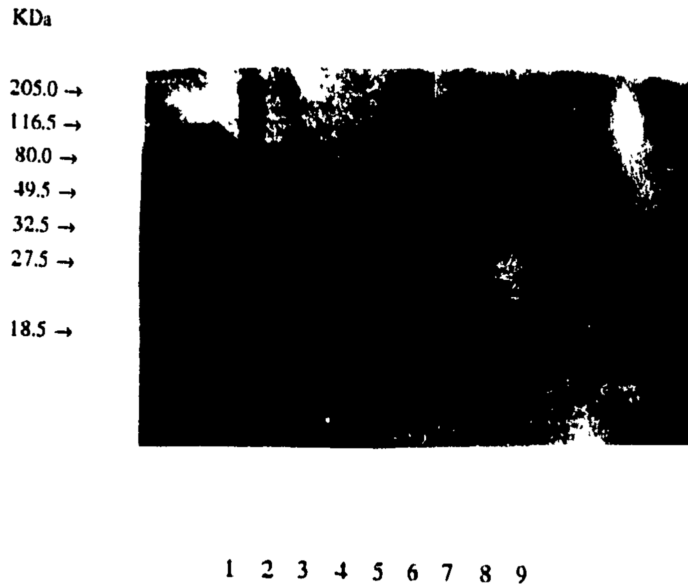


Fig. R10. SDS-PAGE of the purified Lrp

5% PAGE was run following the method in "Molecular Cloning" and the gel stained with Coomassie brilliant blue. Lane 1 was loaded with molecular weight markers and Lane 6 and lane 7 with the cell extract of wild-type strain and of the wild-type carrying the Lrp plasmid. Lane 2, 3, 4, 5, 8, and 9 were loaded with the particular fractions from the Lrp purification column indicated in Fig. R9.

This figure represents one experiment and is typical of many.

2-1c-2. Gel retardation experiments

Gel mobility shift experiments were performed in order to compare binding of Lrp with *serA* fragments from the mutant plasmids with binding to that of the wild-type one. End-labelled *serA* fragments carrying different mutations were derived from these plasmids, and incubated with differing amounts of Lrp protein.

The fragment from mutant UP1-2 showed less binding affinity for Lrp than the wild-type *serA* fragment, (Fig. R11). When the end-labelled UP1-2 fragment was incubated with different amounts of purified Lrp protein, followed by polyacrylamide gel electrophoresis, it was very weakly retarded with 10 ng of Lrp, (Fig. R11, A, Lane 3), whereas the wild-type fragment was retarded much more with the same amount of Lrp (Fig. R11, B, Lane 3). With 25 ng of Lrp, the wild-type fragment was retarded most of the DNA fragment and the UP1-2 substitution was only retarded part of the fragment. (Fig. R11, B, Lane 4 and A, Lane 4). This correlates with the fact that *in vivo* expression from this mutant as measured by β-galactosidase activities was also severely decreased (Table R3, expt. 3, 58%). This suggests that the decrease in *serA* expression in this mutant was caused by a decreased binding of Lrp to the *serA* promoter region.

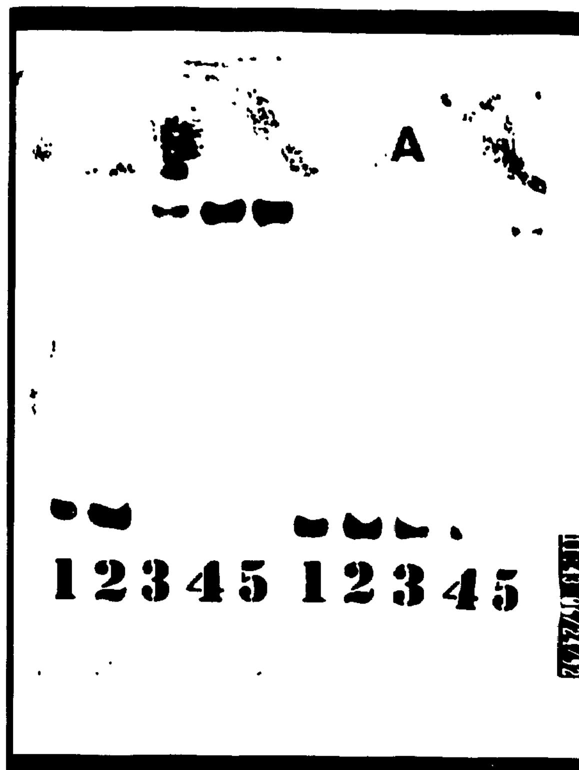


Fig. R11. The gel retardation analysis on the *serA* promoter mutant UP1-2

(A) The 380 bp fragment from UP1-2 was labelled with ^{32}P, incubated with increasing concentrations of Lrp protein, Lane 1, no lrp protein was added. Lane 2 through 5 represent incubations with 5, 10, 25, and 50 ng of Lrp protein, respectively. (B) Lane 1 through 5 represent the same experiment using wild-type *serA* fragment.

