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Sequences Affecting the Expression of the Escherichia
Coli LeuB Gene in Yeast

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A Thesis

in

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ABSTRACT

Sequences Affecting the Expression of the <u>Escherichia Coli</u>
<u>LeuB</u> Gene in Yeast

Sheida Bonyadi

The physical location of a gene in relation to other
genetic material may influence its expression. Variations
in the expression of a gene as a function of changes in its
genomic location are called "position effects". It was
previously shown that the functional expression of the
<u>Escherichia</u> <u>coli</u> <u>leuB</u> gene in <u>Saccharomyces</u> <u>cerevisiae</u>, was
subject to "position effects". This thesis characterized
the ability of specific yeast DNA sequences to affect <u>leuB</u>
expression in a "position effect" dependent manner.

The major findings of this report are : (I) A second
promoter upstream of a promoter can prevent its expression
in yeast. (II) The ability of a second promoter to prevent
expression is dependent upon its orientation, that is, it
has no effect when directing transcription away from the
downstream promoter but it abolishes expression when
directing transcription towards it. (III) The insertion of
a yeast terminator sequence between two promoter sequences
restores expression from the downstream promoter.

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INTRODUCTION

Several studies have shown that gene expression can be dependent upon genomic location. The genomic location dependent expression of a gene is termed "position effect". Although several authors have described "position effects", the molecular mechanism underlying this phenomena is not clearly understood.

This introduction is divided into three sections. In the first section I will explain how postion effects were first recognized and differentiated from other mutations which affect gene expression. I will also review the literature relating to the analysis of the mechanisms causing position effects. In the second section I will describe some recent experiments which have utilized recombinant DNA methods to study position effects. Finally, in the third section, I will provide the background data for my own thesis work and present the rationale for the experiments I have performed.

I) POSITION EFFECTS

(a) <u>Chromosomal Rearrangements Cause Position Effects</u>

By 1950, it was clear that certain <u>Drosophila melanogaster</u> mutants resulted because a gene's position was changed (Lewis, 1950). Lewis investigated a series of

Drosophila mutants which affected a number of loci
including those involved in the development of eye colour.
He examined a large number of Drosophila genes that
were known to demonstrate a mosaic phenotype when genomic
rearrangements resulted in the close association of these
genes with heterochromatin. In Drosophila, the gene
responsible for the development of eye colour is carried
on the X chromosome, and red eye colour is considered
normal. One example of a position effect associated
with a genomic rearrangement resulted from a translocation
between the fourth and the X chromosome. This translocation
resulted in a white variegated eye colour morphology.
Cytological analysis of this mutant showed that the
heterochromatin portion of the fourth chromosome had
been fused to euchromatin of the X chromosome. Furthermore,
he showed that often other genes in close proximity to the
point of rearrangement were also affected. Lewis concluded
that these uncommon forms of mutations, termed "variegation
of a gene" were intrachromosomal phenomena, and were not
due to structural heterozygosity. Although the underlying
mechanism responsible for the observed position effects was
not clear, he postulated that specific portions of
heterochromatin material influenced the variegated
phenotype.

In 1956, McClintock reported on certain heritable

alterations in maize which were clearly associated with.
chromosomal rearrangements. She hypothesized that these
chromosomal rearrangements resulted from the action of two
elements, the dissociation element (Ds) and the activator
factor (Ac). The Ds was responsible for the chromosome
breakage that gave rise to translocations, inversions,
deletions, and ring chromosomes. The Ac element was
necessary for the activation of the Ds element. Not only do
these genetic elements (Ds and Ac), when present together,
cause chromosomal rearrangements but they bring about
changes in gene expression, because the genomic environment
adjacent to genes is altered.

(b) Insertion Elements Cause Position Effects

In E. coli the genes for the enzymes catalyzing the
three reactions which convert D-galactose to glucose-6-
phosphate are closely linked. Furthermore, these three
genes are part of a single operon (Kalckar et al, 1959).
The three genes are coordinately inducible by D-galactose.
Sadler et al (1967) showed that a class of spontaneous
mutants which mapped near the gal operon had extreme polar
effects on the expression of the genes distal to the
mutated region. Close examination of these mutants showed
that they were not due to base substitutions, frameshifts
or deletions. Eventually, density-gradient analysis
(Shapiro et al, 1969) of lambda transducing phage, carrying

these extreme polar mutations and the wild type gal operon,
found that the phage carrying the polar mutation had higher
densities than the phage which carried the wild type
galactose operon. Thus it was concluded that additional DNA
had been inserted into the gal operon and was responsible
for the strong polarity and the unusual genetic properties.
Similar mutations were reported in the E. coli lac operon
(Malamy et al, 1972). Fiandt et al, 1972, showed that
insertions or transposable genetic elements can integrate
into the E. coli lac operon in either orientation and
consequently affect the expression of the adjacent gene(s)
in a polarity dependent fashion.

(c) Position Dependent Gene Expression and Cell
    Differentiation

    S. cerevisiae haploids normally exist in one of two
mating types called "a" and "alpha". The mating type of a
haploid cell is determined by a single locus which is
located near the centromere of chromosome III and is called
the MAT locus. The MAT locus can exists in two forms, MATa
and MATalpha. Although there is only one mating type locus
per haploid genome, normal wild type haploids can change
mating type as often as once per cell generation. This
alternation of mating type is refered to as mating type
interconversion. Studies on mating type interconversion in
S. cerevisiae (Hicks et al., 1977) introduced a new

mechanism of cell differentiation, which involved position dependent gene expression. This work showed that additional silent copies of the mating type locus, called HMLalpha and HMRa, were present near the left and right ends of the same chromosome (III) that contained the expressed MAT locus. These silent copies of the mating type locus which encoded "alpha" and "a" cell types respectively, are activated only after transposition to the mating type locus (MAT). This suggested that the expressed locus has an additional essential element which leads to the expression of the adjacent information. Alternatively, the silent copies contain additional information which prevents their expression. Although the mechanism involved was not clearly understood, the authors proposed that another gene, the HO gene, behaves like an activator of the silent HMRa and HMLalpha sequences. Activity of the HO gene causes transposition of the distally located silent copies into the MAT locus, consequently replacing the resident mating type gene. Therefore, mating type interconversion is an example of how gene location can dramatically affect gene expression.

(d) Transcriptional Interference

"Transcriptional interference" has been proposed as the cause of position effects seen in avian retroviruses (Cullen et al, 1984). Such interference is believed to be due to the presence of extensive overlaps between

regions involved in the initiation and termination of transcripts expressed from the integrated proviruses. In the presence of a transcriptionally active upstream 5' LTR (long terminal repeat), the downstream 3' LTR of an integrated provirus is unable to act as a promoter of transcription. To study this observation, proviral clones were constructed in which the upstream and downstream LTRs could control the transcription of genes whose level of expression could be detected and measured independently in transformed avian cells. The results showed that clones in which transcription from the upstream LTR terminated before reaching the downstream LTR, showed a large increase in transcription promoted by the 3' LTR. Therefore, 3' LTR activity was subject to position effects exerted by the 5' LTR in avian retroviruses.

In addition to the classic promoter elements such as the polymerase binding sites which are immediately 5' to the gene, more recent emphasis on defining transcriptional regulatory signals has revealed a set of promoter elements such as enhancer elements and upstream activating sequences which cause position like effects.

Benoist et al, 1981, showed that the A+T rich region known as TATA box or Hogness box in eukaryotes and its analogue in prokaryotes, the Pribnow box, was essential

for precise initiation of transcription of SV40 early genes. However, removal of sequences upstream from the TATA region abolished gene expression. These results showed that a tandem repeat of 72 bp, upstream from the TATA box (now known as the enhancer or activator element), was essential for the transcriptional expression of the SV40 early genes. Furthermore, Banerji et al, 1981, showed that the expression of cloned rabbit Beta-globin was increased 200 fold when the 72 base pair enhancer element from SV40 was cloned adjacent to it. This element was able to increase the efficiency of transcription independent of its orientation and its position (ie. 1400 bp upstream or 3300 bp downstream from the Beta-globin gene transcriptional initiation site).

Similar to the enhancer elements found in higher eukaryotes, are the upstream activator sequences (UAS) found in yeast. These elements act over long distances when placed upstream from the transcriptional initiation site (Struhl et al, 1982). Deletion of a gene's associated UAS element leads to poor transcriptional expression of the gene. Experiments performed by Guarente, 1983, on the iso-1-cytochrome c (CYC1) gene of yeast, showed that deletion of a repeated DNA sequence (UAS), centered about 275 bp upstream from the transcriptional initiation sites, results in approximately a 200-fold decrease in the -

expression of the CYC1 gene. Furthermore, substitution of
the CYC1 UAS with the GAL10 UAS restored CYC1 expression.
Detailed experiments by many researchers have shown that
UAS elements are involved in gene regulation. Located
adjacent to a gene they not only optimize the
transcriptional efficiency but also regulate gene
expression at the level of transcription.

Another example is the regulation of the yeast GAL1 and
GAL10 genes by their UAS. In Saccharomyces cerevisiae, the
GAL1, GAL7, and GAL10 genes (which encode the enzymes for
utilization of galactose) are located near the centromere of
chromosome II (Bassel et al., 1971). The GAL1 and GAL10
genes are separated by approximately 600 base pairs of
spacer DNA. This spacer DNA contains sequences which have
been identified as the GAL upstream activator sequence
(UASG). These two gene are divergently transcribed (St.John
et al., 1981). Deletion mapping experiments performed by
West et al., 1984, showed that a 75 bp nucleotide sequence
located approximately in the middle of the 600 base pair
intergene region, identified as UASG', was equally
essential for maximal activity of both genes. They
concluded that a 15-bp dyad-symmetrical sequence and a
second 15-bp dyad having partial homology to the first,
within the 75 bp UASG', was the binding site for GAL4 gene
product which activates expression of both the GAL1 and

GAL10 genes. Therefore, the effect of these elements, ie. enhancers and UASs, on gene expression is reminiscent of position effects.

II)POSITION EFFECTS RESULTING FROM DNA CLONING

A position effect can also be caused by cloning, adjacent to one another, molecules of DNA that are not usually juxtaposed. Struhl (1981^a) reported position effects resulting from DNA cloning experiments which involved cloning of the yeast TRP1 and HIS3 genes adjacent to one another in a yeast shuttle vector. The resulting plasmid yRP7-Sc2715 carried the entire yeast TRP1 gene and the HIS3 gene from which the promoter region was severed. On this plasmid the two genes were cloned in opposite orientation, having their 5' ends adjacent to a 375 base pair fragment of the vector spacer DNA. When this plasmid was introduced into a yeast his3, trp1 strain, the transformants showed a His$^-$, Trp$^+$ phenotype. However, another plasmid, yRP7-Sc2715' which carried the HIS3 containing fragment in the opposite orientation, reading towards the TRP1 gene, conferred a His$^+$, Trp$^-$ phenotype on the transformants. The results suggested that the vector sequences on yRP7-Sc2715' affected chromatin structure in such a way that allowed the cloned HIS3 DNA fragment, which lacked the 5' end, to be expressed. The results on the orientation dependent expression of the entire TRP1

gene, also suggested the involvement of chromatin structure in expression of this gene. TRP1 gene was not expressed when transcription of HIS3 fragment was reading towards the TRP1 gene on yPR7-Sc2715'. Therefore it was concluded that both HIS3 and TRP1 were subject to position effects.

III.) POSITION DEPENDENT EXPRESSION OF THE ESCHERICHIA COLI LEUB GENE IN YEAST

This thesis describes a series of experiments which attempt to determine both the sequences and the mechanism responsible for the position dependent expression of the Escherichia coli leuB gene in yeast. To provide the necessary background, I will briefly describe what was known about the expression of leuB in yeast before I started the research reported here.

The E. coli leucine operon consists of four genes, leu A-B-C and D. A lesion in the leuB gene abolished expression of the enzyme Beta-isopropylmalate dehydrogenase (Kessler et al, 1969). This enzyme catalyzes the oxidative decarboxylation of beta-isopropylmalate to form alpha-keto-isocaproic acid, an intermediate in the biosynthesis of leucine in both E. coli and yeast (Ratzkin et al, 1977). In yeast this enzyme is the product of the LEU2 gene (Satyanarayana et al, 1968).

Genetic complementation of E. coli leuB mutants by the yeast LEU2 gene (Ratzkin et al, 1977) suggested that either the same or similar transcriptional control signals were recognized by both E. coli and S. cerevisiae. Alternatively, fortuitous transcriptional and translational information adjacent the yeast LEU2 gene were utilized by E. coli.