Another mutated *serA* fragment which showed changed binding affinity is UP2-1. It showed that the binding affinity of the UP2-1 fragment was also reduced in comparison with that of the wild-type (Fig. R12, A, Lane 3, the UP2-1 fragment with 10 ng of Lrp and B, Lane 3, the wild-type *serA* fragment with 10 ng of Lrp). This is consistent with the *in vivo* expression experiments showing the UP2-1 substitution caused a nearly total loss of the *serA* promoter expression. In addition, according to the sequence result, the UP2-1 mutation was located in the -10 region of P2 promoter, which is also the Lrp binding region according to the DNase I footprint results of Lin (Lin, 1992c). Thus the phenomenon can be explained as the mutation affecting the binding affinity of Lrp to *serA* promoter, so that the Lrp could not activate the transcription initiation of the promoter.

Some other mutated fragments were also tested for the binding affinity. The results did not show detectable affinity change (Fig. R13).

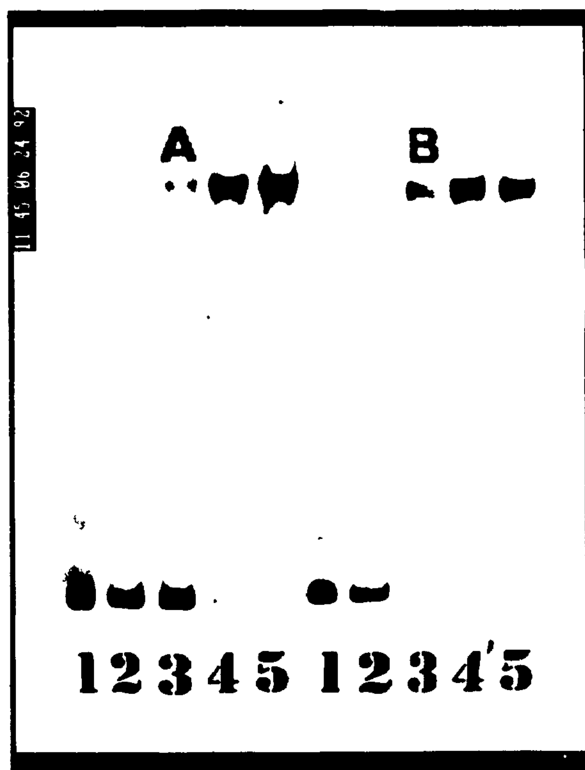


Fig. R12. The gel retardation analysis on the *serA* promoter mutant UP2-1

The experiment was the same as that in Fig. R11, except that the UP2-1 fragment replaced UP1-2 fragment as substrate.

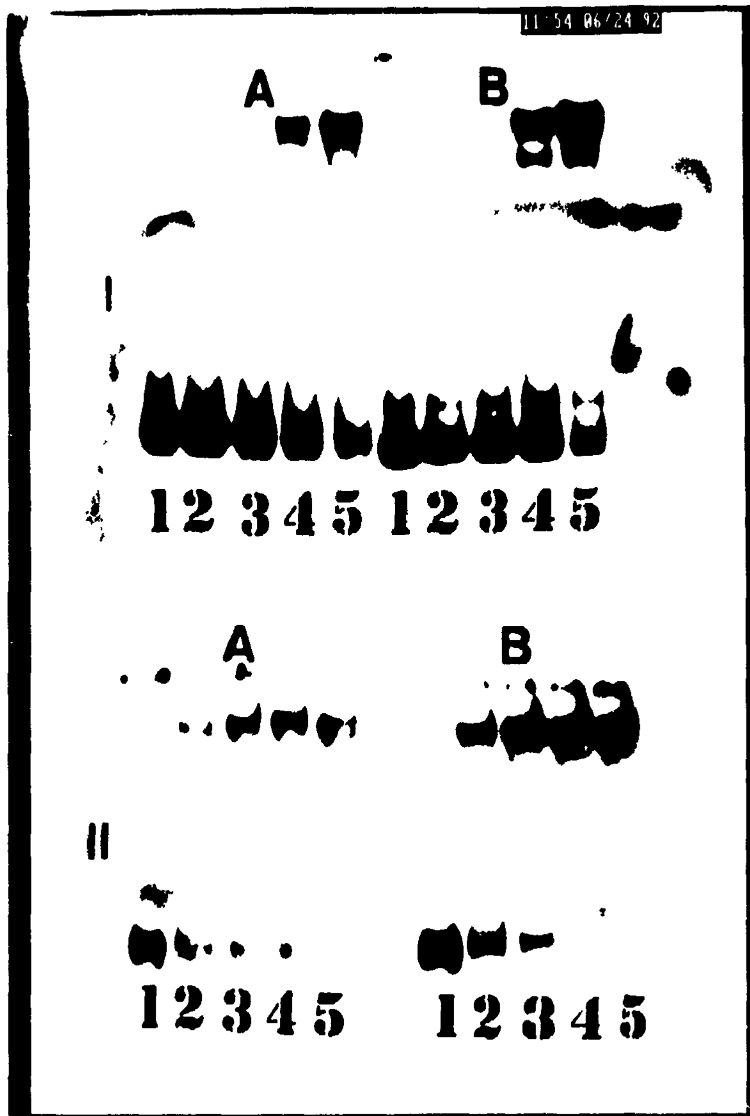


Fig. R13. The gel retardation analysis on the *serA* promoter mutants UATG1 and UATG2

The experiment was the same as that in Fig. R11, except that the UATG1 (Fig. R13-I) or UATG2 (Fig. R13-II) fragment replaced UP1-2 fragment as substrate.