. Since the LEU2 gene of S. cerevisiae could complement E. coli leuB mutations, the leuB gene of E. coli might be functionally expressed in yeast. Storms et al (1981) showed that the leuB gene could be functionally expressed in yeast. However, functional expression of the leuB gene, within a shuttle vector, was dependent upon the orientation of the E. coli DNA encoding the leuB gene. Therefore, vector sequences adjacent the cloned leuB gene were affecting its expression in yeast. The structure of some of the plasmids used in these earlier studies are presented in Figure 1. The results of the orientation dependent expression of the leuB gene are similar to those reported by Struhl, 1981 [a] (see above). These results suggest two possible explanations: (I) Vector sequences encoding elements essential for expression are appropriately positioned in pEH25 but not in pEH26. (II) Vector sequences encoding an element preventing expression are appropriately positioned to prevent expression in pEH26 but not in pEH25.

Figure 1

Structure of plasmids pEH25, pEH26 and pRS27 (Storms,
1981). Regions with vertical lines represent pBR322 DNA.
AP represents a functional ampicillin resistant gene, TC'
represents a non-functional tetracycline resistant region.
The arrow indicates an intact Tc promoter and the direction
of transcription from this promoter. Regions marked by two
thin lines represent yeast plasmid 2-um DNA. A single thick
line represents the E. coli leu DNA. A, B, and C represent
genes of the E.coli leucine operon; and D' represents
truncated A and D genes. The position of EcoRI, HindIII,
and BamHI restriction endonuclease sites are indicated by
E, H, and B, respectively. The ability of yeast
transformants to express the plasmid borne leuB gene is
indicated by leu+ and leu-, respectively. The size of each
plasmid is presented in kilobases (Kb). (a) pEH25 carries
the E. coli leucine operon, flanked by BamHI restriction
sites. (b) pEH26 is identical with pEH25, except for the
orientation of the leucine operon. (c) pRS27 is derived
from pEH26 by removing the HindIII fragment 5' to the
leucine operon.

Fig. 1

pEH 25
15 Kb
leu
D C B A
Tc´   Ap   2μm
E HB   H   B   E   E
leu⁺ (a)

pEH 26
15 Kb
leu
A B C D
Tc´   Ap   2μm
E HB   H   B   E   E
leu⁻ (b)

pRS 27
11.4 Kb
leu
A B C D´
Tc´   Ap   2μm
E H   B   E   E
leu⁺ (c)
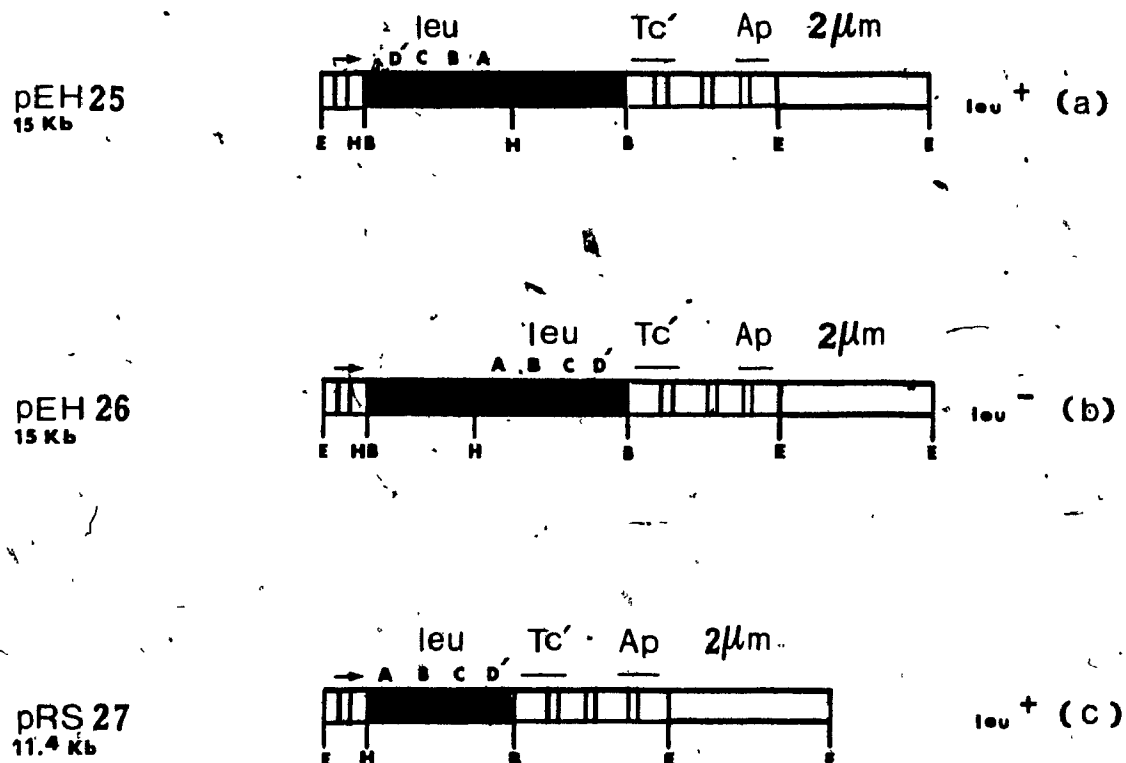
To determine which of these two explanations was correct, the sequences upstream from the _leuB_ gene on plasmid pEH26 was removed. This was done by deleting the _Hind_III fragment. The derived plasmid was pRS27 (see Fig. 1). Plasmid pRS27 was able to transform yeast strain AH22 to leucine prototrophy. This suggested that sequences upstream from the _leuB_ gene were able to prevent expression and that these sequences were deleted or inactivated by removal of the _Hind_III fragment. Nevertheless, it remained unclear what sequences were responsible for the position dependent expression of _leuB_ in yeast. The objectives of the research presented in this thesis were two fold: (I) To test the ability of known yeast sequences to alter _leuB_ expression in a position dependent fashion. (II) To determine how these sequences were able to alter _leuB_ expression.

MATERIALS AND METHODS

Yeast and Bacterial Strains

The _S. cerevisiae_ strains used were AH22 (can-1, leu2-3, leu2-112, his4-5-14) and LL20 (leu2-3, leu2-112, his3-11, his3-15) from G.R.Fink (Cornell University); and LL20-15 (trp1, leu2-3-112) from R.Ord (Concordia University). The _E.coli_ strain used was JF1754 (hsdr, lac, gal, metB, leuB, hisB436) (R.K.Storms, 1981).

Plasmids

The previously constructed plasmids used were pRS83, pYF91 and yRP7 (Table 1). Plasmid pRS83 is derived from plasmid pYF85 (Storms et al., 1979). pYF85 is a pBR322 derivative carrying portion of the yeast 2-μm circle DNA. pRS83 is capable of replication in both _E.coli_ (because it contains pBR322) and yeast (because it contains the 3.6 Kilobase EcoRI fragment from the 2-μm yeast plasmid). Plasmid pRS83 carries a HindIII-BamHI fragment from the _E.coli_ leucine operon, which encodes the leucine region informationm for B and C genes, and truncated A and D (i.e. leu A'B C D'). This plasmid also contains an 850 base pair HindIII fragment which harbors the 3' end of the yeast HIS3 gene, along with the yeast DED1 promoter region (see Fig. 2a). Plasmid pYF91 is a plasmid derived from pYF85

Table 1

plasmids	features [a]	source [b]
pYF91	pBR322 Ori; yeast 2-μm, and yeast <u>LEU2</u> gene (13.4 Kb).	Storms et al (1979)
yRP7	pBR322 Ori; yeast 2-μm, and the yeast <u>TRP1</u> gene.	Struhl et al (1981) [b]
pRS83	pYF85 Ori; and <u>E.coli</u> leucine operon (4 Kb, A'B C D'), yeast <u>HIS3</u> 5' end, and yeast <u>DED1</u> 5' end (Fig. 2a).	Storms et al (1981)
pRS87	pYF85 Ori. Identical with pRS83, except for the orientation of the leucine operon (Fig. 2a).	Storms et al (1981)
pSB11	pRS83 Ori; and the yeast <u>TRP1</u> gene from yRP7 (Fig. 2b).	this study
pSB30 pSB20	pSB11 Ori; and they both lack the yeast <u>DED1</u> 5' end (Fig. 2b).	this study
pSB151	pSB30 Ori; and the yeast <u>LEU2</u> 5' end from pYF91 (Fig. 2b).	this study
pSB12	pSB30 Ori; and it is identical to pSB151, except for the orientation of the yeast <u>LEU2</u> (Fig.2b).	this study

a/ Features pertinent to the search presented in this thesis.

b/ The references give detailed description of the plasmids listed.

by insertion of the yeast LEU2 gene (Storms et al, 1979). Plasmid yRP7 contains pBR322, the yeast 2-μm circle, and the yeast TRP1 sequences (Struhl et al, 1981[b]).

The plasmids constructed for this study are pSB11, pSB20, pSB30, pSB12 and pSB151 (Fig. 2b, Table 1). Plasmid pSB11 was constructed by insertion of the TRP1 gene from yRP7 into pRS83. Plasmids pSB20 and pSB30 are derived from pSB11, by excision of the yeast DED1 promoter region. Plasmids pSB12 and pSB151 are derived from pSB30, which carry the yeast LEU2 promoter region from pYF91. These two plasmids are identical except for the orientation of the inserted LEU2 promoter region.

MEDIA

YEPD, rich medium, consists of 2% bactopeptone (DIFCO), 1% yeast extract and 4% glucose. 2 x YNBD, selective medium, consists of 1.34% yeast nitrogen base (without amino acids, Difco), 4% glucose and 10 μg/ml of the required amino acids (Osley and Hereford, 1981). LB broth consists of 1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, 0.002 M MgSO4, 0.0001 M CaCl2 and 0.2% glucose.

GROWTH OF YEAST STRAINS

Strains were grown at 30°C in an incubator room, in 25 mls of medium in a 250 ml side armed flask. Adequate aeration was maintained by vigorous shaking and the use of

cotton plugs for closing of the flasks. For routine growth,
cells were grown in minimal media (2 x YNBD) plus the
required amino acids. A saturated 2ml overnight culture (1
$.x 10^8$ cells/ml), was used to inoculate 25 ml of medium at
approximately $1 x 10^5$ cells/ml, and the growth of the
culture was followed by measuring iturbidity with
a Klett colorimeter. All strains were maintained on 2
x YNBD media plates with the required amino acids (2%
agar), with routine restreaking to confirm phenotypes.

YEAST TRANSFORMATION

Yeast cells were transformed with plasmid DNA, using
the LiCl method (Ito et al., 1983). Late log phase cells
(approximately $2 x 10^7$ cells/ml), growing in YEPD media,
were harvested by centrifugation, washed once in TE (1 mM
EDTA, 10 mM Tris-HCl, pH 7) and resuspended in TE at about $2 x 10^8$ cells/ml. An equal volume of cells and of 0.2 M LiCl
were mixed together and the mixture was incubated for 1 hour
in a shaking water bath, at 30°C. Plasmid DNA (1-2 ug) was
then added and the cells were incubated at 30°C for 30
minutes. The cell samples were diluted with an equal volume
of 70% polyethylene glycol-4000 (J.T. Baker Chemical Co.)
and incubated at 30°C for 1 hour, without shaking. Samples
were washed twice with distilled deionized water, and plated
on selective media at about $1 x 10^6$ cells/plate. After 4 to
5 days of incubation at 30°C, distinct colonies appeared.

Transformation frequency was usually greater than 10^3/μg of DNA.

E. COLI GROWTH AND TRANSFORMATION

E.coli strain JF1754 was transformed using the CaCl2 method (Mandel and Higa, 1970). Transformants were selected for by plating on LB media containing either ampicillin or tetracycline at final concentrations of 40 μg/ml and 15 μg/ml, respectively. These colonies were then restreaked on selective M9 agar plates to purify the transformants.

RESTRICTION ENDONUCLEASES AND DNA MODIFYING ENZYMES

Restriction endonucleases, T4 ligase, SI nuclease and alkaline phosphatase were purchased from either Boehringer Mannheim (Montreal, Canada) or Amersham (Montreal, Canada), and were used as recommended by the supplier.

DNA, when treated with any of the above enzymes other than T4 ligase, was purified by phenol-chloroform extraction. The DNA was then recovered by ethanol precipitation and dissolved in TE (10 mM Tris-1 mM EDTA, pH 8 buffer), or deionized water.

DNA PREPARATIONS

The rapid isolation of plasmid DNA from E. coli was performed using a modification of the alkaline lysis method of Birnboim and Doly (1979). Large scale isolation of

plasmid DNA, using the ethidium bromide CsCl density gradient method was performed as described by Clewell and Helinski (1970).

S. cerevisiae DNA was isolated using modification of the method of Maniatis et al (1982). Mid-log phase cells growing in minimal medium (2 x YNBD), were harvested, washed and resuspended in 1M sorbitol. After adding 2-3% glosulase (Dupont laboratories), the samples were incubated at 37°C for 1 hour, washed 3 x with 1 M sorbitol and resuspended in lysis mix (50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl pH 8) and incubated at room temperature for 5 minutes. Two volumes of a freshly prepared solution of 0.2 N NaOH and 1% SDS was added to the sample, gently mixed and kept on ice for 5 minutes. Sodium acetate (5 M) was added to a final concentration of 0.5 M and the samples were vortexed gently. After vortexing at 4°C, the samples were centrifuged at 12,000 xg for 10 min. The supernatant was transfered to a fresh tube and phenol-chloroform (1:1) extraction performed, followed by ethanol precipitation. The resulting yeast DNA was resuspended in TE buffer.

RNA ISOLATION

Total yeast RNA was isolated by a modification of the method described by Thomas (1980). First, cultures were grown to mid log phase in media which selected for plasmid

maintenance. Before harvesting, cycloheximide (10 mg/ml) was added at a final concentration of 100 ug/ml, to the cell culture. Cells were harvested by centrifugation, washed with ice cold breaking buffer (0.5 M NaCl, 0.2 M Tris-HCl pH 7.5 and 0.01 M EDTA), and resuspended in 0.2 mls of breaking buffer. Then an equal volume of phenol-chloroform-isoamylalcohol mixture (50% phenol containing 0.2% beta-mercaptoethanol, 49.8% chloroform and 0.2% isoamyl-alcohol), containing 0.3% SDS and 0.2 mls of glass beads were added to the samples. These samples were vortexed for 3-5 minutes at 4° C. Following vortexing the samples were centrifuged and the supernatant was transfered to a fresh tube containing chloroform-isoamylalcohol. The sample was vortexed for 10-15 seconds and the supernatant was transfered to a fresh tube and the purification process with chloroform-isoamylalcohol was repeated until no debris was visible at the interphase. The RNA was then ethanol precipitated and dissolved in diethylpyrocarbonate (0.1%) treated, deionized and autoclaved water.

GEL ELECTROPHORESIS OF RNA AND TRANSFER TO MEMBRANE

The fractionation of RNA samples was performed essentially as described by McMaster and Carmichael, 1977. Up to 20 ug of RNA was denatured by incubation in 16 ul containing 1 M deionized glyoxal, 50% dimethylsulfoxide and

0.01 M NaHPO4 (pH 7.0) at 50°C for 60 minutes. After denaturation, 4 µl of sterile loading buffer (50% Glycerol, 0.01 M NaHPO4, pH 7.0, and 0.4% bromophenol blue), was added and the RNA samples loaded on a horizontal 1.0% agarose gel. Electrophoresis was carried out in a 0.01 M NaHPO4 buffer (pH 7.0), at 50 volts for 6 hours. After electrophoresis, the fractionated RNAs were then transfered to nitrocellulose membranes, as described by Thomas (1980). The gel was placed in contact with the membrane filter and blotted with 20 x SSC blotting buffer (3 M NaCl and 0.3 M trisodium citrate) overnight. The blots were dried at room temperature and baked in a vacuum oven at 80° C for 2 hours.

RNA Hybridization

The RNA blots were prehybridized overnight at 42°C in buffer containing 50% formamide, 5 x SSC (3 M NaCl and 0.3 M trisodium citrate), 50 mM sodium phosphate at pH 6.5, sonicated denatured salmon sperm DNA at 250 µg/ml, and 0.02% each bovine serum albumine, ficoll, and polyvinylpyrolidone. The hybridization buffer contained 4 parts of fresh prehybridization buffer and 1 part of 50% dextran sulfate. A nick translation kit was purchased from Amersham and was used as recommended by the supplier to prepare ^{32}P-labeled DNA probes. The nick translated probe was then denatured at 100°C for 10 minutes, rapidly cooled

on ice and added to the hybridization buffer, and the
blots were hybridized for about 20 hours at 42°C. Following
hybridization, the RNA blots were washed with four changes
of 2 x SSC and 0.1% sodium dodecyl sulfate for 5 minutes
each at room temperature, and washed with two changes of
0.1 x SSC and 0.1% sodium dodecyl sulfate for 15 minutes
each at 50°C. The damp blots were wrapped in saran wrap and
exposed to X-ray film at -70°C, using an intensifying
screen (DUPONT).

RESULTS

CONSTRUCTION OF PLASMIDS pRS83 AND pRS87 AND THEIR EXPRESSION IN YEAST

As described in the introduction, pRS27 could transform yeast strain AH22 (leu2) to leucine prototrophy but pEH26 could not, we wanted to know whether other known sequences could alter leuB expression when placed upstream of the leuB gene. First, plasmids pRS83 and pRS87 were constructed as described in the legend to Figure 2a. These two plasmids contain an 850 bp yeast fragment carrying the yeast DED1 gene's promoter. In plasmid pRS83 this promoter directs transcription towards the leuB gene while in pRS87, this promoter directs transcription away from the leuB gene. pRS83 was not capable of transforming strain AH22 (leu2) to prototrophy, while pRS87 could transform strain AH22 to prototrophy at high frequency (Table 2). This difference could be because: a) pRS83 was unable to replicate autonomously in yeast, while pRS87 could replicate autonomously. b) The leuB gene present in pRS83 could not be functionally expressed in yeast but the leuB gene present in pRS87 could be expressed.

To distinguish between these two possibilities, a derivative of pRS83 carrying the yeast TRP1 gene which would serve as an additional selectable marker in yeast,

Figure 2a

Structure of plasmids pGY21, pRS27, pGY33, pGY34, pRS83, and pRS87. The position of <u>BamHI</u>, <u>HindIII</u>, <u>BglII</u>, <u>EcoRI</u>, <u>XhoI</u>, and <u>ClaI</u> restriction endonuclease sites are indicated by B, H, G, E, X, and C, respectively. Regions with vertical lines represent pBR322 sequences (see Fig. 1 for more detail). The solid thick line represents <u>E. coli</u> leucine operon sequences (see Figure 1 for the detail). Regions marked by two thin lines represent yeast 2-um sequences. leu$^+$ and leu$^-$ indicate whether the <u>leuB</u> gene is expressed or not expressed, respectively, in yeast. Each plasmid size is presented in kilobases (Kb). a) Structure of plasmid pGY21, a pBR322 derivative with two tandemly repeated copies of the 1.8 Kb <u>BamHI</u> fragment of yeast DNA, containing the yeast <u>HIS3</u> gene inserted into the <u>BamHI</u> site of pBR322. The arrows indicate the location and direction of transcription for the duplicated <u>HIS3</u> and <u>DED1</u> sequences. The region with diagonal lines, flanked by <u>HindIII</u> sites, is the fragment cloned into <u>HindIII</u> site on pRS27 to generate pGY33 and pGY34. In plasmid pGY33 the <u>HindIII</u> fragment has been cloned in the orientation which results in the <u>HIS3</u> promoter directing transcription away from the <u>leuB</u> gene. In plasmid pGY34 the <u>HindIII</u> fragment has been cloned so that the <u>HIS3</u> promoter directs transcription towards the

Fig. 2a (cont'd)

leuB gene. Small "p" and small "t" indicate the promoter and
terminator regions of a gene, respectively.   e & f)
Structure of pRS87 and pRS83. These two plasmids were
derived from pGY33 and pGY34 respectively, by BglII
digestion followed by ligation. In the resulting plasmids
the DED1 promoter directs transcription towards the leuB
gene in pRS83 and away from the leuB gene in pRS87.

Fig. 2a
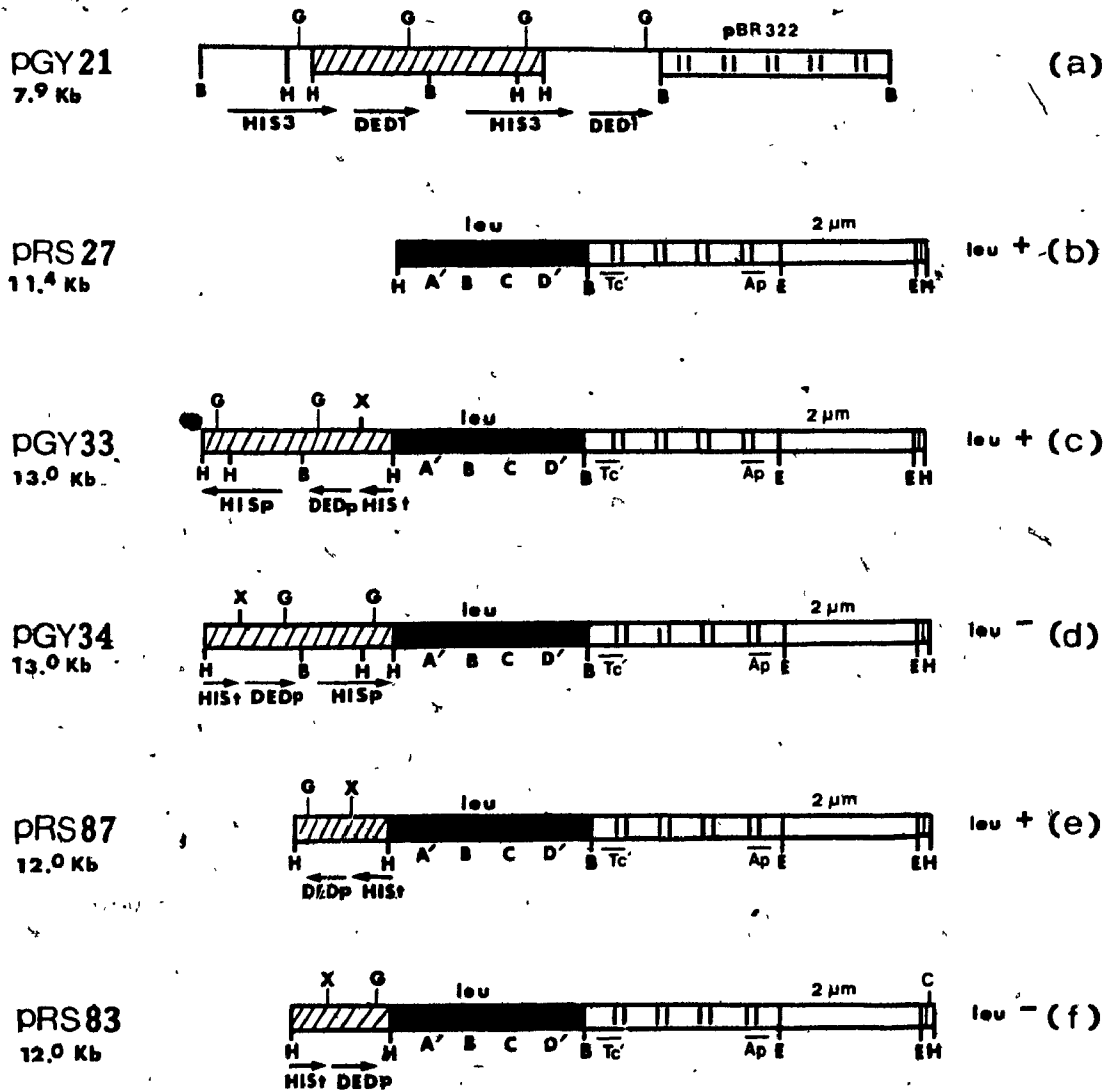
Table 2

plasmid	transformation frequency [a]	growth rate [b]
pRS27	1×10^3	8 hours
pGY33	1×10^3	8 hours
pGY34	0	ND [c]
pRS83	0	ND
pRS87	1×10^3	8 hours

a) Frequency given in transformants (to leu prototrophy) per ug of plasmid DNA used.

b) Growth rates (as doubling time) are for yeast strain AH22 transformed with the indicated plasmids, grown in minimal medium without leucine. The growth rate for untransformed yeat strain AH22, grown in rich media is approximately 2 hrs.

c) ND, not determined.

was constructed. Thus if the plasmid could replicate
autonomously, it should be able to transform yeast strain
LL20-15 (trp1-leu2) to tryptophan prototrophy at a high
frequency.