2-2. The Expression and Regulation of the Two Separated *serA* Promoters P1 and P2

2-2a. The Construction of *serA*P1 and *serA*P2 *lacZ* Fusion Plasmids

serA contains two promoters, P1 and P2. Lrp binds to *serA* promoter region and regulates the expression of these two promoters differently (Lin, 1992c). In this study, the two *serA* promoters, P1 and P2 were separately amplified by PCR and subcloned into the pRS415 vector to permit regulatory studies of each promoter *in vivo* (Fig. R14), as explained in methods.

As shown in the diagram, to subclone the separated *serA* promoters, P1 and P2, each cloned individual promoter would lose some regulatory region of wild-type *serA* promoter. P1 promoter will lose the Lrp high affinity binding site which is located in P2 region, and P2 promoter will lose the low affinity binding site which is near P1 promoter. The deletions may affect the expression of the promoters.

2-2b. Expression Studies of Separated P1 and P2 Promoters

Regulation of expression from P1 and P2 separately was very different. Lrp activated at P1 (Table R5, expt. 1, column E, 37%) and repressed at P2, (Table R5, expt. 2, column E, 391%). This data is consistent with the primer extension

experiments which measured transcript accumulation (Lin, 1992c).

Leucine had very different effects on the two *serA* promoters, repressing transcription from P1 (Table R5, expt. 1, column D, 78%) and activating at P2 (Table R5, expt. 2, column D, 181%).

Together these experiments indicate that the *serA* gene has two promoters, P1 and P2. Promoter P1 is used in the presence of Lrp and the activation is partially reversed by leucine. Initiation at the other promoter, P2, is repressed by Lrp and the repression is partially reversed by leucine.

Expression from either P1 or P2 was greatly reduced as compared to that of the entire promoter (Table R5, 3530, 690 *vs* Table R1, 24,300).

The experiments reported here show that the P2 promoter region was required along with the P1 promoter to obtain full expression from P1. The P1 promoter, without the extended upstream region, has much decreased expression. It is clear then that both P1 and P2 are needed for full wild-type transcription of *serA*.

Fig. R14 The subcloned regions of the *serA*P1 and P2 promoters

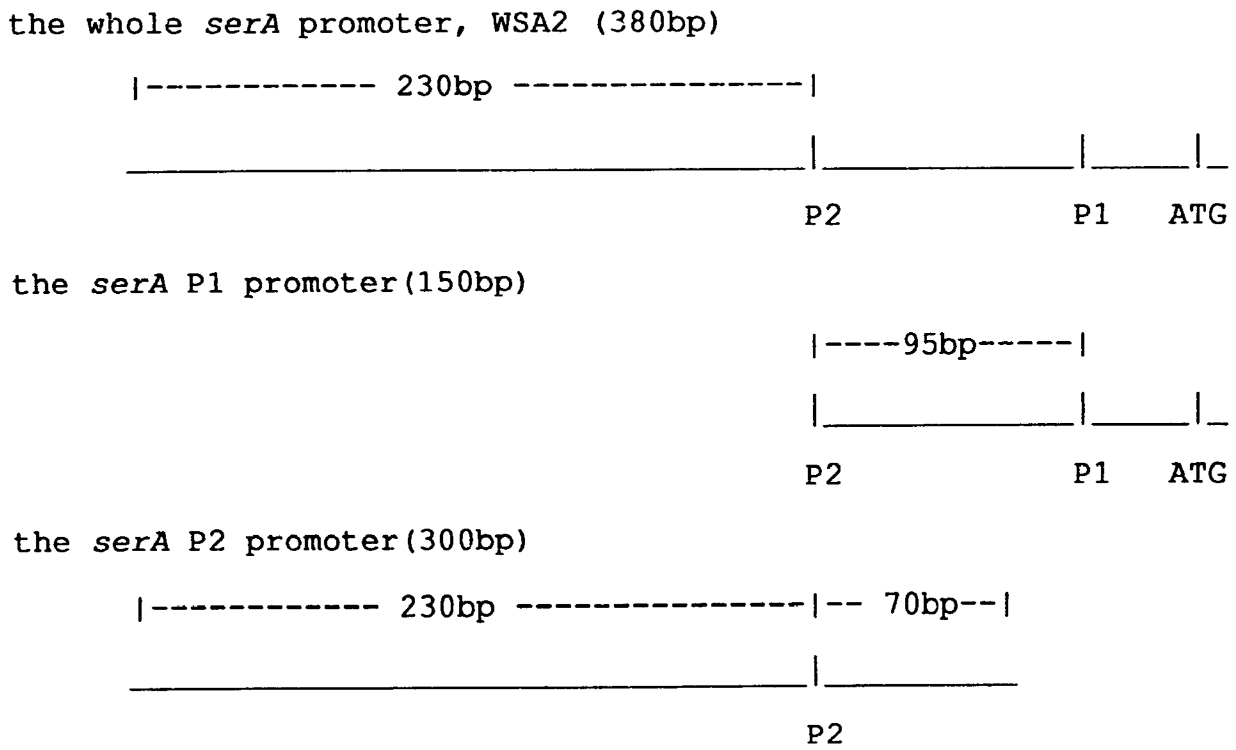


Fig. R14. The subcloned regions of the *serA*P1 and P2 promoters

The two promoters were subcloned independently from pWSA2 into pRS415. The 150 bp fragment carrying P1 runs from the +10 downstream end of pWSA2 to 95 bp upstream of the P1 transcription start site, the point at which P2 transcription usually starts. The 300 bp P2 fragment starts 70 bp downstream from the P2 transcription start site, and extends upstream to the same point as pWSA2, 230 bp upstream of P2.

Table R5. Expression from the individual *serA* promoters

Experiment			ß-galactosidase			D	E
			A	B	C		
Host strain			WT		*lrp*⁻	Eff. of leu B/A(%)	Eff.of *lrp*⁻ C/A(%)
Addition to medium			0	leu	0		
Expt.	Plasmid	Description					
1	SAI	P1 promoter(-140 to +10)[a]	3530	2770	1300	78	37
2	SAII	P2 promoter(-370 to -70)	690	1250	2420	181	351

Expressed as in Table R1.

a: bases are numbered from the A of ATG as +1

Discussion

We have studied the mechanism of *serA* gene regulation by Lrp. Lrp is a protein that transcriptionally regulates the expression of a large number of genes. Exogenous L-leucine can cooperate with Lrp and affect its functions. *SerA* which codes for the first enzyme in serine biosynthesis pathway, 3-phosphoglycerate dehydrogenase, is regulated by Lrp and L-leucine.

Part 1. Lrp Regulation studies on the wild-type *serA* promoter

1-1. L-serine biosynthesis plays a central role in *Escherichia coli* cell metabolism

In *Escherichia coli,* the availability of serine is absolutely essential for the metabolism of several diverse classes of cellular components. During growth on glucose, as much as 15% of the assimilated carbon in *E. coli* involves serine or its metabolites (Pixer, 1964). L-serine is synthesized from 3-phosphoglyceric acid (PGA), an intermediate of the Embden Meyerhof Parnas (EMP) pathway, via three primary enzymes: 3-phosphoglycerate dehydrogenase(*serA*), 3-phosphoserine aminotransferase(*serC*), and 3-phosphoserine phosphatase(*serB*). Serine is required for a large number of

biosynthetic reactions as a three-carbon precursor in the synthesis of cysteine, methionine and tryptophan, and a two-carbon unit for the formation of glycine and purine. In addition, serine and glycine are the only sources of one-carbon units for biosynthetic and methylation reactions.

The regulation of serine biosynthesis differs from that of other amino acids. The expression of the first enzyme in this pathway, *serA*, is not repressed by the end-product, instead serine only inhibits the enzyme activity of the *serA* gene product. In fact, it never totally shuts down serine biosynthesis; a certain level of serine inside the cell is always needed to provide C1 units, and to support other metabolic reactions.

How is serine biosynthesis regulated inside the cell? Usually, metabolic systems are transcriptionally regulated at the first enzyme in a pathway. The biosynthesis of serine is also regulated by the first enzyme in that pathway (*serA*). It was shown recently that the *serA* gene contains two promoters, P1 and P2. The P1 promoter is activated by Lrp. In the presence of exogenous L-leucine, this activation is partially reversed. P2 promoter is repressed by Lrp. The Lrp protein, therefore, has at least two binding sites on the *serA* promoter. The *serA* gene is regulated in such a way that serine biosynthesis is always above the level that allows the cell to function properly.

1-2. Technique for studies of gene regulation

Genetic fusions have provided a powerful tool to study the regulation of gene expression in prokaryotes (Bassford, 1978; Silhavy, 1985). Fusions to the *lacZ* gene of *E. coli* are particularly useful. The activity of the fused *lacZ* gene is quantified by a simple, sensitive assay. A variety of sophisticated genetic techniques are available for the identification and/or selection of mutations that alter the expression of the fused *lacZ* gene. Two types of fusion products, protein fusion and operon fusion, can be constructed. In the protein fusion, a *lacZ* gene lacking both transcription and translation initiation signals is fused in frame to the coding sequence of a target gene. On the other hand, in an operon fusion, a *lacZ* gene containing its own translation start site, but lacking a promoter, is fused to an exogenous promoter. Operon fusions are useful for transcriptional regulatory studies of any gene. Protein fusions are useful for both transcriptional and translational regulatory studies.

1-3. The level of *serA* expression is directly proportional to Lrp concentration

To study the effect of Lrp concentration on *serA* expression, pBAD-*lrp* fusion plasmids, in which the *lrp* gene is under the control of *ara*BAD promoter, were constructed. This construction allows arabinose to regulate *lrp* expression. In this way we are able to control the amount of Lrp in the cell by varying the concentration of arabinose.

The effect of arabinose on the Lrp expression plasmids were first studied. The results indicated that the expression of pBAD promoter was affected not only by arabinose, the regulator of the pBAD promoter, but also by the growth of the cell.

The comparison of the expression of three subcloned pBAD::*lrp* plasmids (MT1, MT3, MTΔ), indicated that a 16-base-pair sequence, immediately upstream from the *lrp* ATG, was necessary for *lrp* expression. This is because the pBAD promoter itself (in the pBAD18 plasmid) did not contain a proper ribosome binding site, which is required for translation of a gene. The 16-base-pair upstream region of *lrp* does have a ribosome binding site for the expression of the protein. The induction of the pBAD promoter in LB was much higher than that in minimum glucose medium. This is because glucose represses the expression of the *ara* promoter and

arabinose uptake.