CONSTRUCTION OF A pRS83 DERIVATIVE CARRYING THE YEAST TRP1 GENE

The procedure used to construct the TRP1 containing
derivative of pRS83 is shown in Figure 2b. Plasmid yRP7
carries the yeast TRP1 gene (Fig. 2b, Struhl, 1981[b]).
The TRP1 gene is contained on a 1.2 Kb fragment flanked by
BglII and BamHI restriction endonuclease recognition sites.
This fragment also contains the 5' portion of the
tetracycline (Tc) resistance encoding region of yRP7.
First, plasmid yRP7 was digested with restriction
endonucleases BamHI and BglII, and pRS83 was digested with
BamHI. The resulting digested DNAs were then treated with
phenol, ethanol precipitated, mixed and ligated, using the
procedures as described in the materials and methods
section. The ligated DNA was then used to transform E. coli
strain JF1754 (leuB), and ampicillin resistant (Amp[r])
leucine prototrophs were selected. This should select
transformants harboring either pRS83 or derivatives of
pRS83 which contained an additional DNA fragment from yRP7.
To select transformants harboring pRS83 with the TRP1
containing BglII-BamHI fragment, the ampicillin resistant,

Figure 2b

Structure of plasmids used for this thesis. Position of
BamHI, HindIII, BglII, EcoRI, XhoI, and ClaI restriction
endonuclease sites are indicated by B, H, G, E, X, and C,
respectively. Regions with vertical lines represent pBR322
DNA (see Fig. 1 for more detail). E. coli leucine operon
sequences are indicated by thick solid lines (see Fig. 1
for more detail). The yeast 2-um sequences are indicated by
two thin lines. leu$^+$ and leu$^-$ indicate whether the plasmid
borne leuB gene is or is not expressed in yeast. TRP$^+$
indicates the plasmid harbors a functional TRP1 gene and
LEU2$^+$ indicates the plasmid harbors a functional LEU2 gene.
The size of each plasmid is indicated in kilobases (Kb).
Arrows indicate the location and orientation of the plasmid
sequences pertinent to this thesis. (a) Structure of
plasmid yRP7 (Struhl et al, 1981^b). This plasmid carries the
yeast TRP1 gene, marked by crosses. (b) Plasmid pRS83.
Construction of this plasmid is explained, in detail, in
Figure 2a. As Fig. 2a indicates, the region with diagonal
lines represents the yeast HIS3 3' sequences (t) and the
DED1 5' sequences (p). (c) Structure of plasmid pSB11.
pSB11 carries the 1.2 Kb TRP1 containing BglII to BamHI
fragment from yRP7 inserted into the BamHI site of pRS83.
The resulting plasmid encodes the yeast TRP1 gene and a
functional Tc$^+$ region. (d) Structure of pSB30. This plasmid

Fig. 2b (cont'd)

is generated by excision of the XhoI-BglII (340 bp)

fragment from pSB11. The 340 bp XhoI-BglII fragment

consists of the TATA box, transcriptional start sites and

the N terminal codon of the DED1 gene (Fig. 4, K. Struhl,

1985). (e) Plasmid pSB20 was generated by removal of the

DED1 5' region in pSB11. However, the size of the excised

fragment is larger than that in pSB30 and is approximately

440 bp. (f) Plasmid pYF91 (Storms et al, 1979) harbors the

yeast LEU2 gene, which is found within a 6.5 Kb fragment.

This 6.5 Kb fragment is indicated by two thin lines and

dots. (g) Plasmid pSB151 is generated by insertion of the

ClaI fragment carrying 5' region of the LEU2 gene from

pYF91 into the ClaI site of pSB30. This region (LEU2p) is

upstream from the yeast HIS3 terminator sequences and

directs transcription towards the HIS3 terminator sequence.

(h) Plasmid pSB12 is identical to pSB151 except for the

orientation of the inserted ClaI fragment containing LEU2p.

The LEU2p promoter directs transcription away from the HIS3

terminator sequence. •

Fig. 2b

yRP7
5.79 Kb
(a) TRP +

pRS83
12.0 Kb
(b) leu −

pSB11
13.2 Kb
(c) leu − TRP +

PSB30
12.86 Kb
(d) leu + TRP +

pSB20
12.76 Kb
(e) leu + TRP +

pYF91
13.4 Kb
(f) LEU2+

pSB151
14.66 Kb
(g) leu + TRP +

pRS12
14.66 Kb
(h) leu + TRP +
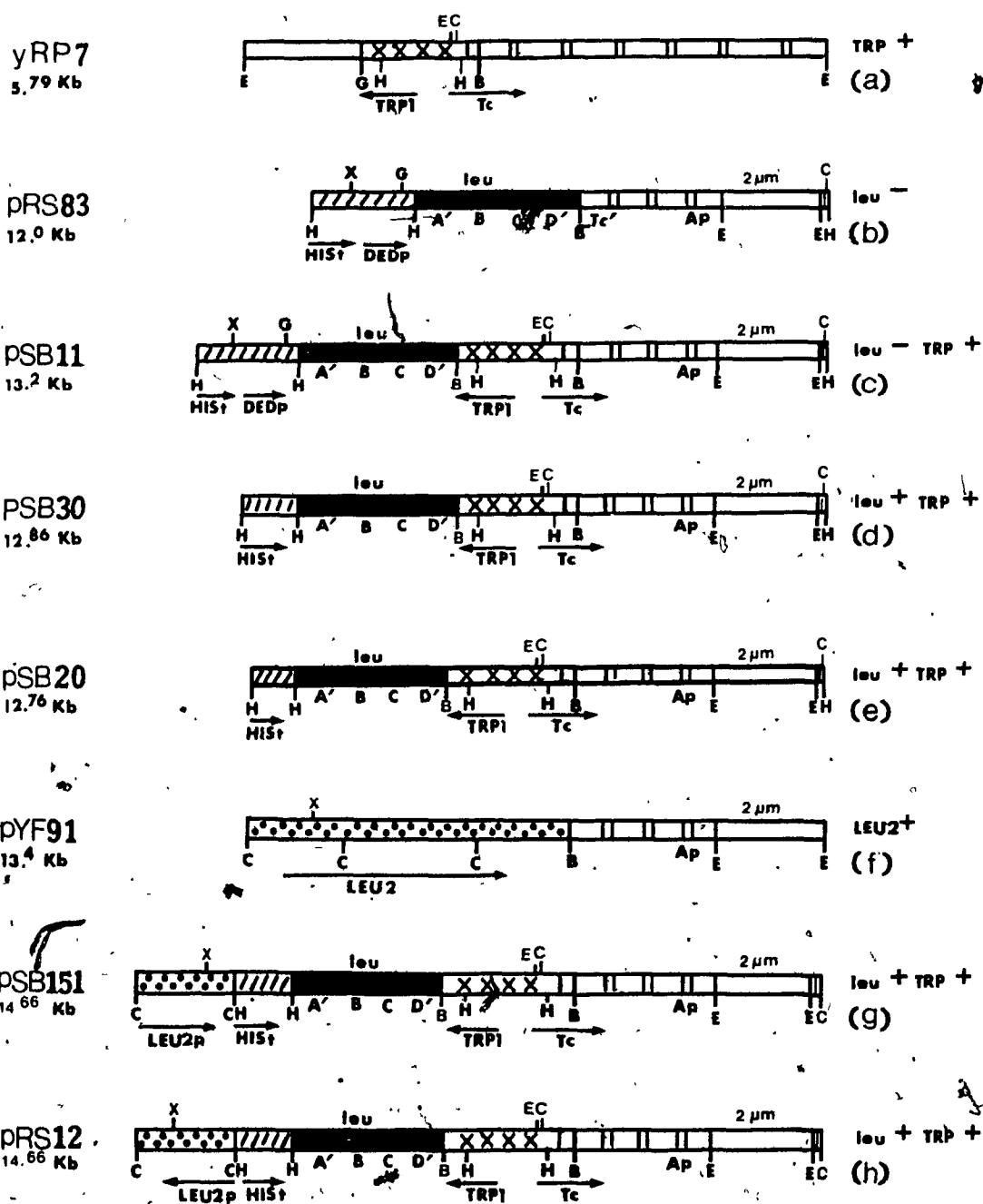
leucine independent transformants were replicated to L-Broth plates containing tetracycline. Presence of tetracycline selects for cells harboring pRS83 containing the TRP1 gene, because insertion of the BamHI-BglII fragment in the orientation shown in Figure 2b (c) would generate a functional Tc resistance encoding region. The results obtained are shown in Table 3. Several $E.$ coli transformants with the correct phenotype (Amp^r, Tc^r and leu$^+$) were chosen for detailed characterization of the plasmids they contained. Plasmid DNA isolated from five independent transformants was digested with restriction endonuclease HindIII followed by fractionation by agarose gel electrophoresis. The five plasmids isolated contained 4 HindIII recognition sites. Furthermore, they all had the expected pattern of the restriction endonuclease generated fragments (see Fig. 3). Plasmid pSB11 was chosen for further restriction endonuclease analysis. All the results obtained confirmed the insertion of the TRP1 gene into pRS83 to generate the plasmid pSB11 (Fig. 2b).

EXPRESSION OF pSB11 IN YEAST

Yeast strain LL20-15 (trp1 leu2) was transformed with pSB11 DNA (see methods and materials). Several trp$^+$ transformants were picked and their growth was examined in the presence and absence of leucine in minimal medium, using the trp$^+$ phenotype to select for maintenance of pSB11

Table 3

plasmid DNA	E.coli transformants/ ug DNA		replica plates
	media condition		
	+ + Amp leu	+ − Amp leu	+ − Tc leu
ligated DNA [a]	ND [c]	$>1 \times 10^3$	32
yRP7 [b]	$>4 \times 10^5$	ND	ND

a/ E. coli strain JF1754 (leuB)was transformed with the ligated DNAs (see the text for the detail). The transformed cells were plated on minimal medium agar plates with ampicillin (Amp) and without leucine (leu), and replica plated on minimal medium agar plates, containing tetracycline (Tc) and without leucine.

b/. Cells transformed with yRP7 (see Fig. 2b), were plated on minimal medium agar plates containing Amp and Leu which selelcted for the plasmid and reflected the transformation frequency (positive control).

ND: Not determined.

Figure 3

Restriction endonuclease HindIII digestion patterns of plasmid DNA samples. From left to right, lane 1) HindIII digested yRP7 (the control plasmid). Three bands are present, two large fragments and the small 1.2 Kb fragment which contains the TRP1 gene (Fig. 2b). Lane 2) HindIII digested pRS83 (the control plasmid). Two bands, a 11.2 KB band and an 850 bp band are present (see Fig. 2b). Lanes 3 to 7 are not relevant and should be ignored. Lanes 8 to 12) Plasmid DNAs isolated from five independent Ap^r Tc^r E.coli transformants obtained with the ligated DNA (Table 2). As Figure 2b indicates insertion of the BamHI-BqlII fragment from yRP7 into pRS83 would generate a plasmid with four HindIII sites. Digestion of this plasmid with HindIII should generate four fragments of approximately 7.5 Kb, 4.2 Kb, an 850 bp identical to the small fragment of HindIII digested pRS83 (in lane 2), and a 644 bp identical to the small fragment of HindIII digested yRP7 (in lane 1). These fragments are all identifiable in lanes 8 to 12.