The expression of *serA* in the presence of the MT3 (3 mutations in the 16-base region) plasmid in LB was studied. By comparing the experimental data of the expression of a pBAD*lrp*::*lacZ* plasmid, and the expression of *serA*::*lacZ* carrying the pBAD::*lrp* plasmid, we can conclude that the expression of *serA* is proportional to the amount of Lrp.

The transcription of *sdaA* in *E. coli* is under negative control by Lrp and the repression is partially reversed by L-leucine. The *in vitro* studies demonstrated that the promoter regions of *sdaA* binds to Lrp and the presence of L-leucine reduces the binding of Lrp to the promoters (Lin, 1992c). In these studies, we demonstrate that with an increase in the amount of Lrp, the level of expression of *sdaA* gene was decreased.

The results suggested that Lrp may work as a direct factor on the regulation of its target genes.

Part 2. The regulation and binding studies on *serA* promoter

The promoter is the DNA sequence upstream to the gene's coding region and is essential for transcription initiation. It usually contains a consensus sequence which is recognized by RNA polymerase, and some regulatory DNA elements. Mutations within the RNA polymerase binding site usually cause a decrease in the level of transcription. The regulatory DNA elements are also important and/or essential for maintaining promoter function. They provide the binding sites for regulatory proteins and possibly maintain the necessary DNA structure. The expression of a particular gene is dependent on the nature of the promoter and its regulatory protein.

serA contains two promoters (P1 and P2). It provides both the consensus sequence for RNA polymerase recognizing and binding, and operators for Lrp binding. Lrp functions as an activator for *serA* P1 and a repressor for *serA* P2.

The function of a repressor is to decrease transcription initiation. The classical repression mechanism in prokaryotes is that the operators overlap the RNA polymerase binding site so that the repressors block access of RNA polymerase to the promoter (Major, 1975). The repressor can also function by interfering with the function of bound RNA polymerase; it may block open-promoter complex isomerization, or it may inhibit

the actual initiation steps (Collado-Vides, 1991). A repressor can also function by binding to a distant duplicated operator site (Collado-Vides, 1991).

In most cases, activators bind near the -35 region of a promoter and increase the rate of transcription initiation (Collado-Vides, 1991).

Binding of a protein to a strong affinity binding site can help a second molecule bind to a weak affinity site (Hochschild, 1986). The high affinity binding site of Lrp on the *ilvIH* promoter is thought to enhance the binding of the low affinity site of Lrp (near the RNA polymerase binding site), and thereby activate transcription initiation (Wang, 1993b).

Changes in the promoter region may alter either the regulatory protein's binding affinity, or the DNA conformation of the promoter; both alterations can affect the expression of the gene. The *serA* regulatory mechanism was studied by analyzing the effect of promoter changes on its expression.

2-1 Techniques used for this study

2-1a Random mutagenesis on the *serA* promoter region

In PCR amplification, the Taq DNA polymerase randomly performs mispairing of bases. The mutation rate of PCR is around 0.1%.(M. Howe, personal communication). This means that on average, PCR amplification generates 1 base mispairing per 1kbp amplified fragment. This allows us to make random mutations on a certain piece of DNA by PCR amplification.

The *serA* promoter fragment, which is 380 bp in size and contains both P1 and P2 promoter, was amplified by PCR method. Presumably, according to the PCR mutagenic activities, in each of three amplified *serA* fragments, there is one mutation. In other words, around 38% of our PCR amplification fragments would contain mutation and most of them should be point mutations.

Based on this assumption, PCR amplification was used to generate mutations on the *serA* fragment. The results of the PCR amplification indicated that the PCR method is a convenient method for making point mutations on a piece of DNA sequence less than 1kb in length. The mutations are randomly located in the entire amplified region and most of the mutations were point mutations.

The PCR amplified *serA* fragments were subcloned into

pBluescript vector, and were sequenced by double-stranded DNA sequencing. The mutations of the *serA* fragment were located. According to the sequencing results, six out of eight mutations are point mutations. The mutagenic frequency of *serA* amplification is therefore around 14% (8/60), much lower than reported (Higuchi, 1989). The *serA* mutations occurred in several regions of the *serA* promoter.

The PCR mutation results demonstrate another fact: that the base changes of most of the mutations by PCR method were G or C replacing A or T. This might be due to differences in the binding affinities of individual base pair. Each G-C pair is held together by three hydrogen bonds, rather than the two holding together each A-T pair. The G-C base pair is therefore somewhat stronger than the A-T base pair. It is possible, given this consideration, that the substitution of G or C might be more favourable for further base pairing, so that the PCR method may generate more GC mutants than AT ones (Results).

To overcome this, the concentration of A and T can be increased in future PCR experiments. The probability of substitution of the four bases can be balanced by adjusting the concentration of the four bases.

2-1b DNA-protein binding assay

Both the regulatory and the protein-DNA binding affinity

studies were carried out with these *serA* promoter mutants. The regulatory studies, as we have already mentioned, used the gene fusion technique. For the protein-DNA binding affinity studies, the gel retardation assay was used (Fried, 1981 and Garner, 1981). The gel retardation assay, a simple and rapid technique, has been widely used in the study of protein-DNA interactions. This method is based on the observation that the electrophoretic mobility of a nucleic acid is altered when a protein is bound to it. The gel retardation method has several advantages compared to other common assays for protein-nucleic acid interactions. First, a specific binding protein can be detected by its effect on mobility even when other binding proteins are present, as in a crude cell extract. Second, the specific nucleic acid target site of a given binding protein can be identified from a mixed population of fragments. Third, because free protein is separated from complexes, only active protein will be present in the complex bands. Fourth, complexes can often be resolved even if they differ only in their stoichiometries or in the physical arrangement of their components. The last two are the most important advantages of the gel retardation method.

2-2. Regulation of the wild-type *serA* promoter

Fragments of the *serA* promoters were subcloned into the operon fusion vector, pRS415. This vector has the advantages of containing unique restriction sites for the introduction of cloned segments, the *lacZ* gene coding region to which the fusion is made, and four tandem copies of the strong transcriptional terminators, *T1* (cloned from the *E. coli rrnB* operon) to block transcription from upstream plasmid promoters. Because of these characteristics, the *serA* promoter which was subcloned into it would be the only functional promoter regulating the expression of the *lacZ* gene and the activities of ß-galactosidase would represent the expression level of the *serA* promoter alone.

For cloning the whole *serA* promoter, two different sizes of the *serA* promoter region were used, to be certain that the subcloned fragment contained all the necessary functional domains for the *serA* promoter retaining its full function. The larger one (WSA1) was 1.26 kbp in size. It was directly subcloned from the *serA* insertion in the pGU2 plasmid. The other fragment (WSA2) was 380 bp in size, and was obtained by removing the coding region from the downstream side (280bp) and part of the far upstream side (600bp) of the 1.26 kbp fragment.

The comparison of the regulation of the chromosomal *serA*

with the plasmids showed that the regulatory patterns were the same, except that the expression level of the plasmids were much higher than that of the chromosomal version (Table R1). This might be due to the difference in copy number. The chromosomal DNA has only one copy of the *serA* gene, whereas the plasmids used in this study were multiple copy plasmids. In any case it is clear that the regions removed in shortening pWSA1 to pWSA2 have no effect on the regulation of transcription. The 380-bp fragment of WSA2 contained all determinants for wild-type *serA* expression. It was used for the further mutagenesis studies.

2-3. Does Lrp stimulate transcription from the *serA* promoter upon binding to the upstream region of the promoter?

These studies were prompted by several considerations. First, *in vivo* expression of the *serA* gene is activated by Lrp and the activation is reversed by exogenous L-leucine (Lin, 1990). Second, *in vitro* experiments show that Lrp binds specifically to the DNA upstream of the *serA* gene (Lin, 1992c). Furthermore, we were interested in the mechanism by which Lrp activates transcription of the *serA* gene. We suspected that Lrp activated the transcription of the *serA* gene by directly interacting with its promoter region. For these reasons, we created mutations on the *serA* promoter

region which may affect the expression of *serA* and the binding affinities with Lrp.

The results with the mutants, UATG1 and UATG2, which mutated the region upstream of ATG, show that mutation in this region did not greatly affect the expression pattern (the expression level is only slightly lower than that of the wild-type). This may be caused by DNA conformational changes in these mutants. The binding affinities of these mutants did not show detectable changes under the conditions at which these experiments were performed. The data suggested that this region was not involved in Lrp activation on the *serA* promoter.

There are four mutants inside the P1 region. The changes in the expressions of these mutants are various. Mutant, DP1-1 which was mutated downstream of P1, shows increased expression in all conditions. UP1-1, which was mutated upstream of P1 shows increased expression in the presence of leucine and in the case of the *lrp*$^{-}$ mutant. The reason for the expression changes in these two mutants may be DNA conformational changes.

The mutation of UP1-2, which is also in the upstream region of P1, severely decreases the *serA* expression in all cases. This mutation also decreases the binding affinity of Lrp to the mutated *serA* fragment. As the mutation site of this mutant is not inside the high affinity binding region of the

Lrp protein, the change in binding affinity may be due to an effect on the cooperation between the two binding region, as suggested by Wang *et al.* for the *ilvIH* promoter (Wang, 1993b) or it may be due to a DNA conformational change which alters the Lrp-DNA binding affinity in the high affinity binding site. Both the Lrp binding affinity changes and the conformational changes may decrease the *serA* expression.

Mutations in the upstream region of P2 also change the regulation pattern of the *serA* promoter. Unlike mutations in P1 region, the mutations in the P2 region share some common features: both UP2-1 and UP2-2 reverse the leucine response from repression to activation; and the Lrp activation level in both cases also decreases somewhat, although the overall expressions of these two mutants are quite different.

The mutation of UP2-1 also decreases the binding affinity of Lrp. As the mutation was at the Lrp high affinity binding region on the footprint (Lin, 1992c), as well as at the P2 RNA polymerase binding region, it is predictable that this mutation may affect Lrp and RNA polymerase binding to this region. Altering the RNA polymerase binding site will affect the expression of the P2 promoter and altering the Lrp binding site may interfere with the Lrp activation of the P1 promoter. The fact that the mutation interferes with the expression of the P1 promoter can be explained by the co-operative binding of Lrp suggested by Wang and Calvo. Due to the decrease in

the binding affinities, Lrp binding at this site could not enhance the binding of the low affinity site near P1, so the Lrp activation of *serA* via the P1 promoter was decreased.