Figure 3
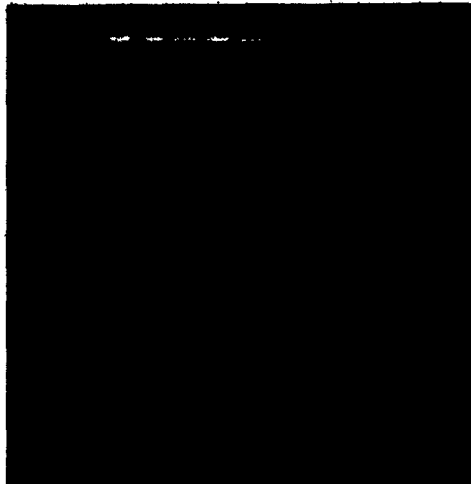
in yeast. The results (Table 4) show that yeast strain LL20-15, transformed with pSB11, is not capable of growth in the absence of leucine. A high transformation frequency of approximately 1×10^3 tryptophan prototrophs/µg of plasmid DNA was obtained. This strongly suggested that pRS83 replicates autonomously in yeast, The following two experiments were carried out to test for the presence of autonomously replicating pSB11 DNA in the trp$^+$ transformants. First plasmid DNA was isolated from the pSB11 yeast transformants and used to transform E. coli strain JF1754 to ampicilin resistance. The results showed that ampicillin resistant colonies obtained were also leu$^+$. Restriction endonuclease mapping of the retrieved plasmids, from yeast, indicated that they were indistinguishable from the original plasmid pSB11. Second, several pSB11 transformants were grown individually under non-selective condition (see methods and materials) for several generations and then tested for their ability to grow on minimal media without tryptophan. The results showed rapid loss of the plasmid in non-selective media. The procedure used was: Yeast transformants of plasmid pSB11 were grown up to saturation, and subcultured five times, using rich liquid medium. The final saturated culture (1×10^8 cells/ml) was diluted and used to plate at about 1×10^3, 1×10^4, 1×10^5 and 1×10^6 cells/plate of media without tryptophan. No tryptophan prototrophs were obtained

Table 4

# of transformants/
ug DNA   replica plates

yeast strain	plasmid DNA	media condition [a]		
		trp$^-$ leu$^+$	trp$^-$ leu$^-$	trp$^+$ leu$^-$
LL20-15	pSB11	1×10^3	$-$ [b]	$-$
(trp1$^-$ leu2$^-$)	yRP7	1×10^3	$-$	$-$
	0	$-$		

a) Three alequotes of competent yeast strain LL20-15 (leu2 trp1) were treated with lug of plasmid pSB11, lug of plasmid yRP7 (positive control which indicates the transformation frequency), and T.E. (negative control). The cells were then plated on media supplemented with leucine, and incubated at 30°C. The trp$^+$ colonies were replica plated on minimal medium and minimal medium with tryptophan, and incubated at 30°C for several weeks.

b) A minus (-) sign indicates no growth.

on any of the plates. For positive control, samples from
the same saturated culture were diluted and plated at
about 100 and 200 cells/plate of rich medium.

INFORMATION UPSTREAM FROM THE LEUB GENE AFFECTS ITS EXPRESSION IN YEAST

As concluded above, pSB11 can replicate in yeast but
does not functionally express the leuB gene. This also
suggests that pRS83 can replicate autonomously in yeast.
Therefore, the reason leucine prototrophs were not obtained
when LL20 (leu2) was transformed with pRS83 or pSB11 was
because pRS83 and pSB11 cannot functionally express leuB in
yeast. Therefore, expression of the cloned leuB gene in
yeast must be affected by sequences present on the shuttle
vector. These results show that leuB expression is
dependent upon plasmid sequences placed 5' to the E. coli
fragment harboring the leuB gene. A comparison of the
upstream information present in plasmids pGY33, pRS27 and
pRS87 which can express leuB in yeast and pRS83, pGY34 and
pSB11 which do not express leuB in yeast clearly shows that
expression depends upon information present immediately
upstream of the leuB gene. It also shows that expression is
dependent upon the orientation of the upstream information.
For example both pGY33 and pGY34 carry the identical 1800
base pair fragment upstream from leuB, but in one plasmid,
pGY33, this fragment is in one orientation and leuB can be

expressed while in the other, pGY34, the 1800 base pair fragment is in the opposite orientation and the leuB gene cannot be expressed. There are two possible explanations for how plasmid sequences could be affecting leuB expression so that plasmids pRS83 and pSB11 cannot express leuB in yeast yet pRS27, pGY33 and pRS87 can express leuB. One explanation is that plasmid sequences adjacent leuB are acting as a positive element allowing leuB expression in pRS27, pGY33 and pRS87 but not in pRS83 and pSB11. The other explanation is that a negative element functions to prevent expression of leuB in pRS83 and pSB11 but does not function in pRS27, pGY33 and pRS87. A comparison of the structure of these plasmids shows that plasmids pRS83 and pGY34 which do not functionally express leuB in yeast, and plasmids pGY33, pRS27 and pRS87, which do functionally express leuB in yeast, all contain identical information downstream from the leuB gene. Therefore, differences in the ability of these plasmids to express leuB in yeast must be as a result of the different information found upstream from the leuB gene.

As noted above the leuB gene on pRS87 can be expressed in yeast but the same gene on pRS83 cannot be expressed in yeast. Again these two plasmids are identical except for the orientation of an 850 base pair fragment that has been cloned into the HindIII site immediately upstream of the

E. coli leuB gene (Fig. 2a).

How can leuB present on plasmids pRS87 and pGY33 be expressed in yeast but not be expressed when present on pRS83 and pGY34. Examination of the structure of these plasmids (Fig. 2a) shows that a yeast promoter directs transcription towards leuB in both pRS83 and pGY34. In pGY34 it is the promoter of the yeast HIS3 gene while in pRS83 it is the promoter of the yeast DED1 gene, which directs transcription towards leuB. Furthermore, in plasmids pGY33 and pRS87 these promoters direct transcription away from leuB. Furthermore, it is possible that in pGY33 and pRS87 the leuB gene is transcribed from its 5' region.

Based on these results I postulated the following hypothesis: Promoter occlusion can prevent leuB expression in yeast. That is transcription from a second yeast promoter upstream from leuB and directing transcription towards leuB prevents transcription of the leuB gene in yeast.

TESTING THE HYPOTHESIS

A) DELETION OF THE DED1 PROMOTER

If promoter occlusion is preventing leuB expression

from pRS83 and the derivative pSB11 then it should be possible to restore leuB expression by deleting the upstream promoter which is responsible for the occlusion of the leuB promoter.

The sequences within the HindIII fragment upstream from the leuB gene, both on pRS83 and pSB11, contain the yeast DED1 promoter region and the 3' portion of the yeast HIS3 gene (Fig. 2b). The DNA sequence of this HindIII fragment is presented in Fig. 4 (Struhl, 1985). According to this Figure The DED1 promoter region, including the TATA box at position 894, transcriptional start sites, and the N terminal codon at position 985, are found between the unique XhoI and BglII restriction endonuclease sites at positions 879 and 1220, respectively. The yeast HIS3 3' end is located upstream from the XhoI site. Excision of the small BglII-XhoI fragment from pSB11 should delete the yeast DED1 promoter, leaving the HIS3 3' portion intact. The following method was used to construct a derivative of pSB11 which was lacking the DED1 promoter region. First pSB11 DNA was digested with XhoI and BglII restriction enzymes (each of these restriction endonucleases recognize single sites within plasmid pSB11). The digested DNA was then purified (see methods and materials) and subjected to SI exonuclease digestion to remove the staggered ends from the endonucleases digested DNA. The DNA was then purified

Figure 4

Nucleotide sequence and transcriptional map of the yeast HIS3 and DED1 gene region (K.Struhl, 1985). The upstream-most HIS3 mRNA start site is designated as nucleotide +1. The mRNA initiation sites are diagrammed as vertical lines connected to horizontal arrows which indicate the direction of transcription. The major HIS3 mRNA termination site is indicated by a vertical line at position +830. The TATA sequences are shown as thin horizontal lines below the sequence. The DED1 promoter region, including the TATA box at position +894, the transcriptional start sites, and the N terminal codon at position +985, are found between the XhoI and BglII restriction endonuclease sites located at positions +879 and +1220, respectively. Plasmid pSB11 harbors the sequences located between the HindIII site at position +515 and the BglII site at position +1220. The position of the HindIII site at +1365 is approximate and according to a previous report by Struhl et al (1981[b]) on the restriction map of this region.

Figure 4

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                                             GATGACTCTT*T~*T*-*TT*CTTAGCGATTGGCATTATCACATAATGAATTATAC  -50

-49 ATTATATAAAGTAATGTGATTTCTTCGAAGAATATACTAAAAAATGAGCAGGCAAGATAAACGAAGGCAAAGATGACAGAGCAGAAAGCC  41
                                                                    MetThrGluGlnLysAla
                                            +1

     LeuValLysArgIleThrAsnGluThrLysIleGlnIleAlaIleSerIle_I,s3.yGlyProLeuAlaIleGluHisSerIlePhePro
  42 CTAGTAAAGCGTATTACAAATGAAACCAAGATTCAGATTGCGATCTCTTTAAAA3CTGGTCCCCTAGCGATAGAGCACTCGATCTTCCCA  131

     GluLysGluAlaGluAlaValAlaGluGlnAlaThrGlnSerGlnValIleAsrValHisThrGlyIleGlyPheLeuAspHisMetIle
 132 GAAAAAGAGGCAGAAGCAGTAGCAGAACAGGCCACACAATCGCAAGTGATTAACGCCACACAGGTATAGGGTTTCTGGACCATATGATA  221

     HisAlaLeuAlaLysHisSerGlyTrpSerLeuIleValGluCysIleGlyAspLeuHisIleAspAspHisHisThrThrGluAspCys
 222 CATGCTCTGGCCAAGCATTCCGGCTGGTCGCTAATCGTTGAGTGCATTGGTGACTTACACATAGACGACCATCACACCACTGAAGACTGC  311
                          Hind III
     GlyIleAlaLeuGlyGlnAlaPheLysGluAlaLeuLeuGlyArgGlyValLysArgPheGlySerGlyPheAlaProLeuAspGluAla
 312 GGGATTGCTCTCGGTCAACCTTTTAAAGAGGCCCTACTGGCGCGTGGAGTAAAAAGG*TTGGATCAGGATTTGCGCCTTTGGATGAGGAA  401
                          Bgl II
     LeuSerArgAlaValValAspLeuSerAsnArgProTyrAlaValValGluLe.GlyLeuGlnArgGluLysValGlyAspLeuSerCys
 402 CTTTCCAGAGCGGTGGTAGATCTTTCGAACAGGCCGTACGCAGTGTCGAAC*TGC*TTGCAAAGGGAGAAAGTAGGAGATCTCTCTTGC  491
                              Hind III
     GluMetIleProHisPheLeuGluSerPheAlaGluAlaSerArgIleThrIe.,.sValAspCysLeuArgGlyLysAsnAspHisHis
 492 GAGATGATCCCCCATTTTCTTGAAAGCTTTGCAGAGGCTAGCAGAAT*ACC*T*CCACGTTGATTGTCTGCGAGGCAAGAATGATCATCAC  581

     ArgSerGluSerAlaPheLysAlaLeuAlaValAlaIleArgGluAlaThrSerProAsnGlyThrAsnAspValProSerThrLysGly
 582 CGTAGTGAGAGTGCGTTCAAGGCTCTTGCGGTTGCCATAAGAGAAGCCACC*CGCCAATGGTACCAACGATGTTCCCTCCACCAAAGGT  671

     ValLeuMet******
 672 GTTCTTATGTAGTGACACCGATTATTTAAAGCTGCAGCATACGA*ATA*A*ACA*GTGTATATATGTATACCTATGAATGTCAGTAAGTA  761

 762 TGTATACGAACAGTATGATACTGAAGATGACAAGGTAATGCATCA*TC*ATA*G*G*CATTCTGAACGAGGCGCGC*T*CCT*T*TCTT  851

     Xhol
 852 TTTGCTTTTC*T*TTTTTTTCTCTTGAACTCGAGAAAAAAAATATAAAAGAGA*GGAGGAACGGGAAAAAGTTAGTTGTGGTGATAGGTG  941

                                                   MetAlaGl**e.SerGluGlnValGlnAsnLeuSerIleAsnAsp
 942 GCAAGTGGTATTCCGTAAGAACAACAAGAAAGCAITTCATATTATGGCTGAAC*GAGCGAACAAGTGCAAAATTTAAGCATCAACGAC  1030

     AsnAsnGluAsnGlyTyrValProProHisLeuArgGlyLysProArgSerAlaArgAsnAsnMetSerAsnTyrAsnAsnAsnAsnGly
1031 AACAACGAGAATGGTTATGTTCCTCCTCACTTAAGAGGGAAAACCAAGAAGTGCCACAAATAACATGAGCAACTACAATAACAACAACGGC  1120