Thus, the mutants which changes the binding affinity of Lrp, such as UP1-2 and UP2-1, cause a severe decrease in the expression of the *serA* promoter. This suggested that the expression of *serA* is directly affected by the Lrp protein. The mutants that affected leucine response are more complicated. There is no specific region which showed clear response to the leucine effect on the *serA* promoter.

2-4. *serA* P1 and P2 promoters are subject to different regulatory effects of Lrp

serA P1 and P2 promoters were subcloned separately into pRS415. Their expressions were then studied.

As mention earlier, a functional promoter requires both the RNA polymerase binding site and the necessary regulatory elements. The regulatory elements of a promoter can provide the binding site for the regulatory protein and/or help the promoter DNA maintain a specific functional conformation.

When cloning the P1 promoter alone, the upstream promoter in front of *serA* P1, the P2 promoter should be deleted. However, the Lrp binding sites overlap with RNA polymerase binding sites of P2 promoter, and the two promoters are near

to each other. When we cut off the P2 promoter, at least part of the Lrp binding sites are also deleted. Therefore, if Lrp regulated *serA* expression by directly binding to the promoter region, then the full activity of P1 promoter would not be able to remain when the P2 promoter was deleted.

The overall expression of the P1 promoter was indeed much lower than that of the whole *serA* promoter. The activation of Lrp on the P1 promoter is also lower than it is on the whole *serA* promoter (Table R1 and R5, 2.7 fold P1 promoter *vs* 13 fold chromosome and 3.6 whole *serA* promoter in the plasmid). In the whole *serA* promoter, the level of activation should be reduced if one considers the repression of the P2 promoter by Lrp. The extent of the leucine effect was also decreased. The results suggested that Lrp binding to the *serA* promoter region is required for P1 promoter expression and activation. In addition, the deletion may cause conformational changes that may also affect the expression.

The P2 promoter was also subcloned into the pRS415 vector. The upstream end of the P2 fragment was the same as SWA2, and the downstream side was 70 bp downstream of P2.

The expression studies on P2 plasmid showed that the expression of the P2 promoter was also lower than the wild-type expression. This promoter was repressed by Lrp.

The expression results from the two separate *serA* promoters, P1 and P2, agreed with the previous primer

extension results in rich medium, in which the expression of *lrp* was repressed, the P1 promoter had only low expression and the P2 promoter was highly expressed; and in minimum medium in which the expression of *lrp* was activated, P1 promoter was highly expressed and P2 had almost no expression. Thus the P1 promoter was activated by Lrp and the P2 promoter was repressed by it.

In fact, the occurrence of two or more promoters in the regulatory regions of genes or operons in *E. coli* is not a new observation. For instance, the *gal* operon which is one of CRP-cAMP regulated genes contains two tandem promoters that initiate transcription at positions five bases apart (Irani, 1989). The binding of the CRP-cAMP complex to the *gal* regulatory region simultaneously represses the expression of the *galP2* promoter and activated the *galP1* promoter. In the *lac* operon, two weak promoters, *lacP2* and *lacP3*, are located 22 and 15 bp upstream of the strong *lacP1* promoter (Malan, 1984 and Xiong, 1990). All three promoters are controlled through one common CRP-cAMP target site, *lacP2* and *lacP1* are repressed by CRP-cAMP, while *lacP3* is activated by CRP-cAMP (Xiong, 1990).

The regulation of *serA* by Lrp is similar to the regulatory system of Crp, especially for the cases of the *gal* and *lac* operons.

There are at least two Lrp binding sites in the *serA*

promoter. The high affinity site, which is in the P2 region, and overlaps with the P2 RNA polymerase binding site, can inhibit the transcription initiation of P2 by blocking the RNA polymerase on this promoter. Another binding site may be near to the P1 promoter, the precise location of Lrp binding in this region has not been determined yet. One possible mechanism for activation of the P1 promoter is that Lrp binding at the P2 region enhances the binding at P1, and activates the transcription initiation of P1.

Part 3. Possible mechanism by which Lrp regulates its target gene expression

3-1. How does Lrp regulate the expression of its target promoters

Lrp activates transcription initiation from some promoters and inhibits expression from others. Studies with some of the genes in the leucine/Lrp regulon show binding of Lrp directly with multiple sites in the promoter region. The binding affinities are quite different among genes, and in the same gene, among different binding sites. DNase I protection experiments have demonstrated that at least in some Lrp repressed promoters, such as *ser*AP2 and *lysU*, the Lrp binding site overlaps with the RNA polymerase binding site (Lin, 1992b and Lin, 1992c). The Lrp protein may repress transcription initiation by blocking of RNA polymerase binding as suggested by Irani, M.(1989).

The DNase I protection experiments and DNA-protein binding experiments have demonstrated that most of the Lrp-regulated promoters studied so far contain multiple Lrp binding sites, and that the Lrp protection site is usually quite large: 70bp in *ilvIH*, (Ricca, 1989), 88 bp in *lysU*, 75 bp in *sdaA*, and 70 bp in *serA*. The DNase I footprinting

experiments of Lrp with its target promoters showed that hypersensitive sites are present within all Lrp binding sites. The DNA bending experiment of the *ilvIH* promoter demonstrated that Lrp protein induced a bending of approximately 52 upon binding to a single binding site. The results suggest that Lrp binding to this region may facilitate the formation of a higher-order nucleoprotein structure, and may activate transcription initiation in a manner similar to that proposed for CRP through the bending of DNA (Reznikoff, 1992).