     GlyTyrAsnGlyGlyArgGlyGlyGlySerPhePheSerAsnAsnArgArgG..,G.y,rGlyAsnG.yGlyPhePheGlyGlyAsnAsn
1121 GGCTAGAACGGTGGCCGTGGCGGTGGCAGCTTCTTTAGCAACAACCGTCGTGGTGGT*ACGGCAACGGTGGTTTCTTCGGTGGAAACAAC  1210
         Bgl II
     GlyGlySerArgSerAsnGlyArgSerGlyGlyArgTrpIleAspGlyLysHissva*roAlaProArgAsnGluLysAlaGlnIleAla
1211 GGTGGCAGCAGATCTAACGGCCGTTCTGGTGGTAGATGGATCGATGGCAAACATGTCCAGCTCCAAGAAACGAAAAGGCCGAGATCGCC  1300

     IlePheGlyValProG.uAspPro
1301 ATATTTGGTGTGCCCGAGGATCC_____Hind III_1365
```

and the blunt ends ligated. Following transformation of
E. coli strain JF1754 with the ligated DNA, ampicillin
resistant leucine prototrophs were selected. To determine
whether the small XhoI-BglII fragment had been excised,
plasmid DNA harbored by the Amp^r leu$^+$ transformants was
isolated and examined by restriction endonuclease analysis.
The desired plasmid should lack both XhoI and BglII site
found in the parent plasmid pSB11. This was confirmed by
restriction endonuclease digestion with excess XhoI and
BglII (see Fig. 5). Furthermore, DNA sequence of this
region predicted that deletion of the information between
the XhoI and BglII site, using the strategy employed above,
would remove exactly 340 base pairs (Fig. 4). The putative
deletion derivative of pSB11 were then digested with
HindIII. The parent plasmid pSB11 should generate four
fragments of approximately 7.5 Kb, 4.2 Kb, 850 bp and 644
bp respectively. The derived plasmid having the small XhoI
and BglII fragment deleted should also generate four
fragments of which three fragments would be identical to
the HindIII digested pSB11 and the 850 bp fragment should
be replaced by 510 bp fragment. A total of 10 isolates were
characterized. The results depicted in Figure 5 shows the
HindIII restriction endonuclease analysis of two isolates
(pSB30 and pSB20) having altered 850 bp HindIII fragments.
This fragment is now 510 bp in pSB30, suggesting that 340
bp has been removed from this pSB11 derivative. In pSB20,

Figure 5.

Restriction endonuclease digestion patterns of plasmid DNA samples. A total of 1 μg of plasmid DNA is present in each lane. Lane 1) pBR322 digested with TaqI, was used as a molecular weight marker. TaqI digestion of pBR322 generates 7 fragments, the smallest fragment of 141 bp is not identifiable on the gel. The size of the other fragments are: 1444 bp, 1307 bp, 475 bp, 368 bp, 315 bp, and 312 bp respectively. Lane 2) pSB11 digested with HindIII which generates four fragments of approximately 7.5 Kb and 4.2 Kb, 850 bp and 644 bp. The 850 bp fragment, the second band from the bottom, carries the HIS3 terminator sequences along with the DED1 promoter region. Lane 3) HindIII digestion of plasmid pSB20 generates three identical bands to those of HindIII digested pSB11 and the fourth band replaced the 850 bp fragment by an approximately 410 bp fragment (the first band from the bottom). Lane 4) pSB30 digested with HindIII which generates three bands that are identical to three of the pSB11 HindIII fragments. The 850 bp fragment has been replaced by a fragment which is approximately 510 bp. Lane 5) pSB30 digested with ClaI which generates two fragments of approximately 7.5 Kb and 5.3 Kb. Lanes 6 & 7) pSB30 digested with ClaI and excess of XhoI and BglII, respectively. Only two bands are present (7.5 Kb and 5.3 Kb). Therefore, there are no XhoI or BglII

Fig. 5 (cont'd)

sites present on pSB30. Lanes 8 to 13 are not related to this experiment.

Figure 5
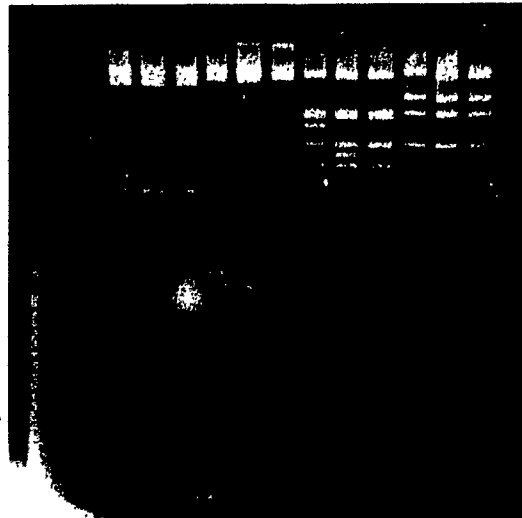
the 850 bp fragment has been changed tp a 410 bp

fragment.

B) EXPRESSION OF pSB30 IN YEAST

Yeast strain LL20-15 (trp1 leu2) was transformed with pSB30 DNA and tryptophan prototrophs selected. Several trp$^+$ transformants were isolated and their ability to grow in minimal media without leucine was tested. These results are presented in Figure 6 and Table 5. The pSB30 transformants, unlike the pSB11 transformants, were able to grow in media without leucine. Therefore, the leuB gene present on pSB30 can be functionally expressed in yeast. This result is consistent with the hypothesis presented earlier. Since removal of the DED promoter region allowed leuB expression, therefore, its presence upstream of the leuB gene and directing transcription through the leuB gene prevented its expression in yeast.

RNA BLOT ANALYSIS OF YEAST TRANSFORMANTS HARBORING pSB11 AND pSB30

. If the DED1 promoter present on pSB11 directs transcription through the leuB gene present on pSB11 it should be possible to detect the resulting transcript using RNA blot analysis. Similarly RNA blot analysis should show that this transcript is not present in the derivatives (pSB30 and pSB20) which have the DED1 promoter deleted.

Figure 6

Growth rate curves of various yeast strains in different media. Cells were grown up to late exponential phase in liquid culture media under condition which selected for the maintenance of the plasmids they contained. The control strain LL20-15, with no plasmid DNA, was grown in rich media. Cells were then subcultured into 25 mls of fresh media in side arm flasks and incubated in a shaker bath. The growth curves were obtained by plotting the turbidity (determined using a Klett machine) versus time. The turbidity values presented are in klett units.

A) The growth rates for pSB11 (x) and pSB30 (+) transformed LL20-15 were identical to that of the control strain LL20-15 (*) when selecting for tryptophan prototrophy (doubling time of approximately 2 hrs). The untransformed control strain LL20-15 (leu2 trp1) was grown in minimal medium supplemented with leucine and tryptophan.

B) The growth curve for pSB30 (#) and pSB151 (^) transformants were identical when selecting for leucine, with a doubling time of approximately 2½ hrs. The pSB11 transformant and the parent strain LL20-15, did not grow in medium without leucine.

Fig. 6

(A)

(B)

51
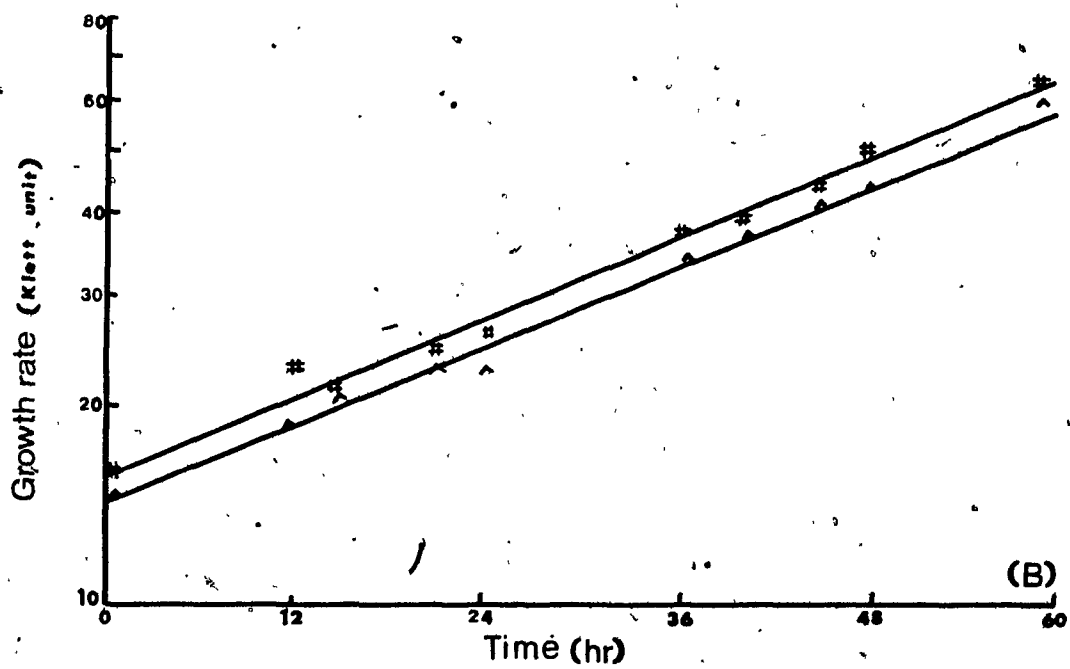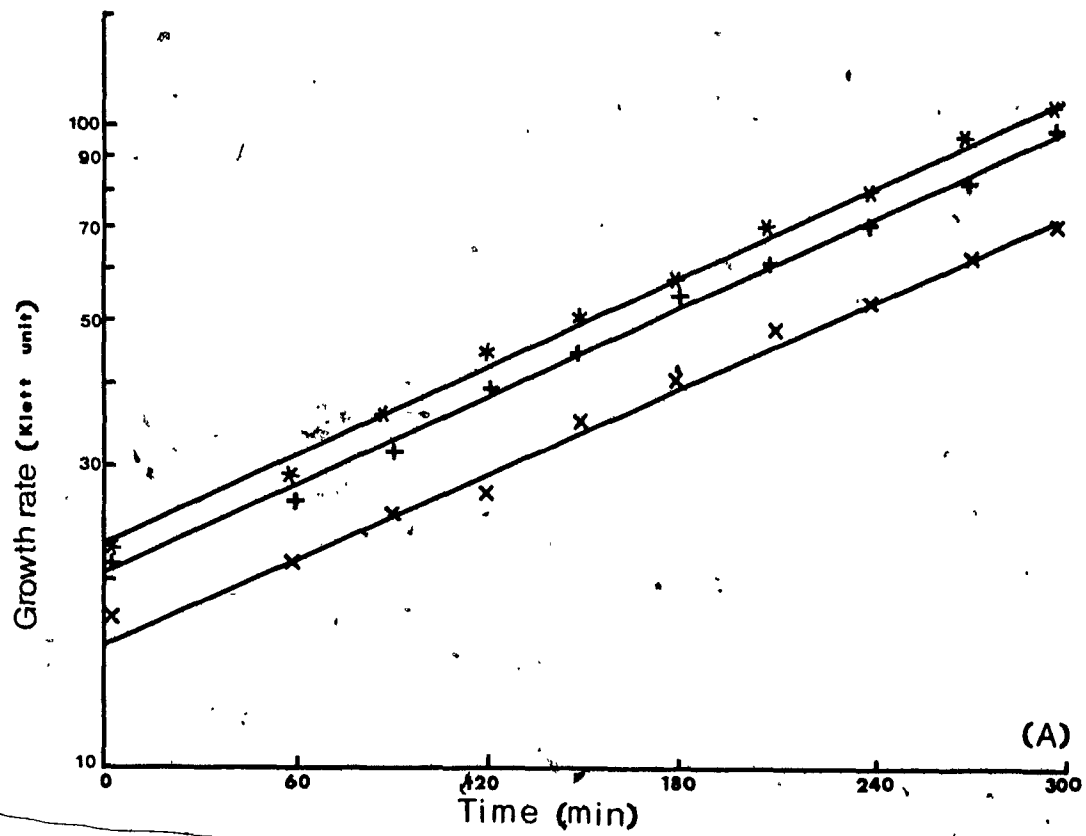
Table 5

Yeast transformants and their growth in different media

strains	plasmid DNA	supplement added to minimal media		
		leucine	tryptophan	nothing
LL20-15	pSB30	+	+	+
(trp1 leu⁻)	pSB20	+	+	+
	pSB151	+	+	+
	pSB11	+	−	−
	YRP7	+	−	−
	0	−	−	−
AH22	pRS83	ND	−	−
(leu⁻)	pRS27	ND	+	+
	pRS87	ND	+	+
	0	−	−	−

a) Depending on the selectable marker present on the plasmids, yeast strains LL20-15 (trp1 leu2) or AH22 (leu2) was used as the plasmid host. Growth is indicated by "+". Negative growth is indicated by "-".

b) No plasmid DNA (0) was used.

ND: not determined.

Total RNA was isolated from yeast transformants harboring pSB11, pSB20 and pSB30. The resulting RNA samples were denatured using glyoxal (McMaster et al., 1977) and fractionated by agarose gel electrophoresis (Fig. 7). The fractionated RNA samples were then transferred to nitrocellulose membrane as described in methods and materials (Thomas, 1980). The resulting RNA blot was then probed with a ^{32}P-labeled leuB specific probe generated by nick translation (Maniatis, 1975). The leuB fragment, flanked by HindIII and BamHI restriction endonucleases sites, was isolated from pRS83. This fragment includes the A'B C D' region of the leucine operon (Fig. 2a). After hybridization and washing, the blot was exposed to X ray film. The resulting autoradiogram (Fig. 8) clearly shows that pSB11 transformants direct the synthesis of a large (approximately 4 Kb) RNA species which is not present in RNA isolated from either pSB20 or pSB30 transformants. Instead, a smaller mRNA (approximately 3 Kb) is detected on the autoradiogram, in both cases.

## HOW DOES AN UPSTREAM PROMOTER PREVENT EXPRESSION

The presence of an upstream promoter could prevent leuB expression in at least two ways. I) Initiation of transcription from an upstream promoter and proceeding through the leuB promoter could prevent transcription of leuB, and therefore prevent expression. II) Presence of a

Figure 7

Total RNA isolates resolved by agarose gel electrophoresis. Samples of total RNA were isolated from the different transformants, and 20 µg of RNA/sample was denatured with glyoxal (see methods and materials). Using 0.9% agarose gel, the RNA isolates were fractionated by gel electrophoresis as described in the methods and materials.

Lane 1) pBR322 DNA digested with TaqI. Lane 2) RNA isolated from Strain LL20-15 grown in minimal media supplemented with leucine and tryptophan. Lane 3) RNA isolated from pSB11 transformed LL20-15, grown in minimal media supplemented with leucine. Lanes 4 & 5) RNA isolated from pSB20 transformed LL20-15, grown in minimal media with and without leucine, respectively. Lanes 6 & 7) RNA isolated from pSB30 transformed LL20-15, grown in minimal media with and without leucine, respectively. Lane 8) Undenatured RNA, used as negative control.

Figure 7
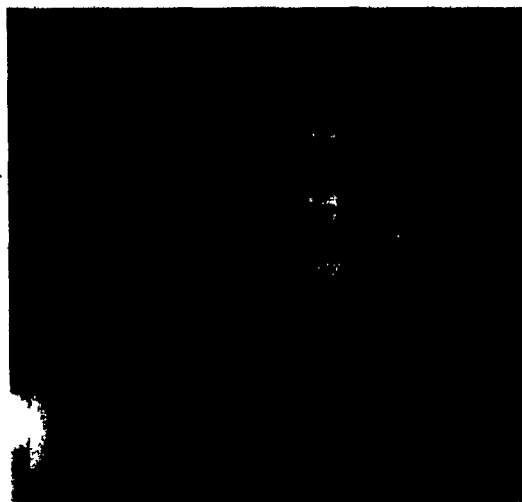
Figure 8

Northern blot analysis of total RNA isolated from LL20-15-
pSB11, LL20-15-pSB20, and LL20-15-pSB30. 20 µg of each RNA
samples isolated from log phase cultures were fractionated
as described for Figure 7, transferred to nitrocellulose
membranes and probed with the 4 Kb leucine operon DNA
fragment from pRS83.

Lane 1) RNA isolated from pSB11 transformed LL20-15
grown in minimal media supplemented with leucine. Lanes 2 &
3) RNA isolated pSB20 transformed LL20-15, grown in minimal
media supplemented with and without leucine, respectively.
Lanes 4 & 5) RNA isolated pSB30 transformed LL20-15, grown
in minimal media supplemented with and without leucine,
respectively.

Figure 8
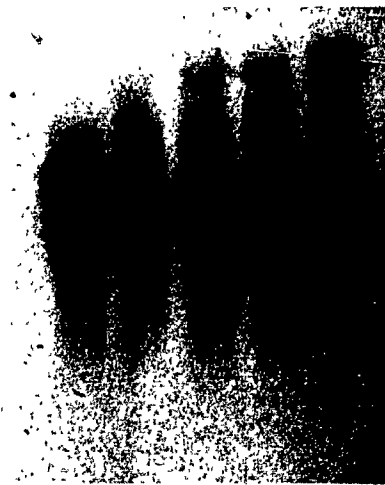
promoter upstream from a second promoter could inhibit binding of RNA polymerase to the downstream promoter (i.e. nucleosome phasing).

Whatever the exact mechanism, If read-through transcription causing "promoter occlusion" prevents the leuB gene expression, then placing a yeast terminator between the upstream promoter and leuB should allow leuB to be expressed in yeast.

CONSTRUCTION OF PLASMID pSB151