The *in vivo* regulation and *in vitro* mechanism studies on Lrp suggest that the Lrp protein should have at least the following three functions: binding to the target promoter, regulating the transcription initiation (this function may be divided into activation and repression) and responding to L-leucine (for some of the genes, this may not be the case). The Lrp protein were predicted to have a helix-turn-helix (HTH) motif at its amino-terminal end (Willins, 1991). This HTH-containing domain in Lrp may be involved in recognizing specific DNA sequence and may function as (the) a DNA binding domain (Harrison, 1990). Lrp may contain other domains as well, such as activation/repression domain(s) and an L-leucine responsive domain. By isolation and characterization of Lrp mutants with *ilvIH* promoter, three kinds of mutations on Lrp were isolated. One kind of mutation alters DNA binding,

another kind affects activation of transcription initiation of the promoter, the third kind of mutant alters response to L-leucine effect (Platko, 1993).

Mutations responding to the DNA binding had changes in Lrp between amino acids 16 to 70, and six of ten of these mutations were within the HTH region; this region is thus called the DNA binding domain. Mutations having low *in vivo* expression of *ilvIH*, but apparently normal DNA binding *in vitro*, had changes in the amino acids between residues 76 and 125, and this region was thus called the activation domain. Mutations that were resistant to the repressive effects of L-leucine had changes in the Lrp amino acid sequence between 108 and 149, and this region was called the L-leucine responsive domain (Platko, 1993).

These functional domains were characterised based on the expression of the *ilvIH* promoter, and there is no further experimental evidence indicating whether those definitions are also suitable for the action of Lrp at other Lrp-regulated promoters.

3-2. Lrp may stimulate transcription from the *serA* promoter by directly interacting with its promoter region

The transcription of the *serA* promoter is under positive control by Lrp (Lin, 1990). The gel retardation experiments

indicate that Lrp binds to at least two sites of the *serA* upstream region. One binding site of Lrp on the *serA* upstream region is related to the P1 promoter region. The binding affinity of this region is much lower than that of the P2 region. One mutation in this region, UP1-2, not only shows severely decreased expression of *serA* but also affects the binding of the promoter with Lrp. This data suggest that the decrease in the expression of *serA* may be due to the decrease in the Lrp binding ability.

Another region with high affinity binding overlaps the proposed -10 to -35 boxes of *serA* P2. In the absent of Lrp, transcription was initiated at both P1 and P2 *in vivo* (Lin, 1992c). The addition of Lrp increased transcription from P1, which is located further downstream from this site, but decreased transcription from P2, which overlaps with this Lrp binding site. The inhibition of *serA* P2 by Lrp may be due to its competition with RNA polymerase for occupation of the P2 promoter (Irani, 1989).

Binding of Lrp to the P2 region may also serve to activate transcription initiation from the P1 promoter. Deletion of this Lrp binding site severely reduced the expression of the P1 promoter (Table R5, plasmid SAI). This may be due to removing any possible cooperation between the two binding sites. Furthermore, mutations inside this binding region which affect binding, also severely reduced the *lrp*

activation.

These results suggested that Lrp activates the P1 promoter and represses the P2 promoter by directly interacting with the high affinity binding site.

3-3. Possible mechanisms by which Lrp Regulates its target gene expression

In *E. coli*, the regulatory protein CRP which binds cAMP can mediate transcription activation of some operons and transcription repression of others. Lrp like CRP can activate transcription of some operons and inhibit transcription of others. In fact, there are many similarities between these two global regulatory proteins (Cossart, 1982, Anderson, 1971, and Willins, 1991). Both Lrp and CRP are small basic relatively abundant proteins that function as a homodimer. Both of them bind to the target promoter (Wu, 1984, and Wang, 1993a) and both of them bend the DNA of their target promoter. Lrp may activate transcription initiation through the bending of DNA or through interaction with RNA polymerase as proposed for the mechanism of CRP-cAMP activation of *lac* expression (Reznikoff, 1992).

Summary

The effect of Lrp on the expression of *serA* was studied *in vivo*. Plasmid MT3, which contains the *lrp* gene coding region under the pBAD promoter, was used to study the effects of varying the cellular concentration of Lrp. The expression of *lrp* in this plasmid was controlled by the amount of arabinose present in the cell. The expression of chromosomal *serA::lacZ* was shown to be proportional to the expression of *lrp* in the MT3 plasmid. The expression of *sdaA* is decreased by increasing the concentration of Lrp.

Using PCR amplification methods, mutations in the *serA* promoter region were made. The regulation of the *serA* promoter was shown to be affected by the mutations. Some of those mutations also affected the *serA* binding affinity with Lrp. Two mutations, UP1-2 and UP2-1, which affect Lrp binding affinity, cause severe reduction of *serA* expression.

The *serA* P1 and P2 promoters were subcloned separately. *In vivo* regulatory studies show that the P1 and P2 promoters are oppositely regulated. The P1 promoter is activated by Lrp and the activation of Lrp is partially reversed by exogenous L-leucine. The P2 promoter is repressed by Lrp and its repression is partially reversed by L-leucine. In addition, it

was shown that the high affinity binding site for Lrp in the P2 region is required for promoter P1 expression and activation.

Thus, the data suggest that Lrp directly regulates *serA* expression through binding to its promoter region.

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