A yeast terminator sequence was placed in the appropriate position by the following construction. Insertion of the promoter region of the yeast LEU2 gene which is flanked by two ClaI restriction sites into the ClaI site on plasmid pSB30 generated plasmid pSB151. This plasmid contains a HindIII fragment encoding the yeast HIS3 terminator region located between the LEU2 promoter region, and the leuB gene (Fig. 2b). The following strategy was employed to construct pSB151. The yeast LEU2 promoter region was isolated from plasmid pYF91 (Storms et al., 1979). The structure of this plasmid is shown in Figure 2b. The 5' end of the LEU2 gene including the promoter region is located on the larger ClaI fragment of this plasmid. The direction of transcription from the LEU2 promoter region is indicated (Fig. 2b) and is transcribed from the XhoI

site towards the ClaI site. Plasmid pYF91 was digested with ClaI and DNA was resolved by polyacrylamide gel electrophoresis. The DNA band which represented the yeast LEU2 promoter region was electroeluted and purified. Because pSB30 has two ClaI sites, the plasmid was partially digested with ClaI (Fig. 9). The partially digested DNA band was electroeluted and subjected to alkaline phosphatase treatment which dephosphorylated the ClaI digested staggered ends of the DNA. The DNA was then purified (see methods and materials) and mixed with the ClaI fragment carrying the LEU2 promoter region and then the DNAs were ligated. The ligated DNA was then used to transform E. coli strain JF1754 and ampicillin resistant leucine prototrophs were selected. Several transformants were chosen for restriction endonuclease analysis of the plasmid they contained. First, restriction endonuclease enzyme ClaI was used to analyze these isolated plasmids. Two plasmids, pSB151 and pSB12, which both contained the desired ClaI fragment were subjected to further restriction endonuclease analysis, using double digestion of the DNA with EcoRI and XhoI. This analysis was used to determine the orientation of the cloned LEU2 promoter containing ClaI fragment. The results are shown in Figure 10. In plasmid pSB151, the resulting pattern of bands indicate that the insert has been cloned in the desired orientation. In this plasmid the LEU2 promoter directs transcription towards

Figure 9

Polyacrylamide gel electrophoresis of partially digested
plasmid pSB30 with ClaI restriction endonuclease. pSB30 was
partially digested, using a fixed amount of ClaI enzyme for
variable amounts of time (see materials and methods). Lane 1)
Not related to this experiment. Lane 2) Uncut pSB30. Lane 3)
Totally digested pSB30 which generates two bands. Lanes 4 to
10) partially digested pSB30.

Figure 9
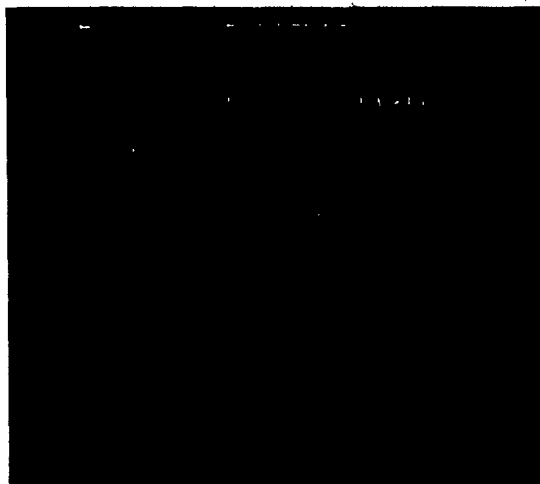
Figure 10

Restriction endonuclease characterization of pSB151 and pSB12. Plasmid pSB12 is identical to plasmid pSB151, except that the 1.8 Kb ClaI insert from pYF91 is present in opposite orientation in these two plasmids.

Lane 1) pRS28, a 13.1 Kb plasmid digested with ClaI. Lane 2) pYF91, digested with ClaI. The second band from the bottom is the 1.8 Kb fragment carrying the yeast LEU2 5' region. Lane 3) Uncut pSB12. Lane 4) pSB12 digested with ClaI. Lane 5) pSB12, digested with XhoI and EcoRI. Lane 6) uncut pSB151. Lane 7) pSB151, digested with ClaI. Lane 8) pSB151, digested with EcoRI and XhoI. According to the restriction map of plasmid pYF91 (R.K. Storms, 1979), if the ClaI fragment on pSB151 is in the orientation where the LEU2 promoter should direct transcription towards the HIS3 sequences, then the smallest fragment resulting from XhoI and EcoRI restriction endonuclease digestion of this plasmid should be approximately 1.2 Kb. This fragment is identifiable in lane 8 (the bottom band). However, the smallest fragment should be approximately 0.6 Kb, if the ClaI fragment is inserted in the opposite orientation. This 0.6 Kb fragment is the bottom band in lane 5 (pSB12).

Figure 10

leuB. Therefore, the isolated pSB151 contained the LEU2
promoter region on the inserted ClaI fragment, reading
towards the terminator sequences on the downstream HindIII
fragment.

EXPRESSION OF pSB151 IN YEAST AND STUDIES ON THE GROWTH RATE

Yeast strain LL20-15 was transformed with pSB151 DNA,
and several trp$^+$ transformants were tested for their
ability to grow in medium lacking leucine. The results in
Table 5 show that the pSB151 yeast transformants were able
to grow in the absence of leucine. Furthermore, the pSB151
transformants grew without leucine at approximately the
same rate as the pSB30 transformants (Fig. 6).

DISCUSSION

In this thesis I have shown that although the E. coli leuB gene can be functionally expressed in yeast, its expression is dramatically affected by sequences which are placed upstream from it. That is, the expression of the leuB gene was dependent upon its physical location relative to other DNA sequences present on the shuttle vector.

pRS83 and Its Derivative p9B11 Are Capable of Autonomous Replication in Yeast

Before initiating the work reported in this thesis, it was known that pRS83 could not transform yeast leu2 cells to leucine prototrophy. To distinguish whether pRS83 was unable to replicate autonomously in yeast, or leuB gene present on pRS83 was not functionally expressed in yeast, a derivative of this plasmid was constructed (pSB11). This derivative harbored the yeast TRP1 gene which served as an additional selectable marker in yeast.

First, it was necessary to clearly demonstrate that the plasmid pSB11 contained the yeast TRP1 gene. Two sets of experiments confirmed the cloning of this gene into pSB11. The first set of experiments involved a detailed restriction endonuclease mapping of pSB11 (Fig. 3). The second experiment involved transforming a Trp yeast strain

to <u>Trp</u> (Table 4). These results confirmed that the desired <u>TRP1</u> containing plasmid had been constructed and that it harbored a functional <u>TRP1</u> gene.

Next it was necessary to determine whether pSB11 could replicate autonomously in yeast. Autonomously replicating plasmids, dependent upon the 2-um sequence for replication in yeast, have the following characteristics: (I) They can transform yeast at high frequency. (II) They are rapidly lost when selection for the plasmid is removed. (III) They can be retrieved in their original state from yeast transformants. The following analysis was performed in order to verify that pSB11 replicated autonomously in yeast. This plasmid and its derivative contain a 3.6 kilobase <u>EcoRI</u> fragment from the 2-um yeast plasmid which allows for its autonomous replication in yeast (Storms et al, 1979). The high frequency of transformation at a rate of approximately 10^3 transformants/ug of plasmid DNA (Table 4), retrieval of the plasmid from the yeast transformants, and loss of transformants ability to grow under selective growth condition after plasmid curing, all confirmed that pRS83 and pSB11 were capable of autonomous replication in yeast.

<u>Read-Through</u> <u>Transcription</u> <u>Initiated</u> <u>from</u> <u>the</u> <u>DED1</u> <u>5'</u>
<u>Region</u> <u>Upstream</u> <u>from</u> <u>leuB</u> <u>Gene</u> <u>Affects</u> <u>Its</u> <u>Expression</u>

A comparison between pRS83 and its derivative, pSB11,
with the parent plasmids (see Figure 2a and 2b), revealed
that the <u>leuB</u> gene was not expressed in the presence of an
upstream yeast promoter region reading towards it. The
observation that transcriptional interference can affect
gene expression of avian leukosis retrovirus genes
(Cullen et al., 1984), was reviewed in the introduction.
Similar results have been reported by Proudfoot (1986).
When two human alpha-globin genes are cloned in tandem,
expression of the downstream alpha gene is dramatically
reduced. The reduced expression is believed to be the
result of transcription initiating from the upstream gene,
reading through, and interfering with the transcription of
the 3' gene. In pRS83 and its derivative pSB11, it is the
promoter of the yeast <u>DED1</u> gene (Fig. 2b) which directs
transcription towards <u>leuB</u>. And in pGY34, it is the
promoter of the yeast <u>HIS3</u> gene which affects the
expression of the <u>leuB</u> gene by directing transcription
towards it. Therefore, it was hypothesized that promoter
occlusion or transcriptional interference prevented <u>leuB</u>
expression in pRS83, the derivative pSB11, and pGY34.

The <u>DED1</u> gene is characterized and reported to be a
gene essential for cell viability in yeast (Struhl,

1985). This gene is located 3' to the yeast HIS3 gene. The nucleotide sequence and transcriptional map of the HIS3 region is presented in Fig. 4. A schematic diagram of the sequences located between the HindIII site at position +515 and a second HindIII site, which is located just beyond the end of the sequenced DNA at position +1365 (Struhl, 1981^{b}), is presented in Fig. 11. The HindIII to BqlII (at positions +515 and +1220 respectively) fragment is located upstream from leuB on pSB11 and the parent plasmid pRS83. As Figure 11 shows, this fragment consists of the HIS3 transcription termination sequences upstream from the XhoI site, and the DED1 regulatory region and part of the coding region of the DED1 gene which are located downstream from the XhoI site.

Excision of the DED1 5' Region from pSB11 Results in Functional Expression of leuB

If read-through transcription, due to the presence of the DED1 promoter region prevented transcription from the leuB gene, then removal of the DED1 promoter from pSB11, should permit expression of the leuB gene. Two plasmids pSB30 (Fig. 2b) and pSB20 were constructed. These should have the DED1 promoter region deleted. These two plasmids were capable of transforming a yeast leu2^{-} strain to leu2^{+} (Table 5), whereas transformants harboring pSB11 could not grow without leucine supplementation. Therefore, it was

Figure 11

Structural organization of the yeast sequences encoding the HIS3 3' end and DED1 5' end. This schematic view of the HIS3 3' end region and DED1 5' end region represents restriction map of the sequences located between HindIII sites, at positions +515 and +1365, on Figure 4. The numbers at the top of the Figure indicate the location of the restriction endonuclease sites for HindIII (H), XhoI (X), and BglII (G), relative to the upstream-most HIS3 mRNA start site (nucleotide +1, according to Figure 4). The HindIII to BglII portion of the region shown is present in pSB11 (Fig. 2b) upstream from leuB gene. The lower portion of the Figure shows an enlarged view of the region surrounding the XhoI site at position +879. This enlarged view indicates the HIS3 translation termination codons (UAA UAG), the spacer sequences between the two genes (black box), the TATA sequence (T) of DED1 gene, and the DED1 translation initiation codon (AUG). Arrows indicate the direction of transcription.

Figure 11
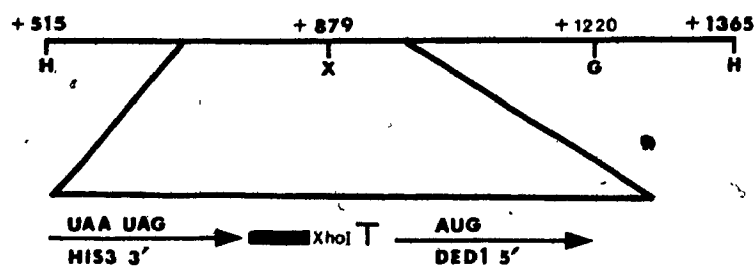
concluded that the <u>DED1</u> promoter region prevented
functional expression of the <u>leuB</u> gene present in pSB11, in
yeast.

Base pairing between complementary sequences of mRNA
and rRNA is required for translation to occur, both in
prokaryotes (Shine and Dalgarno, 1974; Steitz and Jakes,
1975; Sprague, 1977) and eukaryotes. However, 5' terminus
capping after the initiation of transcription is required
for eukaryotic translation to occur efficiently. The
capping occurs by the formation of a 5'-5' pyrophosphate
linkage and a cluster of methyl groups is added to this
structure (Perry and Kelley, 1974). Both et al (1975)
showed that in a wheat germ translation system no protein
synthesis occurs if uncapped mRNA is used. Muthukrishnan et
al., 1976, showed that decapping of reovirus mRNA (the cap
is removed chemically) results in 4-fold reduction in the
ability to bind to wheat germ ribosome. These results
suggest that capping provides a feature for recognition by
ribosomes. Therefore, in eukaryotes translational
initiation requires that the ribosomes bind at the capped
5' region.

Considering the above, if transcription initiating at
the <u>DED1</u> promoter extended through the approximately 1 Kb
of DNA between it and the <u>leuB</u> gene and prevented

transcription of leuB from sequences immediately 5' to leuB, then leuB would only be encoded by mRNA initiating at the DED1 promoter. The DED1 promoted leuB mRNA would have an extended 5' end. In this case, it is unlikely that the yeast translation machinery could initiate translation at a site appropriate for the functional expression of leuB.

RNA Blot Analysis

If expression of the leuB gene contained on pSB11 was affected due to read-through transcription initiating at the DED1 promoter, then it should be possible to detect a large DED1 promoted mRNA species. Furthermore, this RNA should be homologous to the leucine operon sequences downstream from the DED1 promoter. This was shown to be the case in the results presented in Figure 8. These results clearly showed that a mRNA species of approximately 4 Kb which hybridized to the leucine operon probe was present in RNA isolated from pSB11 transformants. This 4 Kb transcript was not present in total RNA isolated from pSB30 and pSB20 transformants. Instead, a smaller mRNA species of approximately 3 Kb in size was detected on the autoradiogram, in both cases. This data supported the hypothesis that expression of the leuB gene was prevented by read-through transcription initiating at the DED1 promoter. Consistant with this interpretation, I found that when the DED1 promoter region was deleted to construct

pSB30 and pSB20, the leuB gene was functionally expressed in yeast (Table 5). Furthermore, the prominent 4Kb RNA species was not detected in the pSB20 and pSB30 transformed LL20-15.

Growth Rate Studies

The growth rate of pSB30 and pSB20 transformed cells was compared in leucine deficient media (Fig. 6). Yeast cells transformed with plasmids pSB30 and pSB20 grew with a doubling time of approximately 22 hrs (in minimal media). In contrast, they grew with a doubling time of approximately 2 hrs when selecting for expression of the plasmid born TRP1 gene. The control strain LL20-15, without plasmid, had a generation time of 2 hrs, when grown in minimal media supplemented with leucine and tryptophan. These results show that in the absence of the yeast DED1 promoter, the leuB gene is functionally expressed, but poorly. This may be due to the fact that major differences exist between eukaryotes and prokaryotes, such as RNA polymerase, ribosomal subunits, transcriptional and translational initiation requirements, post-transcriptional (as discussed above) and post-translational modifications (Ratzkin et al, 1977). For example, in yeast, a given gene can have multiple upstream activator sequences which could all direct transcription initiation at the same position to increase transcriptional efficiency of the gene

(Guarente, 1984). The upstream activator sequences are not found in bacteria. Therefore, it is possible that the prolonged generation time in pSB20, pSB30, and pSB151 transformants grown in media without leucine was due to the fact that E. coli leuB gene was not transcribed as efficiently as its eukaryotic counterpart. Consistent with this interpretation is the work reported by McNeil et al., 1985. This work showed that the cloned E. coli leuB gene is expressed weakly in yeast leu2 mutants, when transcription of the leuB gene is initiated from its own 5' region. However, replacement of the 5' region of the leuB gene by the yeast HIS3 gene (including the transcription and translation initiation signals), results in efficient expression of leuB gene in yeast leu2 mutant and enables it to grow in the absence of leucine at a growth rate comparable to LEU2 transformed stain.

The A' Gene Region

The 3' region of the A gene of the E.coli leucine operon, indicated as A' (approximately 1KB in size), is present upstream from the leuB gene in all the plasmids used in this studies. Since bacterial genes with related function are together in a single operon and are transcribed as one large transcript, the A' region probably does not possess any transcription termination

information for transcripts initiated from sequences
located upstream from it. The results of the RNA blot
analysis supports this postulate (Fig. 8). If the DED1
promoted transcript expressed from pSB11, terminated within
the A gene sequences, then the size of the transcript
detected should be less than 1.5 Kb. However, the RNA blot
analysis showed that the transcript was about 4 Kb (Fig.
8).

Presence of a Yeast Terminator Sequence Between leuB and an Upstream Promoter Can Restore leuB Activity

From the above results it appeared that read-through
transcription initiating at the DED1 promoter prevented
initiation of transcription from sequences adjacent the
leuB gene. This possibility was tested by constructing
plasmid pSB151. pSB151 was constructed by placing a
transcription terminator sequence between a promoter
upstream of leuB and the leuB gene.

The mechanism by which the 3' termini of eukaryotic
mRNA arise are complex (Birchmeier et al, 1982). Studies on
transcription termination in adenovirus type 2 (Nevins et
al, 1980) and simian virus 40 (Ford and Hsu, 1978) have
shown that transcription proceeds past the poly(A) site.
The addition of poly(A) occurs in conjunction with an RNA
chain cleavage rather than transcription termination at

the 3' sequences. Transcriptional termination in animal cells also appears to occur downstream of the poly(A) site (Darnell et al, 1982). A signal sequence of AAUAAA is believed to be important for the processing of the larger precursor molecules and for polyadenylation at the 3' terminus of the mRNA (Fitzgerald and Shenk, 1981). This signal sequence, however, is not present in sea urchin histone mRNAs (Hentschel and Birnstiel, 1980), and mRNAs are not polyadenylated.

In yeast, however, transcription termination and poly A addition appear to be coupled, since all messages are polyadenylated (K.S. Zaret and F. Sherman, 1982). The presence of a conserved repeat sequence TTTTTATA has been shown to be associated with transcription termination (Henikof et al, 1983), this signal sequence is not present in all yeast genes, including HIS3 (Struhl, 1985).

Deletion mapping of the yeast HIS3 terminator regions (Struhl, 1985), has shown that sequences (which are roughly 100-150 bp downstream from the translation termination codons, at position +780 and +830 (Fig. 4) are involved in transcription termination of HIS3 mRNA. These transcription termination regions are present between the LEU2 promoter region and the leuB gene in pSB151.

In this plasmid, presence of the LEU2 promoter region,
upstream from the leuB gene did not abolish leuB
expression. That is, pSB151 transformed LL20-15 strain was
capable of growth in minimal media without leucine (Table
5). Therefore, presence of the yeast HIS3 terminator region
between the two promoters (the LEU2 and the leuB region),
in pSB151, allowed the leuB gene to be functionally
expressed in yeast. This suggested that, in pSB151,
transcripts initiated at the LEU2 promoter region
terminated at the yeast HIS3 terminator region, before
reaching the leuB gene. Thus, the leuB gene was capable of
complementing the pSB151 transformed leu2 cells.

The result of growth studies on pSB151 transformed
LL20-15, grown in media without leucine (Fig. 6), is in
support of the above. In media without leucine,
supplementation, the pSB151 transformed cells doubled at
the same rate observed in pSB30 transformed cells, with an
increase of approximatley 20 hrs in the growth rate,
relative to their growth rate in minimal media supplemented
with leucine. These results suggested that, both in pSB30
and pSB151, transcription initiation of the leuB gene is
promoted from the same region, possibly the leuB 5' region.
Therefore, presence of the HIS3 terminator region between
the two promoters, the leuB promoter and the LEU2 promoter,
in pSB151, could prevent read-through transcription

observed in the parent plasmids pRS83 and pSB11.

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